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**Comparative genomics
of clinically significant
Staphylococcus
species**

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Abstract

Bacteria of the *Staphylococcus* genus colonize a variety of environments and are a causative agent of human and animal infections. Recently, many coagulase-negative members of this genus have been described. They are primarily commensals that can become pathogens under the appropriate conditions. Persons with compromised immune system or inserted foreign bodies are particularly at risk. However, the main significance of coagulase-negative staphylococci for the health care system lies in their large reservoir of antimicrobial resistance determinants, which can disseminate to pathogenic species by horizontal gene transfer.

This thesis investigates isolates from different phylogenetic branches of the genus *Staphylococcus* and its related genus *Macrococcus*, which were collected from clinical, animal and soil samples. These strains were studied using phenotypic, chemotaxonomic and genotypic methods including whole genome sequencing. This led to the description of six novel species of staphylococci and three novel species of macrococci. Also, the information about species found in Antarctica was expanded. Detailed characterization of plasmids, prophages, transposons, and other mobile elements encoding antibiotic resistance has been emphasized. A unique genomic island, closely related to the staphylococcal chromosomal cassette mediating the transmission of methicillin resistance, was found in the genome of *Staphylococcus brunensis*. Comparative genomics has shown that this genomic island also has the potential to accumulate virulence and resistance genes. These results have improved the diagnosis of coagulase-negative staphylococci and provided insights into their phylogeny and the evolution of antibiotic resistance.

Anotace

Bakterie z rodu *Staphylococcus* kolonizují různá prostředí a jsou původci infekcí lidí i zvířat. Nedávno bylo popsáno mnoho koaguláza negativních zástupců toho rodu. Jsou to především komenzálové, kteří se za vhodných podmínek mohou stát patogeny. Ohrožené jsou jimi zejména osoby s oslabeným imunitním systémem nebo cizími tělesy v těle. Hlavní význam koaguláza negativních stafylokoků pro zdravotnictví ale spočívá v jejich rozsáhlém rezervoáru rezistence k antimikrobiálním látkám, které se mohou šířit do patogenních druhů horizontálním genovým přenosem.

Tato práce se zabývá izoláty z různých fylogenetických větví rodu *Staphylococcus* a také jemu příbuzného rodu *Macrococcus*, které byly odebrány z klinických, zvířecích i půdních vzorků. Tyto kmeny byly zkoumány pomocí fenotypových, chemotaxonomických a genotypových metod včetně celogenomového sekvenování. Díky tomu bylo nově popsáno šest druhů stafylokoků a tři druhy makrokoků. Rozšířeny byly i poznatky o druzích vyskytujících se v Antarktidě. Důraz byl dán na detailní charakterizaci plazmidů, profágů, transponzonů a jiných mobilních elementů kódujících rezistence k antibiotikům. V genomu *Staphylococcus brunensis* byl nalezený jedinečný genomový ostrov blízce příbuzný stafylokokové chromozomové kazetě, která je zodpovědná za přenos rezistence k methicilinu. Srovnávací genomikou bylo zjištěno, že i tento genomový ostrov má potenciál akumulovat geny virulence a rezistence. Získané výsledky zlepšily diagnostiku koaguláza negativních stafylokoků a přinesly vhled do jejich fylogeneze a evoluce rezistence k antibiotikům.

Declaration

I declare that I have prepared my thesis independently under the mentorship of the thesis supervisor and using the information sources provided in the thesis.

Brno, February 7, 2024

.....
Vojtěch Kovařovic

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List of Abbreviations

ACME	– arginine catabolic mobile element
ANI	– average nucleotide identity
BSI	– bloodstream infection
CC	– clonal complex
CG	– cytosine and guanine
CoNS	– coagulase-negative staphylococci
CRISPR	– clustered regularly interspaced short palindromic repeats
DDH	– DNA-DNA hybridization
eDNA	– extracellular DNA
FBRI	– foreign body infection
HGT	– horizontal gene transfer
ICE	– integrative and conjugative element
IS	– insertion sequence
MALDI-TOF-MS	– matrix-assisted laser desorption/ionization-time of flight mass spectrometry
MGE	– mobile genetic element
MLST	– multilocus sequence typing
MRSA	– methicillin-resistant <i>Staphylococcus aureus</i>
MSCRAMM	– microbial surface components recognizing adhesive matrix molecules
MSSA	– methicillin-sensitive <i>Staphylococcus aureus</i>
NVIE	– native valve endocarditis
ORF	– open reading frame
PFGE	– pulse field gel electrophoresis
PIA/PNAG	– polysaccharide intercellular adhesin
PICI	– phage inducible chromosomal island
PSM	– phenol soluble modulin

LIST OF ABBREVIATIONS

RM	– restriction modification
rRNA	– ribosomal RNA
SCC	– staphylococcal chromosomal cassette
SIG	– <i>Staphylococcus intermedius</i> group
SNP	– single nucleotide polymorphism
SSI	– surgical site infection
ST	– sequence type
UTI	– urinary tract infection
VRSA	– vancomycin-resistant <i>Staphylococcus aureus</i>
4mC	– N4-methylcytosine
5mC	– 5-methylcytosine
6mA	– N6-methyladenine

1 Introduction to Genus *Staphylococcus*

1.1 Early years of clinical research

In the late 19th century, the Scottish surgeon Alexander Ogston investigated the presence of microorganisms in pus isolated from acute abscesses and whether they caused blood poisoning. He observed the presence of micrococci in such samples and was the first to succeed in obtaining pure cultures by cultivating them in eggs. However, it was found that their virulence varied considerably and that micrococci grew better under anaerobic conditions (Ogston, 1881). Some of these micrococci formed distinct grape-like clusters, as seen in Fig. 1, which gave a name to the genus *Staphylococcus*, in contrast to the streptococci, which formed long chains. Although the name was first proposed by Ogston in 1883, the formal description of the genus *Staphylococcus* was given by Rosenbach initially as *Staphylococcus pyogenes* var. *aureus* and *Staphylococcus pyogenes* var. *albus* named for their color (Rosenbach, 1884). At that time, staphylococci were known to colonize the skin and cause abscesses, acne, osteomyelitis, endocarditis, conjunctivitis, and septicemia. The increasing number of isolates made classification difficult. Seemingly even in pure cultures, a considerable variation in phenotype and virulence was observed after several passages (Winslow and Winslow, 1908). This led to several nomenclature proposals in the following years. Eventually, it was agreed that the pathogenic yellow pigment producing strains would be assigned *Staphylococcus aureus* and the others forming white colonies as *Staphylococcus epidermidis* (Evans, 1916). However, *S. epidermidis* was known to be occasionally pathogenic, so the pigmentation does not indicate pathogenicity, which depends on the production of toxins (Panton, 1937). The hemolytic and coagulase activities were found to be suitable markers to distinguish virulent strains. However, the coagulase test (Loeb, 1903) is easier to perform and was proposed as a routine classification criterion for staphylococci. The pathogenic strains were further characterized by serology or phage typing (Fairbrother, 1940). On the contrary, pus production characteristic of staphylococci infections is not necessary for virulence (Blair, 1939). Coagulase-negative strains (CoNS) were generally considered saprophytic. However, sporadic evidence of severe endocarditis caused by coagulase-negative *S. epidermidis* indiscernible from commensal skin strains has suggested that CoNS should not be dismissed as a contaminant only (Cunliffe *et al.*, 1943). This was later reinforced when a third species was recognized, *Staphylococcus saprophyticus*, which causes urinary tract infections and is resistant to novobiocin (Fairbrother, 1940; Mitchell and Baird-Parker, 1967; Maskell, 1974).

Since the 1920s, staphylococcal infections have been extensively studied, and soon several staphylococcal toxins were isolated, such as exotoxin, enterotoxin, and fibrinolysin (Blair, 1939). In addition, two types of hemotoxins were recognized.

α -hemotoxin, which coagulates rabbit and human blood, and β -hemolysin, active only on sheep erythrocytes (Morgan and Graydon, 1936). Another important cytotoxin was recognized as a causative virulence factor and named Pantan-Valentine leucocidin (Panton *et al.*, 1932), although it had been discovered earlier (Van de Velde, 1894) and many years later described to be encoded by a prophage (Kaneko *et al.*, 1997). These virulence factors enabled *S. aureus* to cause outbreaks and food poisoning. However, pathogenicity was not always expressed. Under the right circumstances, primary pathogenesis proved rapid and lethal, as demonstrated in the Bundaberg outbreak, where twelve of twenty-one children died within hours after being injected with a contaminated diphtheria vaccine (Kellaway *et al.*, 1928).

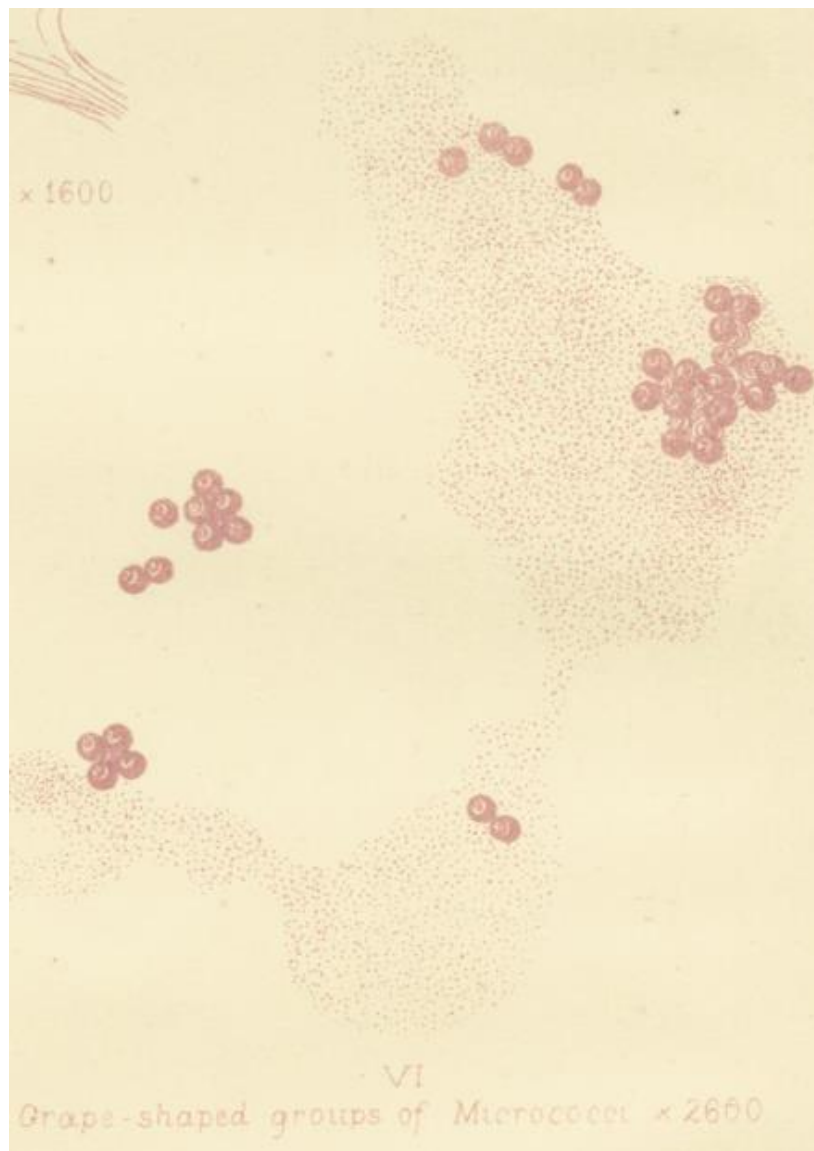


Fig. 1: Ogston's original drawing of cocci

Drawing of grape-shaped groups of micrococci in pus, magnification 2600x (Ogston, 1881, modified)

1.2 The Era of Antibiotics

The severity of staphylococcal infections demanded the search for an effective treatment. Early staphylococcal infections were unsuccessfully treated with sulfonamides such as sulfanilamide or dimethyl sulfanilamide (Mellon *et al.*, 1937). Bacteriophage therapy has been partially successful, with 75–80 % of patients reportedly cured (Larkum, 1929; Sulakvelidze *et al.*, 2001). Although the benefit of bacteriophages is virtually no toxicity to the host, the disadvantage is high phage specificity, which limits their use to a few bacterial strains (Larkum, 1929).

In 1943, the mass production of benzylpenicillin, also known as penicillin G, began. This first widely available antibiotic nearly solved the problem of staphylococcal infections. By the 1940s, most staphylococci were susceptible to penicillin (Brumfitt and Hamilton-Miller, 1989). However, failure of antibiotic therapy and resistant strains were also known to occur (Rammelkamp and Maxon, 1942). In 1947, it was reported that 12.5 % of *Staphylococcus* cultures were resistant to penicillin. It was observed that the resistant strains were more prevalent in hospitalized compared to newly admitted patients. The cause is the selection of resistant strains by penicillin usage. The incidental evidence was provided by a patient with a resistant strain present in an abscess before the treatment (Barber, 1947).

The declining efficacy of penicillin G did not initially cause alarm; infections could usually be treated with semisynthetic derivatives of penicillin such as methicillin or some of the newly discovered antibiotics such as erythromycin, streptomycin, tetracycline, or cephalosporins (Brumfitt and Hamilton-Miller, 1989). However, new methicillin-resistant strains of *S. aureus* (MRSA) soon emerged. The MRSA strains constituted a subpopulation of *S. aureus* and could be obtained from hospital or community isolates or by selection in repeated passages in low antibiotic concentration. *Staphylococcus aureus* had only a limited ability to acquire resistance in vitro. In contrast, the CoNS acquired resistance more readily. In addition, MRSA is cross-resistant to other beta-lactam antibiotics (Stewart, 1961). Methicillin was used sparingly, yet the prevalence of MRSA continued to increase. The distribution of resistant strains was mainly localized and did not depend on using methicillin (Parker and Jevons, 1964). An example of an early MRSA outbreak occurred in March 1961 at Queen Mary's Hospital for Children, Carshalton, Surrey. Methicillin, first used in the second half of 1959 during routine screening, detected MRSA strain in a pediatric patient by application of topical chlorhexidine, neomycin, and bacitracin. Immediately afterward, 25 patient and staff samples were taken. MRSA was detected in another patient; three additional cases were reported a month later. At that moment, it was decided to close the ward and administer preventive topical applications of antibiotics. During the following year, a total of 71 MRSA strains were isolated from 37 persons, which were also resistant to streptomycin and tetracycline. Most were not serious, except one infant died after a surgical wound infection (Stewart and Holt, 1963).

Several more outbreaks followed. Two-thirds were in intensive care units, and MRSA remained an endemic pathogen in over 85 % of hospitals. The initial goal was the eradication of MRSA, but this was ultimately not feasible (Thompson *et al.*, 1982). Nevertheless, even minimal measures to control MRSA are sufficient to lower mortality. Another less apparent benefit is that suppressing MRSA also reduces the usage of vancomycin, which is the drug of choice to treat MRSA infections (Chambers, 1988), and therefore lowers the incidence of vancomycin-resistant *S. aureus* strains (VRSA) (Marshall *et al.*, 2004). Vancomycin is a glycopeptide antibiotic; resistance is based on thicker cell wall (Pereira *et al.*, 2007). Because of that, the VRSA strains are growing slowly and have low potential to cause outbreaks (Lindsay, 2010). The situation became serious when it became apparent that MRSA and VRSA are multiresistant, and in some cases, this made nosocomial infections untreatable (C. Liu and Chambers, 2003). To control these infections, it is necessary to combine many procedures that are partially effective. Preventive treatment can include mupirocin decolonization and the use of disinfectants and gloves by healthcare workers. These approaches have been used by the Netherlands and the Nordic countries in rather aggressive policies, successfully reducing the incidence of MRSA (Vos *et al.*, 2009).

The national preventive strategy should emphasize the effective use of antibiotics in primary care (Livermore, 2009; Marshall *et al.*, 2004). Current Food and Drug Administration guidelines recommend five antibiotics for treating MRSA infections; vancomycin, daptomycin, tigecycline, telavancin, and cefazoline (Maddiboyina *et al.*, 2023). Meta-analysis showed that innate neutrophil immunity is sufficient in healthy persons and low-risk patients to defend organism against *S. aureus*, and antibiotic therapy has negligible effect. Another important point is that only 4–5 % of patients treated empirically with antibiotics are infected with MRSA. Ideally, the antibiotics should be reserved for use in high-risk immunocompromised patients or in cases where the skin barrier has been penetrated; neutrophil deficiency, septic shock, surgical wounds, and catheter-associated infections (Carey *et al.*, 2023; Grundmann *et al.*, 2006). Even the most effective antibiotics can fail in cases of co-infection with multiple strains with different genetic backgrounds and resistance profiles, which is common in cystic fibrosis (Long *et al.*, 2021), or in the presence of small colony variants, which are present in 10–40 % cases of osteomyelitis. *Staphylococcus aureus* also evades antibiotics via intracellular replication (Zelmer *et al.*, 2022). Another issue with antibiotic therapy is that a resistant strain is expected to eventually appear against every antibiotic because there are only a handful of new antibiotics, most of which are just derivatives of previous compounds. Thus, current research is focused on alternatives based on antibiotic-antibody conjugates, bacteriophages, and bacteriotherapy with *Staphylococcus hominis* (Douglas *et al.*, 2023) or *Staphylococcus lugdunensis* (Zipperer *et al.*, 2016). A new therapeutic approach focuses on indirect therapy based on immunometabolites to suppress cytokine storm and antivirulence to act directly on toxins (Howden *et al.*, 2023).

1.3 *Staphylococcus aureus* Clonal Expansion

The molecular basis for methicillin resistance is the *mecA* gene (Matsuhashi *et al.*, 1986), which produces alternative penicillin-binding protein PBP2' (Ubukata *et al.*, 1985). When the vicinity of the *mecA* gene was sequenced for the first time in *S. aureus* N315, it was determined that the gene is part of a larger 51.7 kb genomic island inserted into the *orfX* locus. The element bordered by complex repeats also harbored extra insertion sequence (IS) and the integrated plasmid. However, the region did not match transposon nor prophages, which were known then and was initially designated simply *mec* DNA (Ito *et al.*, 1999). Later it was recognized as a unique mobile genetic element (MGE), which is mobilized with its *ccr* recombinases and called staphylococcal chromosome cassette (SCC). The exact mechanism of SCC*mec* transmission between strains is still unknown and subject to undergoing research (Uehara, 2022) and the most recent addition is the discovery of new alleles of *ccr* recombinases in distinct genomic islands related to SCC (Xiao *et al.*, 2023; Kovařovic *et al.*, 2023).

Three homologues of *mecA* genes are known; a *mecB* found at plasmids (Tsubakishita *et al.*, 2010), *mecC*, that is common in livestock isolates (García-Álvarez *et al.*, 2011), and *mecD* present on resistance island McRI_{mecD} in genomes of *Macrococcus* but not in staphylococci (Schwendener *et al.*, 2017; Mašlaňová *et al.*, 2018). It is important to note that environmental MRSA exists independently of clinical antibiotic usage (Pantůček *et al.*, 2018), and the origin of SCC*mec* itself is likely from a different genus, as native *mecA* genes were found in *Mammaliicoccus* genomes (Schwendener and Perreten, 2022). The research of *S. aureus* strains isolated from hedgehogs hinted at the transmission route of the *mecC* gene. Sequence analysis has shown a surprisingly high percentage of MRSA strains with the *mecC* allele. The possible selection advantage is that hedgehogs are frequently colonized by the fungal dermatophyte *Trichophyton erinaceid*, which produces penicillin (Larsen *et al.*, 2022).

The success of *S. aureus* as a pathogen is mainly due to its ability to acquire SCC*mec* and other mobile genetic elements (MGE) by horizontal gene transfer (HGT), which together account for up to 10–20 % of the genome and are the source of virulence and resistance factors (Lindsay and Holden, 2004; Miyamoto *et al.*, 2020). This means that the total gene pool is much larger than the number of genes in a single cell, a characteristic of an open pangenome (Argemi *et al.*, 2019). As a result, the evolution of *S. aureus* strains is shaped by the propagation of clonal lineages. Each clonal lineage is distinguished by its accessory genome, adapted to different environments and hosts (Lindsay, 2019). Comparative genomics revealed that human clinical isolates are enriched for MGE related genes such as ISs, transposases, and recombinases. More prevalent are also aminoglycoside, beta-lactam, macrolide resistances, and enterotoxins. Environmental isolates are more likely to contain prophages, adhesins, leucocidin, and other toxins and resist streptomycin phenicol, phosphomycin, and the *ermC* gene (Rocha *et al.*, 2023).

Staphylococcus aureus outbreaks are typically caused by a single clonal lineage that can evolve over time. One such successful clone is the well-known USA300 MRSA strain, which originated in the United States of America in the late 1990s. Although it was initially a community-associated strain, it quickly spread and became a dominant clone in hospitals in North America and later globally. *Staphylococcus aureus* USA300 lineages later acquired both chromosomal and plasmid-based resistances to multiple antibiotics. Cases of resistance to vancomycin and daptomycin, the main antibiotics against MRSA, are especially concerning (Enström *et al.*, 2018).

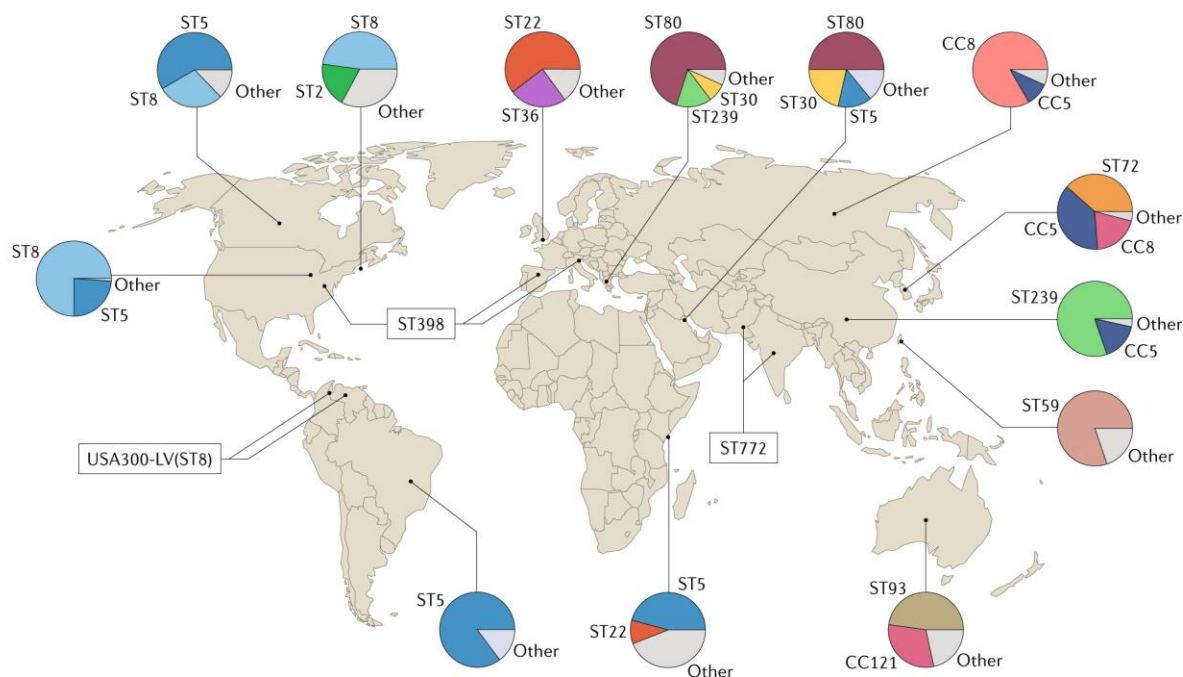


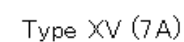
Fig. 2: Global distribution of MRSA clones

The global prevalence of MRSA sequence types and clonal complexes reported in selected studies in Africa, Asia, Australia, Europe, the Middle East, North America, and South America (Turner *et al.*, 2019)

In order to fully grasp the epidemiology of *S. aureus*, it is crucial to trace lineages and analyze associated characteristics. Initially, phage typing was used to track outbreaks. However, more accurate methods with better resolution have replaced it, such as pulse field electrophoresis (PFGE), where genomic DNA is cleaved with the *SmaI* restriction enzyme (Bannerman *et al.*, 1995). Other typing methods include plasmid profile analysis, Southern blotting, and rep-PCR (Trindade *et al.*, 2003). Whole-genome sequencing combined with single nucleotide polymorphisms (SNP) is the most accurate method, enabling the detection of mutations in individual patients as well as tracking outbreaks in real time. However, it is too expensive for routine use (Lindsay, 2014). A solution is to sequence only a subset of representative genes. The current

standard classification is based on multilocus sequence typing (MLST), relying on seven locuses of housekeeping genes with numbered alleles; *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *pti*, and *yqiL*. A unique combination of all seven alleles defines a specific sequence type (ST). If only five or six alleles match, the strains are considered part of the same clonal complex (CC) (Fountain *et al.*, 2019). Often, only one locus is sufficient, a repetitive region of the *spa* gene. The *spa* type is then determined by repeat type and its count (Koreen *et al.*, 2004). More than 920,000 MLST alleles and 8,500 ST are currently deposited in the PubMLST database, although only ten clonal complexes are common (Jolley *et al.*, 2018). Some clonal complexes are clearly more capable than others and were already prevalent as methicillin-susceptible *S. aureus* (MSSA) before SCCmec was acquired. It is possible that some CCs cannot stably incorporate the SCCmec cassette. The first MRSA isolates belonged to CC8 with SCCmec type I (Harkins *et al.*, 2017). The MLST correlates well with the whole genome typing methods (Petit and Read, 2018), and many sequence types are host-specific (Fitzgerald, 2012). Human MRSA is typically from clonal complexes CC1, CC5, CC8, CC30, CC22, and CC45 (Feil *et al.*, 2003; Gomes *et al.*, 2006; Park and Ronholm, 2021); livestock-associated MRSA are then predominantly CC9 or ST398 (Lindsay, 2019). The global prevalence of MRSA types reported by hospitals is shown in Fig. 2. *Staphylococcus aureus* clonal lineages are in constant competition with each other. If a particular clone proves successful, it can quickly replace others. For instance, from 1970 to 2000, the ST239 with type III SCCmec was the most common type in Singapore hospitals. Later, epidemic clone MRSA-15 emerged in the United Kingdom after it acquired resistance to fluoroquinolones and exhibited fast growth. With these properties, the clone spread quickly and was introduced to Singapore as ST22, which became dominant. However, the ST239 clonal lineage did not disappear completely and became more diverse (Hsu *et al.*, 2015).

MRSA nomenclature additionally reflects the SCCmec class. So far, fifteen SCCmec types and twenty-three subtypes were described, as shown in Fig. 3. The different types are determined by the alleles of *ccr* recombinases and the *mec* gene complex. There are two terminologies currently used to describe each type. The first uses Roman numerals I to XV for each type. The other way refers to *ccr* genes by the numbers 1 to 9 and *mec* complexes by the letters A to E. The SCCmec subtypes are determined by additional gene content, primarily in the J1 region and are typically designated by a lowercase letter or number based on the order of their discovery. It is common for many cassettes to be untypeable. This can occur when either *mec* or *ccr* genes are missing, or the SCCmec comprises several elements (Uehara, 2022).



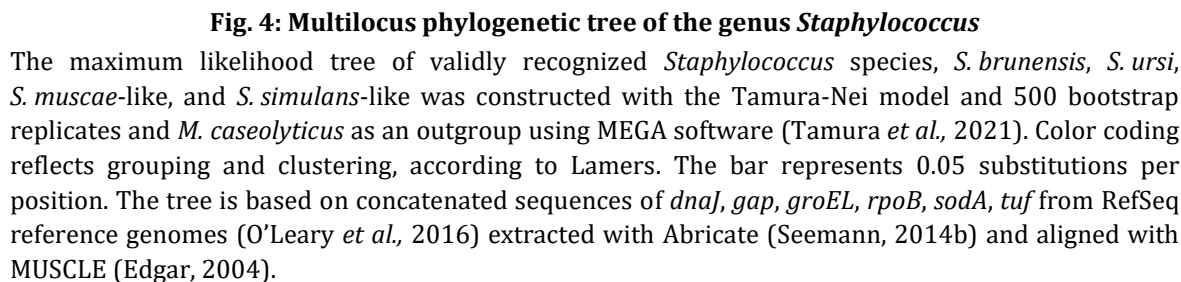
The structure of currently known SCC*mec* types I–XV. The alignment of *mec* and *ccr* complexes is highlighted (IWG SCC, 2009; Baig *et al.*, 2018; Urushibara *et al.*, 2020; Wang *et al.*, 2022).

1.4 Current Taxonomy of the Genus *Staphylococcus*

Much of the research effort in past decades was focused on *S. aureus*, which is indeed the most significant pathogen in the clinical setting, and other species were often considered unimportant contaminants. To study the variation of staphylococcal species, chemotaxonomic methods were used; the composition of cell wall teichoic acids (Baddiley *et al.*, 1962), the presence of menaquinone types (Jeffries *et al.*, 1968), or fatty acid branches (Morrison *et al.*, 1971). Schleifer and Kloos in 1975 analyzed cell wall composition and phenotype of CoNS obtained from the skin of healthy people and discovered several new species; *Staphylococcus capitis*, *Staphylococcus cohnii*, *Staphylococcus haemolyticus*, *Staphylococcus hominis*, *Staphylococcus simulans*, *Staphylococcus warneri* and *Staphylococcus xylosus* (Kloos and Schleifer, 1975; Schleifer and Kloos, 1975). In the following years, other staphylococcus species were described, and evaluating relationships based on DNA became the norm such as cytosine-guanine (CG) content (Rosypal *et al.*, 1966), the DNA–DNA hybridization (DDH) (Wayne *et al.*, 1987) and partial 16S rRNA sequence (Woese and Fox, 1977) allowed for delineation of closely related *Macrococcus* (Kloos *et al.*, 1998).

As molecular biology advanced and improved diagnostic methods, the clinical significance of CoNS was gradually recognized. The number of staphylococcal species continued to grow (Becker *et al.*, 2014). The widespread use of next-generation sequencing expanded the current list of validly recognized species in standing nomenclature to sixty-six. Since 2019, at least one new species has been described each year (Parte, 2014). The availability of complete genomes allowed for a more accurate assessment of relationships with whole genome metrics such as average nucleotide identity (ANI). Based on multilocus phylogeny, the *Staphylococcus* genus was divided into five main branches and fourteen clusters, as depicted in Fig. 4 and Tab. 1 (Lamers *et al.*, 2012).

Among the staphylococcal species, only eleven are coagulase-positive, and they are part of either the *S. aureus* or *Staphylococcus intermedius* cluster, while *Staphylococcus hyicus* and *Staphylococcus agnetis* are coagulase variable. Most other species are coagulase-negative, with *S. lugdunensis*, *S. epidermidis*, and *S. haemolyticus* from the Epidermidis-Aureus group being the most clinically significant (Becker *et al.*, 2014; Pinheiro-Hubinger *et al.*, 2021). *S. lugdunensis* stands out among CoNS for its high virulence, similar to *S. aureus*, and an unusually closed pangenome with fewer MGE (Argemi *et al.*, 2018). *Staphylococcus haemolyticus* and *S. epidermidis* are common commensals but can sometimes cause mild infections as opportunistic pathogens (Becker *et al.*, 2014). Their genome also has fewer virulence factors, but they have open pangenomes that contain a rich pool of resistance determinants (Ikhimiukor *et al.*, 2023). Both species exhibit clonal characteristics like *S. aureus*, although the *S. haemolyticus* is to a lesser extent (Pinheiro-Hubinger *et al.*, 2021).



Several new species related to *S. haemolyticus* have been discovered recently, such as *Staphylococcus caledonicus* isolated from dog skin, *Staphylococcus taiwanensis*, and *Staphylococcus borealis* found in human blood and skin. While *S. taiwanensis* and *S. caledonicus* exhibit phenotypic differences (Newstead *et al.*, 2021; Lin *et al.*, 2022), *S. borealis* is almost indistinguishable from *S. haemolyticus* regarding phenotype, 16S rRNA sequence, and MALDI-TOF-MS profile. A polyphasic approach is necessary to differentiate between the two, involving the analysis of genotype, biochemistry, and fatty acid composition (Pain *et al.*, 2020).

The insufficient discriminatory power of 16S rRNA sequencing also applies to other members of the genus *Staphylococcus* (Hassler *et al.*, 2022), particularly several closely related coagulase-positive species from the *S. intermedius* group (SIG); *Staphylococcus cornubiensis*, *Staphylococcus pseudintermedius*, and *Staphylococcus delphini* are challenging to differentiate (González-Martín *et al.*, 2020). Although these species are animal pathogens, just like other members of the Hyicus-Agnetis group, they can be transmitted to humans. Studies suggest that the prevalence of *S. pseudintermedius*, which is frequently found in domestic dogs, in human infections might be higher than previously reported because it is often misidentified as *S. aureus*, as laboratories often test only coagulase positivity (González-Martín *et al.*, 2020; Moses *et al.*, 2023; Glajzner *et al.*, 2023).

Identifying *S. saprophyticus* is also tricky because most labs use the novobiocin test without standard methods (Raz *et al.*, 2005). *Staphylococcus saprophyticus* is the leading cause of urinary tract infections in young women. Its nasal isolates do not cluster, but the urinary tract isolates are highly clonal (Pinheiro-Hubinger *et al.*, 2021). The other members of the Saprophyticus group are isolated from mixed environments, such as recently *Staphylococcus shinii* from cavies (Cho *et al.*, 2022), *Staphylococcus caeli* from air in rabbit enclosures (MacFadyen, Drigo, *et al.*, 2019) or *Staphylococcus edaphicus* from Antarctic soil (Pantůček *et al.*, 2018). The next Auricularis group contains only a few species, mostly rare finds and includes the latest addition to the genus *Staphylococcus*: *Staphylococcus marylandisciuri* from the Pettenkoferi-Massiliensis cluster. Together with *S. marylandisciuri*, the species *Staphylococcus americanisciuri* was isolated and described from squirrels, but it belongs to the Hyicus-Intermedius group (Vолоkhov *et al.*, 2023). The last major branch is the Simulans group. Although the *S. simulans* itself is known to be a human pathogen, the other species are associated with fermented food (Becker *et al.*, 2020).

The phylogenetic relationships are complex, and opinions change frequently. For instance, some authors disagree with the Massiliensis-Pettenkoferi cluster, according to Lamers, a part of the Saprophyticus group. The alternative phylogeny states that *Staphylococcus massiliensis* should form its own branch instead, and *Staphylococcus pettenkoferi* and *Staphylococcus argensis* are grouped with *Staphylococcus auricularis* (Smith and Andam, 2021), as shown in Fig. 4. Recently, several major changes have been accepted, notably the reclassification of the

Staphylococcus sciuri group as a separate genus *Mammaliicoccus* and updated description species, which were previously considered a subspecies; *Staphylococcus ureilyticus*, *Staphylococcus coagulans*, *Staphylococcus casei*, *Staphylococcus pragensis*, *Staphylococcus croceilyticus* (Madhaiyan *et al.*, 2020). However, the validity of *S. coagulans* has been questioned (Kania, 2022). Other suggested amendments include a proposal of not yet valid species, *Staphylococcus ursi*, from SIG (Perreten *et al.*, 2020), description of several possible new species *Staphylococcus muscae*-like and *Staphylococcus simulans*-like found in camels (Akarsu *et al.*, 2022) or a merging species from *S. aureus* group, predominantly isolated from wounds in tropical countries; *Staphylococcus schweitzeri*, *Staphylococcus singaporensis* and *Staphylococcus roterodami* as clonal variants of single species (Monecke *et al.*, 2022).

Tab. 1: List of validly recognized species of the genus *Staphylococcus*

List of validly recognized species of genus *Staphylococcus* and *S. brunensis*, *S. ursi*, *S. muscae*-like, *S. intermedius*-like grouped according to Lamers classification. The most important human pathogens are highlighted. Coagulase reaction, type strain isolation sources, clinical significance with infection type bloodstream (BSI), foreign body related (FBRI), native valve endocarditis (NVIE), urinary tract infection (UTI), surgical site infection (SSI), number of genomes available in the GenBank database by end of 2023 (Benson *et al.*, 2013), and effective publication or last emendation is shown (Becker *et al.*, 2020; Michels *et al.*, 2021; Bartlett *et al.*, 2022).

Group	Cluster	Species (Coagulase)	Isolation sources	Clinical significance	GenBank	Publication
Auricularis	Auricularis	<i>S. auricularis</i> (-)	Human ear canal; human and animal skin (rare)	Human, BSI (rare)	17	(Kloos and Schleifer, 1983)
		<i>S. argenteus</i> (+)	Human blood	Human	360	(Tong <i>et al.</i> , 2015)
	Aureus	<i>S. aureus</i> (+)	Human and animal skin and blood	Human	89665	(Rosenbach, 1884)
		<i>S. roterodami</i> (+)	Human skin and blood	Human, BSI	4	(Schutte <i>et al.</i> , 2021)
		<i>S. schweitzeri</i> (+)	Animal (monkey) nares	Human and animal	9	(Tong <i>et al.</i> , 2015)
		<i>S. simiae</i> (+)	Animal (monkey) faeces	Not documented	9	(Pantůček <i>et al.</i> , 2005)
		<i>S. singaporensis</i> (+)	Human blood	Human, BSI	6	(Chew <i>et al.</i> , 2021)
Epidermidis-Aureus	Epidermidis	<i>S. capitis</i> (-)	Human scalp and animal skin	Human, FBRI, BSI in neonates	232	(Kloos and Schleifer, 1975)
		<i>S. caprae</i> (-)	Animal skin	Human and animal, FBRI (occasional)	23	(Devriese <i>et al.</i> , 1983)
		<i>S. epidermidis</i> (-)	Human and animal skin; food	Human; FBRI, NVIE, BSI in neonates	4645	(Winslow and Winslow, 1908)
	Haemolyticus	<i>S. saccharolyticus</i> (-)	Human and animal skin and plasma	Human; FBRI, BSI	20	(Kilpper-Bälz and Schleifer, 1981)
		<i>S. borealis</i> (-)	Human skin and blood	Human	14	(Pain <i>et al.</i> , 2020)
		<i>S. brunensis</i> (-)	Human ear canal	Putative human	5	(Kovařovic <i>et al.</i> , 2023)

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Group	Cluster	Species (Coagulase)	Isolation sources	Clinical significance	GenBank	Publication
Epidermidis-Aureus	Haemolyticus	<i>S. caledonicus</i> (-)	Animal skin	Not documented	1	(Newstead <i>et al.</i> , 2021)
		<i>S. croceilyticus</i> (-)	Human ear canal	Human putative	2	(Madhaiyan <i>et al.</i> , 2020)
		<i>S. devriesei</i> (-)	Animal skin, food; milk	Animal	13	(Supré <i>et al.</i> , 2010)
		<i>S. haemolyticus</i> (-)	Human and animal skin; food; milk	Human; FBRI, NVIE, BSI in neonates	625	(Schleifer and Kloos, 1975)
		<i>S. hominis</i> (-)	Human and animal skin	Human; FBRI, BSI in neonates	287	(Kloos and Schleifer, 1975)
		<i>S. petraei</i> (-)	Human skin	Human; BSI	8	(Pantůček <i>et al.</i> , 2013)
		<i>S. pragensis</i> (-)	Human skin	Human, BSI, and wounds	2	(Madhaiyan <i>et al.</i> , 2020)
		<i>S. taiwanensis</i> (-)	Human blood	Human, BSI	1	(Lin <i>et al.</i> , 2022)
	Lugdunensis	<i>S. lugdunensis</i> (-)	Human and animal skin	Human; NVIE/PVIE, FBRI, SSI, wound infection	127	(Freney <i>et al.</i>, 1988)
	Warneri	<i>S. pasteurii</i> (-)	Human and animal skin; food	Human; FBRI (rare), BSI	43	(Chesneau <i>et al.</i> , 1993)
		<i>S. xylosus</i> (-)	Human (rare) and animal skin; food	Human, FBRI (occasional)	88	(Schleifer and Kloos, 1975)
Hyicus-Hyicus-Intermedius	Hyicus	<i>S. agnetis</i> (var)	Animal skin; milk	Animal	62	(Taponen <i>et al.</i> , 2012)
		<i>S. canis</i> (-)	Animal skin	Not documented	1	(Newstead <i>et al.</i> , 2021)
		<i>S. felis</i> (-)	Animal skin	Animal	42	(Igimi <i>et al.</i> , 1989)
		<i>S. hyicus</i> (var)	Animal skin	Human and animal	27	(Devriese <i>et al.</i> , 1978)
		<i>S. chromogenes</i> (-)	Animal skin; milk	Human and animal	165	(Hájek <i>et al.</i> , 1986)
		<i>S. ratti</i> (-)	Animal nares	Not documented	1	(Kovařovic <i>et al.</i> , 2022)
	Intermedius	<i>S. coagulans</i> (-)	Animal ear canal	Human and animal	187	(Madhaiyan <i>et al.</i> , 2020)
		<i>S. cornubiensis</i> (+)	Human skin	Human; skin	1	(Murray <i>et al.</i> , 2018)
		<i>S. delphini</i> (+)	Animal skin	Animal putative	76	(Varaldo <i>et al.</i> , 1988)
		<i>S. intermedius</i> (+)	Animal nares	Human	8	(Hájek, 1976)
		<i>S. lutrae</i> (+)	Animal skin	Animal	2	(Foster <i>et al.</i> , 1997)
		<i>S. pseudintermedius</i> (+)	Human and animal skin, animal lung	Human and animal	4871	(Devriese <i>et al.</i> , 2005)
		<i>S. schleiferi</i> (-)	Human and animal skin and blood	Human; FBRI, wound (rare)	68	(Freney <i>et al.</i> , 1988)
		<i>S. ursi</i> (-)	Animal nares	Not documented	1	(Perreten <i>et al.</i> , 2020)
		<i>S. americanisciuri</i> (-)	Animal faeces	Not documented	1	(Volokhov <i>et al.</i> , 2023)
		<i>S. microti</i> (-)	Animal skin and nares; milk	Animal	3	(Nováková <i>et al.</i> , 2010)
	Muscae	<i>S. muscae</i> (-)	Animal skin	Not documented	4	(Hájek <i>et al.</i> , 1992)
		<i>S. muscae</i> -like	Animal skin	Not documented	7	(Akarsu <i>et al.</i> , 2022)
		<i>S. rostri</i> (-)	Animal skin and nares	Not documented	3	(Riesen and Perreten, 2010)
Saprophyticus	Arlettae-Kloosii	<i>S. arlettae</i> (-)	Animal skin; environmental	Not documented	49	(Schleifer <i>et al.</i> , 1984)
Saprophyticus	Arlettae-Kloosii	<i>S. durrellii</i> (-)	Animal nares	Not documented	1	(Fountain <i>et al.</i> , 2019)

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Group	Cluster	Species (Coagulase)	Isolation sources	Clinical significance	GenBank	Publication
		<i>S. kloosii</i> (-)	Animal skin	Human and animal; FBRI (rare)	12	(Schleifer <i>et al.</i> , 1984)
		<i>S. lloydii</i> (-)	Animal skin and mouth	Not documented	4	(Fountain <i>et al.</i> , 2019)
	Cohnii-Nepalensis	<i>S. cohnii</i> (-)	Human and animal skin	Human; FBRI, BSI	96	(Schleifer and Kloos, 1975)
		<i>S. nepalensis</i> (-)	Animal skin and faeces; human urine	Human	29	(Spergser, 2003)
		<i>S. ureilyticus</i> (-)	Human and animal skin	Human	16	(Madhaiyan <i>et al.</i> , 2020)
	Pettenkoferi-Massiliensis	<i>S. argensis</i> (-)	Environmental	Not documented	9	(Heß and Gallert, 2015)
		<i>S. marylandisciuri</i> (-)	Animal faeces	Not documented	1	(Volokhov <i>et al.</i> , 2023)
		<i>S. massiliensis</i> (-)	Human skin	Human putative	6	(Al Masalma <i>et al.</i> , 2010)
		<i>S. pettenkoferi</i> (-)	Human and animal skin and blood	Human; FBRI	56	(Trülsch <i>et al.</i> , 2007)
	Saprophyticus	<i>S. caeli</i> (-)	Environmental	Not documented	2	(MacFadyen, Drigo, <i>et al.</i> , 2019)
		<i>S. casei</i> (-)	Fermented food; animal skin	Not documented	2	(Madhaiyan <i>et al.</i> , 2020)
		<i>S. edaphicus</i> (-)	Environmental	Not documented	2	(Pantůček <i>et al.</i> , 2018)
		<i>S. equorum</i> (-)	Animal skin; food	Human	111	(Schleifer <i>et al.</i> , 1984)
		<i>S. gallinarum</i> (-)	Animal skin	Human; FBRI (rare)	44	(Devriese <i>et al.</i> , 1983)
		<i>S. pseudoxylosus</i> (-)	Animal skin	Human and animal	26	(MacFadyen, Leroy, <i>et al.</i> , 2019)
		<i>S. saprophyticus</i> (-)	Human and animal skin	Human; UTI, FBRI, NVIE	475	(Fairbrother, 1940)
		<i>S. shini</i> (-)	Plant	Not documented	15	(Cho <i>et al.</i> , 2022)
		<i>S. succinus</i> (-)	Environmental; animal skin; food	Human, BSI (rare)	26	(Lambert <i>et al.</i> , 1998)
		<i>S. xylosus</i> (-)	Human (rare) and animal skin; food	Human, FBRI (occasional)	88	(Schleifer and Kloos, 1975)
Simulans	Simulans-Carnosus	<i>S. carnosus</i> (-)	Food; animal skin	Not documented	23	(Schleifer and Fischer, 1982)
		<i>S. condimenti</i> (-)	Food	Human; FBRI (rare)	11	(Probst <i>et al.</i> , 1998)
		<i>S. debuckii</i> (-)	Animal skin; milk	Animal	1	(Naushad <i>et al.</i> , 2019)
		<i>S. piscifermentans</i> (-)	Food	Not documented	3	(Tanasupawat <i>et al.</i> , 1992)
		<i>S. simulans</i> (-)	Human and animal skin	Human; FBRI (rare)	104	(Kloos and Schleifer, 1975)
		<i>S. simulans-like</i> (?)	Animal skin	Not documented	2	(Akarsu <i>et al.</i> , 2022)

1.5 Epidemiology of Coagulase-Negative Staphylococci

Nasal swab screenings have shown that almost everyone carries at least one type of CoNS. The dominant species is *S. epidermidis*, which colonizes 39–90 % of people. Other common commensals include *S. haemolyticus*, *S. capitis*, *S. hominis*, *S. warneri*, *S. lugdunensis*, *S. saprophyticus*, *S. simulans*, and *Staphylococcus pasteurii*, present in 25–44 % of individuals. Other species were isolated in single cases, although they may be more prevalent in other areas of the body (Becker *et al.*, 2006; Kaspar *et al.*, 2016; Pinheiro-Hubinger *et al.*, 2021). The same species are also clinically significant (Lindsay, 2019); however, the widespread distribution of CoNS as commensals also means they are common contaminants. As most species are also opportunist pathogens, it is challenging to determine their clinical relevance. The colony growth speed measured as time to positivity under 16 hours is a good predictor for bacteremia when combined with speciation with MALDI-TOF-MS. Clinical relevance can be further assessed using other factors, such as the presence of foreign bodies or reaction to therapy, as shown in Fig. 5 (Asai *et al.*, 2021; Michels *et al.*, 2021). Using those criteria, it has been found that *S. epidermidis*, *S. haemolyticus*, and *S. lugdunensis* were true pathogens in 60–80 %, and other CoNS isolates were clinically relevant in 20–80 % of cases. *Staphylococcus capitis*, *Staphylococcus caprae*, and *S. hominis* were more often contaminants (Asai *et al.*, 2021). Proper diagnosis and speciation of CoNS infection is crucial as it is the third most frequent cause of nosocomial infection, following *Escherichia coli* and *S. aureus* (Verway *et al.*, 2022), and is responsible for a third of nosocomial bacteremia cases (Wisplinghoff *et al.*, 2004; Ott *et al.*, 2013; Rosenthal *et al.*, 2021).

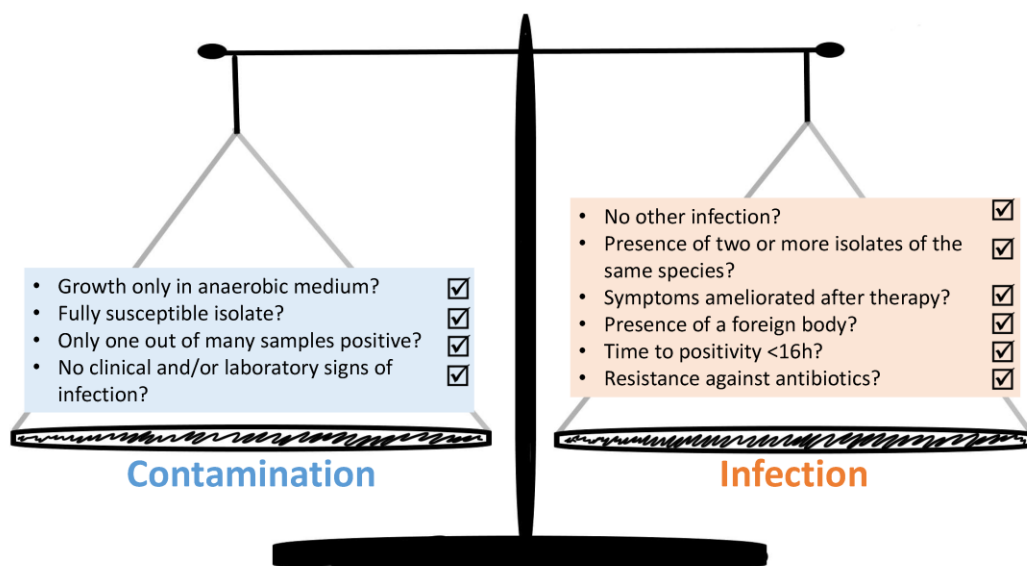


Fig. 5: Diagnostic of CoNS pathogens as the causative agents of diseases

Diagnostic criteria for differentiating true CoNS infection and contaminants (Michels *et al.*, 2021).

Bloodstream CoNS infections affect 1.8 % of intensive care unit patients in the USA (Hurley, 2020), with 19.7 % 30-day mortality (Verway *et al.*, 2022). However, CoNS infections are likely underestimated, which may lead to delays in antibiotics application and, as a consequence, higher mortality (Taylor *et al.*, 2021). To determine the healthcare burden, it is necessary to study the evolution of CoNS and their role as virulence and resistance reservoirs (Becker *et al.*, 2020). A retrospective study has shown that the reported CoNS normally sterile sites such as blood, bone, joints, or cerebrospinal fluid increased from 24 thousand in 2010 to 81 thousand in 2022 due to improvements in diagnostics and reporting (Paranthaman *et al.*, 2023). The study also confirmed the increased risk in preterm newborns and infants up to one year of age (Eichel *et al.*, 2019; Paranthaman *et al.*, 2023). The newborns are particularly vulnerable because of the immature immune system with few phagocytes. It is hypothesized that disease tolerance in the first few days of life allows the acquisition of commensals into the skin microbiome (França, 2023).

The second main high-risk group is seniors over sixty-five with various comorbidities, especially when an indwelling medical device is present. Males are also more often susceptible. All CoNS followed the bimodal distribution except for *Staphylococcus felis*, which was not detected in young children (Paranthaman *et al.*, 2023). Another large study of bloodstream CoNS infections in the Netherlands has reached similar conclusions and further emphasized the importance of identifying the specific species involved. The proportion of present species seemed stable over time, except for newly described species such as *S. pettenkoferi* (Berends *et al.*, 2022). However, certain CoNS species are more commonly associated with specific types of infections. For instance, *S. epidermidis* is the most common species in CoNS endocarditis (Parthasarathy *et al.*, 2020). *Staphylococcus haemolyticus* is frequently found in surgical wound isolates (Pinheiro-Hubinger *et al.*, 2021), while *S. saprophyticus* is often responsible for urinary tract infections (Hovelius and Mårdh, 1984) and *S. lugdunensis*, typically causes soft tissue infections with abscesses, similar to *S. aureus* (Parthasarathy *et al.*, 2020).

Although the number of speciated CoNS has increased from 11 % to 63.2 % since 2010 (Paranthaman *et al.*, 2023), the CoNS are still often reported as a single group (Michels *et al.*, 2021; Berends *et al.*, 2022). This is a problem because the prevalence of antibiotic resistance is high between CoNS, resistance to at least one antibiotic is reported in 55–85 % of CoNS bloodstream isolates (Paranthaman *et al.*, 2023). However, the resistance is unevenly distributed. For instance, 50 – 80 % of *S. epidermidis* and *S. haemolyticus* isolates from the bloodstream exhibited resistance to teicoplanin and macrolides, which is rare in other CoNS. *Staphylococcus haemolyticus*, *S. epidermidis*, and *S. cohnii* also have a higher prevalence of resistance to erythromycin, gentamicin, and methicillin (Berends *et al.*, 2022). The virulent *S. lugdunensis* is, on the other hand, more sensitive to beta-lactam antibiotics (Petti *et al.*, 2008). The speciation with MALDI-TOF-MS is thus a valuable tool for

selecting suitable therapy. However, it should be noted that antibiotic resistance can be heterogeneous during CoNS infection (Berends *et al.*, 2022), and treatment can be complicated by a selection of isogenic resistant population, often in the form of small colony variants (El-Halfawy and Valvano, 2015). In addition to surveillance for CoNS in clinical infections, it is helpful to monitor environmental isolates, especially from livestock and companion animals, under the One Health approach because of the possibility of zoonotic transmission and HGT. Although some species are not pathogenic, they can indirectly affect human health by transferring MGE and antibiotic resistance, such as *SCCmec*. This includes other closely related members of the *Staphylococcaceae* family; *Mammaliicoccus* and *Macrococcus* (Becker, 2021) or even distant taxa such as *Lactobacillales* or *Bacillales* (John *et al.*, 2019). Plasmid and transposon-mediated beta-lactamases *blaZ* are widespread in CoNS isolated from food (Salamandane *et al.*, 2022) and mammaliicocci from cattle (Lienen *et al.*, 2022). However, the native *bla* genes are found in some staphylococci from the Saprophyticus group, although they usually do not confer resistance to penicillin (Schwendener and Perreten, 2022). Comparative analysis of 191 animal CoNS strains from the USA has revealed large reservoirs of phages, plasmids, and antimicrobial resistance genes, allowing the evolution of novel variants with higher virulence, phages, plasmids, and antimicrobial resistance genes, allowing the evolution of novel variants with higher virulence, as with *S. aureus*, the most significant risk is the possible selection of multidrug-resistant strains (Fitzgerald and Holden, 2016; Glajzner *et al.*, 2023).

Unlike the antimicrobial resistance, the CoNS virulence is poorly understood. Compared to *S. aureus*, CoNS have fewer virulence factors (Heilmann *et al.*, 2019). The specific virulence determinants are often microbial surface components recognizing adhesive matrix molecules (MSCRAMM) (Becker *et al.*, 2014). Proteins UafA, Aas, and SdrI from this family are responsible for *S. saprophyticus* affinity to urothelium (Sakinc *et al.*, 2006; Mortimer *et al.*, 2017). The adhesins AtlE, Aap, and Bap also help attach cells to the surface (Heilmann *et al.*, 1997; Hirschhausen *et al.*, 2012). This is a necessary step for biofilm formation, described in Fig. 6. Although not an essential virulence factor, CoNS bacteremia and endocarditis are frequently associated with biofilm formation on the catheter or implant (Heilmann *et al.*, 2019). Biofilm needs a suitable substrate that is rough, hydrophobic, and non-polar. Indwelling medical devices are usually first covered by a host matrix to which bacteria adhere (Herrmann *et al.*, 1988). The best characterized staphylococcal biofilm formation pathway is the *ica* operon, which produces biofilm based on polysaccharide intracellular adhesin (PIA/PNAG). The operon consists of five genes, *icaABCDR*, responsible for the regulation synthesis of PIA/PNAG and its export to the surface (Rohde *et al.*, 2010). Extracellular DNA is in staphylococcal biofilms. The fibrin and other host proteins are embedded in the biofilm matrix (Kwiecinski *et al.*, 2015) and extracellular DNA (Izano *et al.*, 2008). Additional virulence factors such as phenol soluble modulins (PSM) or proteases can disrupt the biofilm and release its particles into the bloodstream (Becker

et al., 2014). The biofilm composition always results from the interaction of host, environment, and other present bacterial species (Donlan, 2002). Because of this, it is challenging to study biofilm properties *in vitro* (Otto, 2018). The fully matured biofilm serves as a barrier against the immune system and antibiotics and promotes HGT between cells (Donlan, 2002). Despite the use of antibiotics that penetrate biofilm, such as rifampicin, the recurring inflammation often requires surgical removal of the implant (Zimmerli and Moser, 2012).

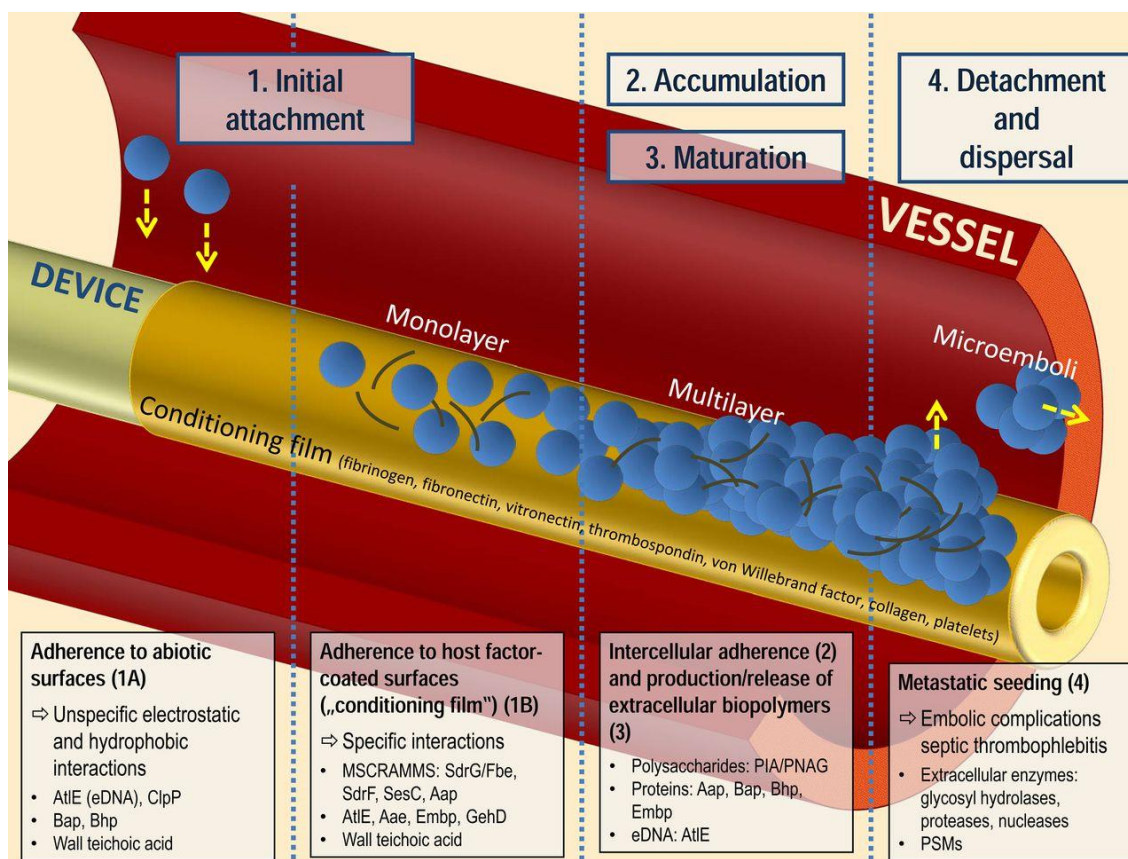


Fig. 6: Stages of biofilm formation

Formation of CoNS biofilm in an intravenous catheter or other foreign body device. In each step, key interactions and virulence genes are listed (Becker *et al.*, 2014)

2 Analysis of Bacterial Genomes

2.1 Genome Sequencing and Assembly

The first complete staphylococcal genomes were two Japanese MRSA strains, *S. aureus* N315 from pharyngeal smear and VRSA *S. aureus* Mu50, were sequenced in 2001 to study mechanism of antibiotic resistance. The ABI PRISM 3,700 genome sequencer generated 63,000–64,000 reads from the plasmid library with random 1–2 kb fragments (Kuroda *et al.*, 2001). The sequencing reads were assembled by Phred/Phrap, which were initially developed for use in the Human Genome Project and were the first programs to improve accuracy with quality scores derived from Sanger sequencing chromatographs and were able to classify chimeras and remove vector sequences (de la Bastide and McCombie, 2007). The gaps were filled by sequencing PCR products to obtain the complete genome. The whole genome analysis then revealed the presence of MGE, transposon Tn554, SCCmec, bacteriophages, and pathogenicity islands harboring toxins. In addition, new candidate virulence factors and the duplication of superantigens were detected (Kuroda *et al.*, 2001). However, first-generation sequencing was too expensive and labor-intensive for widespread use. By 2008, only eighteen staphylococcal genomes had been sequenced, and of these, only a few CoNS: two strains of *S. epidermidis* RP62A and ATCC 12228 (Y.-Q. Zhang *et al.*, 2003; Gill *et al.*, 2005), *S. haemolyticus* JCSC 1435 (Takeuchi *et al.* 2005), and *S. saprophyticus* ATCC 15305 (Kuroda *et al.*, 2005), providing valuable information about evolution and virulence factors (Takeuchi *et al.*, 2005; Wei *et al.*, 2006).

When the second-generation sequencing platforms Roche 454, Illumina, and later IonTorrent became available, the number of genomes multiplied. In 2009 alone, forty-three genomes were deposited into the GenBank database (Benson *et al.*, 2013). The new technology, however, produced short reads. The original assembler algorithms were based on overlap layout consensus, where each read is represented by a single node in the graph with links to other overlapping reads, the longest uninterrupted graph path that constitutes a contig. This requires alignment between each read, and due to the graph complexity, it does not scale well with a growing number of reads. The De Bruijn graph approach was found more suitable for a higher number of short sequencing due to lower computing power requirements (Li *et al.*, 2012). The De Bruijn algorithms differ in partitioning all reads into sequences of fixed length called *k*-mers and constructing graphs from them. This effectively compresses graph size and allows for searching for contigs with the Euler path algorithm, which is easier to perform. Because the reads contain errors, some approximation and simplification are necessary (Miller *et al.*, 2010). The most critical parameter is the *k*-mer length itself. The optimal value depends on genome complexity as well as error rate. Because the ability to resolve repeats is limited by node length, too short a *k*-mer assembly

tends to result in more contigs. On the other hand, long k -mers have a slight overlap, which breaks the graph with dead ends (Wick *et al.*, 2017b). De Bruijn graph assemblers utilize various strategies to solve this problem. The SPAdes assembler uses a consensus of multiple k -mer (Prjibelski *et al.*, 2020), and the Velvet instead uses an optimizer with variable k -mer length. The later assembler was used in the Modernising Medical Microbiology Consortium project, which aimed to obtain genomes of all staphylococcal type strains. In total, sixty four isolates from fifty then valid species were sequenced, some for the first time (Cole *et al.*, 2019). As of December 2023, over a hundred thousand staphylococcal genomes are available in the GenBank database (Sayers *et al.*, 2021), and this number is rapidly increasing, as indicated in Fig. 7. However, the database is biased towards clinically important species, with the majority being *S. aureus*. *Staphylococcus epidermidis* and *S. pseudintermedius* are also present in large numbers, while nine species only have one genome stored in the database (Benson *et al.*, 2013). The falling prices of sequencing have enabled large-scale projects such as 'One Day in Denmark,' where 2009 bacterial isolates from a single day in Danish hospitals were sequenced. Of these, 414 belonged to the genus *Staphylococcus* (Rebelo *et al.*, 2022).

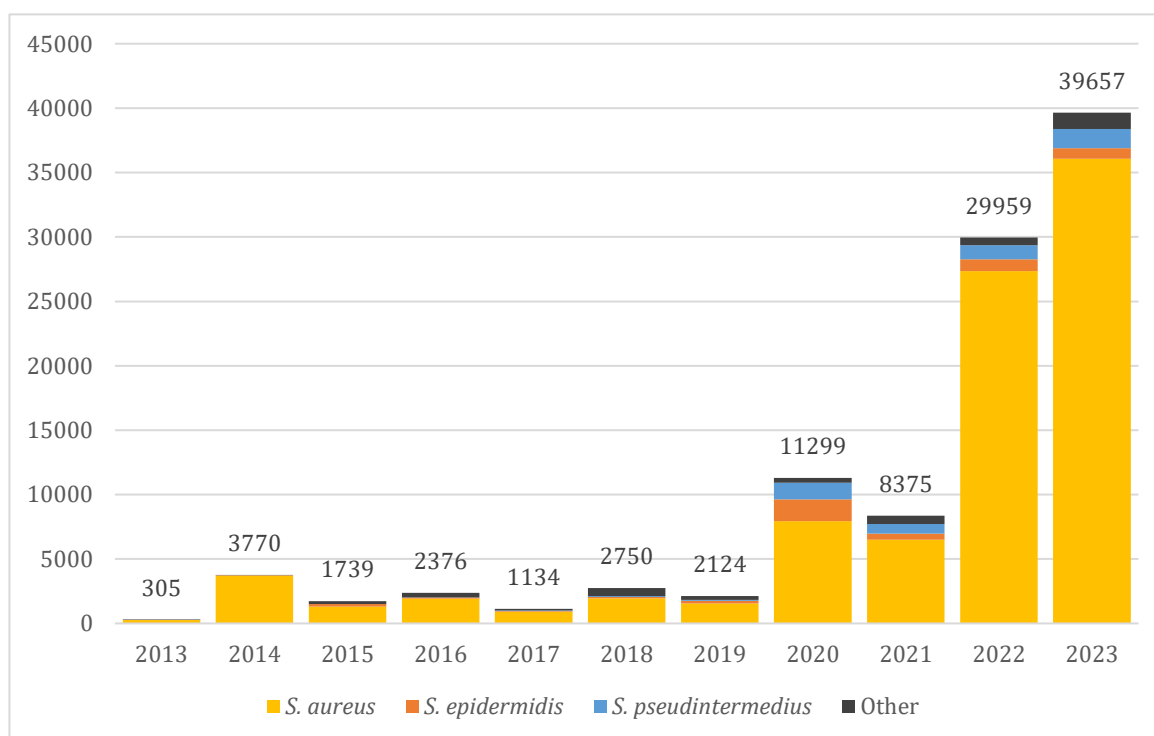


Fig. 7: Trend of increase of the Staphylococcus genome count in GenBank

The graph shows the number of genomes in the GenBank database (Sayers *et al.*, 2021) between 2013–2023. Only 289 genomes were deposited into the database before 2013, and the total count of all genomes is 103,777. Most often, three species are separated and color-coded as described by legend. The total genomes deposited in each year are shown in the above column.

Filling the gaps between contigs with Sanger sequencing of PCR products is time-consuming. Much effort has been put into developing alternative methods such as optical mapping (Latreille *et al.*, 2007) or scaffolding using reference genome (Bosi *et al.*, 2015). The problem of completing bacterial genomes was finally solved with third-generation sequencing. The hybrid assembly combining the error prone long reads produced by Oxford Nanopore with short reads from Illumina almost always results in a complete genome without manual adjustments (Wick *et al.*, 2017a). Due to developments in error correction, the long read only assembly is viable. This has led to the return of overlap layout consensus assemblers such as Flye (Kolmogorov *et al.*, 2019). This has changed the latest approach to hybrid assembly to doing long read assembly first, preferably as consensus from several assemblers, and using short reads only for polishing to improve quality (Wick *et al.*, 2021). It is still advisable to verify the presence of small plasmids in short read-only assembly because they tend to be underrepresented in long reads and may be missing from long read-only assembly (Wick *et al.*, 2017a).

The long-read sequencing method can also directly detect methylated bases in native DNA. In PacBio, the modified bases take longer to react (Clark *et al.*, 2012). The Nanopore technology uses slight variations in electric current to detect the presence of methylated states (Schatz, 2017). The reliability of detection depends on the base modification. PacBio is more accurate with the N4-methylcytosine (4mC) and N6-methyladenine (6mA), and was used in various methylome studies of *Staphylococcus schleiferi* (Misic *et al.*, 2015), *S. epidermidis* (Lee *et al.*, 2019), and collection of multiple staphylococci from camels (Akarsu *et al.*, 2022). However, Nanopore is better at detecting 5-methylcytosine (5mC) and 6mA and in the future, it is expected that the new version of R10 pores will be more accurate and significantly cheaper (Nielsen *et al.*, 2023). Lowering the price would enable deep sequencing to conduct genetic-wide association studies, whole metagenome studies, and a combination of these to understand the relationships between epigenome and interaction between host and MGE (Fitzgerald and Holden, 2016; Nielsen *et al.*, 2023).

2.2 Evolution of Staphylococcal Genomes

Staphylococci have low CG content, ranging from 30 % to 37 % (Rosypal *et al.*, 1966). *Staphylococcus muscae* has the smallest genome, with only 2.1 Mb (Cole *et al.*, 2019), while the largest is the 3.0 Mb genome of *S. shinii* (Cho *et al.*, 2022). The staphylococcal genome contains approximately 2,500–3,000 open reading frames (ORFs) and RNA genes, which are arranged in functionally related operons with a common transcription origin (Lindsay, 2019). The core genome of each staphylococcal species is defined by approximately 1,500–2,000 genes, which are highly conserved (Méric *et al.*, 2015; Chong *et al.*, 2022; K. Zhang *et al.*, 2023). Variation in the core is driven primarily by SNPs. An analysis of 122 genomes from *Staphylococcaceae* and *Gemella* families has revealed seventy-eight distinct SNPs common to *Staphylococcus*, *Macrococcus*, and *Mammaliicoccus*. However, only two unique SNPs were found for genus *Staphylococcus*. These two SNPs were deletions in Dyp-type peroxidase and signal peptidase I (Bello *et al.*, 2023).

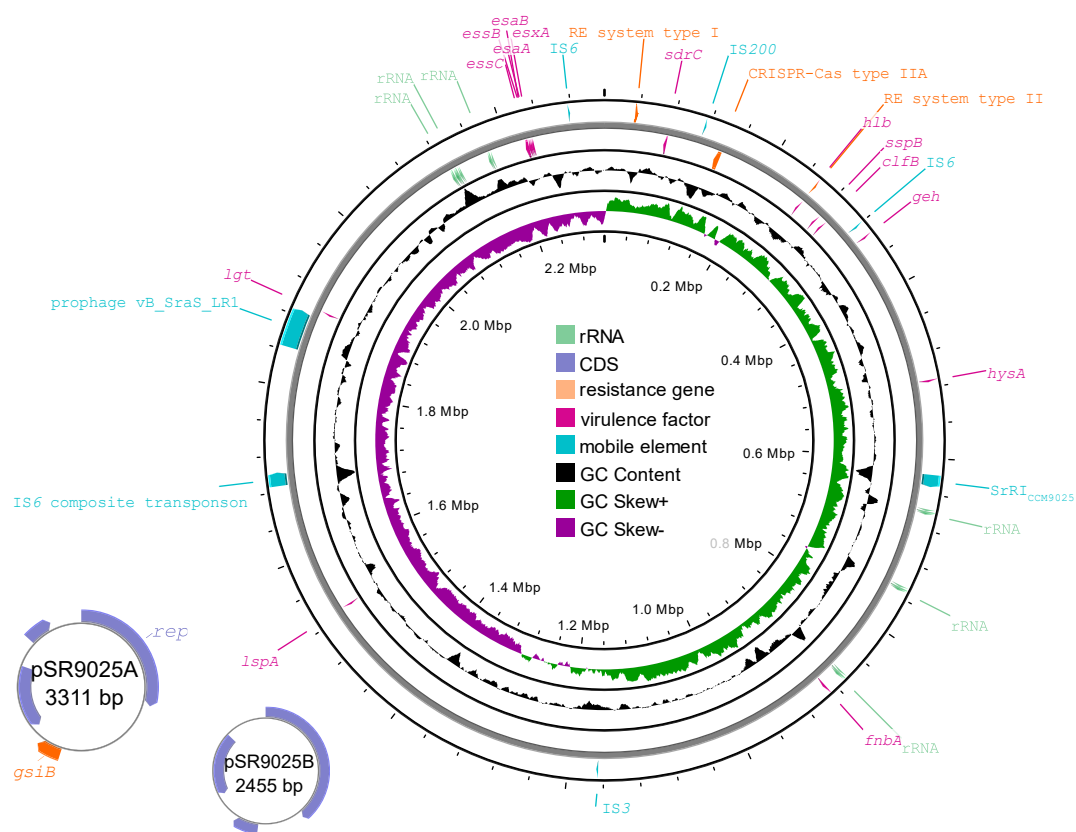


Fig. 8: Genome map of species *Staphylococcus ratti* described in this work

Annotated genome map of the *S. ratti* CCM 9025 chromosome and its plasmids pSR9025A and pSR9025B. The MGE, virulence, resistance, and other genes of interest are shown in the outer circle and color-coded, as shown in the legend. The switch in CG skew from positive to negative value marks the replication origin and terminus. Plasmid sizes are not to scale.

Whole genome sequencing of *S. aureus* outbreaks has shown that epidemic clones are closely related if their core genome shares fewer than 40 SNPs. It has also been determined that the mutation rate is approximately one SNP per genome every 5–10 weeks (Harris *et al.*, 2010; Golubchik *et al.*, 2013; Paterson *et al.*, 2015). The circular chromosome is divided into two replichores by replication origin and terminus, where one replichore is 12% longer, as is seen in Fig. 8. There is considerable bias in gene distribution to the leading strand of DNA (Mira and Pushker, 2007). The cause of genome imbalance is unclear, and it could be due to differences in replication of the lagging strand, large-scale rearrangements, or gene accumulation in the *oriC* environ (Mira and Pushker, 2007; Ziebuhr, 2012). The *oriC* environ is a prominent region in staphylococcal genomes that spans about 500 Mb of chromosome downstream of the replication origin. The region was discovered during the comparative analyses of the first available *S. aureus*, *S. epidermidis*, and *S. haemolyticus* genomes. It was originally defined as a region of contiguous 20 kb windows containing less than 45 % of the ORFs common for staphylococci (Takeuchi *et al.*, 2005). This region is a source of variability as many accessory genes and mobile genetic elements, including *SCCmec*, are located here (Ziebuhr, 2012).

Chromosomal rearrangements in *S. aureus* usually occur by homologous recombination, often associated with hotspots such as SCC, conjugative transposon ICE6013, and genomic island *vSaα*. The recombination rate is unevenly distributed among the clonal complexes. For instance, ST239 arose from large-scale homologous recombination in 20 % of the chromosome, derived from the parental lineages ST30 and ST8 (Robinson and Enright, 2004). There is evidence for recombination in CC1, CC8, and CC25, whereas CC5, CC8, and CC30 lineages are more conservative (Everitt *et al.*, 2014). There are three distinct clades in *S. epidermidis*. Group A has a low recombination rate, while group B has almost none. On the other hand, group C is highly recombinational and may have originated from a hybridization event between groups A and B (Méric *et al.*, 2015). Chromosomal recombination is also prevalent in *S. capitis* (Carter *et al.*, 2018) and *S. haemolyticus*, where it has played an important role in shaping the evolution of pathogenic strains (Takeuchi *et al.*, 2005; Saroha *et al.*, 2022).

The frequent genome rearrangements observed in *S. haemolyticus* are attributed to the unusually high number of ISs present in its genome, which serve as sites for homologous recombination. These small mobile genetic elements are only 1–2 kb in size, encode a single transposase gene, and tend to accumulate in the genomes of CoNS (Loessner *et al.*, 2002). The genome of *S. haemolyticus* JCSC 1435 harbors eighty two ISs, including IS1272, ISSha1, and IS256 (Takeuchi *et al.*, 2005). The IS256 is particularly prevalent in clinical strains of *S. haemolyticus* and *S. epidermidis* (Murugesan *et al.*, 2018) due to its incorporation into other MGEs, where the IS256 provides a promoter for the increased production of antibiotic resistance determinants (Conlon *et al.*, 2004; Couto *et al.*, 2003). The IS257/IS431 transposon, a

member of the IS26 family, is even more widespread due to its unique role in the evolution of SCC, transposons, and plasmids. The IS257/IS431 element is associated with resistance to several antibiotics and heavy metals and has significantly influenced the dissemination of trimethoprim resistance (Firth *et al.*, 2018). Its distinct replication mechanism explains this ability, which involves an unresolved intermediate (Firth and Skurray, 2006) or targeted site-specific recombination (Harmer and Hall, 2020). Both mechanisms produce two direct copies of IS257/IS431, with inserted fragments between them, facilitating the transposition of antibiotic resistance genes (Harmer and Hall, 2020).

Staphylococcal genomes contain many mobile genetic elements such as plasmids, prophages, transposons, resistance and pathogenicity islands, SCC, and other genomic islands, which are horizontally transferred through transformation, transduction, or conjugation. However, the bacteria can naturally take up exogenous DNA (Morikawa *et al.*, 2012; Maree *et al.*, 2022). The most effective method of horizontal gene transfer is transduction, which depends on packaging DNA into phage heads. Phage species classification is governed by the International Committee on Taxonomy of Viruses, which categorizes phages according to morphology, serology, and genomic characteristics. Olivera *et al.* (2019) then divided staphylococcal phages by DNA sequence into five groups. Clusters A, C, and D are lytic phages from the families of the *Podoviridae*, *Myoviridae*, and *Siphoviridae*. Group B contains diverse temperate prophages with significant impact on bacterial evolution (Gummalla *et al.*, 2023). These prophages are *Siphoviridae* with genome sizes of 40-50 kb and with either serine or one of four families of tyrosine integrases (Oliveira *et al.*, 2019). The prophage integrase is also used for detailed classification. In *S. aureus* there are seven major integrases Sa1int–Sa7int, that integrate into their specific *att* sites (Goerke *et al.*, 2009). This typology can be extended by adding representative genes for each phage module; integrase, anti-repressor, replication proteins, dUTPase, portal, tail and amidase (Kahánková *et al.*, 2010). Furthermore, only cluster B phages carry virulence determinants. A notable outlier is the SPβ-like phage, a unique phage that does not belong to any known cluster. It possesses a large genome size of 127 kb and harbors a number of antibiotic resistance genes (Oliveira *et al.*, 2019).

On transduction depends also on phage inducible chromosomal islands (PICI), a family of small 12–27 kb elements, which carry packaging mechanisms similar in structure to prophages and may be originally derived from them (Novick, 2019). Well-known PICIs include SaPI1 and SaPI2 from *S. aureus*, harboring the toxic shock syndrome toxin. Only five integration sites for SaPI have been identified in *S. aureus* (Lindsay *et al.*, 1998). However, CoNS usually carry resistance determinants such as SrRI_{CCM9025} (Kovařovic *et al.*, 2022).

Another way in which genes can be transferred horizontally is by plasmid conjugation. This process relies on the presence of mobilization and transfer genes and

an *oriT* site. Thus, staphylococcal plasmids can be divided into three main groups. The first group, small Class I plasmids, are typically up to 8 kb in size and replicate using the rolling circle mechanism. The second and third groups consist of larger Class II plasmids that are either non-conjugative or replicate, mainly utilizing the theta mechanism. The plasmids can be further classified based on their incompatibility groups, which do not necessarily reflect their evolutionary relationships (Novick, 1989). Classification based on the replication gene *rep* is more reliable. There are seven major classes of *rep* and type plasmids: *Rep_trans*/pT181, *rep1*/pC194, *rep2*/pE194, *repL*/pSN2, *rep_3*/pSK639, *repA_N*/pSK41, *priCT_1*/pCH91. Plasmid size roughly correlates with these *rep* proteins (Kwong *et al.*, 2017). Large plasmids are often composed of modules of mobilization genes or resistance to antimicrobials, which are flanked by IS257/IS431, which helps the cointegration of smaller plasmids and a combination of new ones (Gruss and Ehrlich, 1989; Firth and Skurray, 2006). Non-conjugative plasmids can still be mobilized in the presence of conjugative plasmids and certain genomic islands called integrative and conjugative elements (ICEs). The classification of ICEs can be somewhat unclear because it is not always easy to distinguish between conjugative transposons and integrated plasmids. An interesting example that demonstrates the possible ancestral origin of ICEs is plasmid pG01evolj, which is integrated into the phage-inducible island SaPIbov2. This results in an element with integrative, conjugative, and transduction capabilities (Humphrey *et al.*, 2021).

It is important to remember that MGEs are independent and selfish. Their evolution can be driven by interactions with the host as well as with other MGEs, which are often antagonistic (Horne *et al.*, 2023). For example, the replication of ISs and transposons is strictly regulated (Twiss *et al.*, 2005), and their mobilization is triggered by the arrival of a new plasmid in the cell (Hall *et al.*, 2021; Tansirichaiya *et al.*, 2021). The resistance genes translocated to the plasmid are then rapidly selected due to the higher gene dosage resulting from the multi-copy plasmid (Yao *et al.*, 2022). Complex interactions are observed during transduction, which is also known to transfer plasmids. However, the efficiency is enhanced in the presence of PICI (McCarthy *et al.*, 2014; Fišarová *et al.*, 2021). This mechanism is likely the primary driving force for intra- and interspecies plasmid transfer (Humphrey *et al.*, 2021). It significantly affects the size distribution of plasmids, resulting in two peaks at approximately 11 kb and 40 kb, which correspond to the sizes of PICI and prophages (Smillie *et al.*, 2010). It is also worth noting that the distribution of antimicrobial resistance determinants in bacterial strains reflects their virulome (Colombo *et al.*, 2017).

Intra-species HGT varies between species and is often directional, probably due to barriers to foreign DNA uptake, such as the restriction modification system. Gene flow is common between *Staphylococcus pseudoxylosus* and *S. xylosus*, which also have extensive accessory genomes. Some species, such as *S. epidermidis* or *S. felis*, contain a small number of highly recombinant strains, which may lead to the unpredictable

emergence of new phenotypes (Ikhimiukor *et al.*, 2023). Similar results were observed in another study of 1,876 CoNS genomes (Smith and Andam, 2021). The main barrier in staphylococcal genomes is the restriction modification (RM) systems, which typically consist of methylases that protect bacterial DNA and restriction endonuclease that degrades exogenous DNA (Casadesús and Low, 2006). In *S. aureus*, the RM classes I and IV are the most studied; however, they usually consist only of the restriction endonuclease subunit (Xu *et al.*, 2011), and the RM classes II and III are rare or nonfunctional (M. J. Jones *et al.*, 2015; Sadykov, 2016).

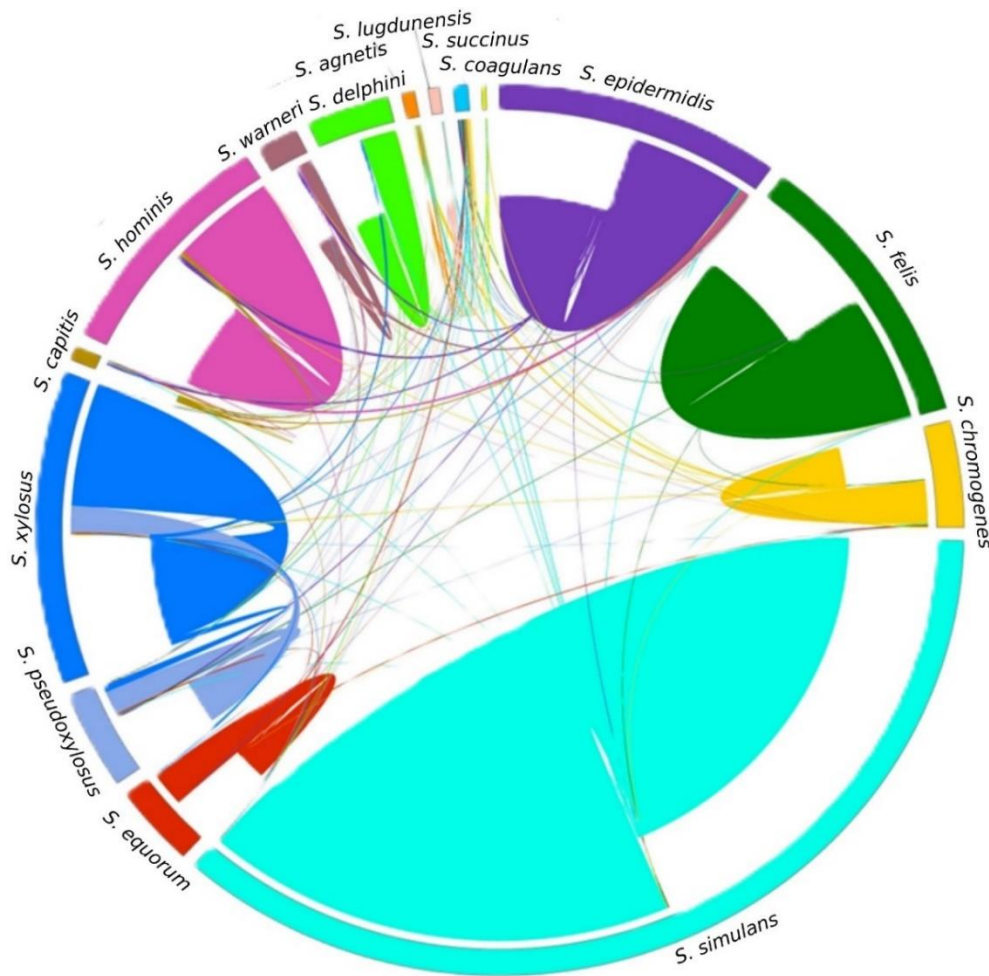


Fig. 9: Gene flow in genomes of coagulase-negative staphylococci

Diagram of Intra and inter-species recombination between 191 animal strains of CoNS. The detected species are shown in the outer circle, proportionally to number of genomes. Inner chords show HGT direction, (Ikhimiukor *et al.*, 2023, modified)

In *S. aureus*, the distribution of RMs is strictly dependent on clonal complexes, with the exception of RMs found on SCC elements. In contrast, *S. epidermidis* exhibits a greater diversity of RMs (Lee *et al.*, 2019). Variable RMs are also present in *S. xylosus*, including active class II and III RMs (Schiffer *et al.*, 2023). Targeted CRISPR-Cas systems in staphylococci are predominantly types I and II. However, they are uncommon in *S. aureus*, and in CoNS, they seem to play a lesser role than RMs and target mainly phage sequences (Moller *et al.*, 2019). Several new bacterial immune systems have recently been described, but their mechanisms are not yet well understood (Moller *et al.*, 2019; Mayo-Muñoz *et al.*, 2023). It remains to be determined whether these systems occur in staphylococci, although Thoreris, Hachiman, Gabija, Lamassu, and Kiwa have orthologs in staphylococcal genomes (Doron *et al.*, 2018).

The current research shows that the HGT can dominate over vertical inheritance in bacterial genomes. The statistical evaluation of biallelic SNPs of *S. aureus* genomes has shown that even the core genome variability is influenced mainly by recombination. The authors of this study propose that every locus has independent phylogeny, although more than half of the SNPs agree with core phylogeny. The core phylogeny does not necessarily show true phylogeny, which would require two assumptions: random recombination and sufficiently large core backbone. Instead, the apparently stable core phylogeny may reflect a population structure determined by distribution of recombination rates. The hypothesis is supported by the fact that the core phylogeny is inferred even when only discordant SNPs are used (Sakoparnig *et al.*, 2021). The concept of the Tree of Life may shift to a statistical network of life (Koonin *et al.*, 2021) with a dynamic pangenome (Medini *et al.*, 2020). Staphylococcal pangenomes are often open (Argemi *et al.*, 2019; Chong *et al.*, 2022; Ikhimiukor *et al.*, 2023), with a large number of accessory genes and, in theory, an unlimited number of rare genes. There is a fundamental difference between the statistical properties of core and accessory genes, which are relatively stable, and a cloud of rare genes, predominantly located on MGE, which are rapidly replaced (Wolf *et al.*, 2016). The acquisition of new genes and the expansion of the accessory genome enables bacteria to adapt to new niches. However, adverse selection is more potent in larger genomes, limiting genome size against unnecessary genes. However, it is important to remember that a reliable analysis requires checking data with experimental biology (Koonin *et al.*, 2021). Although the whole genome SNPs may not provide meaningful insight into the true phylogeny of strains, it is still helpful for clinical management because they allow for tracking outbreaks and identifying clusters (Ahrenfeldt *et al.*, 2017).

2.3 Detection and Annotation of Mobile Genetic Elements

Simply having a genome sequence is insufficient to obtain meaningful information. It needs to be annotated. This entails analyzing the CG content and identifying the origin, terminus, and all genes in their most basic form. In prokaryotic genomes, coding, because of the high percentage of coding content and the absence of introns, it is relatively easy to generate open reading frames and decide which are coding. However, assigning a function to a gene is more complicated and relies on a well curated gene database (Salzberg, 2019). Currently, the most popular annotation software is Prokka (Seemann, 2014a) and PGAP (Tatusova *et al.*, 2016). Prokka is an easy to use command line program whose main advantage is speed, taking only a few minutes per genome on a personal computer. However, Prokka is more conservative, assigning a gene function when the identity to the database is high. On average, about half of the genes are labeled as hypothetical with unknown function (Lobb *et al.*, 2020). This is actually desirable behavior because it limits the propagation of errors in the database. PGAP is part of the GenBank database, taking advantage of the immense collection of millions of genes, and is the de facto standard for new genomes. To ensure quality, it relies on high-quality genomes and suppresses questionable annotations. The older genomes are also reannotated to ensure consistency of position and name (Tatusova *et al.*, 2016). The genes can also be further annotated with more detail as protein motifs, functional sites (P. Jones *et al.*, 2014; Coudert *et al.*, 2023), or signal peptides (Almagro Armenteros *et al.*, 2019) or multiple gene ontologies to determine the overall function and interaction between genes (Ashburner *et al.*, 2000; Kanehisa and Goto, 2000; Huerta-Cepas *et al.*, 2019). There are also specialized databases such as heavy metal resistance (Pal *et al.*, 2014), restriction modification systems, CRISPR-Cas arrays (Couvin *et al.*, 2018), and toxin-antitoxin genes (Xie *et al.*, 2018) or *S. aureus* pangenome (Fuchs *et al.*, 2018). They tend to be small, and although well curated, they are not updated often, if at all.

The main concern for clinical strains is the determination of potential virulence and resistance factors, which are usually found by BLAST searches against either protein or nucleotide-based databases. Since the virulence factors are not well defined among CoNS and the virulence databases typically contain only *S. aureus* sequences, the assigned annotation must be interpreted carefully and the gene context, *e.g.*, location on MGE, must be considered. This method is partially automated by the VFAnalyzer, which is part of the VFDB database (B. Liu *et al.*, 2019). Conversely, the identification of antimicrobial resistance genes is generally reliable, and more databases are available to combine results to improve confidentiality (Zankari *et al.*, 2012; Gupta *et al.*, 2014; Feldgarden *et al.*, 2019; Doster *et al.*, 2020; Alcock *et al.*, 2023). The CARD database also employs a special RGI program, that uses a sequence database but also attempts to predict novel resistance genes based on protein motifs (Alcock *et al.*, 2023).

The precise identification and annotation of MGEs is more complex. Staphylococci have a wide array of MGEs, which may be incomplete (Firth *et al.*, 2018). Even intact elements often contain extensive repetitions, facilitating misassembly or breaking sequences into contigs. A detailed description of MGEs helps to study bacterial evolution, as they often carry virulence, resistance factors, and other genes important for adaption to the environment (Humphrey *et al.*, 2021). For this purpose, BLAST databases are also used, for instance, ISfinder (Siguier *et al.*, 2006), PHASTER (Arndt *et al.*, 2019), ICEberg (M. Liu *et al.*, 2019), ACLAME (Leplae *et al.*, 2010). The popular actively maintained collection of various databases is a web-based CGE suite, including PlasmidFinder, SCCmecFinder, and general MobileElementFinder, which detects and provides a classification of MGE (Johansson *et al.*, 2021). The main limitation of homology-based database searches is the inability to identify novel types of MGEs. Only a handful of programs are capable of performing *ab initio* identification. The Islandviewer4 combines three tools to compare sequences with reference genomes and analyze codon and dinucleotide frequencies that differs in MGEs compared to horizontally descended genes (Bertelli *et al.*, 2017). Another software, MGEfinder, attempts to improve the accuracy of MGE localization by mapping reads to a closely related reference genome, with any additional sequences appearing as gaps in coverage (Durrant *et al.*, 2020). None of the current annotation software is perfect. Although they are reliable in identifying general MGE regions, determining exact borders is difficult and requires manual intervention. In particular complex repeats, such as SCCmec, require experience to resolve. The future trend is to use machine learning, e.g. for phage detection (Sirén *et al.*, 2021). With the growing number of sequenced genomes, it becomes more important to properly prepare the sequence data and simplify the submission of relevant databases. Some efforts have already been made in this direction. For example, the GenBank database requires metadata to be added to each genome, such as the sequencing platform, assembler used, and source host (Benson *et al.*, 2013). The antibiotic resistance database CARD recently modified records to make them accessible to machine learning (Alcock *et al.*, 2023). An ambitious project is the proGenomes3 database, which attempts to consistently annotate all available prokaryotes, nearly one million genomes (Fullam *et al.*, 2023).

2.4 Phylogeny and Comparative Genomics

The growing number of annotated genomes requires fast and scalable algorithms to compute their relationships. The simplest methods cluster related genomes by nucleotide or codon composition. The first such method was laboratory DNA-DNA hybridization (DDH). Digital DDH algorithms are complex and sensitive to incomplete genomes. Instead, the representation of the ANI of whole genome is more practical. The first implementation used the alignments with the BLAST algorithm, which is too slow for large-scale analysis. New tools such as fastANI can analyze tens of thousands of genomes and show that the distribution of ANI is discrete and with species boundaries at 95–96 %. In contrast, interspecies ANI is typically less than 83 % (Jain *et al.*, 2018). The composition-based methods, such as pentamer frequency, can also infer phylogeny for closely related genomes (Nakano *et al.*, 2023). For the construction of the phylogenetic tree, algorithms based on Maximum-Likelihood, which uses a substitution model to find the tree topology with the highest probability, are more commonly used, such as RAxML (Stamatakis, 2014) and FastTree (Price *et al.*, 2010). With these tools, it was possible to construct a phylogenetic tree of ten thousand bacterial and archaeal genomes in reasonable time (Zhu *et al.*, 2019).

As mentioned above, the traditional method for inferring phylogeny is 16S rRNA, which is not accurate enough for closely related species (Hassler *et al.*, 2022). To examine phylogeny relationships in detail, a set of housekeeping genes is more suitable (Gherardi *et al.*, 2018). The *tuf* gene is more divergent than 16S and clusters staphylococci into nine groups: *S. epidermidis*, *S. saprophyticus*, *S. simulans*, *S. intermedius*, *S. hyicus*, *S. auricularis* and *Mammaliicoccus sciuri*. Similar results are obtained for *rpoB*, *hsp60*, and *sodA*. The remaining gene *gap* forms fewer clusters but discriminates very well at the species level (Ghebremedhin *et al.*, 2008). The phylogeny tree shown in Fig. 4 was constructed from their concatenated sequences with the addition of the *dnaJ* gene. A similar approach is used by UBCG, which computes a tree for each of 81 universally present single-copy genes and concatenates them into the single tree (Kim *et al.*, 2021).

Theoretically, it would be best to use maximum whole genome data with SNPs. Practical experiments show that even in a perfect vertical ancestry dataset generated with serial passages of a hypermutator strain of *E. coli*, the inferred phylogenies contain only 50–73 % correct branches. The reason is that some algorithms assume uniform mutation frequency, which is not always true (Ahrenfeldt *et al.*, 2017). However, the assumption of a perfect vertical evolutionary history also does not hold (Kapli *et al.*, 2020). Bacterial genomes are significantly influenced by HGT (Nakano *et al.*, 2023), which can reach up to 40 % of genes of in *Staphylococcus* (Hassler *et al.*, 2022). The recombination rate is also biased towards certain strains and species, as shown in Fig. 9 (Smith and Andam, 2021; Ikhimiukor *et al.*, 2023). One possible solution is to mask the horizontal gene content to focus on only the core genome.

However, the HGT content itself is also worth researching. It usually encompasses MGE and is a considerable source of clinically important features. The HGT affected sequences can therefore be determined by MGE detection or by composition based methods like HGTector (Zhu *et al.*, 2014), DarkHorse (Podell and Gaasterland, 2007), or by the distribution of a given gene in the phylogenetic tree, which takes advantage of the large number of genomes available in the database. One such tool is APP, which marks genes as horizontally transferred if present in less than 30 % of genomes. The drawback is that it does not account for gene loss events. To alleviate this problem, gene presence is also checked at the family level. In the test data set of eighteen genomes, these methods found 45–72 % of the recombinant genes. Each method has its strengths and should be used complementarily (Sengupta and Azad, 2023).

Genes from all strains of a species make up its pangenome, which are usually clustered at the protein level. Thus, special care should be taken to annotate all genomes with the same tool to ensure consistency. Furthermore, to create a non-redundant set of genes, it is necessary to recognize orthologs, which are genes related by vertical descent from paralogs, which are present in copies in a single genome. Paralogs can be acquired horizontally or as a result of duplication events. The web-based OrthoVenn3 distinguishes them based on gene collinearity (Sun *et al.*, 2023). It can also annotate genes with GO ontology terms to examine functional differences between strains (Ashburner *et al.*, 2000).

The annotated genes can be further mapped to metabolic pathways, e.g., in KEGG (Kanehisa and Goto, 2000), making the pangenome an excellent way to compare the presence and absence of clinically relevant genes at CoNS. This is especially true for virulence genes, which are poorly understood. The pangenome analysis pipeline Roary (Page *et al.*, 2015) was therefore used for genome wide association study of a set of 123 clinical and 46 environmental isolates of *S. haemolyticus* to detect potential virulence factors and MGE and found that *sraP* and three variants of *cap* operon, plasmid pSHaeB with *ermC*, IS256, and Tn522, which were predominantly present in clinical strains. The isolates resistant to oxacillin and aminoglycosides, that produced biofilm, were almost exclusively nosocomial, although no *ica* operon was found, other detected resistance factors are shown in Fig. 10 (Pain *et al.*, 2019). Similar Genome wide association studies in *S. epidermidis* and *S. aureus* have failed because of data heterogeneity. Unlike *S. haemolyticus*, *S. aureus* and *S. epidermidis* do not have specialized clinical strains and cause many symptoms (Copin *et al.*, 2018; Pain *et al.*, 2019). Comparing the presence of MGE is, on the other hand, relatively straightforward. A large-scale pangenome study of forty thousand genomes of *S. aureus* has showed that the differences between genomes are mainly phages and plasmids, which can be classified into at least 18 types, according to the *rep* gene. Clinically relevant were primarily SCCmec and arginine catabolic mobile element (ACME).

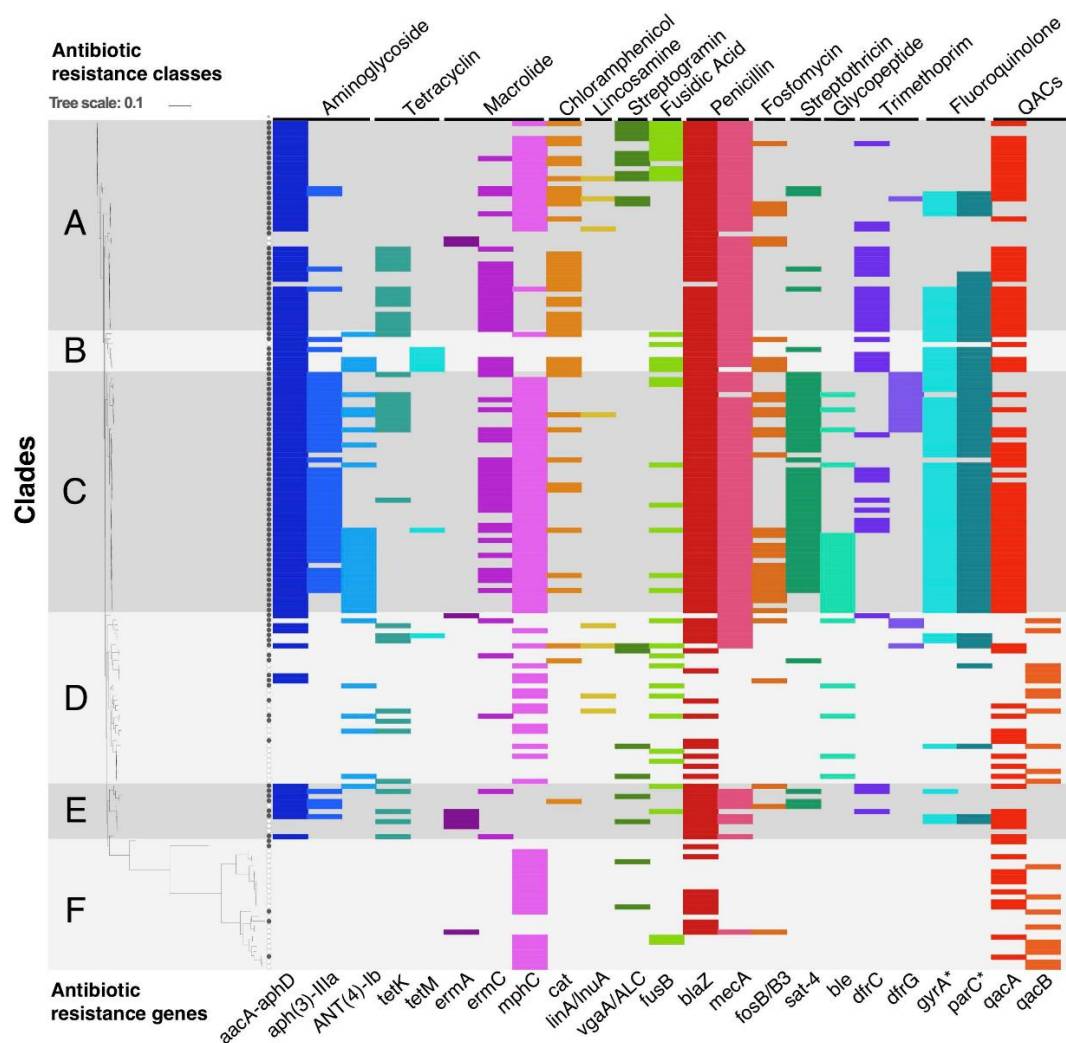


Fig. 10: Distribution of antibiotic resistance determinants *S. haemolyticus*

The presence/absence of antibiotic resistance genes and their antibiotic classes are plotted to the core genome SNP genome tree with the major clades highlighted (Pain *et al.*, 2019)

The results also indicated that small mutations in the global regulators *agr* and *rsp* were essential for virulence. However, the study was limited by a small proportion of reliable complete genomes and database bias towards CC5 and CC8 lineages (Copin *et al.*, 2018). Another useful approach is to combine sequence analysis with laboratory experiments, for example, the transposon mutagenesis library has been used to test MRSA survival in human and porcine blood, resulting in identification of seventy-three genes associated with host adaption and virulence (Ba *et al.*, 2023).

As illustrated by the examples above, comparative genomics has great potential to mine data. However, the relevant metadata is seldom published or available in machine accessible form. Accurate annotation and information on disease, symptoms, and therapy outcomes would enable the elucidation of the mechanisms of pathogenesis (Lindsay, 2014; Copin *et al.*, 2018; Howden *et al.*, 2023).

2.5 Automated Workflows

Bioinformatic analysis of bacterial genomes is a time-consuming process with many computationally expensive steps. The current effort is to create a pipeline encompassing all partial analyses. However, sequencing technologies and bioinformatic algorithms develop so rapidly that any program integrated into such workflow is soon obsolete. For instance, the normalizing read depth (Love *et al.*, 2014) or scaffolding tools (Bosi *et al.*, 2015) are entirely unnecessary for *de novo* sequencing of bacterial genomes when long read sequencing becomes available. An analogy might be the development of genome assemblers, which were initially separate programs to assign quality scores, perform error correction, and finally assemble contigs (de la Bastide and McCombie, 2007). In contrast, contemporary assemblers integrate all steps into a single program. Currently, few pipelines perform complex genome analysis, and they are usually designed for a specific purpose, which may limit their usage. For instance, pipelines intended for clinicians to rapidly identify virulence and resistance factors, such as command line Nullabor (Seemann *et al.*, 2015) or web-based PATRIC (Gillespie *et al.*, 2011). Differently focused is TYGS web, which addresses whole genome-based taxonomy and is helpful for rapid classification of a new strain and determining whether it is a new species and its relationship to other type strains.

A modular approach is often used to ensure that the analysis stays up to date. Instead of a monolithic program, a workflow management system is used to maintain various bioinformatics programs. In the pipeline, where the input and output of each step are well defined, it is easy to add or change specific tools as needed. One such system is Nextflow (Di Tommaso *et al.*, 2017), which is used by Bactopia, a general pipeline for the complete analysis of bacterial genomes (Petit and Read, 2020). The same authors have also developed The Python-based pipeline Staphopia, which specifically designed for the rapid annotation of 42,949 *S. aureus* genomes (Petit and Read, 2018). Another popular workflow manager is web-based Galaxy servers, which is easily accessible to people without programming knowledge. However, more options are available with the command line Snakemake based on Python. It has the advantage of integrated package management systems, that automatically install all the necessary programs for analysis. This makes it easy to move the analysis to another computer, but it also ensures the reproducibility of the analysis as it keeps the correct version of all tools (Mölder *et al.*, 2021). The Fig. 11 presents the workflow scheme developed by the author of this thesis for the analysis of the genome of *Staphylococcus brunensis* (Kovařovic *et al.*, 2023) and, in the previous version, also for *Staphylococcus ratti* (Kovařovic *et al.*, 2022).

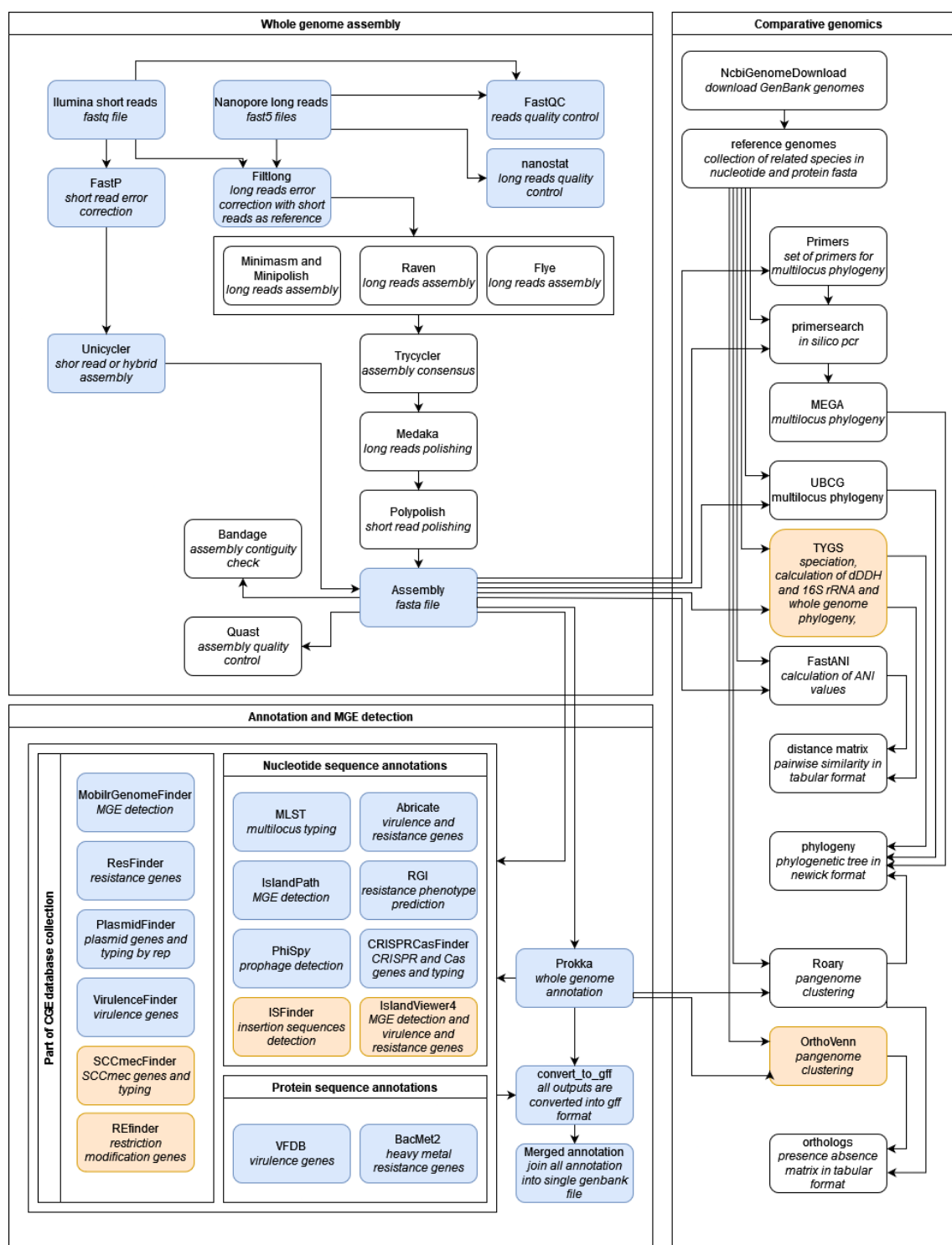


Fig. 11: Snakemake based analysis workflow for preparing expert annotated datasets

Bacterial genome analysis workflow that was used for annotation of *S. brunensis* (Kovařovic *et al.*, 2023) and *S. rattii* (Kovařovic *et al.*, 2022). The blue highlighted boxes are part of an automated pipeline for assembly and annotation (Kovařovic, 2024). The orange highlighted boxes are web-based services.

3 Objectives of the Dissertation Thesis

Molecular diagnostics has significantly enhanced the fast and efficient screening of bacterial isolates from diverse environments. Among these techniques, MALDI-TOF-MS has emerged as a powerful tool for routinely typing clinical strains, leading to the identification of several atypical *Staphylococcus* and *Macrococcus* isolates, which differed from known species and were the focus of this work. However, validation of new species from such isolates requires a thorough analysis of their complete sequences and storage in a database with detailed annotation. The rapid development of sequencing technologies, as well as bioinformatic methods over the last decade, has made it challenging to establish a standard protocol for bioinformatical analysis of bacterial genomes. Although there is no shortage of available programs and analyses for this purpose, processing each can be time-consuming. Thus, most publications describing new bacterial species are limited to genome assembly and initial annotation of genes. Existing bioinformatics pipelines are usually narrowly focused on specific purposes. The main intention was, therefore, to develop a modular pipeline to facilitate an efficient and comprehensive analysis of staphylococcal genomes that provides expert annotated genomes and an integrated overview of multiple outputs of multiple analyses.

Determining the pathogenic potential of CoNS is particularly important due to their increasing role in healthcare-associated infections. Genome sequencing allows the investigation of disease-related genes, often harbored on mobile genetic elements. Identifying the specific genes responsible for antibiotic resistance and virulence factors is also necessary to develop targeted treatments for CoNS infections. The most significant element found in genomes of genera *Staphylococcus*, *Mammaliicoccus*, and *Macrococcus* is the *SCCmec*, which confers resistance to beta-lactam antibiotics. The natural transmission of the *SCCmec* is not yet understood, and its presence in other genomes and evolutionary history is an active area of research. Despite their importance, little is known about virulence factors in species other than *S. aureus*. The genomic islands to which SCC belongs were examined in detail in this work, which should bring new insights into their evolution.

In-depth functional analysis of genes present on mobile elements is crucial to identifying the candidate genes responsible for pathogenicity. Additionally, by comparing the genomes of clinical and environmental species, we can obtain a better understanding of how these bacteria adapt to different environments and how new species described in detail in this work acquire antibiotic resistance and virulence factors.

4 Results and Discussion

The dissertation is presented as an annotated collection of the following publications describing genomes of novel species of *Staphylococcus* and *Macrococcus*, which are attached in order of publication. The bacterial genome assembly and annotation pipeline was also developed during this thesis and is available at Github:

Kovařovic, V. (2024). Snakestaph. *Github*. <https://github.com/kovavo/snakestaph>

Pantůček, R., Sedláček, I., Indráková, A., Vrbovská, V., Mašlaňová, I., **Kovařovic, V.**, Švec, P., Králová, S., Křištofová, L., Kekláková, J., Petráš, P., Doškař, J., 2018. *Staphylococcus edaphicus* sp. nov., Isolated in Antarctica, Harbors the *mecC* Gene and Genomic Islands with a Suspected Role in Adaptation to Extreme Environments. *Applied and Environmental Microbiology* 84, e01746-17.
<https://doi.org/10.1128/AEM.01746-17>

Mašlaňová, I., Wertheimer, Z., Sedláček, I., Švec, P., Indráková, A., **Kovařovic, V.**, Schumann, P., Spröer, C., Králová, S., Šedo, O., Křištofová, L., Vrbovská, V., Füzik, T., Petráš, P., Zdráhal, Z., Ružicková, V., Doškař, J., Pantůček, R., 2018. Description and Comparative Genomics of *Macrococcus caseolyticus* subsp. *hominis* subsp. nov., *Macrococcus goetzii* sp. nov., *Macrococcus epidermidis* sp. nov., and *Macrococcus bohemicus* sp. nov., Novel Micrococci From Human Clinical Material With Virulence Potential and Suspected Uptake of Foreign DNA by Natural Transformation. *Frontiers in Microbiology* 9, 1178.
<https://doi.org/10.3389/fmicb.2018.01178>

Vrbovská, V., **Kovařovic, V.**, Mašlaňová, I., Indráková, A., Petráš, P., Šedo, O., Švec, P., Fišarová, L., Šiborová, M., Mikulášek, K., Sedláček, I., Doškař, J., Pantůček, R., 2019. *Staphylococcus petrasii* diagnostics and its pathogenic potential enhanced by mobile genetic elements. *International Journal of Medical Microbiology* 309, 151355.
<https://doi.org/10.1016/j.ijmm.2019.151355>

Vrbovská, V., Sedláček, I., Zeman, M., Švec, P., **Kovařovic, V.**, Šedo, O., Laichmanová, M., Doškař, J., Pantůček, R., 2020. Characterization of *Staphylococcus intermedius* Group Isolates Associated with Animals from Antarctica and Emended Description of *Staphylococcus delphini*. *Microorganisms* 8, 204.
<https://doi.org/10.3390/microorganisms8020204>

Kovařovic, V., Sedláček, I., Petráš, P., Králová, S., Mašlaňová, I., Švec, P., Neumann-Schaal, M., Botka, T., Gelbíčová, T., Staňková, E., Doškař, J., Pantůček, R., 2022. *Staphylococcus ratti* sp. nov. Isolated from a Lab Rat. *Pathogens* 11, 51.
<https://doi.org/10.3390/pathogens11010051>

Kovařovic, V., Finstrlová, A., Sedláček, I., Petráš, P., Švec, P., Mašlaňová, I., Neumann-Schaal, M., Šedo, O., Botka, T., Staňková, E., Doškař, J., Pantůček, R., 2023. *Staphylococcus brunensis* sp. nov. isolated from human clinical specimens with a staphylococcal cassette chromosome-related genomic island outside of the *rlmH* gene bearing the *ccrDE* recombinase gene complex. *Microbiology Spectrum* 11, 01342-23.
<https://doi.org/10.1128/spectrum.01342-23>

4.1 *Staphylococcus edaphicus* sp. nov., Isolated in Antarctica, Harbors the *mecC* Gene and Genomic Islands with a Suspected Role in Adaptation to Extreme Environments

Background and objectives: The *Staphylococcus* genus is commonly found in various niches, including plants and abiotic surfaces. Most populations are small and transient and result from contamination by animals. However, the highly adaptable *S. xylosus* from the Saprophyticus group is virtually ubiquitous in soil environments and may be able to survive on inorganic compounds only. In January 2013, as part of a monitoring project on psychrotolerant bacteria, two coagulase-negative strains were isolated from rock and sandy soils on James Ross Island in Antarctica. The preliminary 16S rRNA sequencing identified these strains as *S. saprophyticus*, but their biochemical properties differed. Thus, the strains were further investigated to determine genomic characteristics and evolutionary relationships, with a focus on antimicrobial resistance, pathogenicity, and survival traits.

Material and methods: Phenotype, chemotaxonomy, and phylogenomic properties of two strains, CCM 8730T (= P5085T = DSM 104441T) and CCM 8731 (=P5191) were characterized. The whole genome sequence was obtained using IonTorrent PGM and compared to *S. saprophyticus* and *S. xylosus* genomes.

Results and discussion: The genomic analysis revealed that the strains demonstrated considerable phylogenetic distance from their closest relatives at the whole-genome level. The strains contained several genomic islands conferring antibiotic resistance and environmental adaption, including ψ SCCmecP5085 harboring *mec*. This study hypothesizes that it may be an evolutionary advantage against beta-lactam producers such as *Penicillium chrysogenum*, common in the polar environment. The findings also showed that although the overall genome layout was relatively conserved, the pangenome was highly plastic, shaped by horizontal gene transfer. This suggests that *S. edaphicus* can act as a reservoir and mediator for spreading antibiotic resistance.

Conclusion: A novel species, *S. edaphicus* from Antarctica, has been described, and whole genome sequencing was utilized to characterize its mobile genetic elements, which surprisingly had a high number of antibiotic resistance genes.

Significance: This is the first description of *mecC* in a soil isolate. The absence of selection pressure from clinical antibiotic usage on *S. edaphicus* provides insight into the evolution of antibiotic resistance. The potential role as a reservoir also highlights the need for monitoring antimicrobial resistance genes in environmental bacteria.



Staphylococcus edaphicus sp. nov., Isolated in Antarctica, Harbors the *mecC* Gene and Genomic Islands with a Suspected Role in Adaptation to Extreme Environments

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ABSTRACT Two Gram-stain-positive, coagulase-negative staphylococcal strains were isolated from abiotic sources comprising stone fragments and sandy soil in James Ross Island, Antarctica. Here, we describe properties of a novel species of the genus *Staphylococcus* that has a 16S rRNA gene sequence nearly identical to that of *Staphylococcus saprophyticus*. However, compared to *S. saprophyticus* and the next closest relatives, the new species demonstrates considerable phylogenetic distance at the whole-genome level, with an average nucleotide identity of <85% and inferred DNA-DNA hybridization of <30%. It forms a separate branch in the *S. saprophyticus* phylogenetic clade as confirmed by multilocus sequence analysis of six housekeeping genes, *rpoB*, *hsp60*, *tuf*, *dnaJ*, *gap*, and *sod*. Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) and key biochemical characteristics allowed these bacteria to be distinguished from their nearest phylogenetic neighbors. In contrast to *S. saprophyticus* subsp. *saprophyticus*, the novel strains are pyrrolidonyl arylamidase and β -glucuronidase positive and β -galactosidase negative, nitrate is reduced, and acid produced aerobically from D-mannose. Whole-genome sequencing of the 2.69-Mb large chromosome revealed the presence of a number of mobile genetic elements, including the 27-kb pseudo-staphylococcus cassette chromosome *mec* of strain P5085^T (*ψSCCmec_{P5085}*), harboring the *mecC* gene, two composite phage-inducible chromosomal islands probably essential to adaptation to extreme environments, and one complete and one defective prophage. Both strains are resistant to penicillin G, ampicillin, ceftazidime, methicillin, cefoxitin, and fosfomycin. We hypothesize that antibiotic resistance might represent an evolutionary advantage against beta-lactam producers, which are common in a polar environment. Based on these results, a novel species of the genus *Staphylococcus* is described and named *Staphylococcus edaphicus* sp. nov. The type strain is P5085^T (= CCM 8730^T = DSM 104441^T).

IMPORTANCE The description of *Staphylococcus edaphicus* sp. nov. enables the comparison of multidrug-resistant staphylococci from human and veterinary sources evolved in the globalized world to their geographically distant relative from the extreme Antarctic environment. Although this new species was not exposed to the pressure of antibiotic treatment in human or veterinary practice, mobile genetic elements carrying antimicrobial resistance genes were found in the genome. The genomic characteristics presented here elucidate the evolutionary relationships in the *Staphylococcus* genus with a special focus on antimicrobial resistance, pathogenicity, and survival traits. Genes encoded on mobile genetic elements were arranged in unique combinations but retained conserved locations for the integration of mo-

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R.P. and I.S. contributed equally to this work.

bile genetic elements. These findings point to enormous plasticity of the staphylococcal pangenome, shaped by horizontal gene transfer. Thus, *S. edaphicus* can act not only as a reservoir of antibiotic resistance in a natural environment but also as a mediator for the spread and evolution of resistance genes.

KEYWORDS coagulase-negative staphylococci, methicillin resistance, genomics, polyphasic taxonomy, pathogenicity islands, drug resistance evolution, beta-lactams, chromosomal islands, mobile genetic elements, penicillin-binding proteins, phylogenetic analysis

Members of the genus *Staphylococcus* are widespread in nature and occupy a variety of niches (1). As a result of their ubiquity and adaptability, staphylococci are a major group of bacteria inhabiting the skin, skin glands, and mucous membranes of humans, other mammals, and birds. Most environmental sources also contain small, transient populations of staphylococci, many of which are probably contaminants disseminated by human, other mammal, or bird host carriers (2, 3). Moreover, *Staphylococcus succinus* subsp. *succinus* was isolated from plant and soil inclusions in Dominican amber (4). Recently, *Staphylococcus argensis* was isolated from river sediments (5). A small number of species, such as *Staphylococcus xylosus* and *Staphylococcus sciuri*, have occasionally been isolated from soil, beach sand, and natural waters and also from plants (1, 6, 7). They can grow in habitats containing only an inorganic nitrogen source and, thus, might be capable of a free-living existence.

The ubiquity of *S. xylosus* might be explained by its ability to adapt to different environments. Its capacity to colonize biotic and abiotic surfaces is probably due to its ability to form a biofilm (8) and to genes implicated in ecological fitness (9). It has been considered a nonpathogenic commensal organism (10) and has rarely been reported to be associated with infections (11). On the other hand, *Staphylococcus saprophyticus*, the closest phylogenetic relative of *S. xylosus*, is an important opportunistic pathogen, causing human urinary tract infections, wound infections, and septicemia (12). The host range of *S. saprophyticus* varies from humans to lower mammals and birds (6, 13). It has been isolated from the gastrointestinal tract of both humans and animals, as well as from meat and cheese products, vegetables, and the environment (14).

The aim of this study was to investigate the genomic properties and clarify the taxonomic position of two coagulase-negative staphylococcal isolates belonging to the *S. saprophyticus* phylogenetic clade that could not be identified to the species level by common diagnostic techniques. The isolates are notable due to their origin from the Antarctic environment and resistance to beta-lactam antibiotics encoded by a novel pseudo-staphylococcus cassette chromosome *mec* (ϕ SCC*mec*) element.

TAXONOMY

Staphylococcus edaphicus (e.da'phi.cus. Gr. n. edaphos, soil; N. L. masc. adj. *edaphicus*, belonging to soil).

Its cells are Gram stain-positive, spherical or irregular cocci, 880 ± 60 nm in diameter, occurring predominantly in clusters, non-spore forming. Colonies on P agar after 24 h are circular with whole margins, slightly convex, smooth, shiny, whitish, 2 mm in diameter and aerobic. Weak hemolytic activity (production of δ -hemolysin) on blood agar. Good growth at 10°C, 42°C, and in the presence of 11% NaCl; weak growth in the presence of 12% NaCl or at 5°C. No growth at 0 or 45°C. No growth in M9 minimal agar. No growth in thioglycolate medium or in the presence of 14% NaCl on a tryptic soy agar (TSA) plate. Catalase, urease, pyrrolidonyl arylamidase, Voges-Proskauer test (acetoin), nitrate reduction, and hydrolysis of Tween 80 positive. Coagulase, clumping factor, hyaluronidase, thermonuclease, oxidase, arginine dihydrolase, ornithine decarboxylase, and arginine arylamidase negative. Susceptible to polymyxin B (300 IU) and furazolidone (100 μ g), but resistant to bacitracin (0.2 IU) and novobiocin (5 μ g). Susceptible to lysostaphin (200 mg liter⁻¹) and resistant to lysozyme (400 mg liter⁻¹). Hydrolysis of esculin, DNA, and gelatin negative. Butyrate esterase, caprylate esterase,

myristate lipase (weak), β -glucuronidase, acid phosphatase (weak), alkaline phosphatase, and naphthol-AS-BI-phosphohydrolase (7-bromo-3-hydroxy-2-naphthoic-o-anisidide) activities are present, but not leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase, and α -fucosidase activities. Acid is produced from glycerol, ribose, galactose, D-glucose, fructose, mannose, mannitol, sorbitol (weak), *N*-acetylglucosamine, salicin, maltose sucrose, trehalose, and β -gentiobiose, but not from erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, adonitol, β -methyl-D-xyloside, sorbose, rhamnose, dulcitol, inositol, α -methyl-D-mannoside, α -methyl-D-glucoside, amygdaline, arbutine, cellobiose, lactose, melibiose, inulin, melezitose, D-raffinose, glycogen, xylitol, turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2 keto-gluconate, or 5 keto-gluconate. The test result was strain dependent for acid production from starch (CCM 8730^T positive). *S. edaphicus* had the ability to utilize the following carbon sources via respiration as determined by the Biolog GEN III MicroPlate test panel: dextrin, D-maltose, D-trehalose, gentiobiose, sucrose, D-turanose, β -methyl-D-glucoside, D-salicin, *N*-acetyl- β -D-mannosamine, *N*-acetyl neuraminic acid, α -D-glucose, mannose, D-fructose, D-galactose, inosine, D-mannitol, D-arabitol, glycerol, glycyl-L-proline, L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, L-histidine, L-serine, pectin, D-gluconic acid, D-glucuronic acid, glucuronamide, L-lactic acid, Tween 40, acetoacetic acid, acetic acid, and formic acid. The negative utilization tests were D-cellobiose, stachyose, D-raffinose, α -D-lactose, D-melibiose, *N*-acetyl-D-galactosamine, 3-methyl glucose, L-fucose, D-sorbitol, *myo*-inositol, D-glucose-6-phosphate, D-aspartic acid, D-serine, gelatin, L-pyrogutamic acid, D-galacturonic acid, D-galactonic acid lactone, mucic acid, quinic acid, D-saccharic acid, *p*-hydroxy phenylacetic acid, D-malic acid, bromo-succinic acid, γ -amino-butyric acid, α -hydroxy-butyric acid, β -hydroxy-D,L-butyric acid, α -keto butyric acid, and propionic acid. The strain-dependent tests were *N*-acetyl-D-glucosamine, L-rhamnose, D-serine, D-fructose-6-phosphate, rifamycin SV, guanidine HCl, methyl pyruvate, D-lactic acid methyl ester, citric acid, α -keto glutaric acid, and L-malic acid (CCM 8730^T positive in all tests), and D-fucose (CCM 8730^T negative). Chemical sensitivity assays, performed with the Biolog GEN III MicroPlate test panel, exhibited positive growth at pH 6 and in the presence of 1% NaCl, 4% NaCl, 8% NaCl, 1% sodium lactate, nalidixic acid, lithium chloride, potassium tellurite, aztreonam, and sodium butyrate. Negative growth was exhibited at pH 5 and in the presence of fusidic acid, troleandomycin, minocycline, lincomycin, Niaproof 4, vancomycin, tetrazolium violet, tetrazolium blue, and sodium bromate. The tested strains were susceptible to cephalothin, ciprofloxacin, clindamycin, erythromycin, gentamicin, chloramphenicol, imipenem, kanamycin, neomycin, cotrimoxazole, tetracycline, and vancomycin and resistant to penicillin G, ampicillin, ceftazidime, methicillin, cefoxitin, and fosfomycin.

The type strain CCM 8730^T (= P5085^T = DSM 104441^T) was retrieved in January 2013 from fragments of black porous stone at a hill above Cape Lachman, James Ross Island, Antarctica. It had G+C content of 33.3 mol% calculated from whole genomic sequence. The majority of the characteristics of the type strain are in agreement with the general species description; in addition, the CCM 8730^T strain produces acid from starch.

RESULTS AND DISCUSSION

Two isolates were obtained as a by-product in the framework of a project monitoring psychrotolerant bacteria of the phylum *Bacteroidetes* from abiotic sources in James Ross Island, Antarctica (CzechPolar2 project). Gram staining and phase-contrast and transmission electron microscopy showed that the cells are Gram-positive spherical cocci with the typical appearance of staphylococci, without flagella (Fig. 1). Strain P5085^T (= CCM 8730^T) was isolated from stone fragments sampled from a hill above Cape Lachman (GPS coordinates 63°46'58"S 57°47'11"W), and strain P5191 (= CCM 8731) was isolated from sandy soil in the Panorama Pass locality (GPS coordinates 63°48'51"S 57°50'45"W). The phenotypic characteristics of the new isolates are summarized in the species description above.

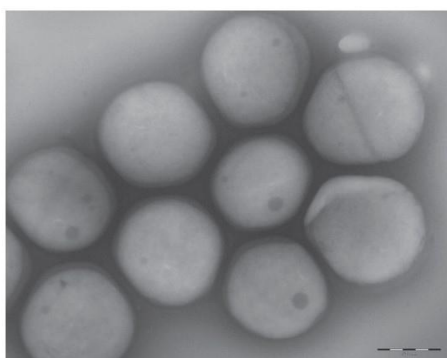


FIG 1 Transmission electron microscopy of type strain *Staphylococcus edaphicus* CCM 8730^T, performed with Morgagni 268D Philips (FEI Company, USA) electron microscope. Negative staining with 2% ammonium molybdate. Bar represents 500 nm (original magnification, $\times 10,000$).

The preliminary identification by sequencing of the 16S rRNA gene assigned both isolates to the *Staphylococcus saprophyticus* species group defined by Takahashi et al. (15). The whole genomic sequence of the *S. saprophyticus* type strain (16) even exhibited indistinguishability from that of the strain CCM 8730^T 16S rRNA gene in two *rrn* operons, but there was a 1-residue difference in each of the three other *rrn* operons (positions 190, 278, and 457). However, the phenotypic results obtained, which are listed in the species description given above, did not allow for the classification of isolates into any known staphylococcal species. The key characteristics differentiating the novel taxon represented by strain CCM 8730^T from the phylogenetically closely related species are shown in Table 1.

The strains were analyzed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) and yielded profiles containing signals in the mass range of 2 to 15 kDa. They did not exhibit significant similarity (BioTyper log score of >1.7) to any of the reference entries belonging to *Staphylococcus* species with valid names included in BioTyper database version 5989 (Bruker Daltonics) and proved *S. saprophyticus* to be the closest relative (see Fig. S1 in the supplemental material).

Three DNA fingerprinting techniques were used to show the differences between the isolates and similarities to related taxa. Repetitive sequence-based PCR (rep-PCR)

TABLE 1 Differentiation of *Staphylococcus edaphicus* sp. nov. from the phylogenetically closest *Staphylococcus* spp.

Test	Result obtained for indicated type strain in this study/from species description ^a :							
	<i>S. edaphicus</i> CCM 8730 ^T	<i>S. xylosus</i> CCM 2738 ^T	<i>S. saprophyticus</i> subsp. <i>saprophyticus</i> CCM 883 ^T	<i>S. saprophyticus</i> subsp. <i>bovis</i> CCM 4410 ^T	<i>S. succinus</i> subsp. <i>succinus</i> CCM 7157 ^T	<i>S. succinus</i> subsp. <i>casei</i> CCM 7194 ^T	<i>S. equorum</i> subsp. <i>equorum</i> CCM 3832 ^T	<i>S. equorum</i> subsp. <i>linens</i> CCM 7278 ^T
Nitrate reduction	+	+/-	-/-	+/+	-/-	+/+	+/+	+/+
Pyrrrolidonyl arylamidase	+	+/-	-/-	W/+	-/-	-/-	-/-	-/-
Voges-Proskauer test (acetoin)	+	-/D	+/+	+/-	-/-	-/-	-/-	-/-
Esculine hydrolysis	-	-/D	-/-	-/-	+/+	+/+	-/D	-/-
β -Glucosidase	-	+/+	-/D	-/D	+/-	+/-	+/-	-/NT
β -Glucuronidase	+	+/+	-/-	-/-	+/+	+/+	W/+	+/+
β -Galactosidase	-	+/+	+/+	+/-	+/+	+/+	W/D	-/-
Acid from mannose	+	+/+	-/-	-/-	+/-	+/+	-/+	+/+

^aSpecies description data are from Schleifer and Bell (17). +, positive reaction; W, weakly positive reaction; -, negative reaction; D, variable reaction; NT, not tested.

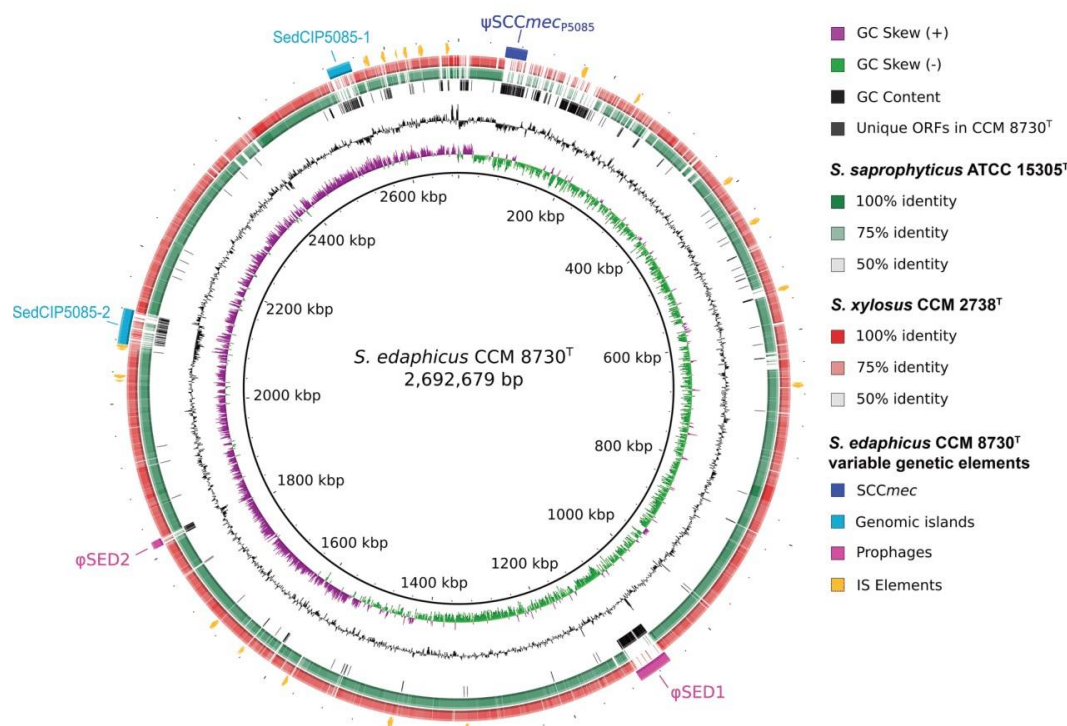


FIG 2 Circular display of *Staphylococcus edaphicus* CCM 8730^T genome (GenBank accession number MRZN000000000) compared to the *Staphylococcus saprophyticus* ATCC 15305^T (GenBank accession number NC_007350) and *Staphylococcus xylosus* CCM 2738^T (GenBank accession number MRZO00000000) genomes. The picture shows (from inner to outer circle) GC skew, mol% G+C, unique regions in CCM 8730^T genome based on BLASTn analysis, and orthologous regions in *S. saprophyticus* ATCC 15305^T genome and *S. xylosus* CCM 2738^T genome based on BLASTn analysis. Outer circle depicts locations of accessory elements in *S. edaphicus* CCM 8730^T genome.

fingerprinting using the (GTG)₅ primer (Fig. S2) and automated ribotyping with the EcoRI restriction enzyme (Fig. S3) showed high genetic similarity between strains CCM 8730^T and CCM 8731, because they gave visually identical fingerprints. At the same time, both DNA fingerprinting techniques also clearly differentiated the strains analyzed from the type strains representing the phylogenetically close *Staphylococcus* spp. Strains CCM 8730^T and CCM 8731 were also undistinguishable by SmaI macrorestriction analysis resolved by pulsed-field gel electrophoresis (Fig. S4).

The fatty acid content of strain CCM 8730^T corresponded with those of phylogenetically related species (Table S1). The fatty acid compositions of strains CCM 8730^T and CCM 8731 were found to be very similar. The major fatty acids (>10%) were C_{15:0} anteiso (51.4%), C_{15:0} iso (13.3%), and C_{17:0} anteiso (12.9%). C_{15:0} and C_{15:0} anteiso were found to be major fatty acids in all members of the *S. saprophyticus* species group. The amount of C_{17:0} anteiso differed slightly between members of this group, only reaching the highest value of 10% in strains CCM 8730^T and CCM 8731 and both subspecies of *S. saprophyticus*. The overall content of other fatty acids was found to be comparable in this clade, with the exception of the *S. succinus* subsp. *succinus* type strain, where C_{13:0} iso and C_{13:0} anteiso were found to be predominant as well (4).

The genome of strain CCM 8730^T was shotgun sequenced, and the contigs were compared with *S. saprophyticus* ATCC 15305^T and *S. xylosus* CCM 2738^T whole-genome sequences (Fig. 2). The size of the draft genome of CCM 8730^T is 2.69 Mb, comprised of 45 contigs of >500 bp (N_{50} = 99,210 bp; 309× mean coverage) with an average G+C content of 33.3 mol%. The sequencing and assembly statistics are shown in Table S2. A total of 2,645 genes were predicted. Comparative analysis of the genes with the

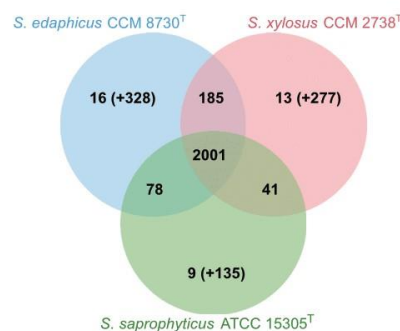
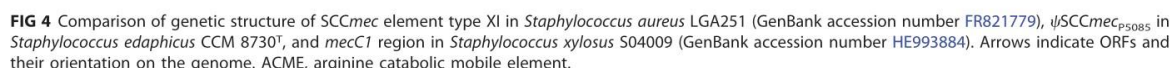


FIG 3 Venn diagram showing orthologous gene clusters for strain *Staphylococcus edaphicus* CCM 8730^T (GenBank accession number MRZN000000000), *Staphylococcus saprophyticus* ATCC 15305^T (GenBank accession number NC_007350), and *Staphylococcus xylosus* CCM 2738^T (GenBank accession number MRZO000000000). The number of singletons specific for each genome is shown in parentheses.

closest relatives identified 2,280 gene clusters and 328 singletons. The majority of the clusters, up to 2,264, are orthologous and shared with either *S. xylosus* or *S. saprophyticus* (Fig. 3). There are 16 unique gene clusters, including 39 open reading frames (ORFs) and 328 singletons specific to the CCM 8730^T strain, localized mainly on variable genetic elements, such as the pseudo-*Staphylococcus* cassette chromosome *mec* (ψ SCC*mec*), two composite phage-inducible chromosomal islands (PICIs), and one complete and one defective prophage (Fig. 2). High heterogeneity in the gene composition compared to those of related species was detected in the region following the ψ SCC*mec*. Genes for a type II restriction modification system similar to Sau3AI were found, as well as a thiamine biosynthesis operon, two predicted protein-coding genes with an LPXTG motif, and genes for a putative PIs cell antiadhesin and cardiolipin synthase. The *pls* gene has been associated with virulence (18), and it has been found adjacent to SCCs present in other staphylococci (19). Cardiolipin synthesis can enhance cell survival under acid or salinity stress conditions (20, 21), and mutations in this gene enhance resistance to daptomycin (22).

The 27,158-bp ψ SCC*mec* element of strain CCM 8730^T (ψ SCC*mec*_{P5085}), bordered by imperfectly matched 27-bp direct repeats, CCGCATCACTTGTGATA(C/T)GCTTC(C/T)CCC, was identified inserted between the *rlmH* gene, encoding rRNA-methyltransferase, and the gene for putative threonyl-tRNA synthetase. This ψ SCC*mec* contains *mec* gene complex class E (*blaZ-mecC-mecR1-mecI*), reflecting the corresponding International Working Group on the Staphylococcal Cassette Chromosome elements (IWG-SCC) designation (23), and it lacks recombinase genes (*ccr*) or their homologues (Fig. 4). The *mecC* gene shares 99% nucleotide identity with that of *Staphylococcus aureus* strain LGA251 (24) and 93% nucleotide identity with the *mecC1* gene of *S. xylosus* strain S04009 (25) and with the *mecC2* of *S. saprophyticus* strain 210 (26). Apart from human and livestock staphylococcal isolates (24), the *mecC* gene was previously identified in isolates from wildlife, including birds and mammals (27–29), and in waste and river waters (30, 31), but to our knowledge, this is the first time the *mecC* gene was identified in an isolate from soil.

Besides the *mecC* gene, very few genes were conserved among ψ SCC*mec*_{P5085} and related SCCs; these were localized mainly in the *mec* gene complex (Fig. 4). This implies that SCC elements might serve for foreign DNA integration and exchange, as suggested for other genomic islands. Downstream from the *mec* gene complex of ψ SCC*mec*_{P5085}, there are 28 predicted ORFs encoding kinase, hydrolase, oxidoreductase, transcriptional regulator, and several hypothetical proteins, whose homologs were identified predominantly in coagulase-negative staphylococcal species. Furthermore, in ψ SCC*mec*_{P5085}, one truncated and other, intact *lpl* genes coding for tandem lipoprotein-like proteins with amino acid similarities of over 89% to those of *S. epidermidis* were identified.



Noticeably lower G+C content, below 29 mol%, clearly leads to the identification of two genomic islands (Fig. 2). The genomic analysis revealed a high resemblance of both islands to the *Staphylococcus aureus* pathogenicity islands (SaPIs), the prototypical members of the PICls (34). The islands of strain CCM 8730^T carry genes necessary for the transfer and autonomous replication of the element, namely, integrase, primase, small subunit of the phage terminase complex, and transcriptional regulators, and were thus classified as PICls. However, the two genomic islands are much longer than SaPIs. The first genomic island, designated SedCIP5085-1 and found in CCM 8730^T, is approximately 45 kb long with more than 70 predicted ORFs. The island is integrated into the transfer-messenger RNA (tmRNA) gene within the attachment sequence site TCCGCG CGTCTCCA(T/C)TATAGAGTCTGCAACC(C/-)AT(T/-)GTGGTTGTGGGCTTTTATTTTGG that corresponds to the attachment site of SaPI_m/νSa3 (35). Two additional copies of the *att* site were found in this island, thus dividing the element into three parts. The first part is 17.9 kb long and resembles canonical SaPI (νSa3) in its gene composition (36). It encodes integrase and contains genes necessary for the transfer, the *fosB* fosfomycin resistance gene homologue (91% amino acid identity with the fosfomycin resistance protein family of *S. saprophyticus*), and several ORFs of unknown function. So far only plasmid-borne fosfomycin resistance genes have been detected in *Staphylococcus* spp., though the possible localization of *fosB* on other mobile genetic elements has been discussed (37). The second part of SedCIP5085-1, 15.4 kb long, is comprised of phage- and SaPI (νSa2)-related genes, but no integrase has been found. It also carries a remnant of the *fosB* gene and other genes of unknown function. The last part of SedCIP5085-1 is 11.7 kb with an indistinct left border, because the bracketing sequence of the *att* site is absent. This part consists of putative genes encoding resistance to arsenic, cadmium, and bleomycin, hypothetical proteins, and a few phage-related genes, such as a transcriptional regulator from the Cro/cI family, which is known to control the switch between the lytic and lysogenic cycle of bacteriophages. The mosaic organization of islands suggests that more than one integration event occurred in the locus.

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contains more than 45 predicted ORFs. The attachment site TATATTATCCCACT CGAT of SedCIP5085-2 is located within the glutamine-hydrolyzing GMP synthase gene, which matches the attachment site for SaPI_{bov1/ν5a2} (35, 38). This genomic island probably enhances the resistance and endurance of the host, because it codes for transporters and transcriptional regulators involved in antibiotic resistance, oxidative stress responses, and the synthesis of virulence factors. It also contains an additional copy of the *cspC* gene, coding for cold shock protein C, which has been found to be expressed strongly after antibiotic, arsenate, and peroxide induction (39). In addition, many insertion sequences from the *IS3* and *IS1182* families were found in this genomic island.

A prophage designated ϕ SED1 (vB_SedS-P5085-1), located downstream from the *tRNA^{Leu}* gene, was identified. The prophage is 44,424 bp long with an average G+C content of 33.36 mol%, which is comparable to the G+C content of the CCM 8730^T strain. The direct repeats of the phage *att* site ATCCCGACCACCGGTAT flank 67 ORFs encoding essential phage genes, which cluster together into functional modules corresponding to those in staphylococcal *Siphoviridae* (40). The lysogeny module starts with phage integrase, which has a tyrosine recombinase XerD domain, AP2-like DNA binding domain, and N-terminal S-adenosylmethionine (SAM)-like domain. There are genes in the DNA replication and transcription regulation modules encoding a DNA primase, helicase, HNH endonuclease domain protein, and putative single-stranded binding protein. The DNA packaging and head and tail morphogenesis modules share 78% nucleotide identity with those of an StB20 phage infecting *Staphylococcus capitis* (41). The holin and amidase from the lysis module exhibit high amino acid identities to *S. saprophyticus* prophage holins and amidases, 95 to 99% and 77 to 85%, respectively. The last gene downstream from the lysis module encodes a glycosyltransferase family 2 protein. The prophage does not carry any genes encoding tRNA or virulence factors. However, another, incomplete ϕ SED2 prophage, 9,241 bp long, bordered by TAGTGT CCTGGGAGG direct repeats, was found in the CCM 8730^T genome. This prophage has an average G+C content of 30.73 mol%, lower than that of its host.

Although the strain CCM 8730^T comes from a geographically isolated polar environment, it carries genes for a surprisingly high number of antimicrobial resistance factors. Resistance to beta-lactam antibiotics is determined by the class E *mec* complex, coding for a beta-lactamase and alternative penicillin-binding MecC protein that is more stable and active at lower temperatures than the MecA protein (42). This might represent an evolutionary advantage against beta-lactam producers, which are common in a polar environment. McRae et al. (43) documented that penicillia are major decomposers and represent an important element of the terrestrial nutrient cycle of Antarctica, particularly since a limited range of other microbial taxa can tolerate the harsh Antarctic climate. Penicillia producing beta-lactams, such as *Penicillium chrysogenum*, have been isolated from sediments of ponds (44), permafrost (45), subglacial ice (46), and oligotrophic and ornithogenic soil in Antarctica (43, 47). Several studies tested for extrolite production and confirmed the antibacterial activity of *Penicillium* species strains isolated in Antarctica (44, 46, 48). This is consistent with the fact that both localities where the strains were isolated are apical parts of the landscape often visited by skua birds in austral summer, and there is a high probability they are colonized by fungi.

The 16S rRNA analysis had limited discriminatory power for identifying the staphylococcal isolates examined, and therefore, their phylogenetic position was assessed using the concatenated multilocus sequence data of six housekeeping genes, *rpoB*, *hsp60*, *dnaJ*, *tuf*, *gap* and *sod*, that are commonly used in phylogenetic studies of *Staphylococcus*, as well as the amino acid sequences of their protein products. The neighbor-joining and maximum-likelihood phylogenetic trees for the six housekeeping genes were very similar and confirmed that the isolates under study represented a well-delineated group within the *S. saprophyticus* phylogenetic clade, clearly separated from known species (Fig. 5).

To evaluate the intergenomic distances between the genome sequences of strain

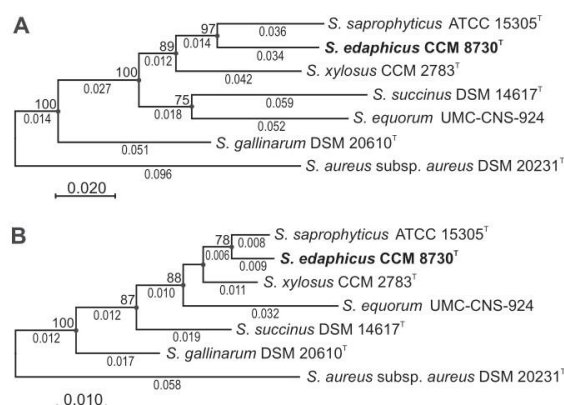


FIG 5 Unrooted maximum-likelihood trees based on concatenated sequences from six multiple loci showing the phylogenetic position of *Staphylococcus edaphicus* sp. nov. The sections of gene sequences used and their protein products correspond to the following gene coordinates of *Staphylococcus aureus* subsp. *aureus* NCTC 8325: 949 to 2748 for *rpoB*, 16 to 1573 for *hsp60*, 1 to 1136 for *dnaJ*, 1 to 1185 for *tuf*, 4 to 1004 for *gap*, and 1 to 597 for the *sod* gene. Bootstrap probability values (percentages of 500 tree replications) greater than 70% are shown at branch points. The evolutionary distances are given as the number of substitutions per site (below the branches). Filled circles indicate that the corresponding nodes are also obtained in the neighbor-joining tree. (A) Evolutionary history inferred from nucleotide sequences by using the maximum-likelihood method based on the Tamura-Nei model. There were a total of 7,175 positions in the final data set. (B) Evolutionary history inferred from amino acid sequences by using the maximum-likelihood method based on the Poisson correction model. There were a total of 2,391 positions in the final data set.

CCM 8730^T and reference type strains belonging to the phylogenetically closest *Staphylococcus* spp., the average nucleotide identity (ANI) and digital DNA-DNA hybridization (dDDH) values were determined (Table S3). The calculated ANI and dDDH values are well below the thresholds of 95 to 96% and 70%, respectively, for species delineation (49). This confirms that strain CCM 8730^T represents a distinct *Staphylococcus* species.

The data from this study demonstrate that although they are highly similar to *S. saprophyticus*, the two isolates represented by strain CCM 8730^T belong to a new taxon that can be distinguished both genotypically and phenotypically from established species of the genus *Staphylococcus*. We suggest classifying these isolates as a novel species for which the name *Staphylococcus edaphicus* is proposed, with the strain P5085^T (= CCM 8730^T = DSM 104441^T) as the type strain.

MATERIALS AND METHODS

Sampling and cultivation. Sampling was carried out by dispersing approximately 1 g of stone fragments or soil in 5 ml of sterile saline solution; 100 μ l of the suspension was spread on an R2A agar plate (Oxoid) and cultivated at 15°C for 5 days. Subsequently, individual morphologically diverse colonies were picked up and purified by repeated streaking on R2A medium at 15°C, and the resulting pure cultures were maintained at –70°C until analyzed. Two Gram stain-positive isolates were obtained. Additional cultivation of these isolates for biotyping and genotyping was performed with cells growing on TSA (Oxoid). The type strains of the closely related staphylococcal taxa were obtained from the Czech Collection of Microorganisms (www.sci.muni.cz/ccm) and were simultaneously investigated in all tests.

Phenotypic characterization. Extensive phenotypic characterization using the commercial kits API 50CH, API ID 32 Staph, and API ZYM (bioMérieux), phenotypic fingerprinting using the Biolog system with the identification test panel GEN III MicroPlate (Biolog), and conventional biochemical, physiological, and growth tests relevant for the genus *Staphylococcus* were done as described previously (50, 51). All phenotypic data presented were from two replicates using commercial kits and three replicates using conventional tests.

Transmission electron microscopy. The surface of the plate containing the bacterial culture was washed off and the bacteria resuspended in distilled water. A 200-mesh Formvar-coated grid was placed on a drop of the suspension for 20 min. Bacterial cells located on the grid were negative stained with 2% ammonium molybdate and treated with UV light. A Morgagni 268D Philips (FEI Company) transmission electron microscope was used to visualize bacterial cells.

Antimicrobial susceptibility tests. The antibiotic resistance pattern was tested by the disc diffusion method on Mueller-Hinton agar (Oxoid). Commercially prepared plates were dried at 35°C for 15 min before use. The direct colony suspension method was used to make a suspension of the organism in saline to the density of a McFarland 0.5 turbidity standard. The antibiotic discs were applied by a disc dispenser (Oxoid) within 15 min of inoculation. Sixteen discs generally used for Gram-positive cocci were applied: ampicillin (10 µg), ceftazidime (10 µg), cefoxitin (30 µg), cephalothin (30 µg), ciprofloxacin (5 µg), clindamycin (2 µg), erythromycin (15 µg), fosfomycin (50 µg), gentamicin (10 µg), chloramphenicol (30 µg), imipenem (10 µg), kanamycin (30 µg), neomycin (10 µg), novobiocin (5 µg), oxacillin (1 µg), penicillin G (1 IU), co-trimoxazole (25 µg), tetracycline (30 µg), and vancomycin (30 µg). The incubation conditions were 16 to 20 h at 36°C in air. The inhibition zone diameters were measured with a caliper. EUCAST/CLSI standards for reading the inhibition zone diameter and interpreting susceptibility to antibiotics were strictly followed (52, 53).

MALDI-TOF MS. For matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF MS), cultures were grown on Columbia blood agar (Oxoid) and transferred with a bamboo toothpick onto an MSP 96 polished steel target plate (Bruker Daltonics). The samples were covered with 1 µl of matrix solution, comprised of a saturated solution of α -cyano-4-hydroxy-cinnamic acid (Bruker) in 50% acetonitrile (Sigma-Aldrich) and 2.5% trifluoroacetic acid (VWR). Each strain was spotted in 10 replicates. Samples were processed using a MALDI-TOF mass spectrometer (Microflex LT; Bruker Daltonics). The mass range of spectra analyzed was 2,000 to 20,000 m/z . Each spectrum was obtained after 300 shots in an automatic acquisition mode. Mass spectra were processed using the software Flex Analysis (version 3.4; Bruker Daltonics). The MALDI-TOF MS-based dendrogram was generated using the software BioTyper (version 3.0; Bruker Daltonics). Signals present in at least 70% of analyses for one sample were transformed into main spectrum projections (MSPs). Afterwards, Pearson coefficients based on the MSPs' similarities were calculated and the dendrogram was generated using the unweighted average linkage algorithm.

FAME analysis. The fatty acid methyl ester (FAME) analysis was performed with cells growing on BBL Trypticase soy agar plates (Becton Dickinson) at 28°C $\pm$ 1°C for 24 h. The extraction of fatty acid methyl esters was performed according to the standard protocol of the Sherlock Microbial Identification System (54). Bacterial biomass was harvested from the third sector of growth on agar plates, where the cultures reached the late-exponential stage of growth according to the four-quadrant streak method (54). Cellular fatty acid extracts were analyzed with an Agilent 7890B gas chromatograph using the rapid Sherlock microbial identification system (MIS) (version 6.2B, MIDI database RTSBA 6.21; MIDI Inc.). The quantities of individual fatty acids are given as percentages of all named fatty acids. Strains CCM 8730^T and CCM 8731^T and the reference strains were tested three times to evaluate the reproducibility of the method.

Genotypic analysis by fingerprinting techniques. Rep-PCR fingerprinting with the (GTG)₅ primer was performed as described previously (55). The automatic ribotyping was performed using a RiboPrinter microbial characterization system (DuPont Qualicon) in accordance with the manufacturer's instructions. Numerical analysis of the fingerprints obtained and dendrogram construction were done using the software BioNumerics 7.6 (Applied Maths). The ribotype patterns were imported into the software BioNumerics using the load samples import script provided by the manufacturer. Pulsed-field gel electrophoresis (PFGE) using SmaI macrorestriction pattern analysis was performed as previously described (56).

Genome sequencing and bioinformatics analysis. Genomic DNA was extracted using a high pure PCR template preparation kit (Roche) according to the manufacturer's recommendations. The concentration of extracted DNA was estimated with a Qubit 2.0 fluorometer using a Qubit double-stranded DNA (dsDNA) broad-range (BR) assay kit (Invitrogen). The partial 16S rRNA gene was sequenced as described previously (57). Whole-genome shotgun (WGS) sequencing was performed using an Ion Torrent Personal Genome Machine (PGM). The purified genomic DNA was used for preparing a 400-bp sequencing library with an Ion plus fragment library kit (Thermo Fisher Scientific). The sample was loaded onto an Ion 316 chip, version 2, and sequenced using an Ion PGM Hi-Q view sequencing kit (Thermo Fisher Scientific). Quality trimming of the reads was performed with the Ion Torrent Suite software (version 5.0.4) with default settings. The assembly computation and error correction were performed using Assembler SPAdes 3.9 (58) with default parameters for Ion Torrent data and k -mer settings of 21, 33, 55, 77, 99, and 127. Contigs were then reordered according to the *S. saprophyticus* ATCC 15305^T reference genome (GenBank accession number NC_007350) (16) using MauveContigMover (59). Ordering was evaluated using the assembly graph visualized in Bandage (60).

Sequences were manipulated and inspected in the cross-platform bioinformatics software Ugene version 1.23.1 (61). For primary analysis, the genome was annotated using RAST (62). Gene content was further examined by BLASTp (63), ISFinder (64), InterProScan (65), CD-Search (66), and OrthoVenn (67). The complete 16S rRNA gene sequence was extracted from WGS data using RNAmmer version 1.2 (68). Phylogenetic analysis from multilocus sequence data was performed using the software MEGA version 7 (69).

DNA homology studies. To calculate the ANI value, the OrthoANI algorithm implemented on the EzBioCloud server (<http://www.ezbiocloud.net/tools/ani>) was used (70). The dDDH values were calculated using the web-based genome-to-genome distance calculator (GGDC) version 2.1 (71), and the recommended formula 2 was taken into account to interpret the results. Whole-genome sequences of related staphylococcal species were obtained from the NCBI database (72–75).

Accession number(s). The GenBank accession number for the 16S rRNA genes of isolate CCM 8730^T is KY315825. The data from WGS of strains *Staphylococcus edaphicus* CCM 8730^T and *Staphylococcus xylosus* CCM 2738^T were recorded in the GenBank WGS project under the accession numbers MRZN00000000 and MRZO00000000.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.01746-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

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I.S. isolated and preliminarily identified the strains. I.S., J.K., and P.P. performed phenotypic characterization. Whole-genome sequencing was performed by V.V., and V.K. and A.I. analyzed the data. Phylogenetic analyses were performed by I.M. and V.V. P.S. performed genotyping by fingerprinting techniques and analyzed the data. S.K. performed FAME analysis. P.P. and L.K. performed MALDI-TOF identification and analyzed the data. R.P. and I.S. designed the study, R.P., I.S., A.I., V.V., I.M., and J.D. wrote the manuscript. All the authors approved the final manuscript.

REFERENCES

- Götz F, Bannerman T, Schleifer K-H. 2006. The genera *Staphylococcus* and *Micrococcus*, p 5–75. In Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E (ed), *The Prokaryotes: Bacteria, Firmicutes, Cyanobacteria*, vol 4. Springer US, New York, NY. https://doi.org/10.1007/0-387-30744-3_1.
- Emmett M, Kloos WE. 1975. Amino acid requirements of staphylococci isolated from human skin. *Can J Microbiol* 21:729–733. <https://doi.org/10.1139/m75-107>.
- Emmett M, Kloos WE. 1979. The nature of arginine auxotrophy in cutaneous populations of staphylococci. *J Gen Microbiol* 110:305–314. <https://doi.org/10.1099/00221287-110-2-305>.
- Lambert LH, Cox T, Mitchell K, Rossello-Mora RA, Del Cueto C, Dodge DE, Orkand P, Cano RJ. 1998. *Staphylococcus succinus* sp. nov., isolated from Dominican amber. *Int J Syst Bacteriol* 48:511–518. <https://doi.org/10.1099/00207713-48-2-511>.
- Hess S, Gallert C. 2015. *Staphylococcus argensis* sp. nov., a hovel staphylococcal species isolated from an aquatic environment. *Int J Syst Evol Microbiol* 65:2661–2665. <https://doi.org/10.1099/ijs.0.000319>.
- Kloos WE. 1980. Natural populations of the genus *Staphylococcus*. *Annu Rev Microbiol* 34:559–592. <https://doi.org/10.1146/annurev.mi.34.100180.003015>.
- Kloos WE, Schleifer KH, Smith RF. 1976. Characterization of *Staphylococcus sciuri* sp. nov. and its subspecies. *Int J Syst Bacteriol* 26:22–37. <https://doi.org/10.1099/00207713-26-1-22>.
- Planchon S, Gaillard-Martinie B, Dordet-Frisoni E, Bellon-Fontaine MN, Leroy S, Labadie J, Hebraud M, Talon R. 2006. Formation of biofilm by *Staphylococcus xylosus*. *Int J Food Microbiol* 109:88–96. <https://doi.org/10.1016/j.jfoodmicro.2006.01.016>.
- Dordet-Frisoni E, Dorchies G, De Araujo C, Talon R, Leroy S. 2007. Genomic diversity in *Staphylococcus xylosus*. *Appl Environ Microbiol* 73:7199–7209. <https://doi.org/10.1128/AEM.01629-07>.
- Irlinger F. 2008. Safety assessment of dairy microorganisms: coagulase-negative staphylococci. *Int J Food Microbiol* 126:302–310. <https://doi.org/10.1016/j.jfoodmicro.2007.08.016>.
- Giordano N, Corallo C, Miracco C, Papakostas P, Montella A, Figura N, Nuti R. 2016. Erythema nodosum associated with *Staphylococcus xylosus* septicemia. *J Microbiol Immunol Infect* 49:134–137. <https://doi.org/10.1016/j.jmii.2012.10.003>.
- Becker K, von Eiff C. 2011. *Staphylococcus*, *Micrococcus*, and other catalase-positive cocci, p 308–330. In Versalovic J, Carroll KC, Funke G, Jorgensen JH, Landry ML, Warnock DW (ed), *Manual of clinical microbiology*, 10th ed. ASM Press, Washington, DC. <https://doi.org/10.1128/9781555816728.ch19>.
- Schleifer KH, Kloos WE. 1975. Isolation and characterization of staphylococci from human skin. 1. Amended descriptions of *Staphylococcus epidermidis* and *Staphylococcus saprophyticus* and descriptions of 3 new species—*Staphylococcus cohnii*, *Staphylococcus haemolyticus*, and *Staphylococcus xylosus*. *Int J Syst Bacteriol* 25:50–61. <https://doi.org/10.1099/00207713-25-1-50>.
- Widerström M, Wiström J, Sjöstedt A, Monsen T. 2012. Coagulase-negative staphylococci: update on the molecular epidemiology and clinical presentation, with a focus on *Staphylococcus epidermidis* and *Staphylococcus saprophyticus*. *Eur J Clin Microbiol Infect Dis* 31:7–20. <https://doi.org/10.1007/s10096-011-1270-6>.
- Takahashi T, Satoh I, Kikuchi N. 1999. Phylogenetic relationships of 38 taxa of the genus *Staphylococcus* based on 16S rRNA gene sequence analysis. *Int J Syst Bacteriol* 49:725–728. <https://doi.org/10.1099/00207713-49-2-725>.
- Kuroda M, Yamashita A, Hirakawa H, Kumano M, Morikawa K, Higashide M, Maruyama A, Inose Y, Matoba K, Toh H, Kuhara S, Hattori M, Ohta T. 2005. Whole genome sequence of *Staphylococcus saprophyticus* reveals the pathogenesis of uncomplicated urinary tract infection. *Proc Natl Acad Sci U S A* 102:13272–13277. <https://doi.org/10.1073/pnas.0502950102>.
- Schleifer K-H, Bell JA. 2009. Genus I. *Staphylococcus* Rosenbach 1884, 18^{AL} (Nom. Cons. Opin 17 Jud. Comm. 1958, 153.), p 392–421. In De Vos P, Garrity GM, Jones D, Krieg NR, Ludwig W, Rainey FA, Schleifer K-H, Whitman WB (ed), *Bergey's manual of systematic bacteriology*, vol 3. The Firmicutes. Springer-Verlag, New York, NY. <https://doi.org/10.1007/978-0-387-68489-5>.

18. Foster TJ, Geoghegan JA, Ganesh VK, Hook M. 2014. Adhesion, invasion and evasion: the many functions of the surface proteins of *Staphylococcus aureus*. *Nat Rev Microbiol* 12:49–62. <https://doi.org/10.1038/nrmicro3161>.
19. Wilson LK, Coombs GW, Christiansen K, Grubb WB, O'Brien FG. 2016. Characterization of a novel staphylococcal cassette chromosome composite island from community-associated MRSA isolated in aged care facilities in Western Australia. *J Antimicrob Chemother* 71:3372–3375. <https://doi.org/10.1093/jac/dkw317>.
20. Ohniwa RL, Kitabayashi K, Morikawa K. 2013. Alternative cardiolipin synthase CIs1 compensates for stalled CIs2 function in *Staphylococcus aureus* under conditions of acute acid stress. *FEMS Microbiol Lett* 338: 141–146. <https://doi.org/10.1111/1574-6968.12037>.
21. Tsai M, Ohniwa RL, Kato Y, Takeshita SL, Ohta T, Saito S, Hayashi H, Morikawa K. 2011. *Staphylococcus aureus* requires cardiolipin for survival under conditions of high salinity. *BMC Microbiol* 11:13. <https://doi.org/10.1186/1471-2180-11-13>.
22. Peleg AY, Miyakis S, Ward DV, Earl AM, Rubio A, Cameron DR, Pillai S, Moellering RC, Jr, Eliopoulos GM. 2012. Whole genome characterization of the mechanisms of daptomycin resistance in clinical and laboratory derived isolates of *Staphylococcus aureus*. *PLoS One* 7:e28316. <https://doi.org/10.1371/journal.pone.0028316>.
23. International Working Group on the Classification of Staphylococcal Cassette Chromosome E. 2009. Classification of staphylococcal cassette chromosome mec (SCCmec): guidelines for reporting novel SCCmec elements. *Antimicrob Agents Chemother* 53:4961–4967. <https://doi.org/10.1128/AAC.00579-09>.
24. García-Alvarez L, Holden MT, Lindsay H, Webb CR, Brown DF, Curran MD, Walpole E, Brooks K, Pickard DJ, Teale C, Parkhill J, Bentley SD, Edwards GF, Girvan EK, Kearns AM, Pichon B, Hill RL, Larsen AR, Skov RL, Peacock SJ, Maskell DJ, Holmes MA. 2011. Methicillin-resistant *Staphylococcus aureus* with a novel *mecA* homologue in human and bovine populations in the UK and Denmark: a descriptive study. *Lancet Infect Dis* 11: 595–603. [https://doi.org/10.1016/S1473-3099\(11\)70126-8](https://doi.org/10.1016/S1473-3099(11)70126-8).
25. Harrison EM, Paterson GK, Holden MT, Morgan FJ, Larsen AR, Petersen A, Leroy S, De Vlieghe S, Perreten V, Fox LK, Lam TJ, Sampimon OC, Zadoks RN, Peacock SJ, Parkhill J, Holmes MA. 2013. A *Staphylococcus xylosus* isolate with a new *mecC* allotype. *Antimicrob Agents Chemother* 57: 1524–1528. <https://doi.org/10.1128/AAC.01882-12>.
26. Malyszko I, Schwarz S, Hauschild T. 2014. Detection of a new *mecC* allotype, *mecC2*, in methicillin-resistant *Staphylococcus saprophyticus*. *J Antimicrob Chemother* 69:2003–2005. <https://doi.org/10.1093/jac/dku043>.
27. Paterson GK, Larsen AR, Robb A, Edwards GE, Pennycott TW, Foster G, Mot D, Hermans K, Baert K, Peacock SJ, Parkhill J, Zadoks RN, Holmes MA. 2012. The newly described *mecA* homologue, *mecA_{LGA251}*, is present in methicillin-resistant *Staphylococcus aureus* isolates from a diverse range of host species. *J Antimicrob Chemother* 67:2809–2813. <https://doi.org/10.1093/jac/dks329>.
28. Monecke S, Gavier-Widen D, Mattsson R, Rangstrup-Christensen L, Lazaris A, Coleman DC, Shore AC, Ehrlich R. 2013. Detection of *mecC*-positive *Staphylococcus aureus* (CC130-MRSA-XI) in diseased European hedgehogs (*Erinaceus europaeus*) in Sweden. *PLoS One* 8:e66166. <https://doi.org/10.1371/journal.pone.0066166>.
29. Loncaric I, Kubber-Heiss A, Posautz A, Stalder GL, Hoffmann D, Rosengarten R, Walzer C. 2013. Characterization of methicillin-resistant *Staphylococcus* spp. carrying the *mecC* gene, isolated from wildlife. *J Antimicrob Chemother* 68:2222–2225. <https://doi.org/10.1093/jac/dkt186>.
30. Porrero MC, Mentaberre G, Sanchez S, Fernandez-Llario P, Casas-Diaz E, Mateos A, Vidal D, Lavin S, Fernandez-Garayzabal JF, Dominguez L. 2014. Carriage of *Staphylococcus aureus* by free-living wild animals in Spain. *Appl Environ Microbiol* 80:4865–4870. <https://doi.org/10.1128/AEM.00647-14>.
31. Porrero MC, Valverde A, Fernandez-Llario P, Diez-Guerrier A, Mateos A, Lavin S, Canton R, Fernandez-Garayzabal JF, Dominguez L. 2014. *Staphylococcus aureus* carrying *mecC* gene in animals and urban wastewater, Spain. *Emerg Infect Dis* 20:899–901. <https://doi.org/10.3201/eid2005.130426>.
32. Babu MM, Priya ML, Selvan AT, Madera M, Gough J, Aravind L, Sankaran K. 2006. A database of bacterial lipoproteins (DOLOP) with functional assignments to predicted lipoproteins. *J Bacteriol* 188:2761–2773. <https://doi.org/10.1128/JB.188.8.2761-2773.2006>.
33. Shahmirzadi SV, Nguyen MT, Götz F. 2016. Evaluation of *Staphylococcus aureus* lipoproteins: role in nutritional acquisition and pathogenicity. *Front Microbiol* 7:1404. <https://doi.org/10.3389/fmicb.2016.01404>.
34. Penadés JR, Christie GE. 2015. The phage-inducible chromosomal islands: a family of highly evolved molecular parasites. *Annu Rev Virol* 2:181–201. <https://doi.org/10.1146/annurev-virology-031413-085446>.
35. Baba T, Takeuchi F, Kuroda M, Yuzawa H, Aoki K, Oguchi A, Nagai Y, Iwama N, Asano K, Naimi T, Kuroda H, Cui L, Yamamoto K, Hiramatsu K. 2002. Genome and virulence determinants of high virulence community-acquired MRSA. *Lancet* 359:1819–1827. [https://doi.org/10.1016/S0140-6736\(02\)08713-5](https://doi.org/10.1016/S0140-6736(02)08713-5).
36. Novick RP, Christie GE, Penadés JR. 2010. The phage-related chromosomal islands of Gram-positive bacteria. *Nat Rev Microbiol* 8:541–551. <https://doi.org/10.1038/nrmicro2393>.
37. Fu Z, Liu Y, Chen C, Guo Y, Ma Y, Yang Y, Hu F, Xu X, Wang M. 2016. Characterization of fosfomycin resistance gene, *fosB*, in methicillin-resistant *Staphylococcus aureus* isolates. *PLoS One* 11:e0154829. <https://doi.org/10.1371/journal.pone.0154829>.
38. Fitzgerald JR, Monday SR, Foster TJ, Bohach GA, Hartigan PJ, Meaney WJ, Smyth CJ. 2001. Characterization of a putative pathogenicity island from bovine *Staphylococcus aureus* encoding multiple superantigens. *J Bacteriol* 183:63–70. <https://doi.org/10.1128/JB.183.1.63-70.2001>.
39. Chanda PK, Mondal R, Sau K, Sau S. 2009. Antibiotics, arsenate and H₂O₂ induce the promoter of *Staphylococcus aureus* *cspC* gene more strongly than cold. *J Basic Microbiol* 49:205–211. <https://doi.org/10.1002/jobm.200800065>.
40. Kahánková J, Pantůček R, Goerke C, Růžicková V, Holochová P, Doškař J. 2010. Multilocus PCR typing strategy for differentiation of *Staphylococcus aureus* siphoviruses reflecting their modular genome structure. *Environ Microbiol* 12:2527–2538. <https://doi.org/10.1111/j.1462-2920.2010.02226.x>.
41. Deghorain M, Bobay LM, Smeesters PR, Bousbata S, Vermeersch M, Perez-Morga D, Dreze PA, Rocha EP, Touchon M, Van Melderen L. 2012. Characterization of novel phages isolated in coagulase-negative staphylococci reveals evolutionary relationships with *Staphylococcus aureus* phages. *J Bacteriol* 194:5829–5839. <https://doi.org/10.1128/JB.01085-12>.
42. Kim C, Milheirico C, Gardete S, Holmes MA, Holden MT, de Lencastre H, Tomasz A. 2012. Properties of a novel PBP2A protein homolog from *Staphylococcus aureus* strain LGA251 and its contribution to the beta-lactam-resistant phenotype. *J Biol Chem* 287:36854–36863. <https://doi.org/10.1074/jbc.M112.395962>.
43. McRae CF, Hocking AD, Seppelt RD. 1999. *Penicillium* species from terrestrial habitats in the Windmill Islands, East Antarctica, including a new species, *Penicillium antarcticum*. *Polar Biol* 21:97–111. <https://doi.org/10.1007/s003000050340>.
44. Montemartini Corte AM, Liotta M, Venturi CB, Calegari L. 2000. Antibacterial activity of *Penicillium* spp. strains isolated in extreme environments. *Polar Biol* 23:294–297. <https://doi.org/10.1007/s003000050447>.
45. Zucconi L, Selbmann L, Buzzini P, Turchetti B, Guglielmin M, Frisvad JC, Onofri S. 2012. Searching for eukaryotic life preserved in Antarctic permafrost. *Polar Biol* 35:749–757. <https://doi.org/10.1007/s00300-011-1119-6>.
46. Sonjak S, Frisvad JC, Gunde-Cimerman N. 2006. *Penicillium* mycobiota in arctic subglacial ice. *Microb Ecol* 52:207–216. <https://doi.org/10.1007/s00248-006-9086-0>.
47. Godinho VM, Gonçalves VN, Santiago IF, Figueredo HM, Vitoreli GA, Schaefer CE, Barbosa EC, Oliveira JG, Alves TM, Zani CL, Junior PA, Murta SM, Romanha AJ, Kroon EG, Cantrell CL, Wedge DE, Duke SO, Ali A, Rosa CA, Rosa LH. 2015. Diversity and bioprospection of fungal community present in oligotrophic soil of continental Antarctica. *Extremophiles* 19:585–596. <https://doi.org/10.1007/s00792-015-0741-6>.
48. Brunati M, Rojas JL, Sponga F, Ciciliato I, Losi D, Gottlieb E, de Hoog S, Genilloud O, Marinelli F. 2009. Diversity and pharmaceutical screening of fungi from benthic mats of Antarctic lakes. *Mar Genomics* 2:43–50. <https://doi.org/10.1016/j.margen.2009.04.002>.
49. Goris J, Konstantinidis KT, Klappenbach JA, Coenye T, Vandamme P, Tiedje JM. 2007. DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. *Int J Syst Evol Microbiol* 57:81–91. <https://doi.org/10.1099/ijs.0.64483-0>.
50. Pantůček R, Švec P, Dajcs JJ, Machová I, Černohlávková J, Šedo O, Gelbíčová T, Mašláňová I, Doškař J, Zdráhal Z, Růžicková V, Sedláček I. 2013. *Staphylococcus petrasii* sp. nov. including *S. petrasii* subsp. *petrasii* subsp. nov. and *S. petrasii* subsp. *croceilyticus* subsp. nov., isolated from human clinical specimens and human ear infections. *Syst Appl Microbiol* 36:90–95. <https://doi.org/10.1016/j.syapm.2012.11.004>.

51. Mannerová S, Pantůček R, Doškař J, Švec P, Snauwaert C, Vancanneyt M, Swings J, Sedláček I. 2003. *Macrocooccus brunensis* sp. nov., *Macrocooccus hajekii* sp. nov. and *Macrocooccus lamae* sp. nov., from the skin of llamas. *Int J Syst Evol Microbiol* 53:1647–1654. <https://doi.org/10.1099/ijs.0.02683-0>.
52. The European Committee on Antimicrobial Susceptibility Testing. 2017. Breakpoint tables for interpretation of MICs and zone diameters, version 7.1. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_7.1_Breakpoint_Tables.pdf.
53. CLSI. 2015. Performance standards for antimicrobial disk susceptibility tests; approved standard, 12th ed. CLSI document M02-A12. Clinical and Laboratory Standards Institute, Wayne, PA. <https://clsi.org/standards/products/microbiology/documents/m02/>.
54. Sasser M. 1990. Identification of bacteria by gas chromatography of cellular fatty acids. MIDI technical note 101. Microbial ID, Inc., Newark, DE.
55. Švec P, Pantůček R, Petráš P, Sedláček I, Nováková D. 2010. Identification of *Staphylococcus* spp. using (GTG)₅-PCR fingerprinting. *Syst Appl Microbiol* 33:451–456. <https://doi.org/10.1016/j.syapm.2010.09.004>.
56. Pantůček R, Götz F, Doškař J, Rosypal S. 1996. Genomic variability of *Staphylococcus aureus* and the other coagulase-positive *Staphylococcus* species estimated by macrorestriction analysis using pulsed-field gel electrophoresis. *Int J Syst Bacteriol* 46:216–222. <https://doi.org/10.1099/00207713-46-1-216>.
57. Pantůček R, Sedláček I, Petráš P, Koukalová D, Švec P, Štětina V, Vancanneyt M, Chrástková L, Vokurková J, Růžicková V, Doškař J, Swings J, Hájek V. 2005. *Staphylococcus simiae* sp. nov., isolated from South American squirrel monkeys. *Int J Syst Evol Microbiol* 55:1953–1958. <https://doi.org/10.1099/ijs.0.63590-0>.
58. Nurk S, Bankevich A, Antipov D, Gurevich AA, Korobeynikov A, Lapidus A, Prjibelski AD, Pyshkin A, Sirotkin A, Sirotkin Y, Stepanauskas R, Clingenpeel SR, Woyke T, Mclean JS, Lasken R, Tesler G, Alekseyev MA, Pevzner PA. 2013. Assembling single-cell genomes and mini-metagenomes from chimeric MDA products. *J Comput Biol* 20:714–737. <https://doi.org/10.1089/cmb.2013.0084>.
59. Rissman AI, Mau B, Biehl BS, Darling AE, Glasner JD, Perna NT. 2009. Reordering contigs of draft genomes using the Mauve aligner. *Bioinformatics* 25:2071–2073. <https://doi.org/10.1093/bioinformatics/btp356>.
60. Wick RR, Schultz MB, Zobel J, Holt KE. 2015. Bandage: interactive visualization of de novo genome assemblies. *Bioinformatics* 31:3350–3352. <https://doi.org/10.1093/bioinformatics/btv383>.
61. Okonechnikov K, Golosova O, Fursov M, UGENE Team. 2012. Unipro UGENE: a unified bioinformatics toolkit. *Bioinformatics* 28:1166–1167. <https://doi.org/10.1093/bioinformatics/bts091>.
62. Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, Formsma K, Gerdes S, Glass EM, Kubal M, Meyer F, Olsen GJ, Olson R, Osterman AL, Overbeek RA, McNeil LK, Paarmann D, Paczian T, Parrello B, Pusch GD, Reich C, Stevens R, Vassieva O, Vonstein V, Wilke A, Zagnitko O. 2008. The RAST Server: rapid annotations using subsystems technology. *BMC Genomics* 9:75. <https://doi.org/10.1186/1471-2164-9-75>.
63. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. *J Mol Biol* 215:403–410. [https://doi.org/10.1016/S0022-2836\(05\)80360-2](https://doi.org/10.1016/S0022-2836(05)80360-2).
64. Siguer P, Perochon J, Lestrade L, Mahillon J, Chandler M. 2006. ISfinder: the reference centre for bacterial insertion sequences. *Nucleic Acids Res* 34:D32–D36. <https://doi.org/10.1093/nar/gkj014>.
65. Jones P, Binns D, Chang HY, Fraser M, Li W, McAnulla C, McWilliam H, Maslen J, Mitchell A, Nuka G, Pesseat S, Quinn AF, Sangrador-Vegas A, Scheremetjew M, Yong SY, Lopez R, Hunter S. 2014. InterProScan 5: genome-scale protein function classification. *Bioinformatics* 30:1236–1240. <https://doi.org/10.1093/bioinformatics/btu031>.
66. Marchler-Bauer A, Derbyshire MK, Gonzales NR, Lu S, Chitsaz F, Geer LY, Geer RC, He J, Gwadz M, Hurwitz DI, Lanczycki CJ, Lu F, Marchler GH, Song JS, Thanki N, Wang Z, Yamashita RA, Zhang D, Zheng C, Bryant SH. 2015. CDD: NCBI's conserved domain database. *Nucleic Acids Res* 43:D222–D226. <https://doi.org/10.1093/nar/gku1221>.
67. Wang Y, Coleman-Derr D, Chen G, Gu YQ. 2015. OrthoVenn: a web server for genome wide comparison and annotation of orthologous clusters across multiple species. *Nucleic Acids Res* 43:W78–W84. <https://doi.org/10.1093/nar/gkv487>.
68. Lagesen K, Hallin P, Rodland EA, Staerfeldt HH, Rognes T, Ussery DW. 2007. Rfam: consistent and rapid annotation of ribosomal RNA genes. *Nucleic Acids Res* 35:3100–3108. <https://doi.org/10.1093/nar/gkm160>.
69. Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol Biol Evol* 33:1870–1874. <https://doi.org/10.1093/molbev/msw054>.
70. Lee I, Kim YO, Park SC, Chun J. 2015. OrthoANI: an improved algorithm and software for calculating average nucleotide identity. *Int J Syst Evol Microbiol* <https://doi.org/10.1099/ijsem.0.000760>.
71. Meier-Kolthoff JP, Auch AF, Klenk HP, Goker M. 2013. Genome sequence-based species delimitation with confidence intervals and improved distance functions. *BMC Bioinformatics* 14:60. <https://doi.org/10.1186/1471-2105-14-60>.
72. Calcutt MJ, Foecking MF, Hsieh HY, Perry J, Stewart GC, Middleton JR. 2013. Genome sequence analysis of *Staphylococcus equorum* bovine mastitis isolate UMC-CNS-924. *Genome Announc* 1:e00840-13. <https://doi.org/10.1128/genomeA.00840-13>.
73. Zhou H, Yao Z, Shi H, Wang B, Li D, Hou J, Ma S. 2017. Draft genome sequence of *Staphylococcus succinus* subsp. *succinus* type strain DSM 14617, isolated from plant and soil inclusions within 25- to 35-million-year-old Dominican amber. *Genome Announc* 5:e01521-16. <https://doi.org/10.1128/genomeA.01521-16>.
74. Shi D, Fang D, Hu X, Li A, Lv L, Guo J, Chen Y, Wu W, Guo F, Li L. 2015. Draft genome sequence of *Staphylococcus gallinarum* DSM 20610^T, originally isolated from the skin of a chicken. *Genome Announc* 3:e00580-15. <https://doi.org/10.1128/genomeA.00580-15>.
75. Shiroma A, Terabayashi Y, Nakano K, Shimoji M, Tamotsu H, Ashimine N, Ohki S, Shinzato M, Teruya K, Satou K, Hirano T. 2015. First complete genome sequences of *Staphylococcus aureus* subsp. *aureus* Rosenbach 1884 (DSM 20231^T), determined by PacBio single-molecule real-time technology. *Genome Announc* 3:e00800-15. <https://doi.org/10.1128/genomeA.00800-15>.

4.2 Description and Comparative Genomics of *Macrococcus caseolyticus* subsp. *hominis* subsp. nov., *Macrococcus goetzii* sp. nov., *Macrococcus epidermidis* sp. nov., and *Macrococcus bohemicus* sp. nov., Novel *Macrococci* From Human Clinical Material With Virulence Potential and Suspected Uptake of Foreign DNA by Natural Transformation

Background and objectives: *Macrococcus* is a genus closely related to staphylococci and the genus *Mammaliicoccus*. *Macrococci* are primarily found in animals and are known to cause veterinary infections. Like staphylococci, they can carry *SCCmec* with *mecB* or another genomic island with the *mecD* gene, conferring resistance to beta-lactam antibiotics. This study examines seven strains of *Macrococcus* sp. that were found in human vaginal smears, wounds, urine, skin, and nails in Czech Republic hospitals between 2000 and 2003. The objective was to investigate the genus *Macrococcus* genomic properties and determine their pathogenicity potential.

Material and methods: The strains CCM 7927^T, CCM 7928, P862, P865, CCM 4927^T, CCM 7099^T, CCM 7099^T, and CCM 7100^T were examined using relevant phenotypic, genotypic, and chemotaxonomic methods. Four types of strains were sequenced on the Ion Torrent platform. Additionally, several prophages were induced and analyzed.

Results and discussion: Polyphasic analysis consistently divided examined strains into two distinct clusters within the genus *Macrococcus*. The group 1 isolates were determined as novel subspecies of *M. caseolyticus* subsp. *hominis*. The group 2 strains are characterized by their structural plasticity, larger genomes, and the presence of several genome islands, including unique SCC-like elements that harbor putative virulence and resistance genes. We hypothesize it is due to the presence of *comG* operon and *dprA* gene, which are associated with natural transformation by exogenous DNA. However, the presence of a new variant of the CRISPR-Cas system with an unusually high number of 90 spacers suggests that the strain CCM 7100^T is also capable of limiting the uptake of foreign DNA.

Conclusion: The study identified novel subspecies, *M. caseolyticus* subsp. *hominis*, and three novel species, *M. goetzii*, *M. epidermidis*, and *M. bohemicus*. Putative virulence and resistance factors were identified, but further studies are needed to investigate their true role. An amended description of the *Macrococcus* genus is also provided.

Significance: This is the first report of *macrococci* isolated from humans. Their highly dynamic genome structure suggests the importance of horizontal gene acquisition in the evolution of pathogens.



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Description and Comparative Genomics of *Macrococcus caseolyticus* subsp. *hominis* subsp. nov., *Macrococcus goetzii* sp. nov., *Macrococcus epidermidis* sp. nov., and *Macrococcus bohemicus* sp. nov., Novel *Macrococci* From Human Clinical Material With Virulence Potential and Suspected Uptake of Foreign DNA by Natural Transformation

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The genus *Macrococcus* is a close relative of the genus *Staphylococcus*. Whilst staphylococci are widespread as human pathogens, macrococci have not yet been reported from human clinical specimens. Here we investigated Gram-positive and catalase-positive cocci recovered from human clinical material and identified as *Macrococcus* sp. by a polyphasic taxonomic approach and by comparative genomics. Relevant phenotypic, genotypic and chemotaxonomic methods divided the analyzed strains into two separate clusters within the genus *Macrococcus*. Comparative genomics of four representative strains revealed enormous genome structural plasticity among the studied isolates. We hypothesize that high genomic variability is due to the presence of a *com* operon, which plays a key role in the natural transformation of bacilli and streptococci. The possible uptake of exogenous DNA by macrococci can contribute to a different mechanism of evolution from staphylococci, where phage-mediated horizontal gene transfer predominates. The described macrococcal genomes harbor

novel plasmids, genomic islands and islets, as well as prophages. Capsule gene clusters, intracellular protease, and a fibronectin-binding protein enabling opportunistic pathogenesis were found in all four strains. Furthermore, the presence of a CRISPR-Cas system with 90 spacers in one of the sequenced genomes corresponds with the need to limit the burden of foreign DNA. The highly dynamic genomes could serve as a platform for the exchange of virulence and resistance factors, as was described for the methicillin resistance gene, which was found on the novel composite SCC*mec*-like element containing a unique *mec* gene complex that is considered to be one of the missing links in SCC evolution. The phenotypic, genotypic, chemotaxonomic and genomic results demonstrated that the analyzed strains represent one novel subspecies and three novel species of the genus *Macrococcus*, for which the names *Macrococcus caseolyticus* subsp. *hominis* subsp. nov. (type strain CCM 7927^T = DSM 103682^T), *Macrococcus goetzii* sp. nov. (type strain CCM 4927^T = DSM 103683^T), *Macrococcus epidermidis* sp. nov. (type strain CCM 7099^T = DSM 103681^T), and *Macrococcus bohemicus* sp. nov. (type strain CCM 7100^T = DSM 103680^T) are proposed. Moreover, a formal description of *Macrococcus caseolyticus* subsp. *caseolyticus* subsp. nov. and an emended description of the genus *Macrococcus* are provided.

Keywords: *Macrococcus*, Gram-positive pathogens, prokaryotic transformation, methicillin resistance, Staphylococcal Cassette Chromosome (SCC), CRISPR-Cas, bacteriophage, plasmids

INTRODUCTION

The genus *Macrococcus*, the closest relative of staphylococci, is currently comprised of eight species (Parte, 2014) which are commonly isolated from animal skin (ponies, horses, cows, llamas, and dogs), from milk or meat products (Kloos et al., 1998; Mannerová et al., 2003; Gobeli Brawand et al., 2017). The phylogenetic relationship to the genus *Staphylococcus* was proven by a genomic study that shows that almost 65% of the predicted genes of *Macrococcus caseolyticus* have the highest homology with genes of staphylococci, and the two genera share many metabolic pathways (Baba et al., 2009). Phylogenetically and genotypically, macrococci are similar to oxidase-positive, novobiocin resistant staphylococci from the *Staphylococcus sciuri* complex (Götz et al., 2006; Švec et al., 2016; Christo-Foroux et al., 2017).

Although none of the species of the genus *Macrococcus* are thought to be human pathogens, there are reports of macrococci associated with animal infections (Gómez-Sanz et al., 2015; Hansen et al., 2015; Cotting et al., 2017). These findings suggest the possible pathogenicity of macrococci. Furthermore, the identification of methicillin-resistant strains of macrococci carrying the staphylococcal cassette chromosome (SCC) in their genome reveals a potential broad host-range dissemination of this resistance element between the genera *Macrococcus* and *Staphylococcus* (Baba et al., 2009; Rubin and Chirino-Trejo, 2010; Tsubakishita et al., 2010; Cicconi-Hogan et al., 2014; Gómez-Sanz et al., 2015; Micheel et al., 2015).

SCC carrying a *mec* complex with *mecA* or *mecC* genes has been described in staphylococci (Paterson et al., 2014; Pantuček et al., 2017). The methicillin resistance of macrococci has been associated with the presence of the *mecB* gene, a

distantly related *mecA* homolog. Tsubakishita et al. (2010) identified the *mec* complex (*mecI*–*mecRI*–*mecB*–*blaZ*) to be part of transposon *Tn6045* integrated downstream of the *orfX* gene on the chromosome of *M. caseolyticus* JCSC7096. The adjacent chromosome cassette recombinase (*ccr*) complex co-excises together with the *mec* complex to form an extrachromosomal SCC*mec* element. The potential for horizontal gene transfer (HGT) is also supported by the identification of the *mec* complex-carrying transposon *Tn6045* on plasmid pMCCL2 (Baba et al., 2009). Similar elements were described in other *M. caseolyticus* strains (Tsubakishita et al., 2010) and an SCC*mec* element independent of *Tn6045* was identified in a chromosome of the recently described *Macrococcus canis* (Gómez-Sanz et al., 2015; Gobeli Brawand et al., 2018).

A new allele of the *mec* gene (*mecD*) conferring resistance to all β-lactam antibiotics, including cephalosporins that are used to treat MRSA infections, was recently discovered in a methicillin-resistant veterinary strain *M. caseolyticus* IMD0819 (Schwendener et al., 2017). The *mecD* gene was localized on a genomic resistance island, whose structure resembles the *Staphylococcus aureus* resistance island. The genomic island contains an integrase gene that shares a close common ancestor with integrases of the genus *Bacillus*. This might indicate possible HGT between species of the families *Bacillaceae* and *Staphylococcaceae* (Schwendener et al., 2017).

Despite the evidence that macrococci take part in HGT and might serve as a reservoir of resistance and virulence determinants for veterinary pathogenic bacteria, very little is known about their genome plasticity and mobile genetic elements (MGE). So far no macrococci from human clinical specimens have been described. In this study, a polyphasic taxonomic approach using phenotypic and genotypic methods has been

used to clarify the taxonomic position of seven *Micrococcus* sp. isolates originating from human clinical material. In four strains, the whole genome sequences were obtained and analyzed with a focus on various MGE and possible virulence factors. In addition, this work contributes to our understanding of the evolutionary relationships of bacterial pathogens in the family *Staphylococcaceae*.

MATERIALS AND METHODS

Bacterial Strains

Seven Gram-stain positive, catalase-positive cocci isolated from human clinical material in different routine clinical laboratories (Table 1) were sent to the Reference Laboratory for Staphylococci (Prague, Czech Republic) for identification. Cells grown on TSA agar (Oxoid) were used for biotyping and genotyping analyses. All isolates were maintained on glass beads at -70°C in BHI broth supplemented with 15% glycerol (v/v). Type strains representing known *Micrococcus* species and reference *M. caseolyticus* strains CCM 8657, CCM 8658, P262, and P885 were obtained from the Czech Collection of Microorganisms (Masaryk University, Brno). *Micrococcus canis* DSM 101690^T (= KM45013^T) was obtained from the Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) and deposited in the Czech Collection of Microorganisms under strain number CCM 8748^T in this work.

Phenotypic Characterization

Extensive phenotypic characterization using API 50CH, API ID 32 Staph and API ZYM (bioMérieux) commercial kits, phenotypic fingerprinting using the Biolog system with the GP2 MicroPlate identification test panel (Biolog) and conventional biochemical, physiological and growth tests relevant for the genus *Staphylococcus* were done as described previously (Mannerová et al., 2003; Pantůček et al., 2005, 2013). Haemolysis was examined on Columbia blood agar (Oxoid) supplemented with 7% sheep blood at 37°C (pH 7.2) after 24 h of incubation in a synergy test with the β -haemolysin-producing strain

Staphylococcus pseudintermedius CCM 4710. The antibiotic resistance pattern was tested by the disc diffusion method on Mueller-Hinton agar (Oxoid). Antimicrobial susceptibility disks generally used for Gram-positive cocci were applied: ampicillin (10 μg), cefoxitin (30 μg), ceftazidime (10 μg), cephalothin (30 μg), ciprofloxacin (5 μg), clindamycin (2 μg), erythromycin (15 μg), gentamicin (10 μg), chloramphenicol (30 μg), imipenem (10 μg), kanamycin (30 μg), neomycin (10 μg), novobiocin (5 μg), oxacillin (1 μg), penicillin G (1 IU), cotrimoxazole (25 μg), tetracycline (30 μg), vancomycin (30 μg). EUCAST/CLSI standards were strictly followed for cultivation and inhibition zone diameter readings (CLSI, 2015; EUCAST, 2017).

Transmission Electron Microscopy

The surface of plates containing bacterial cultures were washed off and resuspended in distilled water. A 200-mesh formvar-coated grid was placed on the drop of suspension for 20 min. Bacterial cells located on the grid were negatively-stained with 2% ammonium molybdate and treated with UV light. A T Morgagni 268D Philips (FEI Company) transmission electron microscope was used to visualize bacterial cells.

Phage lysate obtained after UV-light induction was centrifuged at $4,500 \times g$ for 30 min at 4°C and filtered through $0.45 \mu\text{m}$ pore-sized polyethersulfone syringe filters (Techno Plastic Products) to remove bacterial debris. Phages were pelleted by centrifugation at $54,000 \times g$ for 2.5 h at 4°C in a JA-30.50 Ti rotor (Beckman). Phage pellet resuspended in phage buffer (50 mM Tris.Cl pH 8.0, 10 mM CaCl_2 , 10 mM NaCl) was overlaid onto a preformed CsCl (Sigma) density gradient (1 mL of each 1.45 g/mL 1.50 g/mL, 1.70 g/mL of CsCl in phage buffer) and centrifuged at $194,000 \times g$ for 4 h at 12°C using a SW 55 Ti rotor (Beckman). The fraction containing phages was collected and dialyzed against an excess of phage buffer at 4°C . For electron microscopy the sample was 10x concentrated by centrifugation, fixed by 2.5% (w/v) glutaraldehyde and deposited for 2 min on carbon-coated 400 mesh copper grids. The grids were washed twice on a drop of deionized water for 10 s and stained with 2% (w/v) uranyl acetate. The samples

TABLE 1 | Source of human *Micrococcus* spp. strains characterized in this study.

Species/subspecies	Strain number	Year of isolation	Locality in the Czech Republic	Specimen	Patients' age range (years)	Diagnosis
<i>M. caseolyticus</i> subsp. <i>hominis</i> subsp. nov.	CCM 7927 ^T	2003	Příbram	Vaginal smear	40–45	Acute vaginitis
<i>M. caseolyticus</i> subsp. <i>hominis</i> subsp. nov.	CCM 7928	2003	Příbram	Wound smear	30–35	A wound from surgery after ankle fracture
<i>M. caseolyticus</i> subsp. <i>hominis</i> subsp. nov.	P862	2003	Příbram	Vaginal smear	40–45	Chronic vulvitis
<i>M. caseolyticus</i> subsp. <i>hominis</i> subsp. nov.	P865	2003	Příbram	Urine	66–70	Cervicitis
<i>M. goetzii</i> sp. nov.	CCM 4927 ^T	2000	České Budějovice	Nail feet smear	30–35	Mycose
<i>M. epidermidis</i> sp. nov.	CCM 7099 ^T	2001	České Budějovice	Skin smear	66–70	Mycose
<i>M. bohemicus</i> sp. nov.	CCM 7100 ^T	2003	Strakonice	Knee wound	80–85	A traumatic wound

were analyzed by Tecnai F20 (Thermo Scientific) transmission electron microscope operated at 120 kV.

Phylogenetic Analysis Using 16S rRNA and Housekeeping Genes

The extraction of genomic DNA by phenol-chloroform from cells treated with mutanolysin (Sigma-Aldrich), lysostaphin (Ambi Products LLC), and achromopeptidase (Sigma-Aldrich) was done as described previously (Gevers et al., 2001). Initial 16S rRNA gene amplification by PCR and partial 16S rRNA gene sequencing was performed as described previously by Mannerová et al. (2003). The complete 16S rRNA gene sequence was subsequently extracted from whole-genome shotgun (WGS) data using RNAmmer version 1.2 (Lagesen et al., 2007) and compared with those of other taxa of the genus *Macrococcus*, available in the GenBank database. Pairwise sequence alignment and calculation of similarity values was carried out by a global alignment algorithm, implemented at the EzBioCloud database (Yoon et al., 2017). The evolutionary history was inferred by the neighbor-joining and maximum likelihood methods using bootstrap values based on 1,000 replications with the software MEGA version 7 (Kumar et al., 2016). In addition, phylogenetic analysis was carried out using concatenated partial gene sequences and the corresponding proteins of the *hsp60*, *rpoB*, *tuf*, *dnaJ*, *gap*, and *sod* genes extracted from whole-genome sequencing data of representative strains analyzed in this study and reference sequences of *M. canis* CCM 8748^T (Gobeli Brawand et al., 2018) and *S. sciuri* DSM 20345^T (GenBank accession no. LEOS01000000).

MALDI-TOF MS Analysis

The samples were prepared by the standard extraction protocol described in detail by Freiwald and Sauer (2009). Approximately 5–10 mg of each culture was treated with 1.2 ml of 75% ethanol. After centrifugation and removal of the supernatant, cells were extracted with 25 µl of 70% formic acid followed by the addition of 25 µl of acetonitrile and vortexing at 2,000 rpm for 1 min. The supernatant after centrifugation was deposited in three positions (wells) of the sample plate in volumes of 0.3 µl and, after drying at room temperature, overlaid with 0.3 µl of the MALDI matrix solution. For identification purposes, alpha-cyano-4-hydroxycinnamic acid (CHCA) in water:acetonitrile:TFA (47.5:50:2.5, v/v) mixture was used as a MALDI matrix. For obtaining signals used for cluster analysis, the matrix solution was composed of 12.5 mg/ml ferulic acid in water:acetonitrile:formic acid (50:33:17, v/v), as proposed by Madonna et al. (2000). MALDI-TOF mass spectra were obtained using an Ultraflexxtreme instrument (Bruker Daltonics) operated in linear positive mode under FlexControl version 3.4 software. Nine independent mass spectra were acquired per sample (three mass spectra per well). Signals present in at least seven out of the nine mass spectra were taken into account. Mass spectra were processed using FlexAnalysis (version 3.4; Bruker Daltonics) and BioTyper software (version 3.1; Bruker Daltonics) supplemented with a database of 6903 reference entries (version 7.0.0.0). The mutual similarity between individual mass spectra of the strains and the BioTyper database entries were expressed in the form of

log(scores) obtained using the default settings of the BioTyper software. A MALDI-TOF mass spectra-based dendrogram was constructed using the Pearson's product moment coefficient as a measure of similarity and the unweighted pair group average linked method (UPGMA) as a grouping method.

Whole Cell Protein Fingerprinting

A comparison of whole-cell protein profiles was carried out using an Agilent 2100 Bioanalyzer system with a Protein 230 kit (Agilent Technologies) and numerical analysis of the obtained fingerprints with the software BioNumerics version 7.6 (Applied Maths) was performed as described previously (Švec et al., 2015).

Genotypic Analysis by Fingerprinting Techniques

Rep-PCR fingerprinting with the (GTG)₅ primer was performed as described previously (Švec et al., 2010). The automatic ribotyping with the *EcoRI* restriction endonuclease was performed using the RiboPrinter Microbial Characterization System (DuPont Qualicon) in accordance with the manufacturer's instructions. Numerical analysis of obtained fingerprints and dendrograms construction was done using the software BioNumerics version 7.6. The ribotype patterns were imported into BioNumerics using the load samples import script provided by the manufacturer. Pulsed-field gel electrophoresis (PFGE) using *SmaI* macrorestriction pattern analysis was performed as described by Pantuček et al. (1996).

Chemotaxonomic Analyses

The fatty acids methyl esters analysis (FAME) was performed with cells growing on BBL Tryptone Soya Agar (TSA) plates (Becton, Dickinson and Company) at 37°C for 24 hrs. The extraction of fatty acid methyl esters was performed according to the standard protocol (Sasser, 1990). Cellular fatty acid extracts were analyzed with an Agilent 7890B gas chromatograph by using the Sherlock Identification System (MIS, version 6.2B, MIDI database: RTSBA 6.21, MIDI Inc.).

Biomass and freeze-dried cells for chemotaxonomic studies were obtained after growing on TSA (Oxoid) at 36.5°C for 24 h. Peptidoglycan was isolated and its structure was analyzed according to published protocols (Schumann, 2011). Menaquinones were extracted from lyophilized cells according to the method of Collins et al. (1977) and analyzed by HPLC (Shimadzu LC 20A) as reported earlier (Groth et al., 1996).

Genome Sequencing and Bioinformatics Analysis

The concentration of extracted DNA was estimated with a Qubit 2.0 Fluorometer using a Qubit dsDNA BR assay kit (Invitrogen). WGS sequencing was performed using an Ion TorrentTM Personal Genome Machine (Thermo Fisher Scientific). The purified genomic DNA was used for preparing a 400-bp sequencing library with an Ion Plus Fragment Library Kit (Thermo Fisher Scientific). The sample was loaded onto a 316v2 chip and sequenced using an Ion PGM Hi-Q View sequencing kit (Thermo Fisher Scientific). Quality trimming of the reads was performed with the Ion Torrent Suite Software

version 5.0.4 with default settings. The assembly computation and error correction was performed using the Assembler SPAdes version 3.11.1 pipeline (Nurk et al., 2013) with recommended parameters for Ion Torrent data. Contigs were then re-ordered according to the reference genome *M. canis* CCM 8748^T (Gobeli Brawand et al., 2018) using MauveContigMover (Rissman et al., 2009). Ordering was evaluated using assembly graph visualized in Bandage (Wick et al., 2015). The plasmid contigs were extracted from WGS data based on higher coverage and content of typical plasmid-borne genes. The plasmid DNA was extracted using the NucleoSpin[®] Plasmid Kit (Macherey-Nagel) according to the manufacturer's protocol with the modification that bacteria were lysed with lysostaphin (30 µg/ml, Ambi Products LLC) and with 4U of mutanolysin (Sigma-Aldrich) at 37°C. The complete plasmid sequencing was achieved by a primer-walking approach. Sequences were manipulated and inspected in the cross-platform bioinformatics software Ugene version 1.28.0 (Okonechnikov et al., 2012). For primal analysis, the genome was annotated using RAST (Aziz et al., 2008). Gene content was further examined by Protein BLAST (<https://blast.ncbi.nlm.nih.gov>), ISfinder (Siguier et al., 2006), IslandViewer 4 (Dhillon et al., 2015), and InterProScan (Jones et al., 2014). Protein sequences predicted by RAST annotation were clustered using web-based OrthoVenn with a default cutoff e-value of $1e^{-5}$ and inflation value of 1.5 (Wang et al., 2015), and were searched against the virulence factors database VFDB using an e-value of $1e^{-4}$ (Chen et al., 2016). The presence of CRISPR-Cas systems were tested using CRISPRone (Zhang and Ye, 2017).

DNA Homology Studies

Cells were disrupted by using a Constant Systems TS 0.75 KW (IUL Instruments) and the DNA in the crude lysate was purified by chromatography on hydroxyapatite as described by Cashion et al. (1977). DNA-DNA hybridization (DDH) was carried out as described by De Ley et al. (1970) taking into account the modifications described by Huss et al. (1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with *in-situ* temperature probe (Varian). To evaluate the genomic similarity among type strains, an average nucleotide identity (ANI) and a digital DDH (dDDH) were determined. The dDDH values were calculated using web-based genome-to-genome distance calculator (GGDC) version 2.1 (Meier-Kolthoff et al., 2013). Cluster analysis based on ANI values and a heat-map of ANI identity were obtained using the Python module PYANI script (Pritchard et al., 2016).

Bacteriophage Analyses

The prophages were identified from WGS data by PHASTER (Arndt et al., 2016). Phage induction by UV-irradiation and/or mitomycin C, vertical one-dimensional SDS-PAGE and LC-MS/MS identification of capsid proteins were performed according to Zeman et al. (2017).

GenBank Accession Numbers

The GenBank accession numbers for the 16S rRNA genes of isolates CCM 7927^T, CCM 7928, P862, P865, CCM 4927^T, CCM

7099^T, and CCM 7100^T are MH044686-MH044692. The data from Whole Genome Shotgun projects of strains CCM 3540^T, CCM 7927^T, CCM 4927^T, CCM 7099^T, and CCM 7100^T have been deposited at DDBJ/ENA/GenBank under the accession numbers: PZJF000000000, MJB100000000, MJB000000000, PZJH000000000, and PZJG000000000, respectively, and annotated by the NCBI Prokaryotic Genome Annotation Pipeline.

RESULTS AND DISCUSSION

Phenotypic Characterization

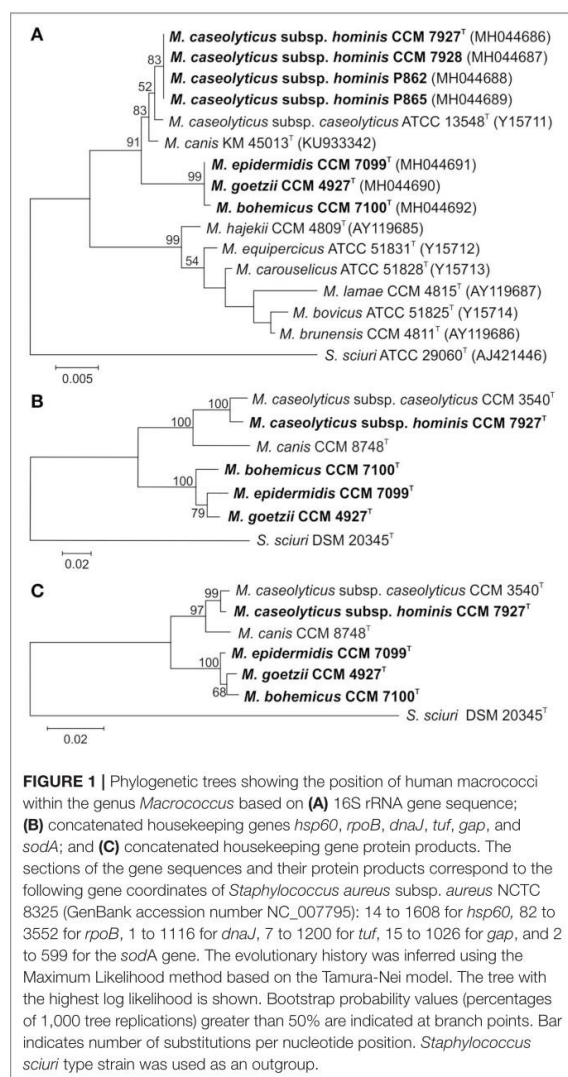
All seven isolates originating from human clinical specimens (Table 1) were observed to be spherical or slightly irregular cocci, occurring predominantly in pairs and clusters, non-spore forming and non-motile. The measured values of their cell size ranged from 1.02 to 1.14 µm in diameter based on transmission electron microscopy (TEM) (Figure S1), i.e., cells that are larger than those of staphylococci. Microscopic and macroscopic morphology and the results of key biochemical and physiological tests relevant for Gram-positive cocci, namely positive catalase and oxidase activity and growth in the presence of 7% NaCl, resistance to bacitracin and novobiocin, but susceptibility to furazolidone and negative growth in the presence of 10% NaCl presumptively identified all seven isolates as *Macrococcus* sp.

Phylogenetic Analyses

The obtained 16S rRNA gene sequences (Figure 1A) and multilocus sequence analyses (MLSA) based on 6 concatenated housekeeping genes, *hsp60*, *rpoB*, *tuf*, *gap*, *sodA*, and *dnaJ* (Figure 1B), and their protein products (Figure 1C) confirmed the phylogenetic position of the isolates within the genus *Macrococcus* and divided the strains into two separate groups. The phylogenetically closest known related species of both groups were strains from a phylogenetic clade including *M. caseolyticus* and *M. canis* (Figure 1). Strains CCM 7927^T, CCM 7928, P862, and P865 representing group 1 had 100% reciprocal identity and 99.8% identity with the 16S rRNA sequence of *M. caseolyticus* CCM 3540^T and *M. canis* CCM 8748^T. Group 2 formed a separate branch in the MLSA and included isolates CCM 4927^T and CCM 7099^T, which have identical 16S rRNA gene sequences and are closely related to strain CCM 7100^T, with 99.9% 16S rRNA gene sequence similarity. The next closest relatives of the group 2 strains were strain CCM 7927^T (99.1%), *M. caseolyticus* CCM 3540^T (99.0%) and *M. canis* CCM 8748^T (98.9%). Despite the high 16S rRNA gene sequence similarities, the MLSA data unambiguously confirmed the differentiation of the new isolates from the phylogenetically closely related known species *M. caseolyticus* and *M. canis* and differentiated all strains of group 2 from each other (Figures 1B,C).

MALDI-TOF MS Identification and Whole Cell Protein Profiling

The analyzed strains and reference type strains of all validly named *Macrococcus* spp. were subjected to a standard MALDI-TOF MS identification procedure including protein extraction, sample deposition with CHCA as the MALDI matrix, and



comparison with a commercially available database (BioTyper, version 7.0.0.0; currently, from *Macrococcus* spp., only entries of *M. caseolyticus* exist in the database). Only strain CCM 3540^T yielded a log(score) greater than 2.000, indicating *M. caseolyticus* species identification with a high confidence. All group 1 strains produced scores between 1.700 and 1.999, which correspond to *M. caseolyticus* species identification with low confidence. The remaining strains did not yield mass spectra with significant similarity to any of the BioTyper database entries [log(scores) lower than 1.699]. The outputs of the cluster analysis based on signals obtained by MALDI-TOF MS analysis using ferulic acid as the MALDI matrix correlate with the other cluster analyses performed (Figure S2); group 1 strains formed a tight cluster with

the *M. caseolyticus* CCM 3540^T strain and *M. canis* CCM 8748^T as the next closest relative, while group 2 strains formed another well-resolved group clearly distinguished from the reference type strains of *Macrococcus* spp.

Whole-cell protein fingerprinting also confirmed the separate position of the closely related group 1 strains and showed their relationship to *M. caseolyticus* strains. The three group 2 strains CCM 4927^T, CCM 7099^T, and CCM 7100^T were placed in a separate cluster, but these three strains exhibited more heterogeneous profiles. The closest profile to the group 2 strains was that of *M. canis* CCM 8748^T. All the analyzed strains were clearly differentiated from all remaining representatives of known *Macrococcus* spp. (Figure S3).

DNA Fingerprinting Analyses

The genomic relatedness among isolated strains and their clonal origin was studied by three DNA fingerprinting techniques, namely automated ribotyping (Figure S4), rep-PCR fingerprinting (Figure S5), and *Sma*I macrorestriction analysis determined by PFGE (Figure S6). Numerical analysis of the obtained fingerprints clearly separated group 1 strains from the remaining macrococci and showed genetic homogeneity within these four strains, revealing visually identical fingerprints by each of these techniques. The group 2 strains CCM 4927^T, CCM 7099^T, and CCM 7100^T were clearly differentiated from the remaining macrococci included in the analysis as well as from each other. The macrorestriction analysis of the strains showed diversity between group 1 and group 2 in their PFGE banding patterns. Group 1 strains exhibited fragments of similar lengths (from 5 kb to 190 kb) to *M. caseolyticus*, in contrast with the macrorestriction patterns of group 2 isolates, for which no significant similarity was found and which were ~20 kb to > 600 kb in size, resembling staphylococci (Figure S6).

Chemotaxonomic Analyses

All chemotaxonomic analyses of the isolates showed characteristics corresponding to those of the other species of the genus *Macrococcus*. FAME analysis (Table S1) showed that group 1 strains have a unique fatty acid composition compared to *M. caseolyticus* CCM 3540^T and *M. canis* CCM 8748^T. The major fatty acids displayed by group 1 were identified as C_{14:0} (27.1%), C_{16:0} N alcohol (13.6%), C_{16:1} ω11c (11.5%), C_{16:0} (9.7%) and C_{18:1} ω9c (22.9%). The group 1 strains contained lower amounts of C_{14:0} iso (1.1%) and C_{16:1} ω11c (11.5%) compared to *M. caseolyticus* CCM 3540^T (7.9% and 29.5%, respectively); moreover, members of group 1 differed by a higher amount of C_{14:0} (27.1%), C_{16:0} (9.7%) and C_{18:3} ω6c (6.9,12) (6.7%) compared to *M. caseolyticus* CCM 3540^T (16.5%, 6.6% and 0%, respectively), confirming their divergence from this type strain.

Strains belonging to group 2 were found to have similar, but not identical fatty acid profiles. Common major fatty acids to all of them were C_{15:0} anteiso (12.3-17.5%), C_{16:0} (8.8-14.9%) and C_{18:1} ω9c (20.2-31.5%). Strain CCM 4927^T differs by the presence of two additional major fatty acids, namely C_{14:0} (11.8%) and C_{16:1} ω9c (11.8%). Strain CCM 7100^T was distinguishable by an additional major fatty acid C_{16:0} (14.9%). Strain CCM 7099^T

differs by an additional major fatty acid C_{18:0} (12.7%) and by a low amount of C_{14:0} (3.6%) compared to the two other species in group 2. Members of group 2 were distinguishable from group 1, *M. caseolyticus* CCM 3540^T and *M. canis* CCM 8748^T based on the presence of C_{15:0} anteiso. Overall, the fatty acid profile of each member of group 2 exhibited a unique fatty acid pattern that represents an important distinctive feature.

The major menaquinone type obtained from the isolates was MK-6, which is in agreement with the data of Gobeli Brawand et al. (2017). The menaquinone profiles of representative strains were found to be as follows: CCM 7927^T [MK-6 and MK-7 (97:0.6)], CCM 4927^T [MK-6, MK-5, and MK-7 (98:1:0.1)], CCM 7099^T [MK-6 and MK-7 (96:0.5)], and CCM 7100^T [MK-6, MK-5, and MK-7 (97:2:0.3)].

The peptidoglycan type of the cell wall of strains CCM 7927^T and CCM 7099^T was determined to be A3α Lys-Gly₃-Ser and so resembled the peptidoglycan type of majority *Macrococcus* spp. members (Schleifer, 2015), while the peptidoglycan type A3α L-Lys-Gly₁₋₂ identified in strains CCM 4927^T and CCM 7100^T was different.

Genome Homology Studies

Both wet-lab DNA-DNA hybridization (DDH) and whole-genome sequencing to compare genome-to-genome distances were performed in order to precisely clarify the taxonomic position of the analyzed human macrococcal isolates (Figure S7). After considering the recommendations of a threshold value of 70% DNA-DNA similarity for the definition of bacterial species by the *ad hoc* committee (Wayne et al., 1987) and 95–96% threshold ANI value (Richter and Rosselló-Móra, 2009; Meier-Kolthoff et al., 2013), the results showed that strain CCM 7927^T representing group 1 belongs to the species *M. caseolyticus*. In contrast, strains CCM 4927^T, CCM 7099^T, and CCM 7100^T were shown to be representatives of three distinct species, clearly differentiated from strains CCM 7927^T and *M. caseolyticus* CCM 3540^T.

Proposal of the New *Macrococcal* Taxa

Based on the above results of overall genome relatedness, a novel subspecies within *M. caseolyticus* and three novel species within the genus *Macrococcus* are proposed. Group 1 strains CCM 7927^T, CCM 7928, P862, and P865, originating from various human clinical materials, formed a coherent cluster for which the name *M. caseolyticus* subsp. *hominis* subsp. nov. is proposed. In accordance with Rule 40d (formerly Rule 46) of the Prokaryotic Code (De Vos and Trüper, 2000; Parker et al., 2015) the remaining strains of *M. caseolyticus* represented by the type strain CCM 3540^T are reclassified as *M. caseolyticus* subsp. *caseolyticus* subsp. nov. Group 2 strains CCM 4927^T, CCM 7099^T and CCM 7100^T represents three distinct species for which the names *Macrococcus goetzii* sp. nov. (type strain CCM 4927^T) and *Macrococcus epidermidis* sp. nov. (type strain CCM 7099^T), both inhabiting human skin, and *Macrococcus bohemius* sp. nov. (type strain CCM 7100^T), isolated from a knee wound, are proposed. The detailed protologues describing these four taxa are given below.

Genome Characteristics

Genomes of the strains CCM 7927^T, CCM 4927^T, CCM 7099^T and CCM 7100^T were shot-gun sequenced and compared with the reference genomes of *M. caseolyticus* subsp. *caseolyticus* subsp. nov. CCM 3540^T, sequenced in this study, and *M. canis* CCM 8748^T (Gobeli Brawand et al., 2018). The sequencing and assembly statistics of the whole genome sequences obtained from the type strains are shown in Table 2. *Macrococcus caseolyticus* subsp. *hominis* subsp. nov. CCM 7927^T with a 2.1-Mb chromosome and G+C content of 36.8 mol% is closely related to the type strain *M. caseolyticus* subsp. *caseolyticus* subsp. nov. CCM 3540^T, differing mainly in the content of variable genetic elements. All strains from group 2 exhibited larger chromosomes than *M. caseolyticus*, ranging from 2.3 to 2.6 Mb, with a notably lower G+C content of 34.0 to 34.2 mol% (Table 2). Their G+C content values lie below the 38–45 mol% range defined by Kloos et al. (1998) for the genus *Macrococcus* using the thermal denaturation method. A comparison of the G+C content in the core genome of the analyzed strains confirmed that this difference is not caused by the presence of accessory genes. Thus *M. goetzii* sp. nov., *M. epidermidis* sp. nov. and *M. bohemius* sp. nov. have similar G+C content to the members of the genus *Staphylococcus* (Schleifer and Bell, 2009).

Examination of the macrococcal pan-genome showed that five macrococcal representatives (CCM 3540^T, CCM 4927^T, CCM 7927^T, CCM 7099^T, and CCM 7100^T) share 1678 orthologous protein clusters (Figure 2). On a pairwise basis, the type strain *M. caseolyticus* subsp. *caseolyticus* subsp. nov. CCM 3540^T shares additional 94 distinctive orthologous groups with *M. caseolyticus* subsp. *hominis* subsp. nov. CCM 7927^T. All group 2 strains share 282 orthologous groups, which is the largest common gene pool in the analyzed sample. Strain CCM 4927^T shared the most with CCM 7099^T, with 164 orthologous groups. Hence these results support the outcomes of the phylogenetic analysis and shows that CCM 4927^T and CCM 7099^T are more related to each other than to strain CCM 7100^T. According to the Gene Ontology (GO) enrichment analysis among the three group 2 strains, five enriched pathways were found: quinone binding, ATP synthesis-coupled electron transport, NADH dehydrogenase (ubiquinone) activity, calcium ion transmembrane transport, and respiratory chain complex, and strains CCM 7099^T and CCM 4927^T had an over-represented lipid metabolic process.

Comparative genomics of human macrococci discovered huge genome structure plasticity, which might give them an advantage in terms of survival in diverse environments (Figure 3). We hypothesize that such genomic variability, particularly in group 2 strains, is attributed to the presence of *com* operons. These operons play a key role in the uptake of exogenous DNA during the transformation of *Streptococcus pneumoniae* (Muschiol et al., 2015) and *Bacillus subtilis* (Chung and Dubnau, 1995) and possibly sustain functionality in macrococci. All sequenced human *Macrococcus* isolates have been found to contain part of the putative *comG* operon (*comGA-comGC*), the complete *comE* (*comEA-comEC*) operon, and the *dprA* gene, which is another key factor in transformations and mediates DNA recombination. This suggests that transformation may lead to the extensive genomic variability and larger genome size of group 2 macrococci.

TABLE 2 | Summary of whole genome sequence characteristics of analyzed *Macrococcus* spp.

Genome	<i>M. caseolyticus</i> subsp. <i>caseolyticus</i> subsp. nov. CCM 3540 ^T	<i>M. caseolyticus</i> subsp. <i>hominis</i> subsp. nov. CCM 7927 ^T	<i>M. goetzii</i> sp. nov. CCM 4927 ^T	<i>M. epidermidis</i> sp. nov. CCM 7099 ^T	<i>M. bohemicus</i> sp. nov. CCM 7100 ^T
WGS Project no.	PZJF00000000	MJB100000000	MJB100000000	PZJH00000000	PZJG00000000
Size (Mb)	2.06	2.10	2.59	2.48	2.30
No. of contigs (>200 bp)	42	37	33	31	29
N50	72 809	189 524	459 952	214 089	260 501
Mean Coverage	67	66	313	264	272
G+C content (mol%)	36.56	36.79	33.95	33.97	34.23
G+C content in core genome (mol%)	36.77	37.02	34.04	34.08	34.32
Coding sequences	2140	2149	2679	2588	2462
No. of prophages	1	1 (φMC1)	2 (φMG1, φMG2)	1 (φME1)	1 (φMB1)
SCC-like elements	ψSCC _{CCM3540}	ψSCC _{CCM7927}	MgCl-ψSCC _{MeC} _{CCM4927}	MeCl-ψSCC _{CCM7099}	MbCl-ψSCC _{CCM7100}
other chromosomal/resistance island	1	1 (McRI _{CCM7927})	–	1 (MeCI _{CCM7099})	2 (MbRI-1 _{CCM7100} , MbRI-2 _{CCM7100})
PICI (Phage-inducible chromosomal island)	3	–	2 (MgCl-1 _{CCM4927} , MgCl-2 _{CCM4927})	–	1 (MbCI _{CCM7100})
No. of plasmids	N/A	1 (pZKMH1)	1 (pZKMG1)	1 (pZKME1)	2 (pZKMB1, pZKMB2)
CRISPR	–	–	–	–	1 (type II-C)
virulence factors	N/A	<i>fbpA^C</i> ; <i>veg^d</i> ; <i>hly-III^F</i> ; <i>cap^f</i>	<i>sdr loci^a</i> ; <i>PIA^b</i> ; <i>fbpA^C</i> ; <i>veg^d</i> ; <i>hly-III^F</i> ; <i>cap^f</i> ; <i>adsA^g</i>	<i>sdr loci^a</i> ; <i>PIA^b</i> ; <i>fbpA^C</i> ; <i>veg^d</i> ; <i>hly-III^F</i> ; <i>cap^f</i>	<i>PIA^b</i> ; <i>fbpA^C</i> ; <i>veg^d</i> ; <i>hly-III^F</i> ; <i>cap^f</i>

^a*sdr loci* are composed of genes *sdrB*, *sdrC* and *sdrD*.^b*ica* locus encoding polysaccharide intercellular adhesin (*PIA*).^cfibronectin binding protein A gene (*fbpA*).^dbiofilm-associated gene (*veg*).^ehemolysin III family gene (*hly-III*).^fcapsule gene clusters (*cap*).^gputative gene for adenine synthase A (*adsA*).

N/A, not analyzed.

–, not detected.

isolates. Conversely the negative lysogenic conversion of the *comG* locus caused by the presence of prophage φMC1 in strain CCM 7927^T from group 1 strains could be responsible for the low accessory genome variability in *M. caseolyticus* subsp. *hominis* subsp. nov. (Figure 4).

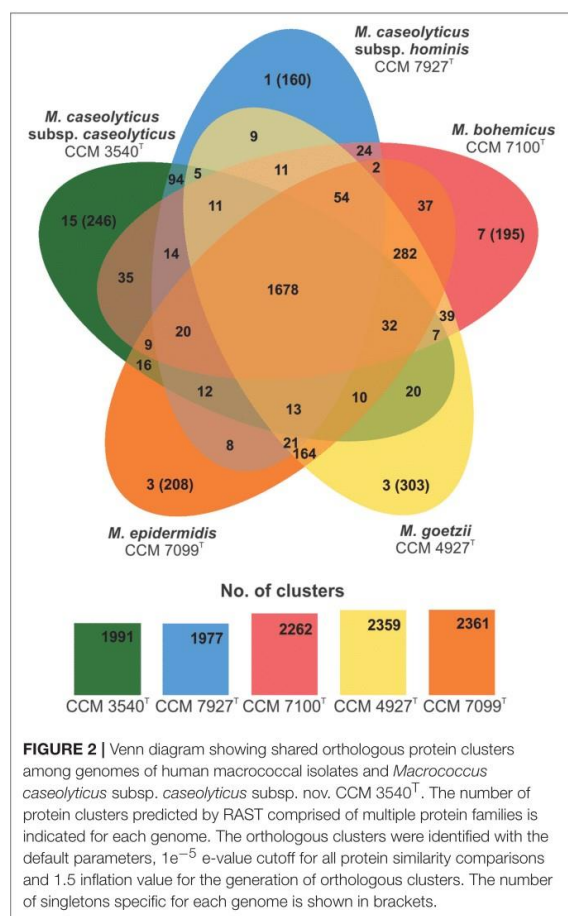
The presence of a CRISPR-Cas system and restriction-modification (R-M) systems in macrococcal genomes corresponds with the need to limit the burden of foreign DNA. Strain CCM 7927^T carries a Type II R-M system with methyltransferase and the endonuclease from the *DpnII* family. Strains CCM 4927^T, CCM 7099^T and CCM 7100^T contain Type I R-M systems consisting of three genes (*hsdM*, *hsdS*, and *hsdR*).

Accessory genes that are not essential in all bacterial strains were identified in the human macrococcal genomes. The highest number of genome-specific proteins, with 303 singletons, was found in the CCM 4927^T genome followed by CCM 3540^T, CCM 7099^T, CCM 7100^T and CCM 7927^T with 246, 208, 195, and 160 singletons, respectively. While accessory genes localized outside MGEs constitute 17% of the predicted coding sequences

of *M. caseolyticus* CCM 3540^T and CCM 7927^T, for the strains CCM 4927^T and CCM 7099^T the accessory genes localized outside MGEs constitute 31–32%, and for strain CCM 7100^T 24%. The higher percentage of accessory genes in strains CCM 4927^T and CCM 7099^T is consistent with their expected ability to receive foreign DNA via transformation, in contrast to strain *M. caseolyticus* subsp. *hominis* subsp. nov. CCM 7927^T, where negative lysogenic conversion of the *comG* locus was identified. Although negative lysogenic conversion was not observed in strain CCM 7100^T, the lower proportion of accessory genes outside MGEs might be caused by the presence of the complete CRISPR-Cas system.

New Variant of Type II-C CRISPR-Cas System

Clustered, regularly interspaced, short, palindromic repeats (CRISPR) loci, together with their CRISPR-associated (Cas) proteins provide the bacteria with adaptive immunity against the invasion of bacteriophages, plasmids, and other MGE. The



CRISPR-Cas system found in *M. bohemicus* sp. nov. CCM 7100^T is demarked by two 36-bp anti-repeats (GAAGCCATTCGCTTCATTCTAAAAGATCATAGTTAA; TCATAGTTCTAAAACACTCGTGCAATCCTACTCTTA). The CRISPR locus is 5,888 bp long, and contains 90 spacers separated by 36-bp-long repetitive sequences (GTTTCACTTCATTCTAAAAGATCATAGTTCTAAAAC). Genes for Cas proteins were found neighboring the CRISPR arrays and were categorized as Cas1, Cas2 and Cas9. Cas1 exhibited 60% amino acid identity with the type II CRISPR-associated endonuclease Cas1 of *S. pseudintermedius* (Ben Zakour et al., 2011); and 57% amino acid identity with the type II CRISPR-associated endonuclease Cas1 of *Staphylococcus simulans* (GenBank accession no. CP023497). Cas2 exhibits 72% amino acid identity with the CRISPR-associated endonuclease Cas2 of *Staphylococcus delphini* (Verstappen et al., 2017). The whole CRISPR-Cas locus exhibited high nucleotide identity (68%) to the type II-C CRISPR-Cas of *S. simulans* strain FDAARGOS_383 (GenBank accession no. CP023497), hence this region was classified as CRISPR-Cas type II-C.

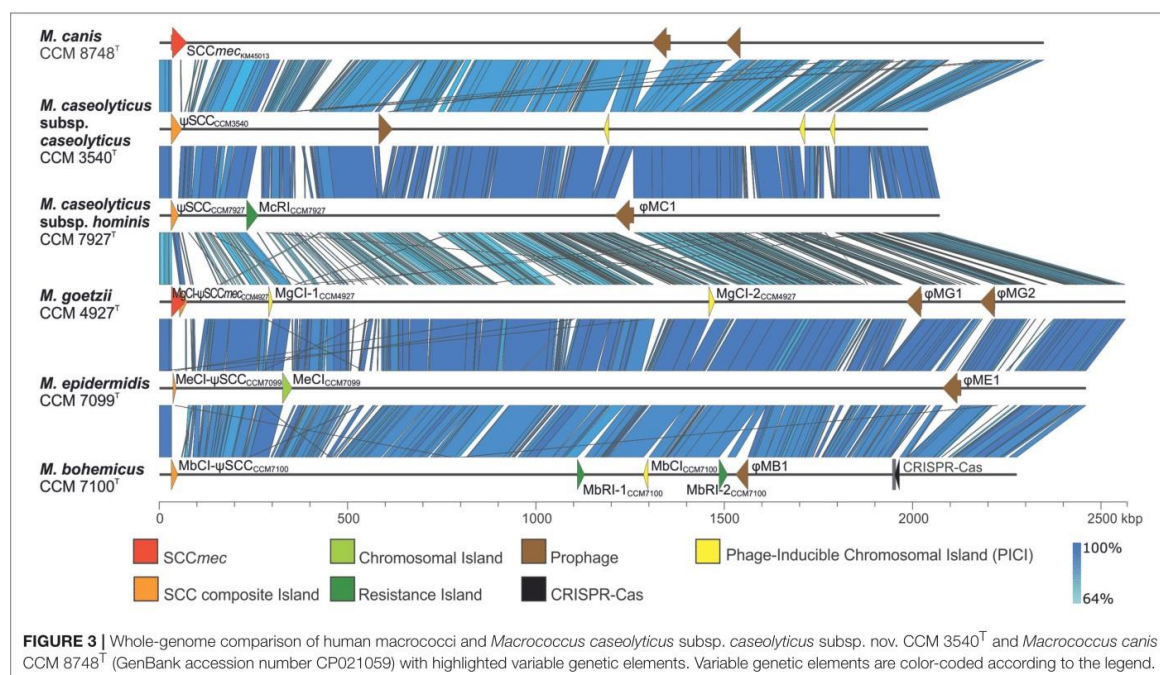
Virulence Factors

The pathogenic potential of bacteria depends on the production of virulence factors and the presence of antimicrobial resistance determinants. Human macrococci, as well as closely related coagulase-negative staphylococci, seem to be a potential reservoir of virulence and resistance genes for staphylococci (Becker et al., 2014). Putative genes carrying the motifs associated with bacterial virulence factors were predicted in analyzed genomes using the virulence factor database (VFDB) (Table 2). The locus encoding polysaccharide intercellular adhesin (PIA) was identified in strains CCM 4927^T, CCM 7099^T, and CCM 7100^T from group 2. PIA has been described as a factor involved in staphylococcal biofilm accumulation (Rohde et al., 2010). Highly heterogenic gene homolog of biofilm synthesis protein poly-beta-1,6-N-acetyl-D-glucosamine synthase (PNAG) have been identified not only across the genus *Staphylococcus*, but also in Gram-negative human pathogens. A putative macrococcal PNAG synthase gene homolog shares the highest amino acid identity (64%) with a protein of *Bacillus onubensis* (Dominguez-Moñino et al., 2018).

The biofilm-associated *veg* gene for a small conserved Veg protein (87 aa) is present in all sequenced macrococcal genomes. Veg protein is conserved among the four sequenced strains and BLAST analysis showed the *veg* gene to be conserved not only among the whole genus *Macrococcus* but also in coagulase-negative staphylococci and other low-G+C Gram-positive bacteria from the phylum *Firmicutes*. The function of the Veg coding gene has been studied in *B. subtilis*, where the transcription of *veg* was increased during exponential growth and sporulation, supporting its function as an additional regulatory protein contributing to the control of matrix production (Lei et al., 2013).

In all sequenced human macrococcal genomes, the putative gene *fbpA* encoding a potential virulence factor, fibronectin binding protein A (FbpA), was identified. This protein is highly conserved across the genus *Macrococcus* with more than 74% amino acid identity. The FbpA protein of strain CCM 4927^T shares 52% amino acid identity and 98% query coverage with the FbpA of *Staphylococcus saprophyticus* (GenBank accession no. WP_061854885) (Mukherjee et al., 2016). The mutual nucleotide identity of the *fbpA* of the strain CCM 4927^T compared to strain CCM 7099^T and CCM 7100^T is 79% and compared to CCM 7927^T is 73%. FbpA is one of the most important adhesins for binding to human fibronectin. Human fibronectin participates in eukaryotic cellular processes, such as adhesion, migration and differentiation, and FbpA is important during staphylococcal and streptococcal infection, since it allows the pathogens to bind to host epithelial cells, facilitates their internalization (Joh et al., 1999) and systemic spread within the host (Christie et al., 2002).

The genome analyses revealed putative capsule gene clusters in all sequenced genomes, composed of putative *cap* genes, which are arranged consecutively. Moreover, strain CCM 7099^T contains two *cap* loci (*cap-1*_{CCM7099}, *cap-2*_{CCM7099}) in its chromosome. Many genes involved in the biosynthesis of the capsule are similar among the members of the genera *Staphylococcus*, *Streptococcus*, and *Bacillus*, with similar capsule gene clusters within the genus *Macrococcus*. The *cap* locus



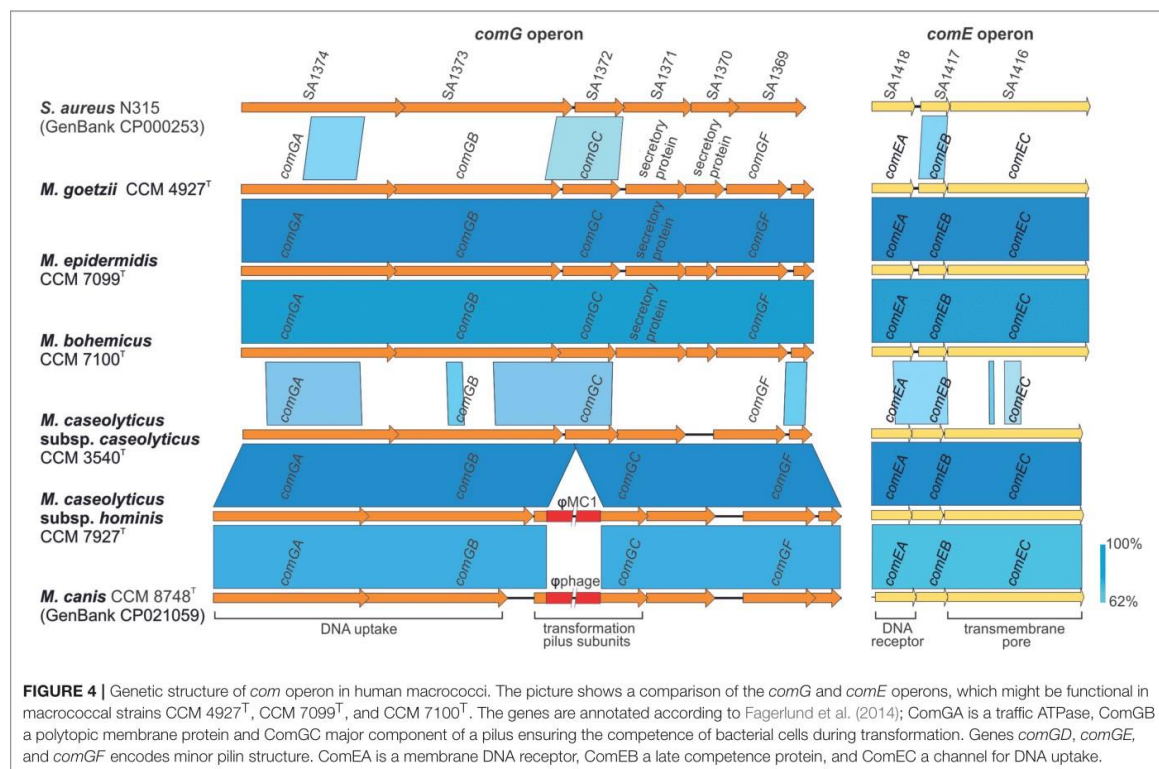
is involved in the expression of the capsule and enhances virulence by phagocytosis evasion. Most staphylococci express the serotype 5 or 8 capsular polysaccharides (Watts et al., 2005). The staphylococcal *cap5* and *cap8* loci are comprised of an ~17.5 kb region with 16 closely linked genes (O’Riordan and Lee, 2004), which is comparable to the genetic structure of the 15 – 18 kb macrococcal *cap* region with 13 to 17 ORFs (Figure 5). The highest similarity (93% nucleotide identity, coverage 89%) of the *cap* locus was observed between *cap-1*_{CCM7099} and *cap*_{CCM4927} from CCM 4927^T. Both regions exhibit partial homology to the staphylococcal serotype 5 *cap* genes (Sau et al., 1997), whereas the *cap-2*_{CCM7099} and *cap*_{CCM7100} from CCM 7100^T, and *cap*_{CCM7927} from CCM 7927^T are homologous to the staphylococcal serotype 8 *cap* genes (Sau and Lee, 1996).

A key aspect of bacterial virulence lies in the ability to target the host cell membrane with many membrane-damaging toxins and peptides. Another putative virulence factor found in all human *Macrococcus* genomes is the predicted membrane channel-forming protein YqfA from the haemolysin III family (*hly-III*). Virulence factor *hly-III* has been described in *Bacillus cereus* to cause erythrocyte lysis as a result of the formation of transmembrane pores (Baida and Kuzmin, 1996). In the multiple sequence alignment of the macrococcal *hly-III* genes, two clusters corresponding to phylogenetic group 1 and group 2 were distinguished. However, the haemolysis determined on Columbia blood agar supplemented with sheep blood was not observed for human macrococci. Further analysis of the *hly* genes found several single-nucleotide polymorphisms that might result in the expression of non-functional haemolysin.

The putative gene for adenosine synthase A (AdsA), which is a critical virulence factor in staphylococci, was found in strains CCM 4927^T and CCM 7099^T. AdsA is a cell wall-anchored enzyme that converts adenosine monophosphate to adenosine (Thammavongsa et al., 2009). Kim et al. (2012) showed that the staphylococcal synthesis of adenosine enabled by AdsA allows the pathogen to escape from phagocytic clearance in blood, to form organ abscesses, and to block host adaptive immune defense mechanisms. AdsA from strain CCM 4927^T exhibited 59% amino acid identity and 74% coverage with that described in *S. aureus* (Thammavongsa et al., 2011). The mutual similarity of AdsA from macrococcal strains CCM 7099^T and CCM 7100^T is 95%.

OriC Environ and Staphylococcal Cassette Chromosome-Like Elements

A comparison of the sequenced *Macrococcus* genomes revealed a high heterogeneity in the region near 3’ *oriC*. This region was found to be distinctive within the genus *Staphylococcus* (Takeuchi et al., 2005) and correspondingly macrococcal genomes are highly diverse in this region (Figure 3). All human macrococcal isolates carry a pseudo *Staphylococcus* chromosome cassette (ψ SCC) inserted into the *rlmH* (*orfX*) gene in the vicinity of the *oriC*. Moreover β -lactam susceptibility testing revealed that strain CCM 4927^T is resistant to β -lactam antibiotics, including oxacillin, thus pointing to the presence of a *mecB* gene, which was indeed identified as part of ψ SCC_{mec}_{CCM4927}. The *mecB* gene of macrococcal origin was recently detected on a plasmid in MRSA strain UKM4229 (Becker et al., 2018), emphasizing



that macrococcal resistance genes could be transferred to staphylococci, especially when the two species share the same habitat.

ψ SCCmec_{CCM4927} is 12,779 bp long, bordered by imperfect 18-bp direct repeats. This element harbors 14 ORFs (Figure 6), including the *mecB* gene, which shares 98% nucleotide identity with other *mecB* genes of sequenced macrococci (Gómez-Sanz et al., 2015). The regulators of the *mecB* transcription, the repressor *mecI* and inducer *mecR1*, were also present within the *mec* gene complex, but this locus as well as the whole genome, did not contain the *blaZ* gene, which is associated with the class E *mec* complex in other macrococci (Tsubakishita et al., 2010). Hence the structure of the complex could be the missing link between the antecedent form of the class E *mec* complex (*mecI*–*mecR*–*mecA*–*blaZ*) and the descendent class A *mec* complex (*mecI*–*mecR*–*mecA*) present in SCCmec type II, III and VIII, as discussed by Tsubakishita et al. (2010). No cassette chromosome recombinase (*ccr*) genes or their homologs were found in ψ SCCmec_{CCM4927}, neither transposase genes were present, so it is uncertain whether this element alone could be transferred.

Three additional copies of the insertion sequence site (ISS) were found in the genomic sequence of strain CCM 4927^T that form the boundaries of three adjacent ψ SCC elements forming a composite island designated MgCI- ψ SCCmec_{CCM4927} composed

of ψ SCCmec_{CCM4927}, ψ SCC1_{CCM4927}, ψ SCC2_{CCM4927}, and ψ SCC3_{CCM4927}.

ψ SCC1_{CCM4927} is 23,281 bp long, carries 24 ORFs and consists of two separate parts. The first part contains hypothetical genes from which three ORFs exhibit 92% sequence identity to genes previously identified as *M. caseolyticus* JCSC5402 chromosomal genes MCCL_0033, MCCL_0034, and MCCL_0035 (Baba et al., 2009). The second part contains a cluster of heavy metal resistance genes with a *cad* operon (*cadDAC* genes), *zntA* gene, *cop* operon (*copYZ*), histidine kinase *hisK* gene and multicopper oxidase *cueO*. This cluster has a G+C content of 35.86 mol%, which is considerably higher than the average G+C content of the rest of the MgCI- ψ SCC_{CCM4927} and *Macrococcus* genome. Based on the gene composition and higher G+C content, we propose that this heavy metal resistance gene cluster is a transposon; it was also found in the ψ SCCs of strains CCM 7100^T and CCM 7927^T. This transposon was probably integrated into the genome independently before the creation of the ψ SCC1_{CCM4927} element, which strengthens the hypothesis that SCCs are prone to the accumulation of MGE and dynamic evolution (Indráková et al., 2016). ψ SCC2_{CCM4927} is a 6,682-bp element carrying the gene for an additional threonine-tRNA-ligase and four genes coding for hypothetical proteins. ψ SCC3_{CCM4927} is 7,899 bp in size and it contains 11 ORFs, comprised of putative isochorismatase, a 3-ketoacyl reductase paralog, and hypothetical proteins.

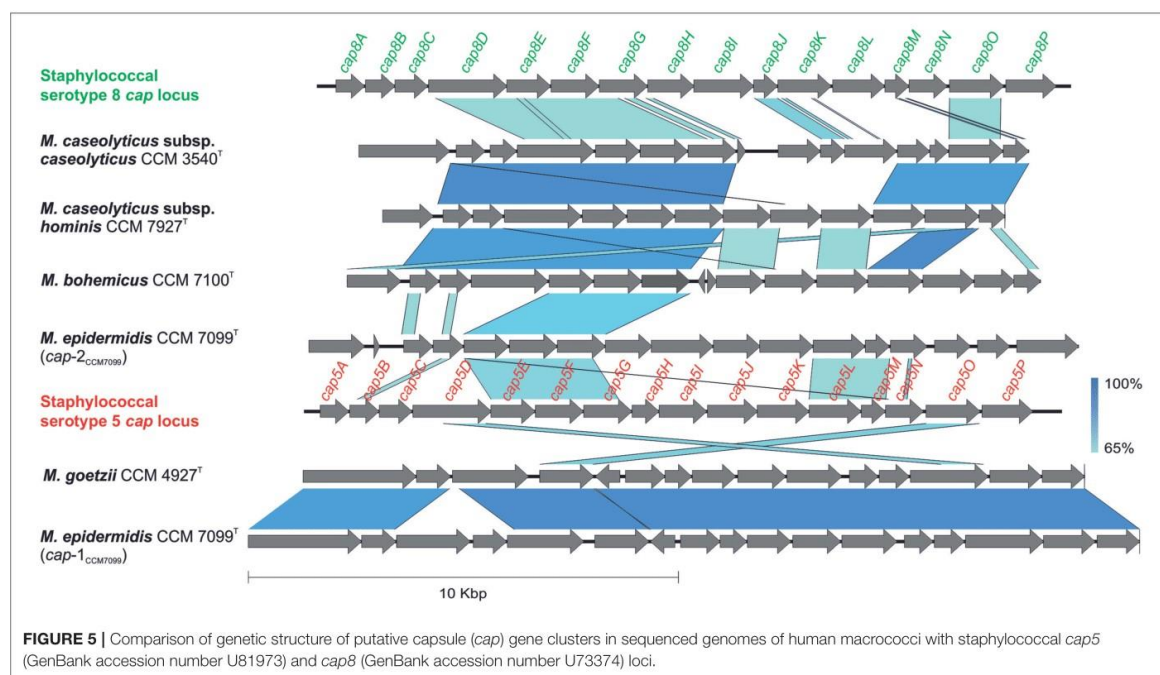


FIGURE 5 | Comparison of genetic structure of putative capsule (*cap*) gene clusters in sequenced genomes of human macrococci with staphylococcal *cap5* (GenBank accession number U81973) and *cap8* (GenBank accession number U73374) loci.

During the *oriC* environ analysis of the strain CCM 7100^T, a composite island MbCI- ψ SCC_{CCM7100} of three ψ SCCs (ψ SCC1_{CCM7100}, ψ SCC2_{CCM7100}, and ψ SCC3_{CCM7100}) was identified (Figure 6). ψ SCC1_{CCM7100} has a length of 19,392 bp and encodes 20 ORFs. Apart from two hypothetical proteins and putative histone acetyltransferase, the ψ SCC1_{CCM7100} element includes a heavy metal resistance transposon comparable to the one found in CCM 4927^T. The second element inserted into the *rlmH* gene of CCM 7100^T is 7,162-bp-long ψ SCC2_{CCM7100}, comprised of 8 ORFs, namely the putative transcriptional regulator from the XRE family, putative HNH endonuclease, and hypothetical proteins. The third element in the region is ψ SCC3_{CCM7100}, which is 8,501 bp long with 12 ORFs coding for daunorubicin resistance protein, putative dehydrogenases, putative arsenic efflux pump protein ArsB and arsenic resistance operon repressor protein ArsR, and several hypothetical proteins.

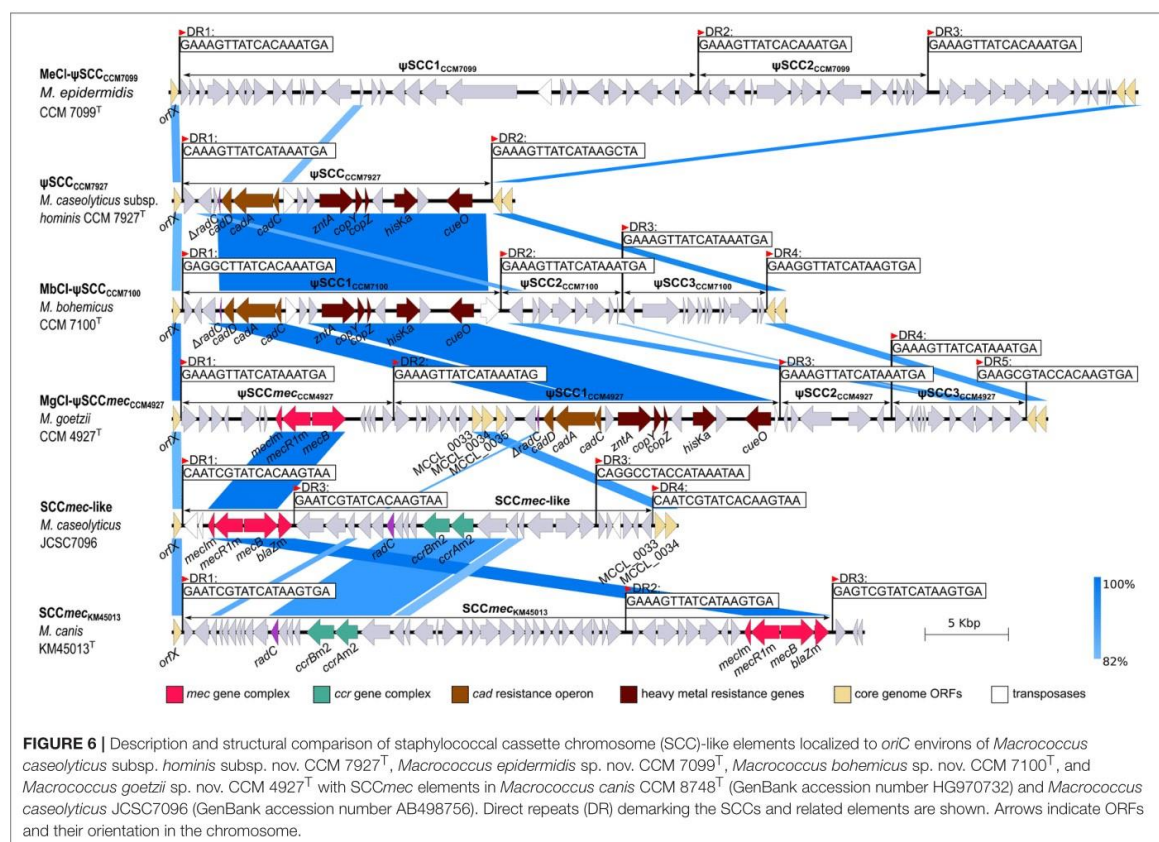
The chromosome of strain CCM 7927^T contained one ψ SCC similar to ψ SCC1_{CCM7100} (Figure 6). The 18,494-bp-long ψ SCC_{CCM7927} carries 20 ORFs and an almost identical transposon to the one detected in strain CCM 7100^T.

Strain CCM7099^T also carries a composite island inserted into the *rlmH* gene. The island is made of two ψ SCCs, 31.0-kb-long ψ SCC1_{CCM7099} and 13.8-kb-long ψ SCC2_{CCM7099}. These two cassettes are not similar in their gene composition to any of the above-described ψ SCCs (Figure 6), and they code for transcriptional regulators, putative resistance (blasticidin S deaminase, bleomycin resistance protein, aminoglycoside transferase) and virulence factors (sortase and LPXTG motif containing protein), and other uncharacterized proteins.

Transposons Harboring Heavy Metal Resistance Genes

The transposon found in MgCl- ψ SCC_{meccCM4927} of strain CCM 4927^T and also within the ψ SCCs of strains CCM 7100^T and CCM 7927^T is presumably inserted into the *radC* gene, whose remnants were found directly upstream of the transposon sequence (Figure 6). Notably, other transposons of *Firmicutes* exhibit a strong preference for the insertion site within *radC* (Müller et al., 2013). The *cadDAC* operon was followed by the transposase from the IS30 family (98% amino acid identity to Tnps from *Macrococcus*, *Enterococcus*, and *Staphylococcus*). A similar *cad* resistance region was found in *S. pseudintermedius* (Chanchaithong et al., 2016). Inverted repeats delimiting the transposon or the second part of the *radC* gene were not found, so the boundary on the right hand-side was determined by the presence of the transposase from the IS4 family in the CCM 7100^T chromosome (66% amino acid identity to Tnp from *Ureibacillus thermosphaericus* and *Lysinibacillus macroides*) and given the presence of two different transposases genes, it is thus possible that the macrococcal transposon is concatenated from two separate transposons that confer heavy metal resistance.

Apart from the transposons found in the *oriC* environ of CCM 4927^T, CCM 7100^T, and CCM 7927^T, another transposon conferring resistance to heavy metals was identified in the genomes of the three above-mentioned human *Macrococcus* strains. The 7.4-kb-long transposon is bordered by insertion sequences with two transposase genes from the IS3 family and apart from lead-, cadmium-, zinc- and mercury/heavy metal-transporting ATPase genes, also contains a resolvase gene and



an *ArsR* transcriptional regulator gene. Inverted repeats in the detected transposon are almost identical to a 123-bp-long sequence in *IS1216E* from *Enterococcus faecium* (Arthur et al., 1997).

Other Chromosomal and Resistance Islands

The resistance island designated *McRI*_{CCM7927} encoding macrolide resistance and harboring a putative gene for chaperone protein *ClpB*, which is part of a stress-induced multi-chaperone system, was found in strain CCM 7927^T (Figure 3). *McRI*_{CCM7927} is integrated into the 3' end of the 30S ribosomal protein S9 gene (*rpsI*) localized upstream of the gene for a hypothetical protein of AAA family ATPase, which is the same locus as the one used by *McRI*_{mecD} carrying the *mecD* gene in two *M. caseolyticus* strains described earlier by Schwendener et al. (2017). *McRI*_{CCM7927} carries 22 ORFs, including an integrase. There is a completely different chromosomal island (*MeCI*_{CCM7099}) in the genome of CCM 7099^T, sharing only the same *att* site and integrase (94% nucleotide similarity) with *McRI*_{CCM7927} (Figure 3). *MeCI*_{CCM7099} does not contain any known resistance or virulence genes. No chromosomal island

was integrated into the *rspI* gene of CCM 7100^T, and even the gene for AAA family ATPase was missing.

Strain CCM 7100^T carries two genomic islands, designated *MbRI*-1_{CCM7100} and *MbRI*-2_{CCM7100}. *MbRI*-1_{CCM7100} is ~19 kb long and carries genes for resistance to heavy metals and copper. This genomic island is integrated into one of the two copies of the gene for LSU ribosomal protein L33p. *MbRI*-2_{CCM7100} is cca 23 kb long and integrated into the *radC* homolog encoding a DNA repair protein (Figure 3). This island harbors the *optrA* gene cluster encoding oxazolidinone/phenicol resistance, which was described in *S. sciuri* (Li et al., 2016), and type I R-M system.

Phage-Induced Chromosomal Islands

MGEs designated phage-inducible chromosomal islands (PICIs) that can contribute to host adaptation and virulence (Penadés and Christie, 2015) were identified in genomes of strains CCM 4927^T and CCM 7100^T (Figure 3). Macroccocal PICIs have a typical modular structure that facilitates their integration, replication and excision such as *S. aureus* pathogenicity islands (SaPIs), the prototype members of these elements. Strain CCM 4927^T has two putative PICIs in the genome. The 11-kb-long island *MgCI*-1_{CCM4927} is

integrated between the genes for hypothetical L-lactate dehydrogenase subunit YkgG and phosphatidylethanolamine N-methyltransferase gene. The 22-bp-long *att* site of MgCI-1_{CCM4927} is TTGTTTTAGATGATAAATAATA. Apart from many hypothetical genes, no virulence or resistance gene homologs were identified. The second 15-kb-long island MgCI-2_{CCM4927} is integrated between the genes for chorismate synthase and nucleoside diphosphate kinase. The 24-bp *att* site of MgCI-2_{CCM4927} is TGCCCTTTTCTGCCCCTTTTCTTA, which is the same *att* site as for the 15-kb-long island MbCI_{CCM7100} located in the genome of strain CCM 7100^T. Although both islands share the integration site and their integrases have 74% mutual amino acid identity, their nucleotide sequences are only similar in 8% of their total length. There is a gene encoding a plasmid antitoxin from the HigA proteins family at the end of the island MbCI_{CCM7100}. The toxin associated with this antitoxin gene was identified on the smaller plasmid pZKMS1 from strain CCM 7100^T.

Prophages

Prophages were identified in all sequenced genomes of *Macrococcus* spp. strains (Table 3). The detected prophages in human *macrococci* exhibit low similarities to any known phage or putative prophage in the databases except for ϕ MC1 (CCM 7927^T). Also, a comparison of all five prophages identified in human *macrococci* uncovered a low level of synteny, only the prophages ϕ MG1 (CCM 4927^T) and ϕ ME1 (CCM 7099^T) exhibit 20% nucleotide identity and have the same integration site in their host genomes (Figure 7A). Although the prophages have unique gene compositions, they follow the typical siphoviral modular structure (Kahánková et al., 2010).

Comparative analysis showed that the phage ϕ MC1 is highly similar in its major genomic modules, including its integrase and integration site, to the prophages of *M. caseolyticus* IMD0819 (Schwendener et al., 2017) and *M. canis* CCM 8748^T (Gobeli Brawand et al., 2018). ϕ MC1 also exhibits high similarity to the ϕ MCCL2 of *M. caseolyticus* JCSC5402 (Baba et al., 2009) (Figure 7B). An important feature of ϕ MC1 is its suspected lysogenic conversion. The phage ϕ MC1 is integrated into the *comGC* gene of the *comG* operon, thus causing its negative

conversion (Figure 4). Two putative immunoglobulin-binding regulators IbrA and IbrB are encoded by the phage genome. IbrA and IbrB are hypothesized to be possible virulence factors in *Escherichia coli* (Sandt et al., 2002) with 33% amino acid and 50% amino acid similarity, respectively, to those harbored in the ϕ MC1 genome. These regulators activate genes for immunoglobulin-binding proteins (Eib) (Rubin et al., 2017). Genes encoding IbrA and IbrB were previously found in the prophages of different distantly related bacterial species (Castillo et al., 2014; Laanto et al., 2015).

Phages from all the strains were induced by UV irradiation. The lysate of *M. caseolyticus* subsp. *hominis* subsp. nov. CCM 7927^T contained phage particles detectable by TEM. Morphological analysis by TEM with negative staining revealed that this bacteriophage, designated ϕ MC1 (vB_McaS_7927), belongs to the *Siphoviridae* family. The phage particles consist of an icosahedral head (B1 morphology) with a flexible, non-contractile tail ending with a base plate. The diameters of the phage head are from 66.5 to 69.5 nm, and tail length is ~310 nm (Figure 8). The structural proteins of the ϕ MC1 phage particles separated by SDS-PAGE and analyzed by mass spectrometry matched a prophage sequence identified in the genome of the monolysogenic host strain CCM 7927^T.

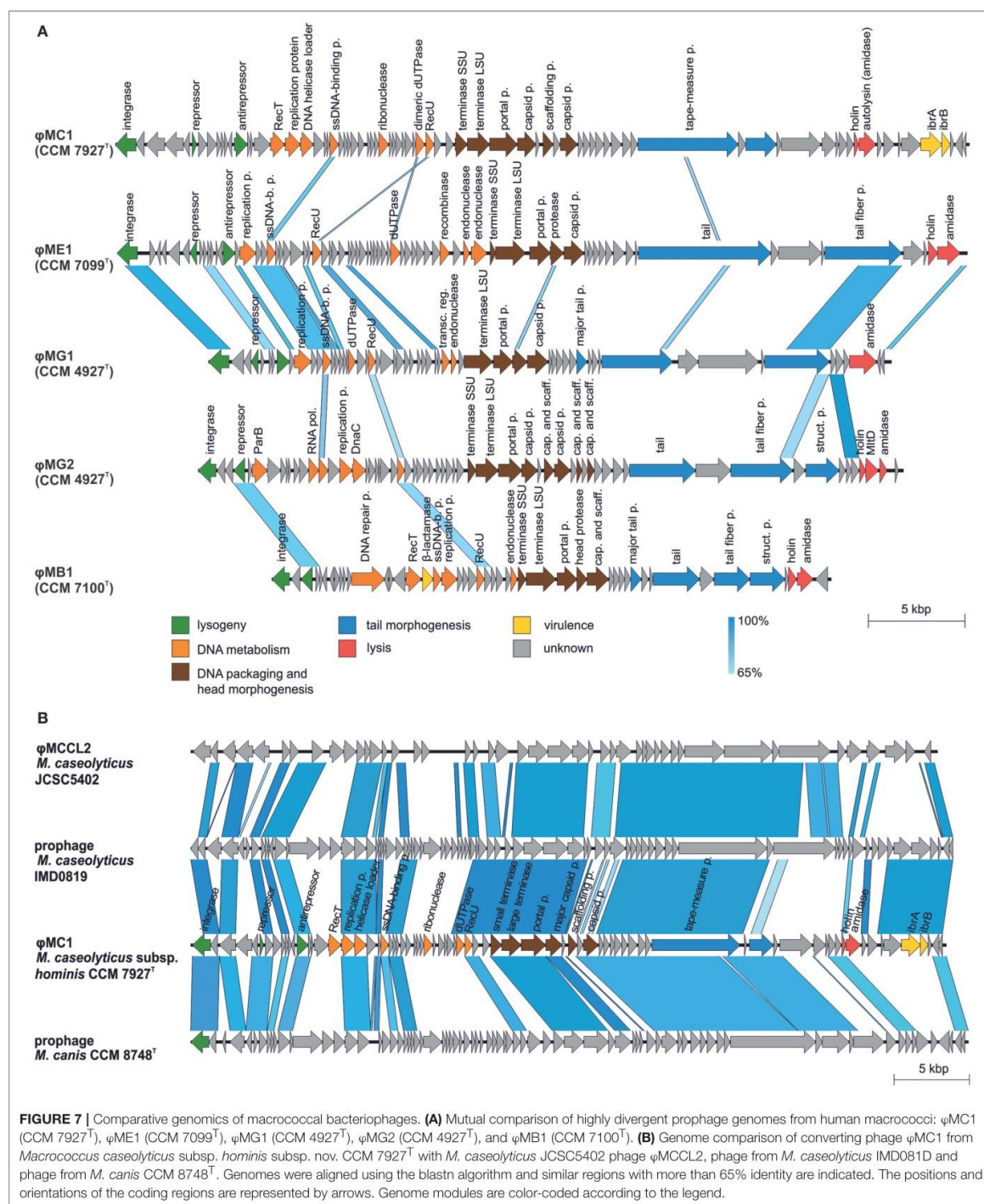
Plasmids

Extrachromosomal plasmids were identified in all sequenced genomes of human *macrococci* (Table 2). All plasmids contain many hypothetical genes without any homologs in the databases.

The 3,842 bp-long plasmid pZKMH1 was identified in the genome of CCM 7927^T. This plasmid carries a transposable element with a small multidrug resistance (*smr*) cassette-like structure, usually found in small non-conjugative plasmids, such as staphylococcal pSK41 (Costa et al., 2013). The cassette encodes two *Smr* proteins conferring resistance to quaternary cation compounds and an associated transcription factor from the ribbon-helix-helix superfamily (Schreiter and Drennan, 2007; Bay and Turner, 2012). These genes could serve as a defense mechanism against active oxygen toxicity and could prevent the oxidative damage of DNA (Dempsey et al., 1983). The same *smr* cassette-like structure was also identified in the chromosomes

TABLE 3 | Prophages identified in genomes of human *macrococci* and their characteristics.

Strain	Phage name	Size (bp)	G+C (mol%)	ORFs	Integration site	<i>att</i> sequence	Similar prophages	
							Host	GenBank accession no.
CCM 7927 ^T	ϕ MC1	50167	34.72	69	<i>comGC</i>	CATTCAATTAAAGT	<i>M. caseolyticus</i> JCSC5402	AP009484
CCM 4927 ^T	ϕ MG1	39954	32.83	58	between <i>TsaD</i> and <i>ABC transporter</i>	CGAACACATGTAC TTGTACACAA	<i>Staphylococcus agnetis</i> 908	CP009623
	ϕ MG2	41282	34.10	60	<i>tRNA-Leu</i>	ACCCCGACCGGTGGTACTA	<i>M. goetzii</i> sp. nov. CCM 4927 (ϕ MG1)	this study
CCM 7099 ^T	ϕ ME1	49155	32.71	73	between <i>TsaD</i> and <i>ABC transporter</i>	TTGTGTACAAGTACATGTG TTCGCGTGGATAA	<i>M. caseolyticus</i> IMD0819	CP021058
CCM 7100 ^T	ϕ MB1	32855	33.87	47	<i>tRNA-Arg</i>	TTAGCGTCTGGGAGG	<i>M. caseolyticus</i> IMD0819	CP021058



of strain CCM 7100^T and newly described species *Auricoccus indicus*, which is distantly related to macrococci (Prakash et al., 2017), hence pointing to the intergeneric horizontal transfer of these resistance genes.

34,802 bp-long pZKMB1 and 69,509 bp-long pZKMB2, low-copy number plasmids from strain CCM 7100^T, encode putative *tra* genes, suggesting their conjugative transfer. Nevertheless, only pZKMB1 harbors other genes necessary for conjugation. The conjugative potential of these plasmids has not been tested due to the absence of suitable selection markers. The conjugation-essential nick region 5'GTGTGTAAGTGCG↓CCCTTG3' that matches the consensus sequence 5'NcgtNtaAgtGCGCcCTta 3' for the RSF10-oriT (Grohmann et al., 2003) was identified in the plasmid pZKMB1. The Mba/MobL superfamily domain of the putative DNA relaxase (TraA) of pZKMB1 possesses two out of three motifs that are typically found in all conjugative DNA relaxases in Gram-positive bacteria (Zechner et al., 2017); motif I with a conserved Tyr residue binds the DNA backbone, and motif III with two His residues is involved in nucleophilic attack at the nick region.

The plasmid pZKMB2 shares short regions with 73% nucleotide identity with the pMCCL2 plasmid from *M. caseolyticus* strain JCSC5402. Unlike pMCCL2 that carries an unusual form of the *mec* gene complex (*mecB-mecR1_m-mecI_m-blaZ_m*) (Tsubakishita et al., 2010), the pZKMB2 plasmid encodes the putative stage V sporulation protein B (SpoVB), responsible for the late stage of spore development (Vasudevan et al., 2009). In strain CCM 7099^T, the 11,867 bp-long plasmid pZKME1 encoding 12 ORFs with unknown function was identified. The small cryptic 1,913-bp-long plasmid pZKMG1 was identified in strain CCM 4927^T. pZKMG1 only encodes a replication protein with 94% amino acid identity to the replication protein of pSTH6 from *S. saprophyticus* (Heir et al., 1998).

Species Description Protologues

Protologues describing the four aforementioned novel taxa are given below. Phenotypic tests distinguishing the novel

macrococcal taxa from the phylogenetically close species *M. canis* and *M. caseolyticus* are presented in Table 4. The ability to use carbon sources via respiration, determined in Biolog GP2 MicroPlate assays is listed in Table S2.

Description of *Macrococcus caseolyticus* subsp. *hominis* subsp. nov.

Macrococcus caseolyticus subsp. *hominis* subsp. nov. (ho'mi.nis L. gen. n. *hominis* of man, indicating that the strains were isolated from humans).

Cells are Gram-stain positive spherical cocci, occurring predominantly in pairs and clusters, non-spore forming and non-motile. Colonies on TSA agar are circular, whole margin, flat, smooth, shiny, 1–2 mm in diameter, aerobic and pigmented a yellowish orange. No haemolytic activity. Grows in the presence of up to 7.5% NaCl, at 15°C (weak) and 48°C but not at 10°C or 50°C. No growth in the presence of 10% or more NaCl and in a thioglycolate medium. Catalase, oxidase, pyrrolidonyl arylamidase, nitrate reduction and Voges-Proskauer test (acetoin) positive. Weak hydrolysis of gelatin. Coagulase, clumping factor, urease, arginine dihydrolase, and ornithine decarboxylase negative. Susceptible to furazolidon (100 µg) and resistant to novobiocin (5 µg) and bacitracin (10 IU). Hydrolysis of esculin and Tween 80 negative. Acid phosphatase, alkaline phosphatase (weak), esterase (C4), esterase lipase (C8), chymotrypsin (weak) and naphthol-AS-Bi-phosphohydrolase positive. Lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-galactosidase, β-galactosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase negative.

TABLE 4 | Phenotypic characteristics that differentiate novel macrococci from phenotypically related *Macrococcus caseolyticus* subsp. *caseolyticus* subsp. nov. and *Macrococcus canis*.

Test	1*	2*	3	4	5	6
Growth at 10°C	+	–	–	+	+	+
Growth at 45°C	+	+	–	+	–	–
Growth in thioglycolate	+	–	–	w	w	+
Yellow pigmented	–	+	–	–	–	–
Chymotrypsin	+	+	w	+	–	+
Acid from: ribose	+	+	–	–	–	–
galactose	+	–	–	+	+	+
mannitol	–	+	+	–	–	–
lactose	+	–	–	+	+	+
salicine	–	–	–	+	+	+
turanose	–	–	–	+	+	+
L-arabinose	–	–	–	+	–	–
melezitose	–	–	–	+	+	–

Strains: 1. *M. caseolyticus* subsp. *caseolyticus* subsp. nov. CCM 3540^T; 2. *M. caseolyticus* subsp. *hominis* subsp. nov. CCM 7927^T; 3. *M. canis* CCM 8748^T; 4. *M. goetzii* sp. nov. CCM 4927^T; 5. *M. epidermidis* sp. nov. CCM 7099^T; 6. *M. bohemicus* sp. nov. CCM 7100^T.

+, positive; w, weak positive; –, negative; *, data valid for type strain as well as subspecies description; all data of type strains were taken from this study

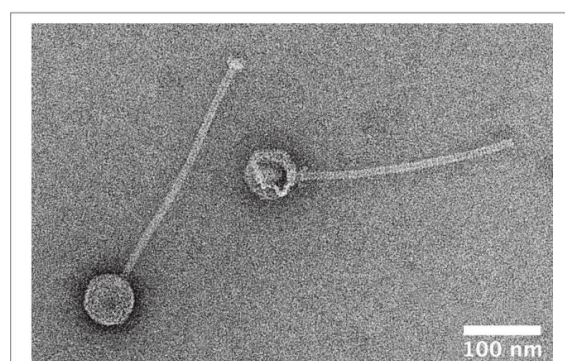


FIGURE 8 | Transmission electron microscopy image of negatively stained particle of phage ϕ MC1 induced from *Macrocococcus caseolyticus* subsp. *hominis* subsp. nov. CCM 7927^T by UV-light.

Acid is produced from glycerol, ribose, D-glucose, D-fructose, mannitol, maltose, sucrose and trehalose. Acid is not produced from erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, adonitol, β -methyl-D-xyloside, galactose, mannose, sorbose, rhamnose, dulcitol, inositol, sorbitol, α -methyl-D-mannoside, α -methyl-D-glucoside, N-acetyl glucosamine, amygdalin, arbutin, salicin, cellobiose, lactose, melibiose, inulin, melezitose, D-raffinose, starch, glycogen, xylitol, β -gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2 keto-gluconate and 5 keto-gluconate. Variable biochemical reactions were obtained for the hydrolysis of DNA (1 of 4 positive), β -glucuronidase (2 of 4) and α -glucosidase (2 of 4). Susceptible to ampicillin, cefoxitin, cephalothin, ciprofloxacin, clindamycin, gentamicin, chloramphenicol, imipenem, kanamycin, neomycin, oxacillin, penicillin G, sulphamethox/trimethoprim (cotrimoxazol), tetracycline and vancomycin. Resistant to erythromycin. The DNA G+C content of the type strain is 36.79 mol% (calculated from WGS). The peptidoglycan type is A3 α Lys-Gly₃-Ser. The major fatty acids are C_{14:0}, C_{16:0} N alcohol, C_{16:1} ω 11c and C_{18:1} ω 9c. The quinone system contains the major component menaquinone MK-6 and minor component MK-7.

Isolated from various human clinical materials. The type strain is CCM 7927^T (= DSM 103682^T). Sequence accession no. of 16S rRNA gene for the type strain is MH044686. Most characteristics of the type strain CCM 7927^T are in agreement with the subspecies description. The strain-dependent test results are as follows: positive β -glucuronidase and negative hydrolysis of DNA and α -glucosidase.

Description of *Macrococcus caseolyticus* subsp. *caseolyticus* subsp. nov.

The subspecies name is created automatically with the same authors as those of *Macrococcus caseolyticus* subsp. *hominis* subsp. nov., in accordance with Rule 40d (formerly Rule 46) of the Prokaryotic Code (De Vos and Trüper, 2000; Parker et al., 2015). The description of *M. caseolyticus* subsp. *caseolyticus* subsp. nov. is based on properties reported previously (Schleifer et al., 1982; Kloos et al., 1998; Mannerová et al., 2003; Schleifer, 2015; Gobeli Brawand et al., 2017) and on phenotypic data determined from the five reference strains included in this study. All strains are gelatin hydrolysis-positive and produce esterase (C4), esterase-lipase (C8) and naphthol-AS-BI-phosphohydrolase. Acid from gluconate, leucine arylamidase and α -glucosidase is variable. Grows in presence of up to 12% NaCl, hydrolysis of Tween 80 and esculin, lipase (C14), valine arylamidase, cysteine arylamidase, trypsin, α -galactosidase, N-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase negative. Acid production from erythritol, D-arabinose, L-xylose, D-adonitol, β -methyl-D-xyloside, L-sorbose, L-rhamnose, dulcitol, inositol, α -methyl-D-mannoside, α -methyl-D-glucoside, N-acetyl glucosamine, amygdaline, arbutine, D-melibiose, inulin, starch, glycogen, xylitol, gentiobiose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, 2 keto-gluconate and 5 keto-gluconate negative.

The type strain CCM 3540^T (= ATCC 13548^T = CCUG 15606^T = CIP 100755^T = DSM 20597^T) was isolated from cow's milk (Schleifer et al., 1982).

Description of *Macrococcus goetzii* sp. nov.

Macrococcus goetzii sp. nov. (goe'tzi.i N.L. masc. gen. n. *goetzii*, of Goetz, named in honor of Prof. Dr. Friedrich Götz, a German microbiologist, for his contribution to the microbiology, physiology, and molecular biology of staphylococci and macrococci).

Cells are Gram-stain positive irregular spherical cocci, occurring predominantly singly and in clusters, non-spore forming and non-motile. Colonies on TSA agar are circular, with whole margins, slightly convex, smooth, shiny, 1–2 mm in diameter, aerobic and non-pigmented. No haemolytic activity. Grows in the presence of up to 7.5% NaCl (weak), at 10°C and 45°C but not at 5°C or 48°C. No growth in the presence of 10% or more NaCl. Weak growth in thioglycolate medium. Catalase, oxidase, nitrate reduction, Voges-Proskauer test (acetoin) and hydrolysis of gelatin positive. Coagulase, clumping factor, urease, arginine dihydrolase, ornithine decarboxylase and pyrrolidonyl arylamidase negative. Susceptible to furazolidon (100 μ g) and resistant to novobiocin (5 μ g) and bacitracin (10 IU). Hydrolysis of esculin, DNA and Tween 80 negative. Acid phosphatase, alkaline phosphatase, esterase (C4), esterase lipase (C8) (weak), chymotrypsin (weak), naphthol-AS-Bi-phosphohydrolase, β -galactosidase (weak) and α -glucosidase (weak) positive. Lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α -galactosidase, β -glucuronidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase negative. Acid is produced from glycerol, L-arabinose, galactose (weak), D-glucose, D-fructose, arbutin, salicin, maltose, sucrose, trehalose, melezitose, β -gentiobiose and D-turanose. Acid is not produced from erythritol, D-arabinose, ribose, D-xylose, L-xylose, adonitol, β -methyl-D-xyloside, mannose, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, α -methyl-D-mannoside, α -methyl-D-glucoside, N-acetyl glucosamine, amygdaline, cellobiose, lactose, melibiose, inulin, D-raffinose, starch, glycogen, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2 keto-gluconate and 5 keto-gluconate. Susceptible to cefoxitin, cephalothin, ciprofloxacin, erythromycin, gentamicin, chloramphenicol, imipenem, kanamycin, neomycin, sulphamethox/trimethoprim (cotrimoxazol), tetracycline and vancomycin. Resistant to ampicillin, clindamycin, oxacillin and penicillin G. The DNA G+C content of the type strain is 33.95 mol% (calculated from WGS). The peptidoglycan type is A3 α L-Lys-Gly_{1–2}. The major fatty acids are C_{14:0}, C_{15:0} anteiso, C_{16:1} ω 11c, C_{16:0}, and C_{18:1} ω 9c. The quinone system contains the major component menaquinone MK-6 and minor components MK-5 and MK-7.

The type strain is CCM 4927^T (= DSM 103683^T). Isolated from human clinical material (swab, nail, mycosis). Sequence accession no. of 16S rRNA gene for the type strain is MH044690.

Description of *Macrococcus epidermidis* sp. nov.

Macrococcus epidermidis sp. nov. (e.pi.der'mi.dis. N.L. n. *epidermis*, outer skin (from Gr. pref. *epi* and Gr. n. *derma*, skin; Gr. n. *epidermis*, -idos, the outer skin); N.L. gen. n. *epidermidis*, of the outer skin).

Cells are Gram-stain positive spherical cocci, occurring predominantly in pairs and clusters, non-spore forming and non-motile. Colonies on TSA agar are circular, with whole margins, convex with condensed centers, lustreless, 1–2 mm in diameter, aerobic and non-pigmented. No haemolytic activity. Grows in the presence of up to 7.5% NaCl (weak), at 10 and 42°C but not at 5 or 45°C. No growth in the presence of 10% or more NaCl. Weak growth in thioglycolate medium. Catalase, oxidase, nitrate reduction, Voges-Proskauer test (acetoin) and hydrolysis of gelatin positive. Coagulase, clumping factor, urease, arginine dihydrolase, ornithine decarboxylase and pyrrolidonyl arylamidase negative. Susceptible to furazolidon (100 µg) and resistant to novobiocin (5 µg) and bacitracin (10 IU). Hydrolysis of esculin, DNA and Tween 80 negative. Acid phosphatase, alkaline phosphatase, esterase (C4), esterase lipase (C8) (weak), α-glucosidase (weak) and naphthol-AS-Bi-phosphohydrolase positive. Lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase negative. Acid is produced from glycerol (weak), galactose, D-glucose, D-fructose, N-acetyl glucosamine, arbutin, salicin, maltose, lactose, sucrose, trehalose, melezitose, β-gentiobiose and D-turanose. Acid is not produced from erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, β-methyl-D-xyloside, mannose, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, α-methyl-D-mannoside, α-methyl-D-glucoside, amygdaline, cellobiose, melibiose, inulin, D-raffinose, starch, glycogen, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2 keto-gluconate and 5 keto-gluconate. Susceptible to ampicillin, cefoxitin, cephalothin, ciprofloxacin, erythromycin, gentamicin, chloramphenicol, imipenem, kanamycin, neomycin, oxacillin, penicillin G, sulphamethox/trimethoprim (cotrimoxazol), tetracycline and vancomycin. Resistant to clindamycin. The DNA G+C content of the type strain is 33.97 mol% (calculated from WGS). The peptidoglycan type is A3α Lys-Gly₃-Ser. The major fatty acids are C_{15:0} anteiso, C_{18:1} ω9c and C_{18:0}. Quinone system contains the major component menaquinone MK-6 and minor component MK-7.

The type strain is CCM 7099^T (= DSM 103681^T). Isolated from human clinical material (swab, mycosis). Sequence accession no. of 16S rRNA gene for the type strain is MH044691.

Description of *Macrococcus bohemicus* sp. nov.

Macrococcus bohemicus sp. nov. (bo.he'mi.cus. N.L. masc. adj. *bohemicus*, pertaining to Bohemia, a region of the Czech Republic where the type strain was isolated).

Cells are Gram-stain positive spherical cocci, occurring predominantly in pairs and tetrads, non-spore forming and non-motile. Colonies on TSA agar are circular, with whole

margins, flat with convex center, lustreless, 1–2 mm in diameter, aerobic and nonpigmented. No haemolytic activity. Grows in the presence of 7.5% NaCl, at 10°C and 42°C but not at 5 or 45°C. Growth in thioglycolate medium. No growth in the presence of 10% or more NaCl. Catalase, oxidase, nitrate reduction, Voges-Proskauer test (acetoin) and hydrolysis of gelatin positive. Coagulase, clumping factor, urease, arginine dihydrolase, ornithine decarboxylase and pyrrolidonyl arylamidase negative. Susceptible to furazolidon (100 µg) and resistant to novobiocin (5 µg) and bacitracin (10 IU). Hydrolysis of esculin, DNA and Tween 80 negative. Acid phosphatase (weak), esterase (C4), esterase lipase (C8), chymotrypsin and naphthol-AS-Bi-phosphohydrolase positive. Lipase (C14), alkaline phosphatase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase negative. Acid is produced from glycerol (weak), galactose, D-glucose, D-fructose, N-acetyl glucosamine, arbutin, salicin, maltose, lactose, sucrose, trehalose, D-raffinose (weak), β-gentiobiose and D-turanose (weak). Acid is not produced from erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, β-methyl-D-xyloside, mannose, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, α-methyl-D-mannoside, α-methyl-D-glucoside, amygdaline, cellobiose, melibiose, inulin, melezitose, starch, glycogen, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2 keto-gluconate and 5 keto-gluconate. Susceptible to ampicillin, cefoxitin, cephalothin, ciprofloxacin, erythromycin, gentamicin, chloramphenicol, imipenem, kanamycin, neomycin, oxacillin, penicillin G, sulphamethox/trimethoprim (cotrimoxazol), tetracycline and vancomycin. Resistant to clindamycin. The DNA G+C content of the type strain is 34.9 mol% (calculated from WGS). Peptidoglycan type is A3α L-Lys-Gly_{1–2}. Major fatty acids are C_{15:0} anteiso, C_{16:0} and C_{18:1} ω9c. Quinone system contains major component menaquinone MK-6 and minor components MK-5 and MK-7.

The type strain is CCM 7100^T (= DSM 103680^T). Isolated from human clinical material (wound, knee). Sequence accession no. of 16S rRNA gene for the type strain is MH044692.

Emended Description of Genus *Macrococcus* Kloos, Ballard, George, Webster, Hubner, Ludwig, Schleifer, Fiedler and Schubert 1998

The phenotypic characteristics are identical to those of the original genus description and additional references (Mannerová et al., 2003; Gobeli Brawand et al., 2017) and the above-mentioned data in subspecies descriptions, except for the following: DNA G+C content is in the range 34–39 mol%.

AUTHOR CONTRIBUTIONS

RP, IS, and IM designed the study. ZW, IS, PŠ, PS, CS, SK, OŠ, LK, VV, TF, and PP performed the experiments; IM, ZW, AI, VK, and OŠ analyzed the data; ZZ, VR and JD contributed the theory and experimental design; IM, ZW, AI, IS, and RP wrote the paper.

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REFERENCES

- Arndt, D., Grant, J. R., Marcu, A., Sajed, T., Pon, A., Liang, Y., et al. (2016). PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic Acids Res.* 44, W16–W21. doi: 10.1093/nar/gkw387
- Arthur, M., Depardieu, F., Gerbaud, G., Galimand, M., Leclercq, R., and Courvalin, P. (1997). The VanS sensor negatively controls VanR-mediated transcriptional activation of glycopeptide resistance genes of Tn1546 and related elements in the absence of induction. *J. Bacteriol.* 179, 97–106. doi: 10.1128/jb.179.1.97-106.1997
- Aziz, R. K., Bartels, D., Best, A. A., Dejongh, M., Disz, T., Edwards, R. A., et al. (2008). The RAST server: rapid annotations using subsystems technology. *BMC Genomics* 9:75. doi: 10.1186/1471-2164-9-75
- Baba, T., Kuwahara-Arai, K., Uchiyama, I., Takeuchi, F., Ito, T., and Hiramatsu, K. (2009). Complete genome sequence of *Macrococcus caseolyticus* strain JCS5402, reflecting the ancestral genome of the human-pathogenic staphylococci. *J. Bacteriol.* 191, 1180–1190. doi: 10.1128/JB.01058-08
- Baida, G. E., and Kuzmin, N. P. (1996). Mechanism of action of hemolysin III from *Bacillus cereus*. *Biochim. Biophys. Acta* 1284, 122–124. doi: 10.1016/S0005-2736(96)00168-X
- Bay, D. C., and Turner, R. J. (2012). Small multidrug resistance protein EmrE reduces host pH and osmotic tolerance to metabolic quaternary cation osmoprotectants. *J. Bacteriol.* 194, 5941–5948. doi: 10.1128/JB.00666-12
- Becker, K., Heilmann, C., and Peters, G. (2014). Coagulase-negative staphylococci. *Clin. Microbiol. Rev.* 27, 870–926. doi: 10.1128/CMR.00109-13
- Becker, K., Van Alen, S., Idelevich, E. A., Schleimer, N., Seggewiss, J., Mellmann, A., et al. (2018). Plasmid-encoded transferable *mecB*-mediated methicillin resistance in *Staphylococcus aureus*. *Emerging Infect. Dis.* 24, 242–248. doi: 10.3201/eid2402.171074
- Ben Zakour, N. L., Bannoehr, J., Van Den Broek, A. H., Thoday, K. L., and Fitzgerald, J. R. (2011). Complete genome sequence of the canine pathogen *Staphylococcus pseudintermedius*. *J. Bacteriol.* 193, 2363–2364. doi: 10.1128/JB.00137-11
- Cashion, P., Holder-Franklin, M. A., McCully, J., and Franklin, M. (1977). A rapid method for the base ratio determination of bacterial DNA. *Anal. Biochem.* 81, 461–466. doi: 10.1016/0003-2697(77)90720-5
- Castillo, D., Espejo, R., and Middelboe, M. (2014). Genomic structure of bacteriophage 6H and its distribution as prophage in *Flavobacterium psychrophilum* strains. *FEMS Microbiol. Lett.* 351, 51–58. doi: 10.1111/1574-6968.12342
- Chanchaithong, P., Prapasarakul, N., Perreten, V., and Schwendener, S. (2016). Characterization of a novel composite staphylococcal cassette chromosome *mec* in methicillin-resistant *Staphylococcus pseudintermedius* from Thailand. *Antimicrob. Agents Chemother.* 60, 1153–1157. doi: 10.1128/AAC.02268-15

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2018.01178/full#supplementary-material>

- Chen, L., Zheng, D., Liu, B., Yang, J., and Jin, Q. (2016). VFDB 2016: hierarchical and refined dataset for big data analysis—10 years on. *Nucleic Acids Res.* 44, D694–D697. doi: 10.1093/nar/gkv1239
- Christie, J., McNab, R., and Jenkinson, H. F. (2002). Expression of fibronectin-binding protein FbpA modulates adhesion in *Streptococcus gordonii*. *Microbiology* 148, 1615–1625. doi: 10.1099/00221287-148-6-1615
- Christo-Foroux, E., Vallaes, T., Loux, V., Dassa, E., Deutscher, J., Wandersman, C., et al. (2017). Manual and expert annotation of the nearly complete genome sequence of *Staphylococcus sciuri* strain ATCC 29059: a reference for the oxidase-positive staphylococci that supports the atypical phenotypic features of the species group. *Syst. Appl. Microbiol.* 40, 401–410. doi: 10.1016/j.syapm.2017.07.002
- Chung, Y. S., and Dubnau, D. (1995). ComC is required for the processing and translocation of ComGC, a pilin-like competence protein of *Bacillus subtilis*. *Mol. Microbiol.* 15, 543–551. doi: 10.1111/j.1365-2958.1995.tb02267.x
- Cicconi-Hogan, K. M., Belomestnykh, N., Gamroth, M., Ruegg, P. L., Tikofsky, L., and Schukken, Y. H. (2014). Prevalence of methicillin resistance in coagulase-negative staphylococci and *Staphylococcus aureus* isolated from bulk milk on organic and conventional dairy farms in the United States. *J. Dairy Sci.* 97, 2959–2964. doi: 10.3168/jds.2013-7523
- CLSI (2015). Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard - 12th Edn. CLSI document M02-A12. Wayne, PA: Clinical and Laboratory Standards Institute.
- Collins, M. D., Pirouz, T., Goodfellow, M., and Minnikin, D. E. (1977). Distribution of menaquinones in actinomycetes and corynebacteria. *J. Gen. Microbiol.* 100, 221–230. doi: 10.1099/00221287-100-2-221
- Costa, S. S., Mourato, C., Viveiros, M., Melo-Cristino, J., Amaral, L., and Couto, I. (2013). Description of plasmid pSM52, harbouring the gene for the Smr efflux pump, and its involvement in resistance to biocides in a methicillin-resistant *Staphylococcus aureus* strain. *Int. J. Antimicrob. Agents* 41, 490–492. doi: 10.1016/j.ijantimicag.2013.01.003
- Cotting, K., Strauss, C., Rodriguez-Campos, S., Rostaher, A., Fischer, N. M., Roosje, P. J., et al. (2017). *Macrococcus canis* and *M. caseolyticus* in dogs: occurrence, genetic diversity and antibiotic resistance. *Vet. Dermatol.* 28, 559–e133. doi: 10.1111/vde.12474
- De Ley, J., Cattoir, H., and Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur. J. Biochem.* 12, 133–142. doi: 10.1111/j.1432-1033.1970.tb00830.x
- De Vos, P., and Trüper, H. G. (2000). Judicial Commission of the International Committee on Systematic Bacteriology; IXth International (IUMS) Congress of Bacteriology and Applied Microbiology. *Int. J. Syst. Evol. Microbiol.* 50, 2239–2244. doi: 10.1099/00207713-50-6-2239
- Demple, B., Halbrook, J., and Linn, S. (1983). *Escherichia coli xth* mutants are hypersensitive to hydrogen peroxide. *J. Bacteriol.* 153, 1079–1082.
- Dhillon, B. K., Laird, M. R., Shay, J. A., Winsor, G. L., Lo, R., Nizam, F., et al. (2015). IslandViewer 3: more flexible, interactive genomic island

- discovery, visualization and analysis. *Nucleic Acids Res.* 43, W104–W108. doi: 10.1093/nar/gkv401
- Dominguez-Moñino, I., Jurado, V., Gonzalez-Pimentel, J. L., Miller, A. Z., Hermosin, B., and Saiz-Jimenez, C. (2018). *Bacillus onubensis* sp. nov., isolated from the air of two Andalusian caves. *Syst. Appl. Microbiol.* doi: 10.1016/j.syapm.2018.01.001
- EUCAST (2017). *Breakpoint Tables for Interpretation of MICs and Zone Diameters*. The European Committee on Antimicrobial Susceptibility Testing. Available online at: http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_7.1_Breakpoint_Tables.pdf
- Fagerlund, A., Granum, P. E., and Havarstein, L. S. (2014). *Staphylococcus aureus* competence genes: mapping of the SigH, ComK1 and ComK2 regulons by transcriptome sequencing. *Mol. Microbiol.* 94, 557–579. doi: 10.1111/mmi.12767
- Freiwald, A., and Sauer, S. (2009). Phylogenetic classification and identification of bacteria by mass spectrometry. *Nat. Protoc.* 4, 732–742. doi: 10.1038/nprot.2009.37
- Gevers, D., Huys, G., and Swings, J. (2001). Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. *FEMS Microbiol. Lett.* 205, 31–36. doi: 10.1111/j.1574-6968.2001.tb10921.x
- Gobeli Brawand, S., Cotting, K., Gomez-Sanz, E., Collaud, A., Thomann, A., Brodard, I., et al. (2017). *Macrococcus canis* sp. nov., a skin bacterium associated with infections in dogs. *Int. J. Syst. Evol. Microbiol.* 67, 621–626. doi: 10.1099/ijsem.0.001673
- Gobeli Brawand, S., Rychener, L., Schwendener, S., Pantucek, R., and Perreten, V. (2018). Complete genome sequence of the type strain of *Macrococcus canis*. *Genome Announc.* 6:e01507–17. doi: 10.1128/genomeA.01507-17
- Gómez-Sanz, E., Schwendener, S., Thomann, A., Gobeli Brawand, S., and Perreten, V. (2015). First staphylococcal cassette chromosome *mec* containing a *mecB*-carrying gene complex independent of transposon Tn6045 in a *Macrococcus caseolyticus* isolate from a canine infection. *Antimicrob. Agents Chemother.* 59, 4577–4583. doi: 10.1128/AAC.05064-14
- Götz, F., Bannerman, T., and Schleifer, K.-H. (2006). “The genera *Staphylococcus* and *Macrococcus*,” in *The Prokaryotes. A Handbook on the Biology of Bacteria. Volume 4: Bacteria: Firmicutes, Cyanobacteria*, eds M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer and E. Stackebrandt (New York, NY: Springer), 5–75.
- Grohmann, E., Muth, G., and Espinosa, M. (2003). Conjugative plasmid transfer in gram-positive bacteria. *Microbiol. Mol. Biol. Rev.* 67, 277–301. doi: 10.1128/MMBR.67.2.277-301.2003
- Groth, I., Schumann, P., Weiss, N., Martin, K., and Rainey, F. A. (1996). *Agrococcus jenensis* gen. nov., sp. nov., a new genus of actinomycetes with diaminobutyric acid in the cell wall. *Int. J. Syst. Bacteriol.* 46, 234–239. doi: 10.1099/00207713-46-1-234
- Hansen, C. M., Meixell, B. W., Van Hemert, C., Hare, R. F., and Hueffer, K. (2015). Microbial infections are associated with embryo mortality in Arctic-nesting geese. *Appl. Environ. Microbiol.* 81, 5583–5592. doi: 10.1128/AEM.00706-15
- Heir, E., Sundheim, G., and Holck, A. L. (1998). The *Staphylococcus qacH* gene product: a new member of the SMR family encoding multidrug resistance. *FEMS Microbiol. Lett.* 163, 49–56. doi: 10.1111/j.1574-6968.1998.tb13025.x
- Huss, V. A., Festl, H., and Schleifer, K. H. (1983). Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Syst. Appl. Microbiol.* 4, 184–192. doi: 10.1016/S0723-2020(83)80048-4
- Indráková, A., Mašláňová, I., Kováčová, V., Doškar, J., and Pantucek, R. (2016). The evolutionary pathway of the staphylococcal cassette chromosome element. *Biologia* 71, 1195–1203. doi: 10.1515/biolog-2016-0156
- Joh, D., Wann, E. R., Kreikemeyer, B., Speziale, P., and Hook, M. (1999). Role of fibronectin-binding MSCRAMMs in bacterial adherence and entry into mammalian cells. *Matrix Biol.* 18, 211–223. doi: 10.1016/S0945-053X(99)00025-6
- Jones, P., Binns, D., Chang, H. Y., Fraser, M., Li, W., McAnulla, C., et al. (2014). InterProScan 5: genome-scale protein function classification. *Bioinformatics* 30, 1236–1240. doi: 10.1093/bioinformatics/btu031
- Kahánková, J., Pantucek, R., Goerke, C., Ruzičková, V., Holochová, P., and Doškar, J. (2010). Multilocus PCR typing strategy for differentiation of *Staphylococcus aureus* siphoviruses reflecting their modular genome structure. *Environ. Microbiol.* 12, 2527–2538. doi: 10.1111/j.1462-2920.2010.02226.x
- Kim, H. K., Thammavongsa, V., Schneewind, O., and Missiakas, D. (2012). Recurrent infections and immune evasion strategies of *Staphylococcus aureus*. *Curr. Opin. Microbiol.* 15, 92–99. doi: 10.1016/j.mib.2011.10.012
- Kloos, W. E., Ballard, D. N., George, C. G., Webster, J. A., Hubner, R. J., Ludwig, W., et al. (1998). Delimiting the genus *Staphylococcus* through description of *Macrococcus caseolyticus* gen. nov., comb. nov. and *Macrococcus equiperficus* sp. nov., and *Macrococcus bovis* sp. no. and *Macrococcus carouselicus* sp. nov. *Int. J. Syst. Bacteriol.* 48, 859–877. doi: 10.1099/00207713-48-3-859
- Kumar, S., Stecher, G., and Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33, 1870–1874. doi: 10.1093/molbev/msw054
- Laanto, E., Bamford, J. K., Ravantti, J. J., and Sundberg, L. R. (2015). The use of phage FCL-2 as an alternative to chemotherapy against columnaris disease in aquaculture. *Front. Microbiol.* 6:829. doi: 10.3389/fmicb.2015.00829
- Lagesen, K., Hallin, P., Rodland, E. A., Staerfeldt, H. H., Rognes, T., and Ussery, D. W. (2007). RNAmmer: consistent and rapid annotation of ribosomal RNA genes. *Nucleic Acids Res.* 35, 3100–3108. doi: 10.1093/nar/gkm160
- Lei, Y., Oshima, T., Ogasawara, N., and Ishikawa, S. (2013). Functional analysis of the protein Veg, which stimulates biofilm formation in *Bacillus subtilis*. *J. Bacteriol.* 195, 1697–1705. doi: 10.1128/JB.02201-12
- Li, D., Wang, Y., Schwarz, S., Cai, J., Fan, R., Li, J., et al. (2016). Co-location of the oxazolidinone resistance genes *optrA* and *cfr* on a multiresistance plasmid from *Staphylococcus sciuri*. *J. Antimicrob. Chemother.* 71, 1474–1478. doi: 10.1093/jac/dkw040
- Madonna, A. J., Basile, F., Ferrer, I., Meetani, M. A., Rees, J. C., and Voorhees, K. J. (2000). On-probe sample pretreatment for the detection of proteins above 15 kDa from whole cell bacteria by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* 14, 2220–2229. doi: 10.1002/1097-0231(20001215)14:23<2220::AID-RCM155>3.0.CO;2-4
- Mannerová, S., Pantucek, R., Doškar, J., Švec, P., Snauwaert, C., Vancanneyt, M., et al. (2003). *Macrococcus brunensis* sp. nov., *Macrococcus hajekii* sp. nov. and *Macrococcus lamae* sp. nov., from the skin of llamas. *Int. J. Syst. Evol. Microbiol.* 53, 1647–1654. doi: 10.1099/ijms.0.02683-0
- Meier-Kolthoff, J. P., Auch, A. F., Klenk, H. P., and Goker, M. (2013). Genome sequence-based species delimitation with confidence intervals and improved distance functions. *BMC Bioinformatics* 14:60. doi: 10.1186/1471-2105-14-60
- Michiel, V., Hogan, B., Koller, T., Warnke, P., Crusius, S., Hinz, R., et al. (2015). Screening agars for MRSA: evaluation of a stepwise diagnostic approach with two different selective agars for the screening for methicillin-resistant *Staphylococcus aureus* (MRSA). *Mil. Med. Res.* 2:18. doi: 10.1186/s40779-015-0046-1
- Mukherjee, A., Chettri, B., Langpoklakpam, J. S., Singh, A. K., and Chattopadhyay, D. (2016). Draft genome sequence of hydrocarbon-degrading *Staphylococcus saprophyticus* strain CNV2, isolated from crude oil-contaminated soil from the Noonmati Oil Refinery, Guwahati, Assam, India. *Genome Announc.* 4:e00370-16. doi: 10.1128/genomeA.00370-16
- Müller, A., Rychli, K., Muhterem-Uyar, M., Zaiser, A., Stessl, B., Guinane, C. M., et al. (2013). Tn6188 - a novel transposon in *Listeria monocytogenes* responsible for tolerance to benzalkonium chloride. *PLoS ONE* 8:e76835. doi: 10.1371/journal.pone.0076835
- Muschol, S., Balaban, M., Normark, S., and Henriques-Normark, B. (2015). Uptake of extracellular DNA: competence induced pili in natural transformation of *Streptococcus pneumoniae*. *Bioessays* 37, 426–435. doi: 10.1002/bies.201400125
- Nurk, S., Bankevich, A., Antipov, D., Gurevich, A., Korobeynikov, A., Lapidus, A., et al. (2013). “Assembling genomes and mini-metagenomes from highly chimeric reads,” in *Research in Computational Molecular Biology. RECOMB 2013. Lecture Notes in Computer Science*, Vol. 7821, eds M. Deng, R. Jiang, F. Sun, and X. Zhang (Berlin, Heidelberg: Springer), 7158–7170.
- Okonechnikov, K., Golosova, O., Fursov, M., and Ugene, Team (2012). Unipro UGENE: a unified bioinformatics toolkit. *Bioinformatics* 28, 1166–1167. doi: 10.1093/bioinformatics/bts091
- O’Riordan, K., and Lee, J. C. (2004). *Staphylococcus aureus* capsular polysaccharides. *Clin. Microbiol. Rev.* 17, 218–234. doi: 10.1128/CMR.17.1.218-234.2004
- Pantucek, R., Götz, F., Doškar, J., and Rosypal, S. (1996). Genomic variability of *Staphylococcus aureus* and the other coagulase-positive *Staphylococcus* species

- estimated by macrorestriction analysis using pulsed-field gel electrophoresis. *Int. J. Syst. Bacteriol.* 46, 216–222. doi: 10.1099/00207713-46-1-216
- Pantuček, R., Sedláček, I., Indráková, A., Vrbovská, V., Mašlanová, I., Kovarovic, V., et al. (2017). *Staphylococcus edaphicus* sp. nov., isolated in Antarctica, harbours *mecC* gene and genomic islands with suspected role in adaptation to extreme environment. *Appl. Environ. Microbiol.* 84:e01746-17. doi: 10.1128/AEM.01746-17
- Pantuček, R., Sedláček, I., Petráš, P., Koukalová, D., Švec, P., Štětina, V., Vancanneyt, M., et al. (2005). *Staphylococcus simiae* sp. nov., isolated from South American squirrel monkeys. *Int. J. Syst. Evol. Microbiol.* 55, 1953–1958. doi: 10.1099/ijs.0.63590-0
- Pantuček, R., Švec, P., Dajcs, J. J., Machová, I., Cernohlávková, J., Šedo, O., et al. (2013). *Staphylococcus petrasii* sp. nov. including *S. petrasii* subsp. *petrasii* subsp. nov. and *S. petrasii* subsp. *croceilyticus* subsp. nov., isolated from human clinical specimens and human ear infections. *Syst. Appl. Microbiol.* 36, 90–95. doi: 10.1016/j.syapm.2012.11.004
- Parker, C. T., Tindall, B. J., and Garrity, G. M. (2015). International code of nomenclature of prokaryotes. *Int. J. Syst. Evol. Microbiol.* doi: 10.1099/ijsem.0.000778. [Epub ahead of print].
- Parte, A. C. (2014). LPSN-list of prokaryotic names with standing in nomenclature. *Nucleic Acids Res.* 42, D613–D616. doi: 10.1093/nar/gkt1111
- Paterson, G. K., Harrison, E. M., and Holmes, M. A. (2014). The emergence of *mecC* methicillin-resistant *Staphylococcus aureus*. *Trends Microbiol.* 22, 42–47. doi: 10.1016/j.tim.2013.11.003
- Penadés, J. R., and Christie, G. E. (2015). The Phage-Inducible chromosomal islands: a family of highly evolved molecular parasites. *Annu. Rev. Virol.* 2, 181–201. doi: 10.1146/annurev-virology-031413-085446
- Prakash, O., Muduli, S., Kumar, R., Kumari, C., Nimonkar, Y., Shouche, Y. S., et al. (2017). Description of *Auricoccus indicus* gen. nov., sp. nov., isolated from skin of human ear. *Int. J. Syst. Evol. Microbiol.* 67, 1212–1218. doi: 10.1099/ijsem.0.001787
- Pritchard, L., Glover, R. H., Humphris, S., Elphinstone, J. G., and Toth, I. K. (2016). Genomics and taxonomy in diagnostics for food security: soft-rotting enterobacterial plant pathogens. *Anal. Methods* 8, 12–24. doi: 10.1039/C5AY02550H
- Richter, M., and Rosselló-Móra, R. (2009). Shifting the genomic gold standard for the prokaryotic species definition. *Proc. Natl. Acad. Sci. U.S.A.* 106, 19126–19131. doi: 10.1073/pnas.0906412106
- Rissman, A. I., Mau, B., Biehl, B. S., Darling, A. E., Glasner, J. D., and Perna, N. T. (2009). Reordering contigs of draft genomes using the Mauve aligner. *Bioinformatics* 25, 2071–2073. doi: 10.1093/bioinformatics/btp356
- Rohde, H., Frankenberger, S., Zahring, U., and Mack, D. (2010). Structure, function and contribution of polysaccharide intercellular adhesin (PIA) to *Staphylococcus epidermidis* biofilm formation and pathogenesis of biomaterial-associated infections. *Eur. J. Cell Biol.* 89, 103–111. doi: 10.1016/j.ejcb.2009.10.005
- Rubin, D., Zhang, W., Karch, H., and Kuczius, T. (2017). Distinct expression of immunoglobulin-binding proteins in Shiga Toxin-producing *Escherichia coli* implicates high protein stability and a characteristic phenotype. *Toxins* 9:153. doi: 10.3390/toxins9050153
- Rubin, J. E., and Chirino-Trejo, M. (2010). Inducibly cefoxitin-resistant *Macrococcus*-like organism falsely identified as methicillin-resistant *Staphylococcus aureus* on CHROMagar with oxacillin. *J. Clin. Microbiol.* 48, 3037–3038. doi: 10.1128/JCM.00519-10
- Sandt, C. H., Hopper, J. E., and Hill, C. W. (2002). Activation of prophage *eib* genes for immunoglobulin-binding proteins by genes from the *lbrAB* genetic island of *Escherichia coli* ECOR-9. *J. Bacteriol.* 184, 3640–3648. doi: 10.1128/JB.184.13.3640-3648.2002
- Sasser, M. (1990). *Identification of Bacteria by Gas Chromatography of Cellular Fatty Acids, MIDI Technical Note 101*. Newark, DE: Microbial ID Inc.
- Sau, S., and Lee, C. Y. (1996). Cloning of type 8 capsule genes and analysis of gene clusters for the production of different capsular polysaccharides in *Staphylococcus aureus*. *J. Bacteriol.* 178, 2118–2126. doi: 10.1128/jb.178.7.2118-2126.1996
- Sau, S., Bhasin, N., Wann, E. R., Lee, J. C., Foster, T. J., and Lee, C. Y. (1997). The *Staphylococcus aureus* allelic genetic loci for serotype 5 and 8 capsule expression contain the type-specific genes flanked by common genes. *Microbiology* 143, 2395–2405. doi: 10.1099/00221287-143-7-2395
- Schleifer, K. H., and Bell, J. A. (2009). “Genus I. *Staphylococcus* Rosenbach 1884, 18AL (Nom. Cons. Opin. 17 Jud. Comm. 1958, 153.)” in *Bergey’s Manual of Systematic Bacteriology, The Firmicutes*, eds P. De Vos, G. M. Garrity, D. Jones, N. R. Krieg, W. Ludwig, F. A. Rainey, K. H. Schleifer, and W. B. Whitman (New York, NY: Springer-Verlag), 392–421.
- Schleifer, K. H., Kilpper-Balz, R., Fischer, U., Faller, A., and Endl, J. (1982). Identification of “*Micrococcus candidus*” ATCC 14852 as a strain of *Staphylococcus epidermidis* and of “*Micrococcus caseolyticus*” ATCC 13548 and *Micrococcus varians* ATCC 29750 as members of a new species, *Staphylococcus caseolyticus*. *Int. J. Syst. Bacteriol.* 32, 15–20. doi: 10.1099/00207713-32-1-15
- Schleifer, K.-H. (2015). “*Macrococcus* (Kloos, Ballard, George, Webster, Hubner, Ludwig, Schleifer, Fiedler and Schubert 1998, 871VP),” in *Bergey’s Manual of Systematics of Archaea and Bacteria*, ed W. B. Whitman (Indianapolis, IN: John Wiley & Sons), 1–6.
- Schreiter, E. R., and Drennan, C. L. (2007). Ribbon-helix-helix transcription factors: variations on a theme. *Nat. Rev. Microbiol.* 5, 710–720. doi: 10.1038/nrmicro1717
- Schumann, P. (2011). Peptidoglycan structure. *Methods Microbiol.* 38, 101–129. doi: 10.1016/B978-0-12-387730-7.00005-X
- Schwendener, S., Cotting, K., and Perreten, V. (2017). Novel methicillin resistance gene *mecD* in clinical *Macrococcus caseolyticus* strains from bovine and canine sources. *Sci. Rep.* 7:43797. doi: 10.1038/srep43797
- Siguier, P., Perochon, J., Lestrade, L., Mahillon, J., and Chandler, M. (2006). ISfinder: the reference centre for bacterial insertion sequences. *Nucleic Acids Res.* 34, D32–D36. doi: 10.1093/nar/gkj014
- Švec, P., Cernohlávková, J., Busse, H. J., Vojtková, H., Pantuček, R., Cnockaert, M., Mašlanová, I., et al. (2015). Classification of strain CCM 4446^T as *Rhodococcus degradans* sp. nov. *Int. J. Syst. Evol. Microbiol.* 65, 4381–4387. doi: 10.1099/ijsem.0.000584
- Švec, P., Pantuček, R., Petráš, P., Sedláček, I., and Nováková, D. (2010). Identification of *Staphylococcus* spp. using (GTG)₅-PCR fingerprinting. *Syst. Appl. Microbiol.* 33, 451–456. doi: 10.1016/j.syapm.2010.09.004
- Švec, P., Petráš, P., Pantuček, R., Doškar, J., and Sedláček, I. (2016). High intraspecies heterogeneity within *Staphylococcus sciuri* and rejection of its classification into *S. sciuri* subsp. *sciuri*, *S. sciuri* subsp. *carinatus* and *S. sciuri* subsp. *rodentium*. *Int. J. Syst. Evol. Microbiol.* 66, 5181–5186. doi: 10.1099/ijsem.0.001493
- Takeuchi, F., Watanabe, S., Baba, T., Yuzawa, H., Ito, T., Morimoto, Y., et al. (2005). Whole-genome sequencing of *Staphylococcus haemolyticus* uncovers the extreme plasticity of its genome and the evolution of human-colonizing staphylococcal species. *J. Bacteriol.* 187, 7292–7308. doi: 10.1128/JB.187.21.7292-7308.2005
- Thammavongsa, V., Kern, J. W., Missiakas, D. M., and Schneewind, O. (2009). *Staphylococcus aureus* synthesizes adenosine to escape host immune responses. *J. Exp. Med.* 206, 2417–2427. doi: 10.1084/jem.20090097
- Thammavongsa, V., Schneewind, O., and Missiakas, D. M. (2011). Enzymatic properties of *Staphylococcus aureus* adenosine synthase (AdA). *BMC Biochem.* 12:56. doi: 10.1186/1471-2091-12-56
- Tsubakishita, S., Kuwahara-Arai, K., Baba, T., and Hiramatsu, K. (2010). Staphylococcal cassette chromosome *mec*-like element in *Macrococcus caseolyticus*. *Antimicrob. Agents Chemother.* 54, 1469–1475. doi: 10.1128/AAC.00575-09
- Vasudevan, P., McElligott, J., Attkisson, C., Betteken, M., and Popham, D. L. (2009). Homologues of the *Bacillus subtilis* SpoVB protein are involved in cell wall metabolism. *J. Bacteriol.* 191, 6012–6019. doi: 10.1128/JB.00604-09
- Verstappen, K. M., Huijbregts, L., Spaninks, M., Wagenaar, J. A., Fluit, A. C., and Duim, B. (2017). Development of a real-time PCR for detection of *Staphylococcus pseudintermedius* using a novel automated comparison of whole-genome sequences. *PLoS ONE* 12:e0183925. doi: 10.1371/journal.pone.0183925
- Wang, Y., Coleman-Derr, D., Chen, G., and Gu, Y. Q. (2015). OrthoVenn: a web server for genome wide comparison and annotation of orthologous clusters across multiple species. *Nucleic Acids Res.* 43, W78–W84. doi: 10.1093/nar/gkv487
- Watts, A., Ke, D., Wang, Q., Pillay, A., Nicholson-Weller, A., and Lee, J. C. (2005). *Staphylococcus aureus* strains that express serotype 5 or serotype 8

- capsular polysaccharides differ in virulence. *Infect. Immun.* 73, 3502–3511. doi: 10.1128/IAI.73.6.3502-3511.2005
- Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A., Kandler, O., Truper, H. G. et al. (1987). Report of the Ad-Hoc-Committee on reconciliation of approaches to bacterial systematics. *Int. J. Syst. Bacteriol.* 37, 463–464. doi: 10.1099/00207713-37-4-463
- Wick, R. R., Schultz, M. B., Zobel, J., and Holt, K. E. (2015). Bandage: interactive visualization of *de novo* genome assemblies. *Bioinformatics* 31, 3350–3352. doi: 10.1093/bioinformatics/btv383
- Yoon, S. H., Ha, S. M., Kwon, S., Lim, J., Kim, Y., Seo, H., et al. (2017). Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. *Int. J. Syst. Evol. Microbiol.* 67, 1613–1617. doi: 10.1099/ijsem.0.001755
- Zechner, E. L., Moncalian, G., and De La Cruz, F. (2017). Relaxases and plasmid transfer in Gram-negative bacteria. *Curr. Top. Microbiol. Immunol.* 413, 93–113. doi: 10.1007/978-3-319-75241-9_4
- Zeman, M., Mašláňová, I., Indráková, A., Šiborová, M., Mikulášek, K., Bendíčková, K., et al. (2017). *Staphylococcus sciuri* bacteriophages double-convert for staphylokinase and phospholipase, mediate interspecies plasmid transduction, and package *mecA* gene. *Sci. Rep.* 7:46319. doi: 10.1038/srep46319
- Zhang, Q., and Ye, Y. (2017). Not all predicted CRISPR-Cas systems are equal: isolated cas genes and classes of CRISPR like elements. *BMC Bioinformatics* 18:92. doi: 10.1186/s12859-017-1512-4

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4.3 *Staphylococcus petrasii* diagnostics and its pathogenic potential enhanced by mobile genetic elements

Background and objectives: Coagulase-negative staphylococci cause infections in immunocompromised patients with medical devices and are often multidrug resistant. The most common being *S. haemolyticus* and related species, including *S. petrasii*, which was initially thought to have four subspecies *S. petrasii* subsp. *petrasii*, *S. petrasii* subsp. *jettensis*, *S. petrasii* subsp. *croceilyticus* and *S. petrasii* subsp. *pragensis*. However, the latter two were recently reclassified as separate species. Despite the importance of the *S. petrasii* complex, little is known about its virulence and epidemiology, as they are frequently misidentified. Therefore, a large set of strains isolated from human clinical material, predominantly from young and older patients, was analyzed to understand pathogenic potential and improve diagnostic.

Material and methods: A set of sixty-five strains belonging to the *S. petrasii* complex were characterized both genotypically and phenotypically. Four type strains: CCM 8418^T, CCM 8494^T, CCM 8421^T, CCM 5829^T, and one additional methicillin-resistant isolate of *S. petrasii* subsp. *jettensis* P5404 were sequenced using Ion Torrent PGM. Additionally, plasmid profiles and induced prophages were analyzed.

Results and discussion: The phylogenomic methods clearly grouped the strains of *S. croceilyticus* and *S. pragensis* but failed to differentiate between the subspecies of *S. petrasii* subsp. *petrasii* and *jettensis*. Comparative genomics analysis has revealed that *S. petrasii* subsp. *jettensis*, linked to bloodstream and implant infections, has accumulated more genes, including large composite SCCmec elements and SP β -like prophage that carry multiple resistance genes. The situation resembles the *S. hominis* subspecies, where one *S. hominis* subsp. *novobiosepticus* has a larger chromosome and is often multiresistant. Regarding virulence, the *S. petrasii* complex is similar to *S. haemolyticus*. Both lack the *ica* operon but present phenol-soluble modulins, suggesting an independent biofilm formation pathway. The analyzed genomes were differentially enriched in pathways for saccharide utilization and contained large plasmids with antimicrobial resistance genes. Some plasmids were also found in the genomes of *S. aureus*, indicating interspecies transfer.

Conclusion: The phylogenetic relationships among the species of the *S. petrasii* complex have been clarified along with the functional organization of its genomes. The MALDI-TOF-MS and PFGE were found to be reliable diagnostic tools.

Significance: This study emphasizes reliable diagnostic of the increasingly reported *S. petrasii* complex to obtain surveillance data, as these species are commonly multidrug-resistant and can serve as reservoirs for horizontal gene transfer.



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Staphylococcus petrasii diagnostics and its pathogenic potential enhanced by mobile genetic elements

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ABSTRACT

Staphylococcus petrasii is recently described coagulase negative staphylococcal species and an opportunistic human pathogen, still often misidentified in clinical specimens. Four subspecies are distinguished in *S. petrasii* by polyphasic taxonomical analyses, however a comparative study has still not been done on the majority of isolates and their genome properties have not yet been thoroughly analysed. Here, we describe the phenotypic and genotypic characteristics of 65 isolates and the results of *de novo* sequencing, whole genome assembly and annotation of draft genomes of five strains. The strains were identified by MALDI-TOF mass spectrometry to the species level and the majority of the strains were identified to the subspecies level by fingerprinting methods, (GTG)₅ repetitive PCR and ribotyping. Macrorestriction profiling by pulsed-field gel electrophoresis was confirmed to be a suitable strain typing method. Comparative genomics revealed the presence of new mobile genetic elements carrying antimicrobial resistance factors such as staphylococcal cassette chromosome (SCC) *mec*, transposons, phage-inducible genomic islands, and plasmids. Their mosaic structure and similarity across coagulase-negative staphylococci and *Staphylococcus aureus* suggest the possible exchange of these elements. Numerous putative virulence factors such as adhesins, autolysins, exoenzymes, capsule formation genes, immunomodulators, the phage-associated *sasX* gene, and SCC-associated spermidine N-acetyltransferase gene, pseudouridine and sorbitol utilization operons might explain clinical manifestations of *S. petrasii* isolates. The increasing recovery of *S. petrasii* isolates from human clinical material, the multi-drug resistance including methicillin resistance of *S. petrasii* subsp. *jettensis* strains, and virulence factors homologous to other pathogenic staphylococci demonstrate the importance of the species in human disease.

1. Introduction

Coagulase-negative staphylococci (CoNS) are considered to be typical opportunists (Heilmann et al., 2019), having a substantial impact on human life and health, particularly in immunocompromised patients (Becker et al., 2014). Antibiotic resistance has been documented to be more common in CoNS than in *S. aureus* (Brzychczy-Włoch et al., 2013; Otto, 2013). Developments in diagnostics have led to the frequent detection of *Staphylococcus petrasii* in human specimens and suggested that it is a clinically relevant species. Based on genotypic and phenotypic analyses, four subspecies *S. petrasii* subsp. *petrasii*, *S. petrasii* subsp.

jettensis, *S. petrasii* subsp. *croceilyticus* and *S. petrasii* subsp. *pragensis* were described (De Bel et al., 2014, 2013; Pantůček et al., 2013; Švec et al., 2015). *S. petrasii* belongs to a phylogenetic clade including *Staphylococcus haemolyticus* and *Staphylococcus hominis* (Pantůček et al., 2013). The well-studied and phylogenetically related *S. haemolyticus* is known for its historically early acquisition of resistance to methicillin and glycopeptide antibiotics (Froggatt et al., 1989). It is possible that *S. haemolyticus* played an important role in the diversification of staphylococcal cassette chromosome *mec* (SCC*mec*) (Cavanagh et al., 2014; Zong et al., 2011), which led to an alarming prevalence of methicillin-resistant *S. haemolyticus* clinical isolates (Bouchami et al., 2012; Panda

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et al., 2016). In contrast, SCC of *S. hominis* exhibited a limited clonality and a low genetic diversity (Bouchami et al., 2011). The properties of both species confirm they are significant opportunistic human pathogens (Barros et al., 2012; Chaves et al., 2005).

Although *S. petrasii* was described 6 years ago (Pantucek et al., 2013), there is limited knowledge of its pathogenicity, genome structure, mobile genetic elements (MGE) carrying virulence and drug resistance genes, and systems limiting the spread of these elements. A set of sixty-five strains originating from human clinical material was analysed in this study with a focus on improving *S. petrasii* diagnostics and the description of the species' diversity. Whole genomes of type strains of four *S. petrasii* subspecies and one additional methicillin-resistant isolate were sequenced and characterized in detail for a better understanding of its pathogenic potential.

2. Materials and methods

2.1. Bacterial strains

S. petrasii strains were collected from a set of CoNS referred by routine clinical laboratories for identification to the National Reference Laboratory for Staphylococci, National Institute of Public Health (NRL/St) (Prague, Czech Republic) as clinically significant agents isolated from clinical materials and from previous taxonomic studies (Table S1, Fig. S1) (De Bel et al., 2013; Pantucek et al., 2013; Svec et al., 2015). The possibility that strains were skin contaminants was excluded by disinfection before the blood collection and analysis of patients' skin microflora. Reference strains are available in the Czech Collection of Microorganisms (CCM) at Masaryk University (Brno, Czech Republic). The phenotypic identification of the isolates was done using commercial identification kits STAPHYtest 24 with ErbaExpert software (Erba Lachema, Brno, Czech Republic) and API Staph (bioMérieux, Marcy l'Etoile, France) according to the manufacturers' instructions.

2.2. Matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis

The samples were prepared by the standard extraction protocol described in detail by Freiwald and Sauer (Freiwald and Sauer, 2009). MALDI-TOF mass spectra were obtained using an UltrafleXtreme instrument (Bruker Daltonics, Bremen, Germany) operated in linear positive mode using the software FlexControl version 3.4. Signals present in at least seven out of nine independent mass spectra acquired per sample were taken into account. Mass spectra were processed using FlexAnalysis (version 3.4; Bruker Daltonics) and BioTyper software (version 3.1; Bruker Daltonics) supplemented with database version 8.0.0.0 (7854 entries). The mutual similarity between individual mass spectra of the strains and the Biotyper database entries were expressed in the form of log(scores) obtained using the default settings of the Biotyper software.

2.3. Genotypic analysis by fingerprinting techniques

Repetitive PCR fingerprinting with the (GTG)₅ primer ((GTG)₅-REP-PCR) was performed as described previously (Svec et al., 2010). The automatic ribotyping was performed using the RiboPrinter Microbial Characterization System (DuPont Qualicon, Wilmington, DE) in accordance with the manufacturer's instructions. Numerical analysis of the obtained fingerprints and dendrogram construction was done using the software BioNumerics version 7.6 (Applied Maths, Kortrijk, Belgium) and compared to the in-house CCM database of reference strains. The ribotype patterns were imported into BioNumerics using the load samples import script provided by the manufacturer.

Macrorestriction analysis with *Sma*I (New England BioLabs, Ipswich, MA) resolved by pulsed-field gel electrophoresis (PFGE) was performed as described previously (Pantucek et al., 1996). PFGE was

done using a CHEF-Mapper system (Biorad, Hercules, CA) with 1.2% agarose gel (Serva, Heidelberg, Germany) in 1 × Tris-acetate-EDTA (TAE) buffer for 20 h at 14 °C. Pulse times of 2 s to 12 s for 14 h at 5.5 V and 30 s to 45 s for 6 h at 6 V were used. The strain *S. petrasii* subsp. *petrasii* CCM 8418^T was used as a reference every sixth line of each gel. The gels were stained in 0.3% ethidium bromide for 3 h and photographed under UV light. Digital images were stored electronically as TIFF files and analyzed with BioNumerics version 7.6 software. Dendrograms were constructed using the Dice similarity coefficient (with 0.5% tolerance and 0.5% optimization) and the unweighted pair group method with arithmetic averages (UPGMA) for clustering.

2.4. Antimicrobial susceptibility tests

The antibiotic resistance was tested by the disc diffusion method on Mueller-Hinton agar (Oxoid, Basingstoke, UK) using seventeen antibiotics generally used for Gram-positive cocci (Oxoid): cefoxitin (30 µg), ciprofloxacin (5 µg), clindamycin (2 µg), erythromycin (15 µg), co-trimoxazole (25 µg), fusidic acid (10 µg), gentamicin (10 µg), rifampicin (5 µg), tigecycline (15 µg), mupirocin (5 µg), tetracycline (30 µg), chloramphenicol (30 µg), linezolid (10 µg), benzylpenicillin (1U), teicoplanin (30 µg), vancomycin (30 µg), and ceftaroline (5 µg). The incubation conditions were 16–20 h at 36 °C in air. EUCAST/CLSI standards for reading the inhibition zone diameter and interpreting susceptibility to antibiotics were strictly followed (CLSI, 2018; EUCAST, 2017). The presence of the methicillin resistance genes *mecA*, *mecB* and *mecC* was examined by PCR amplification with the primers SA-MECA165 and SAMECA1482 for *mecA* (Wu et al., 1998), *mecA*_{LGA251}MultiFP and *mecA*_{LGA251}MultiRP for *mecC* (Stegger et al., 2012), and *mecB*-F (GGGACTATAGACGCATCAAC) and *mecB*-R (CCA GTAACACTCTTCTCATGC) designed in this study for *mecB* gene.

2.5. *rpoB* gene sequencing

Partial *rpoB* gene amplification was performed as described previously (Mellmann et al., 2006). PCR amplicons were sequenced by Sanger sequencing with the primers 1418F and 1876R in Eurofins Genomics (Ebersberg, Germany). Phylogenetic analyses were performed with the software MEGA version 7 (Kumar et al., 2016).

2.6. Plasmid profiling

The plasmid DNA was extracted using a NucleoSpin[®] Plasmid Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol with the modification that bacteria were lysed with 30 µg/ml of lysostaphin (Ambi Products, Lawrence, NY) at 37 °C. Undigested plasmid DNA was separated by agarose gel electrophoresis as described previously (Kuntova et al., 2012).

2.7. Genome sequencing and assembly

Genomic DNA was isolated and whole genomic sequencing was performed using an Ion Torrent[™] Personal Genome Machine (Thermo Fisher Scientific, Carlsbad, CA). The purified genomic DNA was used for preparing a 400-bp sequencing library with an Ion Plus Fragment Library Kit (Thermo Fisher Scientific) as described previously (Pantucek et al., 2018). The sample was loaded onto a 318v2 chip and sequenced using an Ion PGM Hi-Q sequencing kit (Thermo Fisher Scientific). Quality trimming of the reads were performed with the Ion Torrent Suite Software version 5.0.4 with default settings. The assembly computation and error correction was performed using the assembler SPAdes version 3.11.1 pipeline (Nurk et al., 2013) with parameters adjusted for Ion Torrent data. Contigs were then re-ordered according to the reference genome *S. haemolyticus* JCS1435 (Takeuchi et al., 2005) using MauveContigMover (Rissman et al., 2009). Ordering was evaluated using an assembly graph visualized in Bandage (Wick et al.,

2015). Plasmid contigs were extracted from WGS data based on higher coverage and content of typical plasmid-borne genes.

2.8. Bioinformatics analyses

Sequences were manipulated and inspected in the cross-platform bioinformatics software Ugene version 1.28.0 (Okonechnikov et al., 2012). For primary analysis, the genome was annotated using RAST (Aziz et al., 2008). Predicted protein sequences were clustered using web-based OrthoVenn with a default cutoff e-value of $1e^{-5}$ and an inflation value of 1.5 (Wang et al., 2015). Functional genome annotation was further performed using several databases; the NCBI BLAST service (blast.ncbi.nlm.nih.gov/Blast.cgi) and InterProScan (Jones et al., 2014). MGE were predicted using IslandViewer 4 (Dhillon et al., 2015), CRISPRone (Zhang and Ye, 2017), VIRFAM (Lopes et al., 2014) and ISfinder (Siguier et al., 2006). Antibiotic resistance determinants were screened with ResFinder (Zankari et al., 2012) and the RGI prediction tool (Jia et al., 2017). Virulence factors were searched against the databases VFDB (Chen et al., 2016a) and VirulenceFinder (Joensen et al., 2014), and nucleotide sequences of previously described candidate virulence genes of *Staphylococcus epidermidis* and *S. haemolyticus* (Gill et al., 2005; Takeuchi et al., 2005). To calculate the ANI value, the OrthoANI algorithm implemented on the EzBioCloud server was used (Lee et al., 2016). The dDDH values were calculated using the web-based genome-to-genome distance calculator (GGDC) version 2.1 (Meier-Kolthoff et al., 2013).

2.9. Sequence accession numbers

The whole-genome shotgun projects of strains CCM 8418^T, CCM 8421^T, CCM 8494^T, P5404, and CCM 8529^T have been deposited at GenBank under the accession numbers SRJE000000000, SRJF000000000, SRLF000000000, SRLS000000000, and SRPJ000000000 respectively. Partial *rpoB* gene sequences of strains P4324, P6625, P6635 and P6644 have been deposited at GenBank under the accession numbers MK649779, MK649780, MK649781 and MK649782, respectively.

2.10. Bacteriophage induction and characterization

Prophages were induced from the genome-sequenced strains with mitomycin C as described previously (Pulverer et al., 1974). Phage φSPJ1-int3 (Vb_Spe5_5404) from *S. petrasii* subsp. *jettensis* strain P5404 was propagated on *S. petrasii* subsp. *jettensis* P5402 and further characterized. The phage lysate was purified by zonal centrifugation in a CsCl gradient as described previously (Maslanova et al., 2013). Proteomic analysis of the purified phage was performed by liquid chromatography - mass spectrometry (LC-MS) using an RSLCnano system (Thermo Fisher Scientific) on-line connected to an Impact II Ultra-High Resolution Qq-Time-of-Flight mass spectrometer (Bruker) as described previously (Zeman et al., 2017).

2.11. Electron and cryo-electron microscopy

Negative-stained samples were prepared by staining in 2% uranyl acetate on copper grids coated with a 12-nm continuous carbon layer. Cryo samples were prepared by vitrification of the bacteriophage solution (at a concentration of 10^{10} PFU ml⁻¹) on Quantifoil grids by plunge freezing in liquid ethane using a ThermoFisher Scientific VitroBot Mark IV. The samples were observed with a ThermoFisher Scientific Tecnai F20 electron microscope operated at 200 kV at a magnification of 29,000 ×.

2.12. PCR detection of *cas1*, *cas2* and *cas9* genes

Conventional singleplex PCR were carried out in 25 µl reaction mixtures (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 200 µM dNTPs),

including 0.2 µM each of primers and 1.5 unit of Taq DNA polymerase (New England Biolabs), and of 50 ng of purified DNA. Primers *cas1F* (ACAGTTGAAGTGGGAYGAAG) and *cas1R* (ACCATCAATTTCACWC-GMGCA), *cas2F* (ATGTTTGATYTRCCTGTAGAAAC) and *cas2R* (AATG-CTCTAACATGYCCTTC) or *cas9F* (AGAGGYGCGCGTAGATTAAA) and *cas9R* (AAWGGACTTCCTTCGCCAGG) gave rise to 502 bp, 194 bp or 515 bp products, respectively. Cycling conditions were 5 min denaturation at 96 °C, followed by 25 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s.

3. Results

3.1. Source and identification of *Staphylococcus petrasii*

The specimens were recovered from human clinical material, mainly inpatients and a limited number of outpatients, predominantly from young children and older patients in which the pathogenesis of infections was similar as for other CoNS species (Table 1). Over the last 20 years, 3% of CoNS in the NRL/St were retrospectively identified as *S. petrasii* (Fig. S1). Since the majority of the strains were originally misidentified in routine clinical microbiology laboratories, we proposed suitable biochemical tests for the differentiation of *S. petrasii* subspecies from biochemically similar *Staphylococcus warneri*, *S. haemolyticus* and *Staphylococcus auricularis* (Fig. 1).

Specifically, the identification of *S. petrasii* strains was enabled by establishing MALDI-TOF MS as a microbial diagnostic tool. Of the 65 analyzed strains, 6 strains were identified with high confidence and log (score) > 2.0, and 37 with low confidence and log(score) 1.7–2.0, both indicating their assignment as Biotyper database entries *Staphylococcus* sp. 10w414721 RLH, *Staphylococcus* sp. 901400083 LBK, *Staphylococcus* sp. 4 PIM and *Staphylococcus* sp. CS 331_1BRB (all these database strains were assigned as *S. petrasii* in the recently released update of the MBT 8468 MSP Library, version 9.0.0.0). The remaining 22 strains yielded log(score) < 1.7, indicating no significant similarity to any of the database entries. Simply extending the Biotyper database with *S. petrasii* subsp. *petrasii* CCM 8418^T mass spectra resulted in the correct identification of 61 strains with log(score) > 2.0, while only four strains (among them three *S. petrasii* subsp. *pragensis*) were identified with log(score) 1.7–2.0. The MALDI-TOF MS-based cluster analysis (Fig. S2) revealed distinct clusters of *S. petrasii* subsp. *croceilyticus* and *S. petrasii* subsp. *pragensis* strains, while the remaining two subspecies were unresolved. For subspecies identification, further extension of the Biotyper database with *S. petrasii* subsp. *croceilyticus* CCM 8421^T and *S. petrasii* subsp. *pragensis* P6089 resulted in the correct assignment of all strains to the corresponding subspecies. Type strain *S. petrasii* subsp. *pragensis* CCM 8529^T was not found to be a suitable database entry. However, due to the absence of mass spectral features distinctive for *S. petrasii* subsp. *petrasii* and *S. petrasii* subsp. *jettensis*, no database extension that resulted in the correct identification of these two subspecies was designable.

Fingerprinting-based techniques (GTG)₅-REP-PCR and automated ribotyping that were previously shown to be effective for subspecies differentiation in staphylococci (Svec et al., 2010) confirmed strain identification at the subspecies level, except for strains P4324, P5414, P6635, P6625, and P6644 (Figs. S3 and S4). Partial *rpoB* gene sequencing was performed in the unassigned isolates and only strain P6644 was classified as *S. petrasii* subsp. *pragensis* (Fig. S5), while the rest remained unidentified to the subspecies level.

3.2. PFGE typing of *Staphylococcus petrasii*

S. petrasii isolates exhibited 61 different PFGE patterns of 7–14 *Sma*I restriction endonuclease fragments (Fig. 2), demonstrating a high clonal diversity and simultaneously confirming PFGE to be an efficient typing technique. The isolates were separated into three major clusters corresponding to *S. petrasii* subsp. *pragensis*, *S. petrasii* subsp.

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Table 1
Origin of *Staphylococcus petrasii* strains characterized in this study.

Material	Patient age group	Number of strains for <i>S. petrasii</i> subspecies ^a					Diagnoses
		SPP	SPJ	unassigned	SPC	SPR	
blood culture	0–10	1	–	–	–	–	low birth weight
	40–59	6	2	–	1	2	cerebral hemorrhage, abdominal pain, fever, secondary malignant neoplasm of lymph nodes, acute respiratory failure, gastritis, epilepsy, pancreatitis, sepsis
	60–100	6	6	–	–	–	hospitalization at ICU, stroke, fever, asthmatic bronchitis, hematuria, pneumonia, sepsis, graphite fibrosis of lung
purulence, wound	0–10	1	1	–	–	–	festering wound, low birth weight
	11–39	–	–	–	–	1	finger lesion
	40–59	1	–	–	–	3	multiple head trauma, appendicitis, head abscess
	60–100	1	1	–	–	1	festering wound, cellulitis of torso
skin or catheter swab	70–90	2	2	–	–	–	cellulitis of finger, burn
	0–10	1	–	–	1	–	urinary tract infection
	40–59	1	–	–	–	–	acute cystitis
	60–100	–	1	–	–	3	malignant neoplasm of breast, malignant neoplasm of kidney, hyperplasia of prostate
vaginal swab	20–40	2	–	–	–	1	high-risk pregnancy, acute inflammation of vagina
ejaculate	50–60	–	–	–	–	1	chronic prostatitis
ear swab	0–10	2	–	1	3	–	neonatal bacterial sepsis, otitis externa
	20–40	–	–	–	1	–	otitis externa
	60–100	–	1	–	–	–	otitis externa
eye swab	50–60	–	–	1	–	–	–
biopsy	50–60	–	1	1	–	–	–
nose swab	20–40	1	–	–	–	–	carrier
hospital environment	–	1	2	–	–	–	–
unknown human origin	–	–	–	1	–	–	–

^a SPP, *S. petrasii* subsp. *petrasii*; SPC, *S. petrasii* subsp. *croceilyticus*; SPJ, *S. petrasii* subsp. *jettensis*; SPR, *S. petrasii* subsp. *pragensis*.

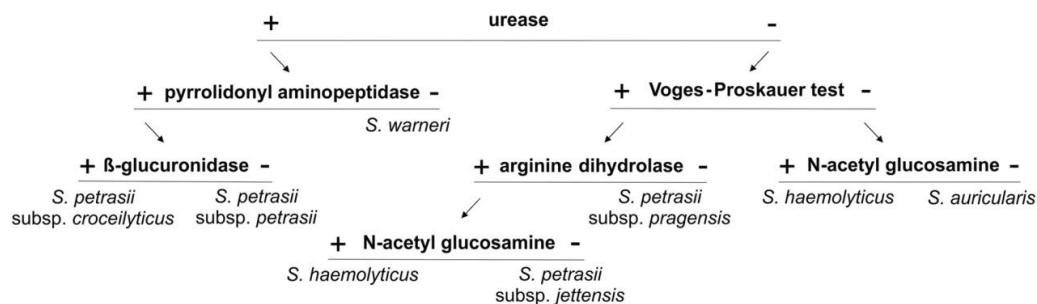


Fig. 1. Biochemical differentiation between *Staphylococcus petrasii* subspecies and phylogenetically related *Staphylococcus haemolyticus* and phenotypically similar *Staphylococcus auricularis* and *Staphylococcus warneri*, performed using commercially available kits. *S. petrasii* strains give a positive reaction in pyrrolidonyl aminopeptidase and Voges-Proskauer tests and do not produce acid from *N*-acetyl glucosamine. The four *S. petrasii* subspecies are easily differentiated from each other by urease, β -glucuronidase and arginine dihydrolase activity tests.

croceilyticus and a cluster of closely related subspecies *S. petrasii* subsp. *petrasii* and *S. petrasii* subsp. *jettensis* at a similarity level of 45%. *S. petrasii* subsp. *jettensis* formed a distinct clade separating at a similarity level of 65% (Fig. 2). Four pairs of strains isolated in the same hospital showed indistinguishable PFGE patterns (Fig. 2), but three pairs differed in their plasmid and antibiotic resistance profiles, indicating that they were not identical clones.

3.3. Antimicrobial susceptibility

Twenty-three percent of the strains were multi-drug resistant, exhibiting resistance to three or more key therapeutic antibiotics, mostly in *S. petrasii* subsp. *jettensis* isolates (Fig. 2). The most prevalent was resistance to benzylpenicillin (63%), erythromycin (28%), clindamycin (22%) and tetracycline (15%). Twenty-eight percent of *S. petrasii* strains did not exhibit resistance to any of the tested antibiotics. Nine *S. petrasii* subsp. *jettensis* strains were resistant to ceftazidime and *mecA*-positive. The *mecA* gene was detected only in subspecies *S. petrasii* subsp. *jettensis*, while *mecB* and *mecC* genes were not detected in any of the tested strains. Of the 9 methicillin resistant strains, strain P6318 was resistant

to anti-MRSA 5th generation cephalosporins when using ceftazidime disks; additional three strains exhibited intermediate susceptibility. Plasmid profiling showed variable plasmid content in all strains (Fig. 2), represented by one to four plasmids classified into 4 classes according to Novick (Novick, 1990).

3.4. Genome characteristics and comparative genomics

The whole genome sequences of type strains *S. petrasii* subsp. *petrasii* CCM 8418^T, *S. petrasii* subsp. *croceilyticus* CCM 8421^T, *S. petrasii* subsp. *jettensis* CCM 8494^T, and *S. petrasii* subsp. *pragensis* CCM 8529^T and one additional methicillin-resistant *S. petrasii* subsp. *jettensis* P5404 isolate from a fatal case of bronchopneumonia were annotated and characterized (Table 2). The comparison of *S. petrasii* with closely related *S. haemolyticus* showed that *S. petrasii* genomes share 78% orthologous protein clusters, as illustrated by Venn diagram (Fig. 3). The accessory genome of *S. petrasii* subsp. *jettensis* strains is larger than in the other subspecies (Table 2). Distinctive operons encoding saccharide intake transporters and various efflux systems were identified in the *oriC* environ of *S. petrasii* subspecies. The strain *S. petrasii* subsp. *petrasii* CCM

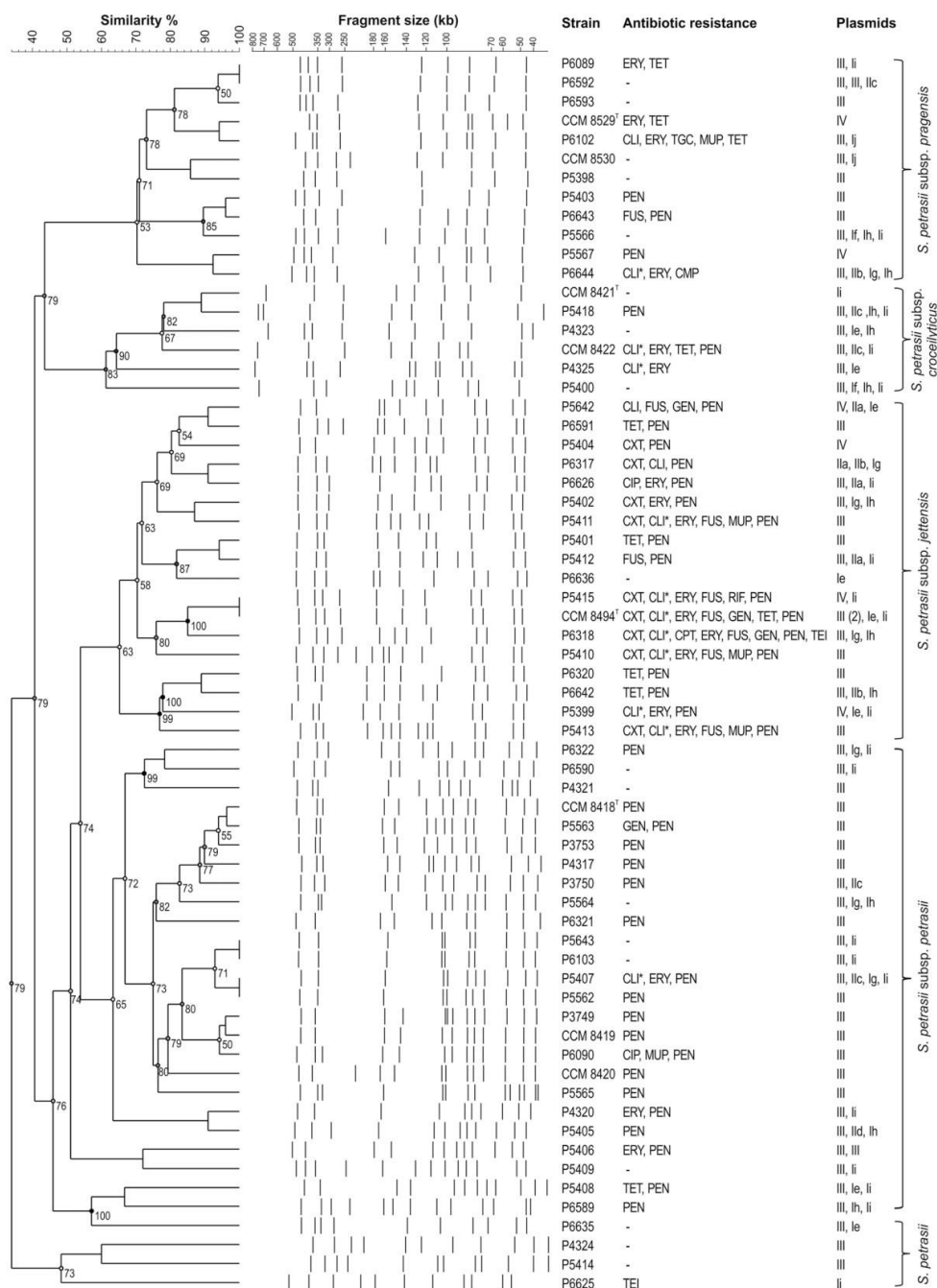


Fig. 2. Dendrogram representing similarity of PFGE patterns of *Smal*-digested genomic DNA of 65 *Staphylococcus petrasii* strains constructed by UPGMA cluster analysis with Dice similarity coefficients. Antibiotic resistance and plasmid profiles identified in these strains are shown. Plasmids were classified into categories based on their size (I: 1–4.9 kb, II: 5–8 kb, III: 15–30 kb and IV: > 30 kb) and to subgroups designated by lower-case letters based on the different patterns resolved by agarose gel electrophoresis. Antibiotics: CIP - ciprofloxacin, CLI - clindamycin, CLI* - possible inducible resistance for clindamycin, CMP - chloramphenicol, CPT - ceftaroline, CXT - ceftazidime, ERY - erythromycin, FUS - fusidic acid, GEN - gentamicin, MUP - mupirocin, PEN - benzylpenicillin, RIF - rifampicin, TEI - teicoplanin, TET - tetracycline, and TGC - tigecycline.

Table 2
Summary of whole genome sequence characteristics of analyzed *Staphylococcus petrasii* strains.

Genome	<i>S. petrasii</i> subsp. <i>petrasii</i> CCM 8418 [†]	<i>S. petrasii</i> subsp. <i>petrasii</i> CCM 8421 [†]	<i>S. petrasii</i> subsp. <i>croceolyticus</i> CCM 8494 [†]	<i>S. petrasii</i> subsp. <i>jettensis</i> P5404	<i>S. petrasii</i> subsp. <i>pragensis</i> CCM 8529 [†]
WGS Project no.	SRJE000000000	SRJF000000000	SRJF000000000	SRLS000000000	SRPJ000000000
Size (bp)	2478122	2371527	2709607	2620440	2447231
Contigs > 500 bp	12	43	54	55	18
N50	879073	148481	140928	145653	396835
GC content (mol%)	33.32	33.31	33.24	33.31	33.06
GC content of core genome (mol%)	33.44	33.34	33.46	33.39	33.22
Total genes	2462	2322	2670	2559	2438
Protein coding sequences	2381	2240	2588	2482	2366
Genes with clusters of orthologous groups	2246	2132	2362	2362	2197
Genes on mobile genetic elements and the genome percentage they represent	165 (6%)	37 (2%)	360 (12%)	270 (9%)	200 (7%)
Prophages	qSPP-int1 (43.1 kb)	–	qSPJ-int3 (45.2 kb), qSPP-like (125 kb)	qSPJ-int2 (42.9 kb), qSPJ-int3 (45.1 kb)	qSPR-int2 (40.9 kb)
SCC elements	SCC-CCM8418 (27 kb)	–	SpCl-SCCmec-CCM8494 (68.9 kb)	SpCl-SCCmec-P5404 (102.5 kb)	ψSCC-CCM8529 (43 kb)
Plasmids	pVVSPI (class III)	SpCl-SCC-CCM8421 (35.3 kb) pVVSPI (class I)	pVVSJ1 (class I), pVVSJ2 (class I), pVVSJ3 (class III), pVVSJ4 (class III) SpCl-1-CCM8494 (16.3 kb)	pVVSJ5 (class IV)	pVVSRI (class IV)
Phage-inducible chromosomal islands	SpCl-1-CCM8418 (12.4 kb), SpCl-2-CCM8418 (16.3 kb)	–	–	–	SpCl-1-CCM8529 (15.6 kb), SpCl-2-CCM8529 (13.6 kb), SpCl-3-CCM8529 (14.2 kb)
Other chromosomal island	–	–	–	–	SpCl-4-CCM8529 (12 kb)
Transposons	–	–	–	–	–
IS elements	–	–	–	–	–
RM systems	type I (incomplete - <i>hsdSR</i>), type IV (<i>mcrBC</i>)	–	–	–	–
CRISPR	–	–	–	–	–

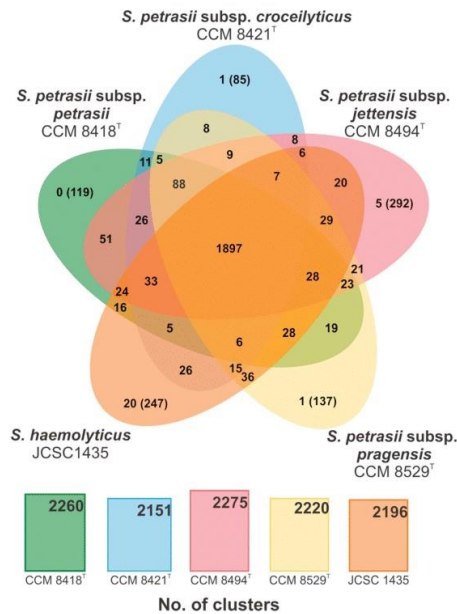


Fig. 3. Venn diagram representing orthologous protein clusters shared between genomes of type strains of *Staphylococcus petrasii* subspecies and *Staphylococcus haemolyticus* JCSC1435 (Takeuchi et al., 2005). The number of singletons for each genome is shown in brackets.

8418^T carries putative phosphotransferase transporter systems (PTSs) for mannitol and mannose in the *oriC* environ. In strain *S. petrasii* subsp. *croceilyticus* CCM 8421^T, pathways for saccharide conversion to pyruvate were predicted, including major facilitator superfamily hexose transporter or glycoside-pentoside-hexuronide transporter. Strains *S. petrasii* subsp. *jettensis* CCM 8494^T and P5404 differ from the other subspecies in the presence of an operon with predicted glutathione transporter and gamma-glutamyl transferase gene involved in cysteine biosynthesis.

Comparative genome analyses revealed the presence of many MGE as the major source of genome variability (Fig. 4). Plasmids, genomic islands including SCC, transposons, and a ϕ SP β -like prophage provided the strains of *S. petrasii* with resistance genes and genes promoting survival in the environment (Table 3). Additionally, SCC-like elements and the ϕ SP β -like prophage carry genes enhancing the virulence of the strains (Table 4). Other virulence factors such as global regulators, exoenzymes and surface proteins promoting biofilm formation and host immune system evasion are mainly found in the core genome and are similar to the virulence genes of *S. haemolyticus*.

SCC elements are present in all analysed genomes, inserted in the *rlmH* (*orfX*) gene (Table 2, Fig. 4). *S. petrasii* subsp. *petrasii* CCM 8418^T and *S. petrasii* subsp. *pragensis* CCM 8529^T have a single cassette (Fig. 5). The SCC_{CCM8418} element harbours the *ccrA5B3* gene complex, whereas in the ψ SCC_{CCM8529} element the *ccr* genes are absent. *S. petrasii* subsp. *croceilyticus* CCM 8421^T and both *S. petrasii* subsp. *jettensis* strains CCM 8494^T and P5404 carry composite chromosomal islands (CIs) (Fig. 5). SpCI-SCC_{CCM8421} has a mosaic structure composed of 17.6-kb SCC1_{CCM8421} and 17.7-kb SCC2_{CCM8421}, which harbour the *ccr* gene complexes *ccrA4B4* and *ccrC*, respectively. The largest CIs are present in *S. petrasii* subsp. *jettensis* and both CIs confer methicillin resistance by carrying *mecA* gene complexes. SpCI-SCC_{mec}CCM8494 is composed of a

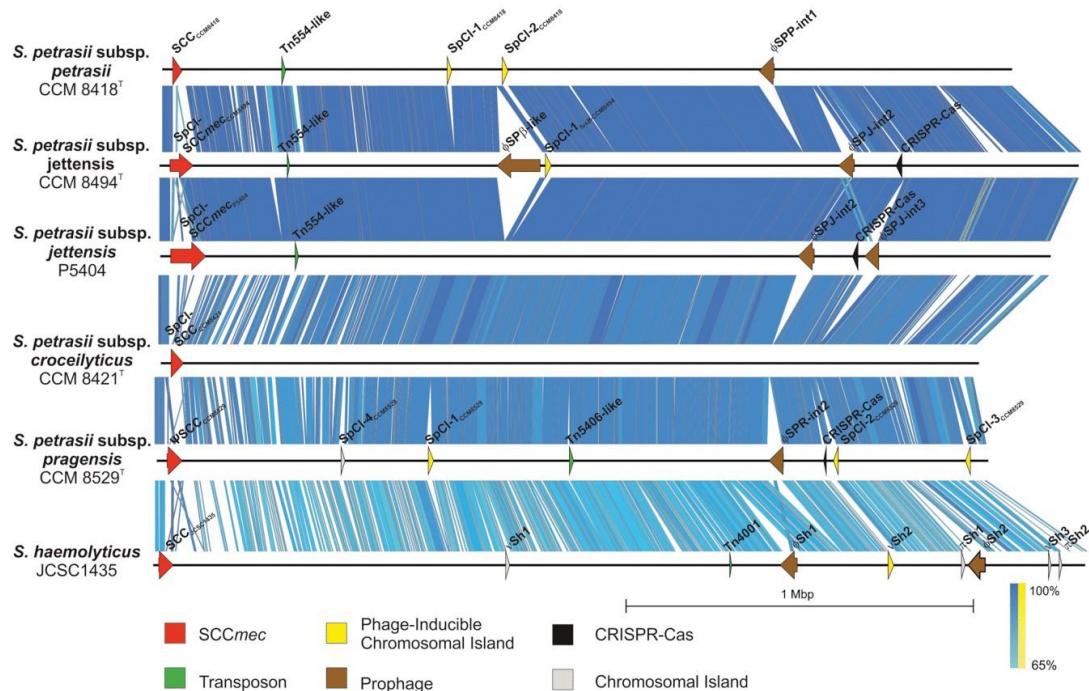


Fig. 4. Whole-genome comparison of *Staphylococcus petrasii* subsp. *petrasii* CCM 8418^T (GenBank accession number [SRJ000000000](https://www.ncbi.nlm.nih.gov/nuccore/SRJ000000000)), *S. petrasii* subsp. *croceilyticus* CCM 8421^T ([SRJ000000000](https://www.ncbi.nlm.nih.gov/nuccore/SRJ000000000)), *S. petrasii* subsp. *jettensis* CCM 8494^T ([SRL000000000](https://www.ncbi.nlm.nih.gov/nuccore/SRL000000000)) and P5404 ([SRLS000000000](https://www.ncbi.nlm.nih.gov/nuccore/SRLS000000000)), *S. petrasii* subsp. *pragensis* CCM 8529^T ([SRPJ000000000](https://www.ncbi.nlm.nih.gov/nuccore/SRPJ000000000)) and *Staphylococcus haemolyticus* JCSC1435 ([AP006716](https://www.ncbi.nlm.nih.gov/nuccore/AP006716)) with a focus on variable genetic elements. Conserved regions with more than 65% homology are indicated in blue, inversions in yellow as determined by blastn.

Table 3
Antibiotic resistance genes and response to environmental stimuli genes, their functions and locations on mobile genetic elements.

Mechanism	Gene(s)	Function	Strain and location on mobile genetic elements				
			CCM 8418 ^T	CCM 8421 ^T	CCM 8494 ^T	P5404	CCM 8529 ^T
Antibiotic resistance	AAC(6)-Ie-APH(2'')-Ia	aminoglycoside antibiotic resistance	–	–	qSPβ-like	–	–
	APH(3)-IIIa	aminoglycoside antibiotic resistance	–	–	qSPβ-like	–	–
	ANT(6)-Ia	aminoglycoside antibiotic resistance	–	–	qSPβ-like	–	–
	ANT(4)-Ib	aminoglycoside antibiotic resistance	–	–	–	pVVSJ4	–
	blaZ	penam antibiotic resistance	–	–	–	Tn554-like, pVVSJ5	–
	mecA	penam, monobactam, carbapenem, cephamycin, cephalosporin antibiotic resistance	Tn554-like	–	SCCmec _{CCM8494}	–	–
	ermC	streptogramin antibiotic, macrolide antibiotic, lincosamide antibiotic resistance	–	–	pVVSJ2	–	–
	fusB	fusidic acid resistance	–	–	SpCl-1 _{fusB-CCM8494}	–	–
	msrA	streptogramin antibiotic, macrolide antibiotic resistance	–	–	–	pVVSJ5	pVVSRI
	mphC	macrolide antibiotic resistance	–	–	–	pVVSJ5	pVVSRI
	tet(K)	tetracycline antibiotic resistance	–	–	–	–	pVVSRI
	mupA	mupirocin resistance	–	–	–	pVVSJ5	–
	vgaA	streptogramin antibiotic resistance	–	–	–	–	Tn5406-like
	qacC	multidrug efflux system	–	–	–	–	pVVSRI
	qacC	multidrug efflux system	–	–	–	–	–
	arsCBAD	arsenic resistance operon	–	pVVSJ1	–	pVVSJ5	–
	cadD	cadmium transporter	–	–	–	–	–
Response to environmental stimuli	cadX	transcriptional regulator, cadmium resistance	SCC _{CCM8418}	–	pVVSJ4	pVVSJ5	qSCC _{CCM8529}
	merR	transcriptional regulator, mercuric ion resistance	pVVSJ1	–	pVVSJ4	pVVSJ5	pVVSRI
	mnh	manganese transporter	pVVSJ1	–	pVVSJ4	–	–
	sulP	sulphate transporter family protein	–	–	pVVSJ3	pVVSJ5	–
	USP	universal stress protein	–	–	pVVSJ3	–	pVVSRI
	LTRs	LysR-type transcriptional regulators	–	–	pVVSJ3	–	pVVSRI
	sunT	ABC-type bacteriocin transporter	–	–	–	–	–
	trkG	potassium pump component	pVVSJ1	–	–	pVVSJ5	–
	trkG	potassium pump component	–	–	–	–	SpCl-1 _{CCM8529}
	RunP pathway	formaldehyde detoxification	–	–	pVVSJ3	pVVSJ5	pVVSRI

Table 4

Candidate virulence factors found in sequenced *Staphylococcus petrasii* strains representing homologs with known and previously predicted virulence factors.

Function and role	Gene(s)	Product	Strain and location				
			CCM 8418 ^T	CCM 8421 ^T	CCM 8494 ^T	P5404	CCM 8529 ^T
Global regulation system	<i>agrA-D</i>	response regulator	ch.	ch.	ch.	ch.	ch.
		accessory gene regulator B	ch.	ch.	ch.	ch.	ch.
		receptor histidine kinase	ch.	ch.	ch.	ch.	ch.
		agrD protein	ch.	ch.	ch.	ch.	ch.
	<i>sarARVZ</i>	staphylococcal accessory regulators	ch.	ch.	ch.	ch.	ch.
	<i>arlRS</i>	two component regulatory system	ch.	ch.	ch.	ch.	ch.
Surface adhesins and biofilm formation	<i>rot</i>	repressor of toxins	ch.	ch.	ch.	ch.	ch.
	<i>sigB</i>	alternative sigma factor	ch.	ch.	ch.	ch.	ch.
	<i>fbe</i>	fibrinogen binding protein	ch.	ch.	ch.	ch.	ch.
	<i>ebpS</i>	elastin binding protein	ch.	ch.	ch.	ch.	ch.
	<i>atlE</i>	bifunctional alutolysin	ch.	ch.	ch.	ch.	ch.
	<i>sasADH</i>	cell wall surface anchors	ch.	ch.	ch.	ch.	ch.
MSCRAMMs	<i>mntABC</i>	transporter	ch.	ch.	ch.	ch.	ch.
	<i>sdrC</i>	serine-aspartate repeat proteins	ch.	ch.	ch.	ch.	ch.
Exoenzymes	<i>sdrH</i>	serine-aspartate repeat proteins	ch.	ch.	ch.	ch.	ch.
	<i>hlyIII</i>	hemolysin III	ch.	ch.	ch.	ch.	ch.
	<i>clpP</i>	clp protease, proteolytic subunit	ch.	ch.	ch.	ch.	ch.
	<i>clpBCX</i>	clp protease, ATP binding subunits	ch.	ch.	ch.	ch.	ch.
	<i>htrA</i>	htrA like protease, putative	ch.	ch.	ch.	ch.	ch.
	<i>spLE/spIF</i>	serine protease	ch.	ch.	ch.	ch.	ch.
	<i>rseP</i>	zinc metalloprotease	ch.	ch.	ch.	ch.	ch.
	<i>lysM</i>	putative peptidoglycan binding domain	ch.	ch.	ch.	ch.	ch.
	<i>nanA</i>	sialic acid lyase	ch.	ch.	ch.	ch.	ch.
	<i>pldB</i>	lysophospholipase	ch.	ch.	ch.	ch.	ch.
	<i>lipA</i>	lipase/esterase	ch.	ch.	ch.	ch.	ch.
	<i>aes</i>	acetyl sterase	ch.	ch.	ch.	ch.	ch.
Capsule formation	<i>sbcCD</i>	exonucleases	ch.	ch.	ch.	ch.	ch.
	<i>capA-P</i>	capsule biosynthesis proteins	ch.	ch.	ch.	ch.	ch.
	<i>oatA</i>	o-acetyltransferase	ch.	ch.	ch.	ch.	ch.
	<i>cpsE</i>	glycosyltransferase	ch.	ch.	ch.	ch.	ch.
Immunomodulation	<i>psmβ1β2</i>	phenol-soluble modulins β1 and β2	ch.	ch.	ch.	ch.	ch.
	<i>psmγ/hld</i>	delta toxin	ch.	ch.	ch.	ch.	ch.
	<i>isaAB</i>	immunodominant antigens	ch.	ch.	ch.	ch.	ch.
	<i>sceD</i>	immunodominant antigen	ch.	ch.	ch.	ch.	ch.
	<i>pls</i>	plasmin-sensitive surface protein	–	–	SpCI-SCCmec _{CCM8494}	SpCI-SCCmec _{P5404}	ψSCC _{CCM8529}
Other virulence and putative virulence genes	<i>sasX</i>	surface-anchored protein	–	–	φSPβ-like	–	–
	<i>abpE</i>	flavin transferase	–	SpCI-SCC _{CCM8421}	–	–	ψSCC _{CCM8529}
	<i>flaR</i>	flavin reductase	–	SpCI-SCC _{CCM8421}	–	–	ψSCC _{CCM8529}
	<i>norB</i>	nitric oxide reductase	–	SpCI-SCC _{CCM8421}	–	–	ψSCC _{CCM8529}
	<i>speG</i>	spermidine N-acetyltransferase	SCC _{CCM8418}	SpCI-SCC _{CCM8421}	–	–	ψSCC _{CCM8529}
	<i>yeiMCN</i>	pseudouridine utilization pathway	–	SpCI-SCC _{CCM8421}	SpCI-SCCmec _{CCM8494}	SpCI-SCCmec _{P5404}	–
	<i>srIBEMRD</i>	sorbitol utilization operon	–	–	–	SpCI-SCCmec _{P5404}	–
	LTTRs	LysR-type transcriptional regulators	–	–	–	–	pVVSRI
	<i>cstABR</i>	hydrogen sulphide detoxification cluster	–	–	SpCI-SCCmec _{CCM8494}	SpCI-SCCmec _{P5404}	–

Legend: ch., chromosome.

29.9-kb SCCmec_{CCM8494} type VIII (4A) and SpCI-SCCmec_{P5404} harbours a 38.3-kb SCCmec_{P5404} type V (5C2&5). Also, both CIs have almost the same 39.0-kb SCC element designated SCC_{P5404} and/or SCC_{CCM8494} with recombinases *ccrA1B3*. SpCI-SCCmec_{P5404} has an additional 25.2-kb SCC_{sorbitol-P5404} element with *ccrA1B1* genes and a sorbitol phosphotransferase operon. SCCs contain putative virulence genes for plasmin-sensitive surface proteins (*pls*) and spermidine N-acetyltransferase (*speG*), a cluster of *apbE*, flavin reductase, and *norB* and a

cluster of *yeiMNC* for the uptake and catabolism of pseudouridine (Table 4, Fig. 5).

All sequenced genomes except that of *S. petrasii* subsp. *croceilyticus* contain prophages with a typical siphoviral modular structure (Fig. S6). Three phage integrase types were identified, determining prophage insertion into tRNA-Arg-TCT (φSPJ-int1), tRNA-Ser-GCT (φSPJ-int2 and φSPJ-int3) or near the CRISPR locus (φSPJ-int3) (Fig. 4). Double lysogenic host strain *S. petrasii* subsp. *jettensis* P5404 produced viable

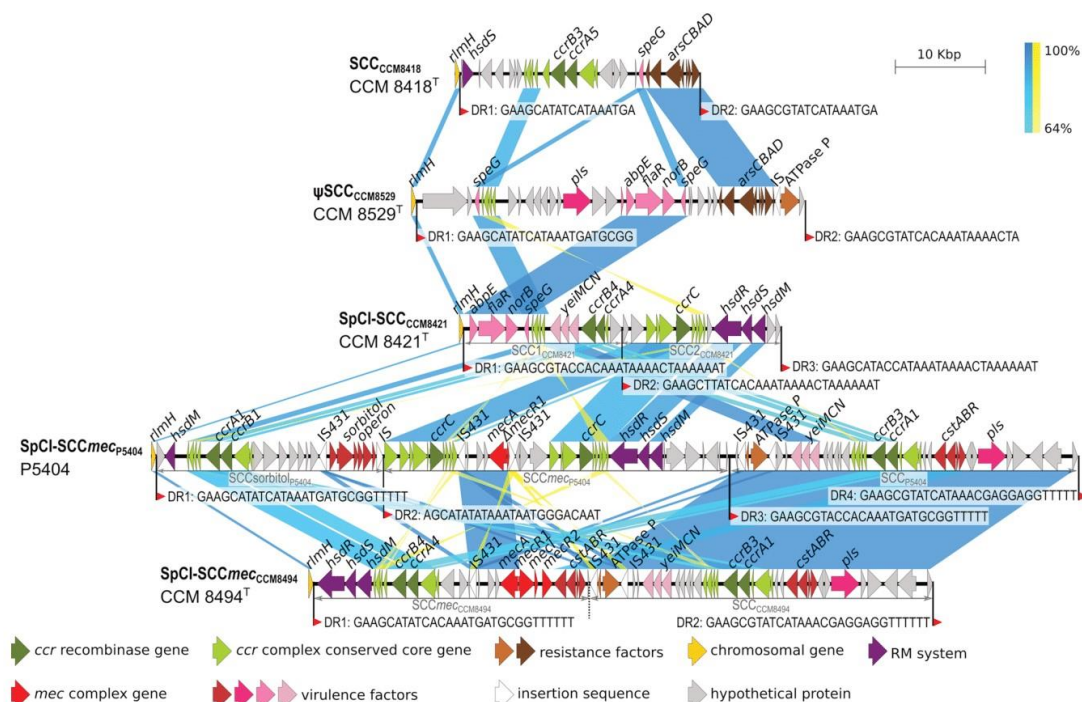


Fig. 5. Comparative analysis of staphylococcal cassette chromosome (SCC) inserted in *rlmH* gene harboured by *Staphylococcus petrasii* subspecies. Predicted coding sequences (ORFs) are depicted in the direction of transcription as arrows. Core genes of the SCC elements, genes for virulence factors, insertion sequences and genes for RM system are annotated. Sequences of direct repeats are shown below the ORF maps. Conserved regions with more than 64% homology are indicated in blue as determined by blastn.

Siphoviridae phage particles (Fig. 6) and LC-MS analysis of the phage structural proteins found a match with the prophage ϕ SPJ-int3 (Vb_SpeS_5404). In the *S. petrasii* subsp. *jettensis* CCM 8494^T genome, a 125-kb-long ϕ SP β -like converting prophage integrated into a gene encoding a YeeE/YedE family protein was found (Fig. 4). The prophage carries aminoglycoside resistance genes and a *sasX* gene homologue considered to be a virulence factor.

Six types of phage-inducible chromosomal islands (PICIs) with lengths of 12–16 kb contributing to host adaptation and virulence were identified (Table 2, Fig. 4). SpCI-1_{fusB-CCM8494} carries the fusidic acid family resistance determinant *fusB*. This island has the common *att* insertion site AATGACATAA with *S. petrasii* subsp. *petrasii* SpCI-2_{CCM8418} in the *groEL* gene encoding a heat shock protein. Insertion site (*att*) TTGAATACGT in the lactose operon is shared by *S. petrasii* subsp. *petrasii* SpCI-1_{CCM8418} and *S. petrasii* subsp. *pragensis* SpCI-1_{CCM8529}, which harbour a *trkG* gene homologue encoding a putative potassium transporter component. *S. petrasii* subsp. *pragensis* SpCI-2_{CCM8529} and SpCI-3_{CCM8529} are integrated into the tmRNA gene *ssrA* and glutamine amidotransferase protein gene *guaA*, respectively. The novel chromosomal island SpCI-4_{CCM8529} with putative genes for methylation with a potential role in DNA protection and repair was found in the strain CCM 8529^T (Fig. 4, Table 2).

Extrachromosomal plasmids were identified in all the sequenced genomes (Table 2), encoding antimicrobial resistance genes enabling survival in adverse environments (Table 3). Small *S. petrasii* plasmids pVVSJ1, pVVSJ2, and pVVSJ3 encode multidrug efflux system *qacC* or erythromycin resistance *ermC* genes (Table 3). Plasmids pVVSJ1, pVVSJ3, and pVVSJ4 carried a *traA* relaxase gene homologue that participates in conjugation. The plasmids pVVSJ3, pVVSJ5, and pVVSJ6 carry the formaldehyde detoxification operon with the ribulose

monophosphate pathway (RuMP) (Fig. S7). Plasmid pVVSJ3 encode membrane protein TcaA, whose inactivation may lead to teicoplanin resistance (McCallum et al., 2007).

Type II-C CRISPR-Cas systems were identified in the genomes of the strains *S. petrasii* subsp. *jettensis* CCM 8494^T and P5404 and *S. petrasii* subsp. *pragensis* CCM 8529^T (Table 2, Fig. 4). Genes *cas1*, *cas2*, and *cas9* identified in all *S. petrasii* subsp. *pragensis* and *S. petrasii* subsp. *jettensis* strains by PCR screening indicate the prevalence of the CRISPR-Cas system in these two subspecies. The CRISPR locus of *S. petrasii* subsp. *jettensis* is 497 bp long, with 7 spacers separated by eight 36-bp-long repeats GTTTCACCTTATACCTAAAAATTACAGAGTACTAAAC that were identical in both sequenced strains. The CRISPR locus of *S. petrasii* subsp. *pragensis* CCM 8529^T is 1684 bp long, with 25 spacers separated by 26 36-bp-long repeats GTTTCACCTTATACCTAAAAATTACAGAGTACTAAAC. The *hsdMSR* and *mcrBC* restriction-modification (RM) systems present in all strains (Table 2) are another protection mechanism against DNA uptake. The *S. petrasii* subspecies share probably new type IV RM system based on the low homology with known *mcrBC* genes.

4. Discussion

Since the pathogenic significance and nosocomial spread of CoNS is increasing (Yu et al., 2017), it is particularly important to analyse the virulence potential and epidemiology of blood culture isolates and infections associated with the use of medical devices. One third of *S. petrasii* isolates originated from such specimens, dominated by *S. petrasii* subsp. *petrasii* and *S. petrasii* subsp. *jettensis*, indicating their importance in human disease. The precise identification of *S. petrasii* subspecies can be a prediction of clinical significance, especially due to the multiple drug resistance and methicillin resistance of *S. petrasii*

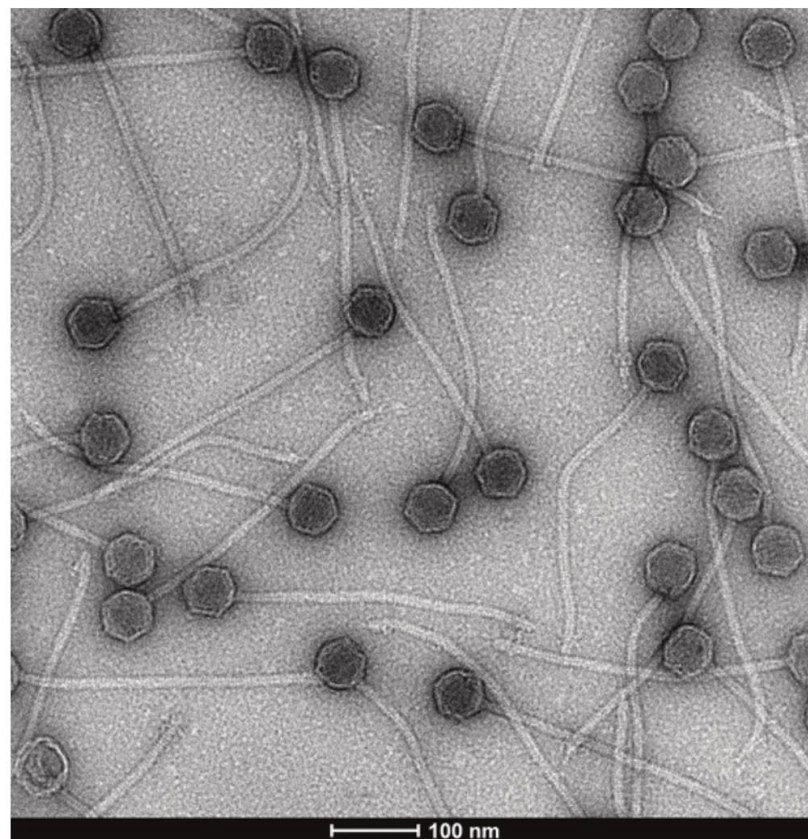


Fig. 6. Transmission electron microscopy image of negatively stained particles of phage qSPJ1-int3 (Vb_SpeS_5404) induced from *Staphylococcus petrasii* subsp. *jettensis* strain P5404 with mitomycin C. The phage particles consist of an icosahedral head (B1 morphology) with diameter 60 nm, flexible, non-contractile 335 nm long and 14 nm wide tail ending with a grape-shaped base plate. The base plate is 40 nm long and 22 nm wide at its upper part.

subsp. *jettensis*. MALDI-TOF MS, PFGE typing, and genome comparison concluded that *S. petrasii* subsp. *petrasii* and *S. petrasii* subsp. *jettensis* are closely related. However, *S. petrasii* subsp. *jettensis* has accumulated more genes in the accessory genome compared to all other subspecies. This could be analogous to the relationship between two *S. hominis* subspecies, where *S. hominis* subsp. *novobiosepticus* exhibits a larger chromosome (Kloos et al., 1998) and significantly higher number of antibiotic resistance genes than *S. hominis* subsp. *hominis* (Fitzgibbon et al., 2001). Unambiguous distinguishing between *S. petrasii* subsp. *petrasii* and *S. petrasii* subsp. *jettensis* is possible through biochemical tests, ribotyping and (GTG)₅-REP-PCR. On the other hand, all the performed analyses demonstrated clear differentiation of *S. petrasii* subsp. *croceilyticus* and *S. petrasii* subsp. *pragensis*, both from each other and from the other subspecies. Both taxa might be reclassified in the future to different species based on the obtained average nucleotide identity (ANI) values (Table S2).

The comparative genome analysis enables the assessment of the pathogenic potential, mechanisms of host adaptation and survival in the environment, and understanding the evolutionary events associated with horizontal gene transfer (HGT). The *S. petrasii* virulence factors and MGE *att* sites are similar to *S. haemolyticus*, reflecting their evolutionary relatedness. Similar to *S. haemolyticus*, the *S. petrasii* genomes lack the *ica* operon essential for biofilm formation, yet the presence of genes for phenol-soluble modulins promoting biofilm dissemination suggests the existence of an *ica*-independent pathway (Arciola et al.,

2015; Panda and Singh, 2018). Genes in the *oriC* environ, which was earlier described as highly variable in staphylococci (Takeuchi et al., 2005), play a crucial role in pathogenesis and adaptation to various host environments. The locus contains various saccharide/pentose uptake systems that enable the utilization of specific substrates. These systems distinguish one staphylococcal species from another, and can be considered to be virulence factors (Siebold et al., 2001; Zuniga et al., 2005). Mannitol-specific PTSs in the *oriC* environ of *S. petrasii* subsp. *petrasii* CCM 8418^T exhibited similarity to that of *S. haemolyticus* and *S. aureus* with 86.5% and 73.6% nucleotide identity, respectively, and this locus is likely horizontally transferred (Takeuchi et al., 2005; Zuniga et al., 2005).

SCC elements located in the *oriC* environ of *S. petrasii* have an extensive mosaic structure with regions homologous to SCC elements from other *Staphylococcus* species supporting the role of HGT in their formation. Despite the fact that nowadays *mecC* is more frequently reported from CoNS (Harrison et al., 2014, 2013; Pantucek et al., 2018) and *mecB* was found in staphylococci (Becker et al., 2018), only *mecA*-carrying SCCmec were detected in the *S. petrasii* strains. The SCC_{CCM8418} element has a *ccr* complex similar to *S. haemolyticus* SH32 (Yu et al., 2014) and has a similar heavy-metal-resistance gene structure to *S. aureus* type IX SCCmec (Li et al., 2011). The major part of SpCI-SCC_{CCM8421} corresponds to the CI in MRSA isolate M06/0171 (Kinnevey et al., 2013). Significant similarities in gene organization and the presence of a sorbitol-specific PTS system were found between the SpCI-

SCCmec_{P5404} and *S. aureus* SCCmec_{WA-MRSA-40-CI} (Wilson et al., 2016). The cluster of SCC-harboured genes *apbE*, *flaR*, *norB*, and *speG* probably promote survival in host cells and confer resistance to host-derived polyamines (Joshi et al., 2011; Morozov et al., 2018; Truong-Bolduc et al., 2011). *Pls* genes can promote the immune system evasion (Werbeck et al., 2007) and the genes *yeiMNC* might be beneficial in urinal or ocular infections, as was suggested previously (Sadaka et al., 2014). Most of the *S. petrasii* SCC elements possessed RM systems or their remnants similar to other staphylococci, which could stabilize the SCC elements in the genome (Hanssen and Sollid, 2006; Ito et al., 2004).

The pathogenic potential of the strains is enhanced by the presence of additional MGE in the genomes. The ϕ SP β -like prophage in *S. petrasii* subsp. *jettensis* CCM 8494^T harbouring the *sasX* gene has almost 99% nucleotide identity with other ϕ SP β -like prophages, including that of *S. aureus* TW20 (Holden et al., 2010). Li et al. (2012) showed that the *sasX* gene, which is a homologue of *sesl* from *S. epidermidis*, plays a key role in colonization and immune evasion. Compared to *S. aureus* pathogenicity islands, no virulence factors were predicted on *S. petrasii* PICs that carry many unique ORFs of unknown functions. The only identified resistance gene was *fusB* localized on SpCI-1_{fusB-CCM8494}, exhibiting a similar structure to the SeRI_{fusB-704} of *S. epidermidis* (Chen et al., 2011). As was found in the *S. haemolyticus* genome (Takeuchi et al., 2005), the presence of IS elements leads to frequent genome rearrangements, that were also observed in strains from the *S. petrasii* complex before its valid taxonomic description (Bartell et al., 2008).

Plasmids that were ubiquitously present in the analyzed set of *S. petrasii* strains carried genes for antibiotic and heavy metal resistance or genes for the response to environmental stimuli. Such an association between heavy metal and antibiotic resistance has been reported earlier, identified mostly in environmental isolates (Baker-Austin et al., 2006) but also in clinical specimens (Zhai et al., 2016). Large plasmids exhibited presence of numerous IS6 family elements establishing their modular structure. Conserved plasmid segments encoding RuMP and its reverse reaction pathway, which mediate formaldehyde fixation to ribulose-5-phosphate and further metabolism, are proposed to participate in the detoxification of formaldehyde generated by the host metabolism or during heme degradation (Chen et al., 2016b). The same metabolic pathway was observed in modular plasmids of *S. aureus* and other clinically important pathogens (Chen et al., 2016b).

The very high prevalence of CRISPR-Cas systems among *S. petrasii* subsp. *jettensis* and *S. petrasii* subsp. *pragensis* strains is unusual in comparison with CoNS reviewed previously (Rossi et al., 2017). The CRISPR-Cas system might thus protect the host against foreign DNA. The spacers in both subspecies target the sequence of *Siphoviridae* phages of different CoNS (Deghorain et al., 2012; Liu et al., 2018). This could be connected with their occurrence in environments with various bacterial populations in which their bacteriophages participate in HGT. Phages use as their receptors wall teichoic acids (WTA), which composition is crucial for efficient phage mediated HGT (Winstel et al., 2013). When analysing *S. petrasii* WTA-encoding genes, similarities of *tagO*, *mnaA*, cluster *tagAHGBXD-*pbp4*-tagF*, *tagNV* and *dtlABCD* loci were found to related CoNS. The highest nucleotide similarity 75–85% for *tag* genes and 80–90% for *dtl* genes was found with their homologues from *S. hominis*, *S. lugdunensis* and *S. haemolyticus*, which have the WTA composition Gro-GlcNAc (α) or (α + β) (Endl et al., 1984). The strain CCM 8529^T lacks the *tagN* gene suggesting difference in glycosylation. We hypothesize that WTA composition does not exclude phage mediated HGT.

These results provide an insight into the functional organization of the *S. petrasii* genomes. The presence of many putative virulence factors and antimicrobial resistance genes indicates that this species could be confirmed to be a significant opportunistic pathogen, as occurred with *Staphylococcus lugdunensis* (Argemi et al., 2017). The frequent similarities of *S. petrasii* MGE with other CoNS and *S. aureus* indicate that HGT is important in the spread of the elements or their parts. The reliable *S.*

petrasii diagnostics proposed here and its genomic characteristics enable clinical microbiologists to interpret the significance of the species in clinical samples and obtain surveillance data for studying its epidemiology.

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Declaration of Competing Interest

All the authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.ijmm.2019.151355>.

References

- Arciola, C.R., Campoccia, D., Ravaoli, S., Montanaro, L., 2015. Polysaccharide inter-cellular adhesin in biofilm: structural and regulatory aspects. *Front. Cell. Infect. Microbiol.* 5, 7. <https://doi.org/10.3389/fcimb.2015.00007>.
- Argemi, X., Hansmann, Y., Riegel, P., Prevost, G., 2017. Is *Staphylococcus lugdunensis* significant in clinical samples? *J. Clin. Microbiol.* 55, 3167–3174. <https://doi.org/10.1128/JCM.00846-17>.
- Aziz, R.K., Bartels, D., Best, A.A., DeJongh, M., Disz, T., Edwards, R.A., Formsma, K., Gerdes, S., Glass, E.M., Kubal, M., Meyer, F., Olsen, G.J., Olson, R., Osterman, A.L., Overbeek, R.A., McNeil, L.K., Paarmann, D., Paczian, T., Parrello, B., Pusch, G.D., Reich, C., Stevens, R., Vassieva, O., Vonstein, V., Wilke, A., Zagnitko, O., 2008. The RAST server: rapid annotations using subsystems technology. *BMC Genomics* 9, 75. <https://doi.org/10.1186/1471-2164-9-75>.
- Baker-Austin, C., Wright, M.S., Stepanauskas, R., McArthur, J.V., 2006. Co-selection of antibiotic and metal resistance. *Trends Microbiol.* 14, 176–182. <https://doi.org/10.1016/j.tim.2006.02.006>.
- Barros, E.M., Ceotto, H., Bastos, M.C.F., dos Santos, K.R.N., Giambiagi-deMarval, M., 2012. *Staphylococcus haemolyticus* as an important hospital pathogen and carrier of methicillin resistance genes. *J. Clin. Microbiol.* 50, 166–168. <https://doi.org/10.1128/Jcm.05563-11>.
- Bartell, J.G., Dajcs, J.J., Ward, D.O., Zentz, E.B., Briska, A.M., Stroman, D.S., 2008. Characterization of staphylococcal isolates by traditional methods and optical mapping. In: 108th ASM General Meeting. American Society for Microbiology, Boston, MA.
- Becker, K., Heilmann, C., Peters, G., 2014. Coagulase-negative staphylococci. *Clin. Microbiol. Rev.* 27, 870–926. <https://doi.org/10.1128/Cmr.00109-13>.
- Becker, K., van Alen, S., Idelovich, E.A., Schleimer, N., Seggewiss, J., Mellmann, A., Kaspar, U., Peters, G., 2018. Plasmid-encoded transferable *mecB*-mediated methicillin resistance in *Staphylococcus aureus*. *Emerg. Infect. Dis.* 24, 242–248. <https://doi.org/10.3201/eid2402.171074>.
- Bouchami, O., Ben Hassen, A., de Lencastre, H., Miragaia, M., 2011. Molecular epidemiology of methicillin-resistant *Staphylococcus hominis* (MRSHo): low clonality and reservoirs of SCCmec structural elements. *PLoS One* 6, e21940. <https://doi.org/10.1371/journal.pone.0021940>.
- Bouchami, O., Ben Hassen, A., de Lencastre, H., Miragaia, M., 2012. High prevalence of *mec* complex C and *crcC* is independent of SCCmec type V in *Staphylococcus haemolyticus*. *Eur. J. Clin. Microbiol. Infect. Dis.* 31, 605–614. <https://doi.org/10.1007/>

- s10096-011-1354-3.
- Brzychczy-Wloch, M., Borszewska-Kornacka, M., Gulczynska, E., Wojkowska-Mach, J., Sulik, M., Grzebyk, M., Luchter, M., Heczko, P.B., Bulanda, M., 2013. Prevalence of antibiotic resistance in multi-drug resistant coagulase-negative staphylococci isolated from invasive infection in very low birth weight neonates in two Polish NICUs. *Ann. Clin. Microb. Antimicrob.* 12, 41. <https://doi.org/10.1186/1476-0711-12-41>.
- Cavanagh, J.P., Hjerde, E., Holden, M.T.G., Kahlke, T., Klingenberg, C., Flaegstad, T., Parkhill, J., Bentley, S.D., Sollid, J.U.E., 2014. Whole-genome sequencing reveals clonal expansion of multiresistant *Staphylococcus haemolyticus* in European hospitals. *J. Antimicrob. Chemother.* 69, 2920–2927. <https://doi.org/10.1093/jac/dku271>.
- Chaves, F., Garcia-Alvarez, M., Sanz, F., Alba, C., Otero, J.R., 2005. Nosocomial spread of a *Staphylococcus hominis* subsp. *novobiosepticus* strain causing sepsis in a neonatal intensive care unit. *J. Clin. Microbiol.* 43, 4877–4879. <https://doi.org/10.1128/JCM.43.9.4877-4879.2005>.
- Chen, H.J., Tsai, J.C., Hung, W.C., Tseng, S.P., Hsueh, P.R., Teng, L.J., 2011. Identification of *fusB*-mediated fusidic acid resistance islands in *Staphylococcus epidermidis* isolates. *Antimicrob. Agents Chemother.* 55, 5842–5849. <https://doi.org/10.1128/AAC.00592-11>.
- Chen, L.H., Zheng, D.D., Liu, B., Yang, J., Jin, Q., 2016a. VFDB 2016: hierarchical and refined dataset for big data analysis-10 years on. *Nucleic Acids Res.* 44, D694–D697. <https://doi.org/10.1093/nar/gkv1239>.
- Chen, N.H., Djoko, K.Y., Veyrier, F.J., McEwan, A.G., 2016b. Formaldehyde stress responses in bacterial pathogens. *Front. Microbiol.* 7, 257. <https://doi.org/10.3389/fmicb.2016.00257>.
- CLSI, 2018. Performance Standards for Antimicrobial Disk Susceptibility Tests: Approved Standard - 13th Ed. CLSI Standard M02. Clinical and Laboratory Standards Institute, Wayne, PA. <https://clsi.org/standards/products/microbiology/documents/m02/>.
- De Bel, A., Svec, P., Petras, P., Sedlacek, I., Pantucek, R., Echahidi, F., Pierard, D., Vandamme, P., 2014. Reclassification of *Staphylococcus jettensis* De Bel et al. 2013 as *Staphylococcus petrasii* subsp. *jettensis* subsp. nov. and emended description of *Staphylococcus petrasii* Pantucek et al. 2013. *Int. J. Syst. Evol. Microbiol.* 64, 4198–4201. <https://doi.org/10.1099/ijso.0.064758-0>.
- De Bel, A., Van Hoorde, K., Wybo, I., Vandoorslaer, K., Echahidi, F., De Brandt, E., Schumann, P., Ieven, M., Soetens, O., Piérard, D., Vandamme, P., 2013. *Staphylococcus jettensis* sp. nov., a coagulase-negative staphylococcal species isolated from human clinical specimens. *Int. J. Syst. Evol. Microbiol.* 63, 3250–3256. <https://doi.org/10.1099/ijso.0.044438-0>.
- Deghorain, M., Bobay, L.M., Smeesters, P.R., Bousbata, S., Vermeersch, M., Perez-Morga, D., Dreze, P.A., Rocha, E.P.C., Touchon, M., Van Melderen, L., 2012. Characterization of novel phages isolated in coagulase-negative staphylococci reveals evolutionary relationships with *Staphylococcus aureus* phages. *J. Bacteriol.* 194, 5829–5839. <https://doi.org/10.1128/Jb.01085-12>.
- Dhillon, B.K., Laird, M.R., Shay, J.A., Winsor, G.L., Lo, R., Nizam, F., Pereira, S.K., Wagglechner, N., McArthur, A.G., Langille, M.G.I., Brinkman, F.S.L., 2015. IslandViewer 3: more flexible, interactive genomic island discovery, visualization and analysis. *Nucleic Acids Res.* 43, W104–W108. <https://doi.org/10.1093/nar/gkv401>.
- Endl, J., Seidl, P.H., Fiedler, F., Schleifer, K.H., 1984. Determination of cell-wall teichoic acid structure of staphylococci by rapid chemical and serological screening methods. *Arch. Microbiol.* 137, 272–280. <https://doi.org/10.1007/Bf00414557>.
- EUCAST, 2017. Breakpoint Tables for Interpretation of MICs and Zone Diameters. The European Committee on Antimicrobial Susceptibility Testing. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v.7.1.Breakpoint_Tables.pdf.
- Fitzgibbon, J.E., Nahvi, M.D., Dubin, D.T., John Jr., J.F., 2001. A sequence variant of *Staphylococcus hominis* with a high prevalence of oxacillin and fluoroquinolone resistance. *Res. Microbiol.* 152, 805–810. [https://doi.org/10.1016/S0923-2508\(01\)01264-5](https://doi.org/10.1016/S0923-2508(01)01264-5).
- Freiwald, A., Sauer, S., 2009. Phylogenetic classification and identification of bacteria by mass spectrometry. *Nat. Protoc.* 4, 732–742. <https://doi.org/10.1038/nprot.2009.37>.
- Froggatt, J.W., Johnston, J.L., Galetto, D.W., Archer, G.L., 1989. Antimicrobial resistance in nosocomial isolates of *Staphylococcus haemolyticus*. *Antimicrob. Agents Chemother.* 33, 460–466. <https://doi.org/10.1128/Aac.33.4.460>.
- Gill, S.R., Fouts, D.E., Archer, G.L., Mongodin, E.F., Deboy, R.T., Ravel, J., Paulsen, I.T., Kolonay, J.F., Brinkac, L., Beanan, M., Dodson, R.J., Daugherty, S.C., Madupu, R., Angiuoli, S.V., Durkin, A.S., Haft, D.H., Vamathevan, J., Khouri, H., Utterback, T., Lee, C., Dimitrov, G., Jiang, L., Qin, H., Weidman, J., Tran, K., Kang, K., Hance, I.R., Nelson, K.E., Fraser, C.M., 2005. Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. *J. Bacteriol.* 187, 2426–2438. <https://doi.org/10.1128/JB.187.7.2426-2438.2005>.
- Hanssen, A.M., Sollid, J.U.E., 2006. *SCCmec* in staphylococci: genes on the move. *FEMS Immunol. Med. Microbiol.* 46, 8–20. <https://doi.org/10.1111/j.1574-695X.2005.00009.x>.
- Harrison, E.M., Paterson, G.K., Holden, M.T., Ba, X., Rolo, J., Morgan, F.J., Pichon, B., Kearns, A., Zadoks, R.N., Peacock, S.J., Parkhill, J., Holmes, M.A., 2014. A novel hybrid *SCCmec-mecC* region in *Staphylococcus sciuri*. *J. Antimicrob. Chemother.* 69, 911–918. <https://doi.org/10.1093/jac/dkt452>.
- Harrison, E.M., Paterson, G.K., Holden, M.T., Morgan, F.J., Larsen, A.R., Petersen, A., Leroy, S., De Vlieghe, S., Perreten, V., Fox, L.K., Lam, T.J., Sampimon, O.C., Zadoks, R.N., Peacock, S.J., Parkhill, J., Holmes, M.A., 2013. A *Staphylococcus xylois* isolate with a new *mecC* allotype. *Antimicrob. Agents Chemother.* 57, 1524–1528. <https://doi.org/10.1128/AAC.01882-12>.
- Heilmann, C., Ziebuhr, W., Becker, K., 2019. Are coagulase-negative staphylococci virulent? *Clin. Microbiol. Infect.* 25, 1071–1080. <https://doi.org/10.1016/j.cmi.2018.11.012>.
- Holden, M.T.G., Lindsay, J.A., Corton, C., Quail, M.A., Cockfield, J.D., Pathak, S., Batra, R., Parkhill, J., Bentley, S.D., Edgeworth, J.D., 2010. Genome sequence of a recently emerged, highly transmissible, multi-antibiotic- and antiseptic-resistant variant of methicillin-resistant *Staphylococcus aureus*, sequence type 239 (TW). *J. Bacteriol.* 192, 888–892. <https://doi.org/10.1128/Jb.01255-09>.
- Ito, T., Ma, X.X., Takeuchi, F., Okuma, K., Yuzawa, H., Hiramatsu, K., 2004. Novel type V staphylococcal cassette chromosome *mec* driven by a novel cassette chromosome recombinase, *ccrC*. *Antimicrob. Agents Chemother.* 48, 2637–2651. <https://doi.org/10.1128/AAC.48.7.2637-2651.2004>.
- Jia, B., Raphenya, A.R., Alcock, B., Wagglechner, N., Guo, P., Tsang, K.K., Lago, B.A., Dave, B.M., Pereira, S., Sharma, A.N., Doshi, S., Courtot, M., Lo, R., Williams, L.E., Frye, J.G., Elsayegh, T., Sardar, D., Westman, E.L., Pawlowski, A.C., Johnson, T.A., Brinkman, F.S., Wright, G.D., McArthur, A.G., 2017. CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. *Nucleic Acids Res.* 45, D566–D573. <https://doi.org/10.1093/nar/gkw1004>.
- Joensen, K.G., Scheutz, F., Lund, O., Hasman, H., Kaas, R.S., Nielsen, E.M., Aarestrup, F.M., 2014. Real-time whole-genome sequencing for routine typing, surveillance, and outbreak detection of verotoxigenic *Escherichia coli*. *J. Clin. Microbiol.* 52, 1501–1510. <https://doi.org/10.1128/JCM.03617-13>.
- Jones, P., Binns, D., Chang, H.Y., Fraser, M., Li, W.Z., McAnulla, C., McWilliam, H., Maslen, J., Mitchell, A., Nuka, G., Pesseat, S., Quinn, A.F., Sangrador-Vegas, A., Scheremetjew, M., Yong, S.Y., Lopez, R., Hunter, S., 2014. InterProScan 5: genome-scale protein function classification. *Bioinformatics* 30, 1236–1240. <https://doi.org/10.1093/bioinformatics/btu031>.
- Joshi, G.S., Spontak, J.S., Klapper, D.G., Richardson, A.R., 2011. Arginine catabolic mobile element encoded *speG* abrogates the unique hypersensitivity of *Staphylococcus aureus* to exogenous polyamines. *Mol. Microbiol.* 82, 9–20. <https://doi.org/10.1111/j.1365-2958.2011.07809.x>.
- Kinnevey, P.M., Shore, A.C., Brennan, G.I., Sullivan, D.J., Ehrlich, R., Monecke, S., Slickers, P., Coleman, D.C., 2013. Emergence of sequence type 779 methicillin-resistant *Staphylococcus aureus* harboring a novel pseudo staphylococcal cassette chromosome *mec* (SCC*mec*)-SCC-SCC*CRSPR* composite element in Irish hospitals. *Antimicrob. Agents Chemother.* 57, 524–531. <https://doi.org/10.1128/AAC.01689-12>.
- Kloos, W.E., George, C.G., Olgiate, J.S., Van Pelt, L., McKinnon, M.L., Zimmer, B.L., Muller, E., Weinstein, M.P., Mirrett, S., 1998. *Staphylococcus hominis* subsp. *novobiosepticus* subsp. nov., a novel trehalose- and N-acetyl-D-glucosamine-negative, novobiocin- and multiple-antibiotic-resistant subspecies isolated from human blood cultures. *Int. J. Syst. Bacteriol.* 48, 799–812. <https://doi.org/10.1099/00207173-48-3-799>.
- Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33, 1870–1874. <https://doi.org/10.1093/molbev/msw054>.
- Kuntova, L., Pantucek, R., Rajova, J., Ruzickova, V., Petras, P., Maslanova, I., Doskar, J., 2012. Characteristics and distribution of plasmids in a clonally diverse set of methicillin-resistant *Staphylococcus aureus* strains. *Arch. Microbiol.* 194, 607–614. <https://doi.org/10.1007/s00203-012-0797-y>.
- Lee, I., Ouk Kim, Y., Park, S.C., Chun, J., 2016. OrthoANI: an improved algorithm and software for calculating average nucleotide identity. *Int. J. Syst. Evol. Microbiol.* 66, 1100–1103. <https://doi.org/10.1099/ijsem.0.000760>.
- Li, M., Du, X., Villaruz, A.E., Diep, B.A., Wang, D.C., Song, Y., Tian, Y.R., Hu, J.H., Yu, F.Y., Lu, Y., Otto, M., 2012. MRSA epidemic linked to a quickly spreading colonization and virulence determinant. *Nat. Med.* 18, 816–819. <https://doi.org/10.1038/nm.2692>.
- Li, S., Skov, R.L., Han, X., Larsen, A.R., Larsen, J., Sorum, M., Wulf, M., Voss, A., Hiramatsu, K., Ito, T., 2011. Novel types of staphylococcal cassette chromosome *mec* elements identified in clonal complex 398 methicillin-resistant *Staphylococcus aureus* strains. *Antimicrob. Agents Chemother.* 55, 3046–3050. <https://doi.org/10.1128/AAC.01475-10>.
- Liu, P., Peng, F., Hu, Q., Tang, Y., Kang, H., Tong, T., 2018. The induction, purification, whole genome sequencing and comparative genome analysis of a novel *Staphylococcus haemolyticus* bacteriophage IME-SA4. *J. Chin. Phys.* 20, 546–550. <https://doi.org/10.3760/cma.j.issn.1008-1372.2018.04.018>.
- Lopes, A., Tavares, P., Petit, M.A., Guerois, R., Zinn-Justin, S., 2014. Automated classification of tailed bacteriophages according to their neck organization. *BMC Genomics* 15, 1027. <https://doi.org/10.1186/1471-2164-15-1027>.
- Maslanova, I., Doskar, J., Varga, M., Kuntova, L., Muzik, J., Maluskova, D., Ruzickova, V., Pantucek, R., 2013. Bacteriophages of *Staphylococcus aureus* efficiently package various bacterial genes and mobile genetic elements including *SCCmec* with different frequencies. *Environ. Microbiol. Rep.* 5, 66–73. <https://doi.org/10.1111/j.1758-2229.2012.00378.x>.
- McCallum, N., Brassinga, A.K.C., Sifri, C.D., Berger-Bächi, B., 2007. Functional characterization of TcaA: minimal requirement for teicoplanin susceptibility and role in *Caenorhabditis elegans* virulence. *Antimicrob. Agents Chemother.* 51, 3836–3843. <https://doi.org/10.1128/AAC.00722-07>.
- Meier-Kolthoff, J.P., Auch, A.F., Klenk, H.P., Goker, M., 2013. Genome sequence-based species delimitation with confidence intervals and improved distance functions. *BMC Bioinformatics* 14, 60. <https://doi.org/10.1186/1471-2105-14-60>.
- Mellmann, A., Becker, K., von Eiff, C., Kockevoet, U., Schumann, P., Harmsen, D., 2006. Sequencing and staphylococci identification. *Emerg. Infect. Dis.* 12, 333–336. <https://doi.org/10.3201/eid1202.050962>.
- Morozov, G.I., Porat, N., Kushnir, T., Najmudeen, H., Adawi, A., Chalifa-Caspi, V., Benisty, R., Ohayon, A., Liron, O., Azriel, S., Malka, I., Dotan, S., Portnoi, M., Piotrowski, A.A., Kafka, D., Hajaj, B., Fishilevich, T., Shagan, M., Tal, M., Ellis, R., Morrison, D.A., Mitchell, A.M., Mitchell, T.J., Dagan, R., Yesilkaya, H., Nebenzahl, Y.M., 2018. Flavin reductase contributes to pneumococcal virulence by protecting

RESULTS AND DISCUSSION

- from oxidative stress and mediating adhesion and elicits protection against pneumococcal challenge. *Sci. Rep.* 8, 314. <https://doi.org/10.1038/s41598-017-18645-8>.
- Novick, R.P., 1990. *Molecular Biology of the Staphylococci*. VCH Publishers, New York.
- Nurk, S., Bankevich, A., Antipov, D., Gurevich, A.A., Korobeynikov, A., Lapidus, A., Prjibelski, A.D., Pyshkin, A., Sirotkin, A., Sirotkin, Y., Stepanauskas, R., Clingenpeel, S.R., Woyke, T., McLean, J.S., Lasken, R., Tesler, G., Alekseyev, M.A., Pevzner, P.A., 2013. Assembling single-cell genomes and mini-metagenomes from chimeric MDA products. *J. Comput. Biol.* 20, 714–737. <https://doi.org/10.1089/cmb.2013.0084>.
- Okonechnikov, K., Golosova, O., Fursov, M., UGENE team, 2012. Unipro UGENE: a unified bioinformatics toolkit. *Bioinformatics* 28, 1166–1167. <https://doi.org/10.1093/bioinformatics/bts091>.
- Otto, M., 2013. Coagulase-negative staphylococci as reservoirs of genes facilitating MRSA infection: staphylococcal commensal species such as *Staphylococcus epidermidis* are being recognized as important sources of genes promoting MRSA colonization and virulence. *Bioessays* 35, 4–11. <https://doi.org/10.1002/bies.201200112>.
- Panda, S., Kar, S., Sharma, S., Singh, D.V., 2016. Multidrug-resistant *Staphylococcus haemolyticus* isolates from infected eyes and healthy conjunctivae in India. *J. Glob. Antimicrob. Resist.* 6, 154–159. <https://doi.org/10.1016/j.jgar.2016.05.006>.
- Panda, S., Singh, D.V., 2018. Biofilm formation by ica-negative ocular isolates of *Staphylococcus haemolyticus*. *Front. Microbiol.* 9, 2687. <https://doi.org/10.3389/fmicb.2018.02687>.
- Pantucek, R., Gotz, F., Doskar, J., Rosypal, S., 1996. Genomic variability of *Staphylococcus aureus* and the other coagulase-positive *Staphylococcus* species estimated by macro-restriction analysis using pulsed-field gel electrophoresis. *Int. J. Syst. Bacteriol.* 46, 216–222. <https://doi.org/10.1099/00207713-46-1-216>.
- Pantucek, R., Sedlacek, I., Indrakova, A., Vrbovska, V., Maslanova, I., Kovarovic, V., Svec, P., Kralova, S., Kristofova, L., Keklakova, J., Petras, P., Doskar, J., 2018. *Staphylococcus edaphicus* sp. nov., isolated in Antarctica, harbors the *mecC* gene and genomic islands with a suspected role in adaptation to extreme environments. *Appl. Environ. Microbiol.* 84, e01746–17. <https://doi.org/10.1128/AEM.01746-17>.
- Pantucek, R., Svec, P., Dajcs, J.J., Machova, I., Cernohlavkova, J., Sedo, O., Gelbicova, T., Maslanova, I., Doskar, J., Zdrahal, Z., Ruzickova, V., Sedlacek, I., 2013. *Staphylococcus petrasii* subsp. nov. including *S. petrasii* subsp. nov. and *S. petrasii* subsp. *erocellyticus* subsp. nov., isolated from human clinical specimens and human ear infections. *Syst. Appl. Microbiol.* 36, 90–95. <https://doi.org/10.1016/j.syapm.2012.11.004>.
- Pulverer, G., Pillich, J., Klein, A., Krivankova, M., 1974. Studies on lysogeny of *Staphylococcus albus*. *Med. Microbiol. Immunol.* 159, 115–119. <https://doi.org/10.1007/BF02123723>.
- Rissman, A.I., Mau, B., Biehl, B.S., Darling, A.E., Glasner, J.D., Perna, N.T., 2009. Reordering contigs of draft genomes using the Mauve aligner. *Bioinformatics* 25, 2071–2073. <https://doi.org/10.1093/bioinformatics/btp356>.
- Rossi, C.C., Souza-Silva, T., Araujo-Alves, A.V., Giambiagi-deMarval, M., 2017. CRISPR-Cas systems features and the gene-reservoir role of coagulase-negative staphylococci. *Front. Microbiol.* 8, 1545. <https://doi.org/10.3389/fmicb.2017.01545>.
- Sadaka, A., Palmer, K., Suzuki, T., Gilmore, M.S., 2014. *In vitro* and *in vivo* models of *Staphylococcus aureus* endophthalmitis implicate specific nutrients in ocular infection. *PLoS One* 9, e110872. <https://doi.org/10.1371/journal.pone.0110872>.
- Siebold, C., Flukiger, K., Beutler, R., Erni, B., 2001. Carbohydrate transporters of the bacterial phosphoenolpyruvate: sugar phosphotransferase system (PTS). *FEBS Lett.* 504, 104–111. [https://doi.org/10.1016/S0014-5793\(01\)02705-3](https://doi.org/10.1016/S0014-5793(01)02705-3).
- Siguier, P., Perchon, J., Lestrade, L., Mahillon, J., Chandler, M., 2006. ISfinder: the reference centre for bacterial insertion sequences. *Nucleic Acids Res.* 34, D32–D36. <https://doi.org/10.1093/nar/gkj014>.
- Stegger, M., Andersen, P.S., Kearns, A., Pichon, B., Holmes, M.A., Edwards, G., Laurent, F., Teale, C., Skov, R., Larsen, A.R., 2012. Rapid detection, differentiation and typing of methicillin-resistant *Staphylococcus aureus* harbouring either *mecA* or the new *mecA* homologue *mecA_{16A251}*. *Clin. Microbiol. Infect.* 18, 395–400. <https://doi.org/10.1111/j.1469-0699.2011.03715.x>.
- Svec, P., De Bel, A., Sedlacek, I., Petras, P., Gelbicova, T., Cernohlavkova, J., Maslanova, I., Cnockaert, M., Varbanovova, I., Echahidi, F., Vandamme, P., Pantucek, R., 2015. *Staphylococcus petrasii* subsp. *pragensis* subsp. nov., occurring in human clinical material. *Int. J. Syst. Evol. Microbiol.* 65, 2071–2077. <https://doi.org/10.1099/ij.s.0.000220>.
- Svec, P., Pantucek, R., Petras, P., Sedlacek, I., Novakova, D., 2010. Identification of *Staphylococcus* spp. using (GTG)₅-PCR fingerprinting. *Syst. Appl. Microbiol.* 33, 451–456. <https://doi.org/10.1016/j.syapm.2010.09.004>.
- Takeuchi, F., Watanabe, S., Baba, T., Yuzawa, H., Ito, T., Morimoto, Y., Kuroda, M., Cui, L.Z., Takahashi, M., Ankai, A., Baba, S., Fukui, S., Lee, J.C., Hiramatsu, K., 2005. Whole-genome sequencing of *Staphylococcus haemolyticus* uncovers the extreme plasticity of its genome and the evolution of human-colonizing staphylococcal species. *J. Bacteriol.* 187, 7292–7308. <https://doi.org/10.1128/Jb.187.21.7292-7308.2005>.
- Truong-Bolduc, Q.C., Bolduc, G.R., Okumura, R., Celino, B., Bevis, J., Liao, C.H., Hooper, D.C., 2011. Implication of the NorB efflux pump in the adaptation of *Staphylococcus aureus* to growth at acid pH and in resistance to moxifloxacin. *Antimicrob. Agents Chemother.* 55, 3214–3219. <https://doi.org/10.1128/AAC.00289-11>.
- Wang, Y., Coleman-Derr, D., Chen, G.P., Gu, Y.Q., 2015. OrthoVenn: a web server for genome wide comparison and annotation of orthologous clusters across multiple species. *Nucleic Acids Res.* 43, W78–W84. <https://doi.org/10.1093/nar/gkv487>.
- Werbeck, C., Becker, K., Mellmann, A., Juuti, K.M., von Eiff, C., Peters, G., Kusela, P.I., Friedrich, A.W., Sinha, B., 2007. Staphylococcal chromosomal cassette *mec* type I, *spa* type, and expression of Pls are determinants of reduced cellular invasiveness of methicillin-resistant *Staphylococcus aureus* isolates. *J. Infect. Dis.* 195, 1678–1685. <https://doi.org/10.1086/517517>.
- Wick, R.R., Schultz, M.B., Zobel, J., Holt, K.E., 2015. Bandage: interactive visualization of *de novo* genome assemblies. *Bioinformatics* 31, 3350–3352. <https://doi.org/10.1093/bioinformatics/btv383>.
- Wilson, L.K., Coombs, G.W., Christiansen, K., Grubb, W.B., O'Brien, F.G., 2016. Characterization of a novel staphylococcal cassette chromosome composite island from community-associated MRSA isolated in aged care facilities in Western Australia. *J. Antimicrob. Chemother.* 71, 3372–3375. <https://doi.org/10.1093/jac/dkw317>.
- Winstel, V., Liang, C., Sanchez-Carballo, P., Steglich, M., Munar, M., Broker, B.M., Penades, J.R., Nubel, U., Holst, O., Dandekar, T., Peschel, A., Xia, G., 2013. Wall teichoic acid structure governs horizontal gene transfer between major bacterial pathogens. *Nat. Commun.* 4, 2345. <https://doi.org/10.1038/ncomms3345>.
- Wu, S.W., de Lencastre, H., Tomasz, A., 1998. Genetic organization of the *mecA* region in methicillin-susceptible and methicillin-resistant strains of *Staphylococcus sciuri*. *J. Bacteriol.* 180, 236–242.
- Yu, D., Pi, B., Chen, Y., Wang, Y., Ruan, Z., Otto, M., Yu, Y., 2014. Characterization of the staphylococcal cassette chromosome composite island of *Staphylococcus haemolyticus* SH32, a methicillin-resistant clinical isolate from China. *PLoS One* 9, e87346. <https://doi.org/10.1371/journal.pone.0087346>.
- Yu, W.Q., Kim, H.K., Rauch, S., Schneewind, O., Missiakas, D., 2017. Pathogenic conversion of coagulase-negative staphylococci. *Microbes Infect.* 19, 101–109. <https://doi.org/10.1016/j.micinf.2016.12.002>.
- Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., Aarestrup, F.M., Larsen, M.V., 2012. Identification of acquired antimicrobial resistance genes. *J. Antimicrob. Chemother.* 67, 2640–2644. <https://doi.org/10.1093/jac/dks261>.
- Zeman, M., Maslanova, I., Indrakova, A., Siborova, M., Mikulasek, K., Bendickova, K., Plevka, P., Vrbovska, V., Zdrahal, Z., Doskar, J., Pantucek, R., 2017. *Staphylococcus sciuri* bacteriophages double-convert for staphylokinase and phospholipase, mediate interspecies plasmid transduction, and package *mecA* gene. *Sci. Rep.* 7, 46319. <https://doi.org/10.1038/srep46319>.
- Zhai, Y., He, Z., Kang, Y., Yu, H., Wang, J., Du, P., Zhang, Z., Hu, S., Gao, Z., 2016. Complete nucleotide sequence of pH11, an IncHI2 plasmid conferring multi-antibiotic resistance and multi-heavy metal resistance genes in a clinical *Klebsiella pneumoniae* isolate. *Plasmid* 86, 26–31. <https://doi.org/10.1016/j.plasmid.2016.04.001>.
- Zhang, Q., Ye, Y., 2017. Not all predicted CRISPR-Cas systems are equal: isolated cas genes and classes of CRISPR like elements. *BMC Bioinformatics* 18, 92. <https://doi.org/10.1186/s12859-017-1512-4>.
- Zong, Z.Y., Peng, C.H., Lu, X.J., 2011. Diversity of SCC_{mec} elements in methicillin-resistant coagulase-negative staphylococci clinical isolates. *PLoS One* 6, e20191. <https://doi.org/10.1371/journal.pone.0020191>.
- Zuniga, M., Comas, I., Linaje, R., Monedero, V., Yebra, M.J., Esteban, C.D., Deutscher, J., Perez-Martinez, G., Gonzalez-Candelas, F., 2005. Horizontal gene transfer in the molecular evolution of mannose PTS transporters. *Mol. Biol. Evol.* 22, 1673–1685. <https://doi.org/10.1093/molbev/msi163>.

4.4 Characterization of *Staphylococcus intermedius* Group Isolates Associated with Animals from Antarctica and Emended Description of *Staphylococcus delphini*

Background and objectives: The species of the *S. intermedius* group (SIG) are frequent commensals and opportunistic pathogens of animals and occasionally humans. The most widespread species is *S. pseudintermedius* with many hosts. However, little is known about the staphylococcal colonization of animals living in the harsh Antarctic environment. This is further complicated by variable phenotype and makes species identification unreliable. This study analyses 22 SIG isolates collected from 2013–2019 from healthy seals and birds on James Ross and Seymour islands as part of the Cultivable Fecal Bacteria Communities study in Antarctica. The focus was on *S. delphini*, described in 1988, based on two isolates from dolphins, which were later isolated from unrelated animals and humans.

Material and methods: In total, 363 samples were screened to obtain 6 *S. delphini* and 16 *S. pseudintermedius* isolates, which were characterized based on genotype and phenotype. Two strains of *S. delphini* P5747 and P6156 and one strain of *S. pseudintermedius* P8688 were sequenced using Illumina NextSeq.

Results and discussion: *Staphylococcus delphini* isolates were found exclusively in Adélie penguins and *S. pseudintermedius* in Weddell seals. The phenotypes of both species differed from the original description. The colonization of seals by *S. pseudintermedius* confirms its occurrence in all families of the suborder *Caniformia* and supports hypothesized long-term coevolution. The comparative analysis revealed differences in extracellular proteins, metabolic pathways, and presence of exfoliative toxin among Antarctic strains of *S. delphini*, which likely play a role in host adaptation and immune evasion. Furthermore, several novel genomic islands were characterized: SdPICI-1, SdCI_{sec} encoding enterotoxin C SdCI_{lukSF-1, ula}, SdCI_{serine protease}, and composite transposon conferring resistance to arsen and chromate.

Conclusion: *Staphylococcus pseudintermedius* is globally distributed at a broader range than presumed. *Staphylococcus delphini* strains are variable and have pathogenic potential due to presence of several toxins. The partial sequencing of rpoB, repPCR, and MALDI-TOF-MS consistently and accurately separated SIG species.

Significance: This is the first report of *S. delphini* and *S. pseudintermedius* isolated from Antarctica wildlife. However, the predicted virulence. Factors needed to be studied to determine the actual function and possible risk of zoonotic infections to humans. The emended description of *S. delphini* is also provided.



Article

Characterization of *Staphylococcus intermedius* Group Isolates Associated with Animals from Antarctica and Emended Description of *Staphylococcus delphini*

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Abstract: Members of the genus *Staphylococcus* are widespread in nature and occupy a variety of niches, however, staphylococcal colonization of animals in the Antarctic environment has not been adequately studied. Here, we describe the first isolation and characterization of two *Staphylococcus intermedius* group (SIG) members, *Staphylococcus delphini* and *Staphylococcus pseudintermedius*, in Antarctic wildlife. *Staphylococcus delphini* were found exclusively in Adélie penguins. The report of *S. pseudintermedius* from Weddell seals confirmed its occurrence in all families of the suborder Caniformia. Partial RNA polymerase beta-subunit (*rpoB*) gene sequencing, repetitive PCR fingerprinting with the (GTG)₅ primer, and matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry gave consistent identification results and proved to be suitable for identifying SIG members. Comparative genomics of *S. delphini* isolates revealed variable genomic elements, including new prophages, a novel phage-inducible chromosomal island, and numerous putative virulence factors. Surface and extracellular protein distribution were compared between genomes and showed strain-specific profiles. The pathogenic potential of *S. delphini* was enhanced by a novel type of exfoliative toxin, trypsin-like serine protease cluster, and enterotoxin C. Detailed analysis of phenotypic characteristics performed on six Antarctic isolates of *S. delphini* and eight reference strains from different animal sources enabled us to emend the species description of *S. delphini*.

Keywords: *Staphylococcus delphini*; *Staphylococcus pseudintermedius*; Antarctica; mobile genetic elements; surface proteins; exfoliative toxin; Adélie penguin; Weddell seal

1. Introduction

Staphylococci are a major group of bacteria inhabiting the skin, skin glands, and mucous membranes of humans, other mammals, and birds, as well as the environment due to their ubiquity and adaptability [1]. However, there is very limited information available on staphylococcal isolates from Antarctica. Researchers have occasionally isolated staphylococcal strains from Antarctic environmental samples [2–6] and also from several animals obtained during their health evaluation, but they were mostly only classified to the genus level, such as isolates from whale wound lesions [7], a fish stomach [8], lesions on two dead Adélie penguins [9], or skin swabs of Weddell seals [10].

To the best of our knowledge, no member of the *Staphylococcus intermedius* group (SIG), which includes important veterinary pathogens, has been reported so far from animals in Antarctica. Originally, most isolates of SIG were classified as *S. intermedius*, before their subdivision into SIG members [11,12] comprised of the following four closely related but distinct coagulase-positive species: *S. intermedius* [13], *S. pseudintermedius* [14], *S. delphini* [15], and a human-originated *S. cornubiensis* [16]. The phenotypic and biochemical differentiation between the members of this group is complex and difficult and can result in unreliable species identification [17]. These species can be interchangeably misidentified as each other. Since SIG species are very similar to *Staphylococcus aureus* based on their clinical manifestations and biochemical characteristics such as coagulase positivity, SIG human clinical isolates have probably been misidentified as *S. aureus* in the past [18]. Protein profiling through matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) seems to be the most reliable method to differentiate between SIG species [19,20]. Other effective methods for SIG diagnostics are sequencing of the housekeeping genes *nuc* [21], *sodA*, and *hsp60* [22], and for the discrimination of *S. pseudintermedius* by means of the *pta* housekeeping gene [23] or multilocus sequence typing (MLST) [24], as well as whole-genome sequencing [25,26].

SIG members are common colonizers of animal mucosal surfaces and are considered to be opportunistic pathogens in many infections of different animal hosts. Staphylococcal isolates from dogs previously identified as *S. intermedius* belong almost exclusively to the species *S. pseudintermedius*, while true *S. intermedius* are found predominantly in pigeons [11,12]. *S. pseudintermedius* is recognized as an important causative agent of pyoderma, dermatitis, and otitis externa in dogs, cats, horses, and other animals [27]. However, its host range is not restricted only to non-human animals, but colonization and zoonotic infections of humans are increasingly recognized [28–34]. The taxonomic description of *S. delphini*, in 1988 [15], was based on two strains isolated from purulent skin lesions of two dolphins. Recently, developments in diagnostics have led to the detection of *S. delphini* in a broad range of animals [11,12,35] and one reported human case, probably of zoonotic origin [36]. According to the multilocus sequence analysis of the *sodA*, *hsp60*, and *nuc* genes, *S. delphini* is divided into two phylogenetically distinct clades; group A closely related to the *S. delphini* type strain, and group B related to the *S. pseudintermedius* type strain [12]. The large number of different host associations suggests that multiple ecovars can exist among *S. delphini* isolates.

The aim of this study was to describe the SIG isolates from mammals and birds on James Ross and Seymour Islands in Antarctica, with a focus on *Staphylococcus delphini*. The SIG strains were characterized in detail by phenotypic and genotypic techniques and based on the results an emended description of *S. delphini* was provided.

2. Materials and Methods

2.1. Bacterial Strains and Their Biochemical Characterization

Bacterial strains were collected over the years from 2013 to 2019 on James Ross Island and Seymour Island, Antarctica (Figure 1). The sampling was a part of the Cultivable Fecal Bacteria Communities study, which was part of the CzechPolar project. Sampling intensity varied considerably with geographic location and weather conditions. The key element in the distribution of sampling intensity was the ice-free coast of James Ross and Seymour Islands. There are no seal colonies occupying littoral zone of both islands and samples were taken from occasionally found seals sunning on the coast. Penguin rookeries are in Seymour Island but only the droppings were collected in this territory for protection of the birds. Additional samples from penguins were taken by random sampling when penguins occurred on the coast. All samples originated from live animals only and the sampling procedure was fast to prevent any stress to the animals. The samples from the beak and cloaca of Adélie penguins (*Pygoscelis adeliae*) (82 specimens), the fresh droppings of South polar skua (*Stercorarius maccormicki*) (14 specimens), and kelp gull (*Larus dominicanus*) (11 specimens), and the anus and mouth of Weddell seals (*Leptonychotes weddellii*) (244 specimens), and Southern elephant

seals (*Mirounga leonina*) (12 individuals) were collected using the swab/transport tube system E-Swab (Dispolab, Brno, Czech Republic) and cultured on Mannitol Salt Agar (HiMedia Laboratories, Mumbai, India) at 35 °C for several days in the laboratory at J.G. Mendel Base, James Ross Island. Colonies with suspected staphylococcal morphology were picked daily, kept at 4 °C and transferred to the Czech Republic for further analyses.

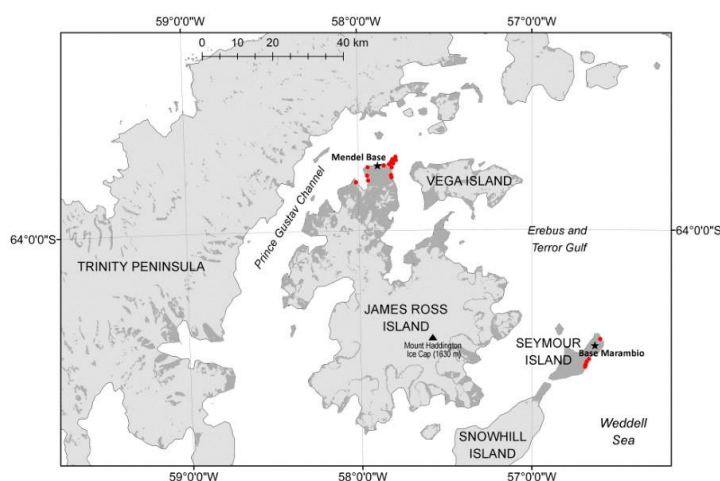


Figure 1. Sampling localities on James Ross Island and Seymour Island. Red mark, location of sampling site; asterisk, location of Antarctic base station; dark grey color, unglaciated area.

Type strains of *S. delphini* CCM 4115^T, *S. intermedius* CCM 5739^T, *S. pseudintermedius* CCM 7315^T, and *S. cornubiensis* CCM 8997^T were acquired from the Czech Collection of Microorganisms (Brno, Czech Republic). Reference strains of *S. delphini*: Nono (=CCM 4184) /dolphin/, CCM 2618 (=CCUG 51769) /mink/, P12548, P12549, and P12550 (=16-9169-2, 17-7762-1, and 18-3863-5, respectively) /all from minks/ [37], P12456 (=HT 2030677) /camel/, P12457 and P12458 (=8086 and 9106) /both from horses/ [11] and CCM 8998 (=MI 18-1587) /human/ [36] were described previously and kindly provided by the authors.

The phenotypic characterization of SIG strains was performed using the commercial kits API 50 CH and API ZYM (bioMérieux, Craponne, France) and by conventional physiological, biochemical, and growth tests discriminative for the genus *Staphylococcus* as described previously [2].

2.2. Partial 16S rRNA and RNA polymerase beta-subunit (*rpoB*) Gene Sequencing and Phylogenetic Analysis

Partial 16S rRNA gene amplification was performed as described previously [38]. PCR amplicons were sequenced with primer 553L in Eurofins Genomics sequencing facility (Ebersberg, Germany). Sequences were identified using the EzTaxon database [39].

Partial RNA polymerase β -subunit (*rpoB*) gene amplification was performed as described previously [40]. PCR amplicons obtained with the primers 1418F and 3554R were sequenced with primers 1418F and 1876R in Eurofins Genomics. Phylogenetic relationships were computed with the software MEGA version 10 [41]. Partial *rpoB* gene sequences were deposited into the GenBank/ENA/DDBJ database under accession numbers MN729216–MN729246.

2.3. Rep-PCR

Repetitive PCR fingerprinting with the (GTG)₅ primer (rep-PCR) was performed as described previously [42]. Numerical analysis of the fingerprints and dendrogram construction was completed using the software BioNumerics version 7.6 (Applied Maths, Kortrijk, Belgium) and compared to

the in-house Czech Collection of Microorganisms rep-PCR database of type and reference strains representing hitherto described *Staphylococcus* spp.

2.4. MALDI-TOF MS

Protein fingerprints by MALDI-TOF MS were acquired with an ultrafleXtreme instrument (Bruker Daltonics, Bremen, Germany) by following the ethanol/formic acid extraction protocol [43]. As many as nine independent mass spectra were acquired for each sample, and only signals present in the minimum of seven of these mass spectra were used for the identification and cluster analysis. The mass spectral data were compared with entries in the latest version of the Biotyper database (version 9.0.0.0, 8468 references) and their mutual similarity was expressed by log(scores), where scores greater than 2.000 correlate to species identification with high confidence, log(score) values between 1.700 and 1.999 correspond to species identification with low confidence, and log(score) values lower than 1.699 result in no identification. A MALDI-TOF MS-based dendrogram was constructed with the software Biotyper (version 3.1, Bruker Daltonics) using the Pearson's product moment similarity coefficient and the unweighted pair group method with arithmetic average (UPGMA) as a grouping method.

2.5. Whole Genome Sequencing and Bioinformatic Analyses

Genomic DNA was isolated using a High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions with the modification of adding 20 µL of lysostaphin (0.5 mg mL⁻¹) for cell lysis. An Illumina NextSeq sequencing platform was used for whole-genome shotgun sequencing of the strains P5747, P6456, and P8688. The purified genomic DNA was used for the preparation of a 500-bp sequencing library with a NEBNext®Ultra™ II DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA). The samples were sequenced using a MID output cartridge in a 150-bp paired-end mode (Illumina, San Diego, CA, USA). The quality of sequencing reads was analyzed with FastQC version 0.11.8 [44]. Bases of lower quality and adapters were trimmed using the Spades version 3.13 implementation of BayesHammer. The de novo assembly of trimmed reads was performed with Spades version 3.13 [45], all k-mers 21 to 127 and follow-up mismatch correction. The package BMap version 38.73 was used to analyze various statistics of genomes [46]. Contigs of described *S. delphini* genomes were reordered according to the reference genome of *S. delphini* strain NCTC 12225^T (GenBank accession no. LR134263, NCTC 3000 Project) by Mauve Contig Mover as a part of Mauve version 2.4 [47]. The sequences of strains P5747, P6456, and P8688 were annotated by Prokka version 1.13.7 [48]. Subcellular localization of proteins of strains was predicted using PSORTb version 3.0 [49]. Proteins assigned to extracellular or cell wall categories were classified to gene ontology by cell2go [50]. Virulence factors were predicted using the VFAnalyzer tool available at the Virulence Factors Database [51]. OrthoVenn2 [52] was used to cluster predicted proteins to orthologous groups. Web-based tools PHASTER [53], CRISPR-Cas++ [54], and GView [55] were employed to find mobile genetic elements and dissimilar regions between analyzed genomes. Sequences were manipulated and examined in the cross-platform software Ugene version 1.31.1 [56]. The multiple sequence alignment was visualised using EasyFig version 2.3 [57].

Whole genome-based phylogenetic analysis was also carried out in order to check the species status of SIG isolates. Average nucleotide identity values (ANI) were calculated with OrthoANIu version 1.2 [58] and digital DNA–DNA hybridization (dDDH) values with the Genome-to-Genome Distance Calculator (GGDC) version 2.1 using the formula 2 recommended for draft genomes [57]. Up-to-date Bacterial Core Gene set (UBCG) software version 3.0 [59] was used to compare 92 core genes between *S. delphini* strains.

Assembly accession numbers of genomes retrieved from NCBI genome database are as follows: 8086 (GCF_000308115.1) [25], NCTC 12225^T (GCF_900636325.1), LMG 22219^T (GCF_001792775.2), NCTC 11048^T (GCF_900458545.1) [60], NW1^T (GCF_900183575.1) [16], 14S03309-1 (GCF_002374115.1), 14S03313-1 (GCF_002374125.1), 14S03318-1 (GCF_002369645.1), and 215100905101-2 (GCF_002369695.1) [26].

The Whole-Genome Shotgun projects of the *S. delphini* strains P5747 and P6456, and *S. pseudintermedius* P8688 have been deposited in DDBJ/ENA/GenBank under the accession numbers WNLD000000000, WNLE000000000, and JAACIP000000000, respectively.

3. Results

3.1. Bacterial Strain Collection and Identification of *Staphylococci*

During the austral summers from 2013 to 2019 on James Ross Island and Seymour Island, Antarctica, a total of 363 animal-related samples were collected in order to isolate staphylococcal species. The swabbed seals and penguins exhibited no disease symptoms at the time of sampling. Pure cultures of Gram-positive, catalase-positive cocci, able to grow in the presence of 12% NaCl, resistant to bacitracin and susceptible to furazolidone were chosen for partial 16S rRNA gene sequencing. In total, 150 *Staphylococcus* spp. strains were identified and most of them classified to the species level (Table S1). The most prevalent staphylococcal isolates were *Staphylococcus haemolyticus* ($n = 28$, predominantly in seals and droppings of skua), *Staphylococcus epidermidis* ($n = 22$, seals only), *Staphylococcus intermedius* group ($n = 22$, seals and penguins), *Staphylococcus sciuri* ($n = 18$, predominantly in penguins and droppings of skua), *Staphylococcus aureus*/*Staphylococcus argenteus*/*Staphylococcus schweitzeri* complex ($n = 14$, in all sources), *Staphylococcus capitis*/*Staphylococcus caprae* complex ($n = 12$, seals only), *Staphylococcus saprophyticus*/*Staphylococcus edaphicus* complex ($n = 9$, predominantly in seals), and *Staphylococcus schleiferi* subsp. *coagulans* ($n = 6$, in all sources).

Twenty-two of the 150 strains were originally identified as members of the *Staphylococcus intermedius* group based on partial 16S rRNA gene sequencing. The strains of SIG were further characterized in detail by genotypic and phenotypic methods and compared with the type strains *S. delphini* CCM 4115^T, *S. intermedius* CCM 5739^T, *S. pseudintermedius* CCM 7315^T, and *S. cornubiensis* CCM 8997^T, as well as with *S. delphini* reference strains CCM 4184, CCM 2618, P12456, P12457, P12458, P12548, P12549, P12550, and CCM 8998 isolated previously from various sources.

3.2. *rpoB* Gene Sequencing

Since 16S rRNA analysis has limited discriminatory power for identifying some staphylococcal species, the phylogenetic position of SIG strains assigned by the 16S rRNA gene sequencing was assessed using the sequence data of the partial *rpoB* gene that was previously shown to be effective for the species differentiation of staphylococci [40,61]. The computed neighbor-joining phylogenetic tree based on the partial *rpoB* gene sequence showed that SIG isolates clustered into two major groups corresponding to *S. delphini* and *S. pseudintermedius* (Figure 2). Strains recovered from Adélie penguins clustered together with the *S. delphini* type strain CCM 4115^T, whereas strains isolated from Weddell seals created a cluster with *S. pseudintermedius* type strain CCM 7315^T.

3.3. Repetitive Sequence-Based PCR (Rep-PCR) Fingerprinting

The repetitive sequence-based PCR (rep-PCR) fingerprinting technique using the (GTG)₅ primer placed the SIG isolates into two clusters corresponding to *S. delphini* and *S. pseudintermedius* at the similarity level of 54% and clearly separated them from *S. intermedius* and *S. cornubiensis* type strains (Figure 3). These results confirmed the *rpoB*-based identification of these strains. *S. delphini* strains exhibited more variable profiles (74% to 100% similarity between strains) than *S. pseudintermedius* (80% to 98%). Five *S. delphini* strains P5747, P5749, P5833, P5835, and P6070 were closely related, while strain P6456 separated from them and clustered together with the *S. delphini* P12458 strain recovered from horse nasal swab.

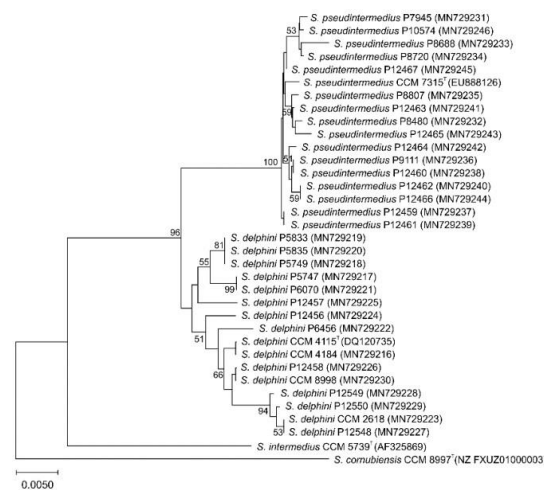


Figure 2. Unrooted neighbor-joining tree based on partial RNA polymerase beta-subunit (*rpoB*) gene sequence comparison, showing the clustering of Antarctic *Staphylococcus intermedius* group isolates and type and reference strains. The percentage of 500 tree replications above 50% in which the associated strains clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tajima-Nei method and are in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 845 positions in the final dataset.

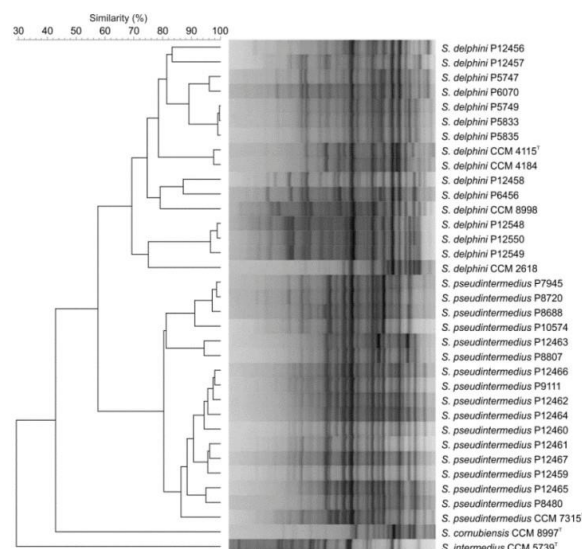


Figure 3. Dendrogram based on cluster analysis of repetitive sequence-based PCR (rep-PCR) fingerprints obtained with (GTG)₅ primer from analyzed isolates and type and reference strains of *Staphylococcus intermedius* group. The dendrogram was calculated with Pearson's correlation coefficients with unweighted pair group method with arithmetic average (UPGMA) clustering method (*r*, expressed as percentage similarity values).

3.4. MALDI-TOF MS

All SIG isolates and reference strains were analyzed by MALDI-TOF MS fingerprinting. Out of the *S. delphini* strains involved in the study, 14 were assigned to *S. delphini* entries of the Biotyper database (six with high confidence and eight with low confidence), while two strains, P5747 and P6070, did not exhibit significant similarity to any of the database entries. All sixteen *S. delphini* strains shared several signals (3787, 4111, 4278, 5302, 6241, 6341, 7438, 8032, 8147, 9028, 9649, and 10506 Da; detected doubly protonated forms of these proteins are not listed), while no peaks characteristic of the strain origin (isolation source/region) were found.

As for *S. pseudintermedius* strains, 13 strains were identified accordingly by using the Biotyper Database (two with high confidence and 11 with low confidence), while the four remaining strains (P7945, P8807, P9111, and P12464) did not exhibit significant similarity to any of the database entries. All 17 *S. pseudintermedius* strains shared several peaks (4278, 5303, 5843, 6241, 6875, 7438, 8032, 8147, 8622, 8649, 9028, 10081, and 10506 Da; detected doubly protonated forms of these proteins are not listed). Seven signals shared by all strains of *S. delphini* and *S. pseudintermedius* (4278, 6241, 7438, 8032, 8147, 9028, and 10506 Da) indicate the close relatedness of these two species. MALDI-TOF MS-based identification outputs correlated with the dendrogram derived from the protein signals (Figure 4). In addition, two *S. delphini* strains, P5747 and P6070, and four *S. pseudintermedius* strains, P7945, P8807, P9111, and P12464 unassigned to *S. delphini* or *S. pseudintermedius* by the MALDI MS-based scoring identification workflow and grouped using cluster analysis (Figure 4) in agreement with the *rpoB*-based and rep-PCR identification (Figure 3).

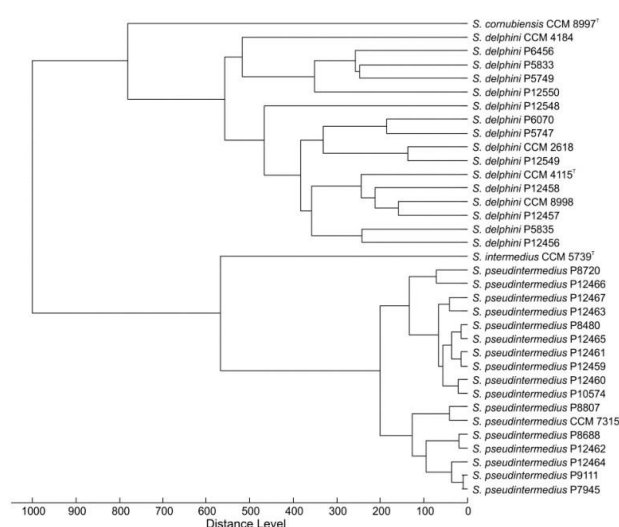


Figure 4. Matrix-assisted laser-desorption/ionization time-of-flight (MALDI-TOF) mass spectra-based dendrogram of analyzed isolates and type and reference strains of *Staphylococcus intermedius* group. The dendrogram was constructed using Pearson's product moment coefficient as a measure of similarity and the unweighted pair group average linked method (UPGMA) as a grouping method.

3.5. Biochemical Identification

The physiological and biochemical properties of SIG Antarctic isolates and 13 strains isolated from other sources were examined (Table 1). Antarctic isolates of both *S. delphini* and *S. pseudintermedius* phenotypically do not correspond to previously described profiles of the species and did not allow their correct classification. Contrary to the original description, all the studied *S. delphini* strains were gelatinase positive, but lecithinase negative and produced acid from trehalose. Coagulase, arginine

dihydrolase, urease, hydrolysis of Tween 80, acid production from glycerol, mannitol, β -gentiobiose, or turanose were strain-dependent for *S. delphini* (Table 1). Similarly, *S. pseudintermedius* from seals differs from the valid description by negative acetoin production and gave variable results for coagulase, arginine dihydrolase, and growth at 45 °C and acid production from mannitol, α -methyl-D-glucoside, and turanose (Table 1). Species from SIG are closely related and phenotypic classification to the species level remains insufficient due to the already reported lack of distinguishable biochemical tests for correct and reliable identification [11,12]. Phenotypic tests applicable for the presumptive species identification of SIG members based on our study, especially for *S. delphini* and *S. pseudintermedius*, are summarized in Table 2.

All strains were positive for the following conventional tests: production of catalase, growth in 12% NaCl, bacitracin resistance, furazolidone and novobiocin susceptibility, production of DNase, pyrrolidonyl arylamidase, nitrate reduction, and growth at 37 °C. Negative for production of oxidase, starch hydrolysis, and growth at 20 °C and 48 °C, production of ornithine decarboxylases, and Voges-Proskauer (acetoin). With the API ZYM kit, positive reactions were obtained for the enzymatic activity of alkaline phosphatase, acid phosphatase, esterase (C4), esterase-lipase (C8), leucine arylamidase and β -galactosidase; and negative reactions for the enzymes lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, naphthol-AS-BI-phosphohydrolase, α -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase, and α -fucosidase. In the API 50 CH tests, all strains gave positive reactions for acid production from glycerol, ribose, galactose, glucose, fructose, mannose, N-acetyl-glucosamine, maltose, lactose, and sucrose. Negative acid production from erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose adonitol, β -methyl-D-xyloside, sorbose, rhamnose, dulcitol, inositol, sorbitol, α -methyl-D-mannoside, amygdalin, arbutin, salicin, cellobiose, melibiose, inulin, melezitose, raffinose, starch, glycogen, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, D-gluconate, 2-keto-gluconate, and 5-keto-gluconate; and negative esculin hydrolysis.

3.6. Comparative Genomic Analysis of *Staphylococcus delphini* Isolates

Two genomes of *S. delphini* Antarctic strains that differed from each other in several characteristics were shotgun sequenced and annotated. Strain P5747 was not successfully identified by the MALDI-TOF MS scoring algorithm, was urease negative, and did not produce acid from lactose; strain P6456 formed a distant cluster in the rep-PCR based dendrogram and *rpoB* gene phylogenetic tree and did not produce acid from mannitol. The size of the draft genomes of strains P5747 and P6456 was 2.54 and 2.65 Mb, comprised of 47 and 104 contigs, with an average G+C content of 38.2 and 38.1 mol%, respectively (Table S2). The genome assemblies were compared to whole-genome sequences of *S. delphini* type strain NCTC 12225^T and horse isolates *S. delphini* 8086 [25] and *S. delphini* 215100905101-2 [26] (Figure 5). Comparative genomic analysis of predicted genes in the above five genomes identified 2460 gene clusters and 596 singletons. The majority of the orthologous clusters (up to 1899) were shared by all the analyzed strains. Fifty-six clusters were unique for Antarctic strains and responsible for plasmid maintenance, phage DNA replication, and a response to phosphate starvation. All non-Antarctic *S. delphini* strains have additional genes for biofilm formation, the regulation of L-carnitine utilization, and a phosphoenolpyruvate-dependent sugar phosphotransferase system. Antarctic *S. delphini* lack the presence of the genes that confer resistance to fosfomycin as compared with the strains of equine origin.

Table 1. Variable phenotypic reactions of *Staphylococcus intermedius* group strains under study.

Species	Strain	Source	Locality	Conventional Test Results										API 50 CH										API ZYM		
				COA	ARG	URE	TWE	GEL	Cas	E-γ	C45	GLY	MAN	SOR	ARB	MDG	LAC	TRE	GEN	TUR	N-PH	α-FLU				
<i>S. delphini</i>	CCM 4115 ^T	dolphin	Italy	+	+	+	-	-	-	+	+	w	+	+	-	-	-	+	-	-	w	-	-	-	-	
<i>S. delphini</i>	CCM 4184	dolphin	Italy	+	w	+	-	-	+	+	+	w	+	+	+	+	-	+	+	-	w	-	-	-	-	
<i>S. delphini</i>	P5747	penguin	Antarctica	w	-	-	w	+	+	-	-	-	w	-	-	-	-	+	+	-	w	-	-	-	-	
<i>S. delphini</i>	P5749	penguin	Antarctica	w	w	+	+	+	+	-	+	w	w	-	-	-	-	+	+	-	w	-	-	-	-	
<i>S. delphini</i>	P5833	penguin	Antarctica	-	-	+	+	+	+	+	+	w	w	-	-	-	-	+	+	-	w	-	-	-	-	
<i>S. delphini</i>	P5835	penguin	Antarctica	-	-	+	+	+	+	+	+	w	w	-	-	-	-	+	+	-	w	-	-	-	-	
<i>S. delphini</i>	P6070	penguin	Antarctica	-	-	-	+	+	+	+	+	-	+	-	-	-	-	+	+	-	w	-	-	-	-	
<i>S. delphini</i>	P6456	penguin	Antarctica	w	-	+	+	+	+	-	+	w	w	-	-	-	-	w	+	-	-	-	-	-	-	
<i>S. delphini</i>	P12456	camel	France	w	w	+	-	+	+	-	-	+	+	+	-	w	-	+	+	+	w	+	-	-	-	
<i>S. delphini</i>	P12457	horse	UK	+	w	+	+	+	+	+	-	w	w	+	-	-	-	w	+	+	w	w	-	-	-	
<i>S. delphini</i>	P12458	horse	UK	w	w	+	+	+	+	+	-	+	+	+	-	-	-	+	+	+	+	+	-	-	-	
<i>S. delphini</i>	CCM 2618	mink	Czechia	+	-	+	+	+	+	+	-	+	+	+	-	-	-	+	+	+	+	+	-	-	-	
<i>S. delphini</i>	P12548	mink	Denmark	w	-	+	-	+	+	+	-	w	-	-	-	-	-	+	+	-	w	-	-	-	-	
<i>S. delphini</i>	P12549	mink	Denmark	w	-	+	+	+	+	+	+	w	-	-	-	-	-	+	+	-	w	-	-	-	-	
<i>S. delphini</i>	P12550	mink	Denmark	w	w	+	+	+	+	+	-	w	+	+	+	-	-	+	+	+	+	+	-	-	-	
<i>S. delphini</i>	CCM 8998	human	USA	+	+	+	+	+	+	-	-	w	+	+	+	-	-	+	+	+	+	+	-	-	-	
<i>S. pseudintermedius</i>	CCM 7315 ^T	cat	Belgium	+	+	+	-	+	+	-	+	+	+	+	-	-	-	+	+	+	+	+	-	-	-	
<i>S. pseudintermedius</i>	P7945	seal	Antarctica	w	w	+	-	+	+	-	-	w	-	-	-	-	-	+	+	+	+	+	-	-	-	
<i>S. pseudintermedius</i>	P8480	seal	Antarctica	+	-	+	-	+	+	-	+	+	+	+	-	-	-	w	+	+	+	+	-	-	-	
<i>S. pseudintermedius</i>	P8688	seal	Antarctica	+	-	+	-	+	+	-	+	+	+	+	-	-	-	w	+	+	+	+	-	-	-	
<i>S. pseudintermedius</i>	P8720	seal	Antarctica	w	+	+	-	+	+	-	+	w	-	-	-	-	-	+	+	+	+	+	-	-	-	
<i>S. pseudintermedius</i>	P8807	seal	Antarctica	+	+	+	-	+	+	-	+	+	+	-	-	-	-	w	+	+	+	+	-	-	-	
<i>S. pseudintermedius</i>	P9111	seal	Antarctica	+	w	+	-	+	+	-	+	+	+	+	-	-	-	w	+	+	+	+	-	-	-	
<i>S. pseudintermedius</i>	P12459	seal	Antarctica	w	-	+	-	+	+	-	+	w	+	-	-	-	-	+	+	+	+	+	-	-	-	
<i>S. pseudintermedius</i>	P12460	seal	Antarctica	-	w	+	-	+	+	-	+	w	+	-	-	-	-	w	+	+	+	+	-	-	-	
<i>S. pseudintermedius</i>	P12461	seal	Antarctica	w	w	+	-	+	+	-	+	+	+	+	-	-	-	w	+	+	+	+	-	-	-	
<i>S. pseudintermedius</i>	P12462	seal	Antarctica	-	-	+	-	+	+	-	+	+	+	+	-	-	-	w	+	+	+	+	-	-	-	
<i>S. pseudintermedius</i>	P12463	seal	Antarctica	-	w	+	-	+	+	-	+	+	+	+	-	-	-	w	+	+	+	+	-	-	-	
<i>S. pseudintermedius</i>	P12464	seal	Antarctica	-	w	+	-	+	+	-	+	+	+	+	-	-	-	w	+	+	+	+	-	-	-	
<i>S. pseudintermedius</i>	P12465	seal	Antarctica	w	w	+	-	+	+	-	+	+	+	+	-	-	-	+	+	+	+	+	-	-	-	
<i>S. pseudintermedius</i>	P12466	seal	Antarctica	w	w	+	-	+	+	-	+	+	+	+	-	-	-	w	+	+	+	+	-	-	-	
<i>S. pseudintermedius</i>	P12467	seal	Antarctica	+	+	+	-	+	+	-	+	+	+	+	-	-	-	w	+	+	+	+	-	-	-	
<i>S. pseudintermedius</i>	P10574	seal	Antarctica	-	+	+	-	+	+	-	+	w	-	-	-	-	-	+	+	+	+	+	-	-	-	
<i>S. intermedius</i>	CCM 5739 ^T	pigeon	Czechia	w	-	+	-	+	+	-	+	+	+	+	-	-	-	+	+	+	+	+	-	-	-	
<i>S. cornuensis</i>	CCM 8997 ^T	human	UK	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	

Legend: COA, coagulase; ARG, arginine dihydrolase; URE, urease; TWE, hydrolysis of Tween 80; GEL, hydrolysis of gelatin; Cas, hydrolysis of casein; E-Y, egg-yolk reaction (lecithinase); C45, growth at 45 °C; GLY, acid from glycerol; MAN, acid from mannitol; SOR, acid from sorbitol; ARB, acid from arbutin; MDG, acid from α-methyl-D-glucoside; LAC, acid from lactose; TRE, acid from trehalose; GEN, acid from β-gentiobiose; TUR, acid from turanose; N-PH, naphthol-AS-BI-phosphohydrolase; α-FU, α-fucosidase; +, positive; -, negative; w, weak.

Table 1. Variable phenotypic reactions of *Staphylococcus intermedius* group strains under study.

Species	Strain	Source	Locality	Conventional Test Results										API 50 CH					API ZYM				
				COA	ARG	URE	TWE	GEL	Cas	E-Y	C45	GLY	MAN	SOR	ARB	MDG	LAC	TRE	GEN	TUR	N-PH	α-FU	
<i>S. delphini</i>	CCM 4115 ^T	dolphin	Italy	+	+	+	-	-	-	+	+	w	+	-	-	+	-	-	w	-	-		
	CCM 4184	dolphin	Italy	+	w	-	-	-	-	+	+	w	+	-	-	+	-	-	w	-	-		
	P5747	penguin	Antarctica	w	-	-	w	+	+	-	-	w	-	-	-	+	-	-	w	-			
	P5749	penguin	Antarctica	w	w	+	+	+	+	-	w	w	-	-	+	+	-	w	-	-			
	P5833	penguin	Antarctica	w	-	+	+	+	+	-	w	+	+	-	-	+	-	w	-	-			
	P5835	penguin	Antarctica	-	-	+	+	+	+	-	w	+	+	-	-	+	-	w	-	-			
	P6070	penguin	Antarctica	w	-	-	+	+	+	-	+	-	+	-	-	+	-	w	-	-			
	P6456	penguin	Antarctica	w	-	+	+	+	+	-	-	-	-	-	-	+	-	-	-	-			
	P12456	camel	France	w	w	+	-	+	+	+	-	w	+	-	-	w	+	+	w	+			
	P12457	horse	UK	+	w	+	+	+	+	-	-	w	+	+	-	-	+	+	w	w			
	P12458	horse	UK	w	w	+	+	+	+	-	w	w	-	-	-	+	+	-	w	-			
	CCM 2618	mink	Czechia	+	-	+	+	+	+	-	+	+	+	-	-	+	-	w	-	w			
	P12548	mink	Denmark	w	-	+	+	+	+	-	+	w	+	-	-	+	+	-	w	-			
	P12549	mink	Denmark	w	-	+	+	+	+	-	+	w	+	-	-	+	+	-	w	-			
	P12550	mink	Denmark	w	w	+	+	+	+	-	+	w	+	-	-	+	+	-	w	-			
	CCM 8998	human	USA	+	+	+	+	+	+	-	-	w	+	+	-	-	+	-	-	-			
	CCM 7315 ^T	cat	Belgium	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	-	-			
	P7945	seal	Antarctica	w	w	+	-	+	+	+	-	w	-	-	-	+	+	-	-	-			
	P8480	seal	Antarctica	+	-	+	-	+	+	+	-	-	-	-	w	+	+	-	-	-			
P8688	seal	Antarctica	+	-	+	-	+	+	+	-	-	w	-	-	+	+	-	w	-				
P8720	seal	Antarctica	w	+	+	-	+	+	+	-	w	-	-	-	+	+	-	-	-				
P8807	seal	Antarctica	+	+	+	+	+	+	+	-	-	-	-	w	+	+	-	w	-				
P9111	seal	Antarctica	+	w	+	-	+	+	+	-	w	+	+	-	+	+	-	w	-				
P12459	seal	Antarctica	w	-	+	+	+	+	+	-	w	+	-	-	w	+	-	-	-				
P12460	seal	Antarctica	-	w	+	-	+	+	+	-	+	+	+	-	+	+	-	+	-				
P12461	seal	Antarctica	w	w	+	-	+	+	+	-	+	+	+	-	+	+	-	+	-				
P12462	seal	Antarctica	-	-	+	-	+	+	+	-	+	+	+	-	+	+	-	+	-				
P12463	seal	Antarctica	-	w	+	-	+	+	+	-	+	+	+	-	+	+	-	+	-				
P12464	seal	Antarctica	-	w	+	-	+	+	+	-	+	+	+	-	+	+	-	+	-				
P12465	seal	Antarctica	w	w	+	-	+	+	+	-	+	+	+	-	+	+	-	+	-				
P12466	seal	Antarctica	w	w	+	-	+	+	+	-	w	+	+	-	+	+	-	+	-				
P12467	seal	Antarctica	+	w	+	+	+	+	+	-	+	+	+	-	+	+	-	+	-				
P10574	seal	Antarctica	-	+	+	-	+	+	+	-	-	w	-	-	+	+	-	-	-				
<i>S. pseudintermedius</i>	CCM 5739 ^T	pigeon	Czechia	w	-	+	-	+	+	-	+	+	+	-	-	w	+	+	-	-			
<i>S. intermedius</i>	CCM 8997 ^T	human	UK	+	+	+	+	+	+	-	-	w	+	-	-	+	+	-	-	-			

Legend: COA, coagulase; ARG, arginine dihydrolase; URE, urease; TWE, hydrolysis of Tween 80; GEL, hydrolysis of gelatin; Cas, hydrolysis of casein; E-Y, egg-yolk reaction (lecithinase); C45, growth at 45 °C; GLY, acid from glycerol; MAN, acid from mannitol; SOR, acid from sorbitol; ARB, acid from arbutin; MDG, acid from α -methyl-D-glucoside; LAC, acid from lactose; TRE, acid from trehalose; GEN, acid from β -gentiobiose; TUR, acid from turanose; N-PH, naphthol-AS-BI-phosphohydrolase; α -FU, α -fucosidase; +, positive; -, negative; w, weak.

Table 2. Proposal of phenotypic traits suitable for differentiation of *Staphylococcus intermedius* group species.

Species	Strains	TWE	E-Y	MAN	MDG
<i>S. delphini</i>	16 strains	d (63%)	- (6%)	+ (88%)	- (0%)
<i>S. pseudintermedius</i>	17 strains	- (0%)	- (0%)	(-) (29%)	(+) (71%)
<i>S. intermedius</i>	CCM 5739 ^T	-	-	+	w
<i>S. cornubiensis</i>	CCM 8997 ^T	+	+	+	-

Legend: TWE, hydrolysis of Tween 80; E-Y, egg-yolk reaction (lecithinase); MAN, acid from mannitol; MDG, acid from α -methyl-D-glucoside; +, 85% to 100%; (+), 70% to 84%; d, 31% to 69%; (-), 15% to 29%; -, 0% to 14%; w, weak.

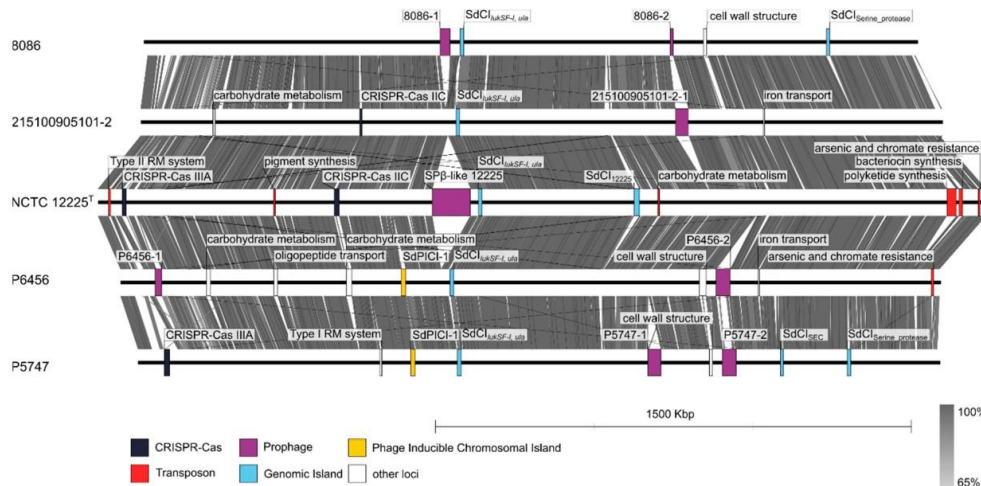


Figure 5. Whole-genome comparison of *Staphylococcus delphini* penguin isolates P5747 (GenBank accession no. WNLD000000000) and P6456 (WNLE000000000) with horse strains 8086 (CAIA000000000), 215100905101-2 (MWUT000000000), and type strain NCTC 12225^T from a dolphin (LR134263). Genomic regions with major differences between neighboring genomes and significant mobile genetic elements are highlighted in rectangles and are color-coded according to the legend. Prophage genomes 8086-1, 8086-2, and P6456-1 were located on different contigs, and therefore are not complete in the whole-genome comparison. Conserved regions with more than 65% homology are indicated with different shades of grey as determined by blastn.

Variability in *S. delphini* genome structure is mostly associated with mobile genetic elements and numerous genomic islets (Table S2 and Figure 5). The genomes of *S. delphini* strains P5747 and P6456 contain predicted plasmid contigs, prophages, a novel *S. delphini* phage-inducible chromosomal island (PICI) designated SdPICI-1 inserted between the chaperone GroEL and glucosamine-6-phosphate N-acetyltransferase genes, and genomic island designated SdCI_{SEC} encoding staphylococcal enterotoxin C (SEC) and putative proteins involved in its transfer. *Staphylococcus delphini* strain P5747 harbours CRISPR type IIIA, a distinctive gene cluster for purine metabolism, genes encoding sphingomyelinase C, sialidase B, putative genes for capsular polysaccharide synthesis located near the *oriC* region, and trypsin-like serine protease gene cluster. A genomic island containing bi-component leukocidin locus *lukSF-I* and the L-ascorbate transport and utilization (*ula*) operon was observed in all the analyzed genomes. The genome of *S. delphini* strain P6456 contains a gene for a novel exfoliative toxin (locus tag: FMF08_11915) in the *oriC* environ, gene clusters for the intake and metabolism of saccharides and oligopeptides, and a composite transposon for arsenic and chromate resistance.

The genes for virulence factors, surface and extracellular proteins are dispersed throughout *S. delphini* genomes. Majority of these proteins have similar distribution to other SIG members (Table S3).

The comparative analysis showed three major differences in those proteins from *S. delphini* strains and representative SIG members originating from different hosts as follows: (i) variability of serine proteases including exfoliative toxin-like proteins, (ii) different polysaccharide capsule synthesis proteins, and (iii) extensive variability of putative proteins with unknown function containing a LPXTG motif anchoring to cell wall peptidoglycan (Table S3).

To evaluate the intergenomic distances between the *S. delphini* genomes, ANI and dDDH values were determined (Table 3). The calculated ANI values among analyzed *S. delphini* genomes ranged from 96.28% to 98.66%, and unambiguously confirmed the position of both strains P5747 and P6456 as *S. delphini* species and at the same time showed that *S. pseudintermedius* and *S. intermedius* type strains are well below the thresholds of 95% to 96% for species delineation [62]. The calculated dDDH values among *S. delphini* were relatively low and indicated an extensive variability that could represent specialization to specific hosts. As a further extension to genome-based phylogeny the Up-to-date Bacterial Core Gene (UBCG) tool was used to compare 92 core genes among analyzed *S. delphini* genomes (Figure 6). This analysis confirmed that the strains do not divide to distinctive lineages according to their animal host.

Table 3. Intergenomic distances between the genomes of *Staphylococcus intermedius* group strains in percent, represented by average nucleotide identity (ANI) and digitally derived genome-to-genome distances (GGD) emulating DNA–DNA hybridization values.

Strain	P5747		P6456		NCTC 12225 ^T		215100905101-2		8086		NCTC 11048 ^T		LMG 22219 ^T		NW1 ^T	
	GGD	ANI	GGD	ANI	GGD	ANI	GGD	ANI	GGD	ANI	GGD	ANI	GGD	ANI	GGD	ANI
<i>S. delphini</i> P5747	-	-	70.2	96.3	70.5	96.5	77.4	97.6	85.6	98.4	35.8	88.6	55.1	93.9	33.5	87.4
<i>S. delphini</i> P6456	70.2	96.3	-	-	87.5	98.7	69.5	96.3	69.0	96.3	35.5	88.4	51.3	93.1	33.2	87.2
<i>S. delphini</i> NCTC 12225 ^T	70.5	96.5	87.5	98.7	-	-	70.4	96.6	70.1	96.4	35.9	88.6	51.2	93.1	33.5	87.4
<i>S. delphini</i> 215100905101-2	77.4	97.6	69.5	96.3	70.4	96.6	-	-	78.4	97.6	35.7	88.6	54.7	93.7	33.6	87.8
<i>S. delphini</i> 8086	85.6	98.4	69.0	96.3	70.1	96.4	78.4	97.6	-	-	35.6	88.6	54.9	93.9	33.6	87.5
<i>S. intermedius</i> NCTC 11048 ^T	35.8	88.6	35.5	88.4	35.9	88.6	35.7	88.6	35.6	88.7	-	-	34.9	88.0	36.2	88.6
<i>S. pseudintermedius</i> LMG 22219 ^T	55.1	93.9	51.3	93.1	51.2	93.1	54.7	93.7	54.9	93.9	34.9	88.0	-	-	32.9	87.2
<i>S. cornubiensis</i> NW1 ^T	33.5	87.4	33.2	87.2	33.5	87.4	33.6	87.8	33.6	87.6	36.2	88.6	32.9	87.2	-	-

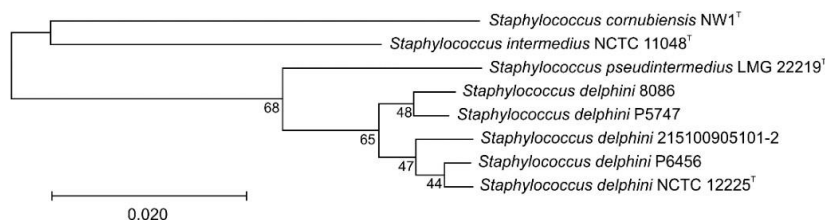


Figure 6. Core gene set phylogenetic tree of *Staphylococcus delphini* strains from different animals (penguin isolates P5747 and P6456, horse strains 8086 and 215100905101-2, and a dolphin strain NCTC 12225^T). The phylogenetic tree was constructed using Up-to-date bacterial core gene set (UBCG) concatenated alignment of 92 core genes. A total of 86,031 nucleotide positions were used. Maximum likelihood phylogenetic tree was inferred using Fasttree version 2.1.10 using GTR + CAT model. Gene support indices are given at branching points (maximal possible value is 92). Bar, 0.1 substitution per position.

4. Discussion

The analyzed SIG strains were collected in several consecutive years at three different locations. Species identification based on previously evaluated 16S ribosomal DNA-based identification [63] showed the presence of 16 staphylococcal taxa. Some of the recently described staphylococcal species shared high similarity in their 16S rRNA sequence and were distinguished on the basis of whole-genome sequencing, therefore they are identified as species complexes. Among the most prevalent staphylococcal species isolated from Antarctic wildlife were *S. haemolyticus*, *S. epidermidis*, and *S. sciuri*, which have been previously collected in Antarctic environmental samples [4–6,64].

In this study, several strains of *S. aureus* were also collected, which corresponds with the findings of Van Elk et al. [65], who recovered an *S. aureus* isolate from Antarctic southern elephant seal and suggested that it could be a host species-specific and coevolved variant which is not closely related to *S. aureus* strains from terrestrial species, based on MLST analysis.

To the best of our knowledge, this is the first report of the isolation and characterization of *S. delphini* and *S. pseudintermedius* from Antarctic animals, penguins and seals. SIG species, thus, seem to be ubiquitous and the most common coagulase-positive staphylococci recovered from non-human animals, in which they can act as opportunistic pathogens and cause a variety of infections. *Staphylococcus delphini* has been previously isolated from a broad range of phylogenetically unrelated mammals (dolphins, cows, horses, camels, and mustelids). The isolation of *S. delphini* from the Antarctic Adélie penguin is the only report from birds except for rare isolates from pigeons [12]. *S. pseudintermedius* is a predominant opportunistic pathogen of canine hosts, and the clonal diversity and broad geographic distribution of *S. pseudintermedius* suggests that it has coevolved with the suborder Caniformia ("dog-like" carnivorans) for a long time in evolutionary terms [11,66,67]. Aarestrup [67] isolated SIG strains from the skin of healthy members of six phylogenetic groups within Caniformia, but not successfully from the seal family (*Phocidae*). The report of the occurrence of *S. pseudintermedius* in Weddell seals supports the theory of *S. pseudintermedius* coevolution with Caniformia, and it is evident that the bacteria can coexist with their hosts in the extreme polar environment. It is likely that SIG species shared a common ancestor and evolved with their specific host adaptation, analogously to the relationship between *S. aureus* and its sister primate-associated species *Staphylococcus simiae* [68,69] or *Staphylococcus schweitzeri* [70]. In addition, this study concluded that members of SIG are distributed worldwide and inhabit an even wider range of hosts than was previously thought.

Differentiating between species belonging to SIG is problematic. In addition, some strains previously described as *S. intermedius* have been reassigned to other species within SIG [12,71]. Identification based on conventional biochemical, physiological, and growth tests in a routine microbiology laboratory is difficult, since SIG members share many phenotypic characteristics, the expression of many biochemical properties is variable, and taxonomic descriptions were done with various identification sets utilizing different reaction substrates. We evaluated the performance of molecular-based and phenotype-based techniques for the ability to differentiate and identify members of SIG, including Antarctic isolates. The study demonstrated that *rpoB* gene sequencing and rep-PCR gave consistent results and differentiated the isolates into distinctive clusters corresponding to individual species within SIG. Cluster analysis based on MALDI-TOF MS protein data was able to separate the *S. pseudintermedius* and *S. delphini* Antarctic isolates, place them together with corresponding type strains, and create separate clusters from *S. intermedius* and *S. cornubiensis* type strains. Unlike a recent report [72], mass spectrometry was unable to identify two Antarctic isolates of *S. delphini* using a MALDI-TOF MS-based scoring identification workflow.

The Antarctic *S. delphini* and *S. pseudintermedius* isolates phenotypically differ from both the type and used reference strains. The type strain of *S. delphini* is described as negative for trehalose and thermonuclease. Because the species description of *S. delphini* was based on only two strains, in our study we tested a set of Antarctic isolates supplemented with *S. delphini* reference strains from different animals, including a human isolate. All Antarctic *S. delphini* strains were positive for acid production from trehalose, similarly to Sasaki et al. [12] who also reported some *S. delphini* strains to be positive for trehalose and thermonuclease. Moreover, in our study we found positive gelatinase activity among *S. delphini* isolates and reference strains, which contradicted their species description, as well as lecithinase production, which was positive for the type strain but negative for the tested strains. It is possible that strains from diverse animals represent distinctive ecotypes with specific ecological adaptations. Variation in phenotype, borderline dDDH values between strains (Table 3), and previously reported polymorphisms in housekeeping genes [12] suggest that more subspecies exists within *S. delphini* species.

Similar to *S. aureus* [73,74], the major differences in the *S. delphini* genomes are due to variable genetic elements. Various prophages with low similarity to known phage sequences in available databases were found in all strains. Two different types of wall teichoic acids synthesis loci were found in the analyzed genomes. Since wall teichoic acids serve as receptors for bacteriophage adsorption [74] and the phage adsorption is sufficient for efficient transduction [75] we hypothesize that differences in phage receptors can drive the diversification of *S. delphini* genomes. The staphylococcal cassette chromosome *mec*, which was described in methicillin-resistant *S. pseudintermedius* [76], was not detected in any of the analyzed *S. delphini* genomes. The presence of CRISPR/Cas and the type of *cas* genes was not related to the animal source of the isolation, thus the loci could have been acquired independently. *Staphylococcus aureus* pathogenicity islands (SaPI) are well described representatives of PICI and contribute to horizontal gene transfer [77]. In contrast to SaPI, *S. delphini* PICI SdPICI-1 was only observed in strains isolated from penguins and contained genes for phage-related head structural proteins and both terminase subunits, which has been observed in some PICIs of Gram-negative bacteria [78]. The finding of genomic island harboring leukocidin encoding genes *lukFS-I* among all analyzed strains confirms its ancient origin [25] and also its preservation among *S. delphini* strains in the polar environment. Virulence potential of all analyzed *S. delphini* strains is enhanced by the presence of SEC encoding genes, which have been previously detected in *S. pseudintermedius* isolates and associated with canine pyoderma [79,80]. Distribution of other toxin genes is variable among the strains (Table S3). Putative exfoliative toxin from P6456 strain has 53% amino acid identity with *S. pseudintermedius* exfoliative toxins EXI and ExpB [81,82]. Furthermore, an island with multiple paralog genes encoding trypsin-like serine proteases related to exfoliative toxins [83] was found in strain P5747 (Figure 5 and Table S3), being a possible virulence factor.

Staphylococcus delphini genomes of the type strain, the strains representing two different phylogenetic branches of equine origin and the selected genomes of SIG isolates from other animals, were used for the comparative genomic analysis with focus on virulence, cell wall anchored proteins, and extracellular proteins (Table S3). These proteins most likely play a role in adaptation to the host and in a defence against host immunity. Homologs of *S. delphini* surface proteins were found in other SIG species, but many proteins were strain-specific, suggesting specific adaptation and virulence. However, there was no distinctive pattern of surface proteins corresponding to individual animal hosts. The location of these genes for surface proteins was predominantly on the chromosomes and not on the mobile genetic elements indicating both common ancestry and differential gene loss among strains as well as gene gain via horizontal transfer events. Despite numerous putative virulence factors found in analyzed genomes of *S. delphini*, further studies are needed to consider their role in animal pathogenesis or the possible risk of zoonotic infection to humans.

5. Emended Description of *Staphylococcus delphini* (Varaldo et al., 1988)

The description is based on the original description and observations of six isolates from penguins and eight reference strains from animals and human (Table 1). The morphological, biochemical, and physiological characteristics are generally those of the *S. delphini* description [15] with the exception of gelatin and lecithin hydrolysis and trehalose acidification, which were strain dependent. In contrast to type strain CCM 4115^T and reference strain CCM 4184, both from dolphins [15], all the studied *S. delphini* strains were gelatinase positive, but lecithinase negative and produced acid from trehalose. Below are the mentioned emended positive and negative test results not included in the original description.

Acid is produced from glycerol, ribose, galactose, and N-acetyl-glucosamine (API 50 CH). Esterase (C4), esterase-lipase (C8), acid phosphatase, pyrrolidonyl arylamidase, leucine arylamidase, and β -galactosidase positive (API ZYM). Resistant to bacitracin (0.04 U) and susceptible to furazolidone (100 μ g). Negative oxidase, ornithine decarboxylase, esculin and starch hydrolysis, and growth at 20 °C. Acid production is negative from erythritol, D-arabinose, D-xylose, adonitol, β -methyl-D-xyloside, sorbose, rhamnose, dulcitol, inositol, sorbitol, α -methyl-D-mannoside, α -methyl-D-glucoside, amygdalin, arbutin, salicine, cellobiose, melibiose, inulin, melezitose, raffinose, starch, glycogen,

D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-keto-gluconate, and 5-keto-gluconate (API 50 CH). No enzymatic activity found for lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, α -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, N-acetyl- β -glucosaminidase, and α -mannosidase (API ZYM). Variable reactions are shown in Table 1. Isolated from mucous membranes of different animals, occasionally humans. G + C content of 38 mol% calculated from whole genomic sequence.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2076-2607/8/2/204/s1>: Table S1: *Staphylococcus* spp. strains identified in animal-related samples from James Ross Island and Seymour Island, Antarctica, Table S2: Comparison of selected genomic features of *Staphylococcus delphini* strains P5747 and P6456 from penguins, and *S. delphini* strains from other hosts, Table S3: Distribution of virulence factors, surface and extracellular proteins among the *Staphylococcus delphini* strains from different hosts and from the reference and type strains of other SIG species.

Author Contributions: Design of the study and conceptualization, R.P., I.S., and V.V.; sampling, isolation and preliminary identification of the strains, I.S. and M.L.; performance of experiments and investigations, V.V., I.S., P.Š., and O.Š.; analysis of data, V.V., I.S., M.Z., P.Š., V.K., and R.P.; writing—original draft preparation, V.V., M.Z., and I.S.; writing—review and editing, V.V., R.P., I.S., P.Š., and J.D.; visualization, M.Z., P.Š., V.K., and R.P.; supervision, R.P.; funding acquisition, R.P., I.S., and J.D. All authors have read and agreed to the published version of the manuscript.

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References

- Götz, F.; Bannerman, T.; Schleifer, K.-H. The Genera *Staphylococcus* and *Micrococcus*. In *The Prokaryotes. Volume 4: Bacteria: Firmicutes, Cyanobacteria*; Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., Stackebrandt, E., Eds.; Springer Science: New York, NY, USA, 2006; Volume 4, pp. 5–75.
- Pantůček, R.; Sedláček, I.; Indráková, A.; Vrbovská, V.; Mašláňová, I.; Kovařovic, V.; Švec, P.; Králová, S.; Křištofová, L.; Kekláková, J.; et al. *Staphylococcus edaphicus* sp. nov., isolated in Antarctica, harbors the *mecC* gene and genomic islands with a suspected role in adaptation to extreme environments. *Appl. Env. Microbiol.* **2018**, *84*. [CrossRef]
- Shivaji, S.; Begum, Z.; Shiva Nageswara Rao, S.S.; Vishnu Vardhan Reddy, P.V.; Manasa, P.; Sailaja, B.; Prathiba, M.S.; Thamban, M.; Krishnan, K.P.; Singh, S.M.; et al. Antarctic ice core samples: Culturable bacterial diversity. *Res. Microbiol.* **2013**, *164*, 70–82. [CrossRef] [PubMed]
- Peeters, K.; Hodgson, D.A.; Convey, P.; Willems, A. Culturable diversity of heterotrophic bacteria in Forlidas Pond (Pensacola Mountains) and Lundstrom Lake (Shackleton Range), Antarctica. *Microbiol. Ecol.* **2011**, *62*, 399–413. [CrossRef] [PubMed]
- Arenas, F.A.; Pugin, B.; Henriquez, N.A.; Arenas-Salinas, M.A.; Diaz-Vasquez, W.A.; Pozo, M.F.; Munoz, C.M.; Chasteen, T.G.; Perez-Donoso, J.M.; Vasquez, C.C. Isolation, identification and characterization of highly tellurite-resistant, tellurite-reducing bacteria from Antarctica. *Polar Sci.* **2014**, *8*, 40–52. [CrossRef]
- Leiva, S.; Alvarado, P.; Huang, Y.; Wang, J.; Garrido, I. Diversity of pigmented Gram-positive bacteria associated with marine macroalgae from Antarctica. *FEMS Microbiol. Lett.* **2015**, *362*, fnv206. [CrossRef] [PubMed]

7. Sieburth, J.M. Gastrointestinal microflora of Antarctic birds. *J. Bacteriol.* **1959**, *77*, 521–531. [[CrossRef](#)] [[PubMed](#)]
8. Vazquez, S.C.; Merino, L.N.R.; McCormack, W.P.; Fraile, E.R. Protease-producing psychrotrophic bacteria isolated from Antarctica. *Polar Biol.* **1995**, *15*, 131–135. [[CrossRef](#)]
9. Nievas, V.F.; Leotta, G.A.; Vigo, G.B. Subcutaneous clostridial infection in Adelie penguins in Hope Bay, Antarctica. *Polar Biol.* **2007**, *30*, 249–252. [[CrossRef](#)]
10. Mellish, J.; Tuomi, P.; Hindle, A.; Jang, S.; Horning, M. Skin microbial flora and effectiveness of aseptic technique for deep muscle biopsies in Weddell seals (*Leptonychotes weddellii*) in McMurdo Sound, Antarctica. *J. Wildlife Dis.* **2010**, *46*, 655–658. [[CrossRef](#)]
11. Bannoehr, J.; Ben Zakour, N.L.; Waller, A.S.; Guardabassi, L.; Thoday, K.L.; van den Broek, A.H.M.; Fitzgerald, J.R. Population genetic structure of the *Staphylococcus intermedius* group: Insights into agr diversification and the emergence of methicillin-resistant strains. *J. Bacteriol.* **2007**, *189*, 8685–8692. [[CrossRef](#)]
12. Sasaki, T.; Kikuchi, K.; Tanaka, Y.; Takahashi, N.; Kamata, S.; Hiramatsu, K. Reclassification of phenotypically identified *Staphylococcus intermedius* strains. *J. Clin. Microbiol.* **2007**, *45*, 2770–2778. [[CrossRef](#)] [[PubMed](#)]
13. Hájek, V. *Staphylococcus intermedius*, a new species isolated from animals. *Int. J. Syst. Bacteriol.* **1976**, *26*, 401–408. [[CrossRef](#)]
14. Devriese, L.A.; Vancanneyt, M.; Baele, M.; Vanechoutte, M.; De Graef, E.; Snauwaert, C.; Cleenwerck, I.; Dawyndt, P.; Swings, J.; Decostere, A.; et al. *Staphylococcus pseudintermedius* sp. nov., a coagulase-positive species from animals. *Int. J. Syst. Evol. Microbiol.* **2005**, *55*, 1569–1573. [[CrossRef](#)] [[PubMed](#)]
15. Varaldo, P.E.; Kilpperbalz, R.; Biavasco, F.; Satta, G.; Schleifer, K.H. *Staphylococcus delphini* sp. nov., a coagulase-positive species isolated from dolphins. *Int. J. Syst. Bacteriol.* **1988**, *38*, 436–439. [[CrossRef](#)]
16. Murray, A.K.; Lee, J.; Bendall, R.; Zhang, L.; Sunde, M.; Schau Slettemeas, J.; Gaze, W.; Page, A.J.; Vos, M. *Staphylococcus cornubiensis* sp. nov., a member of the *Staphylococcus intermedius* group (SIG). *Int. J. Syst. Evol. Microbiol.* **2018**, *68*, 3404–3408. [[CrossRef](#)]
17. Bond, R.; Loeffler, A. What's happened to *Staphylococcus intermedius*? Taxonomic revision and emergence of multi-drug resistance. *J. Small Anim. Pr.* **2012**, *53*, 147–154. [[CrossRef](#)]
18. Viau, R.; Hujer, A.M.; Hujer, K.M.; Bonomo, R.A.; Jump, R.L.P. Are *Staphylococcus intermedius* infections in humans cases of mistaken identity? A case series and literature review. *Open Forum Infect. Dis.* **2015**, *2*, ofv110. [[CrossRef](#)]
19. Decristophoris, P.; Fasola, A.; Benagli, C.; Tonolla, M.; Petrini, O. Identification of *Staphylococcus intermedius* group by MALDI-TOF MS. *Syst. Appl. Microbiol.* **2011**, *34*, 45–51. [[CrossRef](#)]
20. Murugaiyan, J.; Walther, B.; Stamm, I.; Abou-Elnaga, Y.; Brueggemann-Schwarze, S.; Vincze, S.; Wieler, L.H.; Lubke-Becker, A.; Semmler, T.; Roesler, U. Species differentiation within the *Staphylococcus intermedius* group using a refined MALDI-TOF MS database. *Clin. Microbiol. Infect.* **2014**, *20*, 1007–1014. [[CrossRef](#)]
21. Sasaki, T.; Tsubakishita, S.; Tanaka, Y.; Sakusabe, A.; Ohtsuka, M.; Hirotsuki, S.; Kawakami, T.; Fukata, T.; Hiramatsu, K. Multiplex-PCR method for species identification of coagulase-positive staphylococci. *J. Clin. Microbiol.* **2010**, *48*, 765–769. [[CrossRef](#)]
22. Lee, J.; Murray, A.; Bendall, R.; Gaze, W.; Zhang, L.H.; Vos, M. Improved detection of *Staphylococcus intermedius* group in a routine diagnostic laboratory. *J. Clin. Microbiol.* **2015**, *53*, 961–963. [[CrossRef](#)] [[PubMed](#)]
23. Slettemeas, J.S.; Mikalsen, J.; Sunde, M. Further diversity of the *Staphylococcus intermedius* group and heterogeneity in the *MboI* restriction site used for *Staphylococcus pseudintermedius* species identification. *J. Vet. Diagn. Invest.* **2010**, *22*, 756–759. [[CrossRef](#)] [[PubMed](#)]
24. Solyman, S.M.; Black, C.C.; Duim, B.; Perreten, V.; van Duijkeren, E.; Wagenaar, J.A.; Eberlein, L.C.; Sadeghi, L.N.; Videla, R.; Bemis, D.A.; et al. Multilocus sequence typing for characterization of *Staphylococcus pseudintermedius*. *J. Clin. Microbiol.* **2013**, *51*, 306–310. [[CrossRef](#)] [[PubMed](#)]
25. Ben Zakour, N.L.; Beatson, S.A.; van den Broek, A.H.M.; Thoday, K.L.; Fitzgerald, J.R. Comparative genomics of the *Staphylococcus intermedius* group of animal pathogens. *Front. Cell. Infect. Microbiol.* **2012**, *2*, 44. [[CrossRef](#)] [[PubMed](#)]
26. Verstappen, K.M.; Huijbregts, L.; Spaninks, M.; Wagenaar, J.A.; Fluit, A.C.; Duim, B. Development of a real-time PCR for detection of *Staphylococcus pseudintermedius* using a novel automated comparison of whole-genome sequences. *PLoS ONE* **2017**, *12*, e0189520. [[CrossRef](#)]

27. Bannoehr, J.; Franco, A.; Iurescia, M.; Battisti, A.; Fitzgerald, J.R. Molecular diagnostic identification of *Staphylococcus pseudintermedius*. *J. Clin. Microbiol.* **2009**, *47*, 469–471. [\[CrossRef\]](#) [\[PubMed\]](#)
28. Borjesson, S.; Gomez-Sanz, E.; Ekstrom, K.; Torres, C.; Gronlund, U. *Staphylococcus pseudintermedius* can be misdiagnosed as *Staphylococcus aureus* in humans with dog bite wounds. *Eur. J. Clin. Microbiol.* **2015**, *34*, 839–844. [\[CrossRef\]](#)
29. Lainhart, W.; Yarbrough, M.L.; Burnham, C.A. The brief case: *Staphylococcus intermedius* group—look what the dog dragged in. *J. Clin. Microbiol.* **2018**, *56*, e00839-17. [\[CrossRef\]](#)
30. Hanselman, B.A.; Kruth, S.A.; Rousseau, J.; Weese, J.S. Coagulase positive staphylococcal colonization of humans and their household pets. *Can. Vet. J.* **2009**, *50*, 954–958.
31. Ishihara, K.; Shimokubo, N.; Sakagami, A.; Ueno, H.; Muramatsu, Y.; Kadosawa, T.; Yanagisawa, C.; Hanaki, H.; Nakajima, C.; Suzuki, Y.; et al. Occurrence and molecular characteristics of methicillin-resistant *Staphylococcus aureus* and methicillin-resistant *Staphylococcus pseudintermedius* in an academic veterinary hospital. *Appl. Env. Microbiol.* **2010**, *76*, 5165–5174. [\[CrossRef\]](#)
32. Sasaki, T.; Kikuchi, K.; Tanaka, Y.; Takahashi, N.; Kamata, S.; Hiramatsu, K. Methicillin-resistant *Staphylococcus pseudintermedius* in a veterinary teaching hospital. *J. Clin. Microbiol.* **2007**, *45*, 1118–1125. [\[CrossRef\]](#)
33. Soedarmanto, I.; Kanbar, T.; Ulbegi-Mohyla, H.; Hijazin, M.; Alber, J.; Lammler, C.; Akineden, O.; Weiss, R.; Moritz, A.; Zschock, M. Genetic relatedness of methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) isolated from a dog and the dog owner. *Res. Vet. Sci.* **2011**, *91*, E25–E27. [\[CrossRef\]](#) [\[PubMed\]](#)
34. Van Hoovels, L.; Vankeerberghen, A.; Boel, A.; Van Vaerenbergh, K.; De Beenhouwer, H. First case of *Staphylococcus pseudintermedius* infection in a human. *J. Clin. Microbiol.* **2006**, *44*, 4609–4612. [\[CrossRef\]](#) [\[PubMed\]](#)
35. Guardabassi, L.; Schmidt, K.R.; Petersen, T.S.; Espinosa-Gongora, C.; Moodley, A.; Agerso, Y.; Olsen, J.E. *Mustelidae* are natural hosts of *Staphylococcus delphini* group A. *Vet. Microbiol.* **2012**, *159*, 351–353. [\[CrossRef\]](#) [\[PubMed\]](#)
36. Magleby, R.; Bemis, D.A.; Kim, D.; Carroll, K.C.; Castanheira, M.; Kania, S.A.; Jenkins, S.G.; Westblade, L.F. First reported human isolation of *Staphylococcus delphini*. *Diagn. Microbiol. Infect. Dis.* **2019**, *94*, 274–276. [\[CrossRef\]](#)
37. Nikolaisen, N.K.; Lassen, D.C.K.; Chriel, M.; Larsen, G.; Jensen, V.F.; Pedersen, K. Antimicrobial resistance among pathogenic bacteria from mink (*Neovison vison*) in Denmark. *Acta. Vet. Scand.* **2017**, *59*, 60. [\[CrossRef\]](#)
38. Novakova, D.; Sedlacek, I.; Pantucek, R.; Stetina, V.; Svec, P.; Petras, P. *Staphylococcus equorum* and *Staphylococcus succinus* isolated from human clinical specimens. *J. Med. Microbiol.* **2006**, *55*, 523–528. [\[CrossRef\]](#)
39. Yoon, S.H.; Ha, S.M.; Kwon, S.; Lim, J.; Kim, Y.; Seo, H.; Chun, J. Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. *Int. J. Syst. Evol. Microbiol.* **2017**, *67*, 1613–1617. [\[CrossRef\]](#)
40. Mellmann, A.; Becker, K.; von Eiff, C.; Keckevoet, U.; Schumann, P.; Harmsen, D. Sequencing and staphylococci identification. *Emerg. Infect. Dis.* **2006**, *12*, 333–336. [\[CrossRef\]](#)
41. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549. [\[CrossRef\]](#)
42. Švec, P.; Pantůček, R.; Petráš, P.; Sedláček, I.; Nováková, D. Identification of *Staphylococcus* spp. using (GTG)₅-PCR fingerprinting. *Syst. Appl. Microbiol.* **2010**, *33*, 451–456. [\[CrossRef\]](#)
43. Freiwald, A.; Sauer, S. Phylogenetic classification and identification of bacteria by mass spectrometry. *Nat. Protoc.* **2009**, *4*, 732–742. [\[CrossRef\]](#) [\[PubMed\]](#)
44. Wingett, S.W.; Andrews, S. FastQ Screen: A tool for multi-genome mapping and quality control. *F1000 Research* **2018**, *7*, 1338. [\[CrossRef\]](#) [\[PubMed\]](#)
45. Nurk, S.; Bankevich, A.; Antipov, D.; Gurevich, A.A.; Korobeynikov, A.; Lapidus, A.; Prjibelski, A.D.; Pyshkin, A.; Sirotkin, A.; Sirotkin, Y.; et al. Assembling single-cell genomes and mini-metagenomes from chimeric MDA products. *J. Comput. Biol.* **2013**, *20*, 714–737. [\[CrossRef\]](#) [\[PubMed\]](#)
46. Bushnell, B. *BBMap Short-Read Aligner, and Other Bioinformatics Tools*; Lawrence Berkeley National Lab.: Berkeley, CA, USA; Available online: <http://sourceforge.net/projects/bbmap/> (accessed on 1 March 2019).
47. Darling, A.E.; Mau, B.; Perna, N.T. progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. *PLoS ONE* **2010**, *5*, e11147. [\[CrossRef\]](#)
48. Seemann, T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* **2014**, *30*, 2068–2069. [\[CrossRef\]](#)

49. Yu, N.Y.; Wagner, J.R.; Laird, M.R.; Melli, G.; Rey, S.; Lo, R.; Dao, P.; Sahinalp, S.C.; Ester, M.; Foster, L.J.; et al. PSORTb 3.0: Improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. *Bioinformatics* **2010**, *26*, 1608–1615. [\[CrossRef\]](#)
50. Yu, C.S.; Cheng, C.W.; Su, W.C.; Chang, K.C.; Huang, S.W.; Hwang, J.K.; Lu, C.H. CELLO2GO: A web server for protein subCELLular LOcalization prediction with functional gene ontology annotation. *PLoS ONE* **2014**, *9*, e99368. [\[CrossRef\]](#)
51. Liu, B.; Zheng, D.; Jin, Q.; Chen, L.; Yang, J. VFDB 2019: A comparative pathogenomic platform with an interactive web interface. *Nucleic Acids Res.* **2019**, *47*, D687–D692. [\[CrossRef\]](#)
52. Xu, L.; Dong, Z.; Fang, L.; Luo, Y.; Wei, Z.; Guo, H.; Zhang, G.; Gu, Y.Q.; Coleman-Derr, D.; Xia, Q.; et al. OrthoVenn2: A web server for whole-genome comparison and annotation of orthologous clusters across multiple species. *Nucleic Acids Res.* **2019**, *47*, W52–W58. [\[CrossRef\]](#)
53. Arndt, D.; Grant, J.R.; Marcu, A.; Sajed, T.; Pon, A.; Liang, Y.; Wishart, D.S. PHASTER: A better, faster version of the PHAST phage search tool. *Nucleic Acids Res.* **2016**, *44*, W16–W21. [\[CrossRef\]](#)
54. Couvin, D.; Bernheim, A.; Toffano-Nioche, C.; Touchon, M.; Michalik, J.; Néron, B.; Rocha, E.P.C.; Vergnaud, G.; Gautheret, D.; Pourcel, C. CRISPRCasFinder, an update of CRISPRFinder, includes a portable version, enhanced performance and integrates search for Cas proteins. *Nucleic Acids Res.* **2018**, *46*, W246–W251. [\[CrossRef\]](#) [\[PubMed\]](#)
55. Petkau, A.; Stuart-Edwards, M.; Stothard, P.; Van Domselaar, G. Interactive microbial genome visualization with GView. *Bioinformatics* **2010**, *26*, 3125–3126. [\[CrossRef\]](#) [\[PubMed\]](#)
56. Okonechnikov, K.; Golosova, O.; Fursov, M. Unipro UGENE: A unified bioinformatics toolkit. *Bioinformatics* **2012**, *28*, 1166–1167. [\[CrossRef\]](#) [\[PubMed\]](#)
57. Sullivan, M.J.; Petty, N.K.; Beatson, S.A. Easyfig: A genome comparison visualizer. *Bioinformatics* **2011**, *27*, 1009–1010. [\[CrossRef\]](#)
58. Yoon, S.H.; Ha, S.M.; Lim, J.; Kwon, S.; Chun, J. A large-scale evaluation of algorithms to calculate average nucleotide identity. *Antonie van Leeuwenhoek* **2017**, *110*, 1281–1286. [\[CrossRef\]](#)
59. Na, S.I.; Kim, Y.O.; Yoon, S.H.; Ha, S.M.; Baek, I.; Chun, J. UBCG: Up-to-date bacterial core gene set and pipeline for phylogenomic tree reconstruction. *J. Microbiol.* **2018**, *56*, 280–285. [\[CrossRef\]](#)
60. Cole, K.; Foster, D.; Russell, J.E.; Golubchik, T.; Llewellyn, M.; Wilson, D.J.; Crook, D.; Paul, J.; Modernising Medical Microbiology Consortium. Draft genome sequences of 64 type strains of 50 species and 25 subspecies of the genus *Staphylococcus* Rosenbach 1884. *Microbiol. Resour. Announc.* **2019**, *8*, e00062-19. [\[CrossRef\]](#)
61. Stepan, J.; Pantucek, R.; Doskar, J. Molecular diagnostics of clinically important staphylococci. *Folia Microbiol.* **2004**, *49*, 353–386. [\[CrossRef\]](#)
62. Richter, M.; Rossello-Mora, R. Shifting the genomic gold standard for the prokaryotic species definition. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 19126–19131. [\[CrossRef\]](#)
63. Becker, K.; Harmsen, D.; Mellmann, A.; Meier, C.; Schumann, P.; Peters, G.; von Eiff, C. Development and evaluation of a quality-controlled ribosomal sequence database for 16S ribosomal DNA-based identification of *Staphylococcus* species. *J. Clin. Microbiol.* **2004**, *42*, 4988–4995. [\[CrossRef\]](#)
64. Ah Tow, L.; Cowan, D.A. Dissemination and survival of non-indigenous bacterial genomes in pristine Antarctic environments. *Extremophiles* **2005**, *9*, 385–389. [\[CrossRef\]](#) [\[PubMed\]](#)
65. van Elk, C.E.; Boelens, H.A.M.; van Belkum, A.; Foster, G.; Kuiken, T. Indications for both host-specific and introduced genotypes of *Staphylococcus aureus* in marine mammals. *Vet. Microbiol.* **2012**, *156*, 343–346. [\[CrossRef\]](#) [\[PubMed\]](#)
66. Wayne, R.K. Molecular evolution of the dog family. *Trends Genet.* **1993**, *9*, 218–224. [\[CrossRef\]](#)
67. Aarestrup, F.M. Comparative ribotyping of *Staphylococcus intermedius* isolated from members of the *Canoidea* gives possible evidence for host-specificity and co-evolution of bacteria and hosts. *Int. J. Syst. Evol. Microbiol.* **2001**, *51*, 1343–1347. [\[CrossRef\]](#) [\[PubMed\]](#)
68. Pantůček, R.; Sedláček, I.; Petráš, P.; Koukalová, D.; Švec, P.; Štětina, V.; Vancanneyt, M.; Chrástínová, L.; Vokurková, J.; Růžicková, V.; et al. *Staphylococcus simiae* sp. nov., isolated from South American squirrel monkeys. *Int. J. Syst. Evol. Microbiol.* **2005**, *55*, 1953–1958. [\[CrossRef\]](#) [\[PubMed\]](#)
69. Suzuki, H.; Lefebvre, T.; Bitar, P.P.; Stanhope, M.J. Comparative genomic analysis of the genus *Staphylococcus* including *Staphylococcus aureus* and its newly described sister species *Staphylococcus simiae*. *BMC Genom.* **2012**, *13*, 3. [\[CrossRef\]](#)

70. Tong, S.Y.; Schaumburg, F.; Ellington, M.J.; Corander, J.; Pichon, B.; Leendertz, F.; Bentley, S.D.; Parkhill, J.; Holt, D.C.; Peters, G.; et al. Novel staphylococcal species that form part of a *Staphylococcus aureus*-related complex: the non-pigmented *Staphylococcus argenteus* sp. nov. and the non-human primate-associated *Staphylococcus schweitzeri* sp. nov. *Int. J. Syst. Evol. Microbiol.* **2015**, *65*, 15–22. [\[CrossRef\]](#)
71. Fitzgerald, J.R. The *Staphylococcus intermedius* group of bacterial pathogens: species re-classification, pathogenesis and the emergence of methicillin resistance. *Vet. Derm.* **2009**, *20*, 490–495. [\[CrossRef\]](#)
72. Canver, M.C.; Tekle, T.; Compton, S.T.; Callan, K.; Burd, E.M.; Zimmer, B.L.; Bemis, D.A.; Carroll, K.C.; Westblade, L.F. Performance of five commercial identification platforms for identification of *Staphylococcus delphini*. *J. Clin. Microbiol.* **2019**, *57*, e00721–19. [\[CrossRef\]](#)
73. Chua, K.Y.; Stinear, T.P.; Howden, B.P. Functional genomics of *Staphylococcus aureus*. *Brief Funct. Genom.* **2013**, *12*, 305–315. [\[CrossRef\]](#)
74. Ingmer, H.; Gerlach, D.; Wolz, C. Temperate phages of *Staphylococcus aureus*. *Microbiol. Spectr.* **2019**, *7*. [\[CrossRef\]](#)
75. Maslanova, I.; Stribna, S.; Doskar, J.; Pantucek, R. Efficient plasmid transduction to *Staphylococcus aureus* strains insensitive to the lytic action of transducing phage. *FEMS Microbiol. Lett.* **2016**, *363*, fnw211. [\[CrossRef\]](#) [\[PubMed\]](#)
76. Pires Dos Santos, T.; Damborg, P.; Moodley, A.; Guardabassi, L. Systematic review on global epidemiology of methicillin-resistant *Staphylococcus pseudintermedius*: Inference of population structure from multilocus sequence typing data. *Front. Microbiol.* **2016**, *7*, 1599. [\[CrossRef\]](#) [\[PubMed\]](#)
77. Novick, R.P.; Christie, G.E.; Penades, J.R. The phage-related chromosomal islands of Gram-positive bacteria. *Nat. Rev. Microbiol.* **2010**, *8*, 541–551. [\[CrossRef\]](#) [\[PubMed\]](#)
78. Fillol-Salom, A.; Martinez-Rubio, R.; Abdulrahman, R.F.; Chen, J.; Davies, R.; Penades, J.R. Phage-inducible chromosomal islands are ubiquitous within the bacterial universe. *ISME J.* **2018**, *12*, 2114–2128. [\[CrossRef\]](#)
79. Becker, K.; Keller, B.; von Eiff, C.; Bruck, M.; Lubritz, G.; Etienne, J.; Peters, G. Enterotoxigenic potential of *Staphylococcus intermedius*. *Appl. Env. Microbiol.* **2001**, *67*, 5551–5557. [\[CrossRef\]](#)
80. Hendricks, A.; Schuberth, H.-J.; Schueler, K.; Lloyd, D.H. Frequency of superantigen-producing *Staphylococcus intermedius* isolates from canine pyoderma and proliferation-inducing potential of superantigens in dogs. *Res. Vet. Sci.* **2002**, *73*, 273–277. [\[CrossRef\]](#)
81. Iyori, K.; Hisatsune, J.; Kawakami, T.; Shibata, S.; Murayama, N.; Ide, K.; Nagata, M.; Fukata, T.; Iwasaki, T.; Oshima, K.; et al. Identification of a novel *Staphylococcus pseudintermedius* exfoliative toxin gene and its prevalence in isolates from canines with pyoderma and healthy dogs. *FEMS Microbiol. Lett.* **2010**, *312*, 169–175. [\[CrossRef\]](#)
82. Futagawa-Saito, K.; Makino, S.; Sunaga, F.; Kato, Y.; Sakurai-Komada, N.; Ba-Thein, W.; Fukuyasu, T. Identification of first exfoliative toxin in *Staphylococcus pseudintermedius*. *FEMS Microbiol. Lett.* **2009**, *301*, 176–180. [\[CrossRef\]](#)
83. Nishifuji, K.; Sugai, M.; Amagai, M. Staphylococcal exfoliative toxins: “Molecular scissors” of bacteria that attack the cutaneous defense barrier in mammals. *J. Derm. Sci.* **2008**, *49*, 21–31. [\[CrossRef\]](#)



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4.5 *Staphylococcus ratti* sp. nov. Isolated from a Lab Rat

Background and objectives: *Staphylococcus hyicus*, *S. agnetis*, and *Staphylococcus chromogenes* form a clade within the Hyicus-Intermedius group and are important pathogens of livestock and poultry and can be transmitted to animal keepers. The dominant *Staphylococcus* species in lab rats are *S. xylosus*, *S. aureus*, and *S. cohnii*. It is currently unknown which species colonize the wild rat. In 2003, a strain was isolated from a nostril swab of a healthy rat prior to a biological experiment at the Unit for Biomedicine and Welfare of Laboratory Animals in Prague, Czech Republic. Although assigned to the Hyicus-Intermedius group, the strain's partial *rpoB* sequence and MALDI-TOF MS profile differed from known species. Thus, further investigation of the strain was conducted, focusing on detailed chemotaxonomic and genomic characterization.

Material and methods: A polyphasic approach was used to describe strain NRL/St 03/464^T (= CCM 9025^T = LMG 31873^T = DSM 111348^T). The complete genome was assembled using a combination of Illumina Nextseq and ONT Minion sequences.

Results and discussion: The NRL/St 03/464^T strain belongs to a distinct branch of the Hyicus-Agnetis complex, with *S. chromogenes* being its closest relative. However, its genome identity was well below the species threshold. The strain was also reliably distinguished by chemotaxonomic and pangenome profiles. Whole genome sequencing and annotation identified several novel mobile genetic elements, which may provide selective advantages, enhancing their survival and adaptability. The presence of phage inducible genomic island, SrRI_{CCM9025}, is particularly interesting. It harbors virulence factor *virE* and composite transposon derived from the IS3 family. Both elements contain several genes associated with modulating antibiotic resistance. We hypothesize that these genes are responsible for beta-lactamase resistance as the strain is resistant to penicillin. Other mobile genetic elements include a prophage and two small plasmids designated as pSR9025A, which contains a stress response gene *gsiB*, and pSR9025B, whose function is currently unknown. A similar plasmid, pLNU9, was found in *S. chromogenes*, suggesting the possibility of horizontal gene flow.

Conclusion: *Staphylococcus ratti*, sp. nov. was described based on a single isolate from a laboratory rats. Genome analysis has revealed the presence of new genomic islands. Further study is needed to verify their suspected role in penicillin resistance.

Significance: The study emphasizes the importance of exploring new staphylococcal species in animals and investigating new antibiotic resistance mechanisms. This is crucial in species with zoonotic potential or act as gene reservoirs for emerging pathogens.

Article

Staphylococcus ratti sp. nov. Isolated from a Lab Rat

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Abstract: Staphylococci from the *Staphylococcus intermedius-Staphylococcus hyicus* species group include numerous animal pathogens and are an important reservoir of virulence and antimicrobial resistance determinants. Due to their pathogenic potential, they are possible causative agents of zoonoses in humans; therefore, it is important to address the properties of these strains. Here we used a polyphasic taxonomic approach to characterize the coagulase-negative staphylococcal strain NRL/St 03/464^T, isolated from the nostrils of a healthy laboratory rat during a microbiological screening of laboratory animals. The 16S rRNA sequence, MALDI-TOF mass spectrometry and positive urea hydrolysis and beta-glucuronidase tests clearly distinguished it from closely related *Staphylococcus* spp. All analyses have consistently shown that the closest relative is *Staphylococcus chromogenes*; however, values of digital DNA-DNA hybridization <35.3% and an average nucleotide identity <81.4% confirmed that the analyzed strain is a distinct *Staphylococcus* species. Whole-genome sequencing and expert annotation of the genome revealed the presence of novel variable genetic elements, including two plasmids named pSR9025A and pSR9025B, prophages, genomic islands and a composite transposon that may confer selective advantages to other bacteria and enhance their survival. Based on phenotypic, phylogenetic and genomic data obtained in this study, the strain NRL/St 03/464^T (= CCM 9025^T = LMG 31873^T = DSM 111348^T) represents a novel species with the suggested name *Staphylococcus ratti* sp. nov.

Keywords: laboratory rat; *Staphylococcus*; Hyicus-Intermedius species group; taxonomy; whole genome sequencing; variable genetic element; genomic island



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1. Introduction

Staphylococci are opportunistic pathogens widespread in nature; they are mainly isolated from the skin, skin glands, and mucous membranes of various animals [1], and less often from the blood of diseased animals [2]. The nasal cavity of many mammalian species is inhabited by distinctive staphylococcal species. Studies of *Staphylococcus* spp. distribution in wild rodents have shown the presence of predominantly coagulase-negative staphylococci, including *Staphylococcus xylosus*, *Staphylococcus equorum*, *Staphylococcus succinus*, *Staphylococcus saprophyticus* and *Mammaliicoccus* spp. [3,4]. The species composition of staphylococcal populations in wild and laboratory rats is largely unknown. The predominant species in laboratory rats is *S. xylosus*, followed by *Staphylococcus aureus* and

Staphylococcus cohnii [5]. Recent studies have shown that rats are reservoir of various *S. aureus* clonal lineages, including methicillin-resistant strains [6]. On the other hand, murine *S. aureus* isolates exhibit host adaptation [7]. Both mice and rats are an animal infection model for studying *S. aureus* pathogenicity [8,9]. In wound infections, rats eliminated bacteria faster and more rapidly organized the inflammatory response than other rodents [10].

Similarly to the main pathogen *S. aureus*, some species in the *Staphylococcus intermedius-Staphylococcus hyicus* phylogenetic complex can also catalyze the polymerization of fibrinogen to fibrin through the enzyme coagulase, thereby contributing to the appearance of purulent foci—abscesses [11]. Besides coagulase, they also have other virulence factors, such as staphylococcal β -hemolysin (sphingomyelinase), a typical product of the species *Staphylococcus pseudintermedius* [12]. Staphylococcal species belonging to this complex are opportunistic pathogens of various animal species. *S. pseudintermedius*, *Staphylococcus coagulans*, and *Staphylococcus canis* occur as carriers in dogs and other carnivores [13–16], and when skin integrity is disrupted, they can cause skin and soft tissue infections or external ear otitis. *Staphylococcus ursi* was isolated from healthy black bears [17]. *Staphylococcus felis* is a common cat commensal and a potential urinary pathogen [18]. However, they can also be zoonotic pathogens, causing skin problems in humans; a typical example is a septic wound after a dog bite or cat scratch [19,20].

Staphylococcus hyicus forms a separate phylogenetic clade together with *Staphylococcus agnetis* and *Staphylococcus chromogenes*. *S. hyicus* causes exudative epidermitis in pigs, known as greasy pig disease, due to the expression of exfoliative toxins that selectively digest porcine desmoglein 1 [21]. Zoonotic infections of the bloodstream have been reported to be caused by *S. hyicus*, as has spondylodiscitis in animal keepers [22,23]. *S. agnetis* was primarily isolated from cows with mastitis [24], but recent findings demonstrate that *S. agnetis* may be associated with diseases and mortality in broiler chickens [25,26]. *S. chromogenes* is globally the most common cause of intramammary infections of dairy cows [27] and has also been reported in both healthy and diseased pigs [28], goats [29] and poultry [5]. There are also reports of nasal carriers of *S. chromogenes* among farm workers [30].

The prevalence of human infections caused by species of the *S. intermedius/S. hyicus* complex is low; however, due to their phenotypic similarity, the capture of some species may be underestimated in studies that did not use molecular techniques. In this work we provide a polyphasic characterization of an isolate of *Staphylococcus* sp. NRL/St 03/464^T that was cultured during a microbiological examination of nasal swabs from healthy rats before a biological experiment at the Unit for Biomedicine and Welfare of Laboratory Animals, National Institute of Public Health (Prague, Czech Republic).

2. Results and Discussion

2.1. Phylogenetic Analyses

The isolate CCM 9025^T (= NRL/St 03/464^T) was assigned by a partial sequencing of the 16S rRNA gene to the *Staphylococcus* genus and previously defined Hyicus-Intermedius species group [31]; however, its biochemical profile, MALDI-TOF MS pattern and partial RNA polymerase subunit beta (*rpoB*) gene sequence did not allow for its classification into any known staphylococcal species. Therefore, a polyphasic taxonomic study was conducted which was focused on a detailed characterization of a new *Staphylococcus* species.

The obtained complete 16S rRNA gene sequence of strain CCM 9025^T was compared to those of other taxa from the Hyicus-Intermedius species group of the *Staphylococcus* genus. The closest relatives were *S. chromogenes* (99.3% similarity), *S. agnetis* (99.2%) and *S. hyicus* (99.1%); other species were below 98% similarity. The topology of the maximum likelihood phylogenetic tree constructed with 16S rRNA gene sequences was similar to that of the neighbor-joining tree (Figure 1a).

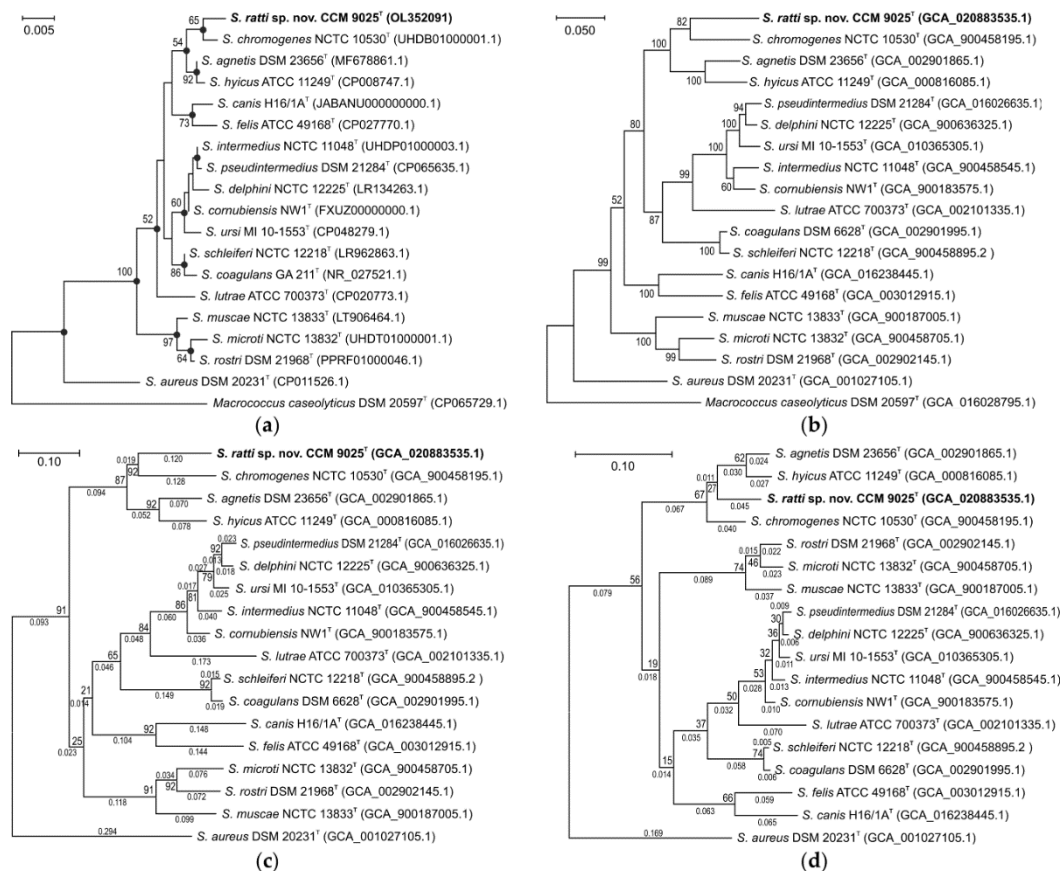


Figure 1. Evolutionary analyses of *S. hyicus*-*S. intermedius* phylogenetic complex including *S. ratti* sp. nov. (a) Unrooted phylogenetic tree based on complete 16S rRNA gene sequences extracted from whole-genomic sequencing data (GenBank accession numbers are in parentheses). The evolutionary history was inferred by using the maximum likelihood method and Tamura–Nei model. Filled circles indicate that the corresponding nodes were also obtained in the tree constructed by the neighbor-joining method. The percentage of 500 tree replications above 50% in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 1551 positions in the final dataset. (b) Unrooted maximum likelihood tree based on multilocus sequence analysis of concatenated nucleotide sequences from six loci, *rpoB*, *hsp60*, *dnaJ*, *tufA*, *gap* and *sodA*, extracted from whole genome assemblies (accession numbers are in parentheses). There were a total of 3972 positions in the final dataset. Bootstrap probability values (percentages of 500 tree replications) greater than 50% are shown at branch points. The evolutionary distances are given as the number of substitutions per site. (c) Nucleotide core gene set phylogenetic tree of *S. ratti* sp. nov. and phylogenetically related species. (d) Protein sequence-based phylogenetic tree of the core gene set of *S. ratti* sp. nov. and phylogenetically related species. Trees (c) and (d) were constructed using up-to-date bacterial core gene set (UBCG; concatenated alignment of 92 core genes). The maximum likelihood tree was inferred using RAxML software and set to 100 replicates. Gene support indices are given at branching points (maximal possible value is 92). Bar, 0.1 substitution per position.

Because the 16S rRNA analysis has limited discriminatory power for the identification of some staphylococcal species, the phylogenetic position of the new isolates was also assessed using the concatenated multilocus sequence data of six routinely used housekeeping genes (Figure 1b). The maximum likelihood phylogenetic trees for the housekeeping genes including the closest relatives had a very similar topology which corresponded to that of the 16S rRNA gene tree, clearly separated the novel isolate CCM 9025^T from the established species and which confirmed *S. chromogenes* as the closest relative. The significant phylogenetic distance from the related staphylococcal species at the whole-genome level, with an average nucleotide identity (ANI) of <81.4% and digital DNA-DNA hybridization (dDDH) of <35.3%, were below the species delineation thresholds, which are 95–96% and 70%, respectively [32]. This confirmed that strain CCM 9025^T represents a distinct *Staphylococcus* species named *Staphylococcus ratti* sp. nov. It forms a separate branch in the *S. hyicus* phylogenetic clade, as also shown by protein coding core genome analysis using the up-to-date bacterial core gene (UBCG) (Figure 1c,d).

2.2. Phenotypic, Genotypic and Chemotaxonomic Characteristics

Cells of strain CCM 9025^T are irregular spherical cocci with diameter 705 ± 55 nm ($n = 100$) (Figure 2). The strain grew well on common media for staphylococci and was mesophilic and moderately halophilic, with the ability to hydrolyze biomacromolecules (gelatin, DNA). Carbohydrates seldom served as the source of carbon. The detailed phenotype data are subsequently mentioned in the species description in the text; here we only specify several notable results. Interestingly, the hyaluronidase test result was positive for CCM 9025^T, which is a rare feature for coagulase-negative staphylococcal species. During biotyping, a few contradictory results depending on the tested conditions were found. Firstly, the Voges–Proskauer test (acetoin) was negative in a standard tube test, but positive with a commercial VPtest strip containing pyruvic acid instead of glucose. The second inconsistency was related to an enzymatic β -glucuronidase test included in the commercial kits, when β -glucuronidase was positive for CCM 9025^T in STAPHYtest 24, but negative in API ZYM, probably due to different substrates being used for enzyme detection.

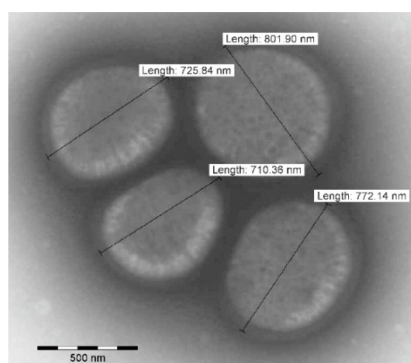


Figure 2. Transmission electron microscopy image of type strain *Staphylococcus ratti* sp. nov. CCM 9025^T negatively stained with ammonium molybdate. Bar represents 500 nm (original magnification $\times 10,000$).

The tests distinguishing novel species from closely related staphylococci of the *S. hyicus* group are shown in Table 1, and these tests enable the correct identification of CCM 9025^T at the species level.

Profiling analysis by matrix-assisted laser desorption/ionization—time-of-flight mass spectrometry (MALDI-TOF MS) is routinely used for bacterial identification. A distinct MALDI-TOF MS pattern is therefore a useful phenotypic feature in describing new species. Strain CCM 9025^T generated a consistent MALDI-TOF MS profile containing signals in

the mass range of 2–10 kDa, which was not related to any of those *Staphylococcus* species already represented in the commercial database at the time of testing. After the manual inclusion of profiles from 24 analyses of the strain CCM 9025^T to the in-house database, the strain was re-analyzed and matched the new pattern with a score of 2.6, whereas the next closest species was *S. schleiferi* with a score of 1.2, which is far below the species identification threshold.

Table 1. Phenotypic differentiation of *Staphylococcus ratti* sp. nov. from closely related *Staphylococcus* spp. type strains.

Feature	<i>S. ratti</i> sp. nov. CCM 9025 ^T	<i>S. agnetis</i> CCM 8869 ^T	<i>S. hyicus</i> CCM 2368 ^T	<i>S. chromogenes</i> CCM 3387 ^T
Pigment	white	white	white	orange
Growth at 15 °C	—	—	+	+
Growth in 15% NaCl	+	+	+	—
Hemolysis	+	—	—	—
Voges–Proskauer test ¹	—	+	—	—
Acid from trehalose	+	—	+	+
Tween 80 hydrolysis	—	+	+	—
Alkaline phosphatase	+	—	+	+
β-glucuronidase ²	+	w	+	—
Urease	+	—	—	+

+, positive; w, weakly positive; —, negative; all data were taken from this study. ¹ Tube test; ² STAPHYtest 24 kit.

Staphylococci are easily differentiated from other Gram-positive cocci at the genus level by cellular fatty acid analysis, which is also one of the recommended tests at the species level. The cellular fatty acid profile of strain CCM 9025^T revealed 4 major fatty acids (FAs) (≥10%), iso-C_{15:0} (35.0%), anteiso-C_{15:0} (24.1%), iso-C_{17:0} (10.8%) and anteiso-C_{17:0} (10.2%), similar to other validly named *Staphylococcus* spp. [33]. Comparison to the closest related *Staphylococcus* spp. showed qualitatively similar profiles of FAs with quantitative differences between the compared type strains (Table 2). Qualitative differences in iso-C_{15:0}, iso-C_{17:0} and C_{20:0} clearly distinguish strain CCM 9025^T from *S. chromogenes* CCM 3387^T, and lower amounts of anteiso-C_{17:0} are specific for *S. agnetis* CCM 8869^T. *S. hyicus* CCM 2368^T has the most similar FA profile to CCM 9025^T, but can be distinguished by lower amounts of branched C_{17:0} FAs and a higher amount of iso-C_{15:0}.

Table 2. Cellular fatty acid composition (as a percentage of the total) of *S. ratti* sp. nov. CCM 9025^T, *S. chromogenes* CCM 3387^T, *S. agnetis* CCM 8869^T and *S. hyicus* CCM 2368^T. Values of less than 1% are not shown.

Fatty Acid	<i>S. ratti</i> sp. nov. CCM 9025 ^T	<i>S. chromogenes</i> CCM 3387 ^T	<i>S. agnetis</i> CCM 8869 ^T	<i>S. hyicus</i> CCM 2368 ^T
iso-C _{14:0}	TR	1.0	TR	TR
C _{14:0}	TR	TR	1.0	1.0
iso-C _{15:0}	35.0	16.5	43.9	33.7
anteiso-C _{15:0}	24.1	36.4	25.1	32.5
iso-C _{16:0}	1.5	1.5	TR	1.2
C _{16:0}	1.5	1.5	2.4	2.7
iso-C _{17:0}	10.8	6.0	9.2	7.4
anteiso-C _{17:0}	10.2	13.4	4.8	6.9
C _{18:0}	2.6	5.5	2.9	4.0
iso-C _{19:0}	4.4	3.2	2.1	2.1
anteiso-C _{19:0}	2.3	2.9	TR	1.0
C _{20:0}	5.8	10.4	6.1	6.2

TR, trace amounts < 1%. Cultivation on TSBA medium for 24 h at 37 °C.

The major respiratory quinone in strain CCM 9025^T was MK-7 (95.7%). Menaquinones MK-6 and MK-8 were also detected as minor components of the electron transport system. The identification of MK-7 as the major component of the quinone system is in accordance with the genus characteristics, as members of the genus *Staphylococcus* reveal the presence of unsaturated menaquinones, typically with six, seven or eight isoprene units [33,34]. Analysis of the peptidoglycan structure revealed a cross-linkage type A structure of A3α L-Lys-Gly₃₋₄ similar to the type A11.2 structure [35], with the molar amino acid ratio 2.0 Ala:3.4 Gly:1.0 Glu:0.8 Lys. The type A11.2 peptidoglycan structure was also identified

in the closest related species *S. chromogenes*, *S. agnetis* and *S. hyicus*, as described by Schumann [35]. Unlike CCM 9025^T and *S. agnetis*, *S. chromogenes* and *S. hyicus* were found to also contain minor amounts of serine, likely substituting for some glycine in the interpeptide bridge [24,33,36].

A DNA fingerprinting technique using repetitive sequence-based PCR (rep-PCR) fingerprinting with the (GTG)₅ primer, previously shown to be suitable for the simultaneous detection and differentiation of *Staphylococcus* spp., was used to demonstrate the difference of strain CCM 9025^T from related taxa. The rep-PCR clearly distinguished the analyzed strain from the type strains representing the phylogenetically close *Staphylococcus* spp. (Figure 3).

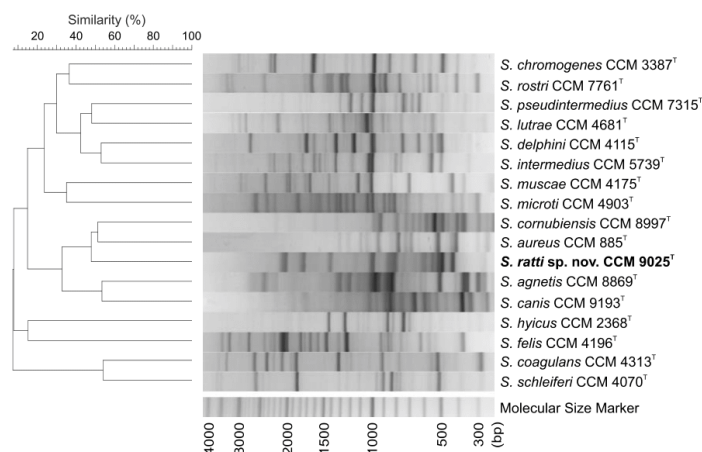


Figure 3. Distinct DNA banding patterns obtained in identification of *Staphylococcus ratti* sp. nov. and the type strains of related species based on repetitive PCR fingerprinting with (GTG)₅ primer. The dendrogram based on cluster analysis of rep-PCR fingerprints was calculated with Pearson's correlation coefficients with an unweighted pair group method with arithmetic average (UPGMA) clustering method (*r*, expressed as percentage similarity values).

2.3. Whole Genome Characterization of *Staphylococcus ratti* sp. nov.

The genome of *S. ratti* sp. nov. type strain CCM 9025^T was sequenced using Illumina and Oxford Nanopore platforms. The size of the complete chromosome assembly is 2.3 Mb with a mean coverage of 500-fold. Based on the NCBI automated annotation pipeline, a total of 2198 CDSs were identified in the genome, of which 2150 were protein-encoding genes. A total of 82 genes for RNAs were identified in the genome, including 59 tRNAs, 19 rRNAs including 7 (5S), 6 (16S) and 6 (23S), and 4 ncRNAs. Two plasmid sequences named pSR9025A (3311 bp) and pSR9025B (2455 bp) were assembled and annotated as separate extrachromosomal replicons. Plasmid pSR9025A encodes the gene for the Rep protein, which shares 100% amino acid identity with the Rep protein gene in p908 of *S. agnetis* [37] and a *gsiB* (glucose starvation-inducible protein B) gene homologue which is involved in a stress response. Further short similar regions were identified in the plasmids of many other coagulase-negative staphylococci. The plasmid pSR9025B is a cryptic plasmid similar (75.5% identity and 27% coverage) to the pLNU9 plasmid of *S. chromogenes* [38]. This is an indication of interspecies transfer of these types of variable genetic elements (VGEs).

Comparative genomic analysis of *S. ratti* with the two type strains *S. chromogenes* NCTC 10530^T and *S. hyicus* ATCC 11249^T revealed the presence of additional VGEs, including insertion sequence elements, a composite transposon, one prophage, and a genomic island (Figure 4).

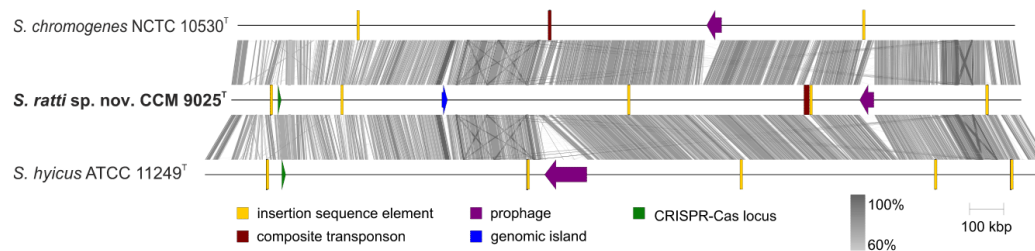


Figure 4. Whole-genome alignment of *Staphylococcus chromogenes* NCTC 10530^T (assembly accession no. GCA_900458195.1), *Staphylococcus ratti* sp. nov. CCM 9025^T (GCA_020883535.1) and *Staphylococcus hyicus* ATCC 11249^T (GCA_000816085.1) chromosomes. Blast map shows nucleotide sequence identity above 60%. The location of variable genetic elements is color coded as in the legend.

Type I restriction-modification (RM) system genes were found downstream of *orfX*, but no evidence of staphylococcal cassette chromosome (SCC) integration was found. Type II RM system genes are localized near a *cap* operon. The clustered, regularly interspaced, short, palindromic repeats (CRISPR)/CRISPR-associated gene (Cas) system was identified in the same location in both the *S. ratti* and *S. hyicus* genomes. The genes for predicted virulence factors, surface and extracellular proteins found in *S. ratti* CCM 9025^T genome are shown in Table 3.

Table 3. Candidate virulence factors predicted in *Staphylococcus ratti* sp. nov. Locus tags in the genome representing homologs with known and previously predicted virulence factors are shown.

Function and Role	Virulence Factors	Related Genes	Prediction in CCM 9025 ^T Genome
Adherence	Clumping factor B	<i>clfB</i>	LN051_01230
	Fibronectin binding proteins	<i>fnbA</i>	LN051_04265
	Ser-Asp rich fibrinogen-binding proteins	<i>sdrC</i>	LN051_00305
Enzymes	Cysteine protease	<i>sspB</i>	LN051_01195
	Hyaluronate lyase	<i>hysA</i>	LN051_02175
	Lipase	<i>geh</i>	LN051_01425
	Thermonuclease	<i>nuc</i>	LN051_06665
Secretion system	Type VII secretion system	<i>esaA</i>	LN051_10885; LN051_10890
		<i>esaB</i>	LN051_10875
		<i>essC</i>	LN051_10865
		<i>esxA</i>	LN051_10895
Surface protein anchoring	Lipoprotein diacylglyceryl transferase	<i>lgt</i>	LN051_09220
	Lipoprotein-specific signal peptidase II	<i>lspA</i>	LN051_07315
Immune evasion	Capsule	Undetermined	LN051_02355; LN051_03230; LN051_07970
Toxin	β-hemolysin	<i>hlyB</i>	LN051_01075

The accessory genome is often associated with virulence and antimicrobial resistance and has an important role in the ability of species to colonize particular hosts or persist in the environment. To determine the genomic diversity within all *Hyicus-Intermedius* species group representatives, the pangenome was analyzed. Type strains within the group have shown an extensive accessory genome whose profile correlates well with the phylogenetic relationship of individual species (Figure 5). The comparison indicates a distinct accessory genes group which is shared with *S. hyicus*, *S. chromogenes* and *S. agnetis* species (Figure 5).

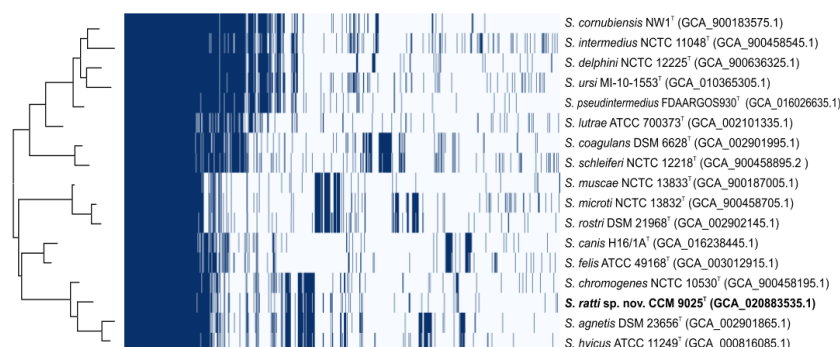


Figure 5. Pangenome analysis of the Hyicus-Intermedius species group showing genomic diversity within the type strains. Gene clusters ($n = 6056$) distinguished at 70% blastp identity were grouped by Roary. The pangenome matrix including 992 core genes present in <16 genomes, 2360 shell genes present in 2–15 genomes and 2704 cloud genes present in <2 genomes where the genes were either present or absent is visualized by the Roary plot on the right. The evolutionary insights between species based on the pangenome is shown by the tree on the left.

The genome harbors various types of insertion sequences (ISs) from the IS200/IS605, IS3 and IS6 families. In addition to the above ISs, a 9.1-kb composite transposon with 28.3% G+C content is integrated into the genome and flanked by two elements from the IS3 family (Figure 4). The transposon encodes a gene for the radical S-adenosyl-L-methionine (SAM) enzyme (LN051_08115), which is involved in a number of metabolic processes, including post-transcriptional and post-translational modifications, and a gene encoding a YcaO-like protein (LN051_08100) which is responsible for the synthesis of thiazole/oxazole-modified microcin antibiotics [39]. The SagB/ThcOx family dehydrogenase gene (LN051_08090) for a membrane-associated N-acetylglucosaminidase that cleaves polymerized glycan strands to their physiological length and a major facilitator superfamily (MFS) transporter gene (LN051_08095), are also part of the transposon. Since these genes are linked to modulating antibiotic resistance in methicillin-resistant *S. aureus* [40], we hypothesize that these genes may be responsible for the penicillin resistance of this strain.

One prophage designated vB_SraS_LR1 with a typical siphoviral modular structure is integrated at the 13-bp-long putative *att* site 5' AAAATCAACYTTT 3' adjacent to the tRNA^{Arg} gene (locus tag LN051_09185) and exhibits 75.9% identity and 43% coverage with the *S. hyicus* phage EW (= RG = NCTC 9856) [41], which was previously misclassified according to its genomic sequence [42] as an *S. aureus* bacteriophage belonging to the *Phietavirus* genus [43]. Moderate similarity was found (77.3% identity and 30% coverage) to the *S. hyicus* phage PMBT9 from the *Siphoviridae* family [44].

A 15-kb long phage-inducible chromosomal island designated SrRICCM9025 was identified in the genome of CCM 9025^T (Figure 4). It has 30.1% G+C content and harbours a site-specific integrase (LN051_02785), IS6 family transposase (LN051_02790), the gene *virE* encoding virulence-associated E family protein (LN051_02795), and new putative phosphotransferase genes which may be related to antimicrobial resistance. SrRICCM9025 exhibits partial sequence similarity to the *Macrococcus* island McRI_{msr} [45] in the proteins for integration and replication but otherwise demonstrates a distinct gene structure (Figure 6). It is integrated adjacent to the *lacA*, *lacB*, *lacD*, *lacG* genes and genes for lactose/cellobiose-specific phosphotransferase system genes, which are required for galactose 6-phosphate isomerase activity, described as part of the *S. aureus* lactose operon *lacABCDFEG* [46].

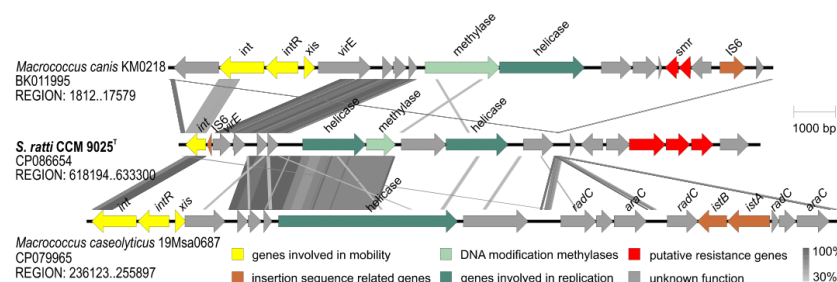


Figure 6. Structure comparison of the phage-inducible chromosomal island SrRI_{CCM9025} from *Staphylococcus ratti* CCM 9025^T, the partial sequence of McRI_{msr}-like island from *Macrococcus canis* KM0218 and the homologous genomic region from *Macrococcus caseolyticus* 19Msa0687. GenBank accession numbers and depicted regions are indicated below the species designation. The Blast map shows protein identities above 30%. The genes are color coded by their function as in the legend.

Despite the fact that mobile elements were found in the genome, the identification of the CRISPR/Cas system in the *S. ratti* CCM 9025^T genome is consistent with the need to limit the uptake of foreign DNA. Strain CCM 9025^T carries a 431-bp-long CRISPR loci with 6 spacers flanked by 36-bp-long direct repeats (DR: GTTTTAGTACTCTGTAATTT-TAGTATAAGGTATTC) and putative *cas* genes encoding CRISPR-associated endonuclease Cas9 and the proteins Cas1, Cas2 and Csn1 typical for CRISPR system type II-A. Cas9 exhibited 79% amino acid identity to the type II CRISPR-associated Cas9 of *S. agnetis* (WP_107390356) [47] and *S. chromogenes* (WP_145399953), and 74% amino acid identity to Cas9 of *S. hyicus* ATCC 11249^T (WP_167696241) [48]. Spacers target bacteriophage-related sequences, but no significant similarity to staphylococcal phage genomes was found, indicating a gap in the knowledge of phages infecting this host.

2.4. Description of *Staphylococcus ratti* sp. nov.

Staphylococcus ratti (rat'ti. L. gen. n. *ratti* of the rat) cells are Gram-stain-positive spherical cocci occurring predominantly in pairs and clusters. They are non-spore-forming and non-motile. Colonies on tryptic soy agar (TSA) are circular with a whole margin and are flat, smooth, shiny, 1–2 mm in diameter, aerobic and white. They demonstrate positive hemolytic activity on blood agar. Growth occurs in the presence of 15% NaCl at 20 °C and 45 °C, but not at 15 °C or 48 °C. Cells contain catalase, arginine dihydrolase, urease, β-glucuronidase, and hyaluronidase, and demonstrate nitrate reduction and hydrolysis of gelatin and DNA. Hydrolysis of esculin and Tween 80 is negative. Cells demonstrate weak anaerobic growth in thioglycolate medium and are coagulase-, thermostable nuclease-, oxidase-, pyrrolidonyl arylamidase-, Voges-Proskauer test (acetoin)- and ornithine decarboxylase-negative. Cells are susceptible to furazolidone (100 µg) and resistant to bacitracin (10 IU). Cells are acid phosphatase-, alkaline phosphatase-, esterase (C4)-, esterase lipase (C8)- and α-chymotrypsin (weak)-positive in the API ZYM kit. Cells are lipase (C14)-, leucine arylamidase-, valine arylamidase-, cystine arylamidase-, trypsin-, naphthol-AS-Bi-phosphohydrolase-, α-galactosidase-, β-galactosidase-, α-glucosidase-, β-glucosidase-, N-acetyl-β-glucosaminidase-, α-mannosidase- and α-fucosidase-negative with the API ZYM kit. Acid is produced from glycerol, ribose, galactose (weak), D-glucose, D-fructose, mannose, N-acetyl glucosamine, lactose, sucrose, and trehalose. Acid is not produced from erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, adonitol, β-methyl-D-xyloside, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, α-methyl-D-mannoside, α-methyl-D-glucoside, amygdaline, arbutine, salicin, cellobiose, maltose, melibiose, inulin, melezitose, D-raffinose, starch, glycogen, xylitol, β-gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2 keto-gluconate, and 5 keto-gluconate. Cells are susceptible to ceftazidim, cephalothin, erythromycin, gentamicin, chloramphenicol, imipenem, kanamycin, neomycin, novobiocin, oxacillin, trimethoprim,

sulphamethoxazole/trimethoprim (cotrimoxazole), tetracycline rifampicin, vancomycin and fusidic acid. Cells are resistant to ampicillin, clindamycin, penicillin G, and polymyxin B. Ciprofloxacin resistance is intermediate. Cells are susceptible to lysostaphin but resistant to lysozyme.

Utilisation of D-trehalose, sucrose, β -methyl-D-glucoside, N-acetyl-D-glucosamine, N-acetyl neuraminic acid, α -D-glucose, D-mannose, D-fructose, glycerol, D-glucose-6-PO₄, L-histidine, L-serine, pectin and acetic acid is positive, and utilisation of D-turanose is borderline with Biolog MicroPlate GEN III, protocol A. There is negative utilisation of dextrin, D-maltose, D-cellobiose, gentiobiose, stachyose, D-raffinose, α -D-lactose, D-melibiose, D-salicin, N-acetyl- β -D-mannosamine, N-acetyl-D-galactosamine, D-galactose, 3-methyl glucose, D-fucose, L-fucose, L-rhamnose, inosine, D-sorbitol, D-mannitol, D-arabitol, myo-inositol, D-fructose-6-PO₄, D-aspartic acid, D-serine, gelatin, glycyl-L-proline, L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, L-pyrogutamic acid, D-galacturonic acid, D-galactonic acid lactone, D-gluconic acid, D-glucuronic acid, glucuronamide, mucic acid, quinic acid, D-saccharic acid, p-hydroxy phenylacetic acid, methyl pyruvate, D-lactic acid methyl ester, L-lactic acid, citric acid, α -keto glutaric acid, D-malic acid, L-malic acid, bromo-succinic acid, tween 40, γ -amino-butyric acid, α -hydroxy-butyric acid, β -hydroxy-D,L-butyric acid, α -keto butyric acid, acetoacetic acid, propionic acid and formic acid.

The peptidoglycan is of the type A3 α L-Lys-Gly₃₋₄, MK-7 is the major menaquinone, and the major fatty acids are iso-C_{15:0}, anteiso-C_{15:0}, iso-C_{17:0} and anteiso-C_{17:0}.

The type strain is NRL/St 03/464^T (= CCM 9025^T = LMG 31873^T = DSM 111348^T), isolated in 2003 from a swab from the nostrils of a healthy laboratory rat. The DNA G+C content of the type strain is 36.1 mol%, calculated from the whole genomic sequence. The GenBank/ENA/DDBJ accession number for the 16S rRNA gene is OL352091. The complete genome sequence is available under GenBank accession number CP086654.

3. Materials and Methods

3.1. Bacterial Strains and Cultivation

The isolate NRL St 03/464^T was isolated from a single animal and maintained as glycerol stock at -70°C until analyzed. Reference strains of the phylogenetic relatives *S. agnetis* CCM 8869^T, *S. hyicus* CCM 2368^T, and *S. chromogenes* CCM 3387^T were obtained from the Czech Collection of Microorganisms (Brno, Czech Republic). All cultivations were performed at 30°C for 24 h unless stated otherwise in the test specifications. Type strain *Staphylococcus ratti* NRL/St 03/464^T has been deposited as publicly accessible in the Czech Collection of Microorganisms (CCM), the German Collection of Microorganisms and Cell Cultures (DSMZ), and the BCCM/LMG Bacteria collection.

3.2. Phenotypic Characterization

Extensive phenotypic characterization using the commercial kits STAPHYtest 24 (Erba Lachema, Brno, Czechia) and API ZYM (bioMérieux, Marcy l'Etoile, France), phenotypic fingerprinting using the Biolog system with the identification test panel GEN III MicroPlate (Biolog, Hayward, CA, USA), and conventional biochemical, physiological, and growth tests relevant for the genus *Staphylococcus* were done as described previously [49–51]. The antibiotic resistance pattern was tested by the disc diffusion method on Mueller–Hinton agar (Oxoid, Basingstoke, UK). A set of discs (Oxoid) generally used for Gram-positive cocci were applied: ampicillin (10 μg), oxacillin (1 μg), ceftazidime (30 μg), cephalothin (30 μg), ciprofloxacin (5 μg), clindamycin (2 μg), erythromycin (15 μg), gentamicin (10 μg), chloramphenicol (30 μg), imipenem (10 μg), kanamycin (30 μg), neomycin (10 μg), novobiocin (5 μg), penicillin G (1 IU), rifampicin (5 μg), trimethoprim (5 μg), cotrimoxazole (25 μg), tetracycline (30 μg), vancomycin (30 μg), fusidic acid (10 μg) and polymyxin B (300 U). EUCAST standards and manufacturer's recommendations (Oxoid) were strictly followed for cultivation and inhibition zone diameter measurement [52].

3.3. Transmission Electron Microscopy

A 200-mesh carbon/formvar-coated grid was placed on a drop of suspension of bacteria in water for 20 min. Bacterial cells located on the grid were negatively stained with 2% ammonium molybdate and treated with UV light. A Morgagni 268D Philips (ThermoFisher Scientific, Amsterdam, The Netherlands) transmission electron microscope was used to visualize bacterial cells.

3.4. Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) Analysis

MALDI-TOF MS was performed in automatic acquisition mode as described previously [53] in a Microflex LT MALDI-TOF spectrometer (Bruker Daltonics, Bremen, Germany) by using MBT Compass 4.1 software (Bruker Daltonics). Identification was made using the Bruker's database MBT Compass Library Revision L 2020 (9607 MSP).

3.5. Chemotaxonomic Analyses

Cellular fatty acids (FAs) were extracted from all compared *Staphylococcus* strains grown on the Trypticase soy broth agar (TSBA) plates under the same cultivation conditions with a cultivation temperature of 37 °C for 24 h to reach the late-exponential stage of growth according to the four-quadrant streak method. The extraction of cellular FAs was performed according to the standard protocol recommended by the MIDI Microbial Identification System [54]. Extracted FAs were identified using an Agilent 7890B gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) according to the Standard Protocol of the Sherlock Identification System (MIDI Sherlock version 6.2, MIDI database RTSBA version 6.21).

Isolation and structure analyses of the peptidoglycan were performed according to published protocols and some modifications [35,55,56]. In brief, the amino acid composition of total hydrolysate (4 N HCl at 100 °C for 16 h) of the peptidoglycan was analyzed by gas chromatography/mass spectrometry (protocol 10 [35]). The partial hydrolysate (4 N HCl, 100 °C, 45 min) of the peptidoglycan was analyzed by high-resolution liquid chromatography mass spectrometry (LC-MS) as described in [35,55]. Enantiomeric analysis was performed by liquid chromatography as described recently [56].

Respiratory quinones were extracted and analyzed as described previously [57]. Their identity was confirmed by mass spectrometry as described previously [55].

3.6. Genotypic Analysis by (GTG)₅-PCR Fingerprinting

Rep-PCR fingerprinting using the (GTG)₅ primer was carried out according to Švec et al. [58]. Numerical analysis of the (GTG)₅-PCR fingerprints was done using BioNumerics version 7.6 (Applied Maths, Kortrijk, Belgium).

3.7. Phylogenetic Analysis Based on 16S rRNA, Housekeeping Genes and Core Genome

The 16S rRNA gene sequences were amplified from crude boiled cell extracts and sequenced by Sanger sequencing in the Eurofins MWG Operon sequencing facility (Ebersberg, Germany) with previously described primers [59]. The partial *rpoB* gene was sequenced as described previously [60]. Initial identification of the strain to the genus level was based on pairwise sequence alignment and calculation of similarity values with the algorithm used in the EzBioCloud database v.2021.07.07 [61]. 16S rRNA gene sequences obtained from PCR products were aligned with those extracted from WGS data using RNAmmer version 1.2 [62]. The multilocus sequence data of six housekeeping genes that are commonly used in phylogenetic studies of the *Staphylococcus* genus were extracted from whole-genome sequence assemblies of type strains available in the NCBI Assembly resource [63], including the NCTC 3000 project (<https://www.sanger.ac.uk/resources/downloads/bacteria/nctc/> accessed on 27 October 2021) and FDA-ARGOS project [64]. The partial gene sequences used correspond to the following gene coordinates of *S. aureus*: 1420..1974 for *rpoB*, 270..826 for *groEL*, 23..911 for *dnaJ*, 49..929 for *gap*, 383..1032 for *tufA*, and 50..480 for the *sodA* gene.

The phylogenetic analyses were performed with the software MEGA X [65]. Genetic distances were corrected using the Tamura–Nei model [66], and the evolutionary history was inferred using the maximum likelihood (ML) and neighbor-joining (NJ) methods using a bootstrap test based on 500 replications [67]. The up-to-date bacterial core gene (UBCG) pipeline version 3.0 was used for whole-genome phylogenetic analysis based on core gene sequences [68].

The ANI and dDDH values were calculated using the web-based genome-to-genome distance calculator (GGDC) version 3.0 [69] and FastANI [70], respectively.

3.8. Genome Sequencing and Bioinformatics Analyses

Total genomic DNA was extracted using a GenElute Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA) from pure culture colonies cultivated on Colombia sheep blood agar (Oxoid). The preparation of DNA libraries with a Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) and whole-genome sequencing on the Illumina platform were conducted externally (LGC Genomics, Berlin, Germany) using 2×150 bp paired-end reads on the NextSeq sequencing platform (Illumina).

For sequencing using the Oxford Nanopore platform, bacterial DNA was isolated as described previously [71]. The library was prepared using the SQK-RBK004 rapid barcoding kit (Oxford Nanopore Technologies, Oxford, UK) according to the manufacturer's instructions. Libraries were sequenced with FLO-MIN106 flow cells (R9.4.1) in a MinION device (Oxford Nanopore Technologies, Oxford, UK). The device was controlled with the software MinKNOW version 4.1.2 (Oxford Nanopore Technologies, Oxford, UK).

Basecalling, demultiplexing and barcode trimming were performed using standalone ONT Guppy software version 5.0.11 using the config file dna_r9.4.1_450bps_sup.cfg with the default minimum q-score threshold, i.e., 10. The MinION reads were subsequently filtered by mapping to Illumina reads using Filtlong version 0.2.1 (<https://github.com/rrwick/Filtlong> accessed on 20 September 2021) with a minimum length of 1500 bp and quality threshold set to 95%. Only data that exceeded these thresholds was used in the assembly. The quality of reads was assessed with FastQC version 0.11.9 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc> accessed on 20 September 2021) and NanoStat [72]. Complete bacterial genome sequences were obtained using a hybrid assembly with Unicycler version 0.4.9 [73] using SPAdes version 3.12.0 [74], and the parameters chosen were bold mode and k-mers 21,55,77,99,127. The resulting assembly was polished with Pilon version 1.24 [75].

For pangenome analysis, the complete genomes were initially annotated with Prokka version 1.14.6 [76] and clustered with Roary [77]; the results were then visualized with the script roary_plots.py, which is provided in the Roary package. Further, the genome was annotated using the NCBI Prokaryotic Genome Annotation Pipeline [78]. Sequences were manipulated and inspected in the cross-platform bioinformatics software Ugene version 38.1 [79]. The multiple sequence alignment was visualised using EasyFig version 2.2.5 [80]. Gene content was further examined manually with NCBI BLAST (<https://blast.ncbi.nlm.nih.gov> accessed on 4 November 2021), and VGEs were identified with PHASTER [81], PhiSpy version 3.4 [82], IslandViewer 4 [83], and ISFinder [84]. The CRISPR/Cas system was characterized with CRISPRCasTyper [85]. Virulence factors were predicted using the VFAnalyzer tool available at the Virulence Factors Database [86].

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References

- Götz, F.; Bannerman, T.; Schleifer, K.-H. The Genera *Staphylococcus* and *Micrococcus*. In *The Prokaryotes*; Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., Stackebrandt, E., Eds.; Springer: New York, NY, USA, 2006; pp. 5–75. [\[CrossRef\]](#)
- Nováková, D.; Pantůček, R.; Hubálek, Z.; Falsen, E.; Busse, H.-J.; Schumann, P.; Sedláček, I. *Staphylococcus microti* sp. nov., isolated from the common vole (*Microtus arvalis*). *Int. J. Syst. Evol. Microbiol.* **2010**, *60*, 566–573. [\[CrossRef\]](#)
- Hauschild, T. Phenotypic and genotypic identification of staphylococci isolated from wild small mammals. *Syst. Appl. Microbiol.* **2001**, *24*, 411–416. [\[CrossRef\]](#)
- Hauschild, T.; Slizewski, P.; Masiewicz, P. Species distribution of staphylococci from small wild mammals. *Syst. Appl. Microbiol.* **2010**, *33*, 457–460. [\[CrossRef\]](#) [\[PubMed\]](#)
- Shimizu, A.; Ozaki, J.; Kawano, J.; Saitoh, Y.; Kimura, S. Distribution of *Staphylococcus* species on animal skin. *J. Vet. Med. Sci.* **1992**, *54*, 355–357. [\[CrossRef\]](#) [\[PubMed\]](#)
- Raafat, D.; Mrochen, D.M.; Al'Sholui, F.; Heuser, E.; Ryll, R.; Pritchett-Corning, K.R.; Jacob, J.; Walther, B.; Matuschka, F.R.; Richter, D.; et al. Molecular epidemiology of methicillin-susceptible and methicillin-resistant *Staphylococcus aureus* in wild, captive and laboratory rats: Effect of habitat on the nasal *S. aureus* population. *Toxins* **2020**, *12*, 80. [\[CrossRef\]](#)
- Mrochen, D.M.; Grumann, D.; Schulz, D.; Gumz, J.; Trube, P.; Pritchett-Corning, K.; Johnson, S.; Nicklas, W.; Kirsch, P.; Martelet, K.; et al. Global spread of mouse-adapted *Staphylococcus aureus* lineages CC1, CC15, and CC88 among mouse breeding facilities. *Int. J. Med. Microbiol.* **2018**, *308*, 598–606. [\[CrossRef\]](#) [\[PubMed\]](#)
- Hanses, F.; Roux, C.; Dunman, P.M.; Salzberger, B.; Lee, J.C. *Staphylococcus aureus* gene expression in a rat model of infective endocarditis. *Genome Med.* **2014**, *6*, 93. [\[CrossRef\]](#) [\[PubMed\]](#)
- Power, M.E.; Olson, M.E.; Domingue, P.A.; Costerton, J.W. A rat model of *Staphylococcus aureus* chronic osteomyelitis that provides a suitable system for studying the human infection. *J. Med. Microbiol.* **1990**, *33*, 189–198. [\[CrossRef\]](#)
- Donnelly, T.M.; Stark, D.M. Susceptibility of laboratory rats, hamsters, and mice to wound infection with *Staphylococcus aureus*. *Am. J. Vet. Res.* **1985**, *46*, 2634–2638.
- Devriese, L.A. Staphylococci in healthy and diseased animals. *J. Appl. Microbiol.* **1990**, *69*, 71S–80S. [\[CrossRef\]](#)
- Bannoehr, J.; Guardabassi, L. *Staphylococcus pseudintermedius* in the dog: Taxonomy, diagnostics, ecology, epidemiology and pathogenicity. *Vet. Dermatol.* **2012**, *23*, 253–e52. [\[CrossRef\]](#) [\[PubMed\]](#)
- Devriese, L.A.; Vancanneyt, M.; Baele, M.; Vaneechoutte, M.; De Graef, E.; Snauwaert, C.; Cleenwerck, I.; Dawyndt, P.; Swings, J.; Decostere, A.; et al. *Staphylococcus pseudintermedius* sp. nov., a coagulase-positive species from animals. *Int. J. Syst. Evol. Microbiol.* **2005**, *55*, 1569–1573. [\[CrossRef\]](#) [\[PubMed\]](#)
- Igimi, S.; Takahashi, E.; Mitsuoka, T. *Staphylococcus schleiferi* subsp. *coagulans* subsp. nov., isolated from the external auditory meatus of dogs with external ear otitis. *Int. J. Syst. Bacteriol.* **1990**, *40*, 409–411. [\[CrossRef\]](#) [\[PubMed\]](#)
- Newstead, L.L.; Harris, J.; Goodbrand, S.; Varjonen, K.; Nuttall, T.; Paterson, G.K. *Staphylococcus caledonicus* sp. nov. and *Staphylococcus canis* sp. nov. isolated from healthy domestic dogs. *Int. J. Syst. Evol. Microbiol.* **2021**, *71*, 004878. [\[CrossRef\]](#) [\[PubMed\]](#)
- Vrbovská, V.; Sedláček, I.; Zeman, M.; Švec, P.; Kovačovic, V.; Šedo, O.; Laichmanová, M.; Doskař, J.; Pantůček, R. Characterization of *Staphylococcus intermedius* group isolates associated with animals from Antarctica and emended description of *Staphylococcus delphini*. *Microorganisms* **2020**, *8*, 204. [\[CrossRef\]](#) [\[PubMed\]](#)
- Perreten, V.; Kania, S.A.; Bemis, D. *Staphylococcus ursi* sp. nov., a new member of the ‘*Staphylococcus intermedius* group’ isolated from healthy black bears. *Int. J. Syst. Evol. Microbiol.* **2020**, *70*, 4637–4645. [\[CrossRef\]](#)

18. Worthing, K.; Pang, S.; Trott, D.J.; Abraham, S.; Coombs, G.W.; Jordan, D.; McIntyre, L.; Davies, M.R.; Norris, J. Characterisation of *Staphylococcus felis* isolated from cats using whole genome sequencing. *Vet. Microbiol.* **2018**, *222*, 98–104. [\[CrossRef\]](#)
19. Guardabassi, L.; Loeber, M.E.; Jacobson, A. Transmission of multiple antimicrobial-resistant *Staphylococcus intermedius* between dogs affected by deep pyoderma and their owners. *Vet. Microbiol.* **2004**, *98*, 23–27. [\[CrossRef\]](#)
20. Boerlin, P.; Eugster, S.; Gaschen, F.; Straub, R.; Schawalder, P. Transmission of opportunistic pathogens in a veterinary teaching hospital. *Vet. Microbiol.* **2001**, *82*, 347–359. [\[CrossRef\]](#)
21. Fudaba, Y.; Nishifuji, K.; Andresen, L.O.; Yamaguchi, T.; Komatsuzawa, H.; Amagai, M.; Sugai, M. *Staphylococcus hyicus* exfoliative toxins selectively digest porcine desmoglein 1. *Microb. Pathog.* **2005**, *39*, 171–176. [\[CrossRef\]](#)
22. Casanova, C.; Iselin, L.; von Steiger, N.; Droz, S.; Sendi, P. *Staphylococcus hyicus* bacteremia in a farmer. *J. Clin. Microbiol.* **2011**, *49*, 4377–4378. [\[CrossRef\]](#)
23. Foissac, M.; Lekaditi, M.; Loutfi, B.; Ehrhart, A.; Dauchy, F.A. Spondylodiscitis and bacteremia due to *Staphylococcus hyicus* in an immunocompetent man. *Germes* **2016**, *6*, 106–110. [\[CrossRef\]](#)
24. Taponen, S.; Supre, K.; Piessens, V.; Van Coillie, E.; De Vliegher, S.; Koort, J.M.K. *Staphylococcus agnetis* sp. nov., a coagulase-variable species from bovine subclinical and mild clinical mastitis. *Int. J. Syst. Evol. Microbiol.* **2012**, *62*, 61–65. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Poulsen, L.L.; Thofner, I.; Bisgaard, M.; Olsen, R.H.; Christensen, J.P.; Christensen, H. *Staphylococcus agnetis*, a potential pathogen in broiler breeders. *Vet. Microbiol.* **2017**, *212*, 1–6. [\[CrossRef\]](#) [\[PubMed\]](#)
26. Al-Rubaye, A.A.; Couger, M.B.; Ojha, S.; Pummill, J.F.; Koon, J.A., 2nd; Wideman, R.F., Jr.; Rhoads, D.D. Genome analysis of *Staphylococcus agnetis*, an agent of lameness in broiler chickens. *PLoS ONE* **2015**, *10*, e0143336. [\[CrossRef\]](#) [\[PubMed\]](#)
27. Devriese, L.A.; Baele, M.; Vaneechoutte, M.; Martel, A.; Haesebrouck, F. Identification and antimicrobial susceptibility of *Staphylococcus chromogenes* isolates from intramammary infections of dairy cows. *Vet. Microbiol.* **2002**, *87*, 175–182. [\[CrossRef\]](#)
28. Andresen, L.O.; Ahrens, P.; Daugaard, L.; Bille-Hansen, V. Exudative epidermitis in pigs caused by toxigenic *Staphylococcus chromogenes*. *Vet. Microbiol.* **2005**, *105*, 291–300. [\[CrossRef\]](#) [\[PubMed\]](#)
29. Andrews, A.H.; Lampport, A. Isolation of *Staphylococcus chromogenes* from an unusual case of impetigo in a goat. *Vet. Rec.* **1997**, *140*, 584. [\[CrossRef\]](#)
30. Schmidt, T.; Kock, M.M.; Ehlers, M.M. Diversity and antimicrobial susceptibility profiling of staphylococci isolated from bovine mastitis cases and close human contacts. *J. Dairy Sci.* **2015**, *98*, 6256–6269. [\[CrossRef\]](#)
31. Lamers, R.P.; Muthukrishnan, G.; Castoe, T.A.; Tafur, S.; Cole, A.M.; Parkinson, C.L. Phylogenetic relationships among *Staphylococcus* species and refinement of cluster groups based on multilocus data. *BMC Evol. Biol.* **2012**, *12*, 171. [\[CrossRef\]](#)
32. Goris, J.; Konstantinidis, K.T.; Klappenbach, J.A.; Coenye, T.; Vandamme, P.; Tiedje, J.M. DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. *Int. J. Syst. Evol. Microbiol.* **2007**, *57*, 81–91. [\[CrossRef\]](#)
33. Schleifer, K.-H.; Bell, J.A. *Staphylococcus*. In *Bergey's Manual of Systematics of Archaea and Bacteria*, Online ed.; Whitman, W.B., Ed.; Wiley & Sons and Bergey's Manual Trust: Hoboken, NJ, USA, 2015. [\[CrossRef\]](#)
34. Nahaie, M.R.; Goodfellow, M.; Minnikin, D.E.; Hájek, V. Polar lipid and isoprenoid quinone composition in the classification of *Staphylococcus*. *J. Gen. Microbiol.* **1984**, *130*, 2427–2437. [\[CrossRef\]](#)
35. Schumann, P. Peptidoglycan Structure. *Methods Microbiol.* **2011**, *38*, 101–129. [\[CrossRef\]](#)
36. Devriese, L.A.; Hajek, V.; Oeding, P.; Meyer, S.A.; Schleifer, K.H. *Staphylococcus hyicus* (Sompolinsky 1953) comb. nov. and *Staphylococcus hyicus* subsp. *chromogenes* subsp. nov. *Int. J. Syst. Bacteriol.* **1978**, *28*, 482–490. [\[CrossRef\]](#)
37. Shwani, A.; Adkins, P.R.F.; Ekesi, N.S.; Alrubaye, A.; Calcutt, M.J.; Middleton, J.R.; Rhoads, D.D. Whole-genome comparisons of *Staphylococcus agnetis* isolates from cattle and chickens. *Appl. Environ. Microbiol.* **2020**, *86*, e00484–20. [\[CrossRef\]](#) [\[PubMed\]](#)
38. Luthje, P.; von Kockritz-Blickwede, M.; Schwarz, S. Identification and characterization of nine novel types of small staphylococcal plasmids carrying the lincosamide nucleotidyltransferase gene *lnu(A)*. *J. Antimicrob. Chemother.* **2007**, *59*, 600–606. [\[CrossRef\]](#)
39. Burkhart, B.J.; Schwalen, C.J.; Mann, G.; Naismith, J.H.; Mitchell, D.A. YcaO-dependent posttranslational amide activation: Biosynthesis, structure, and function. *Chem. Rev.* **2017**, *117*, 5389–5456. [\[CrossRef\]](#) [\[PubMed\]](#)
40. Chan, Y.G.; Frankel, M.B.; Missiakas, D.; Schneewind, O. SagB glucosaminidase is a determinant of *Staphylococcus aureus* glycan chain length, antibiotic susceptibility, and protein secretion. *J. Bacteriol.* **2016**, *198*, 1123–1136. [\[CrossRef\]](#)
41. Dean, B.A.; Williams, R.E.O.; Hall, F.; Corse, J. Phage typing of coagulase-negative staphylococci and micrococci. *Epidemiol. Infect.* (J. Hyg.) **1973**, *71*, 261–270. [\[CrossRef\]](#)
42. Kwan, T.; Liu, J.; DuBow, M.; Gros, P.; Pelletier, J. The complete genomes and proteomes of 27 *Staphylococcus aureus* bacteriophages. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 5174–5179. [\[CrossRef\]](#) [\[PubMed\]](#)
43. Gutierrez, D.; Adriaenssens, E.M.; Martinez, B.; Rodriguez, A.; Lavigne, R.; Kropinski, A.M.; Garcia, P. Three proposed new bacteriophage genera of staphylococcal phages: “3alikevirus”, “77likevirus” and “Phietaikevirus”. *Arch. Virol.* **2014**, *159*, 389–398. [\[CrossRef\]](#)
44. Tetens, J.; Sprotte, S.; Thimm, G.; Wagner, N.; Brinks, E.; Neve, H.; Holzel, C.S.; Franz, C.M.A.P. First molecular characterization of *Siphoviridae*-like bacteriophages infecting *Staphylococcus hyicus* in a case of exudative epidermitis. *Front. Microbiol.* **2021**, *12*, 653501. [\[CrossRef\]](#)
45. Schwendener, S.; Dona, V.; Perreten, V. The novel macrolide resistance genes *mef(D)*, *msr(F)*, and *msr(H)* are present on resistance islands in *Macrococcus canis*, *Macrococcus caseolyticus*, and *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **2020**, *64*, e00160–20. [\[CrossRef\]](#)

46. Rosey, E.L.; Oskouian, B.; Stewart, G.C. Lactose metabolism by *Staphylococcus aureus*: Characterization of *lacABCD*, the structural genes of the tagatose 6-phosphate pathway. *J. Bacteriol.* **1991**, *173*, 5992–5998. [\[CrossRef\]](#)
47. Naushad, S.; Barkema, H.W.; Luby, C.; Condas, L.A.; Nobrega, D.B.; Carson, D.A.; De Buck, J. Comprehensive phylogenetic analysis of bovine non-aureus staphylococci species based on whole-genome sequencing. *Front. Microbiol.* **2016**, *7*, 1990. [\[CrossRef\]](#)
48. Haft, D.H.; Selengut, J.; Mongodin, E.F.; Nelson, K.E. A guild of 45 CRISPR-associated (Cas) protein families and multiple CRISPR/Cas subtypes exist in prokaryotic genomes. *PLoS Comput. Biol.* **2005**, *1*, e60. [\[CrossRef\]](#)
49. Pantůček, R.; Švec, P.; Dajcs, J.J.; Machová, I.; Černohlavková, J.; Šedo, O.; Gelbíčová, T.; Mašlaňová, I.; Doškař, J.; Zdráhal, Z.; et al. *Staphylococcus petrasii* sp. nov. including *S. petrasii* subsp. *petrasii* subsp. nov. and *S. petrasii* subsp. *croceilyticus* subsp. nov., isolated from human clinical specimens and human ear infections. *Syst. Appl. Microbiol.* **2013**, *36*, 90–95. [\[CrossRef\]](#) [\[PubMed\]](#)
50. Mannerová, S.; Pantůček, R.; Doškař, J.; Švec, P.; Snauwaert, C.; Vancanneyt, M.; Swings, J.; Sedláček, I. *Macroccoccus brunensis* sp. nov., *Macroccoccus hajekii* sp. nov. and *Macroccoccus lamae* sp. nov., from the skin of llamas. *Int. J. Syst. Evol. Microbiol.* **2003**, *53*, 1647–1654. [\[CrossRef\]](#) [\[PubMed\]](#)
51. Mašlaňová, I.; Wertheimer, Z.; Sedláček, I.; Švec, P.; Indráková, A.; Kovařovic, V.; Schumann, P.; Spröer, C.; Králová, S.; Šedo, O.; et al. Description and comparative genomics of *Macroccoccus caseolyticus* subsp. *hominis* subsp. nov., *Macroccoccus goetzii* sp. nov., *Macroccoccus epidermidis* sp. nov., and *Macroccoccus bohemicus* sp. nov., novel macrococci from human clinical material with virulence potential and suspected uptake of foreign DNA by natural transformation. *Front. Microbiol.* **2018**, *9*, 1178. [\[CrossRef\]](#) [\[PubMed\]](#)
52. EUCAST. Breakpoint Tables for Interpretation of MICs and Zone Diameters. The European Committee on Antimicrobial Susceptibility Testing: Version 6.0. 2016. Available online: <https://www.eucast.org> (accessed on 10 June 2019).
53. Pantůček, R.; Sedláček, I.; Indráková, A.; Vrbová, V.; Mašlaňová, I.; Kovařovic, V.; Švec, P.; Králová, S.; Křištofová, L.; Kekláková, J.; et al. *Staphylococcus edaphicus* sp. nov., isolated in Antarctica, harbors the *mecC* gene and genomic islands with a suspected role in adaptation to extreme environments. *Appl. Env. Microbiol.* **2018**, *84*, e01746–17. [\[CrossRef\]](#)
54. Sasser, M. *Identification of Bacteria by Gas Chromatography of Cellular Fatty Acids*, MIDI Technical Note 101, Revision July 2006 ed.; MIDI Inc.: Newark, DE, USA, 1990.
55. Schumann, P.; Kalensee, F.; Cao, J.; Criscuolo, A.; Clermont, D.; Kohler, J.M.; Meier-Kolthoff, J.P.; Neumann-Schaal, M.; Tindall, B.J.; Pukall, R. Reclassification of *Haloactinobacterium glaciecola* as *Occultella glaciecola* gen. nov., comb. nov., of *Haloactinobacterium album* as *Ruania alba* comb. nov., with an emended description of the genus *Ruania*, recognition that the genus names *Haloactinobacterium* and *Ruania* are heterotypic synonyms and description of *Occultella aeris* sp. nov., a halotolerant isolate from surface soil sampled at an ancient copper smelter. *Int. J. Syst. Evol. Microbiol.* **2021**, *71*, 004769. [\[CrossRef\]](#)
56. Kämpfer, P.; McInroy, J.A.; Clermont, D.; Neumann-Schaal, M.; Criscuolo, A.; Busse, H.-J.; Glaeser, S.P. *Leucobacter soli* sp. nov., from soil amended with humic acid. *Int. J. Syst. Evol. Microbiol.* **2021**, *71*, 005156. [\[CrossRef\]](#) [\[PubMed\]](#)
57. Vieira, S.; Huber, K.J.; Neumann-Schaal, M.; Geppert, A.; Luckner, M.; Wanner, G.; Overmann, J. *Usitatibacter rugosus* gen. nov., sp. nov. and *Usitatibacter palustris* sp. nov., novel members of *Usitatibacteraceae* fam. nov. within the order *Nitrosomonadales* isolated from soil. *Int. J. Syst. Evol. Microbiol.* **2021**, *71*, 004631. [\[CrossRef\]](#) [\[PubMed\]](#)
58. Švec, P.; Pantůček, R.; Petráš, P.; Sedláček, I.; Nováková, D. Identification of *Staphylococcus* spp. using (GTG)₅-PCR fingerprinting. *Syst. Appl. Microbiol.* **2010**, *33*, 451–456. [\[CrossRef\]](#) [\[PubMed\]](#)
59. Pantůček, R.; Sedláček, I.; Petráš, P.; Koukalová, D.; Švec, P.; Štětina, V.; Vancanneyt, M.; Chrástínová, L.; Vokurková, J.; Růžicková, V.; et al. *Staphylococcus simiae* sp. nov., isolated from South American squirrel monkeys. *Int. J. Syst. Evol. Microbiol.* **2005**, *55*, 1953–1958. [\[CrossRef\]](#)
60. Mellmann, A.; Becker, K.; von Eiff, C.; Keckevoet, U.; Schumann, P.; Harmsen, D. Sequencing and staphylococci identification. *Emerg. Infect. Dis.* **2006**, *12*, 333–336. [\[CrossRef\]](#)
61. Yoon, S.H.; Ha, S.M.; Kwon, S.; Lim, J.; Kim, Y.; Seo, H.; Chun, J. Introducing EzBioCloud: A taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. *Int. J. Syst. Evol. Microbiol.* **2017**, *67*, 1613–1617. [\[CrossRef\]](#)
62. Lagesen, K.; Hallin, P.; Rodland, E.A.; Staerfeldt, H.H.; Rognes, T.; Ussery, D.W. RNAmmer: Consistent and rapid annotation of ribosomal RNA genes. *Nucleic Acids Res.* **2007**, *35*, 3100–3108. [\[CrossRef\]](#)
63. Kitts, P.A.; Church, D.M.; Thibaud-Nissen, F.; Choi, J.; Hem, V.; Sapojnikov, V.; Smith, R.G.; Tatusova, T.; Xiang, C.; Zherikov, A.; et al. Assembly: A resource for assembled genomes at NCBI. *Nucleic Acids Res.* **2016**, *44*, D73–D80. [\[CrossRef\]](#)
64. Sighting, H.; Minogue, T.; Yan, Y.; Stefan, C.; Hall, A.; Tallon, L.; Sadzewicz, L.; Nadendla, S.; Klimke, W.; Hatcher, E.; et al. FDA-ARGOS is a database with public quality-controlled reference genomes for diagnostic use and regulatory science. *Nat. Commun.* **2019**, *10*, 3313. [\[CrossRef\]](#)
65. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549. [\[CrossRef\]](#) [\[PubMed\]](#)
66. Tamura, K.; Nei, M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.* **1993**, *10*, 512–526. [\[CrossRef\]](#)
67. Felsenstein, J. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **1985**, *39*, 783–791. [\[CrossRef\]](#)
68. Na, S.I.; Kim, Y.O.; Yoon, S.H.; Ha, S.M.; Baek, I.; Chun, J. UBCG: Up-to-date bacterial core gene set and pipeline for phylogenomic tree reconstruction. *J. Microbiol.* **2018**, *56*, 280–285. [\[CrossRef\]](#)

69. Meier-Kolthoff, J.P.; Carbasse, J.S.; Peinado-Olarte, R.L.; Göker, M. TYGS and LPSN: A database tandem for fast and reliable genome-based classification and nomenclature of prokaryotes. *Nucleic Acids Res.* **2021**, *49*, gkab902. [\[CrossRef\]](#)
70. Jain, C.; Rodriguez, R.L.; Phillippy, A.M.; Konstantinidis, K.T.; Aluru, S. High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. *Nat. Commun.* **2018**, *9*, 5114. [\[CrossRef\]](#)
71. Fišarová, L.; Botka, T.; Du, X.; Mašláňová, I.; Bárdy, P.; Pantůček, R.; Benešik, M.; Roudnický, P.; Winstel, V.; Larsen, J.; et al. *Staphylococcus epidermidis* phages transduce antimicrobial resistance plasmids and mobilize chromosomal islands. *mSphere* **2021**, *6*, e00223-21. [\[CrossRef\]](#)
72. De Coster, W.; D'Hert, S.; Schultz, D.T.; Cruts, M.; Van Broeckhoven, C. NanoPack: Visualizing and processing long-read sequencing data. *Bioinformatics* **2018**, *34*, 2666–2669. [\[CrossRef\]](#)
73. Wick, R.R.; Judd, L.M.; Gorrie, C.L.; Holt, K.E. Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Comput. Biol.* **2017**, *13*, e1005595. [\[CrossRef\]](#)
74. Bankevich, A.; Nurk, S.; Antipov, D.; Gurevich, A.A.; Dvorkin, M.; Kulikov, A.S.; Lesin, V.M.; Nikolenko, S.I.; Pham, S.; Prjibelski, A.D.; et al. SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* **2012**, *19*, 455–477. [\[CrossRef\]](#)
75. Walker, B.J.; Abeel, T.; Shea, T.; Priest, M.; Abouelliel, A.; Sakthikumar, S.; Cuomo, C.A.; Zeng, Q.; Wortman, J.; Young, S.K.; et al. Pilon: An integrated tool for comprehensive microbial variant detection and genome assembly improvement. *PLoS ONE* **2014**, *9*, e112963. [\[CrossRef\]](#)
76. Seemann, T. Prokka: Rapid prokaryotic genome annotation. *Bioinformatics* **2014**, *30*, 2068–2069. [\[CrossRef\]](#) [\[PubMed\]](#)
77. Page, A.J.; Cummins, C.A.; Hunt, M.; Wong, V.K.; Reuter, S.; Holden, M.T.; Fookes, M.; Falush, D.; Keane, J.A.; Parkhill, J. Roary: Rapid large-scale prokaryote pan genome analysis. *Bioinformatics* **2015**, *31*, 3691–3693. [\[CrossRef\]](#) [\[PubMed\]](#)
78. Li, W.; O'Neill, K.R.; Haft, D.H.; DiCuccio, M.; Chetvernin, V.; Badretdin, A.; Coulouris, G.; Chitsaz, F.; Derbyshire, M.K.; Durkin, A.S.; et al. RefSeq: Expanding the Prokaryotic Genome Annotation Pipeline reach with protein family model curation. *Nucleic Acids Res.* **2021**, *49*, D1020–D1028. [\[CrossRef\]](#)
79. Okonechnikov, K.; Golosova, O.; Fursov, M.; The UGENE Team. Unipro UGENE: A unified bioinformatics toolkit. *Bioinformatics* **2012**, *28*, 1166–1167. [\[CrossRef\]](#) [\[PubMed\]](#)
80. Sullivan, M.J.; Petty, N.K.; Beatson, S.A. Easyfig: A genome comparison visualizer. *Bioinformatics* **2011**, *27*, 1009–1010. [\[CrossRef\]](#)
81. Arndt, D.; Grant, J.R.; Marcu, A.; Sajed, T.; Pon, A.; Liang, Y.; Wishart, D.S. PHASTER: A better, faster version of the PHAST phage search tool. *Nucleic Acids Res.* **2016**, *44*, W16–W21. [\[CrossRef\]](#)
82. Edwards, R.; Decewicz, P.; Katelyn; Daniel, S.; Laurasisk. linsalrob/PhiSpy: Dropped prophages (v.4.2.19). In *Zenodo*; CERN: Geneva, Switzerland, 2021. [\[CrossRef\]](#)
83. Bertelli, C.; Laird, M.R.; Williams, K.P.; Simon Fraser University Research Computing Group; Lau, B.Y.; Hoar, G.; Winsor, G.L.; Brinkman, F.S.L. IslandViewer 4: Expanded prediction of genomic islands for larger-scale datasets. *Nucleic Acids Res.* **2017**, *45*, W30–W35. [\[CrossRef\]](#)
84. Siguié, P.; Perochon, J.; Lestrade, L.; Mahillon, J.; Chandler, M. ISfinder: The reference centre for bacterial insertion sequences. *Nucleic Acids Res.* **2006**, *34*, D32–D36. [\[CrossRef\]](#)
85. Russel, J.; Pinilla-Redondo, R.; Mayo-Munoz, D.; Shah, S.A.; Sorensen, S.J. CRISPRCasTyper: Automated identification, annotation, and classification of CRISPR-Cas loci. *CRISPR J.* **2020**, *3*, 462–469. [\[CrossRef\]](#)
86. Liu, B.; Zheng, D.; Jin, Q.; Chen, L.; Yang, J. VFDB 2019: A comparative pathogenomic platform with an interactive web interface. *Nucleic Acids Res.* **2019**, *47*, D687–D692. [\[CrossRef\]](#) [\[PubMed\]](#)

4.6 *Staphylococcus brunensis* sp. nov. isolated from human clinical specimens with a staphylococcal cassette chromosome-related genomic island outside of the *rimH* gene bearing the *ccrDE* recombinase gene complex

Background and objectives: *Staphylococcus haemolyticus* is a common nosocomial pathogen that often causes infections in wounds and the bloodstream. The prevalence of its multidrug resistance and pool of diverse mobile genetic elements is of particular concern. Primarily studied is the *SCCmec* encoding resistance to beta-lactams; however, its evolutionary history and mechanism of transmission are not yet well understood. Due to the widespread usage of MALDI-TOF MS in diagnostics, new species related to *S. haemolyticus*, such as *S. taiwanensis* and *S. borealis*, have recently been recovered from human clinical material. This study analyzed five unidentified strains obtained from human ear swabs, wounds, and bile samples between 2016 and 2022.

Material and methods: Phylogenomic methods, chemotaxonomy, extensive biotyping, sequencing with Illumina Nextseq and ONT Minion were used to characterize five strains NRL/St 16/872^T (=CCM 9024^T), NRL/St 19/737 (=CCM 9023), NRL/St 18/288 (=P1256), NRL/St 21/187 (=P13332), NRL/St 22/194 (=P13326).

Results and discussion: Phylogenomic analysis revealed that new isolates are closely related to the *S. petrasii* complex. The comparative analysis showed differences in mobile genetic elements, including ψ SCC, prophages, plasmids, transposons, insertion elements and a unique genomic island *SbC_{IccrDE}*, which contains a core region homologous to the *ccr* recombinase gene complex of *SCCmec*. Due to the low amino acid identity with canonical Ccr recombinases, we propose classification as a new allotype *ccrDE*. A survey conducted in the GenBank database has discovered genomes of *S. haemolyticus*, *S. aureus*, *S. hominis*, and *S. borealis* harbored the same genomic island, consistently integrated into the *rimL* gene, but varied in the presence of RM systems, antibiotic resistance genes, and other plasmid-like sequences.

Conclusion: The novel species *S. brunensis* isolated from clinical material was characterized in detail. Three strains harbored a previously undescribed genomic island related to *SCCmec*.

Significance: The description of *SbC_{IccrDE}* is crucial to understanding the origin and evolution of SCC-like elements. It may also represent a primordial element capable of accumulating virulence and drug resistance factors, the spread of which into established pathogens such as *S. aureus* would threaten the healthcare system.



Staphylococcus brunensis sp. nov. isolated from human clinical specimens with a staphylococcal cassette chromosome-related genomic island outside of the *rlmH* gene bearing the *ccrDE* recombinase gene complex

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ABSTRACT Novel species of coagulase-negative staphylococci, which could serve as reservoirs of virulence and antimicrobial resistance factors for opportunistic pathogens from the genus *Staphylococcus*, are recognized in human and animal specimens due to advances in diagnostic techniques. Here, we used whole-genome sequencing, extensive biotyping, MALDI-TOF mass spectrometry, and chemotaxonomy to characterize five coagulase-negative strains from the *Staphylococcus haemolyticus* phylogenetic clade obtained from human ear swabs, wounds, and bile. Based on the results of polyphasic taxonomy, we propose the species *Staphylococcus brunensis* sp. nov. (type strain NRL/St 16/872^T = CCM 9024^T = LMG 31872^T = DSM 111349^T). The genomic analysis revealed numerous variable genomic elements, including staphylococcal cassette chromosome (SCC), prophages, plasmids, and a unique 18.8 kb-long genomic island SbCl_{ccrDE} integrated into the ribosomal protein L7 serine acetyltransferase gene *rimL*. SbCl_{ccrDE} has a cassette chromosome recombinase (*ccr*) gene complex with a typical structure found in SCCs. Based on nucleotide and amino acid identity to other known *ccr* genes and the distinct integration site that differs from the canonical methyltransferase gene *rlmH* exploited by SCCs, we classified the *ccr* genes as novel variants, *ccrDE*. The comparative genomic analysis of SbCl_{ccrDE} with related islands shows that they can accumulate virulence and antimicrobial resistance factors creating novel resistance elements, which reflects the evolution of SCC. The spread of these resistance islands into established pathogens such as *Staphylococcus aureus* would pose a great threat to the healthcare system.

IMPORTANCE The coagulase-negative staphylococci are important opportunistic human pathogens, which cause bloodstream and foreign body infections, mainly in immunocompromised patients. The mobile elements, primarily the staphylococcal cassette chromosome *mec*, which confers resistance to methicillin, are the key to the successful dissemination of staphylococci into healthcare and community settings. Here, we present a novel species of the *Staphylococcus* genus isolated from human clinical material. The detailed analysis of its genome revealed a previously undescribed genomic island, which is closely related to the staphylococcal cassette chromosome and has the potential to accumulate and spread virulence and resistance determinants. The island harbors a set of conserved genes required for its mobilization, which we recognized as novel cassette chromosome recombinase genes *ccrDE*. Similar islands were revealed not only in the genomes of coagulase-negative staphylococci but also in *S. aureus*. The comparative genomic study contributes substantially to the understanding of the evolution and pathogenesis of staphylococci.

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Vojtěch Kovařovic and Adéla Finstrlová contributed equally to this article. To determine the order of the authors we flipped the coin.

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Staphylococci, mainly coagulase-positive *Staphylococcus aureus*, are the leading cause of a broad spectrum of diseases in humans and animals. Over the last three decades, coagulase-negative staphylococcal species (CoNS), with the most significant being *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, and *Staphylococcus hominis*, have been recognized as opportunistic pathogens, especially in immunocompromised patients. CoNS are a frequent cause of nosocomial infections related to catheters or medical devices aided by their ability to form a biofilm (1). Additionally, the gene pool of substrate utilization pathways and resistance determinants enables CoNS to occupy various niches, providing favorable conditions for the emergence of multidrug-resistant CoNS and their subsequent spread in healthcare environments (2).

The adaptation of *S. haemolyticus* strains to diverse environments is facilitated by frequent recombination among numerous insertion sequences (IS_{Sha1}) (3). Therefore, the standard microbial and molecular diagnostic tools have limited discriminatory power to reliably distinguish species closely related to *S. haemolyticus* (4). Only recently, molecular diagnostics approaches, mainly in-depth whole-genome characterization, have assigned atypical *S. haemolyticus* strains isolated from clinical specimens into the new species *Staphylococcus borealis* (5) and *Staphylococcus taiwanensis* (6). The core genome phylogeny also led to the reclassification of the *S. petrasii* phylogenetic complex (7, 8), which now consists of three species—*S. petrasii*, *S. croceilyticus*, and *S. pragensis* (9). All these species were isolated from various human biological samples, mainly from wounds, eye and ear infections, urinary infections, and blood samples (10).

The versatility of CoNS is associated with a significant reservoir of mobile genetic elements (MGEs). Notably, the methicillin resistance encoded by the staphylococcal chromosomal cassette (SCC) *mec* (SCC*mec*) significantly complicates healthcare and increases the need to use second-line antimicrobial drugs to treat staphylococcal infections (11). Apart from *mec* genes responsible for methicillin resistance, SCC*mec* carries numerous genes for virulence, such as phenol-soluble modulins (PSM-*mec*), plasmin, or heavy-metal and other resistance genes contributing to the survival of these strains in an environment (12–14). It is suspected that the SCC*mec* originates from CoNS species (15), but the natural mechanism of SCC transmission is still unknown. It can be transferred intra- and even interspecies by transduction (16), conjugation (17), or natural competence (18). The transfer of SCC*mec* is mediated by *ccr* chromosome cassette genes of the serine recombinase family. Three phylogenetically distinct *ccr* genes, namely *ccrA*, *ccrB*, and *ccrC*, have been delineated with nucleotide identities below 50%. These recombinases recognize a specific *att* site in the bacterial *rlmH* gene for ribosomal RNA large-subunit methyltransferase H (19). The recombinases CcrA and CcrB function together as a heterotetramer in the specific excision of the SCC (20), whereas the CcrC recombinase enables mobilization of the element without another recombinase (21).

The recent increase in the association of CoNS with nosocomial infections and improvements in diagnostic approaches make it possible to recognize other often overlooked species of this group related to human diseases. Investigation of these CoNS as a pool of genes for antimicrobial resistance and virulence can reveal the molecular mechanisms that lead to these opportunistic pathogens' evolution, adaptation, and success. This article reports the polyphasic characterization of five *Staphylococcus* sp. isolates from human clinical material to delineate a novel species. Whole-genome sequence analyses of the strains revealed a remarkable non-SCC genomic island harboring *ccrDE* cassette chromosome recombinase types.

RESULTS

Phylogenetic relationship of the strains

Five unidentified *Staphylococcus* sp. strains were collected from various human clinical specimens from both mixed culture and monoculture between 2016 and 2022 (Table 1) and transferred to the National Reference Laboratory for Staphylococci (National Institute of Public Health, Prague) for long-term storage and further study. The phylogenetic analysis of complete 16S rRNA gene sequences consistently placed the five isolates in *S. haemolyticus* cluster group defined previously (22). The closest relatives were *Staphylococcus petrasii*, *Staphylococcus croceilyticus*, and *Staphylococcus pragensis*, with 16S rRNA gene sequence similarities ranging from 99.80 to 99.59%, while other species were below 99.4% similarity. The topology of the neighbor-joining (NJ) phylogenetic tree constructed with 16S rRNA gene sequences was similar to that of the maximum likelihood (ML) tree (Fig. 1A). Because the 16S rRNA analysis has limited discriminatory power in the genus *Staphylococcus* (5, 6, 23), the phylogenetic position of the new isolates was also assessed using the concatenated multilocus sequence data of six routinely used conserved housekeeping genes: *rpoB*, *hsp60*, *dnaJ*, *tufA*, *gap*, and *sodA* for discrimination of staphylococcal species (Fig. 1B). The ML phylogenetic tree for the housekeeping genes had a very similar topology to that of the 16S rRNA gene tree and to the additional phylogenetic trees constructed using the up-to-date bacterial core gene (UBCG) at the nucleotide and protein level (Fig. 1C and D).

The whole-genome phylogenetic distance from the related staphylococcal taxa with an ANI value of <92.3% (Table S3) and digital DNA-DNA hybridization (dDDH) of <45.6% determined the closest species *S. petrasii*; however, the values were below the species delineation thresholds, which are 95–96% and 70%, respectively (24). The whole-genome phylogeny thus confirmed that the five isolates represent a distinct *Staphylococcus* species designated *Staphylococcus brunensis* sp. nov.

Growth, morphological, biochemical, and chemotaxonomical characterization of analyzed isolates

All five isolates exhibited Gram-positive stain, irregular cells ranging in diameter from 433 nm to 1,210 nm (Fig. S1) arranged in pairs, tetrads, and clusters. They grew very well on

TABLE 1 Origin of the strains of *Staphylococcus brunensis* sp. nov. characterized in this study

Strain	Date of isolation	Locality	Specimen	Sex	Age	Diagnosis	Other microflora
NRL/St ^a 16/872 ^T = CCM ^b 9024 ^T = LMG ^c 31,872 ^T = DSM ^d 111349 ^T	Oct 2016	Karlovy Vary	Swab of ear	M	3 y	Acute otitis externa	Monoculture
NRL/St ^a 19/737 = CCM ^b 9023	Jul 2019	Prague	Wound pus	M	32 y	Surgical wound infection	<i>S. aureus</i>
NRL/St ^a 18/288 = P12563	Mar 2018	Lyon	Wound pus	M	51 y	Leg wound infection NA ^e	
NRL/St ^a 21/187 = P13332	Jul 2021	Karlovy Vary	Bile	M	83 y	Cholelithiasis and cholecystitis	<i>E. coli</i> , <i>Klebsiella pneumoniae</i> , <i>Citrobacter freundii</i> , and <i>Enterococcus faecalis</i>
NRL/St ^a 22/194 = P13326	Apr 2022	Prague	Swab of ear	M	6 m	Acute otitis media	Monoculture

^aNRL/St, National Reference Laboratory for Staphylococci, National Institute for Public Health, Prague.

^bCCM, Czech Collection of Microorganisms.

^cLMG, Bacteria Collection at the Laboratorium voor Microbiologie Universiteit Gent.

^dDSM, German Collection of Microorganisms and Cell Cultures.

^eNA, not analyzed.

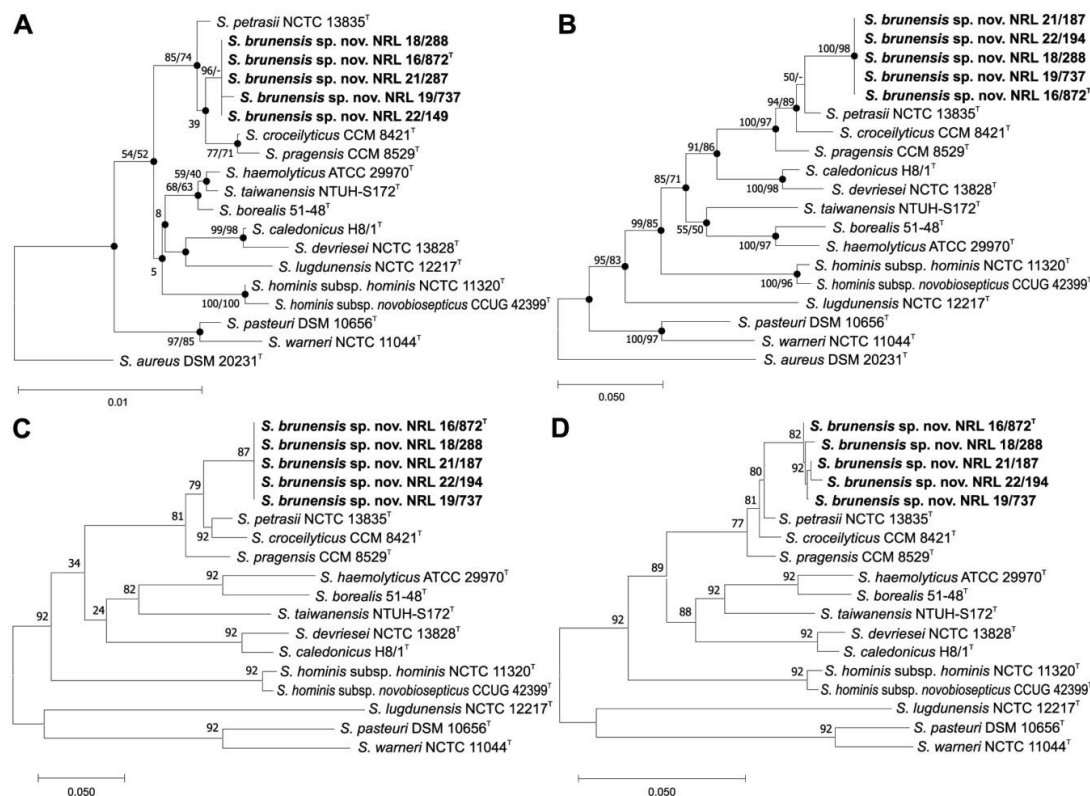


FIG 1 Evolutionary analyses of *Staphylococcus brunensis* sp. nov. and its closest relatives. GenBank accession numbers of used sequences are listed in Table S1. (A) Evolutionary history inferred using neighbor-joining (NJ) method based on complete 16S rRNA gene sequences extracted from whole-genome assemblies. Filled circles indicate that the corresponding nodes were also identified from analysis by the maximum likelihood (ML) method. The percentage of replicate trees above 50% in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches for the NJ and ML methods. *S. aureus* DSM 20231^T was used as an outgroup. The evolutionary distances are in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair. There were a total of 1,552 positions in the final data set. (B) Unrooted ML tree based on multilocus sequence analysis of concatenated nucleotide sequences from six loci—*rpoB*, *hsp60*, *dnaJ*, *tufA*, *gap*, and *sodA*—were extracted from whole-genome assemblies. Filled circles indicate that the corresponding nodes were also identified from analysis by the NJ method. There were a total of 3,952 positions in the final data set. Bootstrap probability values (percentages of 500 tree replications) greater than 50% are shown at branch points. The evolutionary distances are given as the number of substitutions per site. (C) Nucleotide sequence-based and (D) protein sequence-based phylogenetic tree of the concatenated alignment of 92 core genes constructed using up-to-date bacterial core gene (UBCG) set. The ML tree was inferred using RAXML software and 100 replicates. The threshold for the gene support index was set to 94% for the nucleotide and 80% for the protein-based tree. Gene support indices are given at branching points (the maximal possible value is 92). The bar indicates the number of substitutions per position.

tryptone soy agar (TSA), Columbia agar with blood, plate count agar, P agar, and nutrient agar, and did not grow in a thioglycollate medium. The Congo red agar method showed negativity in the production of polysaccharide intercellular adhesin (PIA) associated with biofilm formation. Phenotypic identification based on bacitracin resistance and sensitivity to furazolidone, positive catalase test, growth in the presence of NaCl above 10%, and microscopic morphology assigned five isolates as *Staphylococcus* sp. In contrast to the main characteristics of staphylococci, isolate NRL/St 19/737 exhibited atypical negative catalase activity. Test-dependent results were also observed for the Voges-Proskauer (VP) test (acetoin) and for β -glucuronidase. With detection of acetoin production, we only obtained positive results in all five strains when pyruvic acid served as substrate using the commercial VP test (Erba Lachema) instead of glucose as substrate for the

TABLE 2 Differentiation of *Staphylococcus brunensis* sp. nov. from closely related staphylococci occurring in human clinical material

Test ^d	Result obtained for indicated type strain ^a /result from species description						
	<i>S. brunensis</i> sp. nov. ^b	<i>S. petrasii</i> CCM 8418 ^T	<i>S. croceilyticus</i> CCM 8421 ^T	<i>S. pragensis</i> CCM 8529 ^T	<i>S. haemolyticus</i> CCM 2737 ^T	<i>S. borealis</i> CCM 9145 ^T	<i>S. taiwanensis</i> CCM 9267 ^T
Arginine dihydrolase	+	+/+	+/+	-/-	+/+	+/+	+/+
Voges-Proskauer test	+	+/+	+/+	+/+	w/+	-/-	+/+
Urease	-	+/-	+/+	-/-	-/-	+/+	+/+
β-Glucuronidase ^c	+	-/-	+/+	-/-	+/-	+/-	-/-
DNA hydrolysis	-	w/d	-/-	+/+	+/-	w/-	-/-
Acid from: lactose	+	-/-	-/-	-/-	-/-	-/-	-/-
Galactose	+	-/-	-/-	-/-	+/-	-/nt	-/-
Mannose	-	+/+	-/-	-/-	-/-	-/-	-/-
Ribose	-	-/w	w/w	-/-	-/-	+/+	+/+
D-arabinose	-	-/-	+/+	-/-	-/-	-/-	-/-
N-Acetylglucosamine	-	-/-	-/-	-/-	+/+	+/-	-/-
Pale yellow pigment	-	-/-	+/+	-/-	-/-	+/+	-/-

^aAll data were taken from this study (in two replications).^bPresented data are uniform for all isolates of *S. brunensis* sp. nov.^cSTAPHYtest 24 kit.^d+, positive; -, negative; w, weak reaction; d, 11–89% strains positive; nt, not tested.

conventional VP test. Similar results were obtained with the β-glucuronidase test, where all isolates were positive in the STAPHYtest 24 kit, but negative in the API ZYM kit due to a different substrate for enzyme detection. The differentiation of novel staphylococcal isolates from similar and/or closely related staphylococci occurring in human clinical material is shown in Table 2. The species *S. petrasii*, *S. pragensis*, and *S. haemolyticus* were phenotypically the most similar taxa to the aforementioned isolates. Strain-dependent utilization results are specified in Table S4. Complete characteristics of *S. brunensis* sp. nov. are stated in the protologue given subsequently.

Antibiotic susceptibility testing showed that all five strains are susceptible to cefoxitin, clindamycin, gentamicin, chloramphenicol, linezolid, oxacillin, rifampicin, tobramycin, trimethoprim, sulphamethox/trimethoprim (cotrimoxazole), tetracycline, and fusidic acid. Susceptibility to ciprofloxacin and levofloxacin was intermediate. Susceptibility to ampicillin, penicillin G, tigecycline, and erythromycin was strain-dependent (Table S4).

By using cluster analysis of matrix-assisted laser-desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) protein profiles, all five *S. brunensis* sp. nov. strains were separated into a coherent cluster distinguished from phylogenetically related

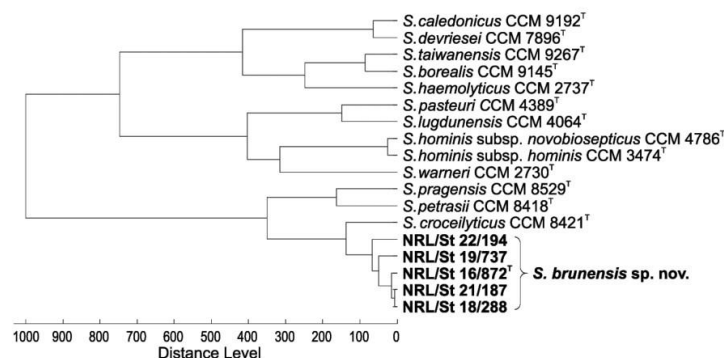


FIG 2 Dendrogram based on MALDI-TOF MS profiles of *Staphylococcus brunensis* sp. nov. and other phylogenetically related species. The dendrogram was generated using the correlation distance measure with the average linkage algorithm (UPGMA) settings of the software BioTyper version 3.1 (Bruker Daltonics).

Staphylococcus spp., as shown in Fig. 2. All five strains share 35 signals within the m/z range 2–11 kDa, while five of these signals ($m/z = 4130, 6660, 7614, 8258, \text{ and } 10625$) were found to be specific for *S. brunensis* sp. nov., being absent in the MALDI-TOF MS protein profiles of a comprehensive set of 65 *S. petrasii*, *S. croceilyticus*, and *S. pragensis* strains analyzed previously (10).

The chemotaxonomic analyses of type strain *S. brunensis* NRL/St 16/872^T showed predominantly menaquinone-7 (MK-7, 95%) and a small amount of MK-6 (4%) and MK-8 (1%). The major fatty acids were C 15:0 anteiso (38.6%) and C 17:0 anteiso (19.6%), followed by C 19:0 anteiso (8.3%), C 15:0 iso (6.3%), C 17:0 iso (6.9%), C 18:0 (6.8%), C 19:0 iso (4.4%), and C 20:0 (3.0%). A small amount of C 16:0 iso (1.8%), C 16:0 (1.2%), and C 18:0 iso (1.6%) fatty acids were also present. The detected peptidoglycan type is A3 α (L-Lys-Gly₃₋₄, A11.2). All chemotaxonomic data are in-line with previous reported patterns (7).

DNA fingerprinting of *S. brunensis* sp. nov.

Screening of the investigated bacterial group by rep-PCR fingerprinting with primer (GTG)₅ showed their genotypic coherence. All five isolates had visually similar fingerprints, which were grouped into a single cluster and separated from the other entries in the in-house fingerprint database, which includes members of all recognized species of the genus *Staphylococcus*, including type strains of phylogenetically closely related

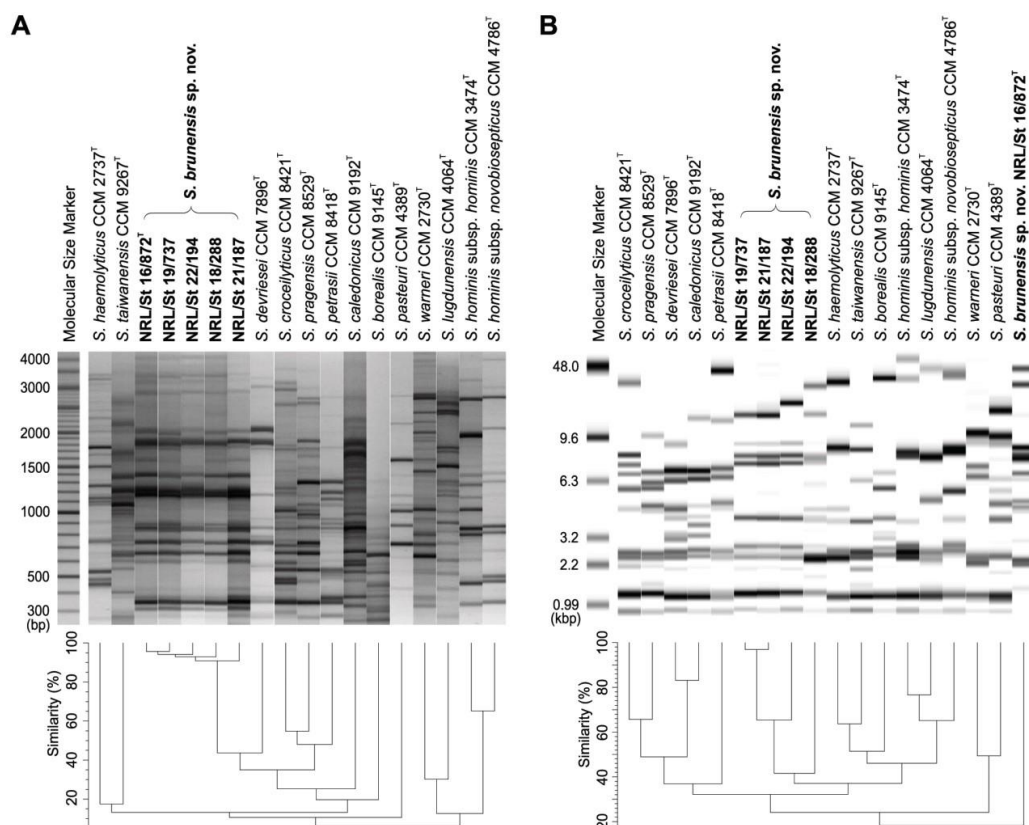


FIG 3 Cluster analysis of rep-PCR fingerprints and ribotype patterns obtained from *Staphylococcus brunensis* sp. nov. strains and the type strains of phylogenetically closely related *Staphylococcus* species. (A) Dendrogram based on (GTG)₅-PCR fingerprints. (B) Dendrogram based on cluster analysis of EcoRI ribotype patterns obtained using a RiboPrinter system. The dendrograms were calculated with Pearson's correlation coefficients with the UPGMA clustering method (r , expressed as percentage similarity values).

species (Fig. 3A). The obtained results also suggest that this rapid and easy-to-perform method can be used for the identification of *S. brunensis* sp. nov., similarly to how we demonstrated for the identification of *Staphylococcus* spp. in our previous studies (8, 23, 25).

In contrast to the rep-PCR method, automated ribotyping with the restriction enzyme *EcoRI* revealed heterogeneity among the five examined strains (Fig. 3B). Three isolates, NRL/St 16/872^T, NRL/St 22/194, and NRL/St 18/288, exhibited unique fingerprint patterns that allowed differentiation at the strain level and indicated that they were not clonally related. The remaining two strains, NRL/St 19/737 and NRL/St 21/187, had visually identical ribotypes, despite being isolated in 2019 and 2021, respectively, at different locations and were therefore not considered clonally related. These results suggest that automated ribotyping with *EcoRI* can separate isolates of *S. brunensis* sp. nov. at the strain level, although some strains may have similar ribotype patterns. However, a reliable assessment of the discriminatory power of this technique for typing *S. brunensis* sp. nov. requires the analysis of a more significant number of strains from different localities and sources.

Genome characterization of *S. brunensis* sp. nov.

The comparison of sequenced genomes (Table S5) revealed a high degree of similarity between the isolates (Fig. 4). The *S. brunensis* sp. nov. genomes were 2.5–2.6 Mb long with GC content 33.3–33.4% encoding 2,500–2,700 CDS, 61–62 tRNAs, and 19 rRNAs. The pangenome consists of 2,230 core and 416 accessory, and 569 unique genes in total. The sequenced genomes differ in variable genomic elements, which constitute 5–8% of the genome and are associated with virulence and antimicrobial resistance genes, predominantly located at plasmids. All *S. brunensis* sp. nov. genomes possess a type III CRISPR-Cas complex with 15–20 variable spacers, some of which target siphoviral prophages as determined by blastn search. The core gene *kat* encoding catalase in the strain NRL/St 19/737 (locus tag MT339_07575) has a 1 bp deletion in homopolymer polyA, which introduces a premature stop codon at the 3' end of the gene. It is possible that the catalase-negative phenotype of the strain is associated with the loss of function of this gene, although a truncated protein without the first 22 amino acids could be produced.

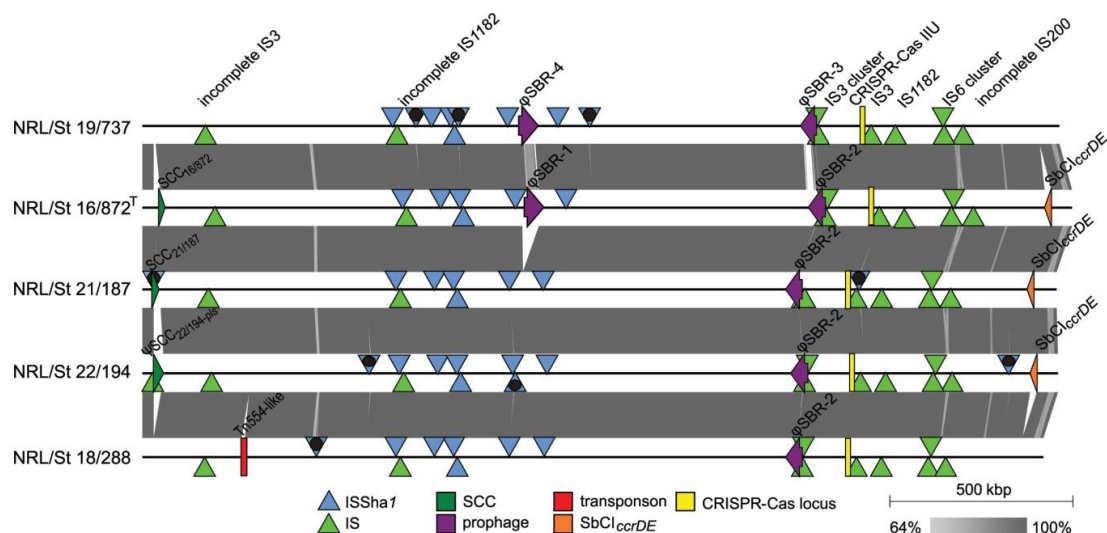


FIG 4 Genome comparison of *Staphylococcus brunensis* sp. nov. isolates. Mobile genetic elements are shown and color coded as in the legend. The ISSha1 loci that are divergent for the respective strains are marked with black circles. Only nucleotide blast hits above 64% identity and longer than 2 kb are shown.

TABLE 3 Plasmid contigs of *Staphylococcus brunensis* sp. nov. grouped by replication gene and gene content

Replication genes	Strain	Size (kb)	Antimicrobial resistance	Virulence factors	Saccharide utilization genes	Mobilization/toxin-anti-toxin systems
<i>rep39</i>	NRL/St 16/872 ^T	31.2	<i>copZ</i> , <i>csrB</i> , <i>qacR</i> , <i>qacA</i>	<i>clp</i>	-	<i>mobA</i>
	NRL/St 18/288	21.9	<i>copZ</i> , <i>csrB</i> , <i>arsM</i> , <i>qacR</i> , <i>qacA</i>	-	-	<i>fstP</i>
	NRL/St 21/187	22.9	<i>copZ</i> , <i>copA</i> , <i>csrB</i> , <i>arsR</i> , <i>arsM</i>	<i>clp</i>	-	<i>mobA</i>
<i>rep20</i>	NRL/St 16/872 ^T	27.2	<i>arsR</i> , <i>arsM</i>	<i>isaB</i> , <i>essG</i>	-	<i>mobC</i> , <i>relaxaseP</i> , <i>mobP</i>
	NRL/St 19/737 and NRL/St 18/288	33.9	<i>arsR</i> , <i>arsM</i>	<i>isaB</i> , <i>essG</i>	<i>mgo</i> , <i>hxlR</i> , <i>hxlA</i> , <i>hxlI</i>	<i>mobC</i> , <i>relaxaseP</i> , <i>mobP</i>
	NRL/St 21/187	22.9	<i>qacR</i> , <i>qacA</i>	<i>isaB</i> , <i>essG</i>	-	-
<i>rep39</i> , <i>rep5b</i> , <i>rep21</i> , <i>rep10</i> , <i>rep20</i>	NRL/St 19/737	32.0	<i>blaZ</i> , <i>blaR</i> , <i>blaI</i> , <i>ermC</i> , <i>cadX</i> , <i>cadA</i> , <i>cadD</i>	-	-	<i>mobC</i> , <i>relaxaseP</i> , <i>mobP</i>
<i>rep21</i>	NRL/St 16/872 ^T , NRL/St 18/288, NRL/St 21/187, and NRL/St 22/194	2.8	<i>cadD</i> , <i>cadX</i>	-	-	-
<i>rep13</i>	NRL/St 16/872 ^T and NRL/St 21/187	2.6	-	-	-	-
<i>rep5b</i>	NRL/St 18/288	7.9	-	-	-	<i>mobC</i> , <i>relaxase P</i>
<i>rep10</i>	NRL/St 22/194	44.7	<i>ermC</i>	-	-	-

The *S. brunensis* sp. nov. genomes harbor several large plasmids, which can be grouped by the presence of either *rep39* or *rep20* gene. Due to high sequence variability and mosaicism caused by interspersed IS431, it was not possible to determine a complete consensus sequence for all plasmids. The plasmid contigs comprise beta-lactam, heavy metal, and disinfectant resistance as well as virulence and saccharide utilization genes as shown in Table 3. All analyzed strains harbor a small *rep21* plasmid conferring cadmium resistance. The strain NRL/St 22/194 contains an additional *rep10* plasmid harboring the *ermC* gene similar to the pUSA05-1 plasmid from *S. aureus* (26). These two small plasmids are integrated into the *rep39*-type plasmid contig of strain NRL/St 19/737. Furthermore, two cryptic plasmids, one containing the *rep13* gene and the other containing *rep5b* gene were identified (Table 3).

The resistance to penicillin in *S. brunensis* sp. nov. NRL/St 18/288 is encoded by the *blaZ* gene located at a 9 kb Tn554-like transposon (Fig. 4) integrated into the chromosomal *isaB* gene. The chromosomes of *S. brunensis* sp. nov. harbor several copies of various full and partial IS elements from the IS3, IS6, IS30, IS1182, IS200/IS605, and ISha1 families (Fig. 4). The IS431 flanked composite transposon localized downstream of the *cspC* gene contains the gene for a short-chain dehydrogenase associated with survival in stress conditions (27). Each strain carries six to eight ISha1 copies integrated in six conserved and six variable loci, usually adjacent to rRNA genes. One copy of ISha1 is inserted in the SCC element of *S. brunensis* sp. nov. NRL/St 21/287 strain (Fig. 4).

The *S. brunensis* sp. nov. strains have one or two complete prophages in their genomes integrated into two different 18 bp *att* sites. The ΦSBR-1 prophage of NRL/St 16/872^T is integrated in the *att* site AATCCCTTACTTCCCGTT, located in the tRNA-Ser(gga) gene. The other strains encompass one or two CRISPR spacers homologous to the ΦSBR-1, and thus are immune to infection by this phage. The same *att* site is used by ΦSBR-4 in the genome of NRL/St 19/737. Both prophages are 45.4 kb long and share 79.3% nt identity. The next *att* site, AATCCCTCCGTTTCCGTT in tRNA-Ser(gct), is occupied by either ΦSBR-2 or ΦSBR-3. Prophage ΦSBR-2, which is 47 kb long, is integrated into the genomes of strains NRL/St 16/872^T, NRL/St 19/288, and NRL/St 21/287. The strain NRL/St 19/737 harbors a 43.5 kb ΦSBR-3 prophage, which shares 79% nt identity with ΦSBR-2 genome along 57% of its length; the difference is mainly in the morphogenesis module. Both ΦSBR-2 and ΦSBR-3 carry a putative accessory virulence factor sialic acid transporter (28) in the lysis gene module.

Two *S. brunensis* sp. nov. strains NRL/St 16/872^T and NRL/St 21/287 comprise an approximately 18.2 kb-long genomic island in the rRNA methyltransferase H gene *rlmH*

(orfX). The genomic island was almost identical in both strains; the only difference is the presence of an insertion sequence ISSha1 in strain NRL/St 21/287. The island is bordered by imperfect 18 bp-long direct repeats GAAGC(A/G)TATCATAA(G/A)TGA and harbor *ccrA1B2* genes; thus, according to the rules of the IWG-SCC (29) the element was designated SCC_{NRL/St 16/872}. The most similar element to SCC_{NRL/St 16/872} is a composite island in genome of *S. hominis* C34847 (30), sharing 95.2% nt identity along 75% of their length. The SCC_{CCM9024} carries genes for the type I restriction-modification (RM) system, sharing high homology with genes encoding a restriction (*hsdR*) and methylation (*hsdM*) subunit with the RM system of *S. hominis* C34847 and *S. aureus* NTUH-4729 (31). Interestingly, the *putP* gene, which encodes a sodium proline symporter, was wedged between the *ccrB2* gene and a set of three short hypothetical genes conserved in the *ccr* gene complex, thus disrupting the canonical organization of the complete *ccr* gene complex. Similarly, the *putP* gene and the flanking sequence from the *ccr* gene complex were found in SCC elements of various CoNS, *S. epidermidis* I1PPP121 (GenBank accession no. MH188479), *S. haemolyticus* BC05211 (KX181861), *S. hominis* J6 (LT963442), and *S. hominis* J11 (LT963438), and in plasmids of *S. warneri* 16A (CP031267) and *S. pasteurii* 3C (CP031281), pointing to a high degree of recombination occurring in staphylococci.

The strain NRL/St 22/194 also harbors an SCC element inserted into the *rlmH* gene bordered by the 21 bp imperfect repeat GG(C/A)GAAGC(A/G)TATCATAA(G/A)GTGA. The 26.6 kb pseudo-SCC element named ψ SCC_{NRL/St 22/194-pls} has no recombinase genes, but carries several virulence genes, including the gene encoding plasmin sensitive protein (*pls*), poly(glycerol-phosphate) α -glycosyltransferase (*tagE*), and UDP-N-acetylmuramate-L-alanine ligase (*murF*), which are also found in *S. aureus* composite island SCCmec_{WAMRSA40} (32).

Novel genomic island harboring cassette chromosome recombinase genes *ccrDE*

The strains NRL/St 16/872^T, NRL/St 21/187 and NRL/St 22/194 harbor a mobile element of size 18.8 kb with cassette chromosome recombinase genes, integrated in the *rimJ/rimL* gene orthologous to the the ribosomal-protein-serine acetyltransferase gene *rimL* in *Escherichia coli* and *ydaF* in *Bacillus subtilis* (UniProt accession no. P13857 and P96579, respectively). The island designated *S. brunensis* chromosomal island *ccrDE* (SbCl_{ccrDE}) harbors another homologue of *rimL* with 70% nt identity to the original *rimL*; thus, it may complement the function of the truncated *rimL* gene. SbCl_{ccrDE} is bordered by the tetranucleotide motif GAAA. Additionally, the 19 bp direct repeat ATTCCACAATGAAATCCAT was found on the integration site in *rimL* and inside the *rimL* homologue on the island, which suggests a complex recombination event.

SbCl_{ccrDE} possesses a cluster of genes homologous to the *ccr* gene complex from SCC elements. The core of the *ccr* complex consists of two *ccr* genes, similar to SCC elements type IV or II. Additional genes in the *ccr* complex, that is the putative primase *polA*, cassette chromosome helicase *cch2*, three short proteins with the domains SAUGI, DUF960, and DUF1643, and a hypothetical protein (Fig. 5), were homologous to those in SCCmec type V, which has only one *ccrC* gene in the *ccr* complex. SbCl_{ccrDE} *ccr* genes share more than 98% DNA sequence identity to *ccrA8B9* recombinases (Table S6) discovered recently in the *S. haemolyticus* genome (33). The values of nucleotide identity to currently known *ccrA1-7*, *ccrB1-8*, and *ccrC1-2* genes from SCCs range from 38.0 to 53.3% (Table S6). Although it is slightly above the threshold of 50.0% for the definition

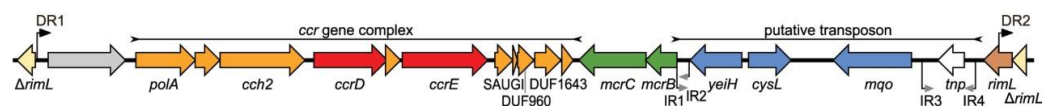


FIG 5 Annotated map of genes and possible functions in genomic island SbCl_{ccrDE} harbored by *Staphylococcus brunensis* sp. nov. Genes are labeled according to known or putative function. Sequence of direct repeats DR1, DR2: 5'-ATTCCACAATGAAATCCAT-3'; sequence of inverted repeats IR1-IR4: 5'-TGGTTCGTGTC-AAAAGT-3'.

of a gene variant according to the rules of IWG-SCC (29), the amino acid (aa) identity level of *SbCl_{CCRDE}* recombinases to known *CcrA*, *CcrB*, and *CcrC* reaches a maximum of 39.9%, which is substantially lower than aa identity among *Ccr* allotypes, which range from 50.9 to 92.3%. Therefore, based on the borderline nucleotide identities and low protein identities to *CcrA*, *CcrB*, and *CcrC* allotypes, we propose designating the *SbCl_{CCR}* recombinases as new allelic types *ccrD* and *ccrE* and reclassifying the described *ccrA8B9* recombinases accordingly (33). The *CcrDE* recombinases are able to excise the *SbCl_{CCRDE}* element from its insertion sequence site, which has been detected by sequence read alignment and PCR analysis (Fig. S2).

Apart from the *ccr* gene complex, the island *SbCl_{CCRDE}* carries the 5-methylcytosine-specific restriction enzyme *mcrB* and specificity subunit *mcrC* genes for the putative RM system (Fig. 5), commonly found on SCC elements, chromosomal islands, and prophages that help to maintain the mobile element in the genome (34). Approximately 5.8 kbp of the *SbCl_{CCRDE}* is occupied by a putative transposon bordered by a perfect 17 bp inverted repeat of TGGTTCTGTGCAAGT. The transposon has one copy of the insertion sequence from the IS6 family (sharing 95% nt identity to IS431_{mec}) and accessory genes encoding putative membrane protein (*yeiH*), putative transcriptional regulator (*cysL*), and malate chinoxine oxidoreductase (*mgo*) (Fig. 5), which are frequently found on plasmids.

We surveyed the GenBank database for sequences resembling *SbCl_{CCRDE}*. In addition to *S. haemolyticus* BC5211 (33), we found related genomic islands with *ccrDE* in the genomes of *S. haemolyticus* (GenBank accession nos. CP033814 and CP102568), *S. hominis* subsp. *hominis* K1 (CP020618), and *S. borealis* GDY8P80P (35), and in *S. aureus* ER04332 and ER11327 (36), and related clones. The island is consistently inserted in the *rimL* gene, in a locus downstream of the conserved *metE* gene (Fig. 6A), which is 17–56 kb counterclockwise from the replication origin in CoNS species. However, in *S. aureus* strains, the *rimL* gene is located at the end of the *oriC* environ due to large-scale chromosomal inversion. The variable region of the islands comprises genes for an RM system (Fig. 6A). In *S. borealis* GDY8P80P, the island harbors many transposons with resistance genes to beta-lactam antibiotics, tetracycline, aminoglycosides, and other antimicrobials. The phylogenetic analysis of *ccrD* and *ccrE* genes from the related genomic islands (Fig. 6B) and the pairwise nucleotide identity comparison (Table S6) clearly distinguished two allotypes designated *ccrD1E1*, present in the genomes of *S. brunensis* sp. nov., *S. haemolyticus*, and *S. aureus*, and *ccrD2E2*, found in the genomes of *S. borealis* and *S. hominis* (Fig. 6B; Table S6).

Taxonomic description of *S. brunensis* sp. nov.

Staphylococcus brunensis (*bru.nen'sis* L. adj. *brunensis* from Bruna, the Roman name of the city of Brno, Czech Republic, where this and other staphylococcal species were first described).

Cells are Gram stain positive cocci, occurring predominantly in pairs and clusters, non-spore-forming, and nonmotile. Colonies on TSA agar are circular, whole margin, flat, smooth, shiny, 2 mm in diameter, aerobic, and white. Hemolytic activity on sheep blood agar, production of delta-hemolysin revealed in synergistic test with a beta-hemolytic producing strain (*S. pseudintermedius* CCM 4710). Growth in the presence of 12% NaCl, at 20°C and 45°C, but not at 15°C and 48°C. They are positive for pyrrolidonyl arylamidase, arginine dihydrolase, and nitrate reduction and negative for coagulase, clumping factor, oxidase, urease, VP test (acetoin, conventional tube test), hyaluronidase, thermostable nuclease, and ornithine decarboxylase; susceptible to furazolidon (100 µg) and novobiocin (5 µg), and resistant to bacitracin (10 IU); partially resistant to lysostaphin (200 mg L⁻¹) and resistant to lysozyme; negative for hydrolysis of esculin, gelatine, DNA, and Tween 80; positive on API ZYM for acid phosphatase, alkaline phosphatase (weak), esterase (C 4), and esterase lipase (C 8); negative on API ZYM for lipase (C 14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. *S. brunensis* sp. nov.

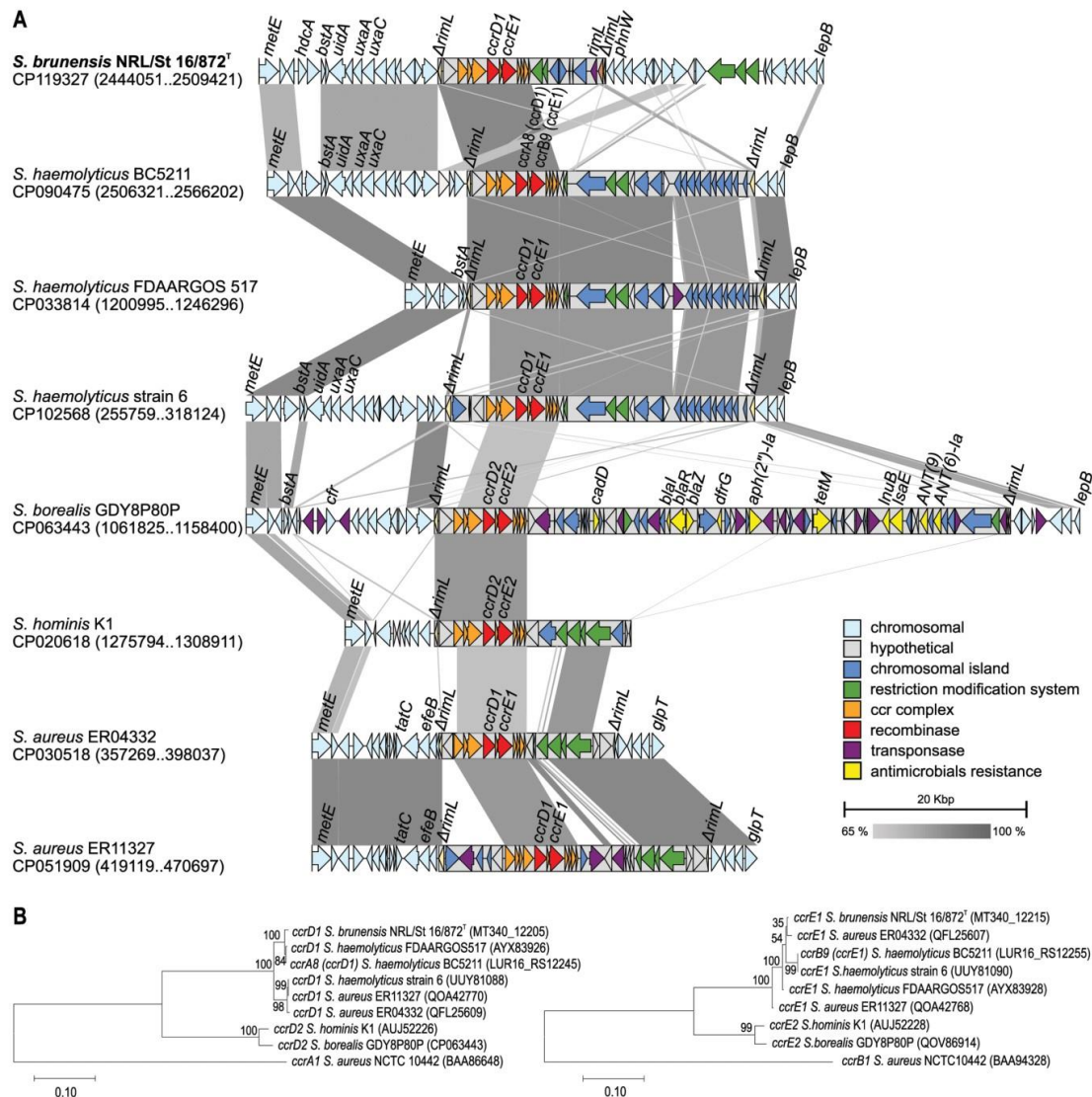


FIG 6 Comparative analysis of chromosomal islands harboring *ccrDE* cassette chromosome recombinases (*CI_{ccrDE}*). (A) Comparison of *CI_{ccrDE}* and flanking regions from different staphylococcal species. The genomic island *CI_{ccrDE}* is highlighted with a gray background. Genes are labeled according to known or putative functions, as shown in the legend. Only nucleotide blast hits above 65% identity and longer than 2 kb are shown. The position of the island is provided in parentheses next to the GenBank accession number. (B) Maximum likelihood trees of nucleotide sequences of *ccrD* and *ccrA8* recombinases with *ccrA1* as an outgroup, and *ccrE* and *ccrB9* recombinases with *ccrB1* as an outgroup, respectively. The trees were constructed using Tamura-Nei model with 500 bootstrap replicates. The evolutionary distances are in the number of base substitutions per site. The protein GenBank accession numbers or locus tags are shown in parentheses.

produce acid from glycerol (weak), galactose, D-glucose, D-fructose, maltose, lactose, sucrose, and trehalose. They do not produce acid from erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, β -methyl-D-xyloside, mannose, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, α -methyl-D-mannoside, α -methyl-D-glucoside, *N*-acetyl glucosamine, amygdaline, arbutine, salicin, cellobiose, melibiose, inulin,

melezitose, D-raffinose, starch, glycogen, xylitol, β -gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-keto-gluconate, and 5-keto-gluconate.

Variable biochemical reactions were obtained for catalase (4 of 5 positive), leucine arylamidase (2 of 5 positive), α -glucosidase (1 of 5 positive), and growth in 15% NaCl (4 of 5 positive) (Table S4).

Utilization (Biolog MicroPlate GEN III, protocol A) is positive for D-maltose, D-trehalose, D-turanose, α -D-glucose, D-fructose, D-galactose, glycerol, L-alanine, L-arginine, L-glutamic acid, L-serine, D-gluconic acid, acetic acid, and formic acid. Negative utilization for D-cellobiose, gentiobiose, stachyose, D-raffinose, D-melibiose, β -methyl-D-glucoside, D-salicin, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, *N*-acetylneuraminic acid, 3-methyl glucose, D-fucose, L-fucose, L-rhamnose, inosine, D-sorbitol, D-mannitol, myo-inositol, D-glucose-6-PO₄, D-aspartic acid, D-serine, gelatin, glycyl-L-proline, L-histidine, D-galacturonic acid, D-galactonic acid lactone, D-glucuronic acid, glucuronamide, mucic acid, quinic acid, D-saccharic acid, *p*-hydroxy phenylacetic acid, D-lactic acid methyl ester, citric acid, α -keto glutaric acid, D-malic acid, bromo-succinic acid, γ -amino-butyric acid, α -hydroxy-butyric acid, β -hydroxy-D,L-butyric acid, and propionic acid.

The type strain is NRL/St 16/872^T (= CCM 9024^T = DSM 111349^T = LMG 31872^T). The major respiratory quinone is menaquinone-7. The major fatty acids are C_{15:0} anteiso and C_{17:0} anteiso. The peptidoglycan type is A3 α (A11.2). The DNA G+C content of strain NRL/St 16/872^T is 33.40 mol%, calculated from the whole-genomic sequence. The species description is based on the characterization of five strains isolated from various human clinical materials. Most of the characteristics of the type strain NRL/St 16/872^T agree with the species description. The GenBank/ENA/DDBJ accession number for the 16S rRNA gene is [OQ401401](#). The complete chromosome sequence of the type strain is available under GenBank accession number [CP119327](#).

DISCUSSION

The recent molecular diagnostic methods and polyphasic taxonomic approach, including whole-genome sequencing, allow for more effective species differentiation of CoNS from various sources. Here, we described *S. brunensis* sp. nov., which occupies similar niches to the closely related species from the *S. petrasii* phylogenetic complex (10). The available isolates of *S. brunensis* sp. nov. were associated with the human ear and wound infections, whereas *S. petrasii* and *S. pragensis* predominated in wounds, blood, or urinary tract infections (Table S7). However, it is challenging for clinicians to determine whether these CoNS are causative agents of human diseases or suspected contaminants associated with the occurrence of these commensal bacteria on human skin.

The pathogenic potential of CoNS is related to immune evasion, invasion of host tissues, and biofilm formation, allowing them to persist on the surfaces of indwelling medical devices and thus cause chronic infections (1). The intercellular adhesion operon (*ica*), biofilm-associated protein (*bap/bhp*), and fibronectin-binding protein genes (*fnbA/fbe*) directly associated with biofilm production in *S. epidermidis* (37) have not been identified in either *S. brunensis* sp. nov. genomes or the other species from *S. petrasii* complex. Likewise, the *ica* operon homologue has not been detected in *S. haemolyticus* (3) and may be missing in this phylogenetic clade. Biofilm formation in the initial phase is influenced by molecules involved in surface adhesion. In staphylococci, these are microbial surface components recognizing adhesion matrix molecules (MSCRAMMs) (38). Homologs of cell wall-anchored serine-aspartate repeat-containing protein genes *sdrC*, *sdrG*, and *sdrH* have been identified in *S. petrasii* genomes (10) but not in *S. brunensis* sp. nov. However, gene homologues for elastin-binding protein (*ebp*), thermonuclease (*nuc*), autolysin E (*atlE*), and gene clusters (*cap5* or *cap8*) involved in the synthesis of capsular polysaccharides have been found in genomes of both *S. petrasii* (10) and *S. brunensis* sp. nov.

A comparison of representative genome sequences revealed that the species *S. brunensis* sp. nov., *S. croceilyticus*, *S. petrasii*, and *S. pragensis* differ only marginally in genomic G+C content, genome size, and the median protein count encoded by the core genome. The major cause of interspecies differences in the *S. petrasii* complex is the accessory genome and variable genetic elements (Fig. S3). The role of these elements is best described in *S. aureus*, where HGT contributes to adaptation and evolution into successful lineages (39–41).

More than 90% of clinical staphylococcal isolates harbor plasmids of various size that can be classified to small multicopy plasmids or larger plasmids carrying several resistance determinants. However, only 5% (42) of staphylococcal plasmids are large multiresistance elements with the ability to mobilize or undergo conjugative transfer. Strains of *S. brunensis* sp. nov. harbor several large plasmids that encode genes for mobilization (*mobA*, *mobC*, *mobP*) and their spread by HGT is possible. The resistance genes are usually cointegrated between two copies of ISs that promote their spread (43). An example is IS431 previously described in plasmids pSK41 and pGO1 (44), harboring linezolid and high-level resistance to vancomycin (45). It is probable that IS431, identified in *S. brunensis* plasmid sequences, is responsible for the mosaic structure of the elements and also promotes the spread of resistance genes across the genus *Staphylococcus*.

The plasmid-borne resistance to antibiotics and disinfectants suggests enhanced survival in healthcare environments. The resistance to beta-lactam (*blaZ*) and macrolide (*ermC*) antibiotics in *S. brunensis* sp. nov. strains correlates with resistance genes on plasmids and the Tn554-like transposon. Resistance to quaternary ammonium compounds (*qacA*), copper and heavy metals, and the hexulose utilization operon (*hxl*) in plasmids of *S. brunensis* sp. nov. suggests adaptation to various ecological niches similar to nosocomial and community-associated isolates of *S. haemolyticus* (46, 47).

The genomes of *S. brunensis* sp. nov. contain more insertion sequence elements than the closest relatives from *S. petrasii* complex (7, 8). The copy number variability of IS implies their recent capture and propagation and increases the genome plasticity among the strains. The most abundant ISSha1 elements with 98% similarity to ISSha1 from *S. haemolyticus* (3) are often located near rRNA genes and may contribute to variable ribotype patterns.

A significant MGE in the genome of *S. brunensis* sp. nov. is the genomic island SbCl_{CCRDE} with unique recombinase genes *ccrD1E1*. Xiao et al. (33) reported the existence of these recombinases as *ccrA8B9* allotypes. Nevertheless, their amino acid sequences that were highly different from other known *ccrAB* variants place them among novel *ccrDE* alleles. This reclassification also made it possible to distinguish another apparent variant *ccrD2E2*, present in *S. hominis* and *S. borealis*. The *ccrDE* genes in all inspected staphylococcal genomes were present on a genomic island related to SbCl_{CCRDE}. Chromosomal islands with *ccrDE* (Cl_{CCRDEs}) share gene structure similar to SCCs, such as conserved *ccr* complex, presence of variable regions, and flanking by direct repeats. However, unlike SCCs, which are canonically inserted in the *rlmH* (*orfX*) gene in the *oriC* environ (48), Cl_{CCRDEs} are inserted in the *rimL* gene. The SCC-like islands with *Ccr* or closely related large serine recombinase were inserted in the *rlmH* in other genera of Gram-positive bacteria, that is the genera *Mammaliicoccus* and *Macroccoccus*, *Enterococcus faecium*, *Bacillus cereus*, *Geobacillus vulcani*, and *Clostridioides difficile* (49–52). Hence, Cl_{CCRDEs} might represent a new class of SCC elements integrated into the *rimL* gene due to the altered specificity of the *ccrDE* recombinases.

The molecular mechanism of the action of *ccrAB* recombinases requires specific sequences of around 60–70 bp and the presence of the central dinucleotide GA in the integration site, which are essential for the integration of the cassettes into the highly conserved *rlmH* gene (20). However, the *ccrAB* recombinases also act on non-canonical less conserved recombination sites (20). Xiao et al. (33) showed that the *ccrDE* recombinases are functional in excision and transfer of an SCC from *rlmH*, but they do not move together with the cassette. Here, we proved that the Cl_{CCRDE} bearing the *ccrDE* genes is excised from the chromosome. Since Cl_{CCRDE} has a similar size as pathogenicity islands

and possesses a set of genes required for its replication (53), the element would perfectly fit into the phage capsid to be spread via transduction, which is a common transmission route of MGE in staphylococci (54, 55). The high sequence conservation of the *ccrDE* gene complex in various species corresponds with their spread through horizontal gene transfer.

The proportion and diversity of Cl_{ccrDE} was low in the sequenced genomes deposited in the public databases, making it extremely difficult to identify the donor of *ccrDE* complex responsible for the formation of this element. Since we found *ccrD1E1* and *ccrD2E2* predominantly in CoNS genomes, it is possible that CoNS species are predisposed to forming this element and it can subsequently be transferred to *S. aureus*. The variable regions of Cl_{ccrDE} are highly diverse in gene content, suggesting that the Cl_{ccrDE} element evolved only recently in independent acquisition events, which are also common in the evolution of *SCCmec* (56) or staphylococcal pathogenicity islands (57). The benefit of Cl_{ccrDE} for the bacterial host might be only marginal, so there is no selective pressure to maintain established element and disseminate it to more strains. The only widespread genes identified in all Cl_{ccrDE} s that help to preserve the element are the RM system genes that differ in the Cl_{ccrDE} s, suggesting that they come from various sources similar to canonical SCC elements (30, 31).

With the high selective pressure exerted on staphylococci by the use of antimicrobials, this element is a perfect candidate for the acquisition and spread of resistance and virulence genes. The *SCCmec* element underlying the successful spread of MRSA clones originated through joining of *ccr* gene complex with *mec* gene complex coming from multiple sources. The first signs of the accumulation of resistance genes in Cl_{ccrDE} were observed in *S. borealis*, previously misidentified as *S. haemolyticus* (35), where the island comprised several transposons. A similar cluster of drug-resistance genes to that in Cl_{ccrDE} of *S. borealis* was also reported in *S. aureus* on an SCC element (58) and a plasmid (59). We conclude that the gene structure of Cl_{ccrDE} indicates its ability to act as a primordial element to accumulate virulence and antimicrobial resistance factors. The spread of the island to established pathogens such as *S. aureus* would thus represent a new threat to the healthcare system.

Conclusion

The identification of the new species *S. brunensis* within the *Staphylococcus* genus expands our understanding of the diversity of coagulase-negative staphylococci. The number of the strains available is still limited, but similar to its closest relative *S. petrasii*, it can be expected that more strains will be captured as better diagnostic methods are developed. Genome analysis of the new isolates has important implications for studying the role of coagulase-negative staphylococci as a reservoir of transmissible genes that can facilitate improved survival in the environment, resistance to antibiotic treatment, or increased virulence following horizontal transfer. Characterization of a previously unexplored genomic island closely related to the SCC indicates the potential for its interspecies transfer enabled by unique *ccrDE* recombinase genes in both coagulase-negative staphylococci and the more clinically significant *S. aureus*. Identifying the new type of MGE thus opens up new possibilities for future research of gene transfer mechanisms in the emergence of multidrug-resistant staphylococcal strains with implications for clinical practice. These findings deepen our understanding of the evolution and pathogenesis of staphylococci, shedding light on how these bacteria acquire and disseminate virulence traits and antibiotic resistance.

MATERIALS AND METHODS

Bacterial strains, cultivation and phenotypic identification, and antimicrobial susceptibility testing

Simultaneously analyzed reference strains of *Staphylococcus* spp. were obtained from the Czech Collection of Microorganisms (CCM, Masaryk University, Brno). The strains grew well in a basic set of staphylococcal media at a temperature of 30–37°C. The morphological, biochemical, and physiological characterization was performed as previously mentioned (7, 60–62). Antimicrobial susceptibility testing by disc diffusion method on Mueller-Hinton agar with adherence to EUCAST guidelines (63) was performed as described previously (60).

Genome sequencing and bioinformatics analyses

The short-read sequencing was conducted for the type strain NRL/St 16/872^T (LGC Genomics, Berlin, Germany). The genomic DNA was isolated with a GenElute Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA) from pure culture colonies cultivated on Colombia sheep blood agar (Oxoid). The library was prepared using a Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) and sequenced externally by LGC Genomics (Berlin, Germany) on the NextSeq platform with 2 × 150 bp reads (Illumina). Genomes of all novel isolates were sequenced by Oxford Nanopore Technology (ONT). The genomic DNA was extracted as described previously (64). Sequencing libraries were prepared using an SQK-RAD004 rapid barcoding kit and sequenced with a FLO-FLG001 cell in a MinION device and MinKnow v21.10.4 software (Oxford Nanopore Technologies, Oxford, UK).

The software Guppy version 6.0.0 (Oxford Nanopore Technologies) with config dna_r9.4.1_450bps_sup.cfg and default settings was used for basecalling, demultiplexing, and barcode trimming. The ONT reads were filtered by quality mapping to Illumina reads using Filtlong version 0.2.1 (<https://github.com/rrwick/Filtlong>) with a minimum length of 1,500 bp and quality threshold set to 95% and mapping on Illumina reads where applicable. The quality of both long and short reads was assessed with FastQC version 0.11.9 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>) and NanoStat (65). Complete chromosome and partial plasmid sequences were obtained using either a hybrid assembly with Unicycler version 0.4.9 (66) or a long-read-only assembly with Tricycler version 0.5.3 (67). The resulting contigs were further polished with Medaka version 1.6.1 (<https://github.com/nanoporetech/medaka>) and Polypolish version 0.5.0 (68). Sequences were manipulated and inspected in the cross-platform bioinformatics software Ugene version 38.1 (69). The genomes were annotated using the NCBI Prokaryotic Genome Annotation Pipeline (70). Nucleotide and protein multiple sequence alignment was performed with Clustal Omega (71). The multiple sequence alignment was visualized using EasyFig version 2.2.5 (72) and IslandCompare version 1.0 (73). Variable genetic content was identified with PhiSpy version 3.4 (74), MobilomeFinder (75), PlasmidFinder (76), IslandViewer 4 (77), ISFinder (78), and SCCmecFinder (79). The CRISPR/Cas system was characterized by CRISPRCasTyper (80). Virulence and resistance genes were predicted using Abricate (<https://github.com/tseemann/abricate>) with the CARD (81), Resfinder (82), and VFDB (83) databases.

Phylogenetic and pangenomic analyses

The partial 16S rRNA gene was sequenced by Sanger sequencing in the Eurofins MWG Operon sequencing facility (Ebersberg, Germany) with previously described primers (84). Whole-genome sequences of related staphylococcal species were obtained from the NCBI database (Table S1). The multilocus sequence data of six housekeeping genes (*rpoB*, *groEL*, *dnaJ*, *tufA*, *sodA*, and *gap*) that are commonly used in phylogenetic studies of the *Staphylococcaceae* were extracted from whole-genome sequence assemblies as follows (gene coordinates of *S. aureus*): 1420..1974 for *rpoB*, 270..826 for *hsp60*, 23..911 for *dnaJ*, 49..929 for *gap*, 383..1032 for *tufA*, and 50..480 for the *sodA* gene. The phylogenetic

analyses were performed with the software MEGA version 11 (85). The UBCG collection of 92 conservative genes (86), the average nucleotide identity (ANI) (87), and digital DNA-DNA hybridization (dDDH) values by the d4 formula using the web-based genome-to-genome distance calculator (GGDC) version 3.0 (88) were used for calculations of overall genome relatedness indices. The pangenome was calculated with the OrthoVenn2 pipeline with proteins clustered at the default threshold (89).

DNA fingerprinting

For genotypic characterization of the investigated bacterial group, fingerprinting by repetitive sequence-based PCR (rep-PCR) with the primer (GTG)₅ (90) and automated ribotyping with the restriction enzyme *EcoRI* were performed. The isolation of DNA for rep-PCR fingerprinting, PCR conditions, and fingerprint analysis were performed as previously described (91). Automated ribotyping was performed using the RiboPrinter microbial characterization system (DuPont Qualicon) according to the manufacturer's instructions. Numerical analysis of rep-PCR fingerprints and *EcoRI* ribotype patterns was performed using BioNumerics version 7.6 (Applied Maths, Belgium). The ribotype patterns were imported into the BioNumerics software using the load samples import script provided by the manufacturer.

PCR analysis of mobilizable genomic island encoding Ccr recombinases

To determine whether a genomic island is mobilizable, we designed primers spanning the excision site and primers targeting the key genes present in the genomic island (Table S2). The PCR reaction was conducted using Quick-Load 2× master mix with standard buffer (New England Biolabs, Ipswich, MA, USA) and a 200 nM concentration of each primer. The genomic island product was further analyzed by Sanger sequencing (Eurofins Genomics, Germany).

Transmission electron microscopy

A 200-mesh carbon/formvar-coated grid was placed on a drop of suspension of bacteria in water for 20 min. Bacterial cells on the grid were negatively stained with 2% ammonium molybdate and treated with UV light. A Morgagni 268D Philips (ThermoFisher Scientific, The Netherlands) transmission electron microscope was used to visualize bacterial cells.

MALDI-TOF MS

Protein fingerprinting by means of MALDI-TOF MS using an Ultraflextreme instrument (Bruker Daltonics, Germany) was conducted after a standard extraction protocol (92). MALDI-TOF mass spectra were obtained using an Ultraflextreme instrument (Bruker Daltonics) operated in linear positive mode using the software FlexControl version 3.4. Signals present in at least seven out of nine independent mass spectra acquired per sample were taken into account. Mass spectra were processed using FlexAnalysis version 3.4 (Bruker Daltonics) and BioTyper version 3.1 software (Bruker Daltonics) supplemented with database version 10.0.0.0 (9,607 entries).

Chemotaxonomic characterization

Respiratory quinones were extracted and analyzed as previously described (93). Identity was confirmed by mass spectrometry, as described by Schumann et al. (94). Analysis of the cellular fatty acid profile was performed using a Microbial Identification System (MIDI, Newark, DE) according to the Standard Protocol of the Sherlock Microbial Identification System software, version 6.1 (95). The fatty acids were identified using gas chromatography–mass spectrometry (GC-MS) according to the study of Vieira et al. (93).

Isolation and structural analysis of the peptidoglycan was performed according to published protocols with some modifications. Briefly, the amino acid composition of the

total hydrolysate (4 N HCl, 100°C for 16 h) of the peptidoglycan was analyzed by GC/MS (protocol 10 by Schumann [96]). The partial hydrolysate (4 N HCl, 100°C, 0.75 h) of the peptidoglycan was analyzed by high-resolution liquid chromatography–mass spectrometry (LC-MS) as described previously (94, 96). Enantiomeric analysis was performed by liquid chromatography as described by reference 97.

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AUTHOR CONTRIBUTIONS

Vojtěch Kovařovic, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review and editing | Adéla Finstrlová, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review and editing | Ivo Sedláček, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review and editing | Petr Petráš, Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Resources, Validation, Writing – original draft, Writing – review and editing | Pavel Švec, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – review and editing | Ivana Mašlaňová, Data curation, Formal analysis, Investigation, Software, Supervision, Validation, Writing – review and editing | Meina Neumann-Schaal, Conceptualization, Data curation, Investigation, Methodology, Resources, Validation, Visualization, Writing – review and editing | Ondrej Šedo, Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Validation, Visualization, Writing – review and editing | Tibor Botka, Funding acquisition, Investigation, Methodology, Software, Validation, Writing – review and editing | Eva Staňková, Data curation, Investigation, Validation, Writing – review and editing | Jiří Doškař, Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review and editing | Roman Pantůček, Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review and editing

DATA AVAILABILITY

The complete genome sequences of new isolates NRL/St 16/872^T, NRL/St 19/737, NRL/St 18/288, NRL/St 21/187, and NRL/St 22/194 have been deposited in GenBank/ENA/DDJB database under accession numbers [CP119327–CP119331](#), [JALGRI000000000](#), [JALGRH000000000](#), [JALGRG000000000](#), and [CP116597–CP116599](#), respectively. The associated BioProject number is [PRJNA779217](#). The accession number for the 16S rRNA gene of the type strain NRL/St 16/872^T is [OQ401401](#).

ADDITIONAL FILES

The following material is available [online](#).

Supplemental Material

Supplemental tables and figures (Spectrum01342-23-S0001.pdf). Tables S1 to S5, Table S7, Figures S1 to S3.

Table S6 (Spectrum01342-23-S0002.xlsx). Pairwise identity matrices of nucleotide and amino acid sequences of cassette recombinases.

REFERENCES

- Heilmann C, Ziebuhr W, Becker K. 2019. Are coagulase-negative staphylococci virulent? Clin Microbiol Infect 25:1071–1080. <https://doi.org/10.1016/j.cmi.2018.11.012>
- Marincola G, Liong O, Schoen C, Abouelfetouh A, Hamdy A, Wencker FDR, Marciniak T, Becker K, Köck R, Ziebuhr W. 2021. Antimicrobial resistance profiles of coagulase-negative staphylococci in community-based healthy individuals in Germany. Front Public Health 9:684456. <https://doi.org/10.3389/fpubh.2021.684456>
- Takeuchi F, Watanabe S, Baba T, Yuzawa H, Ito T, Morimoto Y, Kuroda M, Cui L, Takahashi M, Ankai A, Baba S, Fukui S, Lee JC, Hiramatsu K. 2005. Whole-genome sequencing of *Staphylococcus haemolyticus* uncovers the extreme plasticity of its genome and the evolution of human-colonizing staphylococcal species. J Bacteriol 187:7292–7308. <https://doi.org/10.1128/JB.187.21.7292-7308.2005>
- Chaudhry V, Patil PB. 2020. Evolutionary insights into adaptation of *Staphylococcus haemolyticus* to human and non-human niches. Genomics 112:2052–2062. <https://doi.org/10.1016/j.ygeno.2019.11.018>
- Pain M, Wolden R, Jaén-Luchoro D, Salvà-Serra F, Iglesias BP, Karlsson R, Klingenberg C, Cavanagh JP. 2020. *Staphylococcus borealis* sp. nov., isolated from human skin and blood. Int J Syst Evol Microbiol 70:6067–6078. <https://doi.org/10.1099/ijsem.0.004499>
- Lin Y-T, Hung W-C, Wan T-W, Li H, Lee T-F, Hsueh P-R, Teng L-J. 2022. *Staphylococcus taiwanensis* sp. nov., isolated from human blood. Int J Syst Evol Microbiol 72. <https://doi.org/10.1099/ijsem.0.005262>
- Pantůček R, Švec P, Dajcs JJ, Machová I, Černohlávková J, Šedo O, Gelbičová T, Mašlaňová I, Doškař J, Zdráhal Z, Růžicková V, Sedláček I. 2013. *Staphylococcus petrasii* sp. nov. including *S. petrasii* subsp. *petrasii* subsp. nov. and *S. petrasii* subsp. *croceilyticus* subsp. nov., isolated from human clinical specimens and human ear infections. Syst Appl Microbiol 36:90–95. <https://doi.org/10.1016/j.syapm.2012.11.004>
- Švec P, De Bel A, Sedláček I, Petráš P, Gelbičová T, Černohlávková J, Mašlaňová I, Cnockaert M, Varbanovová I, Echahidi F, Vandamme P, Pantůček R. 2015. *Staphylococcus petrasii* subsp. *pragensis* subsp. nov., occurring in human clinical material. Int J Syst Evol Microbiol 65:2071–2077. <https://doi.org/10.1099/ijse.0.000220>
- Madhaiyan M, Wirth JS, Saravanan VS. 2020. Phylogenomic analyses of the *Staphylococcaceae* family suggest the reclassification of five species within the genus *Staphylococcus* as heterotypic synonyms, the promotion of five subspecies to novel species, the taxonomic reassignment of five *Staphylococcus* species to *Mammalicoccus* gen. nov., and the formal assignment of *Nosocomiicoccus* to the family *Staphylococcaceae*. Int J Syst Evol Microbiol 70:5926–5936. <https://doi.org/10.1099/ijsem.0.004498>
- Vrbovská V, Kovařovic V, Mašlaňová I, Indráková A, Petráš P, Šedo O, Švec P, Fišarová L, Šiborová M, Mikulášek K, Sedláček I, Doškař J, Pantůček R. 2019. *Staphylococcus petrasii* diagnostics and its pathogenic potential enhanced by mobile genetic elements. Int J Med Microbiol 309:151355. <https://doi.org/10.1016/j.ijmm.2019.151355>
- Uehara Y. 2022. current status of staphylococcal cassette chromosome *mec* (SCCmec). Antibiotics 11:86. <https://doi.org/10.3390/antibiotics11010086>
- Qin L, McCausland JW, Cheung GYC, Otto M. 2016. PSM-Mec-A virulence determinant that connects transcriptional regulation, virulence, and antibiotic resistance in staphylococci. Front Microbiol 7:1293. <https://doi.org/10.3389/fmicb.2016.01293>
- Bleiziffer I, Eikmeier J, Pohlentz G, McAulay K, Xia G, Hussain M, Peschel A, Foster S, Peters G, Heilmann C. 2017. The plasmin-sensitive protein pls in methicillin-resistant *Staphylococcus aureus* (MRSA) is a glycoprotein. PLoS Pathog 13:e1006110. <https://doi.org/10.1371/journal.ppat.1006110>
- Chen H-J, Lin Y-T, Hung W-C, Tsai J-C, Hsueh P-R, Teng L-J. 2016. Distribution of staphylococcal cassette chromosome (SCC) *mec* element types in fusidic acid-resistant *Staphylococcus epidermidis* and identification of a novel SCC7684 element. Antimicrob Agents Chemother 60:5006–5009. <https://doi.org/10.1128/AAC.00231-16>
- Tsubakishita S, Kuwahara-Arai K, Sasaki T, Hiramatsu K. 2010. Origin and molecular evolution of the determinant of methicillin resistance in staphylococci. Antimicrob Agents Chemother 54:4352–4359. <https://doi.org/10.1128/AAC.00356-10>
- Scharn CR, Tenover FC, Goering RV. 2013. Transduction of staphylococcal cassette chromosome *mec* elements between strains of *Staphylococcus aureus*. Antimicrob Agents Chemother 57:5233–5238. <https://doi.org/10.1128/AAC.01058-13>
- Ray MD, Boundy S, Archer GL. 2016. Transfer of the methicillin resistance genomic island among staphylococci by conjugation. Mol Microbiol 100:675–685. <https://doi.org/10.1111/mmi.13340>
- Maree M, Thi Nguyen LT, Ohniwa RL, Higashide M, Msadek T, Morikawa K. 2022. Natural transformation allows transfer of SCCmec-mediated methicillin resistance in *Staphylococcus aureus* biofilms. Nat Commun 13:2477. <https://doi.org/10.1038/s41467-022-29877-2>
- Boundy S, Safo MK, Wang L, Musayev FN, O'Farrell HC, Rife JP, Archer GL. 2013. Characterization of the *Staphylococcus aureus* rRNA methyltransferase encoded by *orfX*, the gene containing the staphylococcal chromosome cassette *mec* (SCCmec) insertion site. J Biol Chem 288:132–140. <https://doi.org/10.1074/jbc.M112.385138>
- Misiura A, Pigli YZ, Boyle-Vavra S, Daum RS, Boocock MR, Rice PA. 2013. Roles of two large serine recombinases in mobilizing the methicillin-resistance cassette SCCmec. Mol Microbiol 88:1218–1229. <https://doi.org/10.1111/mmi.12253>
- Ito T, Ma XX, Takeuchi F, Okuma K, Yuzawa H, Hiramatsu K. 2004. Novel type V staphylococcal cassette chromosome *mec* driven by a novel cassette chromosome recombinase, *crrC*. Antimicrob Agents Chemother 48:2637–2651. <https://doi.org/10.1128/AAC.48.7.2637-2651.2004>
- Takahashi T, Satoh I, Kikuchi N. 1999. Phylogenetic relationships of 38 taxa of the genus *Staphylococcus* based on 16S rRNA gene sequence analysis. Int J Syst Bacteriol 49:725–728. <https://doi.org/10.1099/00207713-49-2-725>
- Pantůček R, Sedláček I, Indráková A, Vrbovská V, Mašlaňová I, Kovařovic V, Švec P, Králová S, Křištofová L, Kekláková J, Petráš P, Doškař J. 2018. *Staphylococcus edaphicus* sp. nov., isolated in Antarctica, harbors the *mecC* gene and genomic islands with a suspected role in adaptation to extreme environments. Appl Environ Microbiol 84:e01746-17. <https://doi.org/10.1128/AEM.01746-17>
- Kim M, Oh HS, Park SC, Chun J. 2014. Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. Int J Syst Evol Microbiol 64:346–351. <https://doi.org/10.1099/ijse.0.059774-0>
- Vrbovská V, Sedláček I, Zeman M, Švec P, Kovařovic V, Šedo O, Laichmanová M, Doškař J, Pantůček R. 2020. Characterization of *Staphylococcus intermedius* group isolates associated with animals from Antarctica and emended description of *Staphylococcus delphini* Microorganisms 8:204. <https://doi.org/10.3390/microorganisms8020204>
- Sabat AJ, Hermelijn SM, Akkerboom V, Juliana A, Degener JE, Grundmann H, Friedrich AW. 2017. Complete-genome sequencing elucidates outbreak dynamics of CA-MRSA USA300 (ST8-spa t008) in an academic hospital of Paramaribo, Republic of Suriname. Sci Rep 7:41050. <https://doi.org/10.1038/srep41050>
- Pumirat P, Boonyuen U, Vanaporn M, Pinweha P, Tandhavanant S, Korbsrisate S, Chantratita N. 2014. The role of short-chain dehydrogenase/oxidoreductase, induced by salt stress, on host interaction of *B. pseudomallei*. BMC Microbiol 14:1. <https://doi.org/10.1186/1471-2180-14-1>

28. North RA, Wahlgren WY, Remus DM, Scalise M, Kessans SA, Dunevall E, Claesson E, Soares da Costa TP, Perugini MA, Ramaswamy S, Allison JR, Indiveri C, Friemann R, Dobson RCJ. 2018. The sodium sialic acid symporter from *Staphylococcus aureus* has altered substrate specificity. *Front Chem* 6:233. <https://doi.org/10.3389/fchem.2018.00233>
29. International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC). 2009. Classification of staphylococcal cassette chromosome mec (SCCmec): guidelines for reporting novel SCCmec elements. *Antimicrob Agents Chemother* 53:4961–4967. <https://doi.org/10.1128/AAC.00579-09>
30. McClure JA, Conly JM, Zhang K. 2021. Characterizing a novel staphylococcal cassette chromosome mec with a composite structure from a clinical strain of *Staphylococcus hominis*, C34847. *Antimicrob Agents Chemother* 65:e00777-21. <https://doi.org/10.1128/AAC.00777-21>
31. Lin Y-T, Tsai J-C, Chen H-J, Hung W-C, Hsueh P-R, Teng L-J. 2014. A novel staphylococcal cassette chromosomal element, SCCfusC, carrying *fusC* and *speG* in fusidic acid-resistant methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother* 58:1224–1227. <https://doi.org/10.1128/AAC.01772-13>
32. Wilson LK, Coombs GW, Christiansen K, Grubb WB, O'Brien FG. 2016. Characterization of a novel staphylococcal cassette chromosome composite island from community-associated MRSA isolated in aged care facilities in Western Australia. *J Antimicrob Chemother* 71:3372–3375. <https://doi.org/10.1093/jac/dkv317>
33. Xiao J, Huang J, Xue X, Wang C, Sun Y, Zheng L, Zhao X, Wang X, Zhao X, Xue H. 2023. Novel cassette chromosome recombinases CcrA8B9 catalyse the excision and integration of the staphylococcal cassette chromosome mec element. *J Antimicrob Chemother* 78:440–444. <https://doi.org/10.1093/jac/dkac410>
34. Vasu K, Nagaraja V. 2013. Diverse functions of restriction-modification systems in addition to cellular defense. *Microbiol Mol Biol Rev* 77:53–72. <https://doi.org/10.1128/MMBR.00044-12>
35. Chen L, Hu JX, Liu C, Liu J, Ma ZB, Tang ZY, Li YF, Zeng ZL. 2021. Identification of the multiresistance gene *poxtA* in oxazolidinone-susceptible *Staphylococcus haemolyticus* and *Staphylococcus saprophyticus* of pig and feed origins. *Pathogens* 10:601. <https://doi.org/10.3390/pathogens10050601>
36. Berbel Caban A, Pak TR, Obla A, Dupper AC, Chacko KI, Fox L, Mills A, Ciferri B, Oussenko I, Beckford C, Chung M, Sebra R, Smith M, Conolly S, Patel G, Kasarskis A, Sullivan MJ, Altman DR, van Bakel H. 2020. PathoSPOT genomic epidemiology reveals under-the-radar nosocomial outbreaks. *Genome Med* 12:96. <https://doi.org/10.1186/s13073-020-00798-3>
37. Ziebuhr W, Hennig S, Eckart M, Kränzler H, Batzilla C, Kozitskaya S. 2006. Nosocomial infections by *Staphylococcus epidermidis*: how a commensal bacterium turns into a pathogen. *Int J Antimicrob Agents* 28 Suppl 1:14–20. <https://doi.org/10.1016/j.ijantimicag.2006.05.012>
38. Foster TJ. 2019. The MSCRAMM family of cell-wall-anchored surface proteins of Gram-positive cocci. *Trends Microbiol* 27:927–941. <https://doi.org/10.1016/j.tim.2019.06.007>
39. Lindsay JA. 2019. Staphylococci: evolving genomes. *Microbiol Spectr* 7:GPP3-0071. <https://doi.org/10.1128/microbiolspec.GPP3-0071-2019>
40. Everitt RG, Didelot X, Batty EM, Miller RR, Knox K, Young BC, Bowden R, Auton A, Votintseva A, Larner-Svensson H, Charlesworth J, Golubchik T, Ip CLC, Godwin H, Fung R, Peto TEA, Walker AS, Crook DW, Wilson DJ. 2014. Mobile elements drive recombination hotspots in the core genome of *Staphylococcus aureus*. *Nat Commun* 5:3956. <https://doi.org/10.1038/ncomms4956>
41. Firth N, Jensen SO, Kwong SM, Skurray RA, Ramsay JP, Fischetti VA, Novick RP, Ferretti JJ, Portnoy DA, Braunstein M, Rood JJ. 2018. Staphylococcal Plasmids, Transposable and integrative elements. *Microbiol Spectr* 6:GPP3-0030. <https://doi.org/10.1128/microbiolspec.GPP3-0030-2018>
42. Shearer JES, Wireman J, Hostetler J, Forberger H, Borman J, Gill J, Sanchez S, Mankin A, Lamarre J, Lindsay JA, Bayles K, Nicholson A, O'Brien F, Jensen SO, Firth N, Skurray RA, Summers AO. 2011. Major families of multiresistant plasmids from geographically and epidemiologically diverse staphylococci. *G3 (Bethesda)* 1:581–591. <https://doi.org/10.1534/g3.111.000760>
43. Caryl JA, Smith MCA, Thomas CD. 2004. Reconstitution of a staphylococcal plasmid-protein relaxation complex *in vitro*. *J Bacteriol* 186:3374–3383. <https://doi.org/10.1128/JB.186.11.3374-3383.2004>
44. Kwong SM, Ramsay JP, Jensen SO, Firth N. 2017. Replication of staphylococcal resistance plasmids. *Front Microbiol* 8:2279. <https://doi.org/10.3389/fmicb.2017.02279>
45. Bender J, Strommenger B, Steglich M, Zimmermann O, Fenner I, Lensing C, Dagwadordsch U, Kekulé AS, Werner G, Lauer F. 2015. Linezolid resistance in clinical isolates of *Staphylococcus epidermidis* from German hospitals and characterization of two *cfr*-carrying plasmids. *J Antimicrob Chemother* 70:1630–1638. <https://doi.org/10.1093/jac/dkv025>
46. Pain M, Hjerde E, Klingenberg C, Cavanagh JP. 2019. Comparative genomic analysis of *Staphylococcus haemolyticus* reveals key to hospital adaptation and pathogenicity. *Front Microbiol* 10:2096. <https://doi.org/10.3389/fmicb.2019.02096>
47. Saroha T, Chaudhry V, Patil PB. 2022. Novel insights into the role of the mobilome in ecological diversification and success of *Staphylococcus haemolyticus* as an opportunistic pathogen. *Microb Genom* 8:000755. <https://doi.org/10.1099/mgen.0.000755>
48. Hiramatsu K, Ito T, Tsubakishita S, Sasaki T, Takeuchi F, Morimoto Y, Katayama Y, Matsuo M, Kuwahara-Arai K, Hishinuma T, Baba T. 2013. Genomic basis for methicillin resistance in *Staphylococcus aureus*. *Infect Chemother* 45:117–136. <https://doi.org/10.3947/ic.2013.45.2.117>
49. Mir-Sanchis I, Roman CA, Misiura A, Pigli YZ, Boyle-Vavra S, Rice PA. 2016. Staphylococcal SCCmec elements encode an active MCM-like helicase and thus may be replicative. *Nat Struct Mol Biol* 23:891–898. <https://doi.org/10.1038/nsmb.3286>
50. Sivertsen A, Janice J, Pedersen T, Wagner TM, Hegstad J, Hegstad K, Ellermeier CD. 2018. The *Enterococcus* cassette chromosome, a genomic variation enabler in enterococci. *mSphere* 3:e00402-18. <https://doi.org/10.1128/mSphere.00402-18>
51. Björkeng EK, Tessema GT, Lundblad EW, Butaye P, Willems R, Sollid JE, Sundsfjord A, Hegstad K. 2010. *ccrABEnt* serine recombinase genes are widely distributed in the *Enterococcus faecium* and *Enterococcus casseliflavus* species groups and are expressed in *E. faecium*. *Microbiol* 156:3624–3634. <https://doi.org/10.1099/mic.0.041491-0>
52. Schwendener S, Perreten V. 2022. The *bla* and *mec* families of beta-lactam resistance genes in the genera *Macrococcus*, *Mammaliococcus* and *Staphylococcus*: an in-depth analysis with emphasis on *Macrococcus*. *J Antimicrob Chemother* 77:1796–1827. <https://doi.org/10.1093/jac/dkac107>
53. Bebel A, Walsh MA, Mir-Sanchis I, Rice PA. 2020. A novel DNA primase-helicase pair encoded by SCCmec elements. *Elife* 9:e55478. <https://doi.org/10.7554/eLife.55478>
54. Zeman M, Mašláňová I, Indráková A, Šiborová M, Mikulášek K, Bendíková K, Plevka P, Vrbovská V, Zdráhal Z, Doškař J, Pantůček R. 2017. *Staphylococcus sciuri* bacteriophages double-convert for staphylokinase and phospholipase, mediate interspecies plasmid transduction, and package *mecA* gene. *Sci Rep* 7:46319. <https://doi.org/10.1038/srep46319>
55. Chlebowicz MA, Mašláňová I, Kuntová L, Grundmann H, Pantůček R, Doškař J, van Dijk JM, Buist G. 2014. The staphylococcal cassette chromosome *mec* type V from *Staphylococcus aureus* ST398 is packaged into bacteriophage capsids. *Int J Med Microbiol* 304:764–774. <https://doi.org/10.1016/j.ijmm.2014.05.010>
56. Rolo J, Worning P, Nielsen JB, Bowden R, Bouchami O, Damborg P, Guardabassi L, Perreten V, Tomasz A, Westh H, de Lencastre H, Miragaia M. 2017. Evolutionary origin of the Staphylococcal cassette Chromosome MEC (SCCmec). *Antimicrob Agents Chemother* 61:e02302–16. <https://doi.org/10.1128/AAC.02302-16>
57. Novick RP, Ram G. 2017. Staphylococcal pathogenicity islands-movers and shakers in the genomic firmament. *Curr Opin Microbiol* 38:197–204. <https://doi.org/10.1016/j.mib.2017.08.001>
58. Jiang N, Li J, Feßler AT, Wang Y, Schwarz S, Wu C. 2019. Novel pseudo-staphylococcal cassette chromosome *mec* element (phiSCCmecT55) in MRSA ST9. *J Antimicrob Chemother* 74:819–820. <https://doi.org/10.1093/jac/dky457>
59. Wendlandt S, Li B, Ma Z, Schwarz S. 2013. Complete sequence of the multi-resistance plasmid pV7037 from a porcine methicillin-resistant *Staphylococcus aureus*. *Vet Microbiol* 166:650–654. <https://doi.org/10.1016/j.vetmic.2013.07.017>

60. Kovačović V, Sedláček I, Petráš P, Králová S, Mašlaňová I, Švec P, Neumann-Schaal M, Botka T, Gelbičová T, Staňková E, Doškař J, Pantůček R. 2022. *Staphylococcus rattii* sp. nov. isolated from a lab rat. *Pathogens* 11:51. <https://doi.org/10.3390/pathogens11010051>
61. Mašlaňová I, Wertheimer Z, Sedláček I, Švec P, Indráková A, Kovačović V, Schumann P, Spröer C, Králová S, Šedo O, Křištofová L, Vrboská V, Fůžik T, Petráš P, Zdráhal Z, Ružicková V, Doškař J, Pantůček R. 2018. Description and comparative genomics of *Macroccoccus caseolyticus* subsp. *hominis* subsp. nov., *Macroccoccus goetzii* sp. nov., *Macroccoccus epidermidis* sp. nov., and *Macroccoccus bohemicus* sp. nov., novel macrococci from human clinical material with virulence potential and suspected uptake of foreign DNA by natural transformation. *Front Microbiol* 9:1178. <https://doi.org/10.3389/fmicb.2018.01178>
62. Freeman DJ, Falkner FR, Keane CT. 1989. New method for detecting slime production by coagulase negative staphylococci. *J Clin Pathol* 42:872–874. <https://doi.org/10.1136/jcp.42.8.872>
63. EUCAST. 2022. Breakpoint tables for interpretation of MICs and zone diameters, in press. The European Committee on Antimicrobial Susceptibility Testing, Version 12. Available from: <http://www.eucast.org>
64. Fišarová L, Botka T, Du X, Mašlaňová I, Bárdy P, Pantůček R, Benešik M, Roudnický P, Winstel V, Larsen J, Rosenstein R, Peschel A, Doškař J. 2021. *Staphylococcus epidermidis* phages transduce antimicrobial resistance plasmids and mobilize chromosomal islands. *mSphere* 6:e00223-21. <https://doi.org/10.1128/mSphere.00223-21>
65. De Coster W, D'Hert S, Schultz DT, Cruts M, Van Broeckhoven C. 2018. NanoPack: visualizing and processing long-read sequencing data. *Bioinformatics* 34:2666–2669. <https://doi.org/10.1093/bioinformatics/bty149>
66. Wick RR, Judd LM, Gorrie CL, Holt KE. 2017. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Comput Biol* 13:e1005595. <https://doi.org/10.1371/journal.pcbi.1005595>
67. Wick RR, Judd LM, Cerdeira LT, Hawkey J, Méric G, Vezina B, Wyres KL, Holt KE. 2021. Tricycler: consensus long-read assemblies for bacterial genomes. *Genome Biol* 22:266. <https://doi.org/10.1186/s13059-021-02483-z>
68. Wick RR, Holt KE. 2022. Polypolish: short-read polishing of long-read bacterial genome assemblies. *PLoS Comput Biol* 18:e1009802. <https://doi.org/10.1371/journal.pcbi.1009802>
69. Okonechnikov K, Golosova O, Fursov M, UGENE team. 2012. Unipro UGENE: a unified bioinformatics toolkit. *Bioinformatics* 28:1166–1167. <https://doi.org/10.1093/bioinformatics/bts091>
70. Li W, O'Neill KR, Haft DH, DiCuccio M, Chetvernin V, Badretdin A, Coulouris G, Chitsaz F, Derbyshire MK, Durkin AS, Gonzales NR, Gwazd M, Lanczycki CJ, Song JS, Thanki N, Wang J, Yamashita RA, Yang M, Zheng C, Marchler-Bauer A, Thibaud-Nissen F. 2021. RefSeq: expanding the prokaryotic genome annotation pipeline reach with protein family model curation. *Nucleic Acids Res* 49:D1020–D1028. <https://doi.org/10.1093/nar/gkaa1105>
71. Madeira F, Pearce M, Tivey ARN, Basutkar P, Lee J, Edbali O, Madhusoodanan N, Kolesnikov A, Lopez R. 2022. Search and sequence analysis tools services from EMBL-EBI in 2022. *Nucleic Acids Res* 50:W276–W279. <https://doi.org/10.1093/nar/gkac240>
72. Sullivan MJ, Petty NK, Beatson SA. 2011. Easyfig: a genome comparison visualizer. *Bioinformatics* 27:1009–1010. <https://doi.org/10.1093/bioinformatics/btr039>
73. Bertelli C, Gray KL, Woods N, Lim AC, Tilley KE, Winsor GL, Hoad GR, Roudgar A, Spencer A, Peltier J, Warren D, Raphenya AR, McArthur AG, Brinkman FSL. 2022. Enabling genomic island prediction and comparison in multiple genomes to investigate bacterial evolution and outbreaks. *Microb Genom* 8:mgen000818. <https://doi.org/10.1099/mgen.0.000818>
74. Edwards R, Decewicz P, Katelyn D, Laurasisk S. 2021. Linsalrob/Phispy: Dropped Prophages (v.4.2.19). Zenodo. <https://doi.org/10.5281/zenodo.5070204>
75. Ou H-Y, He X, Harrison EM, Kulasekara BR, Thani AB, Kadioglu A, Lory S, Hinton JCD, Barer MR, Deng Z, Rajakumar K. 2007. MobilomeFINDER: web-based tools for in silico and experimental discovery of bacterial genomic islands. *Nucleic Acids Res* 35:W97–W104. <https://doi.org/10.1093/nar/gkm380>
76. Carattoli A, Zankari E, García-Fernández A, Voldby Larsen M, Lund O, Villa L, Møller Aarestrup F, Hasman H. 2014. In silico detection and typing of plasmids using plasmidfinder and plasmid multilocus sequence typing. *Antimicrob Agents Chemother* 58:3895–3903. <https://doi.org/10.1128/AAC.02412-14>
77. Bertelli C, Laird MR, Williams KP, Simon Fraser University Research Computing Group, Lau BY, Hoad G, Winsor GL, Brinkman FSL. 2017. IslandViewer 4: expanded prediction of genomic islands for larger-scale datasets. *Nucleic Acids Res* 45:W30–W35. <https://doi.org/10.1093/nar/gkx343>
78. Siguié P, Perochon J, Lestrade L, Mahillon J, Chandler M. 2006. Isfinder the reference centre for bacterial insertion sequences. *Nucleic Acids Res* 34:D32–6. <https://doi.org/10.1093/nar/gkj014>
79. Kaya H, Hasman H, Larsen J, Stegger M, Johannesen TB, Allesøe RL, Lemvig CK, Aarestrup FM, Lund O, Larsen AR, Limbago BM. 2018. Scmecfinder, a web-based tool for typing of Staphylococcal cassette Chromosome MEC in *Staphylococcus aureus* using whole-genome sequence data. *mSphere* 3:e00612–17. <https://doi.org/10.1128/mSphere.00612-17>
80. Russel J, Pinilla-Redondo R, Mayo-Muñoz D, Shah SA, Sørensen SJ. 2020. CRISPRCasTyper: automated identification, annotation, and classification of CRISPR-Cas loci. *CRISPR J* 3:462–469. <https://doi.org/10.1089/crispr.2020.0059>
81. Jia B, Raphenya AR, Alcock B, Wagglechner N, Guo P, Tsang KK, Lago BA, Dave BM, Pereira S, Sharma AN, Doshi S, Courtot M, Lo R, Williams LE, Frye JG, Elsayegh T, Sardar D, Westman EL, Pawlowski AC, Johnson TA, Brinkman FSL, Wright GD, McArthur AG. 2017. CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. *Nucleic Acids Res* 45:D566–D573. <https://doi.org/10.1093/nar/gkw1004>
82. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, Aarestrup FM, Larsen MV. 2012. Identification of acquired antimicrobial resistance genes. *J Antimicrob Chemother* 67:2640–2644. <https://doi.org/10.1093/jac/dks261>
83. Chen L, Zheng D, Liu B, Yang J, Jin Q. 2016. VFDB 2016: hierarchical and refined dataset for big data analysis-10 years on. *Nucleic Acids Res* 44:D694–D697. <https://doi.org/10.1093/nar/gkv1239>
84. Pantůček R, Sedláček I, Petráš P, Koukalová D, Švec P, Štětina V, Vancanneyt M, Chrástínová L, Vokurková J, Ružicková V, Doškař J, Swings J, Hájek V. 2005. *Staphylococcus simiae* sp. nov., isolated from South American squirrel monkeys. *Int J Syst Evol Microbiol* 55:1953–1958. <https://doi.org/10.1099/ijs.0.63590-0>
85. Tamura K, Stecher G, Kumar S, Battistuzzi FU. 2021. Mega11: molecular evolutionary genetics analysis version 11. *Mol Biol Evol* 38:3022–3027. <https://doi.org/10.1093/molbev/msab120>
86. Na SI, Kim YO, Yoon SH, Ha SM, Baek I, Chun J. 2018. UBCC: up-to-date bacterial core gene set and pipeline for phylogenomic tree reconstruction. *J Microbiol* 56:280–285. <https://doi.org/10.1007/s12275-018-8014-6>
87. Jain C, Rodriguez-R LM, Phillippy AM, Konstantinidis KT, Aluru S. 2018. High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. *Nat Commun* 9:5114. <https://doi.org/10.1038/s41467-018-07641-9>
88. Meier-Kolthoff JP, Klenk H-P, Göker M. 2014. Taxonomic use of DNA G+C content and DNA-DNA hybridization in the genomic age. *Int J Syst Evol Microbiol* 64:352–356. <https://doi.org/10.1099/ijs.0.056994-0>
89. Xu L, Dong Z, Fang L, Luo Y, Wei Z, Guo H, Zhang G, Gu YQ, Coleman-Derr D, Xia Q, Wang Y. 2019. OrthoVenn2: a web server for whole-genome comparison and annotation of orthologous clusters across multiple species. *Nucleic Acids Res* 47:W52–W58. <https://doi.org/10.1093/nar/gkz333>
90. Versalovic J, Schneider M, de Bruijn FJ, Lupski JR. 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods Mol Cell Biol* 5:25–40.
91. Švec P, Pantůček R, Petráš P, Sedláček I, Nováková D. 2010. Identification of *Staphylococcus* spp. using (GTG)₅-PCR fingerprinting. *Syst Appl Microbiol* 33:451–456. <https://doi.org/10.1016/j.syapm.2010.09.004>
92. Freiwald A, Sauer S. 2009. Phylogenetic classification and identification of bacteria by mass spectrometry. *Nat Protoc* 4:732–742. <https://doi.org/10.1038/nprot.2009.37>
93. Vieira S, Huber KJ, Neumann-Schaal M, Geppert A, Luckner M, Wanner G, Overmann J. 2021. *Usitatibacter rugosus* gen. nov., sp. nov. and *Usitatibacter palustris* sp. nov., novel members of *Usitatibacteraceae* fam

- nov. within the order *Nitrosomonadales* isolated from soil. *Int J Syst Evol Microbiol* 71:004631. <https://doi.org/10.1099/ijsem.0.004631>
94. Schumann P, Kalensee F, Cao J, Criscuolo A, Clermont D, Köhler JM, Meier-Kolthoff JP, Neumann-Schaal M, Tindall BJ, Pukall R. 2021. Reclassification of *Haloactinobacterium glacieicola* as *Occultella glacieicola* gen. nov., comb. nov., of *Haloactinobacterium album* as *Ruania alba* comb. nov., with an emended description of the genus *Ruania*, recognition that the genus names *Haloactinobacterium* and *Ruania* are heterotypic synonyms and description of *Occultella aeris* sp. nov., a halotolerant isolate from surface soil sampled at an ancient copper smelter. *Int J Syst Evol Microbiol* 71:004769. <https://doi.org/10.1099/ijsem.0.004769>
 95. Sasser M. 1990. MIDI Technical Note 101, Revision July 2006 ed, *in press* Identification of bacteria by gas chromatography of cellular fatty acids MIDI Inc, Newark, DE. http://midi-inc.com/pdf/MIS_Technote_101.pdf.
 96. Schumann P. 2011. Peptidoglycan structure. *Methods Microbiol* 38:101–129. <https://doi.org/10.1016/b978-0-12-387730-7.00005-x>
 97. Kämpfer P, McInroy JA, Clermont D, Neumann-Schaal M, Criscuolo A, Busse H-J, Glaeser SP. 2021. *Leucobacter soli* sp. nov., from soil amended with humic acid. *Int J Syst Evol Microbiol* 71:005156. <https://doi.org/10.1099/ijsem.0.005156>

5 Summary

Coagulase-negative staphylococci (CoNS) are a diverse group that inhabit a wide range of ecological niches. Recent novel taxa were predominantly isolated from bloodstream infections. In role of pathogen, they significantly impact human and animal health due to their ability to acquire and transmit antimicrobial resistance genes. The intricate network of horizontal gene transfer (HGT) among genera *Staphylococcus* and related *Micrococcus* facilitates the rapid dissemination of the antimicrobial resistance genes. This constant evolution of pathogens poses a significant challenge to human and animal health, as it necessitates the development of new antimicrobial treatments that can effectively combat emerging multidrug-resistant strains.

As a significant part of a polyphasic taxonomic approach and to overcome limitations in traditional phylogenetic and phenotypic methods, whole genome sequencing was utilized to characterize strains of CoNS and micrococci originating from diverse environments, including Antarctic soil, human clinical materials, and animal hosts. As a result, three new species of CoNS were identified, belonging to different branches of the *Staphylococcus* genus. Comparative genomics revealed that CoNS strains harbored a diverse range of mobile genetic elements (MGEs). These elements play a crucial role in evolution by facilitating the transfer of genes associated with virulence, antibiotic resistance, and environmental adaptation. This thesis also identified several candidate antibiotic resistance genes in phage-inducible resistance islands. The detailed description of present SCC elements and related genomic islands have contributed to our understanding of the evolution and mechanism of transmission of clinically relevant *SCCmec*, which is prevalent in many pathogens.

Reduced sequencing costs lead to an increase in bacterial genomes deposited in databases, but user-friendly tools for genomic analyses for microbiologists are lacking. This work emphasizes the importance of comprehensive annotation of all MGEs to study HGT and evolution, which directly influence pathogenicity, a crucial aspect often neglected in deposited sequences. A pipeline was developed to streamline and unify the specialized annotation of multiple available tools. This pipeline is envisioned as modular, allowing for the seamless integration of emerging bioinformatics methods, which will utilize better sequence databases, advanced algorithms, and neural networks. However, verifying candidate virulence and resistance genes in follow-up laboratory studies remains essential.

References

- Ahrenfeldt, J., Skaarup, C., Hasman, H., Pedersen, A. G., Aarestrup, F. M., and Lund, O. (2017). Bacterial whole genome-based phylogeny: Construction of a new benchmarking dataset and assessment of some existing methods. *BMC Genomics*, 18(1), 19. <https://doi.org/10.1186/s12864-016-3407-6>
- Akarsu, H., Liljander, A., Younan, M., Brodard, I., Overesch, G., Glücks, I., Labroussaa, F., Kuhnert, P., Perreten, V., Monecke, S., Drexler, J. F., Corman, V. M., Falquet, L., and Jores, J. (2022). Genomic Characterization and Antimicrobial Susceptibility of Dromedary-Associated Staphylococcaceae from the Horn of Africa. *Applied and Environmental Microbiology*, 88(21), e01146-22. <https://doi.org/10.1128/aem.01146-22>
- Al Masalma, M., Raoult, D., and Roux, V. (2010). Staphylococcus massiliensis sp. Nov., isolated from a human brain abscess. *International Journal of Systematic and Evolutionary Microbiology*, 60(5), 1066–1072. <https://doi.org/10.1099/ijs.0.006486-0>
- Alcock, B. P., Huynh, W., Chalil, R., Smith, K. W., Raphenya, A. R., Wlodarski, M. A., Edalatmand, A., Petkau, A., Syed, S. A., Tsang, K. K., Baker, S. J. C., Dave, M., McCarthy, M. C., Mukiri, K. M., Nasir, J. A., Golbon, B., Imtiaz, H., Jiang, X., Kaur, K., ... McArthur, A. G. (2023). CARD 2023: Expanded curation, support for machine learning, and resistome prediction at the Comprehensive Antibiotic Resistance Database. *Nucleic Acids Research*, 51(1), 690–699. <https://doi.org/10.1093/nar/gkac920>
- Almagro Armenteros, J. J., Tsirigos, K. D., Sønderby, C. K., Petersen, T. N., Winther, O., Brunak, S., von Heijne, G., and Nielsen, H. (2019). SignalP 5.0 improves signal peptide predictions using deep neural networks. *Nature Biotechnology*, 37(4), 420–423. <https://doi.org/10.1038/s41587-019-0036-z>
- Argemi, X., Hansmann, Y., Prola, K., and Prévost, G. (2019). Coagulase-Negative Staphylococci Pathogenomics. *International Journal of Molecular Sciences*, 20(5), 1215. <https://doi.org/10.3390/ijms20051215>
- Argemi, X., Matelska, D., Ginalski, K., Riegel, P., Hansmann, Y., Bloom, J., Pestel-Caron, M., Dahyot, S., Lebeurre, J., and Prévost, G. (2018). Comparative genomic analysis of Staphylococcus lugdunensis shows a closed pan-genome and multiple barriers to horizontal gene transfer. *BMC Genomics*, 19(1), 621. <https://doi.org/10.1186/s12864-018-4978-1>
- Arndt, D., Marcu, A., Liang, Y., and Wishart, D. S. (2019). PHAST, PHASTER and PHASTEST: Tools for finding prophage in bacterial genomes. *Briefings in Bioinformatics*, 20(4), 1560–1567. <https://doi.org/10.1093/bib/bbx121>
- Asai, N., Sakanashi, D., Suematsu, H., Kato, H., Hagihara, M., Watanabe, H., Shiota, A., Koizumi, Y., Yamagishi, Y., and Mikamo, H. (2021). Clinical characteristics and relevance of coagulase-negative Staphylococci other than S. epidermidis by positive blood culture. *Journal of Microbiology, Immunology and Infection*, 54(4), 632–638. <https://doi.org/10.1016/j.jmii.2020.03.001>

REFERENCES

- Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., Davis, A. P., Dolinski, K., Dwight, S. S., Eppig, J. T., Harris, M. A., Hill, D. P., Issel-Tarver, L., Kasarskis, A., Lewis, S., Matese, J. C., Richardson, J. E., Ringwald, M., Rubin, G. M., and Sherlock, G. (2000). Gene Ontology: Tool for the unification of biology. *Nature Genetics*, 25(1), 25–29. <https://doi.org/10.1038/75556>
- Ba, X., Matuszewska, M., Kalmar, L., Fan, J., Zou, G., Corander, D., Raisen, C. L., Li, S., Li, L., Weinert, L. A., Tucker, A. W., Grant, A. J., Zhou, R., and Holmes, M. A. (2023). High-Throughput Mutagenesis Reveals a Role for Antimicrobial Resistance- and Virulence-Associated Mobile Genetic Elements in *Staphylococcus aureus* Host Adaptation. *Microbiology Spectrum*, 11(2), e04213-22. <https://doi.org/10.1128/spectrum.04213-22>
- Baddiley, J., Buchanan, J. G., Rajbhandary, U. L., and Sanderson, A. R. (1962). Teichoic acid from the walls of *Staphylococcus aureus* H. Structure of the N-acetylglucosaminyldribitol residues. *Biochemical Journal*, 82(3), 439. <https://doi.org/10.1042/bj0820439>
- Baig, S., Johannesen, T. B., Overballe-Petersen, S., Larsen, J., Larsen, A. R., and Stegger, M. (2018). Novel SCCmec type XIII (9A) identified in an ST152 methicillin-resistant *Staphylococcus aureus*. *Infection, Genetics and Evolution*, 61, 74–76. <https://doi.org/10.1016/j.meegid.2018.03.013>
- Bannerman, T. L., Hancock, G. A., Tenover, F. C., and Miller, J. M. (1995). Pulsed-field gel electrophoresis as a replacement for bacteriophage typing of *Staphylococcus aureus*. *Journal of Clinical Microbiology*, 33(3), 551–555. <https://doi.org/10.1128/jcm.33.3.551-555.1995>
- Barber, M. (1947). Staphylococcal Infection due to Penicillin-resistant Strains. *British Medical Journal*, 2(4534), 863–865. <https://doi.org/10.1136/bmj.2.4534.863>
- Bartlett, A., Padfield, D., Lear, L., Bendall, R., and Vos, M. (2022). A comprehensive list of bacterial pathogens infecting humans. *Microbiology*, 168(12). <https://doi.org/10.1099/mic.0.001269>
- Becker, K. (2021). Methicillin-Resistant Staphylococci and Macrococci at the Interface of Human and Animal Health. *Toxins*, 13(1), 61. <https://doi.org/10.3390/toxins13010061>
- Becker, K., Both, A., Weißelberg, S., Heilmann, C., and Rohde, H. (2020). Emergence of coagulase-negative staphylococci. *Expert Review of Anti-Infective Therapy*, 18(4), 349–366. <https://doi.org/10.1080/14787210.2020.1730813>
- Becker, K., Heilmann, C., and Peters, G. (2014). Coagulase-negative staphylococci. *Clinical Microbiology Reviews*, 27(4), 870–926. <https://doi.org/10.1128/CMR.00109-13>
- Becker, K., Pagnier, I., Schuhen, B., Wenzelburger, F., Friedrich, A. W., Kipp, F., Peters, G., and von Eiff, C. (2006). Does Nasal Cocolonization by Methicillin-Resistant Coagulase-Negative Staphylococci and Methicillin-Susceptible *Staphylococcus aureus* Strains Occur Frequently Enough To Represent a Risk of False-Positive Methicillin-Resistant *S. aureus* Determinations by Molecular Methods? *Journal of Clinical Microbiology*, 44(1), 229–231. <https://doi.org/10.1128/jcm.44.1.229-231.2006>

- Bello, S., Mudassir, S. H., Rudra, B., and Gupta, R. S. (2023). Phylogenomic and molecular markers based studies on Staphylococcaceae and Gemella species. Proposals for an emended family Staphylococcaceae and three new families (Abyssicoccaceae fam. Nov., Salinicoccaceae fam. Nov. And Gemellaceae fam. Nov.) harboring four new genera, Lacicoccus gen. Nov., Macrococcoides gen. Nov., Gemelliphila gen. Nov., and Phocicoccus gen. Nov. *Antonie Van Leeuwenhoek*, 116(10), 937–973. <https://doi.org/10.1007/s10482-023-01857-6>
- Benson, D. A., Cavanaugh, M., Clark, K., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J., and Sayers, E. W. (2013). GenBank. *Nucleic Acids Research*, 41(1), 36–42. <https://doi.org/10.1093/nar/gks1195>
- Berends, M. S., Luz, C. F., Ott, A., Andriesse, G. I., Becker, K., Glasner, C., and Friedrich, A. W. (2022). Trends in Occurrence and Phenotypic Resistance of Coagulase-Negative Staphylococci (CoNS) Found in Human Blood in the Northern Netherlands between 2013 and 2019. *Microorganisms*, 10(9), 1801. <https://doi.org/10.3390/microorganisms10091801>
- Bertelli, C., Laird, M. R., Williams, K. P., Simon Fraser University Research Computing Group, Lau, B. Y., Hoad, G., Winsor, G. L., and Brinkman, F. S. (2017). IslandViewer 4: Expanded prediction of genomic islands for larger-scale datasets. *Nucleic Acids Research*, 45(W1), W30–W35. <https://doi.org/10.1093/nar/gkx343>
- Blair, J. E. (1939). *The Pathogenic Staphylococci*. 3(1), 97–146. <https://doi.org/10.1128/br.3.1.97-146.1939>
- Bosi, E., Donati, B., Galardini, M., Brunetti, S., Sagot, M.-F., Lió, P., Crescenzi, P., Fani, R., and Fondi, M. (2015). MeDuSa: A multi-draft based scaffold. *Bioinformatics*, 31(15), 2443–2451. <https://doi.org/10.1093/bioinformatics/btv171>
- Brumfitt, W., and Hamilton-Miller, J. (1989). Methicillin-Resistant Staphylococcus aureus. *New England Journal of Medicine*, 320(18), 1188–1196. <https://doi.org/10.1056/NEJM198905043201806>
- Carey, G. B., Holleck, J. L., Ein Alshaeba, S., Jayakrishnan, R., Gordon, K. S., Grimshaw, A. A., and Gunderson, C. G. (2023). Estimated mortality with early empirical antibiotic coverage of methicillin-resistant Staphylococcus aureus in hospitalized patients with bacterial infections: A systematic review and meta-analysis. *Journal of Antimicrobial Chemotherapy*, 78(5), 1150–1159. <https://doi.org/10.1093/jac/dkad078>
- Carter, G. P., Ussher, J. E., Da Silva, A. G., Baines, S. L., Heffernan, H., Riley, T. V., Broadbent, R., van der Linden, A., Lee, J., Monk, I. R., Stinear, T. P., Howden, B. P., and Williamson, D. A. (2018). Genomic Analysis of Multiresistant Staphylococcus capitis Associated with Neonatal Sepsis. *Antimicrobial Agents and Chemotherapy*, 62(11). <https://doi.org/10.1128/aac.00898-18>
- Casadesús, J., and Low, D. (2006). Epigenetic Gene Regulation in the Bacterial World. *Microbiology and Molecular Biology Reviews*, 70(3), 830–856. <https://doi.org/10.1128/mnbr.00016-06>
- Chambers, H. F. (1988). Methicillin-resistant staphylococci. *Clinical Microbiology Reviews*, 1(2), 173–186. <https://doi.org/10.1128/cmr.1.2.173>

REFERENCES

- Chesneau, O., Morvan, A., Grimont, F., Labischinski, H., and El Solh, N. (1993). *Staphylococcus pasteurii* sp. Nov., Isolated from Human, Animal, and Food Specimens. *International Journal of Systematic Bacteriology*, 43(2), 237–244. <https://doi.org/10.1099/00207713-43-2-237>
- Chew, K. L., Octavia, S., Lai, D., Lin, R. T. P., and Teo, J. W. P. (2021). *Staphylococcus singaporensis* sp. Nov., a new member of the *Staphylococcus aureus* complex, isolated from human clinical specimens. *International Journal of Systematic and Evolutionary Microbiology*, 71(10). <https://doi.org/10.1099/ijsem.0.005067>
- Cho, G.-S., Li, B., Brinks, E., and Franz, C. M. A. P. (2022). Characterization of antibiotic-resistant, coagulase-negative staphylococci from fresh produce and description of *Staphylococcus shinii* sp. Nov. Isolated from chives. *Journal of Microbiology*, 60(9), 877–889. <https://doi.org/10.1007/s12275-022-2100-5>
- Chong, C. E., Bengtsson, R. J., and Horsburgh, M. J. (2022). Comparative genomics of *Staphylococcus capitis* reveals species determinants. *Frontiers in Microbiology*, 13, 1005949. <https://doi.org/10.3389/fmicb.2022.1005949>
- Clark, T. A., Murray, I. A., Morgan, R. D., Kislyuk, A. O., Spittle, K. E., Boitano, M., Fomenkov, A., Roberts, R. J., and Korlach, J. (2012). Characterization of DNA methyltransferase specificities using single-molecule, real-time DNA sequencing. *Nucleic Acids Research*, 40(4), e29. <https://doi.org/10.1093/nar/gkr1146>
- Cole, K., Foster, D., Russell, J. E., Golubchik, T., Llewelyn, M., Wilson, D. J., Crook, D., and Paul, J. (2019). Draft Genome Sequences of 64 Type Strains of 50 Species and 25 Subspecies of the Genus *Staphylococcus* Rosenbach 1884. *Microbiology Resource Announcements*, 8(17), 10.1128/mra.00062-19. <https://doi.org/10.1128/mra.00062-19>
- Colombo, S., Arioli, S., Neri, E., Della Scala, G., Gargari, G., and Mora, D. (2017). Viromes As Genetic Reservoir for the Microbial Communities in Aquatic Environments: A Focus on Antimicrobial-Resistance Genes. *Frontiers in Microbiology*, 8, 1095. <https://doi.org/10.3389/fmicb.2017.01095>
- Conlon, K. M., Humphreys, H., and O’Gara, J. P. (2004). Inactivations of *rsbU* and *sarA* by IS256 Represent Novel Mechanisms of Biofilm Phenotypic Variation in *Staphylococcus epidermidis*. *Journal of Bacteriology*, 186(18), 6208–6219. <https://doi.org/10.1128/jb.186.18.6208-6219.2004>
- Copin, R., Shopsin, B., and Torres, V. J. (2018). After the deluge: Mining *Staphylococcus aureus* genomic data for clinical associations and host–pathogen interactions. *Current Opinion in Microbiology*, 41, 43–50. <https://doi.org/10.1016/j.mib.2017.11.014>
- Coudert, E., Gehant, S., de Castro, E., Pozzato, M., Baratin, D., Neto, T., Sigrist, C. J. A., Redaschi, N., Bridge, A., and The UniProt Consortium. (2023). Annotation of biologically relevant ligands in UniProtKB using ChEBI. *Bioinformatics*, 39(1), btac793. <https://doi.org/10.1093/bioinformatics/btac793>
- Couto, I., Wu, S. W., Tomasz, A., and de Lencastre, H. (2003). Development of Methicillin Resistance in Clinical Isolates of *Staphylococcus sciuri* by Transcriptional Activation of the *mecA* Homologue Native to the Species. *Journal of Bacteriology*, 185(2), 645–653. <https://doi.org/10.1128/jb.185.2.645-653.2003>

- Couvin, D., Bernheim, A., Toffano-Nioche, C., Touchon, M., Michalik, J., Néron, B., Rocha, E. P. C., Vergnaud, G., Gautheret, D., and Pourcel, C. (2018). CRISPRCasFinder, an update of CRISRFinder, includes a portable version, enhanced performance and integrates search for Cas proteins. *Nucleic Acids Research*, 46(W1), W246–W251. <https://doi.org/10.1093/nar/gky425>
- Cunliffe, A. C., Gillam, G. G., and Williams, R. (1943). Bacterial Endocarditis associated with a Coagulase-Negative Staphylococcus albus. *Lancet*, 242(6264), 355. [https://doi.org/10.1016/S0140-6736\(00\)72550-5](https://doi.org/10.1016/S0140-6736(00)72550-5)
- de la Bastide, M., and McCombie, W. R. (2007). Assembling Genomic DNA Sequences with PHRAP. *Current Protocols in Bioinformatics*, 17(1), 11.4.1–11.4.15. <https://doi.org/10.1002/0471250953.bi1104s17>
- Devriese, L. A., Hajek, V., Oeding, P., Meyer, S. A., and Schleifer, K. H. (1978). Staphylococcus hyicus (Sompolinsky 1953) comb. Nov. And Staphylococcus hyicus subsp. Chromogenes subsp. Nov. *International Journal of Systematic Bacteriology*, 28(4), 482–490. <https://doi.org/10.1099/00207713-28-4-482>
- Devriese, L. A., Poutrel, B., Kilpper-Balz, R., and Schleifer, K. H. (1983). Staphylococcus gallinarum and Staphylococcus caprae, Two New Species from Animals. *International Journal of Systematic Bacteriology*, 33(3), 480–486. <https://doi.org/10.1099/00207713-33-3-480>
- Devriese, L. A., Vancanneyt, M., Baele, M., Vaneechoutte, M., De Graef, E., Snauwaert, C., Cleenwerck, I., Dawyndt, P., Swings, J., Decostere, A., and Haesebrouck, F. (2005). Staphylococcus pseudintermedius sp. Nov., a coagulase-positive species from animals. *International Journal of Systematic and Evolutionary Microbiology*, 55(4), 1569–1573. <https://doi.org/10.1099/ijs.0.63413-0>
- Di Tommaso, P., Chatzou, M., Floden, E. W., Barja, P. P., Palumbo, E., and Notredame, C. (2017). Nextflow enables reproducible computational workflows. *Nature Biotechnology*, 35(4), 316–319. <https://doi.org/10.1038/nbt.3820>
- Donlan, R. M. (2002). Biofilms: Microbial life on surfaces. *Emerging Infectious Diseases*, 8(9), 881–890. <https://doi.org/10.3201/eid0809.020063>
- Doron, S., Melamed, S., Ofir, G., Leavitt, A., Lopatina, A., Keren, M., Amitai, G., and Sorek, R. (2018). Systematic discovery of anti-phage defense systems in the microbial pan-genome. *Science (New York, N.Y.)*, 359(6379), eaar4120. <https://doi.org/10.1126/science.aar4120>
- Doster, E., Lakin, S. M., Dean, C. J., Wolfe, C., Young, J. G., Boucher, C., Belk, K. E., Noyes, N. R., and Morley, P. S. (2020). MEGARes 2.0: A database for classification of antimicrobial drug, biocide and metal resistance determinants in metagenomic sequence data. *Nucleic Acids Research*, 48(1), 561–569. <https://doi.org/10.1093/nar/gkz1010>
- Douglas, E. J. A., Wulandari, S. W., Lovell, S. D., and Laabei, M. (2023). Novel antimicrobial strategies to treat multi-drug resistant Staphylococcus aureus infections. *Microbial Biotechnology*, 16(7), 1456–1474. <https://doi.org/10.1111/1751-7915.14268>
- Durrant, M. G., Li, M. M., Siranosian, B. A., Montgomery, S. B., and Bhatt, A. S. (2020). A bioinformatic analysis of integrative mobile genetic elements highlights their role in

- bacterial adaptation. *Cell Host & Microbe*, 27(1), 140-153.e9.
<https://doi.org/10.1016/j.chom.2019.10.022>
- Edgar, R. C. (2004). MUSCLE: A multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics*, 5(1), 113. <https://doi.org/10.1186/1471-2105-5-113>
- Eichel, V., Papan, C., and Mutters, N. T. (2019). [Update Hygiene: Prevention of Vascular Catheter-Associated Infections in Premature and Newborn Infants]. *Klinische Padiatrie*, 231(4), 177–182. <https://doi.org/10.1055/a-0883-5350>
- El-Halfawy, O. M., and Valvano, M. A. (2015). Antimicrobial Heteroresistance: An Emerging Field in Need of Clarity. *Clinical Microbiology Reviews*, 28(1), 191–207.
<https://doi.org/10.1128/cmr.00058-14>
- Enström, J., Fröding, I., Giske, C. G., Ininbergs, K., Bai, X., Sandh, G., Tollström, U.-B., Ullberg, M., and Fang, H. (2018). USA300 methicillin-resistant *Staphylococcus aureus* in Stockholm, Sweden, from 2008 to 2016. *PLoS ONE*, 13(11), e0205761.
<https://doi.org/10.1371/journal.pone.0205761>
- Evans, A. C. (1916). The Bacteria of Milk Freshly Drawn from Normal Udders. *The Journal of Infectious Diseases*, 18(5), 437–476. <https://doi.org/10.1093/infdis/18.5.437>
- Everitt, R. G., Didelot, X., Batty, E. M., Miller, R. R., Knox, K., Young, B. C., Bowden, R., Auton, A., Votintseva, A., Larnier-Svensson, H., Charlesworth, J., Golubchik, T., Ip, C. L. C., Godwin, H., Fung, R., Peto, T. E. A., Walker, A. S., Crook, D. W., and Wilson, D. J. (2014). Mobile elements drive recombination hotspots in the core genome of *Staphylococcus aureus*. *Nature Communications*, 5(1), 3956. <https://doi.org/10.1038/ncomms4956>
- Fairbrother, R. W. (1940). Coagulase production as a criterion for the classification of the staphylococci. *The Journal of Pathology and Bacteriology*, 50(1), 83–88.
<https://doi.org/10.1002/path.1700500112>
- Feldgarden, M., Brover, V., Haft, D. H., Prasad, A. B., Slotta, D. J., Tolstoy, I., Tyson, G. H., Zhao, S., Hsu, C.-H., McDermott, P. F., Tadesse, D. A., Morales, C., Simmons, M., Tillman, G., Wasilenko, J., Folster, J. P., and Klimke, W. (2019). Validating the AMRFinder Tool and Resistance Gene Database by Using Antimicrobial Resistance Genotype-Phenotype Correlations in a Collection of Isolates. *Antimicrobial Agents and Chemotherapy*, 63(11), e00483-19. <https://doi.org/10.1128/AAC.00483-19>
- Firth, N., Jensen, S. O., Kwong, S. M., Skurray, R. A., and Ramsay, J. P. (2018). Staphylococcal Plasmids, Transposable and Integrative Elements. *Microbiology Spectrum*, 6(6).
<https://doi.org/10.1128/microbiolspec.gpp3-0030-2018>
- Firth, N., and Skurray, R. A. (2006). Genetics: Accessory Elements and Genetic Exchange. In *Gram-Positive Pathogens* (pp. 413–426). John Wiley & Sons, Ltd.
<https://doi.org/10.1128/9781555816513.ch33>
- Fišarová, L., Botka, T., Du, X., Mašláňová, I., Bárdy, P., Pantůček, R., Benešík, M., Roudnický, P., Winstel, V., Larsen, J., Rosenstein, R., Peschel, A., and Doškař, J. (2021). *Staphylococcus epidermidis* Phages Transduce Antimicrobial Resistance Plasmids and Mobilize Chromosomal Islands. *mSphere*, 6(3). <https://doi.org/10.1128/msphere.00223-21>

- Fitzgerald, J. R. (2012). Livestock-associated *Staphylococcus aureus*: Origin, evolution and public health threat. *Trends in Microbiology*, 20(4), 192–198. <https://doi.org/10.1016/j.tim.2012.01.006>
- Fitzgerald, J. R., and Holden, M. T. G. (2016). Genomics of Natural Populations of *Staphylococcus aureus*. *Annual Review of Microbiology*, 70, 459–478. <https://doi.org/10.1146/annurev-micro-102215-095547>
- Foster, G., Ross, H. M., Hutson, R. A., and Collins, M. D. (1997). *Staphylococcus lutrae* sp. Nov., a New Coagulase-Positive Species Isolated from Otters. *International Journal of Systematic Bacteriology*, 47(3), 724–726. <https://doi.org/10.1099/00207713-47-3-724>
- Fountain, K., Gibbon, M. J., Loeffler, A., and Feil, E. J. (2019). Closed genome sequences of *Staphylococcus lloydii* sp. Nov. And *Staphylococcus durrellii* sp. Nov. Isolated from captive fruit bats (*Pteropus livingstonii*). *International Journal of Systematic and Evolutionary Microbiology*, 71(3). <https://doi.org/10.1099/ijsem.0.004715>
- França, A. (2023). The Role of Coagulase-Negative Staphylococci Biofilms on Late-Onset Sepsis: Current Challenges and Emerging Diagnostics and Therapies. *Antibiotics*, 12(3), 554. <https://doi.org/10.3390/antibiotics12030554>
- Freney, J., Brun, Y., Bes, M., Meugnier, H., Grimont, F., Grimont, P. A. D., Nervi, C., and Fleurette, J. (1988). *Staphylococcus lugdunensis* sp. Nov. And *Staphylococcus schleiferi* sp. Nov., Two Species from Human Clinical Specimens. *International Journal of Systematic Bacteriology*, 38(2), 168–172. <https://doi.org/10.1099/00207713-38-2-168>
- Fuchs, S., Mehlan, H., Bernhardt, J., Hennig, A., Michalik, S., Surmann, K., Pané-Farré, J., Giese, A., Weiss, S., Backert, L., Herbig, A., Nieselt, K., Hecker, M., Völker, U., and Mäder, U. (2018). AureoWiki- The repository of the *Staphylococcus aureus* research and annotation community. *International Journal of Medical Microbiology*, 308(6), 558–568. <https://doi.org/10.1016/j.ijmm.2017.11.011>
- Fullam, A., Letunic, I., Schmidt, T. S. B., Ducarmon, Q. R., Karcher, N., Khedkar, S., Kuhn, M., Larralde, M., Maistrenko, O. M., Malfertheiner, L., Milanese, A., Rodrigues, J. F. M., Sanchis-López, C., Schudoma, C., Szklarczyk, D., Sunagawa, S., Zeller, G., Huerta-Cepas, J., von Mering, C., ... Mende, D. R. (2023). proGenomes3: Approaching one million accurately and consistently annotated high-quality prokaryotic genomes. *Nucleic Acids Research*, 51(1), 760–766. <https://doi.org/10.1093/nar/gkac1078>
- García-Álvarez, L., Holden, M. T., Lindsay, H., Webb, C. R., Brown, D. F., Curran, M. D., Walpole, E., Brooks, K., Pickard, D. J., Teale, C., Parkhill, J., Bentley, S. D., Edwards, G. F., Girvan, E. K., Kearns, A. M., Pichon, B., Hill, R. L., Larsen, A. R., Skov, R. L., ... Holmes, M. A. (2011). Meticillin-resistant *Staphylococcus aureus* with a novel *mecA* homologue in human and bovine populations in the UK and Denmark: A descriptive study. *The Lancet. Infectious Diseases*, 11(8), 595. [https://doi.org/10.1016/S1473-3099\(11\)70126-8](https://doi.org/10.1016/S1473-3099(11)70126-8)
- Ghebremedhin, B., Layer, F., König, W., and König, B. (2008). Genetic Classification and Distinguishing of *Staphylococcus* Species Based on Different Partial gap, 16S rRNA, hsp60, rpoB, sodA, and tuf Gene Sequences. *Journal of Clinical Microbiology*, 46(3), 1019–1025. <https://doi.org/10.1128/JCM.02058-07>

- Gherardi, G., Di Bonaventura, G., and Savini, V. (2018). Chapter 1—Staphylococcal Taxonomy. In V. Savini (Ed.), *Pet-To-Man Travelling Staphylococci* (pp. 1–10). Academic Press. <https://doi.org/10.1016/B978-0-12-813547-1.00001-7>
- Gill, S. R., Fouts, D. E., Archer, G. L., Mongodin, E. F., DeBoy, R. T., Ravel, J., Paulsen, I. T., Kolonay, J. F., Brinkac, L., Beanan, M., Dodson, R. J., Daugherty, S. C., Madupu, R., Angiuoli, S. V., Durkin, A. S., Haft, D. H., Vamathevan, J., Khouri, H., Utterback, T., ... Fraser, C. M. (2005). Insights on Evolution of Virulence and Resistance from the Complete Genome Analysis of an Early Methicillin-Resistant *Staphylococcus aureus* Strain and a Biofilm-Producing Methicillin-Resistant *Staphylococcus epidermidis* Strain. *Journal of Bacteriology*, 187(7), 2426–2438. <https://doi.org/10.1128/JB.187.7.2426-2438.2005>
- Gillespie, J. J., Wattam, A. R., Cammer, S. A., Gabbard, J. L., Shukla, M. P., Dalay, O., Driscoll, T., Hix, D., Mane, S. P., Mao, C., Nordberg, E. K., Scott, M., Schulman, J. R., Snyder, E. E., Sullivan, D. E., Wang, C., Warren, A., Williams, K. P., Xue, T., ... Sobral, B. W. (2011). PATRIC: The Comprehensive Bacterial Bioinformatics Resource with a Focus on Human Pathogenic Species. *Infection and Immunity*, 79(11), 4286–4298. <https://doi.org/10.1128/IAI.00207-11>
- Glajzner, P., Szewczyk, E. M., and Szemraj, M. (2023). Pathogenic potential and antimicrobial resistance of *Staphylococcus pseudintermedius* isolated from human and animals. *Folia Microbiologica*, 68(2), 231–243. <https://doi.org/10.1007/s12223-022-01007-x>
- Goerke, C., Pantucek, R., Holtfreter, S., Schulte, B., Zink, M., Grumann, D., Bröker, B. M., Doskar, J., and Wolz, C. (2009). Diversity of Prophages in Dominant *Staphylococcus aureus* Clonal Lineages. *Journal of Bacteriology*, 191(11), 3462–3468. <https://doi.org/10.1128/jb.01804-08>
- Golubchik, T., Batty, E. M., Miller, R. R., Farr, H., Young, B. C., Larner-Svensson, H., Fung, R., Godwin, H., Knox, K., Votintseva, A., Everitt, R. G., Street, T., Cule, M., Ip, C. L. C., Didelot, X., Peto, T. E. A., Harding, R. M., Wilson, D. J., Crook, D. W., and Bowden, R. (2013). Within-Host Evolution of *Staphylococcus aureus* during Asymptomatic Carriage. *PLOS ONE*, 8(5), e61319. <https://doi.org/10.1371/journal.pone.0061319>
- González-Martín, M., Corbera, J. A., Suárez-Bonnet, A., and Tejedor-Junco, M. T. (2020). Virulence factors in coagulase-positive staphylococci of veterinary interest other than *Staphylococcus aureus*. *Veterinary Quarterly*, 40(1), 118–131. <https://doi.org/10.1080/01652176.2020.1748253>
- Grundmann, H., Aires-de-Sousa, M., Boyce, J., and Tiemersma, E. (2006). Emergence and resurgence of methicillin-resistant *Staphylococcus aureus* as a public-health threat. *The Lancet*, 368(9538), 874–885. [https://doi.org/10.1016/S0140-6736\(06\)68853-3](https://doi.org/10.1016/S0140-6736(06)68853-3)
- Gruss, A., and Ehrlich, S. D. (1989). The family of highly interrelated single-stranded deoxyribonucleic acid plasmids. *Microbiological Reviews*, 53(2), 231–241. <https://doi.org/10.1128/mr.53.2.231-241.1989>
- Gupta, S. K., Padmanabhan, B. R., Diene, S. M., Lopez-Rojas, R., Kempf, M., Landraud, L., and Rolain, J.-M. (2014). ARG-ANNOT, a new bioinformatic tool to discover antibiotic resistance genes in bacterial genomes. *Antimicrobial Agents and Chemotherapy*, 58(1), 212–220. <https://doi.org/10.1128/AAC.01310-13>

- Hajek, V. (1976). *Staphylococcus intermedius*, a New Species Isolated from Animals. *International Journal of Systematic Bacteriology*, 26(4), 401–408. <https://doi.org/10.1099/00207713-26-4-401>
- Hájek, V., Devriese, L. A., Mordarski, M., Goodfellow, M., Pulverer, G., and Varaldo, P. E. (1986). Elevation of *Staphylococcus hyicus* subsp. *chromogenes* (Devriese et al., 1978) to species status: *Staphylococcus chromogenes* (Devriese et al., 1978) comb. nov. *Systematic and Applied Microbiology*, 8(3), 169–173. [https://doi.org/10.1016/S0723-2020\(86\)80071-6](https://doi.org/10.1016/S0723-2020(86)80071-6)
- Hajek, V., Ludwig, W., Schleifer, K. H., Springer, N., Zitzelsberger, W., Kroppenstedt, R. M., and Kocur, M. (1992). *Staphylococcus muscae*, a New Species Isolated from Flies. *International Journal of Systematic Bacteriology*, 42(1), 97–101. <https://doi.org/10.1099/00207713-42-1-97>
- Hall, J. P. J., Wright, R. C. T., Harrison, E., Muddiman, K. J., Wood, A. J., Paterson, S., and Brockhurst, M. A. (2021). Plasmid fitness costs are caused by specific genetic conflicts enabling resolution by compensatory mutation. *PLOS Biology*, 19(10), e3001225. <https://doi.org/10.1371/journal.pbio.3001225>
- Harkins, C. P., Pichon, B., Doumith, M., Parkhill, J., Westh, H., Tomasz, A., de Lencastre, H., Bentley, S. D., Kearns, A. M., and Holden, M. T. G. (2017). Methicillin-resistant *Staphylococcus aureus* emerged long before the introduction of methicillin into clinical practice. *Genome Biology*, 18(1), 130. <https://doi.org/10.1186/s13059-017-1252-9>
- Harmer, C. J., and Hall, R. M. (2020). IS26 Family Members IS257 and IS1216 Also Form Cointegrates by Copy-In and Targeted Conservative Routes. *mSphere*, 5(1), 10.1128/msphere.00811-19. <https://doi.org/10.1128/msphere.00811-19>
- Harris, S. R., Feil, E. J., Holden, M. T. G., Quail, M. A., Nickerson, E. K., Chantratita, N., Gardete, S., Tavares, A., Day, N., Lindsay, J. A., Edgeworth, J. D., de Lencastre, H., Parkhill, J., Peacock, S. J., and Bentley, S. D. (2010). Evolution of MRSA During Hospital Transmission and Intercontinental Spread. *Science*, 327(5964), 469–474. <https://doi.org/10.1126/science.1182395>
- Hassler, H. B., Probert, B., Moore, C., Lawson, E., Jackson, R. W., Russell, B. T., and Richards, V. P. (2022). Phylogenies of the 16S rRNA gene and its hypervariable regions lack concordance with core genome phylogenies. *Microbiome*, 10(1), 104. <https://doi.org/10.1186/s40168-022-01295-y>
- Heilmann, C., Hussain, M., Peters, G., and Götz, F. (1997). Evidence for autolysin-mediated primary attachment of *Staphylococcus epidermidis* to a polystyrene surface. *Molecular Microbiology*, 24(5), 1013–1024. <https://doi.org/10.1046/j.1365-2958.1997.4101774.x>
- Heilmann, C., Ziebuhr, W., and Becker, K. (2019). Are coagulase-negative staphylococci virulent? *Clinical Microbiology and Infection*, 25(9), 1071–1080. <https://doi.org/10.1016/j.cmi.2018.11.012>
- Herrmann, M., Vaudaux, P. E., Pittet, D., Auckenthaler, R., Lew, P. D., Perdreau, F. S., Peters, G., and Waldvogel, F. A. (1988). Fibronectin, Fibrinogen, and Laminin Act as Mediators of

- Adherence of Clinical Staphylococcal Isolates to Foreign Material. *The Journal of Infectious Diseases*, 158(4), 693–701. <https://doi.org/10.1093/infdis/158.4.693>
- Heß, S., and Gallert, C. (2015). *Staphylococcus argensis* sp. Nov., a novel staphylococcal species isolated from an aquatic environment. *International Journal of Systematic and Evolutionary Microbiology*, 65(Pt_8), 2661–2665. <https://doi.org/10.1099/ijs.0.000319>
- Hirschhausen, N., Schlesier, T., Peters, G., and Heilmann, C. (2012). Characterization of the Modular Design of the Autolysin/Adhesin Aaa from *Staphylococcus Aureus*. *PLOS ONE*, 7(6), e40353. <https://doi.org/10.1371/journal.pone.0040353>
- Horne, T., Orr, V. T., and Hall, J. P. (2023). How do interactions between mobile genetic elements affect horizontal gene transfer? *Current Opinion in Microbiology*, 73, 102282. <https://doi.org/10.1016/j.mib.2023.102282>
- Hovelius, B., and Mårdh, P. A. (1984). *Staphylococcus saprophyticus* as a common cause of urinary tract infections. *Reviews of Infectious Diseases*, 6(3), 328–337. <https://doi.org/10.1093/clinids/6.3.328>
- Howden, B. P., Giulieri, S. G., Wong Fok Lung, T., Baines, S. L., Sharkey, L. K., Lee, J. Y. H., Hachani, A., Monk, I. R., and Stinear, T. P. (2023). *Staphylococcus aureus* host interactions and adaptation. *Nature Reviews Microbiology*, 21, 380–395. <https://doi.org/10.1038/s41579-023-00852-y>
- Hsu, L.-Y., Harris, S. R., Chlebowicz, M. A., Lindsay, J. A., Koh, T.-H., Krishnan, P., Tan, T.-Y., Hon, P.-Y., Grubb, W. B., Bentley, S. D., Parkhill, J., Peacock, S. J., and Holden, M. T. (2015). Evolutionary dynamics of methicillin-resistant *Staphylococcus aureus* within a healthcare system. *Genome Biology*, 16(1), 81. <https://doi.org/10.1186/s13059-015-0643-z>
- Huerta-Cepas, J., Szklarczyk, D., Heller, D., Hernández-Plaza, A., Forslund, S. K., Cook, H., Mende, D. R., Letunic, I., Rattei, T., Jensen, L. J., von Mering, C., and Bork, P. (2019). eggNOG 5.0: A hierarchical, functionally and phylogenetically annotated orthology resource based on 5090 organisms and 2502 viruses. *Nucleic Acids Research*, 47(1), 309–D31. <https://doi.org/10.1093/nar/gky1085>
- Humphrey, S., San Millán, Á., Toll-Riera, M., Connolly, J., Flor-Duro, A., Chen, J., Ubeda, C., MacLean, R. C., and Penadés, J. R. (2021). Staphylococcal phages and pathogenicity islands drive plasmid evolution. *Nature Communications*, 12(1), 5845. <https://doi.org/10.1038/s41467-021-26101-5>
- Hurley, J. C. (2020). Incidence of coagulase-negative staphylococcal bacteremia among ICU patients: Decontamination studies as a natural experiment. *European Journal of Clinical Microbiology & Infectious Diseases*, 39(4), 657–664. <https://doi.org/10.1007/s10096-019-03763-0>
- Igimi, S., Kawamura, S., Takahashi, E., and Mitsuoka, T. (1989). *Staphylococcus felis*, a New Species from Clinical Specimens from Cats. *International Journal of Systematic Bacteriology*, 39(4), 373–377. <https://doi.org/10.1099/00207713-39-4-373>
- Ikhimiukor, O. O., Souza, S. S., Marcovici, M. M., Nye, G. J., Gibson, R., and Andam, C. P. (2023). Leaky barriers to gene sharing between locally co-existing coagulase-negative

- Staphylococcus species. *Communications Biology*, 6(1), 482.
<https://doi.org/10.1038/s42003-023-04877-0>
- Ito, T., Katayama, Y., and Hiramatsu, K. (1999). Cloning and Nucleotide Sequence Determination of the Entire mec DNA of Pre-Methicillin-Resistant Staphylococcus aureus N315. *Antimicrobial Agents and Chemotherapy*, 43(6), 1449–1458.
<https://doi.org/10.1128/aac.43.6.1449>
- IWG SCC, I. W. G. on the C. of S. C. C. E. (2009). Classification of staphylococcal cassette chromosome mec (SCCmec): Guidelines for reporting novel SCCmec elements. *Antimicrobial Agents and Chemotherapy*, 53(12), 4961.
<https://doi.org/10.1128/AAC.00579-09>
- Izano, E. A., Amarante, M. A., Kher, W. B., and Kaplan, J. B. (2008). Differential Roles of Poly-N-Acetylglucosamine Surface Polysaccharide and Extracellular DNA in Staphylococcus aureus and Staphylococcus epidermidis Biofilms. *Applied and Environmental Microbiology*, 74(2), 470–476. <https://doi.org/10.1128/AEM.02073-07>
- Jain, C., Rodriguez-R, L. M., Phillippy, A. M., Konstantinidis, K. T., and Aluru, S. (2018). High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. *Nature Communications*, 9(1), 5114. <https://doi.org/10.1038/s41467-018-07641-9>
- Jeffries, L., Cawthorne, M. A., Harris, M., Cook, B., and Diplock, A. T. (1968). Menaquinone Determination in the Taxonomy of Micrococcaceae. *Journal of General Microbiology*, 54(3), 365–380. <https://doi.org/10.1099/00221287-54-3-365>
- Johansson, M. H. K., Bortolaia, V., Tansirichaiya, S., Aarestrup, F. M., Roberts, A. P., and Petersen, T. N. (2021). Detection of mobile genetic elements associated with antibiotic resistance in Salmonella enterica using a newly developed web tool: MobileElementFinder. *Journal of Antimicrobial Chemotherapy*, 76(1), 101–109. <https://doi.org/10.1093/jac/dkaa390>
- John, J., George, S., Nori, S. R. C., and Nelson-Sathi, S. (2019). Phylogenomic Analysis Reveals the Evolutionary Route of Resistant Genes in Staphylococcus aureus. *Genome Biology and Evolution*, 11(10), 2917–2926. <https://doi.org/10.1093/gbe/evz213>
- Jolley, K. A., Bray, J. E., and Maiden, M. C. J. (2018). Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. *Wellcome Open Research*, 3, 124.
<https://doi.org/10.12688/wellcomeopenres.14826.1>
- Jones, M. J., Donegan, N. P., Mikheyeva, I. V., and Cheung, A. L. (2015). Improving Transformation of Staphylococcus aureus Belonging to the CC1, CC5 and CC8 Clonal Complexes. *PLOS ONE*, 10(3), e0119487.
<https://doi.org/10.1371/journal.pone.0119487>
- Jones, P., Binns, D., Chang, H.-Y., Fraser, M., Li, W., McAnulla, C., McWilliam, H., Maslen, J., Mitchell, A., Nuka, G., Pesseat, S., Quinn, A. F., Sangrador-Vegas, A., Scheremetjew, M., Yong, S.-Y., Lopez, R., and Hunter, S. (2014). InterProScan 5: Genome-scale protein function classification. *Bioinformatics*, 30(9), 1236–1240.
<https://doi.org/10.1093/bioinformatics/btu031>

- Kahánková, J., Pantůček, R., Goerke, C., Růžičková, V., Holochová, P., and Doškař, J. (2010). Multilocus PCR typing strategy for differentiation of *Staphylococcus aureus* siphoviruses reflecting their modular genome structure. *Environmental Microbiology*, 12(9), 2527–2538. <https://doi.org/10.1111/j.1462-2920.2010.02226.x>
- Kanehisa, M., and Goto, S. (2000). KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Research*, 28(1), 27–30. <https://doi.org/10.1093/nar/28.1.27>
- Kaneko, J., Kimura, T., Kawakami, Y., Tomita, T., and Kamio, Y. (1997). Panton-valentine leukocidin genes in a phage-like particle isolated from mitomycin C-treated *Staphylococcus aureus* V8 (ATCC 49775). *Bioscience, Biotechnology, and Biochemistry*, 61(11), 1960–1962. <https://doi.org/10.1271/bbb.61.1960>
- Kania, S. A. (2022). Reclassification of *Staphylococcus schleiferi* by Madhaiyan et al. Lacks key supporting data. *International Journal of Systematic and Evolutionary Microbiology*, 72(4), 005289. <https://doi.org/10.1099/ijsem.0.005289>
- Kapli, P., Yang, Z., and Telford, M. J. (2020). Phylogenetic tree building in the genomic age. *Nature Reviews Genetics*, 21(7), 428–444. <https://doi.org/10.1038/s41576-020-0233-0>
- Kaspar, U., Kriegeskorte, A., Schubert, T., Peters, G., Rudack, C., Pieper, D. H., Wos-Oxley, M., and Becker, K. (2016). The culturome of the human nose habitats reveals individual bacterial fingerprint patterns. *Environmental Microbiology*, 18(7), 2130–2142. <https://doi.org/10.1111/1462-2920.12891>
- Kellaway, C. H., Maccallum, P., and Tebbutt, A. H. (1928). Report of the Royal Commission of Inquiry into Fatalities at Bundaberg, together with Appendices. *Report of the Royal Commission of Inquiry into Fatalities at Bundaberg, together with Appendices*.
- Kilpper-Bälz, R., and Schleifer, K. H. (1981). Transfer of *Peptococcus saccharolyticus* Foubert and Douglas to the genus *Staphylococcus*: *Staphylococcus saccharolyticus* (Foubert and Douglas) comb. nov. *Zentralblatt Für Bakteriologie Mikrobiologie Und Hygiene: I. Abt. Originale C: Allgemeine, Angewandte Und Ökologische Mikrobiologie*, 2(4), 324–331. [https://doi.org/10.1016/S0721-9571\(81\)80025-7](https://doi.org/10.1016/S0721-9571(81)80025-7)
- Kim, J., Na, S.-I., Kim, D., and Chun, J. (2021). UBCG2: Up-to-date bacterial core genes and pipeline for phylogenomic analysis. *Journal of Microbiology*, 59(6), 609–615. <https://doi.org/10.1007/s12275-021-1231-4>
- Kloos, W. E., Ballard, D. N., George, C. G., Webster, J. A., Hubner, R. J., Ludwig, W., Schleifer, K. H., Fiedler, F., and Schubert, K. (1998). Delimiting the genus *Staphylococcus* through description of *Macroccoccus caseolyticus* gen. Nov., comb. Nov. And *Macroccoccus equipericus* sp. Nov., *Macroccoccus bovicus* sp. Nov. And *Macroccoccus carouselicus* sp. Nov. *International Journal of Systematic and Evolutionary Microbiology*, 48(3), 859–877. <https://doi.org/10.1099/00207713-48-3-859>
- Kloos, W. E., and Schleifer, K. H. (1975). Isolation and Characterization of *Staphylococci* from Human Skin II. Descriptions of Four New Species: *Staphylococcus warneri*, *Staphylococcus capitis*, *Staphylococcus hominis*, and *Staphylococcus simulans*. *International Journal of Systematic Bacteriology*, 25(1), 62–79. <https://doi.org/10.1099/00207713-25-1-62>

- Kloos, W. E., and Schleifer, K. H. (1983). *Staphylococcus auricularis* sp. nov.: An Inhabitant of the Human External Ear. *International Journal of Systematic Bacteriology*, 33(1), 9–14. <https://doi.org/10.1099/00207713-33-1-9>
- Kolmogorov, M., Yuan, J., Lin, Y., and Pevzner, P. A. (2019). Assembly of long, error-prone reads using repeat graphs. *Nature Biotechnology*, 37(5), 540–546. <https://doi.org/10.1038/s41587-019-0072-8>
- Koonin, E. V., Makarova, K. S., and Wolf, Y. I. (2021). Evolution of Microbial Genomics: Conceptual Shifts over a Quarter Century. *Trends in Microbiology*, 29(7), 582–592. <https://doi.org/10.1016/j.tim.2021.01.005>
- Koreen, L., Ramaswamy, S. V., Graviss, E. A., Naidich, S., Musser, J. M., and Kreiswirth, B. N. (2004). spa Typing Method for Discriminating among *Staphylococcus aureus* Isolates: Implications for Use of a Single Marker To Detect Genetic Micro- and Macrovariation. *Journal of Clinical Microbiology*, 42(2), 792–799. <https://doi.org/10.1128/jcm.42.2.792-799.2004>
- Kovařovic, V. (2024). Snakestaph. *Github*. <https://github.com/kovavo/snakestaph>
- Kovařovic, V., Finstrlová, A., Sedláček, I., Petráš, P., Švec, P., Mašlaňová, I., Neumann-Schaal, M., Šedo, O., Botka, T., Staňková, E., Doškař, J., and Pantůček, R. (2023). *Staphylococcus brunensis* sp. Nov. Isolated from human clinical specimens with a staphylococcal cassette chromosome-related genomic island outside of the rlmH gene bearing the ccrDE recombinase gene complex. *Microbiology Spectrum*, 11(5). <https://doi.org/10.1128/spectrum.01342-23>
- Kovařovic, V., Sedláček, I., Petráš, P., Králová, S., Mašlaňová, I., Švec, P., Neumann-Schaal, M., Botka, T., Gelbíčová, T., Staňková, E., Doškař, J., and Pantůček, R. (2022). *Staphylococcus ratti* sp. Nov. Isolated from a Lab Rat. *Pathogens*, 11(1), 51. <https://doi.org/10.3390/pathogens11010051>
- Kuroda, M., Ohta, T., Uchiyama, I., Baba, T., Yuzawa, H., Kobayashi, I., Cui, L., Oguchi, A., Aoki, K., Nagai, Y., Lian, J., Ito, T., Kanamori, M., Matsumaru, H., Maruyama, A., Murakami, H., Hosoyama, A., Mizutani-Ui, Y., Takahashi, N. K., ... Hiramatsu, K. (2001). Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. *The Lancet*, 357(9264), 1225–1240. [https://doi.org/10.1016/S0140-6736\(00\)04403-2](https://doi.org/10.1016/S0140-6736(00)04403-2)
- Kuroda, M., Yamashita, A., Hirakawa, H., Kumano, M., Morikawa, K., Higashide, M., Maruyama, A., Inose, Y., Matoba, K., Toh, H., Kuhara, S., Hattori, M., and Ohta, T. (2005). Whole genome sequence of *Staphylococcus saprophyticus* reveals the pathogenesis of uncomplicated urinary tract infection. *Proceedings of the National Academy of Sciences of the United States of America*, 102(37), 13272–13277. <https://doi.org/10.1073/pnas.0502950102>
- Kwieceński, J., Kahlmeter, G., and Jin, T. (2015). Biofilm Formation by *Staphylococcus aureus* Isolates from Skin and Soft Tissue Infections. *Current Microbiology*, 70(5), 698–703. <https://doi.org/10.1007/s00284-014-0770-x>
- Kwong, S. M., Ramsay, J. P., Jensen, S. O., and Firth, N. (2017). Replication of Staphylococcal Resistance Plasmids. *Frontiers in Microbiology*, 8, 2279. <https://doi.org/10.3389/fmicb.2017.02279>

REFERENCES

- Lambert, L. H., Cox, T., Mitchell, K., Rossello-Mora, R. A., Del Cueto, C., Dodge, D. E., Orkand, P., and Cano, R. J. (1998). *Staphylococcus succinus* sp. Nov., isolated from Dominican amber. *International Journal of Systematic Bacteriology*, 48(2), 511–518. <https://doi.org/10.1099/00207713-48-2-511>
- Lamers, R. P., Muthukrishnan, G., Castoe, T. A., Tafur, S., Cole, A. M., and Parkinson, C. L. (2012). Phylogenetic relationships among *Staphylococcus* species and refinement of cluster groups based on multilocus data. *BMC Evolutionary Biology*, 12(1), 171. <https://doi.org/10.1186/1471-2148-12-171>
- Larkum, N. W. (1929). Bacteriophage Treatment of *Staphylococcus* Infections. *The Journal of Infectious Diseases*, 45(1), 34–41. <https://doi.org/10.1093/infdis/45.1.34>
- Larsen, J., Raisen, C. L., Ba, X., Sadgrove, N. J., Padilla-González, G. F., Simmonds, M. S. J., Loncaric, I., Kerschner, H., Apfalter, P., Hartl, R., Deplano, A., Vandendriessche, S., Černá Bolfíková, B., Hulva, P., Arendrup, M. C., Hare, R. K., Barnadas, C., Stegger, M., Sieber, R. N., ... Larsen, A. R. (2022). Emergence of methicillin resistance predates the clinical use of antibiotics. *Nature*, 602(7895), 135–141. <https://doi.org/10.1038/s41586-021-04265-w>
- Latreille, P., Norton, S., Goldman, B. S., Henkhaus, J., Miller, N., Barbazuk, B., Bode, H. B., Darby, C., Du, Z., Forst, S., Gaudriault, S., Goodner, B., Goodrich-Blair, H., and Slater, S. (2007). Optical mapping as a routine tool for bacterial genome sequence finishing. *BMC Genomics*, 8(1), 321. <https://doi.org/10.1186/1471-2164-8-321>
- Lee, J. Y. H., Carter, G. P., Pidot, S. J., Guérillot, R., Seemann, T., Gonçalves da Silva, A., Foster, T. J., Howden, B. P., Stinear, T. P., and Monk, I. R. (2019). Mining the Methyloome Reveals Extensive Diversity in *Staphylococcus epidermidis* Restriction Modification. *mBio*, 10(6), 10.1128/mbio.02451-19. <https://doi.org/10.1128/mbio.02451-19>
- Lepplae, R., Lima-Mendez, G., and Toussaint, A. (2010). ACLAME: A CLAssification of Mobile genetic Elements, update 2010. *Nucleic Acids Research*, 38(suppl_1), 57–61. <https://doi.org/10.1093/nar/gkp938>
- Li, Z., Chen, Y., Mu, D., Yuan, J., Shi, Y., Zhang, H., Gan, J., Li, N., Hu, X., Liu, B., Yang, B., and Fan, W. (2012). Comparison of the two major classes of assembly algorithms: Overlap–layout–consensus and de-bruijn-graph. *Briefings in Functional Genomics*, 11(1), 25–37. <https://doi.org/10.1093/bfgp/elr035>
- Lienen, T., Schnitt, A., Hammerl, J. A., Maurischat, S., and Tenhagen, B.-A. (2022). *Mammaliococcus* spp. From German Dairy Farms Exhibit a Wide Range of Antimicrobial Resistance Genes and Non-Wildtype Phenotypes to Several Antibiotic Classes. *Biology*, 11(2), 152. <https://doi.org/10.3390/biology11020152>
- Lin, Y.-T., Hung, W.-C., Wan, T.-W., Li, H., Lee, T.-F., Hsueh, P.-R., and Teng, L.-J. (2022). *Staphylococcus taiwanensis* sp. Nov., isolated from human blood. *International Journal of Systematic and Evolutionary Microbiology*, 72(2). <https://doi.org/10.1099/ijsem.0.005262>
- Lindsay, J. A. (2010). Genomic variation and evolution of *Staphylococcus aureus*. *International Journal of Medical Microbiology*, 300(2), 98–103. <https://doi.org/10.1016/j.ijmm.2009.08.013>

- Lindsay, J. A. (2014). Evolution of *Staphylococcus aureus* and MRSA during outbreaks. *Infection, Genetics and Evolution*, 21, 548–553. <https://doi.org/10.1016/j.meegid.2013.04.017>
- Lindsay, J. A. (2019). Staphylococci: Evolving Genomes. *Microbiology Spectrum*, 7(6), 10.1128/microbiolspec.gpp3-0071-2019. <https://doi.org/10.1128/microbiolspec.gpp3-0071-2019>
- Lindsay, J. A., and Holden, M. T. G. (2004). *Staphylococcus aureus*: Superbug, super genome? *Trends in Microbiology*, 12(8), 378–385. <https://doi.org/10.1016/j.tim.2004.06.004>
- Lindsay, J. A., Ruzin, A., Ross, H. F., Kurepina, N., and Novick, R. P. (1998). The gene for toxic shock toxin is carried by a family of mobile pathogenicity islands in *Staphylococcus aureus*. *Molecular Microbiology*, 29(2), 527–543. <https://doi.org/10.1046/j.1365-2958.1998.00947.x>
- Liu, B., Zheng, D., Jin, Q., Chen, L., and Yang, J. (2019). VFDB 2019: A comparative pathogenomic platform with an interactive web interface. *Nucleic Acids Research*, 47(1), 687–692. <https://doi.org/10.1093/nar/gky1080>
- Liu, C., and Chambers, H. F. (2003). *Staphylococcus aureus* with Heterogeneous Resistance to Vancomycin: Epidemiology, Clinical Significance, and Critical Assessment of Diagnostic Methods. *Antimicrobial Agents and Chemotherapy*, 47(10), 3040–3045. <https://doi.org/10.1128/aac.47.10.3040-3045.2003>
- Liu, M., Li, X., Xie, Y., Bi, D., Sun, J., Li, J., Tai, C., Deng, Z., and Ou, H.-Y. (2019). ICEberg 2.0: An updated database of bacterial integrative and conjugative elements. *Nucleic Acids Research*, 47(1), 660–665. <https://doi.org/10.1093/nar/gky1123>
- Livermore, D. M. (2009). Has the era of untreatable infections arrived? *Journal of Antimicrobial Chemotherapy*, 64(suppl_1), i29–i36. <https://doi.org/10.1093/jac/dkp255>
- Lobb, B., Tremblay, B. J.-M., Moreno-Hagelsieb, G., and Doxey, A. C. (2020). An assessment of genome annotation coverage across the bacterial tree of life. *Microbial Genomics*, 6(3), e000341. <https://doi.org/10.1099/mgen.0.000341>
- Loeb, L. (1903). The Influence of certain Bacteria on the Coagulation of the Blood. *The Journal of Medical Research*, 10(3), 407–419.
- Loessner, I., Dietrich, K., Dittrich, D., Hacker, J., and Ziebuhr, W. (2002). Transposase-Dependent Formation of Circular IS256 Derivatives in *Staphylococcus epidermidis* and *Staphylococcus aureus*. *Journal of Bacteriology*, 184(17), 4709–4714. <https://doi.org/10.1128/jb.184.17.4709-4714.2002>
- Long, D. R., Wolter, D. J., Lee, M., Precit, M., McLean, K., Holmes, E., Penewit, K., Waalkes, A., Hoffman, L. R., and Salipante, S. J. (2021). Polyclonality, Shared Strains, and Convergent Evolution in Chronic Cystic Fibrosis *Staphylococcus aureus* Airway Infection. *American Journal of Respiratory and Critical Care Medicine*, 203(9), 1127–1137. <https://doi.org/10.1164/rccm.202003-0735OC>
- Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, 15(12), 550. <https://doi.org/10.1186/s13059-014-0550-8>

- MacFadyen, A. C., Drigo, I., Harrison, E. M., Parkhill, J., Holmes, M. A., and Paterson, G. K. (2019). *Staphylococcus caeli* sp. Nov., isolated from air sampling in an industrial rabbit holding. *International Journal of Systematic and Evolutionary Microbiology*, 69(1), 82–86. <https://doi.org/10.1099/ijsem.0.003098>
- MacFadyen, A. C., Leroy, S., Harrison, E. M., Parkhill, J., Holmes, M. A., and Paterson, G. K. (2019). *Staphylococcus pseudoxylosus* sp. Nov., isolated from bovine mastitis. *International Journal of Systematic and Evolutionary Microbiology*, 69(8), 2208–2213. <https://doi.org/10.1099/ijsem.0.003416>
- Maddiboyina, B., Roy, H., Ramaiah, M., Sarvesh, C. N., Kosuru, S. H., Nakkala, R. K., and Nayak, B. S. (2023). Methicillin-resistant *Staphylococcus aureus*: Novel treatment approach breakthroughs. *Bulletin of the National Research Centre*, 47(1), 95. <https://doi.org/10.1186/s42269-023-01072-3>
- Madhaiyan, M., Wirth, J. S., and Saravanan, V. S. (2020). Phylogenomic analyses of the Staphylococcaceae family suggest the reclassification of five species within the genus *Staphylococcus* as heterotypic synonyms, the promotion of five subspecies to novel species, the taxonomic reassignment of five *Staphylococcus* species to *Mammaliicoccus* gen. Nov., and the formal assignment of *Nosocomiicoccus* to the family Staphylococcaceae. *International Journal of Systematic and Evolutionary Microbiology*, 70(11), 5926–5936. <https://doi.org/10.1099/ijsem.0.004498>
- Maree, M., Thi Nguyen, L. T., Ohniwa, R. L., Higashide, M., Msadek, T., and Morikawa, K. (2022). Natural transformation allows transfer of SCCmec-mediated methicillin resistance in *Staphylococcus aureus* biofilms. *Nature Communications*, 13(1), 2477. <https://doi.org/10.1038/s41467-022-29877-2>
- Marshall, C., Wesselingh, S., McDonald, M., and Spelman, D. (2004). Control of endemic MRSA—what is the evidence? A personal view. *Journal of Hospital Infection*, 56(4), 253–268. <https://doi.org/10.1016/j.jhin.2004.02.001>
- Maskell, R. (1974). Importance of coagulase-negative staphylococci as pathogens in the urinary tract. *The Lancet*, 303(7867), 1155–1158. [https://doi.org/10.1016/S0140-6736\(74\)90634-5](https://doi.org/10.1016/S0140-6736(74)90634-5)
- Mašlaňová, I., Wertheimer, Z., Sedláček, I., Švec, P., Indráková, A., Kovařovic, V., Schumann, P., Spröer, C., Králová, S., Šedo, O., Křištofová, L., Vrbovská, V., Füzik, T., Petráš, P., Zdráhal, Z., Ružicková, V., Doškař, J., and Pantuček, R. (2018). Description and Comparative Genomics of *Macrococcus caseolyticus* subsp. *Hominis* subsp. Nov., *Macrococcus goetzii* sp. Nov., *Macrococcus epidermidis* sp. Nov., and *Macrococcus bohemicus* sp. Nov., Novel *Macrococci* From Human Clinical Material With Virulence Potential and Suspected Uptake of Foreign DNA by Natural Transformation. *Frontiers in Microbiology*, 9, 1178. <https://doi.org/10.3389/fmicb.2018.01178>
- Matsushashi, M., Song, M. D., Ishino, F., Wachi, M., Doi, M., Inoue, M., Ubukata, K., Yamashita, N., and Konno, M. (1986). Molecular cloning of the gene of a penicillin-binding protein supposed to cause high resistance to beta-lactam antibiotics in *Staphylococcus aureus*. *Journal of Bacteriology*, 167(3), 975–980. <https://doi.org/10.1128/jb.167.3.975-980.1986>

- Mayo-Muñoz, D., Pinilla-Redondo, R., Birkholz, N., and Fineran, P. C. (2023). A host of armor: Prokaryotic immune strategies against mobile genetic elements. *Cell Reports*, 42(7), 112672. <https://doi.org/10.1016/j.celrep.2023.112672>
- McCarthy, A. J., Loeffler, A., Witney, A. A., Gould, K. A., Lloyd, D. H., and Lindsay, J. A. (2014). Extensive Horizontal Gene Transfer during *Staphylococcus aureus* Co-colonization In Vivo. *Genome Biology and Evolution*, 6(10), 2697–2708. <https://doi.org/10.1093/gbe/evu214>
- Medini, D., Donati, C., Rappuoli, R., and Tettelin, H. (2020). The pangenome: A data-driven discovery in biology. *The Pangenome: Diversity, Dynamics and Evolution of Genomes*, 3–20. <https://doi.org/10.1007/978-3-030-38281-0>
- Mellon, R. R., Shinn, L. E., and McBroom, J. (1937). Therapy of Experimental *Staphylococcus* Infections with Sulfonamide Compounds. *Proceedings of the Society for Experimental Biology and Medicine*, 37(3), 563–565. <https://doi.org/10.3181/00379727-37-9642>
- Méric, G., Miragaia, M., de Been, M., Yahara, K., Pascoe, B., Mageiros, L., Mikhail, J., Harris, L. G., Wilkinson, T. S., Rolo, J., Lambie, S., Bray, J. E., Jolley, K. A., Hanage, W. P., Bowden, R., Maiden, M. C. J., Mack, D., de Lencastre, H., Feil, E. J., ... Sheppard, S. K. (2015). Ecological Overlap and Horizontal Gene Transfer in *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Genome Biology and Evolution*, 7(5), 1313–1328. <https://doi.org/10.1093/gbe/evv066>
- Michels, R., Last, K., Becker, S. L., and Papan, C. (2021). Update on Coagulase-Negative *Staphylococci*—What the Clinician Should Know. *Microorganisms*, 9(4), 830. <https://doi.org/10.3390/microorganisms9040830>
- Miller, J. R., Koren, S., and Sutton, G. (2010). Assembly algorithms for next-generation sequencing data. *Genomics*, 95(6), 315–327. <https://doi.org/10.1016/j.ygeno.2010.03.001>
- Mira, A., and Pushker, R. (2007). Genome Architecture and Evolution of Bacterial Pathogens. In *Evolutionary Biology of Bacterial and Fungal Pathogens* (pp. 113–127). John Wiley & Sons, Ltd. <https://doi.org/10.1128/9781555815639.ch12>
- Mitchell, R. G., and Baird-Parker, A. C. (1967). Novobiocin Resistance and the Classification of *Staphylococci* and *Micrococci*. *Journal of Applied Bacteriology*, 30(1), 251–254. <https://doi.org/10.1111/j.1365-2672.1967.tb00296.x>
- Miyamoto, H., Murakami, S., Saito, M., Tamaki, M., Ochi, C., and Iyoda, M. (2020). [Molecular Epidemiology of Methicillin-Resistant *Staphylococcus aureus* (MRSA) by POT Method, Antimicrobial Susceptibility Patterns, and Toxin-Producing Types]. *Rinsho Biseibutsu Jinsoku Shindan Kenkyukai shi = JARMAM: Journal of the Association for Rapid Method and Automation in Microbiology*, 29(2), 45–52.
- Mölder, F., Jablonski, K. P., Letcher, B., Hall, M. B., Tomkins-Tinch, C. H., Sochat, V., Forster, J., Lee, S., Twardziok, S. O., Kanitz, A., Wilm, A., Holtgrewe, M., Rahmann, S., Nahnsen, S., and Köster, J. (2021). Sustainable data analysis with Snakemake (10:33). F1000Research. <https://doi.org/10.12688/f1000research.29032.2>
- Moller, A. G., Lindsay, J. A., and Read, T. D. (2019). Determinants of Phage Host Range in *Staphylococcus* Species. *Applied and Environmental Microbiology*, 85(11), e00209-19. <https://doi.org/10.1128/AEM.00209-19>

REFERENCES

- Monecke, S., Schaumburg, F., Shittu, A. O., Schwarz, S., Mühldorfer, K., Brandt, C., Braun, S. D., Collatz, M., Diezel, C., Gawlik, D., Hanke, D., Hotzel, H., Müller, E., Reinicke, M., Feßler, A. T., and Ehricht, R. (2022). Description of Staphylococcal Strains from Straw-Coloured Fruit Bat (*Eidolon helvum*) and Diamond Firetail (*Stagonopleura guttata*) and a Review of their Phylogenetic Relationships to Other Staphylococci. *Frontiers in Cellular and Infection Microbiology*, 12. <https://doi.org/10.3389/fcimb.2022.878137>
- Morgan, F. G., and Graydon, J. J. (1936). Toxins of the Staphylococcus, with Special Reference to the Estimation of Potency. *Journal of Pathology and Bacteriology*, 43, 385–401. <https://doi.org/10.1002/path.1700430217>
- Morikawa, K., Takemura, A. J., Inose, Y., Tsai, M., Thi, L. T. N., Ohta, T., and Msadek, T. (2012). Expression of a Cryptic Secondary Sigma Factor Gene Unveils Natural Competence for DNA Transformation in *Staphylococcus aureus*. *PLOS Pathogens*, 8(11), e1003003. <https://doi.org/10.1371/journal.ppat.1003003>
- Morrison, S. J., Tornabene, T. G., and Kloos, W. E. (1971). Neutral Lipids in the Study of Relationships of Members of the Family Micrococcaceae. *Journal of Bacteriology*, 108(1), 353–358. <https://doi.org/10.1128/jb.108.1.353-358.1971>
- Mortimer, T. D., Annis, D. S., O'Neill, M. B., Bohr, L. L., Smith, T. M., Poinar, H. N., Mosher, D. F., and Pepperell, C. S. (2017). Adaptation in a Fibronectin Binding Autolysin of *Staphylococcus saprophyticus*. *mSphere*, 2(6), e00511-17. <https://doi.org/10.1128/mSphere.00511-17>
- Moses, I. B., Santos, F. F., and Gales, A. C. (2023). Human Colonization and Infection by *Staphylococcus pseudintermedius*: An Emerging and Underestimated Zoonotic Pathogen. *Microorganisms*, 11(3), 581. <https://doi.org/10.3390/microorganisms11030581>
- Murray, A. K., Lee, J., Bendall, R., Zhang, L., Sunde, M., Schau Sletteameås, J., Gaze, W., Page, A. J., and Vos, M. (2018). *Staphylococcus cornubiensis* sp. Nov., a member of the *Staphylococcus intermedius* Group (SIG). *International Journal of Systematic and Evolutionary Microbiology*, 68(11), 3404–3408. <https://doi.org/10.1099/ijsem.0.002992>
- Murugesan, S., Mani, S., Kuppusamy, I., and Krishnan, P. (2018). Role of insertion sequence element IS256 as a virulence marker and its association with biofilm formation among methicillin-resistant *Staphylococcus epidermidis* from hospital and community settings in Chennai, South India. *Indian Journal of Medical Microbiology*, 36(1), 124–126.
- Nakano, Y., Domon, Y., and Yamagishi, K. (2023). Phylogenetic trees of closely related bacterial species and subspecies based on frequencies of short nucleotide sequences. *PLOS ONE*, 18(4), e0268847. <https://doi.org/10.1371/journal.pone.0268847>
- Naushad, S., Kanevets, U., Nobrega, D., Carson, D., Dufour, S., Roy, J.-P., Lewis, P. J., and Barkema, H. W. (2019). *Staphylococcus debuckii* sp. Nov., a coagulase-negative species from bovine milk. *International Journal of Systematic and Evolutionary Microbiology*, 69(8), 2239–2249. <https://doi.org/10.1099/ijsem.0.003457>
- Newstead, L. L., Harris, J., Goodbrand, S., Varjonen, K., Nuttall, T., and Paterson, G. K. (2021). *Staphylococcus caledonicus* sp. Nov. And *Staphylococcus canis* sp. Nov. Isolated from

- healthy domestic dogs. *International Journal of Systematic and Evolutionary Microbiology*, 71(7). <https://doi.org/10.1099/ijsem.0.004878>
- Nielsen, T. K., Forero-Junco, L. M., Kot, W., Moineau, S., Hansen, L. H., and Riber, L. (2023). Detection of nucleotide modifications in bacteria and bacteriophages: Strengths and limitations of current technologies and software. *Molecular Ecology*, 32(6), 1236–1247. <https://doi.org/10.1111/mec.16679>
- Nováková, D., Pantůček, R., Hubálek, Z., Falsen, E., Busse, H.-J., Schumann, P., and Sedláček, I. (2010). *Staphylococcus microti* sp. Nov., isolated from the common vole (*Microtus arvalis*). *International Journal of Systematic and Evolutionary Microbiology*, 60(3), 566–573. <https://doi.org/10.1099/ijms.0.011429-0>
- Novick, R. P. (1989). Staphylococcal Plasmids and Their Replication. *Annual Review of Microbiology*, 43(1), 537–563. <https://doi.org/10.1146/annurev.mi.43.100189.002541>
- Novick, R. P. (2019). Pathogenicity Islands and Their Role in Staphylococcal Biology. *Microbiology Spectrum*, 7(3). <https://doi.org/10.1128/microbiolspec.gpp3-0062-2019>
- Ogston, A. (1881). Report upon Micro-Organisms in Surgical Diseases. *British Medical Journal*, 1(1054), 369.b2-375. <https://doi.org/10.1136/bmj.1.1054.369>
- O'Leary, N. A., Wright, M. W., Brister, J. R., Ciufu, S., Haddad, D., McVeigh, R., Rajput, B., Robbertse, B., Smith-White, B., Ako-Adjei, D., Astashyn, A., Badretdin, A., Bao, Y., Blinkova, O., Brover, V., Chetvernin, V., Choi, J., Cox, E., Ermolaeva, O., ... Pruitt, K. D. (2016). Reference sequence (RefSeq) database at NCBI: Current status, taxonomic expansion, and functional annotation. *Nucleic Acids Research*, 44(1), 733–745. <https://doi.org/10.1093/nar/gkv1189>
- Oliveira, H., Sampaio, M., Melo, L. D. R., Dias, O., Pope, W. H., Hatfull, G. F., and Azeredo, J. (2019). Staphylococci phages display vast genomic diversity and evolutionary relationships. *BMC Genomics*, 20(1), 357. <https://doi.org/10.1186/s12864-019-5647-8>
- Ott, E., Saathoff, S., Graf, K., Schwab, F., and Chaberny, I. F. (2013). The prevalence of nosocomial and community acquired infections in a university hospital: An observational study. *Deutsches Arzteblatt International*, 110(31–32), 533–540. <https://doi.org/10.3238/arztebl.2013.0533>
- Otto, M. (2018). Staphylococcal Biofilms. *Microbiology Spectrum*, 6(4). <https://doi.org/10.1128/microbiolspec.gpp3-0023-2018>
- Page, A. J., Cummins, C. A., Hunt, M., Wong, V. K., Reuter, S., Holden, M. T. G., Fookes, M., Falush, D., Keane, J. A., and Parkhill, J. (2015). Roary: Rapid large-scale prokaryote pan genome analysis. *Bioinformatics*, 31(22), 3691–3693. <https://doi.org/10.1093/bioinformatics/btv421>
- Pain, M., Hjerde, E., Klingenberg, C., and Cavanagh, J. P. (2019). Comparative Genomic Analysis of *Staphylococcus haemolyticus* Reveals Key to Hospital Adaptation and Pathogenicity. *Frontiers in Microbiology*, 10, 2096. <https://doi.org/10.3389/fmicb.2019.02096>

- Pain, M., Wolden, R., Jaén-Luchoro, D., Salvà-Serra, F., Iglesias, B. P., Karlsson, R., Klingenberg, C., and Cavanagh, J. P. (2020). *Staphylococcus borealis* sp. Nov., isolated from human skin and blood. *International Journal of Systematic and Evolutionary Microbiology*, 70(12), 6067–6078. <https://doi.org/10.1099/ijsem.0.004499>
- Pal, C., Bengtsson-Palme, J., Rensing, C., Kristiansson, E., and Larsson, D. G. J. (2014). BacMet: Antibacterial biocide and metal resistance genes database. *Nucleic Acids Research*, 42(1), 737–743. <https://doi.org/10.1093/nar/gkt1252>
- Panton, P. N. (1937). The Specific Treatment of Staphylococcal Infections. *Proceedings of the Royal Society of Medicine*, 30(5), 515–520. <https://doi.org/10.1177/003591573703000501>
- Panton, P. N., Came, M. B., and Valentine, F. C. O. (1932). Staphylococcal toxin. *The Lancet*, 219(5662), 506–508. [https://doi.org/10.1016/S0140-6736\(01\)24468-7](https://doi.org/10.1016/S0140-6736(01)24468-7)
- Pantůček, R., Sedláček, I., Indráková, A., Vrbovská, V., Mašlaňová, I., Kovařovic, V., Švec, P., Králová, S., Křištofová, L., Kekláková, J., Petráš, P., and Doškař, J. (2018). *Staphylococcus edaphicus* sp. Nov., Isolated in Antarctica, Harbors the *mecC* Gene and Genomic Islands with a Suspected Role in Adaptation to Extreme Environments. *Applied and Environmental Microbiology*, 84(2), e01746-17. <https://doi.org/10.1128/AEM.01746-17>
- Pantůček, R., Sedláček, I., Petráš, P., Koukalová, D., Švec, P., Štětina, V., Vancanneyt, M., Chrastinová, L., Vokurková, J., Růžicková, V., Doškař, J., Swings, J., and Hájek, V. (2005). *Staphylococcus simiae* sp. Nov., isolated from South American squirrel monkeys. *International Journal of Systematic and Evolutionary Microbiology*, 55(5), 1953–1958. <https://doi.org/10.1099/ijms.0.63590-0>
- Pantůček, R., Švec, P., Dajcs, J. J., Machová, I., Černohlávková, J., Šedo, O., Gelbíčová, T., Mašlaňová, I., Doškař, J., Zdráhal, Z., Růžicková, V., and Sedláček, I. (2013). *Staphylococcus petrasii* sp. Nov. Including *S. petrasii* subsp. *Petrasii* subsp. Nov. And *S. petrasii* subsp. *Croceilyticus* subsp. Nov., isolated from human clinical specimens and human ear infections. *Systematic and Applied Microbiology*, 36(2), 90–95. <https://doi.org/10.1016/j.syapm.2012.11.004>
- Paranthaman, K., Wilson, A., Verlander, N., Rooney, G., Macdonald, N., Nsonwu, O., Hope, R., Fleming, P., Hatcher, J., Ogundipe, E., Ratnaraja, N., Wan, Y., Pichon, B., Westrop, S. J., Brown, C. S., and Demirjian, A. (2023). Trends in coagulase-negative staphylococci (CoNS), England, 2010–2021. *Access Microbiology*, 5(6), 000491.v3. <https://doi.org/10.1099/acmi.0.000491.v3>
- Parker, M. T., and Jevons, M. P. (1964). A Survey of Methicillin Resistance in *Staphylococcus Aureus*. *Postgraduate Medical Journal*, 40(Suppl), 170–178. <https://doi.org/10.1136/pgmj.40.suppl.170>
- Parte, A. C. (2014). LPSN—list of prokaryotic names with standing in nomenclature. *Nucleic Acids Research*, 42(1), 613–616. <https://doi.org/10.1093/nar/gkt1111>
- Parthasarathy, S., Shah, S., Raja Sager, A., Rangan, A., and Durugu, S. (2020). *Staphylococcus lugdunensis*: Review of Epidemiology, Complications, and Treatment. *Cureus*, 12(6). <https://doi.org/10.7759/cureus.8801>

- Paterson, G. K., Harrison, E. M., Murray, G. G. R., Welch, J. J., Warland, J. H., Holden, M. T. G., Morgan, F. J. E., Ba, X., Koop, G., Harris, S. R., Maskell, D. J., Peacock, S. J., Herrtage, M. E., Parkhill, J., and Holmes, M. A. (2015). Capturing the cloud of diversity reveals complexity and heterogeneity of MRSA carriage, infection and transmission. *Nature Communications*, 6(1), 6560. <https://doi.org/10.1038/ncomms7560>
- Pereira, P. M., Filipe, S. R., Tomasz, A., and Pinho, M. G. (2007). Fluorescence Ratio Imaging Microscopy Shows Decreased Access of Vancomycin to Cell Wall Synthetic Sites in Vancomycin-Resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, 51(10), 3627–3633. <https://doi.org/10.1128/AAC.00431-07>
- Perreten, V., Kania, S. A., and Bemis, D. (2020). *Staphylococcus ursi* sp. Nov., a new member of the ‘*Staphylococcus intermedius* group’ isolated from healthy black bears. *International Journal of Systematic and Evolutionary Microbiology*, 70(8), 4637–4645. <https://doi.org/10.1099/ijsem.0.004324>
- Petit, R. A., and Read, T. D. (2018). *Staphylococcus aureus* viewed from the perspective of 40,000+ genomes. *PeerJ*, 6, e5261. <https://doi.org/10.7717/peerj.5261>
- Petit, R. A., and Read, T. D. (2020). Bactopia: A Flexible Pipeline for Complete Analysis of Bacterial Genomes. *mSystems*, 5(4), e00190-20. <https://doi.org/10.1128/mSystems.00190-20>
- Petti, C. A., Simmon, K. E., Miro, J. M., Hoen, B., Marco, F., Chu, V. H., Athan, E., Bukovski, S., Bouza, E., Bradley, S., Fowler, V. G., Giannitsioti, E., Gordon, D., Reinbott, P., Korman, T., Lang, S., Garcia-de-la-Maria, C., Raglio, A., Morris, A. J., ... Woods, C. W. (2008). Genotypic Diversity of Coagulase-Negative *Staphylococci* Causing Endocarditis: A Global Perspective. *Journal of Clinical Microbiology*, 46(5), 1780–1784. <https://doi.org/10.1128/jcm.02405-07>
- Pinheiro-Hubinger, L., Moraes Riboli, D. F., Abraão, L. M., Pereira Franchi, E. P. L., and Ribeiro de Souza da Cunha, M. de L. (2021). Coagulase-Negative *Staphylococci* Clones Are Widely Distributed in the Hospital and Community. *Pathogens*, 10(7), 792. <https://doi.org/10.3390/pathogens10070792>
- Podell, S., and Gaasterland, T. (2007). DarkHorse: A method for genome-wide prediction of horizontal gene transfer. *Genome Biology*, 8(2), R16. <https://doi.org/10.1186/gb-2007-8-2-r16>
- Price, M. N., Dehal, P. S., and Arkin, A. P. (2010). FastTree 2 – Approximately Maximum-Likelihood Trees for Large Alignments. *PLOS ONE*, 5(3), e9490. <https://doi.org/10.1371/journal.pone.0009490>
- Prjibelski, A., Antipov, D., Meleshko, D., Lapidus, A., and Korobeynikov, A. (2020). Using SPAdes De Novo Assembler. *Current Protocols in Bioinformatics*, 70(1), e102. <https://doi.org/10.1002/cpbi.102>
- Probst, A. J., Hertel, C., Richter, L., Wassill, L., Ludwig, W., and Hammes, W. P. (1998). *Staphylococcus condimenti* sp. Nov., from soy sauce mash, and *Staphylococcus carnosus* (Schleifer and Fischer 1982) subsp. *Utilis* subsp. Nov. *International Journal of Systematic Bacteriology*, 48(3), 651–658. <https://doi.org/10.1099/00207713-48-3-651>

- Rammelkamp, C. H., and Maxon, T. (1942). Resistance of *Staphylococcus aureus* to the Action of Penicillin. *Proceedings of the Society for Experimental Biology and Medicine*, 51(3), 386–389. <https://doi.org/10.3181/00379727-51-13986>
- Raz, R., Colodner, R., and Kunin, C. M. (2005). Who Are You—*Staphylococcus saprophyticus*? *Clinical Infectious Diseases*, 40(6), 896–898. <https://doi.org/10.1086/428353>
- Rebelo, A. R., Bortolaia, V., Leekitcharoenphon, P., Hansen, D. S., Nielsen, H. L., Ellermann-Eriksen, S., Kemp, M., Røder, B. L., Frimodt-Møller, N., Søndergaard, T. S., Coia, J. E., Østergaard, C., Westh, H., and Aarestrup, F. M. (2022). One Day in Denmark: Comparison of Phenotypic and Genotypic Antimicrobial Susceptibility Testing in Bacterial Isolates From Clinical Settings. *Frontiers in Microbiology*, 13, 804627. <https://doi.org/10.3389/fmicb.2022.804627>
- Riesen, A., and Perreten, V. (2010). *Staphylococcus rostri* sp. Nov., a haemolytic bacterium isolated from the noses of healthy pigs. *International Journal of Systematic and Evolutionary Microbiology*, 60(9), 2042–2047. <https://doi.org/10.1099/ijs.0.012443-0>
- Robinson, D. A., and Enright, M. C. (2004). Evolution of *Staphylococcus aureus* by Large Chromosomal Replacements. *Journal of Bacteriology*, 186(4), 1060–1064. <https://doi.org/10.1128/jb.186.4.1060-1064.2004>
- Rocha, J., Silva, V., Poeta, P., Botelho, J., and Manaia, C. M. (2023). Searching for links between environmental and clinical *mecA*+ *Staphylococcus aureus*: A comparative genomics study. *Science of The Total Environment*, 895, 165078. <https://doi.org/10.1016/j.scitotenv.2023.165078>
- Rohde, H., Frankenberger, S., Zähringer, U., and Mack, D. (2010). Structure, function and contribution of polysaccharide intercellular adhesin (PIA) to *Staphylococcus epidermidis* biofilm formation and pathogenesis of biomaterial-associated infections. *European Journal of Cell Biology*, 89(1), 103–111. <https://doi.org/10.1016/j.ejcb.2009.10.005>
- Rosenbach, F. J. (1884). *Mikro-organismen bei den Wund-Infektions-Krankheiten des Menschen* (pp. 1–150). J.F. Bergmann. <https://doi.org/10.5962/bhl.title.22955>
- Rosenthal, V. D., Chaparro, G. J., Servolo-Medeiros, E. A., Souza-Fram, D., Escudero, D. V. da S., Gualtero-Trujillo, S. M., Morfin-Otero, R., Gonzalez-Diaz, E., Rodriguez-Noriega, E., Altuzar-Figueroa, M. A., Aguirre-Avalos, G., Mijangos-Méndez, J. C., Corona-Jiménez, F., Hernandez-Chena, B. E., Abu-Jarad, M. R., Diaz-Hernandez, E. M., Miranda-Novales, M. G., Vázquez-Rosales, J. G., Aguilar-De-Morós, D., ... Di-Silvestre, G. (2021). An eight-year multicenter study on short-term peripheral intravenous catheter-related bloodstream infection rates in 100 intensive care units of 9 countries in Latin America: Argentina, Brazil, Colombia, Costa Rica, Dominican Republic, Ecuador, Mexico, Panama, and Venezuela. Findings of the International Nosocomial Infection Control Consortium (INICC). *Infection Control & Hospital Epidemiology*, 42(9), 1098–1104. <https://doi.org/10.1017/ice.2020.1373>
- Rosypal, S., Rosypalova, A., and Horejs, J. (1966). The Classification of Micrococci and Staphylococci Based on their DNA Base Composition and Adansonian Analysis. *Journal of General Microbiology*, 44(2), 281–292. <https://doi.org/10.1099/00221287-44-2-281>

- Sadykov, M. R. (2016). Restriction-Modification Systems as a Barrier for Genetic Manipulation of *Staphylococcus aureus*. *Methods in Molecular Biology (Clifton, N.J.)*, 1373, 9–23. https://doi.org/10.1007/7651_2014_180
- Sakinc, T., Kleine, B., and Gatermann, S. G. (2006). SdrI, a Serine-Aspartate Repeat Protein Identified in *Staphylococcus saprophyticus* Strain 7108, Is a Collagen-Binding Protein. *Infection and Immunity*, 74(8), 4615–4623. <https://doi.org/10.1128/iai.01885-05>
- Sakoparnig, T., Field, C., and van Nimwegen, E. (2021). Whole genome phylogenies reflect the distributions of recombination rates for many bacterial species. *eLife*, 10, e65366. <https://doi.org/10.7554/eLife.65366>
- Salamandane, A., Oliveira, J., Coelho, M., Ramos, B., Cunha, M. V., Malfeito-Ferreira, M., and Brito, L. (2022). Enterotoxin- and Antibiotic-Resistance-Encoding Genes Are Present in Both Coagulase-Positive and Coagulase-Negative Foodborne *Staphylococcus* Strains. *Applied Microbiology*, 2(2), 367–380. <https://doi.org/10.3390/applmicrobiol2020028>
- Salzberg, S. L. (2019). Next-generation genome annotation: We still struggle to get it right. *Genome Biology*, 20(1), 92. <https://doi.org/10.1186/s13059-019-1715-2>
- Saroha, T., Chaudhry, V., and Patil, P. B. (2022). Novel insights into the role of the mobilome in ecological diversification and success of *Staphylococcus haemolyticus* as an opportunistic pathogen. *Microbial Genomics*, 8(1), 000755. <https://doi.org/10.1099/mgen.0.000755>
- Sayers, E. W., Bolton, E. E., Brister, J. R., Canese, K., Chan, J., Comeau, D. C., Connor, R., Funk, K., Kelly, C., Kim, S., Madej, T., Marchler-Bauer, A., Lanczycki, C., Lathrop, S., Lu, Z., Thibaud-Nissen, F., Murphy, T., Phan, L., Skripchenko, Y., ... Sherry, S. T. (2021). Database resources of the National Center for Biotechnology Information. *Nucleic Acids Research*, 50(1), 20–26. <https://doi.org/10.1093/nar/gkab1112>
- Schatz, M. C. (2017). Nanopore sequencing meets epigenetics. *Nature Methods*, 14(4), 347–348. <https://doi.org/10.1038/nmeth.4240>
- Schiffer, C. J., Grätz, C., Pfaffl, M. W., Vogel, R. F., and Ehrmann, M. A. (2023). Characterization of the *Staphylococcus xylosus* methylome reveals a new variant of type I restriction modification system in staphylococci. *Frontiers in Microbiology*, 14, 946189. <https://doi.org/10.3389/fmicb.2023.946189>
- Schleifer, K. H., and Fischer, U. (1982). Description of a New Species of the Genus *Staphylococcus*: *Staphylococcus carnosus*. *International Journal of Systematic Bacteriology*, 32(2), 153–156. <https://doi.org/10.1099/00207713-32-2-153>
- Schleifer, K. H., Kilpper-Bälz, R., and Devriese, L. A. (1984). *Staphylococcus arlettae* sp. nov., *S. equorum* sp. nov. and *S. kloosii* sp. nov.: Three New Coagulase-Negative, Novobiocin-Resistant Species from Animals. *Systematic and Applied Microbiology*, 5(4), 501–509. [https://doi.org/10.1016/S0723-2020\(84\)80007-7](https://doi.org/10.1016/S0723-2020(84)80007-7)
- Schleifer, K. H., and Kloos, W. E. (1975). Isolation and Characterization of *Staphylococci* from Human Skin I. Amended Descriptions of *Staphylococcus epidermidis* and *Staphylococcus saprophyticus* and Descriptions of Three New Species: *Staphylococcus cohnii*, *Staphylococcus haemolyticus*, and *Staphylococcus xylosus*.

REFERENCES

- International Journal of Systematic Bacteriology*, 25(1), 50–61.
<https://doi.org/10.1099/00207713-25-1-50>
- Schutte, A. H. J., Strepis, N., Zandijk, W. H. A., Bexkens, M. L., Bode, L. G. M., and Klaassen, C. H. W. (2021). Characterization of *Staphylococcus roterodami* sp. Nov., a new species within the *Staphylococcus aureus* complex isolated from a human foot infection. *International Journal of Systematic and Evolutionary Microbiology*, 71(9).
<https://doi.org/10.1099/ijsem.0.004996>
- Schwendener, S., Cotting, K., and Perreten, V. (2017). Novel methicillin resistance gene *mecD* in clinical *Macroccoccus caseolyticus* strains from bovine and canine sources. *Scientific Reports*, 7. <https://doi.org/10.1038/srep43797>
- Schwendener, S., and Perreten, V. (2022). The *bla* and *mec* families of β -lactam resistance genes in the genera *Macroccoccus*, *Mammaliococcus* and *Staphylococcus*: An in-depth analysis with emphasis on *Macroccoccus*. *Journal of Antimicrobial Chemotherapy*, 77(7), 1796–1827. <https://doi.org/10.1093/jac/dkac107>
- Seemann, T. (2014a). Prokka: Rapid prokaryotic genome annotation. *Bioinformatics*, 30(14), 2068–2069. <https://doi.org/10.1093/bioinformatics/btu153>
- Seemann, T. (2014b). ABRicate. *Github*. <https://github.com/tseemann/abricate>
- Seemann, T., Goncalves da Silva, A., Bulach, D. M., Schultz, M. B., Kwong, J. C., and Howden, B. P. (2015). Nullarbor. *Github*. <https://github.com/tseemann/nullarbor>
- Sengupta, S., and Azad, R. K. (2023). Leveraging comparative genomics to uncover alien genes in bacterial genomes. *Microbial Genomics*, 9(1), mgen000939.
<https://doi.org/10.1099/mgen.0.000939>
- Siguié, P., Perochon, J., Lestrade, L., Mahillon, J., and Chandler, M. (2006). ISfinder: The reference centre for bacterial insertion sequences. *Nucleic Acids Research*, 34(suppl_1), 32–36. <https://doi.org/10.1093/nar/gkj014>
- Sirén, K., Millard, A., Petersen, B., Gilbert, M. T. P., Clokie, M. R. J., and Sicheritz-Pontén, T. (2021). Rapid discovery of novel prophages using biological feature engineering and machine learning. *NAR Genomics and Bioinformatics*, 3(1), lqaa109.
<https://doi.org/10.1093/nargab/lqaa109>
- Smillie, C., Garcillán-Barcia, M. P., Francia, M. V., Rocha, E. P. C., and de la Cruz, F. (2010). Mobility of plasmids. *Microbiology and Molecular Biology Reviews: MMBR*, 74(3), 434–452. <https://doi.org/10.1128/MMBR.00020-10>
- Smith, J. T., and Andam, C. P. (2021). Extensive Horizontal Gene Transfer within and between Species of Coagulase-Negative *Staphylococcus*. *Genome Biology and Evolution*, 13(9), evab206. <https://doi.org/10.1093/gbe/evab206>
- Spergser, J. (2003). *Staphylococcus nepalensis* sp. Nov., isolated from goats of the Himalayan region. *International Journal of Systematic and Evolutionary Microbiology*, 53(6), 2007–2011. <https://doi.org/10.1099/ijms.0.02646-0>
- Stamatakis, A. (2014). RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*, 30(9), 1312–1313.
<https://doi.org/10.1093/bioinformatics/btu033>

- Stewart, G. T. (1961). Changes in Sensitivity of Staphylococci to Methicillin. *British Medical Journal*, 1(5229), 863–866. <https://doi.org/10.1136/bmj.1.5229.863>
- Stewart, G. T., and Holt, R. J. (1963). Evolution of Natural Resistance to the Newer Penicillins. *British Medical Journal*, 1(5326), 308–311. <https://doi.org/10.1136/bmj.1.5326.308>
- Sulakvelidze, A., Alavidze, Z., and Morris, J. G. (2001). Bacteriophage Therapy. *Antimicrobial Agents and Chemotherapy*, 45(3), 649–659. <https://doi.org/10.1128/aac.45.3.649-659.2001>
- Sun, J., Lu, F., Luo, Y., Bie, L., Xu, L., and Wang, Y. (2023). OrthoVenn3: An integrated platform for exploring and visualizing orthologous data across genomes. *Nucleic Acids Research*, 51(1), 397–403. <https://doi.org/10.1093/nar/gkad313>
- Supré, K., De Vlieghe, S., Cleenwerck, I., Engelbeen, K., Van Trappen, S., Piepers, S., Sampimon, O. C., Zadoks, R. N., De Vos, P., and Haesebrouck, F. (2010). Staphylococcus devriesei sp. Nov., isolated from teat apices and milk of dairy cows. *International Journal of Systematic and Evolutionary Microbiology*, 60(12), 2739–2744. <https://doi.org/10.1099/ijs.0.015982-0>
- Takeuchi, F., Watanabe, S., Baba, T., Yuzawa, H., Ito, T., Morimoto, Y., Kuroda, M., Cui, L., Takahashi, M., Ankai, A., Baba, S., Fukui, S., Lee, J. C., and Hiramatsu, K. (2005). Whole-Genome Sequencing of Staphylococcus haemolyticus Uncovers the Extreme Plasticity of Its Genome and the Evolution of Human-Colonizing Staphylococcal Species. *Journal of Bacteriology*, 187(21), 7292–7308. <https://doi.org/10.1128/JB.187.21.7292-7308.2005>
- Tamura, K., Stecher, G., and Kumar, S. (2021). MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution*, 38(7), 3022–3027. <https://doi.org/10.1093/molbev/msab120>
- Tanasupawat, S., Hashimoto, Y., Ezaki, T., Kozaki, M., and Komagata, K. (1992). Staphylococcus piscifermentans sp. Nov., from Fermented Fish in Thailand. *International Journal of Systematic Bacteriology*, 42(4), 577–581. <https://doi.org/10.1099/00207713-42-4-577>
- Tansirichaiya, S., Moyo, S. J., Al-Haroni, M., and Roberts, A. P. (2021). Capture of a novel, antibiotic resistance encoding, mobile genetic element from Escherichia coli using a new entrapment vector. *Journal of Applied Microbiology*, 130(3), 832–842. <https://doi.org/10.1111/jam.14837>
- Taponen, S., Supré, K., Piessens, V., Van Coillie, E., De Vlieghe, S., and Koort, J. M. K. (2012). Staphylococcus agnetis sp. Nov., a coagulase-variable species from bovine subclinical and mild clinical mastitis. *International Journal of Systematic and Evolutionary Microbiology*, 62(1), 61–65. <https://doi.org/10.1099/ijs.0.028365-0>
- Tatusova, T., DiCuccio, M., Badretdin, A., Chetvernin, V., Nawrocki, E. P., Zaslavsky, L., Lomsadze, A., Pruitt, K. D., Borodovsky, M., and Ostell, J. (2016). NCBI prokaryotic genome annotation pipeline. *Nucleic Acids Research*, 44(14), 6614–6624. <https://doi.org/10.1093/nar/gkw569>
- Taylor, S. P., Anderson, W. E., Beam, K., Taylor, B., Ellerman, J., and Kowalkowski, M. A. (2021). The Association Between Antibiotic Delay Intervals and Hospital Mortality Among

- Patients Treated in the Emergency Department for Suspected Sepsis. *Critical Care Medicine*, 49(5), 741. <https://doi.org/10.1097/CCM.00000000000004863>
- Thompson, R. L., Cabezudo, I., and Wenzel, R. P. (1982). Epidemiology of Nosocomial Infections Caused by Methicillin-Resistant *Staphylococcus aureus*. *Annals of Internal Medicine*, 97(3), 309–317. <https://doi.org/10.7326/0003-4819-97-3-309>
- Tong, S. Y. C., Schaumburg, F., Ellington, M. J., Corander, J., Pichon, B., Leendertz, F., Bentley, S. D., Parkhill, J., Holt, D. C., Peters, G., and Giffard, P. M. (2015). Novel staphylococcal species that form part of a *Staphylococcus aureus*-related complex: The non-pigmented *Staphylococcus argenteus* sp. nov. and the non-human primate-associated *Staphylococcus schweitzeri* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, 65(Pt_1), 15–22. <https://doi.org/10.1099/ijls.0.062752-0>
- Trindade, P. A., McCulloch, J. A., Oliveira, G. A., and Mamizuka, E. M. (2003). Molecular techniques for MRSA typing: Current issues and perspectives. *Brazilian Journal of Infectious Diseases*, 7, 32–43. <https://doi.org/10.1590/S1413-86702003000100005>
- Trülsch, K., Grabein, B., Schumann, P., Mellmann, A., Antonenka, U., Heesemann, J., and Becker, K. (2007). *Staphylococcus pettenkoferi* sp. Nov., a novel coagulase-negative staphylococcal species isolated from human clinical specimens. *International Journal of Systematic and Evolutionary Microbiology*, 57(7), 1543–1548. <https://doi.org/10.1099/ijls.0.64381-0>
- Tsubakishita, S., Kuwahara-Arai, K., Baba, T., and Hiramatsu, K. (2010). Staphylococcal Cassette Chromosome mec-Like Element in *Macroccoccus caseolyticus*. *Antimicrobial Agents and Chemotherapy*, 54(4), 1469. <https://doi.org/10.1128/AAC.00575-09>
- Turner, N. A., Sharma-Kuinkel, B. K., Maskarinec, S. A., Eichenberger, E. M., Shah, P. P., Carugati, M., Holland, T. L., and Fowler, V. G. (2019). Methicillin-resistant *Staphylococcus aureus*: An overview of basic and clinical research. *Nature Reviews Microbiology*, 17(4), 203–218. <https://doi.org/10.1038/s41579-018-0147-4>
- Twiss, E., Coros, A. M., Tavakoli, N. P., and Derbyshire, K. M. (2005). Transposition is modulated by a diverse set of host factors in *Escherichia coli* and is stimulated by nutritional stress. *Molecular Microbiology*, 57(6), 1593–1607. <https://doi.org/10.1111/j.1365-2958.2005.04794.x>
- Ubukata, K., Yamashita, N., and Konno, M. (1985). Occurrence of a beta-lactam-inducible penicillin-binding protein in methicillin-resistant staphylococci. *Antimicrobial Agents and Chemotherapy*, 27(5), 851–857. <https://doi.org/10.1128/aac.27.5.851>
- Uehara, Y. (2022). Current Status of Staphylococcal Cassette Chromosome mec (SCCmec). *Antibiotics*, 11(1), 86. <https://doi.org/10.3390/antibiotics11010086>
- Urushibara, N., Aung, M. S., Kawaguchiya, M., and Kobayashi, N. (2020). Novel staphylococcal cassette chromosome mec (SCCmec) type XIV (5A) and a truncated SCCmec element in SCC composite islands carrying *speG* in ST5 MRSA in Japan. *Journal of Antimicrobial Chemotherapy*, 75(1), 46–50. <https://doi.org/10.1093/jac/dkz406>
- Van de Velde, H. (1894). Etude sur le mécanisme de la virulence du *Staphylocoque pyogene*. *La Cellule*, 10(403), 10.

- Varaldo, P. E., Kilpper-Balz, R., Biavasco, F., Satta, G., and Schleifer, K. H. (1988). *Staphylococcus delphini* sp. Nov., a Coagulase-Positive Species Isolated from Dolphins. *International Journal of Systematic Bacteriology*, 38(4), 436–439. <https://doi.org/10.1099/00207713-38-4-436>
- Verway, M., Brown, K. A., Marchand-Austin, A., Diong, C., Lee, S., Langford, B., Schwartz, K. L., MacFadden, D. R., Patel, S. N., Sander, B., Johnstone, J., Garber, G., and Daneman, N. (2022). Prevalence and Mortality Associated with Bloodstream Organisms: A Population-Wide Retrospective Cohort Study. *Journal of Clinical Microbiology*, 60(4), e02429-21. <https://doi.org/10.1128/jcm.02429-21>
- Volokhov, D. V., Zagorodnyaya, T. A., Furtak, V. A., Nattanmai, G., Randall, L., Jose, S., Gao, Y., Eisenberg, T., Delmonte, P., Blom, J., and Mitchell, K. K. (2023). *Streptococcus sciuri* sp. Nov., *Staphylococcus marylandisciuri* sp. Nov. And *Staphylococcus americanisciuri* sp. Nov., isolated from faeces of eastern grey squirrel (*Sciurus carolinensis*). *International Journal of Systematic and Evolutionary Microbiology*, 73(8), 006015. <https://doi.org/10.1099/ijsem.0.006015>
- Vos, M. C., Behrendt, M. D., Melles, D. C., Mollema, F. P. N., de Groot, W., Parlevliet, G., Ott, A., Horst-Kreft, D., van Belkum, A., and Verbrugh, H. A. (2009). 5 years of experience implementing a methicillin-resistant *Staphylococcus aureus* search and destroy policy at the largest university medical center in the Netherlands. *Infection Control and Hospital Epidemiology*, 30(10), 977–984. <https://doi.org/10.1086/605921>
- Wang, W., Hu, Y., Baker, M., Dottorini, T., Li, H., Dong, Y., Bai, Y., Fanning, S., and Li, F. (2022). Novel SCCmec type XV (7A) and two pseudo-SCCmec variants in foodborne MRSA in China. *Journal of Antimicrobial Chemotherapy*, 77(4), 903–909. <https://doi.org/10.1093/jac/dkab500>
- Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E., Stackebrandt, E., Starr, M. P., and Truper, H. G. (1987). Report of the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics. *International Journal of Systematic and Evolutionary Microbiology*, 37(4), 463–464. <https://doi.org/10.1099/00207713-37-4-463>
- Wei, W., Cao, Z., Zhu, Y.-L., Wang, X., Ding, G., Xu, H., Jia, P., Qu, D., Danchin, A., and Li, Y. (2006). Conserved genes in a path from commensalism to pathogenicity: Comparative phylogenetic profiles of *Staphylococcus epidermidis* RP62A and ATCC12228. *BMC Genomics*, 7(1), 112. <https://doi.org/10.1186/1471-2164-7-112>
- Wick, R. R., Judd, L. M., Cerdeira, L. T., Hawkey, J., Méric, G., Vezina, B., Wyres, K. L., and Holt, K. E. (2021). Trycycler: Consensus long-read assemblies for bacterial genomes. *Genome Biology*, 22(1), 266. <https://doi.org/10.1186/s13059-021-02483-z>
- Wick, R. R., Judd, L. M., Gorrie, C. L., and Holt, K. E. (2017a). Completing bacterial genome assemblies with multiplex MinION sequencing. *Microbial Genomics*, 3(10). <https://doi.org/10.1099/mgen.0.000132>
- Wick, R. R., Judd, L. M., Gorrie, C. L., and Holt, K. E. (2017b). Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. *PLOS Computational Biology*, 13(6), e1005595. <https://doi.org/10.1371/journal.pcbi.1005595>

- Winslow, C. E. A., and Winslow, A. R. (1908). *The systematic relationships of the Coccaceae, with a discussion of the principles of bacterial classification*. New York J. Wiley & Sons, Ltd.
- Wisplinghoff, H., Bischoff, T., Tallent, S. M., Seifert, H., Wenzel, R. P., and Edmond, M. B. (2004). Nosocomial bloodstream infections in US hospitals: Analysis of 24,179 cases from a prospective nationwide surveillance study. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, 39(3), 309–317. <https://doi.org/10.1086/421946>
- Woese, C. R., and Fox, G. E. (1977). Phylogenetic structure of the prokaryotic domain: The primary kingdoms. *Proceedings of the National Academy of Sciences of the United States of America*, 74(11), 5088–5090. <https://doi.org/10.1073/pnas.74.11.5088>
- Wolf, Y. I., Makarova, K. S., Lobkovsky, A. E., and Koonin, E. V. (2016). Two fundamentally different classes of microbial genes. *Nature Microbiology*, 2(3), 1–6. <https://doi.org/10.1038/nmicrobiol.2016.208>
- Xiao, J., Huang, J., Xue, X., Wang, C., Sun, Y., Zheng, L., Zhao, X., Wang, X., Zhao, X., and Xue, H. (2023). Novel cassette chromosome recombinases CcrA8B9 catalyse the excision and integration of the staphylococcal cassette chromosome mec element. *Journal of Antimicrobial Chemotherapy*, 78(2), 440–444. <https://doi.org/10.1093/jac/dkac410>
- Xie, Y., Wei, Y., Shen, Y., Li, X., Zhou, H., Tai, C., Deng, Z., and Ou, H.-Y. (2018). TADB 2.0: An updated database of bacterial type II toxin–antitoxin loci. *Nucleic Acids Research*, 46(1), 749–753. <https://doi.org/10.1093/nar/gkx1033>
- Xu, S., Corvaglia, A. R., Chan, S.-H., Zheng, Y., and Linder, P. (2011). A type IV modification-dependent restriction enzyme SauUSI from *Staphylococcus aureus* subsp. *Aureus* USA300. *Nucleic Acids Research*, 39(13), 5597–5610. <https://doi.org/10.1093/nar/gkr098>
- Yao, Y., Maddamsetti, R., Weiss, A., Ha, Y., Wang, T., Wang, S., and You, L. (2022). Intra- and interpopulation transposition of mobile genetic elements driven by antibiotic selection. *Nature Ecology & Evolution*, 6(5), 555–564. <https://doi.org/10.1038/s41559-022-01705-2>
- Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., Aarestrup, F. M., and Larsen, M. V. (2012). Identification of acquired antimicrobial resistance genes. *The Journal of Antimicrobial Chemotherapy*, 67(11), 2640–2644. <https://doi.org/10.1093/jac/dks261>
- Zelmer, A. R., Nelson, R., Richter, K., and Atkins, G. J. (2022). Can intracellular *Staphylococcus aureus* in osteomyelitis be treated using current antibiotics? A systematic review and narrative synthesis. *Bone Research*, 10(1), 1–18. <https://doi.org/10.1038/s41413-022-00227-8>
- Zhang, K., Potter, R. F., Marino, J., Muenks, C. E., Lammers, M. G., Dien Bard, J., Dingle, T. C., Humphries, R., Westblade, L. F., Burnham, C.-A. D., and Dantas, G. (2023). Comparative genomics reveals the correlations of stress response genes and bacteriophages in developing antibiotic resistance of *Staphylococcus saprophyticus*. *mSystems*, 8(6), e00697-23. <https://doi.org/10.1128/msystems.00697-23>

- Zhang, Y.-Q., Ren, S.-X., Li, H.-L., Wang, Y.-X., Fu, G., Yang, J., Qin, Z.-Q., Miao, Y.-G., Wang, W.-Y., Chen, R.-S., Shen, Y., Chen, Z., Yuan, Z.-H., Zhao, G.-P., Qu, D., Danchin, A., and Wen, Y.-M. (2003). Genome-based analysis of virulence genes in a non-biofilm-forming *Staphylococcus epidermidis* strain (ATCC 12228). *Molecular Microbiology*, 49(6), 1577–1593. <https://doi.org/10.1046/j.1365-2958.2003.03671.x>
- Zhu, Q., Kosoy, M., and Dittmar, K. (2014). HGTector: An automated method facilitating genome-wide discovery of putative horizontal gene transfers. *BMC Genomics*, 15(1), 717. <https://doi.org/10.1186/1471-2164-15-717>
- Zhu, Q., Mai, U., Pfeiffer, W., Janssen, S., Asnicar, F., Sanders, J. G., Belda-Ferre, P., Al-Ghalith, G. A., Kopylova, E., McDonald, D., Kosciolk, T., Yin, J. B., Huang, S., Salam, N., Jiao, J.-Y., Wu, Z., Xu, Z. Z., Cantrell, K., Yang, Y., ... Knight, R. (2019). Phylogenomics of 10,575 genomes reveals evolutionary proximity between domains Bacteria and Archaea. *Nature Communications*, 10(1), 5477. <https://doi.org/10.1038/s41467-019-13443-4>
- Ziebuhr, W. (2012). Genome Structure and Variability in Coagulase-Negative Staphylococci. In *Genome Plasticity and Infectious Diseases* (pp. 44–57). John Wiley & Sons, Ltd. <https://doi.org/10.1128/9781555817213.ch4>
- Zimmerli, W., and Moser, C. (2012). Pathogenesis and treatment concepts of orthopaedic biofilm infections. *FEMS Immunology & Medical Microbiology*, 65(2), 158–168. <https://doi.org/10.1111/j.1574-695X.2012.00938.x>
- Zipperer, A., Konnerth, M. C., Laux, C., Berscheid, A., Janek, D., Weidenmaier, C., Burian, M., Schilling, N. A., Slavetinsky, C., and Marschal, M. (2016). Human commensals producing a novel antibiotic impair pathogen colonization. *Nature*, 535(7613), 511–516. <https://doi.org/doi: 10.1038/nature18634>

Appendix A Conference Abstracts

Partial results of the dissertation were presented in the following conference papers, listed in order of presentation from the most recent.

- Mašlaňová, I., **Kovařovic, V.**, Švec, P., Sedláček, I., Pantůček, R. Genome-wide analysis of *Macrococcus* spp. provides insights into high diversity of mobile genetic elements. In The Fifth Meeting of Bergey's International Society for Microbial Systematics (BISMiS). Guangzhou, China, November 6–9, 2023. Abstract Book p. 111. Poster presentation.
- Pantůček, R., **Kovařovic, V.**, Finstrlová, A., Sedláček, I., Petráš, P., Švec, P., Mašlaňová, I., Neumann-Schaal, M., Botka, T., Doškař, J., *Staphylococcus brunensis* sp. nov. from human clinical specimens with SCC-related genomic island outside of the *orfX* (*rlmH*) gene bearing novel *ccrDE* recombinase gene complex. In The Fifth Meeting of Bergey's International Society for Microbial Systematics (BISMiS). Guangzhou, China, November 6–9, 2023. Abstract Book p. 118. Poster presentation.
- Micenková, L., Mašlaňová, I., Sedláček, I., Švec, P., **Kovařovic, V.**, Pěňčík, O., Pantůček, R., Kadlec, M., Křážková, M., Nývlt, D., Hrbáček, F. Bacterial composition of active layer and permafrost on the Ulu Peninsula of James Ross Island, Antarctica. In The Fifth Meeting of Bergey's International Society for Microbial Systematics (BISMiS). Guangzhou, China, November 6–9, 2023. Abstract Book p. 169. Poster presentation.
- Kovařovic, V.**, Sedláček, Petráš, P., Králová, S., Mašlaňová, I., Švec, P., Neumann-Schaal, M., Botka, T., Gelbíčová, T., Staňková, E., Doškař, J., Pantůček, R., *Staphylococcus rattus* sp. nov. isolated from a lab rat. In Genetická konference GSGM 2022 a XXIX. Genetické dny. Slavkov u Brna, Česká republika, October 5.–7, 2022. *Konvoj*, Brno, ISBN 978-80-7302-182-5. Abstract Book p. 65. Poster presentation.
- Pantůček, R., **Kovařovic, V.**, Finstrlová, A., Sedláček, I., Petráš, P., Mašlaňová, I., Švec, P., Meina Neumann-Schaal, Šedo, O., Botka, T. A Doškař, J., *Staphylococcus brunensis* sp. nov. isolated from human clinical specimens with an SCC-related genomic island outside of the *rlmH* gene bearing *ccrDE* recombinase gene complex. In 33rd European Congress of Clinical Microbiology & Infectious Diseases (ECCMID). Copenhagen, Denmark, April 15–18, 2023. Abstract Book p. LB068. Poster presentation.
- Mašlaňová, I., Vrba, D., **Kovařovic, V.**, Pantůček, R., Lysogeny In Staphylococci - Impact of antimicrobial agents on frequency of spontaneous induction of Siphophages In Staphylococcal Diseases Gordon Research Conference - Basic Biological Insights Driving Control of Staphylococcal Infections. Castelldefels, Spain, August 4–9, 2019. Poster presentation.

- Pantůček, R., Vrbovská, V., **Kovařovic, V.**, Indráková, A., Mašlaňová, I., Švec, P., Petráš, P., Doškař, J.. Whole genome sequencing of *Staphylococcus petrasii* shows its significant role in human disease. In Staphylococcal Diseases Gordon Research Conference - Basic Biological Insights Driving Control of Staphylococcal Infections. Castelldefels, Spain, August 4–9, 2019. Poster presentation.
- Mašlaňová, I., Pantůček, R., Sedláček, I., Indráková, A., Vrbovská, V., **Kovařovic, V.**, Švec, P., Králová, S., Petráš, P., Doškař, J.. Genomics of methicillin-resistant *Staphylococcus edaphicus* from Antarctica, harbouring *mecC* gene and genomic islands essential in adaptation to extreme environment. In Spotlight Microbiology Meeting 2018. Tübingen, Germany, Eberhard Karls Universität, November 19–20, 2018. Oral Presentation.
- Vrbovská, V., **Kovařovic, V.**, Šedo, O., Švec, P., Petráš, P., Pantůček, R., Characterization of *Staphylococcus petrasii* using molecular typing methods and whole genome sequencing. In 28th European Congress of Clinical Microbiology and Infectious Diseases. Madrid, Spain, April 21–24, 2018. Abstract Book, P2056. Poster presentation.
- Indráková, Adéla, Mašlaňová, I., Wertheimer, Z., **Kovařovic, V.**, Sedláček, I., Doškař, J., Pantůček, R., Mobile genetic elements including novel SCC*mec* of macrococcal species isolated from human clinical material. In The 18th International Symposium on Staphylococci and Staphylococcal Infections (ISSSI 2018). Copenhagen, Denmark, August 23–26, 2018. Abstract Book, p. 321. Poster presentation.
- Mašlaňová, I., **Kovařovic, V.**, Vrbovská, V., Pantůček, R., Doškař, J.. Identification of prophage types in coagulase-negative *Staphylococcus petrasii* strains. In 5th World Congress on Targeting Phage & Antibiotic Resistance - Phage Therapy and Other Inovative Ideas. Florence, Italy, May 17–18, 2018. Abstracts Book, p. 84. Poster presentation.
- Indráková, A., Mašlaňová, I., Wertheimer, Z., **Kovařovic, V.**, Šiborová, M., Doškař, J., Pantůček, R., Novel temperate phage from *Macroccoccus caseolyticus* isolated from human clinical material changes virulence of its host by negative and positive lysogenic conversion. In EMBO Workshop | Viruses of Microbes 2018. Wroclaw, Poland, July 9–13, 2018. Abstract Book, p. 230. Poster presentation.
- Vrbovská, V., **Kovařovic, V.**, Šedo, O., Petráš, P., Pantůček, R., Molecular typing and genome variability of *Staphylococcus petrasii*. In The Biomania Student Scientific Meeting 2017. Brno, Czech Republic, October 3, 2017. ISBN 978-80-210-8737-8. Masaryk University, Brno; Conference Book, p. 66. Oral Presentation.
- Pantůček, R., Sedláček, I., Indráková, A., Vrbovská, V., **Kovařovic, V.**, Mašlaňová, I., Švec, P., Králová, S., Petráš, P., Doškař, J.. *Staphylococcus edaphicus* sp. nov., isolated in Antarctica, harbouring *mecC* gene and genomic islands essential in adaptation to extreme environments. In The 15th International Congress of Mycology and Eukaryotic Microbiology – International Union of Microbiological Societies (IUMS 2017). Singapore, July 17–21, 2017. Abstract Book, p. 183. Poster presentation.
- Vrbovská, V., Petráš, P., Švec, P., Kýrová K., Šedo, O., **Kovařovic, V.**, Doškař, J., Pantůček, R., Genomic characterization of *Staphylococcus petrasii*. In The 2nd Rapid Microbial NGS and Bioinformatics: Translation Into Practice Conference. Hamburg, Germany, June 9–11, 2016. Abstract Book p. 44. Poster presentation.