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Characterization of rare and recently first described human pathogenic bacteria

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Characterization of rare and recently first described human pathogenic bacteria

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Editorial: Characterization of rare and recently first described human pathogenic bacteria

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Editorial on the Research Topic
Characterization of rare and recently first described human pathogenic bacteria

The vast number of unknown bacteria is estimated to range between 10^7 and 10^9 (Overmann et al., 2017). Therefore, although routine clinical diagnostics are geared towards detecting the known human pathogenic bacteria, it becomes obvious that rare and even new species are observed and reported in clinical routine diagnostics as well. However, the knowledge of their clinical relevance often extends only to sporadic case reports. This confronts the medical microbiologist with considerable problems in individual case consultation, since this information alone is not sufficient to make a clear statement about the pathogenicity of the respective species and consequently whether they are causative for a disease or not. In certain clinical conditions (such as pre-existing immunosuppression) however, these species are nevertheless reported in many laboratories on findings, which also include a resistogram. Furthermore, it should also be noted that there is currently no standardized definition for "rare bacterial pathogens". Following already existing definitions of rare diseases may therefore be the best approach to solve this dilemma. A common feature of the various definitions available is the use of the point prevalence for epidemiological assessment (Nguengang Wakap et al., 2020). According to a recent data analysis conducted by the "orpha-net" network (an association of 37 countries researching rare diseases), almost 6% of the world's population is supposed to be affected by any kind of a rare disease (Nguengang Wakap et al., 2020). Transferring these findings to infectious diseases underlines the assumption that rare pathogenic bacterial species are indeed of clinical relevance. However, since rare human pathogenic bacteria have hardly been systematically researched so far, there is only very little information available on virulence, risk factors for infection, the clinical picture or the status of the patient (including immunosuppression). For this reason research into rare pathogens must be aimed be to work out which species has pathogenic potential for humans and which does

not. It must also be clearly stated here that clinical case reports alone at best help to uncover circumstantial evidence, but ultimately cannot satisfactorily clarify the question of pathogenicity. Therefore, when starting to do research on rare pathogens, it is advisable to first establish a collection of the species of interest derived from clinical samples. Therefore, the first essential aspect to be clarified is the species identification strategy. The chosen method should ensure a high sample throughput while allowing reliable species identification. Based on our previous research, MALDI TOF MS currently seems to be the most suitable method for this purpose (Kostrzewa et al., 2019; Kopf et al., 2021; Bigge et al., 2022). In contrast, methods that use biochemical or metabolic traits for identification have not proven to be entirely reliable (Rudolph et al., 2019; Kopf et al., 2021; Bigge et al., 2022). The third option, which is commonly used in routine diagnostics, is the sequencing of the 16S rRNA gene. This method has also shown to produce reliable results, but is more time-consuming and requires specially trained personnel. For this reason, it is unsuitable for high-throughput examinations and therefore more applicable to confirm questionable identification results. In general, however, it should be noted that the identification accuracy of the respective methods varies for different species. Therefore, in order to determine the actual underlying species, genome-based methods such as the "digital DNA-DNA hybridisation" or calculation of the "average nucleotide identity" (ANI) should be applied (Richter and Rosselló-Móra, 2009; Rudolph et al., 2019; Kopf et al., 2021). The importance of genomic data for species identification has been addressed by several authors in this special edition. Reviewing the literature on the clinical significance of *Shewanella putrefaciens*, Müller et al. for instance pointed out that the species has frequently been misidentified in previous publications and the pathogenic species was assigned to *S. algae*. In addition, Wang et al. reported a patient with cervical carcinoma who died as a result of septic shock. Only with the help of genome sequencing, the authors were finally able to determine that the disease was caused by a yet unknown species of the genus *Peptoniphilus*. The species was subsequently designated as *Peptoniphilus septimus* sp. nov. Furthermore, Monecke et al. described staphylococcal strains from a straw-coloured fruit bat and a diamond firetail and gave a review of their phylogenetic relationships to other staphylococci. They propose that *Staphylococcus roterodami* and *Staphylococcus singaporensis* are distinct clonal complexes of the same species for which they propose the name *S. roterodami*. This species is also a known human pathogen. In addition, to further characterize rare pathogenic bacterial species, phenotypic examinations such as biochemical reactions or antimicrobial susceptibility profiles should be carried out, e.g. as done for initial species descriptions. For example, by comparing phenotypic results with genome data, the molecular basis of antimicrobial resistance can be determined. This approach can be used to describe and explain unusual resistance phenomena. Shittu et al. for instance described for the first time a *Staphylococcus argenteus* isolate in Germany that exhibited high resistance to mupirocin in addition to methicillin resistance. An increase of *S. argenteus* infections has been observed in recent years (Alhussein et al., 2020). In addition, Yao et al. described an *Enterobacter xiangfangensis* isolate that harbors both carbapenemases KPC-2 and OXA-48 and a mobile colistin resistance gene, which is an extreme rarity in this combination. Gaur et al. analyzed a *Chryseobacterium gallinarum* isolate that is highly resistant to colistin. In addition, the regional occurrence of rare species is important from an epidemiologic perspective. To date, there have been no systematic epidemiological studies on rare human pathogenic species. For this reason, it is currently still necessary to rely on the evaluation of published case reports to do this (Kopf et al., 2021). An important contribution to this Research Topic was made by Li et al. who were the first to report a bloodstream infection with *Herbaspirillum huttiense* in China. Of equal relevance is the initial description of previously unknown potential sources of infection. Zautner et al. for instance demonstrated that *Erysipelothrix rhusiopathiae* can be transmitted from animals (in this case pigs and dogs) to humans via bath water and can cause severe infections. Once a sufficient number of sequenced genomes of a species is available, it is advisable to analyze the pangenome using comparative genome analyses. Kopf et al. have impressively demonstrated this for *Wohlfahrtiimonas chitiniclastica*. In addition to the direct investigation of rare isolates, it is important to place the topic of rare human pathogenic bacteria in a broader clinical context in order to understand their significance to various diseases. For example, Felber et al. showed in a retrospective study of appendicitis in pediatric patients that there is no significant difference in complication rates when rare pathogens are detected. However, future prospective multi-centric studies including significantly more patients and isolates will provide more clarity.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

All authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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BRIEF RESEARCH REPORT
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First Report of a Methicillin-Resistant, High-Level Mupirocin-Resistant *Staphylococcus argenteus*

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We describe the identification of a methicillin-resistant, high-level mupirocin-resistant *Staphylococcus argenteus*. The isolate (1801221) was characterized as t6675-ST2250-SCC*mec*IVc, and whole-genome sequencing revealed that the isolate possessed two plasmids. One plasmid (34,870 bp), designated p1_1801221 with *rep23*, harboured the mupirocin resistance (*mupA*) gene. The second plasmid (20,644 bp), assigned as p2_1801221 with *rep5a* and *rep16*, carried the resistance determinants for penicillin (*blaZ*) and cadmium (*cadD*). Phylogenetic analysis revealed that the isolate clustered with the European ST2250 lineage. The overall high similarity of both plasmids in *S. argenteus* with published DNA sequences of *Staphylococcus aureus* plasmids strongly suggests an interspecies transfer. The pathogenic potential, community and nosocomial spread, and acquisition of antibiotic resistance gene determinants, including the *mupA* gene by *S. argenteus*, highlight its clinical significance and the need for its correct identification.

Keywords: identification, methicillin-resistant *Staphylococcus argenteus*, high-level mupirocin resistance, plasmid, whole-genome sequencing (WGS)

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INTRODUCTION

Staphylococcus argenteus and *S. schweitzeri*, with *S. roterodami* and *S. singaporensis*, are recently designated species and assigned to the *Staphylococcus aureus*-related complex (Tong et al., 2015; Chew et al., 2021; Schutte et al., 2021). *S. argenteus* and *S. aureus* demonstrate similar reactions to key biochemical tests for phenotypic characterization with identical 16S rRNA gene sequences (Tong et al., 2015). Hence, it is difficult to distinguish these two species by routine diagnostic methods (Kaden et al., 2018; Tunsjø et al., 2018). Various tools have been developed to differentiate *S. argenteus* from the *S. aureus*-related complex (Becker et al., 2019). They include Matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Chen et al., 2018a) and PCR detection of the nonribosomal peptide synthetase (NRPS) gene (Zhang et al., 2016). *S. argenteus* was previously considered less virulent than *S. aureus* due to the lack of the carotenoid pigment, staphyloxanthin (Holt et al., 2011), which impairs oxidative stress and neutrophil killing (Liu et al., 2005). However, *S. argenteus* possesses similar *S. aureus* virulence determinants (Zhang et al., 2017), including the gene encoding Panton-Valentine leukocidin (PVL) (Chantratita et al., 2016).

There are increasing reports of *S. argenteus* infections worldwide (Chantratita et al., 2016; Alhussein et al., 2020; Diot et al., 2020; Hao et al., 2020; Mitsutake et al., 2020; Eshaghi et al., 2021). *S. argenteus* isolates are generally penicillin-resistant (*blaZ*-positive) (Becker et al., 2019), but in Europe, methicillin-resistant (MR)-*S. argenteus* (>10 isolates) have been identified in Denmark (Hansen et al., 2017), Netherlands (Bank et al., 2021) and Sweden (Hallbäck et al., 2018; Giske et al., 2019). Also, a recent study (Goswami et al., 2021) revealed that of the *S. argenteus* genomes deposited in the public databases, 20% were *mecA*-positive. Becker et al. (2019), in a position paper on the *S. aureus*-related complex, suggested adopting infection prevention and control measures similar to methicillin-resistant *S. aureus* (MRSA) guidelines on a laboratory report of MR-*S. argenteus* in human infections. The application of mupirocin ointment on the mucous membrane (e.g., anterior nares) is an important strategy for decolonizing patients and healthcare personnel with MRSA (Patel et al., 2009). However, the emergence of resistance is associated with unrestricted policies and antibiotic use for long periods in healthcare settings (Hetem and Bonten, 2013). Two levels of *S. aureus* resistance to mupirocin have been elucidated, i.e., low-level and high-level resistance (HmupR) attributed to mutation and the acquisition of plasmids, respectively (Patel et al., 2009). Whereas the prevalence of MRSA with HmupR is 5.9%, 8.0%, and 12.1% in the Americas, Europe, and Asia, respectively (Dadashi et al., 2020), it is entirely unknown in *S. argenteus* until now. We describe the first report of a methicillin-resistant *S. argenteus* that exhibited HmupR.

MATERIALS AND METHODS

Identification of the Methicillin-Resistant, Mupirocin-Resistant *S. argenteus*

The isolate (1801221) was obtained in April 2018 from a human nasal swab and was previously identified as methicillin-resistant *S. aureus* (MRSA) with HmupR. For characterization, it was sent to the National Reference Center for Staphylococci and Enterococci, Robert Koch Institute, Germany. To delineate *S. argenteus* from *S. aureus*, PCR amplification of the NRPS gene (Becker et al., 2019) was performed at the Institute of Medical Microbiology, Münster. The isolate was subjected to antibiotic susceptibility testing (Vitek 2 automated system bioMérieux, Marcy l'Étoile, France). The minimum inhibitory concentration (MIC) to mupirocin was also determined using the gradient diffusion method (E-test, bioMérieux, Marcy l'Étoile, France). Methicillin and mupirocin resistance was confirmed by PCR detection of *mecA* (Murakami et al., 1991) and *mupA* (Nagant et al., 2016). We interpreted the results of the antibiotic susceptibility testing and E-test according to the EUCAST clinical breakpoints (Version 11.0).

Whole-Genome Sequencing

The *S. argenteus* isolate was further processed for whole-genome sequencing (WGS) on a Sequel II platform (Pacific Biosciences Inc., Menlo Park, CA, USA). Before sequencing, we constructed the sequence library using the SMRTbell Express Template Prep Kit 2.0 (Pacific Biosciences Inc.) according to the manufacturer's recommendations. The resulting long-read sequencing data were assembled applying the "Microbial Assembly" pipeline within the SMRT Link software version 9 (Pacific Biosciences Inc.) using default parameters except for the genome size, which was adopted to 2.8 Mb. Then, we utilized the Ridom SeqSphere$^+$ software (version 7, Ridom GmbH, Münster, Germany) to *in silico* predict the antimicrobial resistance and virulence genes and to extract the staphylococcal protein A (*spa*) type and the multilocus sequence type (ST) of the isolate. Also, we used the Plasmid Finder (version 2.1) to identify the replicon sequences (Carattoli et al., 2014). Further analysis, and annotation of the sequences, was performed using the NCBI Prokaryotic Genome Annotation Pipeline software revision 5.3 (Tatusova et al., 2016). A Neighbor-Joining (NJ) tree was constructed using sequences of a global collection of 111 *S. argenteus* (ST2250) isolates. Single nucleotide polymorphisms (SNPs) were extracted from 1,864 core genome genes (Leopold et al., 2014) present in all isolates. The SNPs analysis formed the basis to calculate the NJ tree with default parameters within the Ridom SeqSphere$^+$ software version 7.

RESULTS AND DISCUSSION

The isolate displayed creamy-white colonies with β-haemolysis on Columbia sheep blood agar (CBA, BD, Heidelberg, Germany) (**Figure 1**). MALDI-TOF identification using the MBT compass

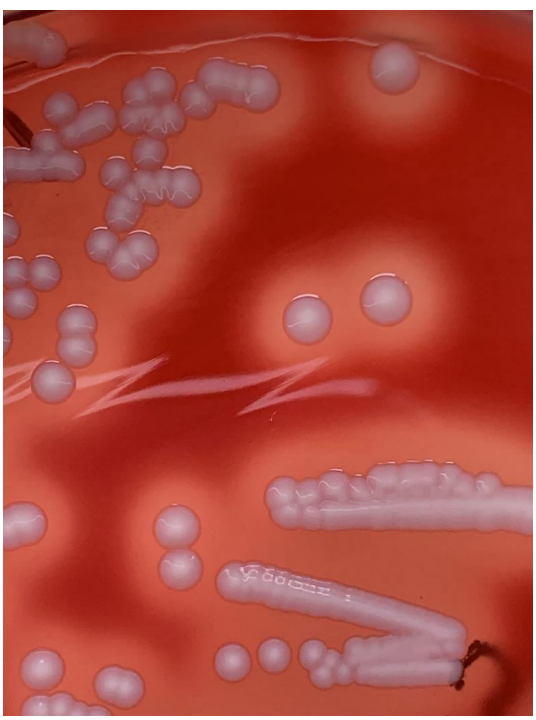

FIGURE 1 | *S. argenteus* (1801221) on Columbia Blood Agar (37°C, 48 hours).

(Version 9) did not distinguish reliably between *S. aureus* (Score: 2.04) and *S. argenteus* (Score: 2.13). However, it was PCR-positive (360bp) for the NRPS gene, indicating that it is *S. argenteus*. Antibiotic susceptibility testing showed that the isolate was resistant to cefoxitin, fosfomycin, mupirocin, and trimethoprim/sulfamethoxazole. The MIC of mupirocin (≥512 µg/ml, E-test) was in agreement with the VITEK result (MIC = ≥512 µg/ml). PCR revealed that the isolate was *mecA* and *mupA*-positive. WGS confirmed the identity of the isolate as *S. argenteus* and its antibiotic resistance phenotype. Also, molecular typing characterized the isolate as t6675-ST2250-SCC*mec*IVc. It was associated with capsule type 8, positive for the immune evasion (*sak*, *scn*) gene cluster, haemolysins (*hld*, *hlgB*, *hly/hla*), and the intracellular adhesion (*icaA*, *icaB*, *icaC*,

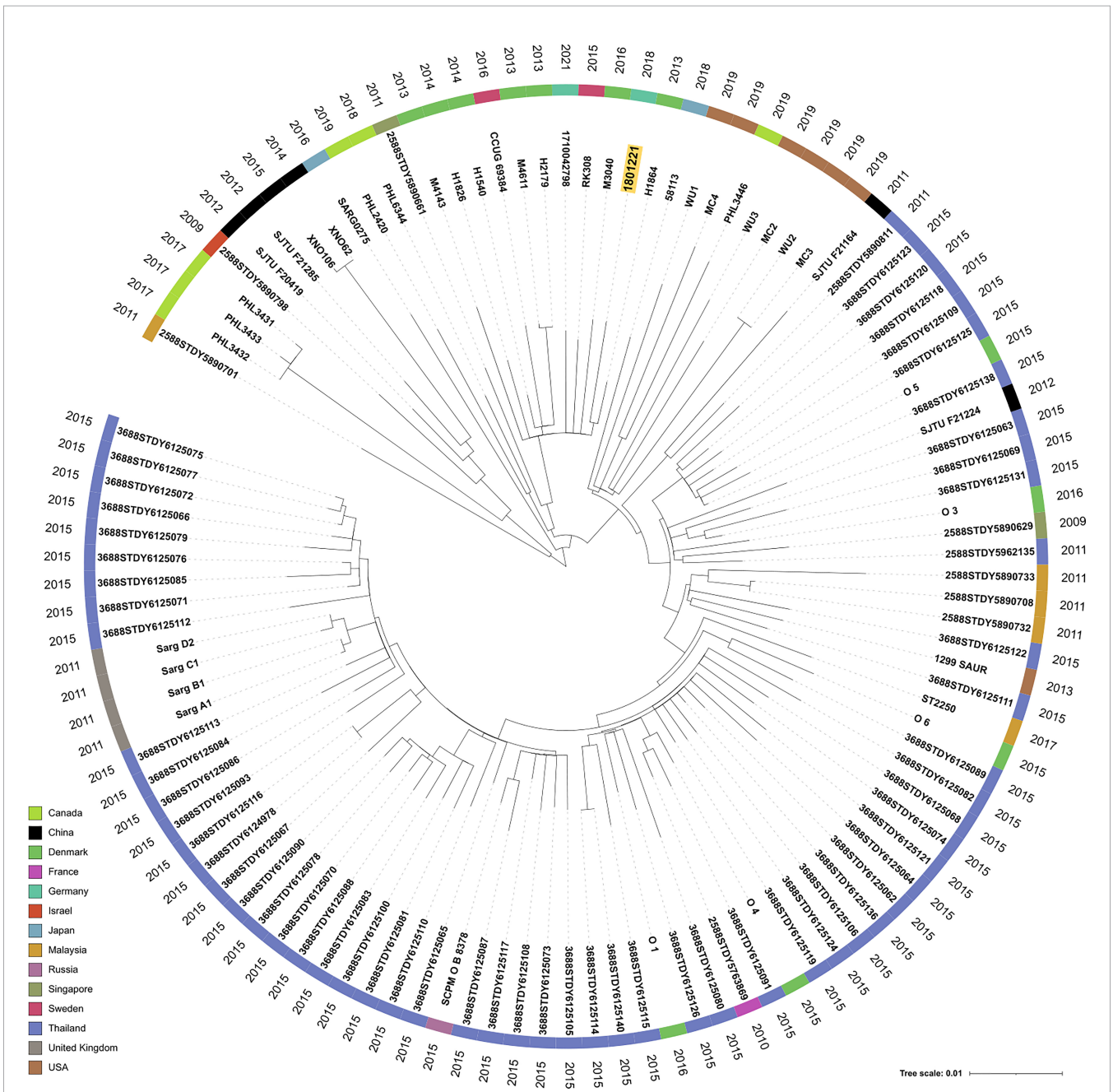

FIGURE 2 | Neighbor-joining (NJ) tree of 111 *S. argenteus* ST2250 global isolates. SNPs (n = 2,177) were extracted from 1,864 core genome genes present in all isolates and formed the basis to calculate the NJ tree with default parameters within the Ridom SeqSphere⁺ software. We used iTOL V. 6 (Letunic and Bork, 2021) to display the tree and metadata of the strains. The leaves of the tree were annotated with the sample names. The colored circle indicates the country of isolation and the outer circle the isolation year, respectively. Isolate 1801221 is highlighted in a yellow box.

icaD, *icaR*) gene operon. The isolate was negative for the PVL-encoding gene.

The *S. argenteus* isolate chromosome was 2,781,166 bp in size, with a GC content of 32.3%, containing 2,650 predicted coding DNA sequences (CDSs). The NJ tree based on 2,177 SNPs from a global collection of all available genomes (as of 8 December 2021) of ST2250 *S. argenteus* isolates (**Supplementary Table**) showed that it clustered with the European ST2250 clade (**Figure 2**). The Plasmid Finder identified sequences of two plasmids with replication (*rep5a* [locus tag 13590 in **Supplementary Figure 1B**], *rep16* [locus tag 13610 in **Supplementary Figure 1B**], and *rep23* [locus tag 13385 in **Supplementary Figure 1A**]) genes, respectively. The larger plasmid (34,870 bp), designated p1_1801221, with *rep23* carried *mupA*. This gene demonstrated 100% sequence identity with the alternative isoleucyl-tRNA synthetase (*ileS-2*) gene conferring HmupR on a conjugative plasmid pPR9 from *S. aureus* (GenBank accession number GU237136). Moreover, the whole plasmid was nearly identical at sequence level with the published plasmid pPR9 (**Figure 3A**) using the BRIG tool (Alikhan et al., 2011). The smaller plasmid (20,644 bp), assigned as p2_1801221, with *rep5a* and *rep16*, harboured the penicillin (*blaZ*) and cadmium (*cadD*) resistance genes. Again, the genes and overall plasmid composition exhibited high homology to *S. aureus* resistance determinants and plasmid. Specifically, *blaZ* showed 99.9% sequence identity with the corresponding gene on pN315 (GenBank accession number AP003139), and the *cadD* gene displayed 100% homology with the resistance determinant on pSAS (GeneBank accession number BX571858). Moreover, the plasmid as a whole was nearly identical to the *S. aureus* plasmid p515718a of strain 515798 (GenBank accession number CP045475) (**Figure 3B**).

S. argenteus was first reported in northern Australia (McDonald et al., 2006) and distinct from *S. aureus* based on the average nucleotide identity of 87.4% and a DNA-DNA hybridization value of 33.5% (Tong et al., 2015). About 10% of *S. aureus* isolates from human infections are non-pigmented (Zhang et al., 2018). Also, *S. argenteus* colonies on blood agar are non-pigmented (creamy-white) due to the lack of the crt*OPQMN* operon responsible for carotenoid pigment, staphyloxanthin (Holt et al., 2011). Hence, *S. argenteus* and non-pigmented *S. aureus* could be indistinguishable on blood agar based on colony morphology and phenotypic tests (coagulase, DNase). This scenario could be a dilemma in the clinical microbiology laboratory (Becker et al., 2019). This study provided evidence on the reliability of the PCR detection of the NRPS gene with WGS in the delineation of *S. argenteus* from *S. aureus*. ST2250 is a global *S. argenteus* clonal group (Eshaghi et al., 2021), and our first report of an isolate in this clone exhibiting HmupR is of public health importance. MRSA with HmupR is a serious problem as decolonization with mupirocin becomes ineffective (Patel et al., 2009). Moreover, HmupR could facilitate the spread of antibiotic resistance through the conjugative transfer of plasmid mediating HmupR with co-mobilization and co-transfer of plasmids encoding other gene determinants (Udo and Jacob, 1998; Pawa et al., 2000). Also, macrolide, gentamicin, tetracycline, and trimethoprim resistance genes have been identified on the same extra-chromosomal element with *mupA* (McDougal et al., 2010). In this study, the identification and high homology of both plasmids identified in

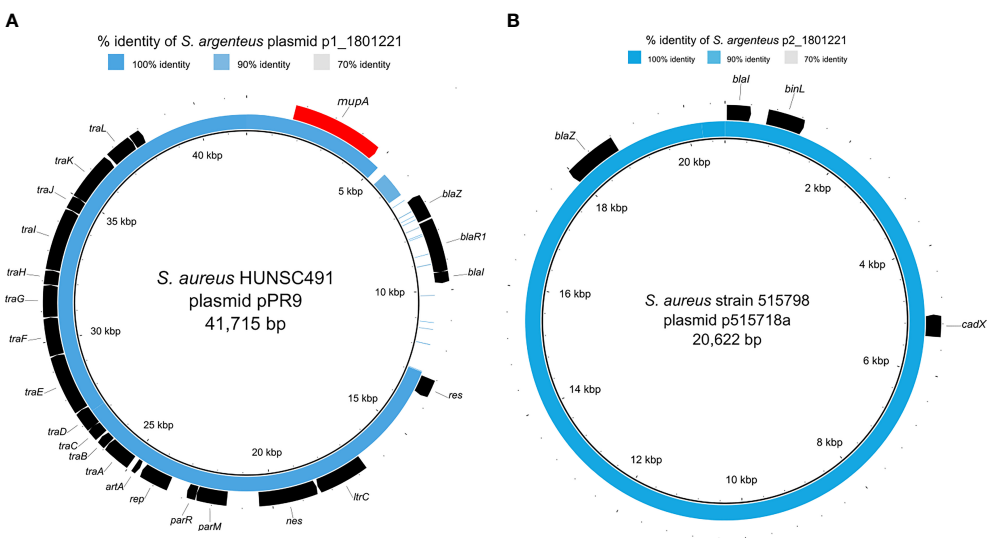

FIGURE 3 | Comparison of *S. argenteus* plasmids with closely related *S. aureus* plasmids. The inner black ring represents the reference sequence, and the blue ring depicts the respective *S. argenteus* plasmid sequence. The outer black ring provides annotation information, i.e., detected ORFs, where the annotation resulted in known genes. The blue color's intensity is related to the sequence similarity, detailed in the **Supplementary Table**. **(A)** depicts the comparison of p1_1801221 and the conjugative *S. aureus* plasmid pPR9 harboring the *mupA* gene encoding for mupirocin resistance (colored in red); **(B)** shows the comparison of p2_1801221 and the *S. aureus* plasmid p515718a harboring *blaZ* and *cadX* encoding for beta-lactam and cadmium resistance, respectively.

S. argenteus with published DNA sequences of *S. aureus* plasmids suggest interspecies transfer.

S. argenteus carriage in the human population (Aung et al., 2017; Senok et al., 2020; Eshaghi et al., 2021; Jauneikaite et al., 2021) and possible person-to-person transmission (Giske et al., 2019; Eshaghi et al., 2021) have been described. Moreover, a study revealed that cases of *S. argenteus* bacteremia were associated with higher mortality than methicillin-susceptible *S. aureus* bacteremia (Chen et al., 2018b). *S. argenteus* with different antibiotic resistance genes have been reported (Aung et al., 2021; Eshaghi et al., 2021), including an isolate with elevated MIC (4μg/ml) to daptomycin and vancomycin in the United States (Hao et al., 2020). Recent studies from China (Chen and Wu, 2020) and Japan (Wakabayashi et al., 2021) have also identified *S. argenteus* from retail foods and an emerging bovine mastitis pathogen in Thailand (Pumipuntu, 2019). We could not ascertain if the study individual received mupirocin or not. Nonetheless, these increasing reports and the capacity of *S. argenteus* to harbor resistance gene determinants (including *mupA*) with its repertoire of virulence factors highlight the need for its delineation from *S. aureus* and correct identification. Therefore, enhanced surveillance is vital to understanding the significance of *S. argenteus* in clinical and non-clinical settings.

DATA AVAILABILITY STATEMENT

The whole-genome sequence project for the S. argenteus; isolate (1801221) has been deposited in NCBI under the bioproject accession number PRJNA764657 with sequence accession numbers CP083805-CP083807 for the chromosome and the two plasmids.

AUTHORS CONTRIBUTIONS

AS, FL-N, BS, and FS designed the research. AS, M-TN, SB, and AM performed the experiments. AS, SB, and AM analyzed the data. AS wrote the initial draft of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.860163/full#supplementary-material

Supplementary Figures 1a and 1b | Circular illustration of the two *S. argenteus* plasmids and annotation of antibiotic resistance genes.

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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

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The phylogenetic tree of the *Staphylococcus aureus* complex consists of several distinct clades and the majority of human and veterinary *S. aureus* isolates form one large clade. In addition, two divergent clades have recently been described as separate species. One was named *Staphylococcus argenteus*, due to the lack of the "golden" pigment staphyloxanthin. The second one is *S. schweitzeri*, found in humans and animals from Central and West Africa. In late 2021, two additional species, *S. roterodami* and *S. singaporensis*, have been described from clinical samples from Southeast Asia. In the present study, isolates and their genome sequences from wild Straw-coloured fruit bats (*Eidolon helvum*) and a Diamond firetail (*Stagonopleura guttata*, an estrildid finch) kept in a German aviary are described. The isolates possessed staphyloxanthin genes and were closer related to *S. argenteus* and *S. schweitzeri* than to *S. aureus*. Phylogenetic analysis revealed that they were nearly identical to both, *S. roterodami* and *S. singaporensis*. We propose considering the study isolates, the recently described *S. roterodami* and *S. singaporensis* as well as some Chinese strains with MLST profiles stored in the PubMLST database as different clonal complexes within one new species. According to the principle of priority we propose it should be named *S. roterodami*. This species is more widespread than previously believed, being observed in West Africa, Southeast Asia and Southern China. It has a zoonotic connection to bats and has been shown to be capable of causing skin and soft tissue infections in humans. It is positive for

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staphyloxanthin, and it could be mis-identified as *S. aureus* (or *S. argenteus*) using routine procedures. However, it can be identified based on distinct MLST alleles, and "*S. aureus*" sequence types ST2470, ST3135, ST3952, ST3960, ST3961, ST3963, ST3965, ST3980, ST4014, ST4075, ST4076, ST4185, ST4326, ST4569, ST6105, ST6106, ST6107, ST6108, ST6109, ST6999 and ST7342 belong to this species.

Keywords: *Staphylococcus aureus*, *Staphylococcus schweitzeri*, *Staphylococcus argenteus*, *Staphylococcus singaporensis*, *Staphylococcus roterodami*, whole genome sequencing (WGS), DNA microarray

INTRODUCTION

The phylogenetic tree of bacteria traditionally considered *Staphylococcus (S.) aureus* consists of several distinct clades. Most human and veterinary *S. aureus* isolates from all over the world form one large clade. In addition, two divergent clades have recently been elevated to the status of species (Tong et al., 2015; Becker et al., 2019). One has been named *S. argenteus*, due to its lack of the "golden" carotenoid pigment staphyloxanthin (Holt et al., 2011) regarded as a unique property of *S. aureus*. Isolates assigned or re-assigned to this new species have been described in many countries including Australia (Ng et al., 2009), Thailand (Indrawattana et al., 2019; Pumipuntu, 2019), Laos (Yeap et al., 2017), Cambodia (Ruimy et al., 2009), Myanmar (Aung et al., 2019), Japan (Ohnishi et al., 2018; Aung et al., 2019; Kitagawa et al., 2020), China and Taiwan (Chen et al., 2018), on Indian Ocean islands such as the Comoros or Mayotte (Dupieux et al., 2015), Gabon (Schuster et al., 2017), Trinidad and Tobago (Monecke et al., 2014) and Brazil (Rossi et al., 2020). Sporadic isolates also have been identified in the United Arab Emirates (Senok et al., 2020), several European countries (Dupieux et al., 2015; Rigaill et al., 2018; Tång Hallbäck et al., 2018; Diot et al., 2020; Kukla et al., 2020; Söderquist et al., 2020) as well as in Canada and the United States of America (Eshaghi et al., 2021). These observations could be associated with travel and migration. Aside from the lack of the carotenoid pigment gene cluster, *S. argenteus* isolates carry the same genes as *S. aureus*, albeit they occur as distinct allelic variants (Monecke et al., 2010), and its genes are located in the genome following the same order as in *S. aureus*. Traditional PCR-based multilocus sequence typing (MLST) can be performed on *S. argenteus* using slightly modified primers (Ng et al., 2009; Ruimy et al., 2009; Holt et al., 2011) and as in *S. aureus*, the resulting sequence types (ST) cluster into closely related groups known as clonal complexes (CCs). Moreover, CC affiliation also correlates with the presence or absence of certain genomic islands essentially identical to their counterparts in *S. aureus*. Thus, ST1223, ST1850 (formerly ST75), ST2198, ST2250, ST2596/2793 (Aung et al., 2019; Hsu et al., 2020), ST2854 and ST4587 can be regarded as founders of homonymous CCs. Some mobile genetic elements from *S. aureus* have also been identified in *S. argenteus*. This includes SCC*mec* IV and V elements that carry the methicillin/beta-lactam resistance gene *mecA*, phages harbouring the Panton-Valentine leukocidin gene (Dupieux et al., 2015; Aung et al., 2017; Senok et al., 2020) and the pathogenicity-island-borne *tst1* [encoding toxic shock syndrome toxin 1 (Aung et al., 2017)]. *S. argenteus* can asymptomatically be carried in the nares. It also can cause the same types of infections as *S. aureus* (Becker et al., 2019), i.e., skin and soft tissue infections (Ohnishi et al., 2018), osteomyelitis (Rigaill et al., 2018) or endoprosthesis infections (Diot et al., 2020; Söderquist et al., 2020) and sepsis (Chen et al., 2018; Kitagawa et al., 2020). *S. argenteus* also has been implicated in food poisoning (Suzuki et al., 2017). Some lineages of *S. argenteus* have been identified in animals such as rabbits (Indrawattana et al., 2019), dairy cattle (Pumipuntu, 2019; Rossi et al., 2020) and a wild gorilla (Schuster et al., 2017).

The other entity, *S. schweitzeri* consists of several sequence types (ST1857, ST1872, ST1873, ST1874, ST2022, ST2058, ST2059, ST2067, ST2071, ST2463, ST2464, ST2465, ST2467, ST3952, ST3958, ST3960, ST3961, ST3962, ST3963, ST3980, ST4316, ST4326, ST5117, ST5600 and ST5602). The alleles of core genome genes of *S. schweitzeri* are distinct from those of *S. aureus* and *S. argenteus*. However, published genome sequences of *S. schweitzeri* and experiments with DNA microarrays (Okuda et al., 2016) indicate that certain genomic island genes (*agr* alleles, capsule type, *egc, cna, seh*, carotenoid locus genes) closely resemble their *S. aureus* counterparts. The presence of these genes in *S. schweitzeri* is related to their CC affiliation, as also noted in *S. aureus*. So far, *S. schweitzeri* has been observed in four different regions. It was first identified in Gabon (Tong et al., 2015) from where it was also reported in other studies (Schaumburg et al., 2012; Schaumburg et al., 2015; Okuda et al., 2016) as well as in Côte d'Ivoire (Schaumburg et al., 2012; Schaumburg et al., 2015), Nigeria (Akobi et al., 2012) and the Democratic Republic of Congo, DRC (Schaumburg et al., 2015). Most of these isolates originated from non-human primates (Schaumburg et al., 2012) or from "bush-meat", i.e., poached or hunted wildlife sold on local markets (Schaumburg et al., 2015). Some isolates from healthy humans have been identified, suggesting that humans carry this lineage sporadically and asymptomatically (Tong et al., 2015; Schaumburg et al., 2015; Okuda et al., 2016). In Nigeria, *S. schweitzeri* was recovered from faecal samples of the Straw-coloured fruit bats (*Eidolon helvum*) on the premises of a university campus (Akobi et al., 2012). This observation caused concerns of zoonotic transmission as these isolates were also detected on fomites in the same university (Shittu et al., 2020). However, a transmission of *S. schweitzeri* from animals to humans has not yet been observed. A large study from the DRC, Gabon and Côte d'Ivoire investigated rural populations and did not identify *S. schweitzeri* among humans despite close contact with bushmeat and wildlife (Schaumburg et al., 2015). The

pathogenicity of *S. schweitzeri* remains unclear as humans were found to be colonised rather than infected (Schaumburg et al., 2015; Okuda et al., 2016). However, *in vitro* experiments suggest that *S. schweitzeri* is as virulent as *S. aureus* (Grossmann et al., 2021). Generally, one might assume that it is a zoonotic species that might asymptomatically colonise humans (Becker et al., 2019) and appears to be restricted to Central/West Africa. However, more data are needed to assess the distribution and a possible clinical significance of *S. schweitzeri.*

In autumn 2021, two new species of "*S. aureus*-like" staphylococci were described from human samples. These were named *S. roterodami* and *S. singaporensis* (Schutte et al., 2021; Chew et al., 2021). A single isolate of *S. roterodami* was identified from an infected wound of a Dutch traveller returning from Bali, Indonesia, prompting bacteriological investigations and genome sequencing (Schutte et al., 2021). A study (Chew et al., 2021) investigating a possible presence of *S. argenteus/schweitzeri* in Singapore identified 37 *S. argenteus* and six "unknowns" assigned to five novel STs and described as a new species, *S. singaporensis*. Four of these six isolates were associated with skin and soft tissue infections.

We describe a group of animal isolates submitted to the authors´ laboratories for characterisation as suspected *S. argenteus* or *S. schweitzeri.* These were characterised and sequenced. We also review their relationship to *S. aureus, S. argenteus, S. schweitzeri, S. roterodami* and *S. singaporensis.*

MATERIAL AND METHODS

Animals and Isolates

Seven isolates originated from faecal samples of the Straw-coloured fruit bat (*Eidolon helvum*), collected on a university campus in Ile-Ife, Nigeria, for earlier studies (Akobi et al., 2012; Olatimehin et al., 2018). The eighth isolate was recovered from a pulmonary specimen of a captive Diamond firetail (*Stagonopleura guttata*), an estrildid finch. The bird was kept in an aviary in a zoological collection in Berlin, Germany. The carcass of the deceased bird was submitted for necropsy. Lung tissue samples revealing disseminated white to yellowish miliary lesions were subsequently forwarded for microbiological investigations with suspected avian mycobacteriosis, and indeed pulmonary smears were positive for acid-fast bacilli.

An overview of isolates and typing data is provided in **Table 1**. The isolates were characterised by microarray (see below and **Supplemental File 1**). Three isolates, two from bats and the one from the finch were selected for phenotypic characterisation and whole-genome sequencing (WGS).

Antimicrobial Susceptibility Testing and Biochemical Tests

Antimicrobial susceptibility testing was performed by the Vitek 2 automated system (bioMérieux, Nuertingen, Germany; **Supplemental File 2**) using the AST-P608 panel (benzylpenicillin, oxacillin, cefoxitin, gentamicin, tobramycin, ciprofloxacin, levofloxacin, moxifloxacin, erythromycin, clindamycin incl. inducible resistance, linezolid, teicoplanin, vancomycin, tetracycline, fosfomycin, nitrofurantoin, fusidic acid, mupirocin, rifampicin) according to manufacturer´s instructions. EUCAST clinical breakpoints for *S. aureus* (https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_11.0_Breakpoint_Tables.pdf) were applied to classify the isolates as susceptible, intermediate or resistant. *S. aureus* ATCC® 29213 served as quality control strain. In addition, biochemical tests were performed using the bioMérieux Gram-positive identification card (GP) for the same device.

MLST and *spa* Typing

MLST is based on sequencing seven housekeeping genes, *arcC, aroE, glpF, gmk, pta, tpi* and *yqiL*. Sequencing of PCR products was performed as previously described (Enright et al., 2000), or the sequences of the target genes were extracted from assembled whole-genome sequence data. The sequences were assigned to MLST alleles and STs using the *S. aureus* section (https://pubmlst.org/bigsdb?db=pubmlst_saureus_seqdef&page=sequenceQuery) of the PubMLST website (Jolley et al., 2018).

In addition, *spa* typing was performed as previously described (Harmsen et al., 2003) using repeat definitions and nomenclature as provided on the Ridom website (http://spa.ridom.de/).

Microarray-Based Genotyping

Isolates were characterised using the DNA microarray-based kit (Interarray *S. aureus*, fzmb GmbH, Research Centre for Medical Technology and Biotechnology, Bad Langensalza, Germany). Primer and probe sequences have been published previously (Monecke et al., 2008; Monecke et al., 2011). The array covers 333 different targets related to approximately 170 different genes and their allelic variants allowing detection of virulence and resistance factors. Isolates were assigned to clonal complexes (CCs) by automated comparison to a reference database. The procedures followed the manufacturer's instructions as previously described (Monecke et al., 2008; Monecke et al., 2011). Briefly, *S. aureus* was cultured overnight on Colombia blood agar. DNA extraction was performed after enzymatic lysis. The next step was a multiplexed linear amplification using one specific primer per target. During that non-exponential amplification, biotin-16-dUTP was incorporated into single-stranded amplicons. After incubation and washing, hybridisation was performed to probes immobilised on the array. Hybridisations were detected by adding streptavidin horseradish peroxidase that triggered a localised dye precipitation resulting in a formation of visible spots. Microarrays were then scanned and analysed using an Arraymate (Alere Technologies GmbH (Abbott), Jena, Germany) reading device. A second microarray (Alere, Monecke et al., 2016) was used to detect additional markers (**Supplemental File 1**), including the staphyloxanthin locus.

Illumina Sequencing

Three isolates were subjected to WGS with the Illumina MiSeq platform (Illumina, Inc., San Diego, USA). The whole-cell DNA was extracted using the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany) with some adaptations for staphylococci as described previously (Scholtzek et al., 2019). The libraries for WGS were prepared using the Nextera XT DNA Library

TABLE 1 | Details of animals and isolates described herein (bold font), as well as of related isolates described otherwise or listed in the MLST database.

Isolate ID	Host	Sample type	Collected	Reference	Location	MLST	Comments
BDS-53B	Straw-coloured fruit bat, *Eidolon helvum*	Faecal	2016		Student Union Building, Obafemi Awolowo University, Ile-Ife, Nigeria	ST3965 (272-616-543-190-268-447-389)	
BDS-53E	*Eidolon helvum*	Faecal	2016		Student Union Building, Obafemi Awolowo University, Ile-Ife, Nigeria	ST4326 (272-616-543-190-268-499-537)	*spa* type t16757 (741-12-96-17-16-371)
BDS-54	*Eidolon helvum*	Faecal	2016		Student Union Building, Obafemi Awolowo University, Ile-Ife, Nigeria	ST3963 (272-357-306-190-268-448-548)	
BDS-69C	*Eidolon helvum*	Faecal	2016		Student Union Building, Obafemi Awolowo University, Ile-Ife, Nigeria	ST3952 (272-603-543-190-268-447-37)	*spa* type t17074 (26-22-17-20-17-13-12-17-17-16-16)
BDH-128	*Eidolon helvum*	Faecal	2015	(Olatimehin et al., 2018)	Health Centre, Obafemi Awolowo University, Ile-Ife, Nigeria	ST3961 (272-357-306-190-268-448-277)	*spa* type t16748 (741-12-96-17-16-371)
BDH-147	*Eidolon helvum*	Faecal	2015	(Olatimehin et al., 2018)	Health Centre, Obafemi Awolowo University, Ile-Ife, Nigeria	ST3960 (272-603-543-190-268-447-537)	*spa* type t17079 (26-23-23-13-23-31-29-17-25-16-28-17-25-17-25-16-28)
BDH-157	*Eidolon helvum*	Faecal	2015		Health Centre, Obafemi Awolowo University, Ile-Ife, Nigeria	ST3980 (272-357-306-190-268-448-37)	*spa* type t16747 (741-12-17-17-17-16-371)
Zoo-28	Diamond firetail, *S. guttata*	Pulmonary sample			Tierpark Berlin; Germany	ST7342 (723-888-907-571-868-807-830)	*spa* type t16114 (712-12-713-17-25-16-371)
BDS-92	*Eidolon helvum*	Faecal	2016	PUBMLST ID 32390	Health Centre, Obafemi Awolowo University, Ile-Ife, Nigeria	ST4014 (272-616-543-190-488-447-11)	*spa* type t16757 (742-743)
AOS157Y	Steering wheel of a car	Fomite		(Shittu et al., 2020)	Health Centre, Obafemi Awolowo University, Ile-Ife, Nigeria	ST3961 (272-357-306-190-268-448-277)	
R20	Bat	Faecal	2008	PUBMLST ID 5861	Ile-Ife, Nigeria	ST3135 (349-357-400-240-356-342-389)	
F16	*Eidolon helvum*	Faecal		PUBMLST ID 4779 (Akobi et al., 2012);	Ile-Ife, Nigeria	ST2470 (272-357-306-190-268-270-277)	
EMCR19	Human, *Homo sapiens*	Wound swab		(Schutte et al., 2021), GenBank CAJGUT01	Netherlands/Bali	ST6999 (818-1013-883-553-836-778-939)	*"S. roterodami"*
SS21	*Homo sapiens*			(Chew et al., 2021), GenBank JABWHB	Singapore	ST6105 (722-884-803-214-743-684-828)	*"S. singaporensis"*
SS35	*Homo sapiens*			(Chew et al., 2021), GenBank NZ_JABWPO	Singapore	ST6106 (722-885-805-214-744-685-831)	*"S. singaporensis"*
SS60	*Homo sapiens*			(Chew et al., 2021), GenBank NZ_JABWHF	Singapore	ST6107 (723-886-804-214-745-686-830)	*"S. singaporensis"*
SS87	*Homo sapiens*			(Chew et al., 2021), GenBank NZ_JABWHE	Singapore	ST6108 (722-887-806-481-746-684-829)	*"S. singaporensis"*
SS90 and SS251	*Homo sapiens*			(Chew et al., 2021), GenBank NZ_JABWHD and NZ_JABWHC	Singapore	ST6109 (724-888-807-481-747-684-277)	*"S. singaporensis"*
Sta1873	Food sample			PUBMLST ID 32453	Guangzhou, China	ST4075 (476-4-1-315-500-469-555)	
YNSA-323	Food			PUBMLST ID 32733	Yunnan, China	ST4185 (476-421-562-315-500-469-555)	

(Continued)

TABLE 1 | Continued

Isolate ID	Host	Sample type	Collected	Reference	Location	MLST	Comments
3574A1	Food			PUBMLST ID 33253	Guangzhou, China	ST4569 (532-1-1-315-567-513-617)	
Sta1874	Food			PUBMLST ID 32454	Guangzhou, China	ST4076 (475-4-1-315-499-465-553)	*spa* type t11641
SA1	*Homo sapiens*	Wound swab	2015	PUBMLST ID 32428	Rio de Janeiro, Brazil	ST4051 (403-1-1-190-1-1-1)	Combines CC1 MLST alleles with *S. roterodami*-like *gmk* sequence.
78085	*Homo sapiens*	Skin swab	2011	PUBMLST ID 5812	Denmark	ST3089 (349-57-45-2-7-58-52)	*mecC*-MRSA. Combines CC130 MLST alleles with *S. roterodami*-like *arcC* sequence.
3245	Food sample			PUBMLST ID 33090	Guangzhou, China	ST4466 (5-4-1-315-4-6-3)	Combines CC7 MLST alleles with *S. roterodami*-like *gmk* sequence. *spa* type t796.
TXA, TXBA140, A1404N, A1404W, A1524, A1525, A109, Z1403, K990W	Rhesus, *Macaca mulatta*, Long-tailed macaques, *M. fascicularis*, Southern pig-tailed macaque, *M. nemestrina*	Nasal and wound swabs	2015	(Soge et al., 2016; Roberts et al., 2018), SAMN04362246, SAMN04362247	Seattle, USA (animals imported from Asia)	ST3268 (1-14-430-214-10-303-329)	*spa* type t13638, SCC*mec* V/VT. Combines possibly CC45-like MLST alleles with a *S. roterodami*-like *gmk* sequence.
Several isolates	*M. fascicularis, Homo sapiens*	Surgical site, nasal and perianal swab	2014	(Hsu et al., 2017)	Singapore (imported animals as well as human contacts)	ST2817 (1-14-360-214-10-303-329) and ST3268 (1-14-430-214-10-303-329)	SCC*mec* V. Combines possibly CC45-like MLST alleles with a *S. roterodami*-like *gmk* sequence.
Several isolates	*Macaca mulatta, M. fascicularis*	Faecal	2017	(Li et al., 2020)	Shanghai, China (imported animals)	ST3268 (1-14-430-214-10-303-329)	*spa* type t13638, SCC*mec* V. Combines possibly CC45-like MLST alleles with a *S. roterodami*-like *gmk* sequence.

Preparation Kit (Illumina, Inc., San Diego, USA) according to the manufacturer's recommendations. The 2×300 bp paired-end sequencing in 40-fold multiplexes was performed on the Illumina MiSeq platform (Illumina, Inc., San Diego, USA).

Nanopore Sequencing

Oxford Nanopore Technology (ONT) sequencing of the study isolates, i.e., BDS-53E, BDS-54 and Zoo-28, was performed using two different MinION flow cells (IDs: FL1339 and FAO01531; rev: FLO-MIN106D containing an R9.4.1 pore). Library preparations were done using the 1D genomic DNA by ligation kit (SQK-LSK109, ONT), and the native barcoding expansion kit (EXP-NBD104, ONT) following manufacturer's instructions with minor adaptations. In summary, an AMPure bead (Agencourt AMPure XP, Beckman Coulter, Krefeld, Germany) clean-up step was performed before the library preparation. Potential nicks in DNA and DNA ends were repaired in a combined step using NEBNext FFPE DNA Repair Mix and NEBNext Ultra II End repair/dA-tailing Module (NewEngland Biolabs, Ipswich, USA) by tripling the incubation time. A subsequent second AMPure bead purification was followed by the ligation of sequencing adapters onto prepared ends and a third clean-up step with AMPure beads. An additional barcoding and clean-up step was performed prior to adapter ligation. Sequencing buffer and loading beads were added to the library. At the start of sequencing, an initial quality check of the flow cells showed 1289 (FL1339) and 1388 (FAO01531) active pores. Genomic DNA samples used for loading comprised a total amount of around 25.5 ng per strain (measured by Qubit 4 Fluorometer; ThermoFisher Scientific, Waltham, USA). The sequencing ran for 48 hrs using the MinKNOW software version 20.06.5.

Sequence Assembly and Polishing

For all nanopore data sets, the guppy basecaller (v4.2.2, Oxford Nanopore Technologies, Oxford, UK) translated and trimmed the MinION raw data (fast5) into quality tagged sequence reads (4,000 reads per fastq-file). Filtlong (v0.2.0) was used for bacterial

DNA with a median read quality of 14 and a minimum read length of 1,000 bp to get a smaller and better subset of reads. The median read quality of 15.5 and a N50 read length of approximately 13,000 bp for each sample was highly suitable for assembly. Flye (v2.8.3) was used to assemble the reads to provide high quality contigs. Then, a racon-medaka (4-times racon v1.4.3; 1-time medaka v1.2.0) pipeline was applied for polishing. Moreover, pilon (v1.23) polished the sequences using Illumina sequence data (**Supplemental Files 3/4/5a**). The NCBI Prokaryotic Genome Annotation Pipeline (PGAP version 2021-01-11.build 5132) was used for annotating all assembled contigs in combination with an in-house database of published staphylococcal gene sequences (**Supplemental Files 3/4/5b**).

Phylogenetic Analysis

We selected a panel of 154 core genome markers for tree construction using SplitsTree (Monecke et al., 2021). These genes and genome sequences used for visualisation (**Figure 1**) are listed in **Supplemental File 6**. Inclusion criteria were the presence of the genes in all CCs of *S. aureus/argenteus/schweitzeri* clonal complexes and uniform length in all published genomes. Sequences were concatenated and analysed using SplitsTree 4.0 (Huson and Bryant, 2006) using default settings (characters transformation, uncorrected P; distance transformation, Neighbour-Net; and variance, ordinary least squares).

Analysis of the Core Genome

A comprehensive analysis of existing sequence databases and available literature led to the definition of a set of 2,167 genes (**Supplemental File 7A**) in the core genome of *S. aureus*, *S. argenteus* and *S. schweitzeri*. These genes are almost universally present although in few cases sequences might be absent due to random mutation or sequencing/assembly artefacts. These genes always appeared in the same order within the genome, regardless of the identity of the isolate. In addition, 125 markers from major genomic islands were considered always present or usually present (with presence or absence being linked to species or CC affiliation). They also appear in the same positions within the genome. Genes in this category are the staphyloxanthin gene cluster, the *set/ssl* loci and the *egc* enterotoxin gene cluster. Genomic islands that might occupy variable locations within the genome were not considered. The sequences of these core genome and major genomic island genes were analysed and compared to each other and to reference sequences. A few genomic island genes not present in any of the isolates compared in the present study were excluded. Thus, a total of 2,292 genes were considered representing roughly 2,040,000 nucleotide positions per genome. For each gene, the number of nucleotides different from the comparator isolate was counted and expressed as a percentage of the length of the respective gene (**Supplemental File 7B**). Gaps

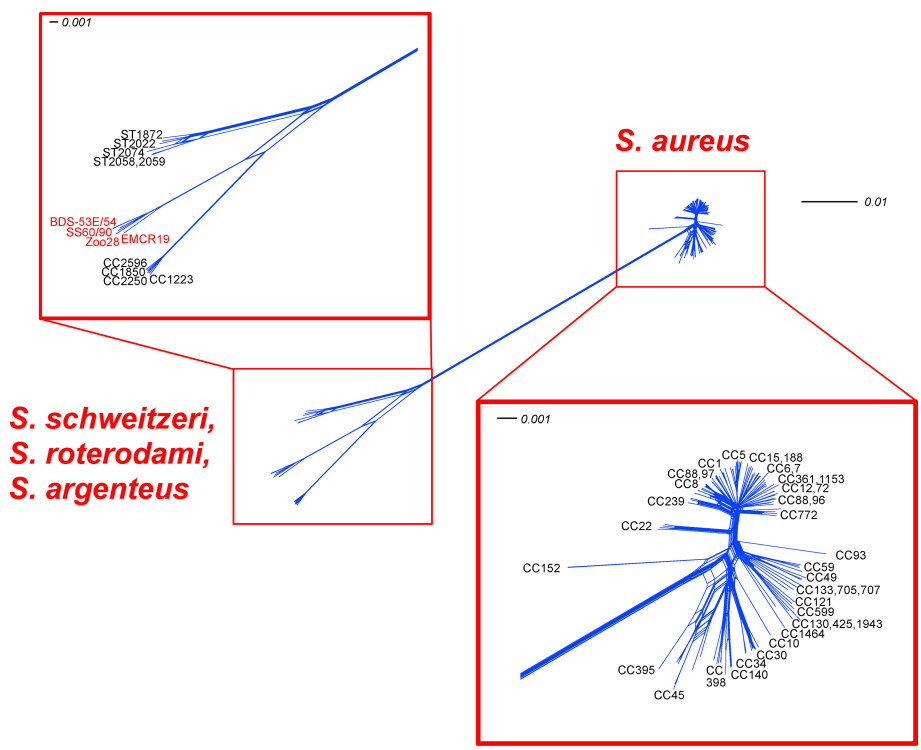

FIGURE 1 | SplitsTree analysis for 154 core genomic markers of the study isolates, "*S. roterodami*" EMCR19 as well as "*S. singaporensis*" SS60 and SS90 compared to diverse *S. aureus, S. argenteus* and *S. schweitzeri* CCs. Note, for the genes that were found inverted in the Zoo-28 genome, reverse complement sequences were used for analysis.

in the alignment of different alleles of a given target gene were treated as mismatches. If a gene was present in one isolate but absent in the other isolate, this percentage was set as 100%. In addition, percentages were plotted over the positions in the genome (**Figure 2**). When comparing two isolates, median values for these percentages of all genes were calculated.

Genome Taxonomy Database Toolkit (GTDB-tk)

We utilized GTDB-tk, a software toolkit for assigning objective taxonomic classifications to bacterial and archaeal genomes to determine if the genomes of the study isolates were novel species (Parks et al., 2018; Parks et al., 2020; Parks et al., 2022). To taxonomically assess the genomes, GTDB-Tk version 1.6.0 (Chaumeil et al., 2019) and the GTDB database version 202 (ref: https://gtdb.ecogenomic.org/stats/r202) containing 254,090 bacteria and 4,316 archaeal genomes) were utilised. Briefly, the target genomes were classified by the GTDB-Tk's "classify" workflow (using the default settings) by placing them into the GTDB's reference tree. We considered the average nucleotide identity (ANI), alignment fraction (AF) and its relative evolutionary divergence (RED) (Scholtzek et al., 2019) to the closely related reference genomes.

RESULTS

Phenotypic Characterisation and Microarray-Based Genotyping

Based on the bioMérieux´ Gram-positive identification card (GP) for VITEK-2, the biochemical test results are summarised in **Table 2** (see also **Supplemental File 2**). Profiles were in accordance with *S. aureus*.

Based on their irregular microarray hybridisation patterns (see **Supplemental File 1**), an assignment of the isolates to any known lineage of *S. aureus* was not possible, suggesting affiliation to either *S. argenteus* or *S. schweitzeri*. However, isolates yielded positive signals for one *crtM* probe and weak signals for *crtP*. Since these probes recognise genes from the staphyloxanthin cluster, which by definition should be absent from *S. argenteus*, the isolates could not be assigned to this species. On the other hand, all three isolates carried *ycjY*, a marker on a genomic island identified in *S. argenteus* and some *S. aureus* lineages (CC12, CC361 and CC398), but absent from any *S. schweitzeri* tested or sequenced. The *orfX*-associated (Holt et al., 2011) *cas1* CRISPR-endonuclease 1 (FR821777.2; pos. 62,418...63,323, which can be observed in *S. argenteus* CC1850 and CC2250) was not detected. The isolates clearly clustered into two distinct putative CCs.

The Nigerian bat isolates were highly similar to each other. They yielded signals with *hld*, *agrC/D*-I and with *S. argenteus*-specific *agr* probes, indicating a presence of an *agr* gene cluster albeit an atypical allele. Similarly, *icaA* was the only *ica* gene detected by the array (while the others were detected by sequencing; see **Supplemental Files 3**, **4**). Capsule genes were not detected by the array although sequencing showed a presence of specific alleles. The *cna* gene was absent, while *sasG* was present. The *ssl* genes (encoding staphylococcal superantigen-like protein locus 1) were not detected by the array although one gene of this cluster was found by sequencing.

The bat isolates could further be subdivided based on reactivities with either *sdrC* or *sdrD* probes. For sequencing, one *sdrC*- and one *sdrD*-positive isolate (BDS-53E and BDS-54, respectively) were selected.

The German isolate Zoo-28 shared *agr* and *ica* reactivities. It differed in the absence of *sasG* and in the allelic variants of several adhesion factors. In contrast to the bat isolates, several *ssl* genes were present (see **Supplemental Files 1**, **5**). It also harboured the leukocidin genes *lukD/E*, which were absent from the bat isolates (although the latter component was identified only by sequencing).

MLST and Phylogenetic Analysis

MLST yielded profiles that are shown in the first half of **Table 1**. Previously published MLST profiles that appeared related are listed in the second half of the table.

An MLST-like approach based on 154 core genomic markers (**Figure 1**; **Supplemental File 6**) led to the clustering of all *S. aureus*, with three major groups and three separate branches. One group comprised CC1, CC5, CC8 and most of the other *S. aureus* lineages. A second one included CC59, CC121 and several, mostly animal-associated, lineages such as CC49, CC130 and CC1464 ("*S. aureus* subsp. *anaerobius*"). The third group consisted mainly of CC30, CC45, CC398. The separate branches, CC22, and more conspicuously, CC93 and CC152, appeared to be more distant from other *S. aureus* lineages.

Another very distant branch consisted of *S. argenteus* lineages (CC1223, CC1850, CC2198, CC2250, CC2596 and CC4587). *S. schweitzeri* (ST1872, ST2022, ST2058, ST2059, ST(206-303-253-142-196-202-197); accession number CCEO01000001-CCEO01000054) was also clearly separate, but much closer to *S. argenteus* than *S. aureus*. The genomes of BDS-53E, BDS-54 and Zoo-28 were closely related to "*S. roterodami*/*singaporensis*" (EMCR19, SS60, SS90). The SplitsTree in **Figure 1** shows that the aforementioned "*S. roterodami*/*singaporensis*" isolates as well as the three sequenced study isolates were located between *S. argenteus* and *S. schweitzeri*.

Sequence Analysis of the Core Genome and Major Genomic Islands

For the Nigerian bat isolates, the order of the predefined core genomic markers and major genomic island markers within the genomes was identical as observed with the published sequences of *S. aureus, S. argenteus* and *S. schweitzeri*. In the isolate Zoo-28, a large part of the genome was found inverted (already in the Nanopore sequence before polishing with Illumina) and integrated further downstream, into the *map/eap* gene, with *map/eap* fragments identified at pos. 1,991,191...1,992,912 and 2,090,178...2,091,407 of the genome. This inverted part of the chromosome contained

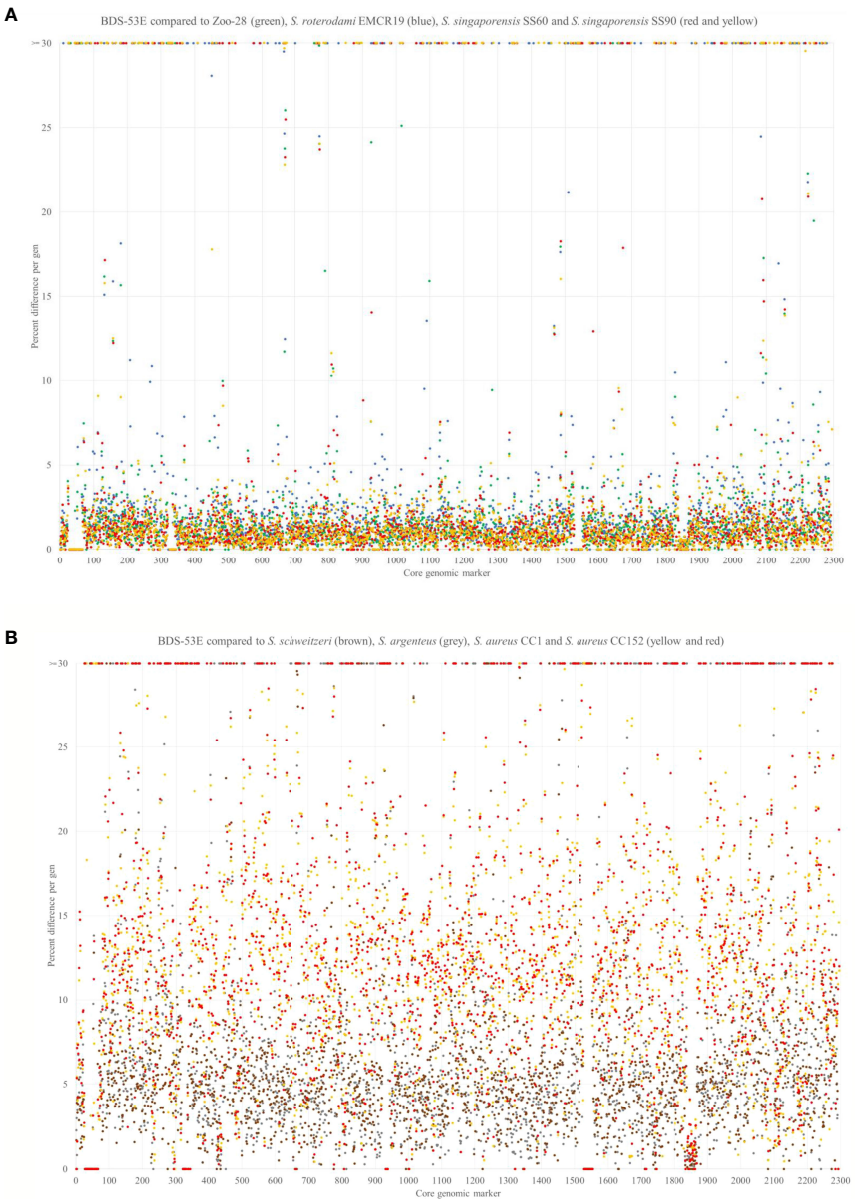

FIGURE 2 | **(A)** SNP analysis comparing 2292 non-motile genes of BDS-53E with the Zoo-28 (green), "*S. roterodami*" EMCR19 (blue) as well as with "*S. singaporensis*" SS60 and SS90 (red and yellow). For each single gene, the number of differences (**Supplemental File 7B**) to the comparator strains was counted and expressed in percentage. For genes that were present in one, but absent in the other isolate, this percentage was set as 100%. Note, for the genes that were found inverted in the Zoo-28 genome, reverse complement sequences were used for analysis. **(B)** SNP analysis comparing 2292 non-motile genes of BDS-53E with *S. schweitzeri* (NCTC13712, LR134304.1, brown), *S. argenteus* (MSHR1132, FR821777.2, grey), *S. aureus* CC1 (MW2, BA000033.2, yellow) and *S. aureus* CC152 (SA17_S6, CP010941.1, red). The genes around pos. 1550 represent a genomic island that is absent in the study isolates (protease genes *splF, splE, splD2, splC, splB, splA*, leukocidin *lukD/E*, lantibiotic epidermin gene cluster). The highly conserved genes around pos. 1850 are those encoding ribosomal proteins.

approximately 69,000 bp or 60 genes from *namA* (NADH: flavin oxidoreductase) to *yrbD* (alanine:cation symporter family protein), including the chromosomal oligopeptide ABC transporter cluster *oppA/oppF/oppD/oppC/oppB*. It was flanked at both sides by transposase genes and genes of yet unknown function resulting in a total size of the insert of about 97 kb. Another transposase gene was identified at the original position where the inserted genes were supposed to be localised.

The analysis and comparison of the core genomic and major genomic island genes of the three isolates and reference sequences indicated that the differences of BDS-53E compared to the reference sequences are somewhat uniformly scattered all across the genome. Moreover, BDS-53E and Zoo-28 are similar to

TABLE 2 | Biochemical profiles, based on results for bioMérieux´ Gram-positive identification card (GP) for VITEK-2.

Pos.	Reaction	Code	BDS-53E	BDS-54	Zoo-28	SS21 (DSM111408)	EMCR19 (DSM111914)
2	D-Amygdalin	AMY	negative	negative	negative	negative	negative
4	Phosphatidylinositol phospholipase C	PIPLC	negative	negative	negative	negative	negative
5	D-Xylose	dXYL	negative	negative	negative	negative	negative
8	Arginine dihydrolase 1	ADH1	**positive**	**positive**	**positive**	**positive**	**positive**
9	b-Galactosidase	BGAL	negative	negative	negative	negative	negative
11	a-Glucosidase	AGLU	**positive**	**positive**	negative	**positive**	negative
13	Ala Phe Pro arylamidase	APPA	negative	negative	negative	negative	negative
14	Cyclodextrin	CDEX	negative	negative	negative	negative	negative
15	L-Aspartate arylamidase	AspA	negative	negative	negative	negative	negative
16	b-Galactopyranosidase	BGAR	negative	negative	negative	negative	negative
17	a-Mannosidase	AMAN	negative	negative	negative	negative	negative
19	Phosphatase	PHOS	**positive**	**positive**	**positive**	**positive**	**positive**
20	Leucine arylamidase	LeuA	negative	negative	negative	negative	negative
23	L-Proline arylamidase	ProA	negative	negative	negative	negative	negative
24	b-Glucaronidase	BGURr	negative	negative	negative	negative	negative
25	a-Galactosidase	AGAL	negative	negative	negative	negative	negative
26	L-Pyrrolidonyl-arylamidase	PyrA	**positive**	**positive**	negative	**positive**	**positive**
27	b-Glucaronidase	BGUR	negative	negative	negative	negative	negative
28	Alanine arylamidase	AlaA	negative	negative	negative	negative	negative
29	Tyrosine arylamidase	TyrA	negative	negative	negative	negative	negative
30	D-Sorbitol	dSOR	negative	negative	negative	negative	negative
31	Urease	URE	**positive**	**positive**	negative	negative	negative
32	Polymyxin B resistance	POLYB	**positive**	**positive**	**positive**	**positive**	**positive**
37	D-Galactose	dGAL	negative	negative	**positive**	**positive**	**positive**
38	D-Ribose	dRIB	**positive**	negative	**positive**	**positive**	negative
39	L-Lactate alkalinisation	lLATk	**positive**	**positive**	**positive**	**positive**	**positive**
42	Lactose	LAC	negative	negative	negative	negative	negative
44	N-Acetyl-D-glucosamine	NAG	negative	negative	negative	negative	negative
45	D-Maltose	dMAL	**positive**	**positive**	**positive**	**positive**	**positive**
46	Bacitracin resistance	BACI	**positive**	**positive**	**positive**	**positive**	**positive**
47	Novobiocin resistance	NOVO	**positive**	negative	negative	negative	negative
50	Growth in 6.5% NaCl	NC6.5	**positive**	**positive**	**positive**	**positive**	**positive**
52	D-Mannitol	dMAN	**positive**	**positive**	**positive**	**positive**	**positive**
53	D-Mannose	dMNE	**positive**	**positive**	**positive**	negative	**positive**
54	Methyl-B-D-glucopyranoside	MBdG	**positive**	**positive**	**positive**	**positive**	**positive**
56	Pullulan	PUL	negative	negative	negative	negative	negative
57	D-Raffinose	dRAF	negative	negative	negative	negative	negative
58	O129 Resistance	O129R	**positive**	**positive**	**positive**	**positive**	**positive**
59	Salicin	SAL	negative	negative	negative	negative	negative
60	Saccharose/sucrose	SAC	**positive**	**positive**	**positive**	**positive**	**positive**
62	D-Trehalose	dTRE	negative	negative	**positive**	**positive**	**positive**
63	Arginine dihydrolase 2	ADH2s	negative	**positive**	negative	negative	negative
64	Optochin resistance	OPTO	**positive**	**positive**	**positive**	**positive**	**positive**

TABLE 3 | GTDB-tk results.

Genome	Closest relative	Average nucleotide identity to closest relative	Alignment fraction to closest relative
BDS-53E	*S. argenteus* (GCF_000236925.1)	93.86	0.91
BDS-54	*S. argenteus* (GCF_000236925.1)	94.01	0.92
Zoo28	*S. argenteus* (GCF_000236925.1)	93.99	0.91

S. roterodami and *S. singaporensis* (**Figure 2**). Using the median values for the differences of all genes to compare sequences of the study isolates, we observed that these sequences and those of *S. roterodami* and *S. singaporensis* differed by approximately 1% (**Figures 2A**, **3**). Furthermore, we observed approximately 5% differences compared to both, *S. argenteus* and *S. schweitzeri*, and of about 11-12% compared to different *S. aureus* CCs (**Figures 2B**, **3**).

Analysis by GTDB-tk

The genome sequences of the study isolates were taxonomically placed in the genus of *Staphylococcus* without a species assignment as their closest relative, *S. argenteus* (GCF_000236925.1), shared only an average nucleotide identity of 93.86% to 94.01% (**Table 3**). The second most closely related species was *S. schweitzeri* (average nucleotide identity, ANI, 93.53 to 93.63), followed by *S. aureus* (ANI,

BDS-53E	BDS-54	Zoo-28	"*S. roterodami*", EMCR19	"*S. singaporensis*", SS60	"*S. singaporensis*", SS90	*S. schweitzeri*, NCTC13712	*S. argenteus*, MSHR1132	*S. aureus* CC152, SA1756	*S. aureus* CC93, JKD6159	*S. aureus* CC1, MW2	
0.00	0.16	1.02	1.38	1.02	1.02	5.22	5.05	11.99	11.51	11.73	BDS-53E
	0.00	1.00	1.38	0.99	1.02	5.20	5.05	11.93	11.53	11.72	BDS-54
		0.00	1.35	0.81	0.82	5.17	4.93	11.86	11.42	11.54	Zoo-28
			0.00	1.35	1.33	5.23	5.07	11.86	11.43	11.65	"*S. roterodami*", EMCR19
				0.00	0.80	5.07	4.96	11.90	11.56	11.67	"*S. singaporensis*", SS60
					0.00	5.16	4.96	11.85	11.62	11.74	"*S. singaporensis*", SS90
						0.00	5.84	10.76	10.85	10.75	*S. schweitzeri*, NCTC13712
							0.00	12.02	11.68	11.83	*S. argenteus*, MSHR1132
								0.00	1.57	1.52	*S. aureus* CC152, SA1756
									0.00	1.05	*S. aureus* CC93, JKD6159
										0.00	*S. aureus* CC1, MW2

FIGURE 3 | Matrix of differences between BDS-53E, Zoo-28, "*S. roterodami*" (EMCR19, CAJGUT01), "*S. singaporensis*" (SS60 and SS90, NZ_JABWHF and NZ_JABWHD), *S. schweitzeri*, (NCTC13712, LR134304.1), *S. argenteus* (MSHR1132, FR821777.2) and three *S. aureus* lineages, CC1 (MW2, BA000033.2), CC93 (JKD6159, CP002114.2) and CC152 (SA17_S6, CP010941.1). The percentages were calculated as explained for **Figure 2** and the image shows the median values over all these 2292 genes for each genome sequence compered to all others.

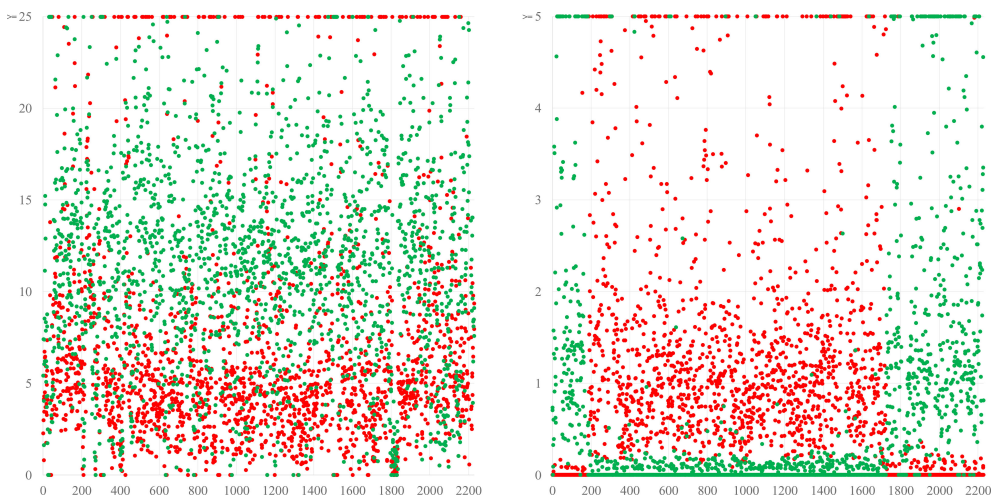

FIGURE 4 | The left diagram shows a simplified version of the blot from **Figure 2**, comparing BDS-53E to the reference sequences of *S. schweitzeri* NCTC13712 (red) and the *S. aureus* CC1 strain MW2 (green). Differences are evenly distributed all across the genome, suggesting a continuous evolution and an accumulation of more mutations compared to *S. aureus* than to *S. schweitzeri* corresponding supposedly to a longer time of separate evolution. The right diagram shows the same analysis for a chimeric strain in which a fragment of "red" origin spanning from approximately position 1750 over *oriC* (pos. 0) to pos. 200 is integrated into a backbone genome of "green" provenance. These are actually *S. aureus* CC140 and CC8, in a ST6610 strain, see (Burgold-Voigt et al., 2021). For the Y-axes, different scales were used because differences between the different species are larger than those between different CCs of *S. aureus*.

88.98 to 89.22). *S. singaporensis* and *S. roterodami* are not (yet) covered by the database.

Resistance Genes and Antimicrobial Susceptibility Testing

All study isolates were methicillin susceptible and negative by both, array and sequencing, for *mecA/mecC* genes.

Antimicrobial susceptibility testing using the AST-P608 panel showed that BDS-53E was fully susceptible to all compounds tested. BDS-54 was susceptible to all compounds but intermediate to fluoroquinolones. However, in *grlA*, some single nucleotide polymorphisms (SNPs) were either observed in BDS-53E and BDS-54 (E310K, K650N, H767Q) or in the three study isolates and in *S. argenteus* and *S. schweitzeri*. Similarly, most SNPs in the *gyrA* gene separated the three study isolates plus *S. argenteus* and *S. schweitzeri* from *S. aureus* (e.g., E248V, V623I). Previously described mutations (Wang et al., 1998) were not identified.

A distinct result for BDS-53E (from the Gram-positive ID panel) was its novobiocin resistance. As mutations in *gyrB* (CP000253.1; 5,034...6,968) and *parE=grlB* (CP000253.1; 1,292,206...1,294,197) might be related to novobiocin resistance (Fujimoto-Nakamura et al., 2005), the sequences of these genes were analysed. The BDS-53E sequence of *gyrB* showed a unique mutation resulting in a substitution of aspartic acid by valine at amino acid position 494, which was not present neither in the other two study isolates nor in some reference sequences (MSHR1132, MW2, SA17_S6, JKD6159 and NCTC13712). The BDS-53E sequence of *parE=grlB* did not contain unique SNPs although in some positions, SNPs were observed that either all three study isolates shared (I490L), or had in common with *S. argenteus* and *S. schweitzeri*.

BDS-53E and BDS-54 carried *aadK*, a gene encoding an aminoglycoside 6-adenyltransferase gene not covered by the microarrays used. It was nearly identical (in 826 of 846 nt) to CP010526.1, 421,013...421,858. In both isolates, it was localised on putative pathogenicity islands integrated between the gene *guaA* (glutamine-hydrolysing GMP synthase) and Q8NY63 (putative protein), around position 400,000 in the genome. These pathogenicity islands additionally included several genes for "hypothetical proteins", a DNA primase, an integrase and a small terminase subunit.

The isolate Zoo-28 was resistant to benzylpenicillin and tetracycline. A *blaZ/I/R* operon was identified together with *yolD* and *tnpA/B/C* genes as part of a transposon that showed 99.58% identity to Tn*553*, a non-conjugative transposon of the Tn*554* family. This transposon has recently been described in porcine *S. aureus* ST9 from China (Krüger et al., 2021). Like the original Tn*553*, the Tn*553* in Zoo-28 was also integrated into the chromosomal Q5HEJ7/*yolD*-like gene. Tetracycline resistance was attributable to the tetracycline resistance gene *tet*(M). It was accompanied by a Tn*916*-transposase, a Tn*916*-excisionase, a gene encoding D2N5T7 (a conjugative transposon protein), *yddH* (encoding a putative peptidase cell wall hydrolase), *ardA2* (coding for an antirestriction protein) and a couple of hypothetical proteins. The entire transposon was identical to Tn*916* (GenBank, U09422.1), a transposon first described in enterococci but also known from staphylococci such as CC398-MRSA-VT (S0385, GenBank AM990992.1 and 08BA02176, GenBank CP003808.1).

Other Mobile Genetic Elements

All three sequenced study isolates did not harbour SCC*mec* elements. BDS-53E and BDS-54 carried clusters of twelve (BDS-54; see **Supplemental File 4B**) or twenty genes (BDS-53E; see **Supplemental File 3B**) associated with, and located directly downstream of *orfX*. These were automatically annotated as "hypothetical proteins", transposases, helicases, methyltransferases, oxidoreductases and hydrolases. In addition, two of the un-sequenced bat isolates (BDS-69C, BDH-147) were positive in array experiments for B2Y834 (a marker usually associated with SCC*mec* IV A, IV E and IV c) as well as another one (BDH-128) with *ccrA-1*.

Zoo-28 lacked these genes, carrying transposase and helicase genes instead, as well as one gene, B6VQU0, which is otherwise known to occur in CC705 (GenBank AJ938182, pos. 34,735...35,634, downstream of *orfX*), as well as in SCC*mec* IV h/j elements.

Downstream of the SCC integration site, a large genomic island is situated whose gene content is related to CC affiliation. Typical genes of that genomic island, such as *seh* and ORF CM14 (characteristic for, *e.g.*, CC1, CC10, CC34, or CC93 or CC772), were absent from the study isolates. BDS-53E and BDS54 harboured in this position *mcrB* (type IV 5-methylcytosine-specific restriction enzyme subunit B), *mcrC* (subunit C) and Q6GD44 (putative acetyltransferase, GNAT family) genes. Zoo-28 differed, carrying C1PH96 (putative protein, carboxymuconolactone decarboxylase family), *lrpC* (HTH-type transcriptional regulator *Lrp/AsnC* family) and Q6GD44 genes.

BDS-53E and BDS-54 carried prophages that could be assigned to *Siphoviridae* based on sequence similarity to known *S. aureus* phages. In both cases, phages were integrated between the genes encoding a putative protein A5ITW8 and tRNA for serine, approximately at position 1,800,000 in the genomes. Phages were similar but not identical (see **Supplemental Files 3B**, **4B**). Both isolates harboured putative pathogenicity islands as described above.

In Zoo-28, no prophage was identified, but it carried as much as three different pathogenicity islands with genes for "hypothetical proteins", integrases as well as for small terminase subunits. Finally, Zoo-28 carried Tn*916* and Tn*553*-like transposon as discussed above.

There was no evidence for plasmids in the three study isolates.

DISCUSSION

Evolution is a continuous process that does not occur in discrete steps. Thus, the classification of "evolving live forms" into discrete or distinct species is always problematic regardless of whether they are bacteria or higher organisms, such as herring gulls (Liebers et al., 2004), or cervids (Ludt et al., 2004). This distinction cannot be made without a certain element of arbitrariness. This is caused by an emphasis on specific features of the target organism that are considered sufficient to define a species and by the fact that different observers might prioritise different properties. For instance, traditionally, *Shigella*

is a distinct taxonomic entity from *Escherichia* (*E.*) *coli* because of the "severity of dysentery" and its lethality in humans. However, an investigation of *Shigella* gene sequences shows only minimal differences compared to *E. coli* which do not justify its recognition as distinct genus or species (van den Beld and Reubsaet, 2012). For other life forms, taxonomists argue about the fertility of hybrids, or, in the case of the herring gulls mentioned above, mating calls and feet colour (Collinson et al., 2008). A numerical approach to gene analysis might reduce arbitrariness although different "threshold values" must carefully be considered for various clades of life forms.

The numerical approach for assessing nucleotide differences per gene length yielded some interesting results in the present study. First, within *S. aureus*, the median difference for all 2,292 genes considered was only 1 to 1.5%, even when comparing a CC1 reference sequence to such diverse, or deviant, lineages as CC93 or CC152. This observation gives an impression or benchmark for the largest possible difference *within* one established species. Median differences between *S. aureus* and *S. argenteu*s or *S. schweitzeri* were 10-12%, while it was about 5% between *S. argenteus* and *S. schweitzeri*. These data might provide an orientation on the level of distinction of valid species to one another. The study isolates differed from each other by 0.16% to 1.38% but differed from *S. argenteus* and *S. schweitzeri* by about 5% and from diverse *S. aureus* lineages (CC1, CC93, and CC152) by 11-12%. These observations suggest that the study isolates belonged to one distinct species, with the African isolates in one CC and the German zoo isolate in another one. However, a comparison of the genomes of the study isolates to the recently published genomes of the isolates assigned to the new species *S. roterodami* and *S. singaporensis* (Schutte et al., 2021; Chew et al., 2021) yielded median differences of around 1%, and the difference between these two species was about the same.

Our observations provide two options. First, one might conclude that a difference of more than 10% was required for recognition as a full species. Hence, *S. aureus* would be one species, while *S. schweitzeri, S. argenteus, S. roterodami, S. singaporensis* and the study isolates clustered to another one. Second, one might define a median difference of about 5% as a threshold for a species. This is implemented in GTDB-tk where a query genome is regarded as the same species as the closest reference sequence if it falls within an ANI of at least 95% and an AF of 65%. In this case, *S. aureus*, *S. schweitzeri* and *S. argenteus* would be three species, while a forth one comprised both *S. roterodami* and *S. singaporensis*, as well as the study isolates. However, phenotypical tests allow no clear distinction of these isolates from the other members of this "species" raising the question of whether sequence analysis should have priority over biochemical tests or not. Hence, a discussion about the definition of clear criteria for recognition as a discrete species is necessary especially as new technologies and lower costs facilitate sequencing of isolates that could not be sequenced before, including those from faeces of exotic animals, resulting in an unprecedented increase in the number of available genome sequences.

We present evidence for recognising a new species of coagulase- and staphyloxanthin-positive staphylococci positioned between *S. argenteus* and *S. schweitzeri*. The previously described species "*S. roterodami*" and "*S. singaporensis*" as well as isolates described herein all together should be regarded as a single species, *i.e.*, as the fourth one in the *S. aureus* complex in addition to *S. aureus, S. argenteus* and *S. schweitzeri*. According to the principle of priority, that species should be named *S. roterodami* as this name was published first, in September 2021 (Schutte et al., 2021).

This species has a much wider geographical range than previously thought, *i.e*, Nigeria, Southern China, Indonesia, Singapore and possibly Australia.

The majority of isolates described herein and two previously published MLST profiles originate from bat faeces from Nigeria. These two MLST profiles were posted to the MLST database by a Japanese and a British group (ST2470 and ST3135). Sequences are not identical albeit similar, and the sampling location was approximately the same as for the bat isolates described herein. One isolate (Zoo-28) was sampled from an estrildid finch living in a zoo in Germany. While this species is native and endemic to Australia, this individual zoo animal might have been colonised/infected by contact with other animals such as flying foxes kept in close proximity. Unfortunately, we cannot investigate that issue anymore due to alterations to the building and the aviary kept inside. "*S. roterodami*" and "*S. singaporensis*" were isolated from humans returning from Bali or living in Singapore, respectively, as discussed above. Further related STs, ST4075, ST4076, ST4569, were observed in isolates from unspecified food from the Guangzhou region, in the southern part of China. Another food isolate, ST4185 originated from Yunnan, a province in Southwestern China. These four STs share a unique *gmk* allele (*gmk*-315) suggesting affiliation to yet another CC. They also present ordinary *S. aureus*-like *aroE*-alleles and unique, deviant *arcC*-alleles.

S. roterodami is a polymorphic species, consisting of at least nine distinct CCs with the animal isolates described herein constituting two CCs. The Bali isolate originally described as *S. roterodami* represents another one. The six "*S. singaporensis*" isolates could be classified into five different CCs. Differences between the CCs of *S. roterodami* include carriage, or absence, of gene clusters that also define complexes within *S. aureus* or *S. argenteus*. These include *agr* locus genes, the *set/ssl* cluster, the enterotoxin gene cluster *egc, edinB+etD* and *sasG*. The four sequence types from Southern China might represent at least one additional CC.

In addition to the CCs discussed above, there are several STs in the MLST database that appear to be *S. aureus* although they contain one *S. roterodami* MLST allele each in addition to six regular *S. aureus* MLST markers. Whether this was evidence for cross-species hybridisation or chimerism involving *S. aureus* and *S. roterodami*, an accidentally identical accumulation of mutations, or merely technical issues, still needs to be clarified. These STs include ST4051 with *gmk*-190 while the other markers are in accordance with a CC1 profile, ST3089 that

differs from CC130 in *arcC*-0349 and ST4466 that carries *gmk*-0315 although it otherwise resembles CC7. Finally, a MRSA lineage associated with imported macaques (*Macaca sp.*), ST3268/ST2817, was identified in the USA, China, and Singapore (Soge et al., 2016; Hsu et al., 2017; Roberts et al., 2018; Li et al., 2020) in which a *S. roterodami*-like *gmk* allele (*gmk*-214) is present, among other MLST alleles that could be derived from *S. aureus* CC45.

With regard to chimerism, one might argue that the *S. roterodami* complex was a group of chimeric *S. argenteus* isolates that acquired the gene cluster encoding the "golden" carotenoid pigment staphyloxanthin by chromosomal replacement, hybridisation, or chimerism. **Figure 1** contains information rendering that concept rather unlikely. The differences compared to the reference isolates affect all parts of the genome essentially and are distributed evenly across the genome (with the notable exception of a few highly conserved genes encoding ribosomal proteins). Previous work (Nimmo et al., 2015; Burgold-Voigt et al., 2021) showed how a part of the genome of a chimeric isolate genome would match the corresponding part from one parent strain, and differ from the same region of the other one, while this would be conversely for the rest of the genome (**Figure 4**). Therefore, we can assume that the similarities and differences of the *S. roterodami* complex compared to *S. aureus*, *S. argenteus* and *S. schweitzeri* do not result from a large-scale chromosomal replacement or chimerism. Thus, the isolates cannot be considered *S. argenteus* that by chance acquired the staphyloxanthin locus from elsewhere. The more likely explanation was a continuous evolution and accumulation of mutations over time. Based on the median differences calculated for core genomic markers (**Figure 3**), we postulate that the split from *S. aureus* occurred earlier than the one from *S. argenteus* and *S. schweitzeri*.

Identifying *S. aureus, S. argenteus, S. schweitzeri* and *S. roterodami* from humans and/or wild animals in Africa suggest that they could have originated from the continent, disseminating to other parts of the world through human migration. Thus, it would be interesting to screen African wildlife for other possible branches of the staphylococcal phylogenetic tree to understand the co-evolution of humans and animals with their coagulase-positive colonisers/pathogens.

S. roterodami has been found in symptomatically ill humans as well as in wild bats and a captive finch, suggesting a relatively broad zoonotic host spectrum as well as a certain virulence in humans. Besides, it can acquire resistance genes known from other staphylococci such as *blaZ*, *tet*(M), *aadK*, *aacA-aphD* and *aadD* as demonstrated in the study isolates and in those identified in Singapore (Chew et al., 2021). Thus, its clinical relevance could be comparable to that of *S. aureus* or *S. argenteus*.

Further studies should focus on staphylococcal isolates from humans, bats, rodents, birds, and atypical *S. aureus* isolates from Western and Central Africa, Southern and Southeastern Asia as well as Australia. While phenotypic tests might not be conclusive, unique MLST alleles (see **Table 1**) should help identifying *S. roterodami* isolates. However, a non-molecular algorithm for diagnostic procedures to identify the new species from routine diagnostic samples is needed.

DATA AVAILABILITY STATEMENT

The genome sequences of the study isolates were submitted to GenBank. The BioProject accession number is PRJNA810320, BioSamples are SAMN26244312 to 314 and the GenBank accession numbers are CP092781, CP092782 and CP092783. All other data are provided within the manuscript, or as Supplemental Files.

ETHICS STATEMENT

No animal experiments were performed and no animal was sacrificed for this study. No ethical clearance was necessary as no animals were captured and no invasive samples were taken. The strains originated from environmental samples (i.e., bat faeces collected under trees used by wild bats for roosting) and from a *post mortem* sample from a zoo animal submitted for routine diagnostic procedures.

AUTHOR CONTRIBUTIONS

SM designed the study. SM, FS, AS, and ATF wrote the manuscript. FS, AS, ATF, and KM obtained samples and performed experiments (bacteriological work). HH, CD, MR, and SB performed experiments (sequencing). SM, CB, MC, and DH analysed sequence data. EM and DG performed experiments (bacteriological work and arrays). ATF, SS, and RE supervised the work and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.878137/full#supplementary-material

Supplemental File 1 | Hybridisation profiles of study isolates and reference strains (pdf).

Supplemental File 2 | Vitek Data (pdf).

Supplemental File 3 | (A) Genome sequence of BDS-53E (fasta). **(B)** Genes identified within the genome sequence of BDS-53E (fasta).

Supplemental File 4 | (A) Genome sequence of BDS-54 (fasta). **(B)** Genes identified within the genome sequence of BDS-54 (fasta).

Supplemental File 5 | (A) Genome sequence of Zoo-28 (fasta). **(B)** Genes identified within the genome sequence of Zoo-28 (fasta).

Supplemental File 6 | Strains and concatenated Sequences used for the Splitstree analysis.

Supplemental File 7 | (A) Sequences for analyses of strain similarities as shown in and (fasta). **(B)** Percentages difference as used for **Figures 2A, B**.

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BRIEF RESEARCH REPORT
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First Study of Bacteremia Caused by *Herbaspirillum huttiense* in China: A Brief Research Report and Literature Review

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Bacteremia caused by *Herbaspirillum huttiense (H. huttiense)* is relatively rare in positive blood cultures. *H. huttiense* is an opportunistic bacterium in patients with cancer and cirrhosis and has also been described in immunocompromised hosts. In this study, *H. huttiense* was isolated from a patient with repeated chest tightness and chest pain. Smears were prepared, stained, and examined by microscopy. Single colonies were analyzed by Gram staining, matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS), 16S rRNA sequencing and Next-Generation Sequencing (NGS). Antibiotic sensitivity was assessed by agar dilution. Almost all publications on *H. huttiense* infections in the PubMed/ScienceDirect/EBSCO databases were reviewed and summarized. Blood sample culturing yielded white, gelatinous, and slightly raised colonies without hemolytic rings. The bacilli were found to be Gram-negative, and MS results showed 99.2% homology with *H. huttiense*. This was confirmed by 16S rRNA gene sequencing, phylogenetic tree analysis and NGS all of which were homologous with *H. huttiense* in GenBank. Antibiotic susceptibility tests were performed to determine the minimum inhibitory concentrations (MICs) of imipenem, meropenem, piperacillin-tazobactam, and levofloxacin. A comprehensive literature review revealed that *H. huttiense* was an emergent pathogen. After medical treatment, the patient's body temperature returned to normal. This is the first report of bacteremia caused by *H. huttiense* in China. The findings could improve the awareness and attention of the rare pathogenic microorganisms in China.

Keywords: first study, *Herbaspirillum huttiense (H. huttiense)*, bacteremia, prompt identification, antibiotic sensitivity

INTRODUCTION

Herbaspirillum species are non-fermenting, strictly aerobic, Gram-negative curved or helical bacilli that do not have hemolytic rings. They are motile with polar flagella, and oxidase-, urease-, and catalase-positive. *Herbaspirillum* species were first reported by Baldani in 1996 (Baldani et al., 1996; Obradovic et al., 2007; Dobritsa et al., 2010) and widely distributed in the environment. As nitrogen-fixing bacteria, *Herbaspirillum* species inhabit the roots of plants in the rhizosphere and have been found in wells and other ground water (Tayeb et al., 2008;. Gulati et al., 2011; Souza et al., 2013). *Herbaspirillum huttiense (H. huttiense)* is a member of *Herbaspirillum* species and has the same properties. Pathologically, although a number of *Herbaspirillum* species have been identified and studied, only a few *H. huttiense* infections have been reported as human pathogens. For example, Regunath et al. (2015) reported the first case of severe community-acquired pneumonia and bacteremia caused by *H. huttiense* in an immunocompetent adult in the USA and Liu et al. (2019) reported the first case of septicemia caused by *H. huttiense* in Korea (Regunath et al., 2015; Liu et al., 2019). In the present study, we provided the first report of a bacteremia case caused by *H. huttiense* in China. We analyzed the *H. huttiense* clinical isolate by MALDI-TOF MS and 16S rRNA gene sequencing and summarized the prompt identification and results of antibiotic sensitivity.

MATERIALS AND METHODS

Isolation and Characterization of *H. huttiense*

Only one strain of *H. huttiense* was successfully isolated from positive aerobic blood cultures between January 2018 and January 2022 in the Department of Laboratory Medicine in the First Affiliated Hospital of Anhui Medical University. One drop of blood from positive blood cultures was inoculated onto Columbia blood plate medium and the bacteria were cultured aerobically at 37°C and 5% CO_2, followed by Gram staining and identification under microscopy.

MALDI-TOF MS Identification

MALDI-TOF MS identification was performed on a Vitek MS platform by the direct smear method in accordance with the instructions of the manufacturer. After acquiring the spectra, data were transferred to the analysis server which used software algorithms to compare the generated spectrum with the typical spectra in the scientific databases.

16S rRNA Sequencing and Phylogenetic Analysis

The original bacteria were purified and the genomic DNA was extracted. The forward and reverse primers used for PCR amplification of 16S rRNA gene were 27F(5'-AGAGTTTGATC ATGGCTCAG-3') and 1492R(5'-TACGGCTACCTTGTACG ACTT-3'). The reaction procedure was 96°C for 3 min, 96°C for 30 s, 58°C for 30 s, 72°C for 1 min, 35 cycles, and 72°C for 10 min. The sequencing was compared with the 16S rRNA gene sequencing of known bacteria in the Genbank database. The phylogenetic tree was established using the MEGA7.0 software.

Genome Sequencing and Data Assembly

The draft genome sequence of *H. huttiense* was analyzed by NGS. Genome sequencing was performed using the Illumina NovaSeq platform by generating paired-end libraries. Genomic DNA libraries for each isolate were prepared using the TruSeq DNA Sample Preparation Kit (Illumina). Adapter contamination was removed by AdapterRemoval v2 and the reads were filtered by SOAPec v2. The filtered reads were assembled into contigs and scaffolds using A5-miseq v20160825.

In Vitro Antibiotic Sensitivity Test

All antibiotic sensitivity was tested using the agar dilution method.

Literature Review

An electronic search was conducted in the PubMed/ScienceDirect/EBSCO databases using the key words "*Herbaspirillum huttiense*" to systematically search for almost all published literatures.

Case Description

A 72-year-old man was admitted to our hospital and was diagnosed with coronary atherosclerotic cardiopathy, mitral and tricuspid insufficiency and lacunar infarction. The patient was hospitalized for a total of 58 days, from June 15th to August 12th, 2020. During hospitalization, the patient developed a high fever with an axillary temperature reaching 39.2°C. On laboratory investigations, the patient had a procalcitonin (PCT) level of 86.73 ng/mL, a CRP level of 88.17mg/L, a WBC count of 8.89×10^9/L, and a neutrophil percentage of 84.50%. The blood was cultured using a BacT/Alert three dimensional automated blood culture system. Each blood culture consisted of a set of two (aerobic and anaerobic) bottles. Two sets of blood samples were collected from the patient. On July 3rd, two aerobic blood cultures were found positive after 20.8 h. The anaerobic blood cultures remained negative. Subsequent blood cultures were redone, and positive aerobic bacteria were still confirmed as *H. huttiense*. The patient was treated with meropenram and tigecycline for anti-infection. Moxifloxacin and piperacillin-tazobactam were changed when the condition of the patient improved. Finally the patient's body temperature returned to normal and discharged from hospital when cured.

RESULTS

The bacterial colonies appeared white, gelatinous, and slightly raised after 24 h in culture and had diameters between 1 and 1.5 mm without apparent hemolytic rings (**Figure 1**). Stainings showed the bacterial colonies were Gram-negative bacilli

FIGURE 1 | The phenotype of *H. huttiense* after 24 h aerobic culture.

(**Figure 2**). The MALDI-TOF MS results indicated 99.2% homology with *H. huttiense* (**Figure 3**). The 16S rRNA gene sequence was consistent with that of *H. huttiense*. Phylogenetic tree analysis showed that the isolate was present on the same branch as *H. huttiense* (**Figure 4**). The isolates were thus identified as *H. huttiense* for the 16S gene. Imipenem, meropenem, piperacillin-tazobactam and levofloxacin had good antibiotic activities and the MICs results were summarized in **Table 1**. The MIC was defined as the drug concentration that completely inhibited bacterial growth or caused a marked reduction (≥90%) compared with the drug-free control. The draft genome sequence of *H. huttiense* revealed chromosome size was 5.5Mb with a 62.74% G + C content. Automatic annotation revealed 5029 open reading frames (ORFs) covering 50 virulence associated genes and 69 antibiotic resistance associated genes. Virulence associated genes including flagellum-specific ATP synthase protein, flagellar biosynthesis protein, chemotaxis regulatory protein, and purine-binding chemotaxis protein were identified in the VFDB database. Antibiotic resistance associated genes including multidrug efflux system protein, multidrug ABC transporter protein, β-lactamase protein, and DNA topoisomerase protein were detected in the CARD database. The detailed genomic features are listed in **Table 2** (Gan et al., 2020; Yang et al., 2021).

After a comprehensive literature search, it was evident that *H. huttiense* was an obviously rare cause of human infections and

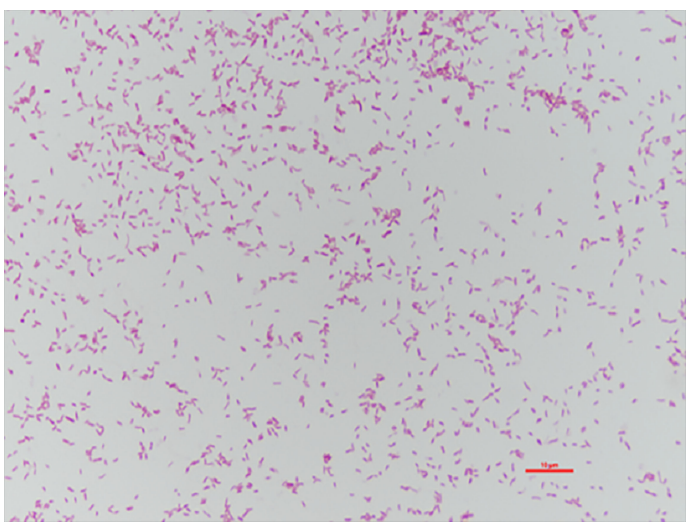

FIGURE 2 | Gram-staining of *H. huttiense* showed Gram-negative bacilli.

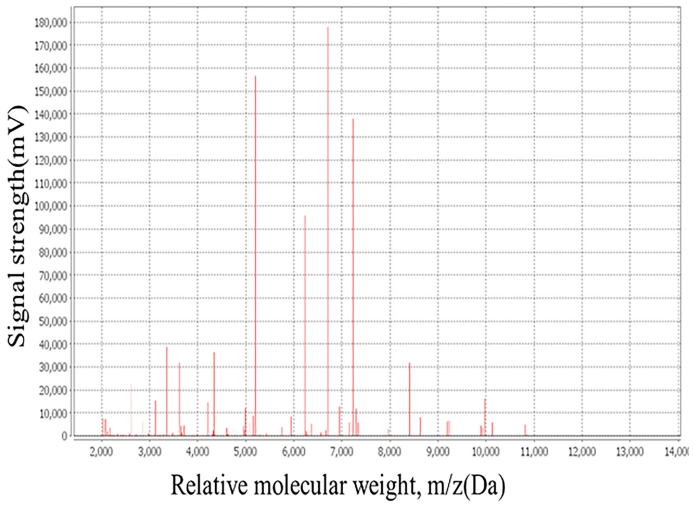

FIGURE 3 | The mass spectra of *H. huttiense* revealed high accuracy.

only 8 cases of *H. huttiense* detected in human samples had been reported. These cases were mainly observed in monomicrobial infections, such as infections of pneumonia (Regunath et al., 2015; Liu et al., 2019), acute myelocytic leukemia (Nurullah et al., 2021), breast cancer (Anonymity, 2021), thrombocytopenia (Berardino et al., 2019; Anonymity, 2020), intraventricular hemorrhage (Hernández et al., 2019), and infective endocarditis (Güngör et al., 2020). Another 7 reports described *H. huttiense* in its natural habitat. As *H. huttiense* was a nitrogen-fixing bacterium and was widely distributed in the environment, it had been investigated in the roots of rice and tea plants (Gulati et al., 2011; Andreozzi et al., 2019), greenhouse tomato seedlings, pineapple and banana crops (Obradovic et al., 2007; González et al., 2019), well water and shallow ice cores (Ding and Akira, 2004; Souza et al., 2013; Chen et al., 2016). The essential data from several of the *H. huttiense* studies were summarized in **Tables 3**, **4**.

DISCUSSION

A review of the literatures of *H. huttiense* infections in human showed that the ages of the patients ranged two months to 93 years old with a male-to-female ratio was 4:3. The gender ratio

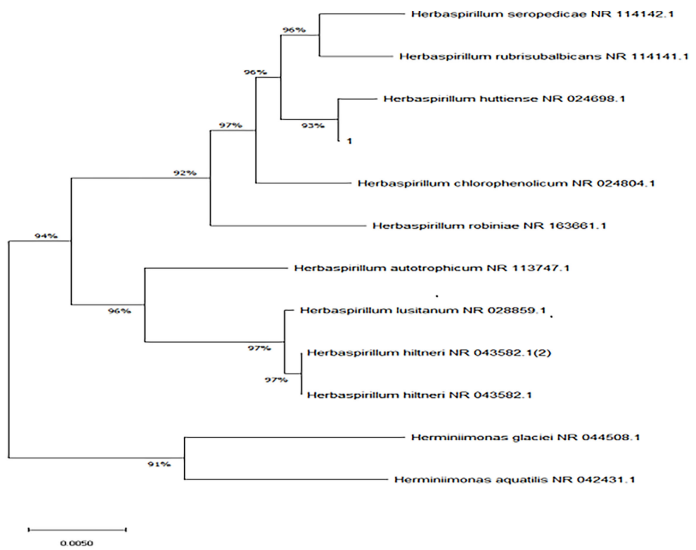

FIGURE 4 | Neighbor-joining phylogenetic tree of *H. huttiense* indicated high homology (1:isolated strain).

TABLE 1 | MIC results of *H. huttiense*.

Antibiotics	MIC (mg/L)
Piperacillin-tazobactam	0.05
Imipenem	0.25
Meropenem	0.25
Levofloxacin	0.5

was balanced. The first case was described in 2015, and another 7 sporadic cases were reported since 2015. Of the eight documented cases, three (3/8) were from Spain, and two each (2/8 and 2/8) respectively came from Turkey and the United States of America. Only one (1/8) originated from Korea. Based on the world prevalence of *H. huttiense*, majority of the reported cases came from Europe, followed by America and Asia. Nonetheless, no cases were reported from Africa, and it was probable that regional differences or underdiagnosis due to lack of technical resources led to this trend. In the present study, we successfully identified the pathogen to the species level using both Biotyper and Vitek MS systems. In terms of the reported cases, four (4/8) was identified on the Bruker Biotyper system and one infection (1/8) was diagnosed using Vitek MS system. Only two cases (2/8) were investigated using 16S rRNA gene sequence. *H. huttiense* infections were usually associated with risk factors, such as pneumonia, hematological system disease and cardiovascular disease. Our study showed that *H. huttiense* infection was associated with cardiovascular disease. In the reported studies, most of *H. huttiense* were isolated from blood, as in the present study.

The cut-off points for the interpretation of *H. huttiense* MICs were essentially in line with the CLSI recommendations for Gram-negative non-fermenters or non-enterobacteriaceae. Güngör et al. (2020) observed that *H. huttiense* was sensitive to teicoplanin, ceftazidime and meropenem. Hernández et al. (2019) reported that *H. huttiense* was sensitive to levofloxacin, ceftazidime, trimethoprim-sulfamethoxazole, minocycline and meropenem and resistant to amikacin and colistin (Hernández et al., 2019; Güngör et al., 2020). Currently, neither CLSI nor EUCAS provided a definite breakpoint for *H. huttiense* and its antibiotic sensitivity test was difficult to perform. In our research, piperacillin-tazobactam, imipenem, meropenem and levofloxacin were found to be effective against *H. huttiense* which was consistent with the results of Güngör ̊s and Hernández ̊s. Antibiotic susceptibility could serve as a means for differentiating *H. huttiense* from *Burkholderia cepacia complex* as the latter was usually multidrug-resistant, whereas the former was not (Berardino et al., 2019).Therefore, there was no definitive consensus reached on the precise antibiotic therapy for this infection and empirical therapy played an important role in the clinical treatment of *H. huttiense* infections. The publications showed that meropenem and piperacillin/tazobactom were the most commonly used clinical drugs and had good clinical effects. Our patient demonstrated a good clinical response due to the treatment with meropenram and tigecycline, followed by moxifloxacin and piperacillin/tazobactam. Of the 50 virulence associated genes detected in all genomes, several categories of flagellum-associated proteins were detected. Flagellar movement could enhance the invasion of bacteria to the host, because the movement was often chemically oriented and thus could avoid harmful environments or move toward the direction of high concentration environments. It was possible that the virulence of *H. huttiense* was flagella-related. Of the 69 antibiotic resistance genes identified, multidrug-resistance associated genes predominated, suggesting that antibiotic resistance of *H. huttiense* might be due to the possession of these multidrug-resistance associated genes.

Many environmental microorganisms have evolved into human pathogens, and *H. huttiense* is one of these. In the past, the isolation *H. huttiense* had proved esspecially challenging as it was easily be misidentified due to its phylogenetic and phenotypic resemblance to other strains. VITEK 2 and other biochemical identification systems had been unable to identify *H. huttiense.*. *H. huttiense* was frequently confused with organisms such as *B. cepacia complex*, *Cupriavidus pauculus, Ralstonia* spp., *or Ochrobactrum anthropic.* These limitations had retarded the investigation and knowledge of *H. huttiense*. However, the recent wide establishment of MALDI-TOF MS in clinical microbiology had resulted in the identification of bacteria and fungi with an accuracy of 90% or higher. MALDI-TOF MS was a spectroscopic method which required a reliable and complete database. The prompt (less than 1 h) identification and high discriminatory power of MALDI-TOF MS made it a useful tool for the characterization of rare bacteria that were previously difficult to identify using routine methods. In addition, the detection probability of rare bacteria was improved by the application of bioMérieux MS scientific research database. In our research, MALDI-TOF MS was used to identify an isolated strain of *H. huttiense* with a confidence of 99.2% in the scientific research database. This is the first report of the identification of *H. huttiense* in China by MALDI-TOF MS technology. The 16S rRNA gene sequencing and NGS were used

TABLE 2 | Genome characteristics of *H. huttiense*.

H. huttiense				
Genome Length	5.5Mb			
G+C content	62.74%			
Num of ORF	5029			
Virulence Factors of Pathogenic Bacteria (Num:50)	Flagellum-specific ATP Synthase Protein	Flagellar Biosynthesis Protein	Chemotaxis Regulatory Protein	Purine-binding Chemotaxis Protein
Antibiotics Resistance Genes (Num:69)	Multidrug Efflux System Protein	Multidrug ABC Transporter Protein	Beta-lactamase Protein	DNA Topoisomerase Protein

TABLE 3 | Cases of *H. huttiense* infections in humans reported in the literatures.

Report year	Age/gender	Country	Diagnosis	Identicification by MALDI-TOF	Molecular investigation	AntibioticTreatment	Type of Infection
2018	93y/M	Korea	Hypotension and Pneumonia.	Bruker Biotyper (Score:2.30)	16S rRNA gene sequence	Meropenem, Colistin, Ceftazidime, Minocycline, and Trimethoprim/ sulfamethoxazole	Polymicrobial
2015	46y/M	USA	Pneumonia	Bruker Biotyper	16S rRNA gene sequence	Ceftriaxone, Azithromycin, Doxycycline, Piperacillin-tazobactam	Monomicrobial
2021	54y/M	Turkey	Acute Myelocytic Leukemia	Bruker Biotyper	No	Meropenem	Monomicrobial
2018	59y/F	Spain	Thrombocythaemia and Pneumonia	MALDI-TOF MS (Brand Not Stated)	16S rRNA gene sequence	Piperacillin-tazobactam	Monomicrobial
2021	64y/F	Spain	Breast Cancer	No	No	Piperacillin/tazobactam, Ceftriaxone	Monomicrobial
2019	Two- month Old /M	Spain	Respiratory Distress and Intraventricular haemorrhage	Bruker Biotyper (more than 2.00)	No	Ceftriaxone, Cefotaxime, Meropenem	Monomicrobial
2020	Neonate [sex and exact age not stated]	USA	Thrombocytopenia	MALDI-TOF MS (Brand Not Stated)	No	Piperacillin/tazobactam	Monomicrobial
2020	11y/F	Turkey	Infective Endocarditis	Vitek MS	No	Teicoplanine, Piperacillin/tazobactom and Meropenem	Monomicrobial

TABLE 4 | Cases of *H. huttiense* infections in natural habitat reported in the literatures.

Report year	Country	Natural habitat
2007	USA	greenhouse tomato seedlings
2019	France	pineapple and banana crops
2019	Italy	rice root
2011	India	tea root
2004	Japan	well water
2013	Brazil	well water
2016	China	shallow ice cores

to verify the MALDI-TOF results and a phylogenetic tree was constructed to confirm the findings. The results of MALDI-TOF MS, 16S rRNA gene sequencing, and NGS were consistent, and achieved high accuracy.

CONCLUSIONS

In conclusion, *H. huttiense* was isolated from one clinical sample and identified by MALDI-TOF MS, 16S rRNA gene sequencing and NGS. MALDI-TOF MS and 16S rRNA gene sequencing represented prompt and accurate detection methods and were completed within 24 h. The isolates had good antibiotic activities to imipenem, meropenem, piperacillin-tazobactam and levofloxacin. This demonstration of the prompt identification of a rare pathogen and its antibiotic activities might increase awareness of these uncommon infections.

DATA AVAILABILITY STATEMENT

The datasets for this article are not publicly available due to concerns regarding participant/patient anonymity. Requests to access the datasets should be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Local Research Ethics committee of the First Affiliated Hospital of Anhui Medical University (Quick-PJ2022-02-14). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XL, XB, GQ, LW, CS, SC, YX, MZ, and ZW conceived and designed the study. YX, MZ, and ZW were responsible for data interpretation. XL and XB wrote the paper. YX, MZ, and ZW revised subsequent versions. GQ, LW, CS, and SC carried out the experimental works in the clinical microbiology laboratory. All authors have read and approved the final manuscript.

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Comparative Genomic Analysis of the Human Pathogen *Wohlfahrtiimonas Chitiniclastica* Provides Insight Into the Identification of Antimicrobial Resistance Genotypes and Potential Virulence Traits

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Recent studies suggest that *Wohlfahrtiimonas chitiniclastica* may be the cause of several diseases in humans including sepsis and bacteremia making the bacterium as a previously underappreciated human pathogen. However, very little is known about the pathogenicity and genetic potential of *W. chitiniclastica*; therefore, it is necessary to conduct systematic studies to gain a deeper understanding of its virulence characteristics and treatment options. In this study, the entire genetic repertoire of all publicly available *W. chitiniclastica* genomes was examined including *in silico* characterization of bacteriophage content, antibiotic resistome, and putative virulence profile. The pan-genome of *W. chitiniclastica* comprises 3819 genes with 1622 core genes (43%) indicating a putative metabolic conserved species. Furthermore, *in silico* analysis indicated presumed resistome expansion as defined by the presence of genome-encoded transposons and bacteriophages. While macrolide resistance genes *macA* and *macB* are located within the core genome, additional antimicrobial resistance genotypes for tetracycline (*tetH, tetB*, and *tetD*), aminoglycosides (*ant(2'')-Ia, aac(6')-Ia*, *aph(3'')-Ib*, *aph(3')-Ia*, and *aph(6)-Id*)), sulfonamide (*sul2*), streptomycin (*strA*), chloramphenicol (*cat3*), and beta-lactamase (*blaVEB*) are distributed among the accessory genome. Notably, our data indicate that the type strain DSM 18708^T does not encode any additional clinically relevant antibiotic resistance genes, whereas drug

resistance is increasing within the *W. chitiniclastica* clade. This trend should be monitored with caution. To the best of our knowledge, this is the first comprehensive genome analysis of this species, providing new insights into the genome of this opportunistic human pathogen.

Keywords: *W. chitiniclastica*, pan-genome, antimicrobial resistance, CRISPR, Acr *(anti-CRISPR)*, *rpoB*, arsenic resistance

INTRODUCTION

Wohlfahrtiimonas chitiniclastica was first isolated from the larvae of *Wohlfahrtia magnifica* (Tóth et al., 2008), an obligate parasitic fly that causes myiasis by depositing eggs and larvae in mammalian wounds in both animals and humans (Robbins and Khachemoune, 2010). *W. chitiniclastica* are Gram-negative, strictly aerobic, non-motile rods. A key feature is strong chitinase activity, which may be an indicator of a symbiotic relationship with its host fly while also playing an important role in metamorphosis (Tóth et al., 2008; Schröttner et al., 2017; Snyder et al., 2020). Apart from the close association between *W. chitiniclastica* and certain flies (Tóth et al., 2008; Cao et al., 2013; Maleki-Ravasan et al., 2020), the bacteria have recently been detected in various habitats around the world such as arsenic-affected soils from Bangladesh (Sanyal et al., 2016), chicken meat purchased in Brazilian supermarkets (Matos et al., 2019), the pancreas of a Zebra in China (Zhou et al., 2016), and human soft tissue infection in Estonia (Kõljalg et al., 2015), to name but a few. Finally, yet importantly, recent studies indicate that *W. chitiniclastica* can be the cause of several diseases in animals (Thaiwong et al., 2014; Diaz-Delgado et al., 2015; Qi et al., 2016) and humans, including sepsis and bacteremia (Almuzara et al., 2011; Kõljalg et al., 2015; Campisi et al., 2015; Suryalatha et al., 2015), making the bacterium a previously underestimated human pathogen (Schröttner et al., 2017). Although the pathogenesis of *W. chitiniclastica* has not been fully elucidated, the bacterium is thought to invade traumatic skin lesions via fly larvae, resulting in severe myiasis and/or wound contamination (Robbins and Khachemoune, 2010; Thaiwong et al., 2014; Schröttner et al., 2017). However, since most clinicians are unfamiliar with this species and conventional methods often lead to misidentification (Kõljalg et al., 2015; Kopf et al., 2021), it can be assumed that *W. chitiniclastica* has been poorly recognized as a possible cause and is even more common than originally thought (Kopf et al., 2021).

To date, NCBI lists 26 genomes of *W. chitiniclastica* strains, 22 of which have been isolated in the course of human disease. In addition, three draft genomes of strains isolated from an animal source have been published (Cao et al., 2013; Zhou et al., 2016; Matos et al., 2019), and annotations revealed genes encoding for macrolide-specific efflux pumps (*macA* and *macB*) (Matos et al., 2019), a $bla_{\text{VEB-1}}$ gene cassette, which confers resistance to ceftazidime, ampicillin, and tetracycline (Zhou et al., 2016), and a genome-encoded 25.9kb intact phage (Matos et al., 2019). However, apart from these preliminary genomic studies, very little is known about the genetic potential of *W. chitiniclastica*, making it necessary to initiate systematic studies in order to gain more insight into its virulence characteristics as well as treatment options. Therefore, in this study, the entire genetic repertoire of all publicly available *W. chitiniclastica* genomes was investigated; including *in silico* characterization of the antibiotic resistome, prophage content, and virulence potential. In addition, we performed a pan-genome analysis to elucidate the major genome features and genetic variability of *W. chitiniclastica*. To the best of our knowledge, this is the first comprehensive genome analysis of *W. chitiniclastica* and allows us to better understand this previously underestimated human pathogen.

MATERIALS AND METHODS

Genomic Strain Collection

All publicly available *W. chitiniclastica* genomes were included in this study (n = 26). These include a total of 14 *W. chitiniclastica* isolates from Dresden (Germany) that have been collected in routine diagnostics over a period of six years (Schröttner et al., 2017; Kopf et al., 2021). These isolates were recovered exclusively from diagnostic cultures analyzed at the Institute for Medical Microbiology and Virology, University Hospital Carl Gustav Carus (Dresden, Germany). Whole-genome sequences were submitted to NCBI GenBank under Acc. Nos JAGIBR000000000-JAGICE000000000, applying the NCBI Prokaryotic Annotation Pipeline PGAP (Tatusova et al., 2016) as previously reported (Kopf et al., 2021). In addition, the remaining publicly available *W. chitiniclastica* genomes at NCBI as of April 2021 (n = 12) were included in this study. The corresponding datasets were retrieved in preassembled nucleotide FASTA files and GenBank files. These include the type strain DSM 18708^{T} (AQXD01000000) (Tóth et al., 2008), BM-Y (LVXD00000000) (Zhou et al., 2016), Strain 20 (LWST01000000) (Matos et al., 2019), SH04 (AOBV01000000) (Cao et al., 2013), and 8 genomes, which were submitted by the Centers for Disease Control and Prevention of the United States but are not associated to a citable publication as far as we know. The latter comprise of ATCC 51249 (NEFL01000000), F6512 (NEFK01000000), F6513 (NEFJ01000000), F6514 (NEFI01000000), F6515 (NEFH01000000), F6516 (NEFC01000000), F9188 (NEFE01000000), and G9145 (NEFC01000000). Isolation source, host, year of isolation, and geographical origins of isolates were taken from published research papers, otherwise estimated using dates/locations on public databases as indicated. Results are displayed in **Table 1**; **Supplementary Table S1**.

TABLE 1 | Overview of the general genome features of the *W. chitiniclastica* genomes analyzed in this study.

Strain	Host	Isolation source	Location	Genome size (bp)	CDSs	rRNA	tRNA	CRISPR	Spacer	Phages	Acr/ Aca
DSM 100374	Homo sapiens	Wound swab	Dresden, Germany	2079313	1961	3	53	2	79	4	4
DSM 100375	Homo sapiens	Wound swab	Dresden, Germany	2103638	1932	3	53	1	8	1	7
DSM 100676	Homo sapiens	Wound swab	Dresden, Germany	2139975	1953	3	51	2	188	3	8
DSM 100917	Homo sapiens	Wound swab	Dresden, Germany	2144768	1955	3	49	2	188	4	8
DSM 105708	Homo sapiens	Wound swab	Dresden, Germany	2084087	1969	3	52	2	15	4	9
DSM 105712	Homo sapiens	Wound swab	Dresden, Germany	2133608	1960	3	49	3	67	3	2
DSM 105838	Homo sapiens	Wound swab	Dresden, Germany	2069521	1910	3	54	3	69	5	9
DSM 105839	Homo sapiens	Wound swab	Dresden, Germany	2123437	1966	3	54	2	41	3	8
DSM 105984	Homo sapiens	Wound swab	Dresden, Germany	2120278	1965	3	49	3	78	3	8
DSM 106597	Homo sapiens	Wound swab	Dresden, Germany	2131555	1966	3	50	3	78	3	8
DSM 108045	Homo sapiens	Wound swab	Dresden, Germany	2090370	1950	3	53	2	38	1	10
DSM 108048	Homo sapiens	Wound swab	Dresden, Germany	2074016	1952	3	54	4	130	2	8
DSM 110179	Homo sapiens	Wound swab	Dresden, Germany	2119644	1965	3	49	3	78	3	8
DSM 110473	Homo sapiens	Wound swab	Dresden, Germany	2126147	1970	3	54	2	53	3	7
DSM 18708^T	Wohlfahrtia magnitica	3rd stage larvae of fly	Mezöfalva, Hungary	1991020	1849	4	45	3	42	1	2
SH04	Chrysomya megacephala	-	Pudong, China	2181980	2132	12	56	3	106	7	10
BM-Y	Zebra	Pancreas	Shenzhen, China	2180519	2029	9	51	2	244	3	11
Strain 20	Chicken	Chicken carcass	Rio de Janeiro, Brazil	2123239	1958	3	48	3	59	1	2
ATCC 51249	Homo sapiens	Arm	New York, USA	2136105	1973	7	48	2	151	2	10
F6512	Homo sapiens	Foot	New York, USA	2120698	1968	7	52	2	40	1	6
F6513	Homo sapiens	Leg	New York, USA	2115422	1975	5	49	2	73	3	6
F6514	Homo sapiens	Oral lesion	New York, USA	2112239	1974	5	49	2	73	3	6
F6515	Homo sapiens	Ankle	New York, USA	2134718	2011	5	50	2	53	6	6
F6516	Homo sapiens	Arm	New York, USA	2071321	1892	7	48	2	39	2	9
F9188	Homo sapiens	Leg wound	Indiana, USA	2127263	1987	7	49	2	106	2	9
G9145	Homo sapiens	Wound	Colorado, USA	2182988	2017	5	51	2	107	2	10

In Silico Genome Analysis

Functional genome analysis was performed using the freely available computational tools with default parameters from January till June 2021, unless indicated otherwise. Preassembled FASTA files were annotated using Prokka (Galaxy version 1.14.6+galaxy0) (Seemann, 2014) and strarmar (Galaxy Version 0.7.2+galaxy0) (Petkau, 2018). Results are displayed in **Figure 3**; **Supplementary Tables S6, S7**. Search for antimicrobial resistance profiling was extended by using the comprehensive antibiotic resistance database CARD (https://card.mcmaster.ca/) (Alcock et al., 2020) retaining "Perfect hit and Strict hit only" and "High-quality/coverage". Results are displayed in **Figure 3**; **Supplementary Table S8**. Phage analysis was performed using PHASTER (PHAge Search Tool Enhanced Release) (https://phaster.ca/) (Arndt et al., 2016). Results are displayed in **Table 1**; **Supplementary Table S2**. Analysis of CRISPR (clustered regularly interspaced short palindromic repeats) and their associated (Cas) proteins was done using the CRISPRCasFinder (Couvin et al., 2018) (https://crisprcas.i2bc.paris-saclay.fr/). Only results with evidence levels 3 and 4 were included in the analysis. Results are displayed in **Table 1**; **Supplementary Table S1**. AcrFinder (http://bcb.unl.edu/AcrFinder/index.php) (Yi et al., 2020) was used for the detection of Anti-CRISPR (Acr) proteins. Results are displayed in **Table 1**; **Supplementary Table S3**.

Phylogenetic Identification

For phylogenomic identification; genomic contigs were submitted to the Type Strain Genome Server at https://tygs.dsmz.de/ (Meier-Kolthoff and Göker, 2019). Gene sequence of the 16S rRNA gene and *rpoB* gene were retrieved from the previous results using Prokka (Galaxy version 1.14.6+galaxy0), and BLAST analysis for the homology of the 16S rRNA gene and the *rpoB* gene for the identification of *W. chitiniclastica* was performed on https://blast.ncbi.nlm.nih.gov (Altschul et al., 1990).

Pan-Genome Assembly and Visualization

Preassembled GenBank files were converted to GFF3 using the 'Genbank to GFF3' converter (Galaxy Version 1.1) (Stajich et al., 2002). Annotated GFF3 files of 26 *W. chitiniclastica* genomes were submitted to Roary (Galaxy Version 3.13.0+galaxy1) (Page et al., 2015) for pan genome analysis using default parameters. A gene-absence-presence data matrix was derived and visualized with Phandango (Hadfield et al., 2018). Results are displayed in **Figure 1**; **Supplementary Tables S4, S5**.

RESULTS

Characterization of Central Genome Features

Over a period of six years, a total of 14 *W. chitiniclastica* strains were recovered from wound swabs in routine diagnostics (**Table 1**) (Kopf et al., 2021). The genome size of these *W. chitiniclastica* isolates, linked in terms of geographic location and isolation source, ranged from 2.07 to 2.14 million bases with an average of 1941 predicted genes (**Table 1**). To broaden the picture, we extended our study to include all *W. chitiniclastica* genomes, which were publicly available at NCBI as of April 2021. Thus, we arrived at 26 genomes comprising 22 isolates from human sources and 4 strains from animal sources (**Table 1**). Genome size ranged from 1.99 to 2.18 million bases with an average of 1967 predicted genes, 51 tRNAs, and five rRNAs. Notably, all isolates from Dresden and Strain 20 have three rRNAs, whereas the other genomes contain an average of seven rRNAs. Interestingly, BM-Y (n = 4) and Strain 20 (n = 2) contain multiple copies of the 16S rRNA gene (**Supplementary Table S1**).

The CRISPRCasFinder (Couvin et al., 2018) was used for the identification of potential genes encoding for CRISPR (clustered regularly interspaced short palindromic repeats) arrays and their associated (Cas) proteins. Because CRISPR arrays of evidence levels 1 and 2 are potentially invalid (Couvin et al., 2018), we focused on the results of evidence levels 3 and 4, which are considered as highly likely candidates. All strains contain CRISPR repeats, spacers, and the cas cluster CAS-Type IF (**Table 1**; **Supplementary Table S1**). On average, the isolates contain two CRISPR and 85 spacers. DSM 108048 stands out with four CRISPR and 130 spacers, while DSM 100375 contains only one potential CRISPR sequence and eight spacers. The highest number of spacers was detected in Strain 20 isolated from an animal source. Interestingly, all *W. chitiniclastica* isolates also contain genomically encoded anti-CRISPR (*acr*) genes (**Table 1**). DSM 108048, SH04, ATCC 51249 and G9145 stand out with 10 genomically encoded Acr proteins, whereas the type strain DSM 18708, Strain 20, and DSM 105712 contain only two sequences. Although some *acr* genes were labeled 'low confidence' (**Supplementary Table S3**), the majority are homologs of known Acr proteins, making the actual presence of potential anti-CRISPR proteins highly likely.

Phylogenetic Identification

Genome-based-taxonomy analysis of all strains revealed correct assignment to *W. chitiniclastica.* Hereby, digital DNA:DNA hybridization (dDDH) values of 74.0-75.2% were computed against the type strain DSM 18708^T and therefore fulfilling the criteria for correct bacterial species identification (Meier-Kolthoff and Göker, 2019) (**Supplementary Table S1**). Construction of a phylogenomic tree based on whole-genome sequences revealed that all strains cluster in one subclade with the type strain DSM 18708^T, and 25 strains form a subspecies using a 79% dDDH threshold (Meier-Kolthoff et al., 2014) (**Figure 2**). Surprisingly, the type strain does not belong to the subspecies. Furthermore, 16S rRNA gene and *rpoB* gene sequences were compared for sequence similarity to other sequences using the BLAST database. All isolates were identified with a minimum of 98% out of 100% sequence identity to corresponding 16S rRNA and *rpoB* reference genes, respectively, leading to the correct assignment of *W. chitiniclastica* (**Supplementary Table S1**). Notably, the additional 16S rRNA gene copies of BM-Y (n = 4) and Strain 20 (n = 2) revealed correct taxonomic assignment and share high homology, ranging from 99.7%-99.9%.

Pan-Genome Construction

We constructed the pan-genome of *W. chitiniclastica* encompassing 26 genomes, which represents the first and largest analysis of this type to date. Roary (Page et al., 2015) was used to cluster the genes encoding complete protein sequences into core (hard core and soft core) and accessory (shell and cloud) genomes. The core genome is shared by every genome tested in this study and its genes are often related to housekeeping functions. It can further be divided into hard core genes, which are defined to be present in >99% of the genomes, and soft core genes, which are present in 95–99%. The accessory genome is shared by a subset of the genomes and is associated with, but not limited to, pathogenicity or environmental adaptation. It is subdivided into shell genes, which are present in 15–95%, and cloud genes, which are found in less than 15% of genomes. The latter include singletons or genes found in only one of the genomes.

The pan-genome of all 26 *W. chitiniclastica* isolates comprises 3819 genes; 1622 core genes (43%), and 2197 accessory genes (57%) (**Figure 1**) with 1117 unique genes (29%) defined as genes only present in one strain. We also observed a remarkable abundance of 1240 (32%) genes with an unknown function. The core genome divides into 1175 hard (31%) and 447 soft core (12%) genes. Notably, 92% of the core genes (1494/1622) encode a known function, while only 8% (128/1622) are assigned to hypothetical proteins. The accessory genome comprises 635 shell (17%) and 1562 cloud (41%) genes. Remarkably, 51% (1122/2197) of the accessory genes code for hypothetical proteins, a fact that highlights the limited characterization of the *W. chitiniclastica* genome.

The majority of core genes encodes for protein families associated with housekeeping functions such as amino acid metabolism, energy production and translation, to name but a few (**Supplementary Table S4**). Genes associated with defense mechanisms such as antimicrobial resistance (AMR) genes are mainly present in the accessory genome (**Supplementary Table S5**). Notably, major multidrug efflux systems, on the other hand, are encoded within the core genome (**Supplementary Table S4**). These include the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily, resistance nodulation-division (RND) family, major facilitator superfamily (MFS), small multidrug resistance (SMR) family, multidrug and toxic compound extrusion (MATE) family, proteobacterial antimicrobial compound efflux (PACE) family, and *p*-aminobenzoyl-glutamate transporter (AbgT) family. In

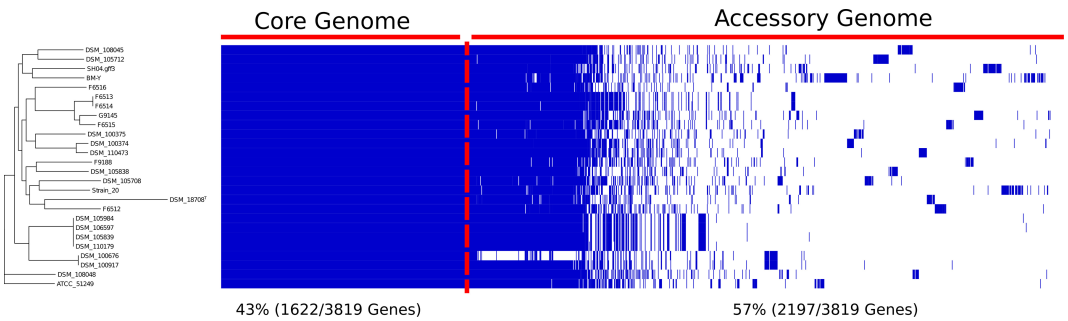

FIGURE 1 | Linearized visualization of W. chitiniclastica pan-genome along with the phylogenetic tree. The pangenome was visualized based on the software Phandango (Hadfield et al., 2018).

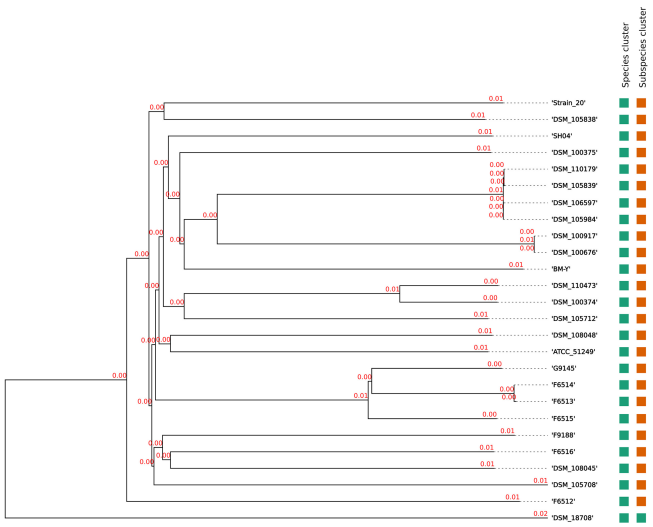

FIGURE 2 | Phylogenomic tree of the *W. chitiniclastica* species and subspecies delineation based on the GBDP phylogenetic analyses retrieved and modified based on the Type (Strain) Genome Server (TYGS). The tree was inferred with FastME 2.1.6.1 (Lefort et al., 2015) from GBDP distances calculated from genome sequences and was subjected to a clustering using established thresholds for delineating species (DDH > 70%) (Meier-Kolthoff et al., 2013) as well as subspecies (DDH > 79%) (Meier-Kolthoff et al., 2014). The branch lengths are scaled in terms of GBDP distance formula d5 and are represented in red numbers. The numbers above branches are GBDP pseudo-bootstrap support values >60% from 100 replications, with an average branch support of 83.0%.

addition, we detected several protein families encoding TRAP transporters and TonB dependent transport systems.

The generated phylogenetic tree of the pan-genome shows three main clades (**Figure 1**). DSM 108048 and ATCC 51249 are clustered in a single lineage (clade 1), and clade 2 is composed of six isolates from Dresden. The remaining isolates are grouped in clade 3, suggesting potential spread and transmission between hosts without any clear geographical links or host association.

Prediction of Arsenic Resistance

Pan-genome analysis revealed two protein families encoding for arsenic resistance proteins within the core genome (**Supplementary Table S4**). Interestingly, genes belonging to the ubiquitous *ars* operon (*arsRDABC*) such as *arsC, arsD, arsA*, and *arsB* (Carlin et al., 1995) were only detected in DSM 100375, DSM 105712, DSM 105838, F6512, F6513, and F6514 (**Figure 3**). In addition, these six isolates contain the gene for the inorganic arsenic efflux pump *acr3* (Fekih et al., 2018).

Prediction of Bacteriophages and Other Mobile Genetic Elements

Prediction of prophage sequences within the *W. chitiniclastica* genomes revealed a total of 75 prophages (**Table 1**), 18 of which are classified as intact (**Supplementary Table S2**). The latter are most likely to be complete and functional, and were found in 16 different genomes all isolated from a human source. The most common intact phage was identified either as Mannheimia (n = 12) or Enterobacteria phage (n = 4) (**Supplementary Table S2**). The remaining prophages were classified as "questionable" (n = 4) and "incomplete" (n = 53). Notably, none of the strains isolated from an animal source contained intact phages.

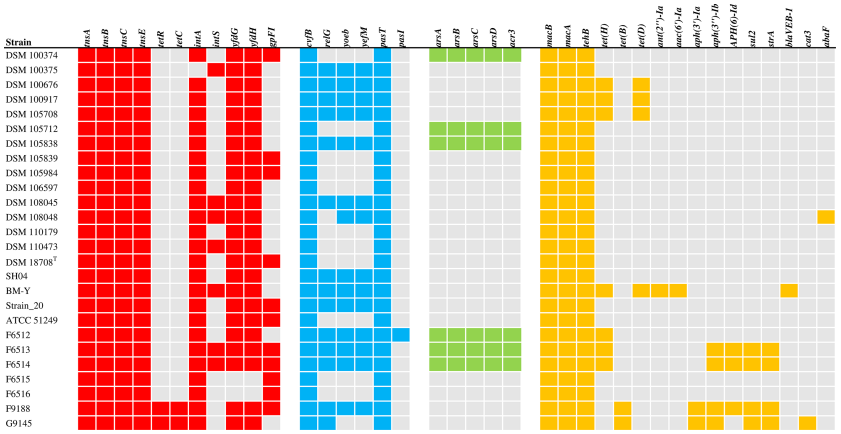

FIGURE 3 | Heat-map visualizing genes derived from mobile genetic elements (red), and genes associated with putative virulence traits (blue), arsenic resistance (green) and antimicrobial resistance (yellow). Cell colors indicate the presence of genes: grey (absence); red, blue, green and yellow (presence).

We then extended our search for the presence of other mobile genetic elements as it can improve the fitness and enables bacteria to acquire new AMR genes (Kottara et al., 2018; Stalder et al., 2019). Each genome contained genes homologous to Tn*7* transposon genes such as *tnsA, tnsB, tnsC*, and *tnsE* (Peters, 2014; Babakhani and Oloomi, 2018) (**Figure 3**). This is consistent with pan-genome analysis, which revealed a ubiquitous presence of a transposase encoding protein family within the core genome (**Supplementary Table S4**). Interestingly, the accessory genome showed numerous additional transposases as well as integrases and phage proteins (**Supplementary Table S5**). Detailed genome analysis based on Prokka confirmed these results (**Supplementary Table S6**). For example, F9188 and G9145 harbor Tn*10* encoded tetracycline resistance genes *tetR* (Smith and Bertrand, 1988) and *tetC* (Babakhani and Oloomi, 2018) (**Figure 3**). Prophage integrase *intA* gene, which is necessary for the integration of the phage into the host genome by site-specific recombination (Kirby et al., 1994), was present in all genomes except DSM 100375. Integrase gene *intS* (Panis et al., 2010), on the other hand, was only detected in seven genomes including DSM 100375. Notably, BM-Y contains five *intS* copies (**Supplementary Table S6**). Additional genome-integrated phage genes such as *yfdh, yfdg* (Rudd, 1999) and *gpFI* (Popovic et al., 2012) were distributed among the *W. chitiniclastica* genomes (**Figure 3**).

In Silico Annotation of Potential Virulence Traits

We analyzed all genomes including the type strain DSM 18708^T with respect to potential virulence traits (**Supplementary Table S6**). To begin with, all isolates harbor the conserved virulence factor B (*cvfB*) (Matsumoto et al., 2007) and genes encoding for active multidrug efflux systems. The latter are one of the major mechanisms of bacterial resistance to drugs. For example, we detected the multidrug resistance transporter gene *mdtA* (Nagakubo et al., 2002), the MATE transporter genes *norM* (Nishino et al., 2006) and *mepA* (Kaatz et al., 2006), the TonB dependent transport genes *tdhA* (Thomas et al., 1998), *exbD*, and *exbB* (Bosch et al., 2002; Holden et al., 2012), and multidrug efflux pumps genes *acrB* and *arcA* (Eicher et al., 2009), to name but a few. Secretion systems are another key element in the pathogenesis of bacterial infections, and all genomes harbor copies of the type II secretion (TS2) genes *xcpT* and *epsF* (Sandkvist, 2001).

Other meaningful elements involved in pathogenicity are toxins. Although *W. chitiniclastica* has not been reported to be a prolific toxin-producing organism, all genomes were manually searched for potential candidates. Based on our *in silico* results, 14 isolates contain the toxin *relG* gene (Korch et al., 2015). Notably, F9188, G9145 and SH04 harbor two copies of *relG*.

Toxin-antitoxin (TA) modules are ubiquitous among bacteria, and are involved in diverse physiological processes including virulence (Lobato-Márquez et al., 2016). Interestingly, the core genome harbors a protein family coding for the type II toxin-antitoxin system RatA (**Supplementary Table S4**), which is described as antisense RNA that blocks the accumulation of the mRNA for the TxpA toxin (Silvaggi et al., 2005). However, the corresponding *txpA* gene appears to be missing. Complete TA modules, on the contrary, can be found in the accessory genome (**Supplementary Table S5**). These include the RelE/ParE family, which encompasses several smaller toxin families including YoeB (Anantharaman and Aravind, 2003). In-depth genome analysis using Prokka (Seemann, 2014) confirmed these results by revealing genes encoding for the TA type II system YefM-YoeB (Norton and Mulvey, 2012) in 14 *W. chitiniclastica* strains (**Figure 3**). Notably, SH04 harbors two copies of *yefM* and *yoeB*. The PasTI is another known TA module (Norton and Mulvey, 2012), and F6512 contains both genes, *pasT* and *pasI*, respectively. The remaining 25 isolates only harbor the *pasT* gene.

In Silico Analysis of Antimicrobial Resistance Genes

In order to perform an thorough and comprehensive search for AMR genes, we extended our previous analysis using the comprehensive antibiotic resistance database CARD (Alcock et al., 2020) retaining 'Perfect' and 'Strict' hits only while excluding 'Loose' hits (**Supplementary Table S8**). Noteworthy, the 'Strict' algorithm represents a flexible sequence variation but lies within the curated BLAST bit score cut-off (Alcock et al., 2020), and by that, making a correct identification highly feasible. The 'Loose' algorithm, on the other hand, works outside of the detection model cut-off to provide detection of new and more distant homologs of AMR genes. Although it could help to identify potential resistance genes and/or shed light on new unknown modifications, *in silico* results based on loose hits should always be taken with caution and require further research. In order to gain high-quality results we decided to restrict the CARD search to 'Perfect' and 'Strict' hits, and combine the outcome with results gained by Prokka (Seemann, 2014) and strarmar (Petkau, 2018). Summarized results are displayed in **Figure 3**, and detailed information can be retrieved from **Supplementary Tables S6, S7**.

Genes coding for macrolide-specific efflux pumps (*macA* and *macB*) (Kobayashi et al., 2000; Yum et al., 2009), and tellurite resistance methyltransferase (*tehB*) (Choudhury et al., 2011) were detected in all 26 genomes. Tetracycline resistant efflux protein *tetH* gene (Roberts, 2005) was found in 7 genomes; notably, BM-Y harbors double *tetH* genes. In addition, DSM 100676, DSM 100917, DSM 105708, and BM-Y contained the tetracycline repressor protein *tetD* gene (Roberts, 2005). G9145 and F9188, on the other hand, harbor the tetracycline efflux protein *tetB* gene, which confers resistance to tetracycline, doxycycline, and minocycline, but not tigecycline (Roberts, 2005). BM-Y contains two aminoglycoside resistance genes encoding for the adenylyltransferase *ant(2")-Ia* (Cox et al., 2015) and acetyltransferase *aac(6')-Ia* (Parent and Roy, 1992). The remaining aminoglycoside resistance genes such as *aph(3")-Ib*, *aph(3')-Ia* (Tauch et al., 2000), and *aph-ld* (Cao et al., 2013)-*Id* (Chiou and Jones, 1995) were detected in F6513, F6515, G9188, and F9188, respectively. Sulfonamide resistance gene *sul2* and streptomyicn resistance gene *strA*, which have been described to be encoded within the same resistance gene cassette (Anantham and Hall, 2012), were detected in F6513, F6514, G9145, and F9188. In addition, BM-Y harbored a gene encoding for the VEB-1 beta-lactamase (Nordmann and Naas, 1994), and G9145 contains the chloramphenicol acetyltransferase *cat3* gene (Vassort-Bruneau et al., 1996). Finally, yet importantly, DSM 108045 contains the antibiotic efflux pump encoding *abaF* gene, which has been reported, when expressed, to increase fosfomycin resistance (Sharma et al., 2017).

DISCUSSION

In the past 12 years, several case reports have shown that *W. chitiniclastica* is capable of causing sepsis and bacteremia in humans labeling this organism as a newly underestimated pathogen. However, little is known about its pathogenicity and genome content. Our current study, analyzing all publicly available *W. chitiniclastica* genomes to date, highlights significant genomic characteristics including potential virulence factors, and AMR genes. Moreover, we provide the first pan genome analysis and shed light on the core features of *W. chitiniclastica*.

Distinct Genomic Characteristics of *W. Chitiniclastica* and Their Effect on the Assessment of Microbial Diversity

Genome size ranged from 1.99 to 2.18 million bases with an average of 1967 predicted genes, 51 tRNAs, and 5 rRNAs. Interestingly, all isolates from Dresden and Strain 20 harbor 3 rRNAs, while the remaining genomes contained an average of 7 rRNAs. It has recently been proposed that multiple rRNA operons confer a selective advantage to respond quickly and grow rapidly in environments characterized by fluctuations in resource availability (Stevenson and Schmidt, 2004). Based on this hypothesis, with 12 and 9 rRNAs, respectively, SH04 and BM-Y should have a fitness advantage when compared to the other isolates. In addition, BM-Y and Strain 20 harbor multiple 16S rRNA gene copies; a fact that has not been reported for any *W. chitiniclastica* strain yet. Although most bacterial genomes exhibit only one or two 16S rRNA genes (Armougom, 2009), some microbes contain multiple or varying numbers (Rainey et al., 1996; Acinas et al., 2004). For example, *Bacillus subtilis* has 10 copies (Stewart et al., 1982) and *Clostridium paradoxum* has up to 15 copies with heterogeneous intervening sequences (Rainey et al., 1996). Those multiple copies are often associated with nucleotide sequence variability (Rainey et al., 1996; Acinas et al., 2004), and/or provide insufficient taxonomic resolution power at the species level or with closely related species (Janda and Abbott, 2007). Our study indicates that the phylogenetic identification of those additional 16S rRNA gene copies of BM-Y and Strain 20 provides correct classification; however it can lead to an overestimation in terms of abundance and diversity composition (Armougom, 2009), a fact that one has to keep in mind within the scope of a thorough microbial community profiling. 16S rRNA-based identification generally faces a number of challenges, including sequencing error (Schirmer et al., 2015), primer bias (Klindworth et al., 2012), and varying discrimination power between variable regions (Sune et al., 2020) and certain bacterial genera (Roux et al., 2011). In light of these drawbacks, an alternative, or at least complementary taxonomic markers, should be considered. Our study indicates that both dDDH and *rpoB* based analysis have proven a worthy identification method for *W. chitiniclastica*. While dDDH is a very costly and time-consuming technique, the *rpoB* gene has emerged as a new marker gene candidate for phylogenetic analyses and identification of bacteria (Adékambi et al., 2009). Although the *rpoB* gene does not have a database currently as comprehensive as that of the 16S rRNA gene (Bondoso et al., 2013), the approach has a number of advantages. For example, *rpoB* excels with an increased phylogenetic resolution on the

genus level or lower, and it is a single-copy protein-encoding gene enabling a phylogenetic analysis on amino acid and nucleotide level (Case et al., 2007; Adékambi et al., 2009). However, since neither the dDDH nor the *rpoB* approach is widely established in clinical routine diagnostics yet, 16S rRNA-based identification most likely remains by far the most frequently used method. Nevertheless, in case of doubtful results, additional methods should be considered as an alternative or complement.

Mobile Genetic Elements and Their Putative Effect in Shaping the Genetic Diversity of *W. Chitiniclastica*

Genome scanning for phages and MGE revealed the ubiquitous presence of transposons and bacteriophages within all *W. chitiniclastica* isolates tested in this study, and we believe that they are some of the key elements in shaping the genetic diversity within the *W. chitiniclastica* clade. Interestingly, all genomes harbor CRISPR-Cas elements, which are described to constitute the adaptive immune system in prokaryotes in order to provide resistance against invasive genetic elements including viruses, plasmids, and transposons (Barrangou et al., 2007). Therefore, in theory, *W. chitiniclastica* should be well equipped against its invasion. However, the ubiquitous distribution of MGEs among prokaryotes suggests that CRISPR systems are not always functional (Grissa et al., 2007), participate in other processes, such as signal transduction and gene regulation (Westra et al., 2014; Faure et al., 2019), and/or have a yet undiscovered function (Aydin et al., 2017). Moreover, many phages have evolved an anti-CRISPR system that inhibits the CRISPR immune response of their host (Landsberger et al., 2018). Although Acr proteins were first discovered in *Pseudomonas* phages and other prophages (Bondy-Denomy et al., 2013), they have also been detected in other prokaryotes such as *Moraxella bovoculi* (Marino et al., 2018). In fact, >30% of *P. aeruginosa* strains carrying a CRISPR-Cas system also encode one or more cognate *acr* genes (van Belkum et al., 2015); therefore, the ubiquitous presence of genome-encoded Acr proteins among *W. chitiniclastica* may partly explain the widespread distribution of MGE and phage-related genes. Although the conditions and extent to which these immunosuppressive genes allow bacteriophages to persist in their bacterial host remain unclear (Landsberger et al., 2018), the balance between CRISPR-Cas immunity and Acr activities may be a central element in shaping the genetic diversity of *W. chitiniclastica* including antimicrobial resistome expansion.

Pan-Genome Composition of *W. Chitiniclastica*

To begin with, the pan-genomic phylogenetic tree indicated no clear host or geographical clustering suggesting a potential spread and transmission. Although six strains isolated from Dresden clustered within a subclade, the analysis might be limited in terms of capturing total diversity due to the fact that the majority are from the same location, and only four are associated with an animal source. With hopefully increasing numbers of available genomes from various locations, we recommend repeating the phylogenetic pan-genome analysis to see whether a certain niche specificity emerges.

The composition of the pan-genome revealed a core genome of 43%, which appears to be conserved when compared to other reported species such as *Clostridium perfringens* (12.6%) (Kiu et al., 2017), *Aliarcobacter butzleri (22%)* (Buzzanca et al., 2021), *Staphylococcus aureus* (32%), *Pseudomonas aeruginosa (26%)* (Costa et al., 2020), *Klebsiella pneumoniae* (26%) and *Salmonella enterica* (16%) (McInerney et al., 2017), to name but a few. Microbes with large and diverse accessory genomes, on the other hand, are considered metabolically versatile species with the ability to migrate to new niches, and to adapt to changing environmental conditions (Rouli et al., 2015; Costa et al., 2020). Accessory genes are often acquired by Horizontal gene transfer (HGT) (Costa et al., 2020), and are related to virulence, antimicrobial defense or confer a fitness advantage (Jordan et al., 2001). Therefore, species with a large and conserved core genome often lack a diverse pool of virulence and AMR factors. This is congruent with recent studies regarding *Bordetella pertussis*. Members of this species have a large core genome (59%) suggesting that due to this low genomic diversity, antibiotics and vaccines are quite effective against this species (Tettelin et al., 2008; Carbonetti, 2016; Costa et al., 2020). This is consistent with recent observations regarding *W. chitiniclastica* isolates, which are described to be susceptible to the majority of known antibiotics with the exception of fosfomycin (Schröttner et al., 2017; Matos et al., 2019), and by that, underlines the assumption that members of this species appear to be metabolically conserved when compared to others. However, as mentioned above, with increasing numbers of available genomes a large-scale pan-genome analysis is recommended to confirm and/or reevaluate the findings.

Further Evidence of a Previously Newly Described Subspecies of *W. Chitiniclastica*

Originally isolated from *Wohlfahrtia magnifica* larvae in Hungary (Tóth et al., 2008), there is increasing evidence that *W. chitiniclastica* inhabits diverse niches and environmental habitats such as soil (Sanyal et al., 2016), humans (Schröttner et al., 2017), other mammals (Thaiwong et al., 2014; Diaz-Delgado et al., 2015), fish (Reddy and Mastan, 2013), and diverse flies (Lysaght et al., 2018; Maleki-Ravasan et al., 2020). In most of these published studies, identification was based on 16S rRNA gene sequence, which is known to lack sufficient resolution to distinguish between closely related species (Roux et al., 2011). The dDDH analysis performed during this work surprised with further evidence of a previously newly described subspecies of *W. chitiniclastica* (Kopf et al., 2021) (**Figure 2**). Originally thought to be the adaptation to a human environment and geographic location (Kopf et al., 2021), this work rather suggest a broad host and environmental range of *W. chitiniclastica*. This observation is also reflected in the pangenomic phylogenetic tree (**Figure 1**). The fact that *W. chitiniclastica* can colonize different species should be

considered an advantage for the bacterium. However, on the other hand, it also poses an increased risk for zoonotic transmission, whose dynamic interactions between humans, animals, and pathogens should be considered in the context of the "One Health" approach (Welch et al., 2007; Cantas and Suer, 2014).

Prediction of Arsenic Resistance

W. chitiniclastica has recently been detected in arsenic-affected soils from Bangladesh (Sanyal et al., 2016) indicating its ability to inhabit different habitats including soil, humans, and animals (Tóth et al., 2008; Thaiwong et al., 2014; Diaz-Delgado et al., 2015; Sanyal et al., 2016; Schröttner et al., 2017; Matos et al., 2019). Arsenic occurs naturally in aquatic and terrestrial environments, and despite its relatively low abundance, the high toxicity of arsenic derivatives is considered a severe problem of public health worldwide (Fekih et al., 2018). In this process, microorganisms are known to play a crucial role in global arsenic geocycles (Zhu et al., 2014) subsequently leading to the ubiquitous presence of arsenic resistance genes among microbes (Fekih et al., 2018). With the presence of arsenic-resistant protein families within the core genome, *W. chitiniclastica* is no exception. However, genes of the common *arsRDABC* operon were only detected in six genomes. Notably, the regulator protein-encoding *arsR* gene, which acts as a repressor of the *arsRDABC* operon in the absence of arsenic (Cai et al., 2009), appears to be missing. These findings indicate the development of a yet unknown regulation and/or arsenic tolerance mechanism distributed among *W. chitiniclastica*. Moreover, these 6 isolates harbor the arsenic efflux pump *acr3* gene (Fekih et al., 2018), which is surprising as most prokaryotic species are described to have either an *arsB* or *acr3* gene (Yang et al., 2012). Notably, arsenic-resistant bacterial isolates from highly arsenic-contaminated soils showed a predominance of *acr3* genes over *arsB* genes (Cai et al., 2009) suggesting that DSM 100375, DSM 105712, DSM 105838, F6512, F6513, and F6514 have a severe fitness advantage in highly enriched arsenic habitats.

In Silico Profiling of Potential Virulence Traits

To detect possible genomic signatures linked to virulence, all genomes were manually searched for genes putatively associated with host-pathogen interaction. The ubiquitous presence of diverse multidrug efflux systems emphasizes a central role in the pathogenesis of *W. chitiniclastica*. For example, the core genome harbors a PACE efflux transporter, which is described to confer resistance to a wide range of biocides used as disinfectants and antiseptics, and are encoded by many Gram-negative human pathogens (Hassan et al., 2018). Other potential virulence components are represented by membrane-associated proteins, like TonB dependent transport systems, which are involved the in virulence of *Shigella dysenteriae, Haemophilus influenzae* and *E. coli*, to name but a few (Reeves et al., 2000; Torres et al., 2001; Morton et al., 2012). Moreover, the ubiquitous presence of the conserved virulence factor B (*cvfB*) suggests a central role in the virulence of *W. chitiniclastica*. Recent studies showed that deletion of CvfB results in reduced virulence in *S. aureus* and decreased production of hemolysin, DNase, and protease (Matsumoto et al., 2007), which further emphasized its importance for pathogenicity. However, in-depth research including target specific manipulations is required to unravel its function in *W. chitiniclastica.*

TA modules are involved in diverse physiological processes providing bacteria with pronounced fitness advantages dependent on toxin expression levels and the specific environmental niche occupied (Ma et al., 2021). These include bacterial adaptation to hostile environments, mediating stress response, stabilization of chromosomal regions, and bacterial survival during infection (Lobato-Márquez et al., 2016). Our *in silico* analysis indicates the presence of the TA system YefM-YoeB (Norton and Mulvey, 2012) in 14 *W. chitiniclastica* strains. This TA module has been described to be involved in the niche-specific colonization, stress resistance, and survival inside the host (Norton and Mulvey, 2012). Although the natural habitat of *W. chitiniclastica* is not well investigated, we could envision that TA systems might be involved in invading different habitats including persistence as part of a polymicrobial infection. Moreover, F6512 harbors a second TA system comprised of the toxin PasT and the antitoxin PasI. The PasTI module assures cell formation in the presence of antibiotics and increases pathogen resistance to nutrient limitation as well as oxidative and nitrosative stresses (Norton and Mulvey, 2012). Notably, the remaining 25 isolates only harbor the *pasT* gene, whose function has recently been reannotated based on new experimental evidence. While it was shown that PasT sustains antibiotic tolerance, and is critical for the formation or survival of ciprofloxacin-tolerant cells, the function of PasTI as a TA system could not be confirmed (Fino et al., 2020). Instead, the supposed toxin PasT is a bacterial homolog of mitochondrial protein Coq10 suggesting a central role in respiratory electron transport by acting as an important accessory factor in the ubiquinone-dependent electron transport chain (Fino et al., 2020). This leaves us to speculate, whether the *pasT* gene of *W. chitiniclastica* is primarily involved in virulence and/or energy production.

The secretion of proteins and toxins have a major role in the pathogenesis of bacterial infections, and several highly specialized pathways have evolved for this purpose such as the T2S system. The latter has been widely discovered in a number of bacterial species including several human pathogens like *Chlamydia trachomatis, Escherichia coli, Klebsiella pneumoniae, Legionella pneumophila, Vibrio cholerae*, and *P. aerunginosa*, to name but a few (Sandkvist, 2001; Durand et al., 2003; Cianciotto and White, 2017). In general, proteins secreted by T2S systems are associated with the destruction of various tissues, cell damage and diseases such as proteases, cellulases, pectinases, phospholipases, lipases, and toxins (Sandkvist, 2001). Based on our *in silico* analysis, *W. chitiniclastica* harbors, for example, a copy of the *xcpT* gene, which encodes for pseudopilin XcpT of the T2S machinery of *P. aeruginosa* (Sandkvist, 2001; Durand et al., 2003). Notably, assembly of the type II pseudopilus also

confers increased bacterial adhesive capabilities (Durand et al., 2003), which raises the question, whether its function in *W. chitiniclastica* is primarily involved in adhesion and/or endotoxin secretion. Unfortunately, the analysis regarding the toxin profile of *W. chitiniclastica* provided limited information. Although some isolates harbor the toxin-encoding gene *relG*, which is described to inhibit mycobacterial growth when expressed independently (Korch et al., 2015), other exotoxin encoding genes appear to be missing or are yet unknown. Based on this observation, *W. chitiniclastica* seems to contain a limited toxin profile when compared to other prolific toxin-producing organisms like *C. perfringens* (Kiu et al., 2017), making the participation of XcpT in bacterial cell adhesion feasible. However, T2S modules are not restricted to exotoxin secretion; in fact, they can export a wide range of substances. For example, in *V. cholerae* the T2S system supports the secretion of cholera toxin, hemagglutinin-protease, and chitinase (Connell et al., 1998; Davis et al., 2000; Sandkvist, 2001). Notably, *W. chitiniclastica* is known to have strong chitinase activity (Tóth et al., 2008) suggesting that T2S systems might be involved in its secretion.

In Silico Analysis of Antimicrobial Resistance Genes

Previous studies have reported *W. chitiniclastica* to be susceptible to the majority of known antibiotics with the exception of fosfomycin (Schröttner et al., 2017; Matos et al., 2019; Kopf et al., 2021). This is in line with our *in silico* analysis, which showed that the majority lacks essential AMR genes suggesting a broad susceptibility against several clinical important antibiotics including β-lactamases and fluoroquinolones. This is congruent with recent case reports, where infections caused by *W. chitiniclastica* were successfully treated with levofloxacin (Schröttner et al., 2017; Bueide et al., 2021) and cephalosporins (Rebaudet et al., 2009; Campisi et al., 2015; Suryalatha et al., 2015; Snyder et al., 2020; Bueide et al., 2021), respectively. However, it should be noted that BM-Y carries a $bla_{\text{VEB-1}}$ gene cassette, thus conferring resistance to ceftazidime and ampicillin as previously reported (Zhou et al., 2016).

Surprisingly, the previously reported fosfomycin resistance (Schröttner et al., 2017; Matos et al., 2019; Kopf et al., 2021) is not reflected within the core resistome profile. DSM 108045 harbors the MFS transporter gene *abaF*, which is described to confer resistance to fosfomycin (Sharma et al., 2017), but apart from several hits for multidrug efflux proteins within the core genome, we did not detect any known fosfomycin resistance genes such *fosA, fosC*, or *fomB* (Silver, 2017), to name but a few. Our results rather indicate a natural fosfomycin resistance most likely based on a yet unknown resistance mechanism as previously anticipated (Kopf et al., 2021). Macrolide resistance genes *macA* and *macB*, on the other hand, are encoded in the core genome. Unfortunately, there are no case studies available, that either support or deny our observation. Nevertheless, based on our *in silico* analysis macrolide resistance appears to be feasible, although further research is still required to uncover the macrolide resistance profile fully. Moreover, *W. chitiniclastica* appears to be resistant to tellurite, which is not surprising, since potassium tellurite was used intensively as an antimicrobial agent in the past, and as a consequence, many Gram-positive and Gram-negative bacteria developed resistance (Valková et al., 2007). Notably, tellurite resistance genes have also been reported to increase oxidative stress resistance in bacteria (Valková et al., 2007), which is another explanation for their core genome presence in *W. chitiniclastica*.

Additional genes involved in antimicrobial defense are distributed within the accessory genome indicating resistome expansion due to enormous selective pressure. For example, *in silico* analysis with respect to aminoglycosides revealed putative resistance in BM-Y, F6513, F6514, F9188 and G9145. Moreover, four isolates harbor sulfonamide resistance genes, while the remaining 22 strains appear to be susceptible. This is in line with recent case reports, in which *W. chitiniclastica* was susceptible to diverse antibiotics including trimethoprim/sulfamethoxazole (Chavez et al., 2017; Katanami et al., 2018; Connelly et al., 2019; Snyder et al., 2020; Bueide et al., 2021), while the first reported case in South Africa surprised with trimethoprim/sulfamethoxazole resistance (Hoffmann et al., 2016). A similar picture was observed for tetracycline resistance, which showed a rather diverse distribution among the isolates. This observation is also reflected in recent case and research studies, in which some isolates were susceptible to tetracyclines (Almuzara et al., 2011; Nogi et al., 2016), and some resistant (Snyder et al., 2020; Kopf et al., 2021). The presence of Tn*10* encoded tetracycline resistance genes *tetR* and *tetC* (Babakhani and Oloomi, 2018) further emphasizes the assumption that the majority of resistance genes within *W. chitinclastica* genomes were required *via* HGT. Notably, our data indicate that the type strain DSM 18708$^{\text{T}}$ does not encode any additional clinical relevant AMR genes, while other strains harbor comparatively more. In particular BM-Y, F6513, F6514, F9188 and G9145 acquired an extended AMR profile; however, this is still limited when compared to other Gram-negative pathogens such as *Acinetobacter lwoffii* (Hu et al., 2011). Nevertheless, there is an increasing incidence of drug resistance within the *W. chitiniclastica* clade, whose development should be observed with caution.

CONCLUSION

The present study provides novel insights on the genetic diversity and pan-genome composition of *W. chitiniclastica* a rare but potential new emerging human pathogen. Our analysis of all publicity available strains indicate a surprisingly conserved pan-genome without clear host or geographical clustering suggesting a potential spread and transmission. However, with an increasing number of available genomes, reanalysis is strongly recommended to confirm and/or reevaluate the findings. *In silico* genome studies revealed first insights into genomic features including putative virulence factors and AMR genes, that potentially influence pathogenicity. Interestingly, no clear toxin profile could be determined suggesting an alternative

virulence profile. Our results could offer advantages in order to identify potential candidates for target specific manipulations and experimental studies to gain deeper insight into the pathogenic lifestyle of this emerging pathogen. With regard to empirical antibiotic therapy, no general validity for the species *W. chitiniclastica* can yet be derived from this study. With increasing numbers of available strains, preferably from different regions, but especially with a clear medical history, the analysis should be repeated and even extended to confirm and/or re-evaluate the results. Overall, our results provide the first overview of the genetic mechanisms and AMR profile of *W. chitiniclastica* that has never been presented in this form, laying the foundation for the best possible therapy.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The study was approved by the Ethics Committee at the Technical University of Dresden (EK 61022019). Written informed consent was not obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

PS had the idea and the concept for the study. AK analyzed the data, and wrote the first version of the manuscript. BB provided the bioinformatic data from the whole genome sequences. BB, TR, SC, FG, and PS contributed text passages for the manuscript. All authors contributed to the revision of the manuscript and approved the present version. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.912427/full#supplementary-material

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Identification of *Peptoniphilus vaginalis*-Like Bacteria, *Peptoniphilus septimus* sp. nov., From Blood Cultures in a Cervical Cancer Patient Receiving Chemotherapy: Case and Implications

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A 39-year-old woman with a 3-year human papillomavirus (HPV) 18 infection history was admitted to the hospital for a 16-day history of vaginal bleeding after sex. She was diagnosed with cervical cancer based on the results of the electronic colposcopy, cervical cytology, microscopy, and magnetic resonance imaging (MRI). Then, she received chemotherapy, with paclitaxel 200 mg (day 1), cisplatin 75 mg (day 2), and bevacizumab 700 mg (day 3) twice with an interval of 27 days. During the examination for the diagnosis and treatment, many invasive operations, including removal of intrauterine device, colposcopy, and ureteral dilatation, were done. After that, the patient was discharged and entered the emergency department about 2.5 months later with a loss of consciousness probably caused by septic shock. The patient finally died of multiple organ failure and bacterial infection, although she has received antimicrobial therapy. The blood cultures showed a monobacterial infection with an anaerobic Gram-positive bacterial strain, designated as SAHP1. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI–TOF MS) indicated that the patient was infected with *Peptoniphilus asaccharolyticus*, while molecular analysis and genome-based taxonomy confirmed the infection with a novel *Peptoniphilus* species that has a close genetic relationship with *Peptoniphilus vaginalis* and proposed provisionally as *Peptoniphilus septimus* sp. nov., which may also act as a commensal of the human vagina. Genomic features of SAHP1 have been fully described, and comparative genomic analysis reveals the known prokaryote relative of *Peptoniphilus septimus* sp. nov. in the genus *Peptoniphilus*. The invasive operations on the genital tract during the diagnosis and treatment of the patient and the tumor tissue damage and bleeding may have a certain

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role in the bloodstream infection. This study casts a new light on the *Peptoniphilus* bacteria and prompts clinicians to include anaerobic blood cultures as part of their blood culture procedures, especially on patients with genital tract tumors. Furthermore, due to the incomplete database and unsatisfying resolution of the MALDI–TOF MS for *Peptoniphilus* species identification, molecular identification, especially whole-genome sequencing, is required for those initially identified as bacteria belonging to *Peptoniphilus* in the clinical laboratory.

Keywords: *Peptoniphilus*, *Peptoniphilus septimus*, *Peptoniphilus vaginalis*, cervical cancer, bloodstream infection, MALDI–TOF, whole-genome sequencing, genome-based taxonomy

INTRODUCTION

The genus *Peptoniphilus* is a group of Gram-positive anaerobic coccus (GPAC), which always acts as normal microbiota that mostly colonizes the mucosal surfaces of the mouth and the gastrointestinal and genitourinary tracts (Murphy and Frick, 2013). It could also be a pathogen for human diseases because of its isolation from various clinical specimens including vaginal discharges and ovarian, peritoneal, and gland abscesses (Murdoch, 1998; Mishra et al., 2012). *Peptoniphilus* species, including *P. vaginalis*, have also reportedly been frequently isolated from vaginal discharges and associated with bacterial vaginosis, indicating that they have a close relationship with the genital tract (Sharma et al., 2014; Petrina et al., 2017). They can also be isolated from ovarian abscesses, retroperitoneal abscesses, and spinal fluid, showing certain pathogenicity in some cases (Puapermpoonsiri et al., 1996; Brook, 1999; Ezaki et al., 2001; Okui et al., 2016; Diop et al., 2016; Diop et al., 2019). Members of *Peptoniphilus* can also cause many severe infections in the peritoneum, osteoarticular skin, lymphocele, breast, bone and joint, soft tissue, surgical site infections, and blood (Fenollar et al., 2006; Dowd et al., 2008; Murphy and Frick, 2013; Seng et al., 2015; Cobo et al., 2017; Verma et al., 2017; Cobo, 2018; Le Bihan et al., 2019; Muller-Schulte et al., 2019; Wan et al., 2021). However, to date, no case of *Peptoniphilus* infection has been reported in patients with genital tract tumors, although many of these bacteria colonize the genitourinary tract (Murphy and Frick, 2013; Diop et al., 2016; Diop et al., 2019). Herein, we report a case of bloodstream monoinfection of *P. vaginalis*-like bacteria in a cervical cancer patient receiving chemotherapy. The molecular identification of the strain indicated that it is a novel *Peptoniphilus* sp. that has a close genetic relationship with *P. vaginalis* and *P. harei*, with *P. vaginalis* the closest, designated as *P. septimus*. Our findings highlight that the prevalence of *Peptoniphilus* bacteria and species in the clinic may have been underestimated. More awareness should be paid regarding the pathogenic potential of these opportunistic bacteria in the genus *Peptoniphilus*. In addition, *P. septimus* may be misidentified as *P. asaccharolyticus* in the clinical laboratory when using the VITEK MS matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI–TOF MS) probably because the two species have similar biochemical characteristics and are difficultly differentiated by proteome phenotyping such as that found between *P. harei* and *P. asaccharolyticus* (Wan et al., 2021), raising the importance of molecular identity, especially the whole-genome sequencing (WGS) for genus *Peptoniphilus* bacteria.

MATERIALS AND METHODS

Ethics Statement

This work was approved by the Ethics Committee of the Seventh Affiliated Hospital, Sun Yat-sen University. No personal identification data or potentially identifiable images were included in this article.

Case Description

A 39-year-old woman was admitted to the hospital for a 16-day history of vaginal bleeding after sex and a 3-year human papillomavirus (HPV) 18 infection on September 6, 2021. The patient has an intrauterine device (IUD) put into the uterus in 2013. For further magnetic resonance imaging (MRI) tests, she has undergone IUD removal and curettage 1 day after admission. No abnormal signs were observed during and after the IUD removal and curettage. Electronic colposcopy and cervical cytology indicated HPV 18 infection and showed that the cervix was withered and small, and the vaginal fornix disappeared. Gray cauliflower polypoid–like vegetation with a diameter of 1 cm was seen at the mouth of the cervix. MRI showed a space-occupying lesion of the cervix (**Figure 1A**). Cervical biopsy specimens showed heterotypic cell-in-cell structures in the tissue, with large and deeply stained nuclei, which were consistent with the cell morphology of poorly differentiated cervical cancer (**Figure 1B**). Immunohistochemistry showed that the cancer cells have strong and diffuse p16 staining, the proportion of Ki67-positive cells was about 60%, and the cells were CK5/6 positive, p63 positive, P40 positive, and CK8/18 positive but ab-pas negative. The patient was finally diagnosed with cervical cancer and planned to receive radiotherapy and chemotherapy. MRI and computed tomography (CT) also showed tumor invasion of the left ureter and effusion of the left ureter and kidney. Thus, she received ureteral dilatation and stent insertion 11 days after admission. Rehydration, antimicrobial, pain relief, and other symptomatic treatment and supportive care were applied to the patient after this operation. The patient's vital signs were stable after the operation, without special discomfort, low back pain, nausea,

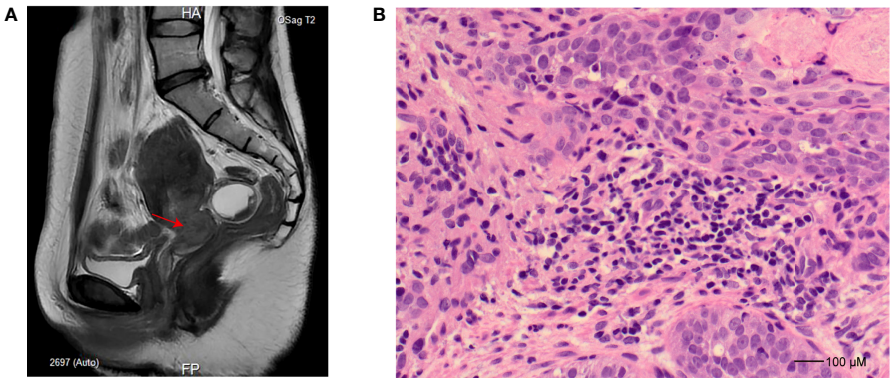

FIGURE 1 | Imaging and histopathology study for the patient. **(A)** Sagittal T2-weighted turbo spin-echo sequence, slightly hyperintense tumor in the cervix (indicated as an arrow). **(B)** Hematoxylin and eosin staining (×40) for cervical biopsy at the time of cervical cancer diagnosis demonstrates poorly differentiated squamous cell carcinoma characterized by pleomorphism and high nuclear-to-cytoplasmic ratios.

vomiting, chills, fever, etc. On September 18, 2021, the patient started to receive chemotherapy treatments with paclitaxel 200 mg (day 1), cisplatin 75 mg (day 2), and bevacizumab 700 mg (day 3). Liver injury after chemotherapy was observed, manifesting as elevated alanine aminotransferase and aspartate aminotransferase (48 U/L and 53 U/L on September 22, 2021, and 85 U/L and 72 U/L on September 23, 2021, respectively; reference normal values are both <40 U/L). After symptomatic treatment, the patient was self-discharged.

On October 11, 2021, the patient was admitted again with a chief complaint of chronic lower abdominal pain and received the second round of chemotherapy treatments during October 14 and October 16. The drug dosage was the same as the first round. The patient was self-discharged on October 18, 2021. The patient experienced a half-month of abdominal pain and diarrhea before the last admission. On December 29, the patient experienced a sudden loss of consciousness without convulsions and foaming at the mouth but with fecal incontinence at 18:31 and entered the emergency department of the hospital. Physical examination showed the disappearance of carotid pulse and weakness of breathing. After cardiopulmonary resuscitation, the heart rate of the patient recovered to about 40 beats/min. Atropine and amiodarone were administered to control the ventricular rate, but cardiac arrest occurred again at 18:46. After a second-round cardiopulmonary resuscitation, the patient's heart was back into a normal rhythm at 18:55. Detailed physical examination showed pulse 130/min (reference normal range is 60–100/min), respirations 45/min (reference normal range is 12–16/min), arterial oxygen saturation (SPO2) 96% (reference normal range is ≥95%), and blood pressure 113/68 mmHg (reference normal range is 90–120/60–90 mmHg). The pupils on both sides were equally large (about 3 mm), the light reflex was dull in the left eye and disappeared in the right eye, the breathing was weak, the abdomen was swollen and soft, and the bowel sound was weak. Laboratory examination showed that white blood cell count was 4.81×10^9/L (reference normal value is $4–10 \times 10^9$/L), C-reactive protein (CRP) was 142.10 mg/L (reference normal value is <10 mg/L), potassium concentration was 2.56 mmol/L (reference normal value range is 3.6–5.2 mmol/L), procalcitonin (PCT) was 81.51 ng/ml (reference normal value range is <0.1 ng/ml), myoglobin (MB) was 2,906.44 ng/ml (reference normal range is 25–72 ng/ml), N-terminal brain natriuretic peptide was 1,030 pg/ml (reference normal range is <125 pg/ml), cardiac troponin I was 0.2667 ng/ml (reference normal range is 0–0.04 ng/ml), indicating a potentiality of bacterial infection and sepsis (Vijayan et al., 2017). Then, the antibiotic treatment was carried out, with imipenem/cilastatin sodium administered at a dose of 1,000/1,000 mg, by intravenous infusion. On December 30, 2021, the patient experienced a rapid drop in blood pressure and recovered after rescue at 2:45. To save the patient more effectively, deep venous catheterization, peripheral arterial catheterization, and temporary central venous catheterization for blood purification had been carried out during 5:50 and 5:51. At 10:34, fiberoptic bronchoscopy and bronchial aspiration were carried out. At 18:00, the laboratory examination showed an elevated PCT level (741.33 ng/ml vs. 81.51 ng/ml) and meropenem was given to the patient at a dose of 1.0 g every 8 h by intravenous infusion to control the infection. On December 31, 2021, a laboratory examination was conducted again to verify the patient's signs. The patient was considered to be suffering from an uncontrolled bacterial infection based on the physical examination and a significant increase in white blood cell count (17.91×10^9/L vs. 7.39×10^9/L, reference normal range is $4–10 \times 10^9$/L) and CRP (267.07 mg/L vs. 184.48 mg/L) (data of December 31 vs. data of December 30). Then, the ascitic fluid and blood of the patient were cultured in the clinical laboratory. A monoinfection of anaerobic bacteria was found based on the blood cultures. After many times of emergency treatment, the patient soon died of multiple organ failure and bacterial infection.

Identification of the Isolate by Culture With MALDI–TOF MS

Ascitic fluid and blood cultures were carried out according to the principles and procedure guidelines for blood cultures raised by the Clinical and Laboratory Standards Institute (CLSI). Briefly,

10 ml of whole blood or ascitic fluid was separately injected into the BacT/ALERT culture media anaerobic bottle and then into the aerobic bottle (bioMérieux, Marcy I'É toile, France). Then, the bottles were put into the BacT/ALERT 3D Microbial Identification System (bioMérieux, Marcy I'É toile, France) for shaking culture at 35°C. The microorganism-positive bottle was then subcultured into the Columbia blood agar and cultured at 35°C for at least 48 h (put into the anaerobic bag or not). Pure colonies of the bacteria on the Columbia blood agar plate were selected for identification by a MALDI–TOF MS system, VITEK MS RUO IVD library (v3.2) (bioMÉrieux, Marcy I'É toile, France), following the manufacturer's instruction.

Antimicrobial Susceptibility Testing

E-test methods were used to determine the minimum inhibitory concentrations (MICs) of various antibiotics against the isolated organisms according to the 2021 CLSI criteria (Humphries et al., 2021). The following seven antibiotics were tested: penicillin G, vancomycin, ampicillin, ceftriaxone, meropenem, clindamycin, and chloramphenicol.

Whole-Genome Sequencing of SAHP1

WGS is a valuable and most accurate tool for the definitive molecular identification of bacterial isolates (Tohya et al., 2022). The pure colonies on the plate were harvested and sent to Guangzhou IGE Biotechnology Ltd., where DNA sequencing was performed on the PromethION 48 platform developed by Oxford Nanopore Technologies using the protocol described elsewhere for WGS (Lu et al., 2016). After genome assembly, correction, and optimization, the final genome of the human blood isolate (designated as SAHP1) was obtained. Coding proteins of SAHP1 were annotated in Clusters of Orthologous Genes (COG), Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Ontology (GO), NCBI Reference Sequence (RefSeq), Pfam, and TIGR-defined protein families (TIGRFAMs) databases.

Molecular Identification of SAHP1 Based on BLAST, Phylogenetic Analysis, Average Nucleotide Identity, Average Amino Acid Identity Calculation, and *In Silico* DNA-DNA Hybridization

For further molecular identification of SAHP1, we performed a set of analyses including the online BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), phylogenetic analysis based on the 16S ribosomal RNA (rRNA) gene, and multilocus sequence analysis (MLSA) to resolve its relationship with other bacterial species (Song et al., 2003; Glaeser and Kämpfer, 2015; Sayers et al., 2021). Average Nucleotide Identity (ANI), Average Amino Acid Identity (AAI) calculation, and *in silico* (also known as digital) DNA-DNA hybridization (iDDH or dDDH) were performed to research whether SAHP1 could be assigned as a new bacterial species (Richter and Rosselló-Móra, 2009; Auch et al., 2010; Thompson et al., 2013; Kim et al., 2014). Reference 16S rRNA gene sequences of the genus *Peptoniphilus* bacteria (all 30 species) were obtained from the NCBI database based on the accession numbers indicated on the website of List of Prokaryotic names with Standing in Nomenclature (LPSN) (https://lpsn.dsmz.de/) (Meier-Kolthoff et al., 2022). These sequences were used for a rough phylogenetic analysis because they were incomplete (~1,300 bp after trimming, 85% of the length of the full 16S rRNA gene for genus *Peptoniphilus*). The whole-genome sequences of the reference/type *Peptoniphilus* strains (19 strains belonging to 16 species) and a strain (*Anaerococcus degeneri* strain FDAARGOS1538) belonging to the family *Peptoniphilaceae* were obtained from the NCBI dataset for genomes (https://www.ncbi.nlm.nih.gov/datasets/genomes/). Full 16S rRNA gene (~1,525 bp) and 6 housekeeping gene sequences (*rpoB*, *gyrA*, *dnaA*, *recA*, *rplE*, and *groL*) of these strains were obtained based on the genome annotation of the NCBI dataset. Genome sequences and gene locus information of the analyzed strains are shown in **Supplementary Table S1**. An unrooted phylogenetic tree of the 16S rRNA gene and the 6 housekeeping genes of SAHP1 and the reference bacterial strains was constructed using MEGA X, inferring the evolutionary history using the neighbor-jointing (NJ) method (Kumar et al., 2018). Bootstrap values were estimated using 1,000 replications. ANI between the two paired strains was obtained by ANI calculator using the OrthoANIu algorithm, an improved iteration of the original OrthoANI algorithm, which uses USEARCH instead of BLAST (Yoon et al., 2017). AAI between the two paired strains was obtained by the CompareM packages (https://github.com/dparks1134/CompareM), which used DIAMOND to perform sequence similarity searches, and gene calling is performed using Prodigal (Hyatt et al., 2010; Yoon et al., 2017). The iDDH was performed using Genome-to-Genome Distance Calculator (GGDC), which could yield the highest correlations with traditional DDH, and values generated by the Genome BLAST Distance Phylogen (GBDP) formula d_4 are recommended as the standard (Meier-Kolthoff et al., 2013; Meier-Kolthoff et al., 2022).

SAHP1 Phylogenetic Inference, Gene Family Construction, and Collinearity Analysis With Its Closest Relative Bacterial Species

To uncover the phylogenetic relationship between SAHP1 and other relative bacterial species, the genome sequences of SAHP1 and 19 other reference *Peptoniphilus* strains we mentioned above were uploaded to the Type (Strain) Genome Server (TYGS) inference for a whole genome-based taxonomic analysis (Meier-Kolthoff et al., 2022). The genomes were compared against all type strain genomes available in the TYGS database *via* the MASH algorithm (Ondov et al., 2016), and close relative type strains were obtained from the TYGS database and auto-implemented in further analysis. Pairwise comparisons of the genome sequences were conducted using the GBDP approach, and intergenomic distances were inferred under the algorithm "trimming" (Meier-Kolthoff et al., 2013). The resulting intergenomic distances were used to infer a balanced minimum evolution tree with branch support *via* FASTME 2.1.6.1 including Subtree Pruning and Regrafting (SPR) postprocessing (Lefort et al., 2015). Branch support was inferred from 100 pseudo-bootstrap replicates each.

For comparative analyses of the orthologous and exclusive genes between SAHP1 and the closest relative genomes, OrthoVenn2, an OrthoMCL-based method that uses DIAMOND (v0.9.24) instead of BLASTP or UBLAST to perform the all-against-all protein sequence comparison, was used (Xu et al., 2019). Collinearity of the conserved and highly orthologous genomic regions was determined and plotted among SAHP1 and its close relative *Peptoniphilus* species, reference strain KhD-2 (*P. vaginalis*), and NCTC13077 (*P. harei*) by using Mauve software (version 2.3.1) with default parameters (Darling et al., 2004).

RESULTS

Characteristics of SAHP1 in the Clinical Laboratory

No bacteria were cultured from the patient's ascitic fluid. The BacT/ALERT 3D Microbial Identification System reported positivity for microorganisms in the anaerobic bottle after 21 h and 37 min of culturing. Anaerobic culture of blood in Columbia agar plates in the anaerobic bag after 48 h revealed pinpoint, smooth, glistening white colonies with blur edges (**Figure 2A**). Gram staining showed Gram-positive cocci (**Figure 2B**). In contrast, the aerobic bottle remained negative after more than 48 h of culture incubation. VITEK MS analysis showed SAHP1 as *P. asaccharolyticus* based on a confidence level of 99.9%, and the spectrum of strain SAHP1 is shown in **Figure 3**. The SAHP1 was susceptible to all antimicrobials tested with variable MICs obtained: penicillin G (0.25 mg/L), vancomycin (0.125 mg/L), ampicillin (0.094 mg/L), ceftriaxone (0.125 mg/L), meropenem (0.004 mg/L), clindamycin (0.75 mg/L), and chloramphenicol (1 mg/L) (**Supplementary Figure S1**).

Molecular Identification of SAHP1 Revealed It as a Novel *Peptoniphilus* Species That Has a Close Relationship with *P. vaginalis* and *P. harei*

In molecular identification *via* 16S rRNA gene BLAST against the nucleotide collection database using the default algorithm parameters, we found that SAHP1 exhibited a 16S rRNA similarity of 95.77%–100% with genus *Peptoniphilus* bacteria (top 20 hints, **Supplementary Figure S2A**). Phylogenetic analysis of partial 16S rRNA gene of SAHP1 and other 30 *Peptoniphilus* species type strains revealed that SAHP1 was closest to *P. vaginalis* strain KhD-2 and *P. harei* strain DSM10020 that had ever been isolated from the human female genital tract or other human clinical specimens (Murdoch et al., 1997; Diop et al., 2016; Diop et al., 2019). The three strains clustered into the same group, and even the same subgroup (**Supplementary Figure S2B**). For accurate species identification, whole-genome sequences of 19 well-documented reference or type strains belonging to 16 species of *Peptoniphilus* were downloaded from NCBI datasets for genomes (https://www.ncbi.nlm.nih.gov/datasets/genomes/). These strains were taxonomy-checked based on the NCBI assembly database report. The full 16S rRNA gene sequence identities of SAHP1 and other strains are shown in **Figure 4A** and **Supplementary Table S2**. Briefly, SAHP1 showed a 99.02% similarity of 16S rRNA with *P. vaginalis* strain KhD-2, a 98.65%–98.69% similarity with three *P. harei* strains (NCTC13077, NCTC13076, FDAARGOS1136), and an 88.65% similarity with the two *P. asaccharolyticus* strains (FDAAROGS1135 and DSM20463). Given that 98.7% of 16S rRNA sequence identity is recommended to delineate a new species in the phylum *Firmicutes* without carrying out DNA-DNA hybridization (Stackebrandt, 2006; Diop et al., 2016), this result indicated that SAHP1 did not belong to *P. asaccharolyticus* and *P. harei* but might belong to *P. vaginalis*. A phylogenetic tree of 21 complete 16S rRNA sequences was constructed using the NJ method. Consistent with the 16S rRNA sequence comparison results, the SAHP1 clustered most closely with *P. vaginalis* strain KhD-2 (**Figure 4B**). Phylogenetic analyses based on the housekeeping gene sequences (single gene or trimmed 10,794-bp concatemers) also showed that SAHP1 formed a most closely cluster with *P. vaginalis* KhD-2 within the genus *Peptoniphilus* (**Figure 4C**, **Supplementary Figure S3**). To further species identification, we adopt more principles to identify SAHP1 as a strain belonging to an existing *Peptoniphilus* species. First, the ANI should be 95%–96% within SAHP1 and an existing species; second, the AAI should be >95% within SAHP1 and an existing species; ultimately,

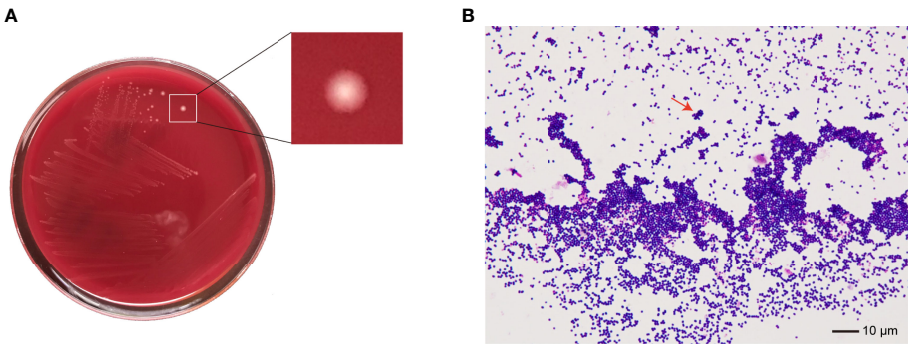

FIGURE 2 | The phenotype of SAHP1. **(A)** Bacterial colonies on the Columbia blood agar after 48 h of incubation under anaerobic conditions. **(B)** Gram staining of bacteria from a positive aerobic blood culture showed Gram-positive cocci (×1,000 magnification) (arrowhead).

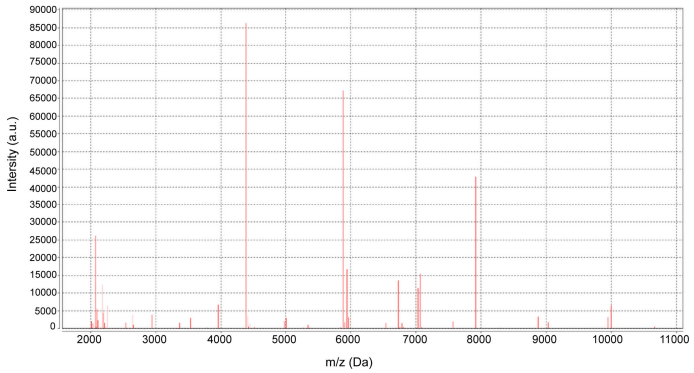

FIGURE 3 | The spectrum of strain SAHP1 *via* the VITEK MS MALDI–TOF MS.

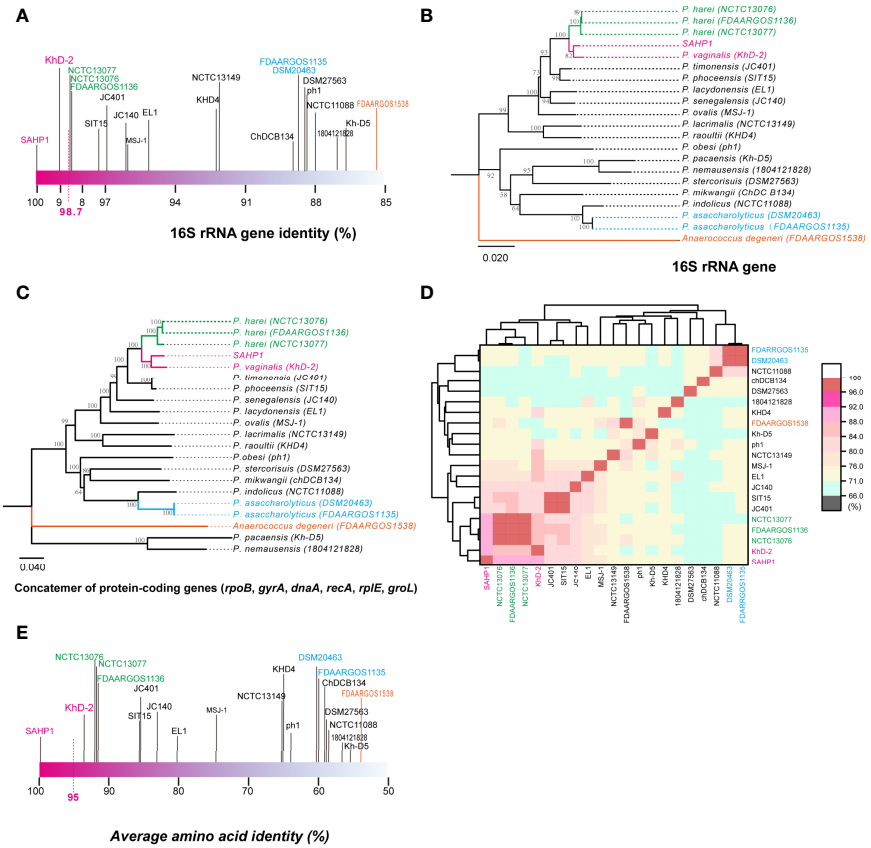

FIGURE 4 | The 16S rRNA gene identity analysis and phylogenetic, ANI, and AAI analyses of SAHP1 with other reference strains in the genus *Peptoniphilus*. **(A)** The 16S rRNA gene identities between SAHP1 and other reference strains of the genus *Peptoniphilus.* Magenta indicates the highest identity of the 16S rRNA gene with SAHP1. Green indicates *P. harei* strains, while blue indicates *P. asaccharolyticus* strains, and orange indicates the *Anaerococcus degeneri* strain that belongs to the same family, the *Peptoniphilaceae*, as those *Peptoniphilus* strains. **(B)** Phylogenetic analysis of full 16S rRNA. **(C)** The MLSA-based phylogenetic tree of strain SAHP1 and other *Peptoniphilus* strains. The tree is based on six housekeeping protein-coding genes. Phylogenetic inference was performed with MEGA X. The bootstrap values of 1,000 replications display the significance of each branch, and those higher than 50% are shown in the taxa clustered in the tree. The tree is drawn to scale, with branch lengths measuring in the number of substitutions per site. **(D)** ANI between SAHP1 and different *Peptoniphilus* strains, and all showed a <92% ANI; the cluster diagram of ANI showed a close relationship among SAHP1 and *P. vaginalis* KhD-2 and the three *P. harei* strains. **(E)** AAI between SAHP1 and other reference strains. Green indicates *P. harei* strains, while blue indicates *P. asaccharolyticus* strains, and orange indicates the *Anaerococcus degeneri* strain that belongs to the same family, the *Peptoniphilaceae*, as those *Peptoniphilus* strains.

iDDH should show >70% similarity within SAHP1 and an existing species (Auch et al., 2010; Thompson et al., 2013; Chun et al., 2018). To our surprise, the highest ANI of SAHP1 against *Peptoniphilus* strains was only 91.93% (against *P. vaginalis* KhD-2), less than the same species threshold of 95%–96%, which indicates that SAHP1 did not belong to *P. vaginalis*, although the ANI heatmap showed a close relationship of SAHP1 with *P. vaginalis* (**Figure 4D**, **Supplementary Table S3**). Similarly, the highest AAI of SAHP1 against *Peptoniphilus* strains was only 93.51% (against *P. vaginalis* KhD-2), less than the threshold of 95% for the same bacterial species (**Figure 4E**, **Supplementary Table S4**). The iDDH of SAHP1 with the reference strains and known type strains in the TYGS database all showed a <70% identity, with the *P. vaginalis* KhD-2 having the highest identity (45.1%, 95% CI 42.5%–47.7%) (**Table 1**). Together, these results indicated that SAHP1 is a novel *Peptoniphilus* sp. that has the closest relationship with *P. vaginalis*. Here, we designated it as *Peptoniphilus septimus* sp. nov.

Genomic Features of SAHP1 and Comparative Genomic Analysis

The genome of *P. septimus* strain SAHP1 is composed of a circular chromosome of 1,917,962 bp (1 chromosome, with a clustered regularly interspaced short palindromic repeats (CRISPR) sequence, a prophage region, and without plasmid) with an overall 34.58% guanine-cytosine (GC) content and 1,804 protein-coding genes, 9 rRNA genes, 43 transfer RNA (tRNA) genes, 27 regulatory noncoding RNA (ncRNA) genes, and 9 other ncRNA genes (**Figure 5A**, **Table 2**). The number of protein-coding genes annotated by the COG, KEGG, GO, Refseq, Pfam, and TIGRFAMs database for SAHP1 is shown in **Supplementary Table S5**. In this study, 1,745 protein-coding genes (95.07%) have predicted functions by COG, while 59 have unknown functions. The properties and the annotated features by COG and GO are summarized in **Supplementary Figure S4**. None of the virulence factors in the SAHP1 was found in the Virulence Factor Database (VFDB). The KEGG only showed that

TABLE 1 | iDDH of SAHP1 with other reference strains.

Query strain	Subject strain	iDDH (d0, in %)	95% CI (d0, in %)	iDDH (d4, in %)*	95% CI (d4, in %)	iDDH (d6, in %)	95% CI (d6, in %)	G+C content difference (in %)	Taxonomy check (by NCBI)
SAHP1	*P. vaginalis (KhD-2)*	66.3	[62.5–70.0]	45.1	[42.5–47.7]	62.7	[59.4–65.9]	0.35	OK
SAHP1	*P. harei (NCTC13076)*	59.6	[55.9–63.1]	35.4	[33.0–37.9]	53.6	[50.5–56.7]	0.83	OK
SAHP1	*P. harei (NCTC13077)*	66.3	[62.4–69.9]	35.0	[32.5–37.5]	58.4	[55.1–61.5]	0.16	OK
SAHP1	*P. harei (FDAARGOS1136)*	61.4	[57.7–65.0]	35.3	[32.9–37.8]	54.9	[51.8–58.0]	0.72	OK
SAHP1	*P. phoceensis (SIT15)*	52.6	[49.1–56.1]	26.8	[24.4–29.3]	44.5	[41.5–47.5]	3.38	OK
SAHP1	*P. timonensis (JC401)*	46.2	[42.9–49.7]	26.2	[23.9–28.7]	39.9	[36.9–42.9]	3.9	OK
SAHP1	*P. senegalensis (JC140)*	37.8	[34.5–41.3]	24.1	[21.8–26.6]	33.2	[30.3–36.3]	2.34	OK
SAHP1	*P. lacydonensis (EL1)*	27.3	[24.0–31.0]	23.2	[20.9–25.6]	25.3	[22.5–28.5]	4.68	OK
SAHP1	*P. ovalis (MSJ-1)*	19.2	[16.1–22.8]	20.5	[18.2–22.9]	18.6	[16.0–21.6]	3.97	OK
SAHP1	*P. raoultii (KHD4)*	14.7	[11.9–18.1]	22.3	[20.0–24.8]	14.9	[12.5–17.8]	2.70	OK
SAHP1	*P. lacrimalis (NCTC13149)*	14.7	[11.8–18.1]	32.9	[30.5–35.4]	15.1	[12.6–17.9]	4.24	OK
SAHP1	*P. obesi (ph1)*	14.6	[11.8–18.1]	35.4	[32.9–37.9]	15.1	[12.6–18.0]	4.44	OK
SAHP1	*P. asaccharolyticus (DSM20463)*	13.7	[11.0–17.1]	29.3	[26.9–31.8]	14.1	[11.7–16.9]	2.29	OK
SAHP1	*P. asaccharolyticus (FDAARGOS1135)*	13.8	[11.0–17.1]	29.5	[27.1–32.0]	14.2	[11.7–17.0]	2.18	OK
SAHP1	*P. nemausensis (1804121828)*	13.6	[10.9–17.0]	47.4	[44.8–50.0]	14.1	[11.7–17.0]	11.18	OK
SAHP1	*P. indolicus (NCTC11088)*	13.3	[10.5–16.6]	23.1	[20.8–25.6]	13.6	[11.2–16.4]	2.92	OK
SAHP1	*P. pacaensis (Kh-D5)*	13.1	[10.3–16.3]	39.6	[37.1–42.1]	13.5	[11.1–16.3]	14.81	OK
SAHP1	*P. mikwangii (ChDCB134)*	13.2	[10.5–16.5]	21.7	[19.5–24.1]	13.6	[11.2–16.3]	3.59	OK
SAHP1	*P. stercorisuis (DSM27563)*	13.2	[10.5–16.5]	18.1	[15.9–20.4]	13.5	[11.1–16.3]	6.75	OK
SAHP1	*Anaerococcus degeneri (FDAARGOS1538)*	13.1	[10.6–16.6]	34.1	[31.6–36.6]	13.7	[11.4–16.5]	0.66	OK
SAHP1	*P. grossensis (ph5)*	45	[41.6–48.4]	25.1	[22.7–27.5]	38.5	[35.5–41.5]	0.70	OK
SAHP1	*P. gorbachii (DSM 21461)*	44.8	[41.5–48.3]	24.8	[22.5–27.3]	38.3	[35.3–41.3]	3.19	OK
SAHP1	*P. urinimassiliensis (Marseille-P3195)*	13.2	[10.5–16.5]	35.8	[33.4–38.4]	13.6	[11.2–16.4]	15.11	OK
SAHP1	*P. rhinitidis (1-13T)*	26.2	[22.8–29.8]	23.0	[20.7–25.4]	24.4	[21.6–27.5]	4.72	Inconclusive
SAHP1	*P. olsenii (DSM 21460)*	16	[13.1–19.5]	22.2	[19.9–24.7]	16.1	[13.5–19.0]	4.59	N/A
SAHP1	*P. duerdenii (ATCC BAA-1640)*	14.6	[11.8–18.0]	32.8	[30.4–35.3]	15.0	[12.5–17.9]	0.37	Inconclusive
SAHP1	*P. koenoeneniae (DSM22616)*	14.2	[11.4–17.6]	30.9	[28.5–33.4]	14.6	[12.1–17.4]	3.26	N/A
SAHP1	*P. coxii (CCUG59622)*	13.8	[11.0–17.1]	37.8	[35.4–40.3]	14.2	[11.8–17.0]	9.99	Inconclusive
SAHP1	*P. ivorii (NCTC13079)*	12.7	[10.1–16.0]	30.4	[28.0–32.9]	13.2	[10.8–15.9]	17.52	Inconclusive
SAHP1	*P. tyrrelliae (CCUG59621)*	37.2	[33.8–40.7]	23.8	[21.5–26.3]	32.6	[29.7–35.7]	2.62	Inconclusive
SAHP1	*P. porci (35-6-1)*	28.0	[24.7–31.7]	23.6	[21.3–26.1]	26.0	[23.1–29.1]	3.37	Inconclusive

**Formula d_4 (also known as GGDC formula 2) sums all identities found in high-scoring segment pairs (HSPs) divided by the overall. The results obtained from d_4 are more robust and recommended as standard ones. N/A indicates not available. Cells filled with gray indicate that the genomic data of these strains were auto-implemented and from the TYGS database.*

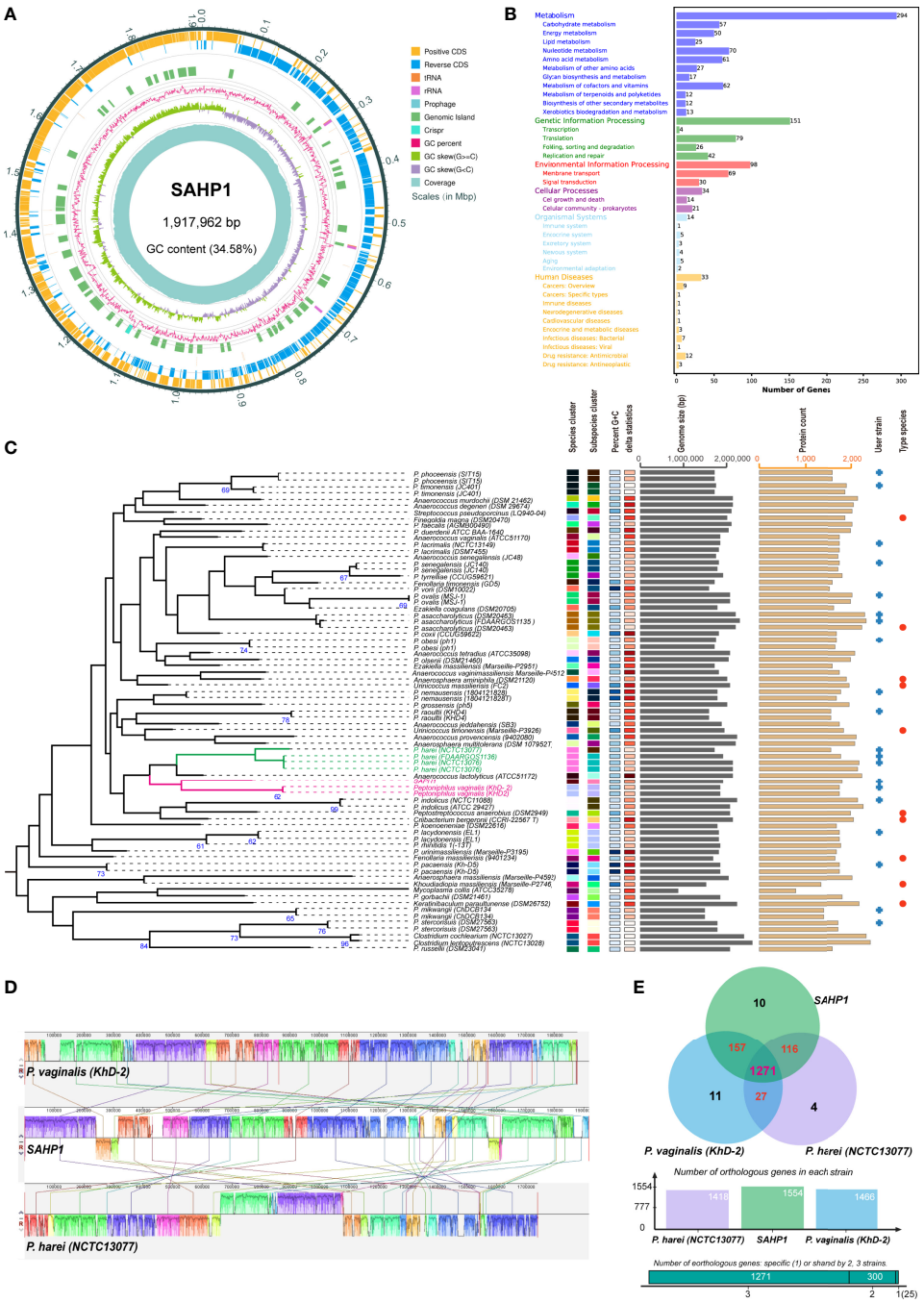

FIGURE 5 | Genomic features of SAHP1 and comparative genomic analysis. **(A)** Graphical circular map of *P. septimus* strain SAHP1 genome. **(B)** Functional categorization of SAHP1 protein-coding genes based on the KEGG. **(C)** The GBDP tree of SAHP1 and other related strains using the genome data from the NCBI database (user strain) and those automatically implemented by the TYGS database. The tree was inferred with FastME 2.1.6.1 from GBDP distances calculated from genome sequences. The branch lengths are scaled in terms of GBDP distance formula d_5. The numbers above the branches are GBDP pseudo-bootstrap support values >60% from 100 replications, with an average branch support of 29.8%. **(D)** Genome-to-genome alignment of *P. septimus* strain SAHP1, *P. vaginalis* strain KhD-2, and *P. harei* strain NCTC13077 using a progressive mauve software and *P. septimus* strain SAHP1 as the reference genome. Boxes with the same color indicate the syntenic regions. Boxes below the horizontal line indicate inverted regions. The colored locally collinear blocks (LCBs) show the conserved and highly similar genomic regions. The white areas inside the colored regions indicate sequence elements specific to one genome that are not aligned. The height of the similarity profile is present inside each block. The colored lines that connect LCBs represent translocations of homologous regions. Blocks above or below the horizontal bar indicate regions that aligned in the forward or reverse orientation, respectively. Rearrangements are shown by colored lines. **(E)** Venn diagram showing the number of orthologous protein-coding genes shared and unique between the three strains SAHP1, KhD-2, and NCTC13077.

TABLE 2 | General features of the genome of *P. septimus* strain SAHP1.

Parameters	Values (n)	% of total*
Genome size (bp)	1,917,962	100
DNA coding region (bp)	1,701,678	88.72
G+C content (bp)	663,137	34.58
Total genes	1,892	100
ncRNA	88	4.65
rRNA	9	10.23
tRNA	43	48.86
Regulatory ncRNA	27	30.68
Other ncRNA	8	9.09
Protein-coding genes	1,804	95.35
Genes with function prediction	1,745	95.07
Genes assigned to COG	1,394	77.27
Genes assigned to KEGG	960	53.21
Genes associated with human disease	33	1.83
Genes associated with bacterial infection	7	0.39
Genes associated with metabolism	294	16.30

**The total is based on either the size of the genome (base pairs) or the total number of genes or protein-coding genes in the annotated genome.*

33 SAHP1 genes were associated with human disease, of which only 7 might take part in the bacterial infection (**Figure 5B**), indicating that *P. septimus* SAHP1 might be a normal microbiota for human beings. Five antibiotic-resistant genes of *P. septimus* SAHP1 were found in the Comprehensive Antibiotic Research Database (CARD), including tetM, ErmA, aad (Puapermpoonsiri et al., 1996), SAT-4, and APH (3')-IIIa, indicating potential tetracycline, macrolide-lincosamide-streptogramin B, aminoglycoside, and streptothricin resistance in SAHP1 (**Supplementary Table 6**).

The GBDP tree based on the whole-genome sequences of the SAHP1 and those closely related type strains in the TYGS database is shown in **Figure 5C**. The *P. septimus* SAHP1 still showed a close genetic relationship with *P. vaginalis* and *P. harei*—the closest with *P. vaginalis*. The *P. septimus* SAHP1 genome is larger than that of the *P. vaginalis* KhD-2 (1,877,211 bp) and *P. harei* NCTC13077 (1,739,102 bp). The collinearity analysis is consistent with the close relatedness among the above three strains (**Figure 5D**). OrthoMCL analysis of the orthologous genes among the three strains showed that 1,271 genes make up the core genome, occupying 79.64% (1,271/1,596) of the pan-genome and 78.96%–89.63% of each genome (**Figure 5E**). In this study, 91.89% (1,428 genes) and 89.25% (1,387 genes) of *P. septimus* SAHP1 genes have orthologs in the *P. vaginalis* KhD-2 and *P. harei* NCTC13077, respectively, and only 10 genes (0.82%) are unique to the *P. septimus* SAHP1 genome (**Figure 5E**).

DISCUSSION

Peptoniphilus may always act as normal microbiota that inhabit human skin and mucosal surfaces. However, many local infections caused by *Peptoniphilus* were found, indicating that they may also act as common pathogens to human beings in some circumstances. The uncommon cause of bacteremia in humans by *Peptoniphilus* confirmed their potential to cause poor clinical outcomes and high rates of mortality, as it was shown that 20% of fatal outcomes were caused by bloodstream infections (Brown et al., 2014). The case we reported in the present study had several unique and interesting clinical characteristics. First, *Peptoniphilus* always acts as one of the causative agents in polymicrobial infections in humans, such as ulcers and osteoarticular and soft tissue infections, while our case revealed a monoinfection of the bloodstream caused by *Peptoniphilus* bacteria, which was rarely reported previously (Brown et al., 2014; Wan et al., 2021). Second, the patient was suffering from a genital tract cancer, cervical cancer, and the causative agent of bloodstream infection was a genetically *P. vaginalis*-like bacterium, which may inhabit the human female genital tract as *P. vaginalis* does, acts as a commensal of the human vagina, and is frequently associated with bacterial vaginosis (Verma et al., 2017). The bacterial characteristics and the case made us deem a possible infection pathway from the present case. The invasive operations in the genital tract of the patient during examinations and treatment (e.g., removal of the IUD, colposcopy, and ureteral dilatation) or the tumor tissue damage and bleeding that were very common in patients with advanced cervical cancer might facilitate the bacteria inhabiting the genital tract and entering the bloodstream (Eleje et al., 2019). The immunosuppressive state caused by the tumor and chemotherapy treatment may impair the neutrophils, monocytes, macrophages, and host immune response to the bacteria in the tissue and also facilitate the bacteria entering the bloodstream (Minasyan, 2017). After entering the bloodstream, bacteria move with blood flow. By mechanisms against reactive oxygen species that are released from the main bactericidal cells in the blood, erythrocytes, the bacteria survive oxidation on the surface of erythrocytes and proliferate and in turn trigger a systemic inflammatory response of the host to the infection, which leads to multifaceted disruption of the finely tuned immunological balance of inflammation and anti-inflammation and finally causes septic shock-like symptoms in the patient who has been seen at the end of the case of the present study (Jarczak et al., 2021). However, this could not exclude other infection pathways, such as the bacteria inhabiting the skin or gastrointestinal tract and migrating to the blood through other ways (e.g., deep venous catheterization, peripheral arterial catheterization, and temporary central venous catheterization). Third, this was the first case of bloodstream monoinfection by *Peptoniphilus* bacteria found in a cervical cancer

patient and contributed to the patient's death, and the novel *Peptoniphilus* species we designated in the present study was genetically close to *P. vaginalis*. The antimicrobial susceptibility of the bloodstream infection agent *P. septimus* SAHP1 showed that it was susceptible to the tested broad-spectrum antibiotics, including penicillin G, vancomycin, ampicillin, ceftriaxone, meropenem, clindamycin, and chloramphenicol. However, genome research revealed the probable resistance of SAHP1 to tetracycline, macrolide-lincosamide-streptogramin B, aminoglycoside, and streptothricin. Thus, clinicians need to pay attention to the possible antibiotic resistance of this bacterium. The E-test showed that SAHP1 was sensitive to clindamycin (MIC = 0.75 mg/L), which seems to be different with the genome research result based on the presence of macrolide-lincosamide-streptogramin B resistance gene, named *ermA*. The ErmA protein dimethylates the A2058 residue of 23S rRNA and in turn impairs the binding of macrolides, lincosamides, and streptogramin B, which accounts for the cross-resistance to these drugs (Petinaki and Papagiannitsis, 2018). Thus, the first is probably that ErmA in the SAHP1 has no or very weak methylase activity due to a relatively low nucleotide sequence identity (82.5% with *Staphylococcus aureus* strain N315 *ermA* gene) and amino acid sequence identity (81.0% with *Staphylococcus aureus* strain N315 ErmA protein). Second, the *ermA* gene expression in the SAHP1 may be inducible, which makes SAHP1 susceptible to 16-membered ring macrolides, lincosamides, and streptogramin B in the absence of 14- and 15-ring macrolides (Petinaki and Papagiannitsis, 2018). Although SAHP1 was shown to be susceptible to meropenem *in vitro*, we found that meropenem might not control the SAHP1 infection in the patient based on the case record. Likely reasons included insufficient meropenem treatment time or the SAHP1 might present in the biofilm or present as L-form in the bloodstream that was common in the sepsis patient, which made it resistant to most of the antibiotics *in vivo* (Minasyan, 2019).

Correctly identifying pathogens is of crucial importance in clinical microbiology and epidemiology research. *Peptoniphilus* spp. are often misidentified by using biochemical methods (Veloo et al., 2011). Due to the limited quantity and accuracy of the MALDI–TOF MS database, the mass spectrometry results may be uncertain when encountering *Peptoniphilus* spp. Evaluations of MALDI–TOF MS for the identification of anaerobic bacteria showed 84%–94.8% accuracy (Barba et al., 2014; Garner et al., 2014; Rodríguez-Sánchez et al., 2016; Li et al., 2019), indicating a dilemma in identifying *Peptoniphilus* species by using the MALDI–TOF MS (Barberis et al., 2022). A previous study by Wan et al. (2021) found misidentification of *P. harei* strain to *P. asaccharolyticus* by the VITEK MS system. We also found a species-level misidentification of SAHP1 caused by the absence of *P. septimus* in the database, emphasizing the importance and urgency of implementing the standard mass spectrum of *P. septimus* in the database.

With the presence of the 16S rRNA gene in almost all bacteria, the role of the "molecular time scale" and the convenience of analysis make it the most popular target for bacterial phylogeny and taxonomy (Janda and Abbott, 2007). Despite the full 16S rRNA gene providing better taxonomic resolution (Johnson et al., 2019), its resolution at the bacterial species level is not satisfying; even if the bacterial strains have >99.7% 16S rRNA gene identity, they may also belong to different species in some cases (e.g., some *Shigella* vs. *Escherichia* strains) (Fox et al., 1992; Devanga Ragupathi et al., 2018). Thus, many other indices that utilized genome data to resolve the genetic relatedness were used for bacterial species identification and taxonomy, including the ANI, AAI, and iDDH (Richter and Rosselló-Móra, 2009; Auch et al., 2010; Thompson et al., 2013; Kim et al., 2014). In the present study, the full 16S rRNA gene of SAHP1 has 99.02% identity with the type strain of *P. vaginalis*. However, taxonomy based on the genomic data (ANI = 91.93%, AAI = 93.51%, and iDDH = 45.1% with its closest relative species type strain KhD-2; all values have not reached the threshold that could be identified as the same species) revealed that SAHP1 was a novel *Peptoniphilus* species. Given that the BLAST results showed that the SAHP1 16S rRNA gene has higher identities (all >99%) with *P. harei* strain TID-12, DCW_SL_32C, and *P. asaccharolyticus* strain 1212-10216, we suspected that the misidentification of *Peptoniphilus* strains may be very common, underlining the importance of molecular species identification methods (e.g., WGS) other than the 16S rRNA gene sequencing.

Genomic information revealed that *P. septimus* strain SAHP1 had none of the virulence factors that were found in the VFDB, and the small number of genes implicated in the infection pathway based on the KEGG annotation implies it as a commensal and opportunistic pathogen, like many other GPACs (Chen et al., 2005; Murphy and Frick, 2013). This could also be deduced from the present case: a woman with a malignant genital tract tumor, an immunosuppressive state, etc. The SAHP1 had a comparable genome size, number of protein-coding genes, and GC contents (0.16%–0.83% difference, **Table 1**) with its close relatives (*P. vaginalis* and *P. harei*). Similar genetic and microbiological properties may have been shared among these species, which were implicated in the collinearity and OrthoMCL analysis, which also implied that the bacteria of the genus *Peptoniphilus* might be relatively evolutionarily conservative (Li et al., 2003). However, a mosaic structure of the GBDP tree (mainly consisting of genus *Peptoniphilus* species) could be observed, which was filled with some other bacterial species in the family *Peptoniphilaceae* such as *Anaerococcus lactolyticus*, *Finegoldia magna*, *Urinicoccus massiliensis*, and *Khoudiadiopia massiliensis*. This may indicate a history of non-vertical inheritance of genetic material in these species and the probable requirement of changes in the nomenclature in the species of the family *Peptoniphilaceae* (Katz, 2021).

CONCLUSIONS

In summary, we have reported a case of a cervical cancer patient receiving chemotherapy monoinfected with *P. vaginalis*-like bacteria in the bloodstream. Based on the probable similar microbiological characteristics of *P. septimus* to other GPACs, clinicians need to pay attention to those patients with genital tract tumors and must include anaerobic blood cultures as part of their blood culture procedures to avoid infection and carry out early antibiotic therapy. The identification procedures of the novel species

P. septimus in the present study reveal the limited and insufficient resolution of MALDI–TOF MS in identifying *Peptoniphilus* bacteria in the clinical laboratory. Missed identification and misidentification of *Peptoniphilus* species might occur many times in the clinical laboratory. The 16S rRNA gene sequencing is required in the clinical laboratory but not adequate, and whole genome-based identification of *Peptoniphilus* bacteria is recommended, as it could provide more detailed information (e.g., AAI, ANI, iDDH) when analyzed with other strains and could catch some "hidden" novel species. These molecular methods may help to better estimate real GPAC prevalence and pathogenicity in the clinic. Genomic features of SAHP1 and comparative genomic analysis among *P. septimus*, *P. vaginalis*, and *P. harei* further confirmed the close genetic relationship of the three bacterial species and might imply comparable microbiological characteristics and pathogenies among them.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/nuccore/CP097885.

AUTHOR CONTRIBUTIONS

X-YZ designed the study and wrote the manuscript. HW, J-LY, CC, YZ, MC, JQ, and ST collected the data of the case, HW and YZ performed the antibiotic subspeciality research, and J-LY cultured the bacteria for WGS and collected the genomic sequence of other bacterial strains. X-YZ analyzed the data and performed graphing and data interpreting. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.954355/full#supplementary-material

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Characterization and Comparative Genomic Analysis of a Highly Colistin-Resistant *Chryseobacterium gallinarum*: a Rare, Uncommon Pathogen

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For the first time, we describe the whole genome of a yellow-pigmented, capsule-producing, pathogenic, and colistin-resistant *Chryseobacterium gallinarum* strain MGC42 isolated from a patient with urinary tract infection in India. VITEK 2 automated system initially identified this isolate as *C. indologenes*. However, 16S rRNA gene sequencing revealed that MGC42 shared 99.67% sequence identity with *C. gallinarum*–type strain DSM 27622. The draft genome of the strain MGC42 was 4,455,926 bp long with 37.08% Guanine-Cytosine (GC) content and was devoid of any plasmid. Antibiotic resistance, virulence, and toxin genes were predicted by implementing a machine learning classifier. Potential homologs of 340 virulence genes including hemolysin secretion protein D, metalloprotease, catalase peroxidases and autotransporter adhesins, type VI secretion system (T6SS) spike proteins, and 27 toxin factors including a novel toxin domain Ntox23 were identified in the genome. Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs of 110 transporter proteins were predicted that were in agreement with moderate efflux activity. Twelve antibiotic resistance genes including two potentially novel putative β-lactamase genes sharing low similarity with known β-lactamase genes were also identified in the genome of this strain. The strain MGC42 was also resistant to several classes of antibiotics along with carbapenems and polymyxin. We also identified mutations in the orthologs of *pmr*B (M384T) and *lpx*D (I66V) that might be responsible for colistin resistance. The MGC42 strain shared 683 core genes with other environmental and clinical strains of *Chryseobacterium* species. Our findings suggest that the strain MGC42 is a multidrug-resistant, virulent pathogen and recommend 16S rRNA gene sequencing to identify clinical specimens of *Chryseobacterium* species.

Keywords: *Chryseobacterium gallinarum*, colistin-resistant, capsular polysaccharide (CPS), β-lactamases, uncommon pathogen, α-hemolysis, comparative genomics

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INTRODUCTION

The frequency of healthcare-associated infections caused by rare or uncommon pathogens like *Chryseobacterium* species has risen over the last decade (Booth, 2014). The high rate of infection of these bacterial pathogens attracts attention for proper management as these are inherently resistance to aminoglycosides, aztreonam, cephalosporin, chloramphenicol, clindamycin, erythromycin, imipenem, penicillin (mezlocillin, piperacillin, and ticarcillin), teicoplanin, and tetracyclines (Hsueh et al., 1997; Sharma et al., 2015). These are chemoorganotrophic, glucose-nonfermenting, non-motile, rod-shaped gram-negative, and emerging clinical pathogenic bacteria ubiquitously detected in soil and water (Hugo et al., 2019). Recently, *C. gleum* and *C. oranimense* are reported as the pathogenic species and linked to ventilator-associated pneumonia, urinary tract infection (UTI) (Rajendran et al., 2016), and cystic fibrosis (Sharma et al., 2015). However, their genetic basis of resistance mechanisms, pathogenicity, and virulence is still poorly known.

Among the *Chryseobacterium* sp., *C. indologenes* has been identified as opportunistic pathogens to nosocomial infections in immunocompromised patients of all ages (Reynaud et al., 2007; Smith et al., 2012). It has been well documented in a variety of illnesses (nosocomial pneumonia, intra-abdominal infection, wound infection, bacteremia, UTI, and cellulitis), particularly in those who were hospitalized with long-term indwelling devices and were exposed to broad-spectrum antibiotics for an extended period (Christakis et al., 2005; Chen et al., 2013). Multidrug resistance *in C. indologenes* has been reported due to increased clinical usage of colistin and tigecycline, which poses a concern to patients who had received substantial antibiotic therapy for an extended period (Chen et al., 2013). Although the source of this infection is unknown, the occurrence of MDR *C. indologenes* has been well documented in seawater and marine fauna (Maravić et al., 2013).

This study aims to describe the whole-genome sequence used to insight the resistome, virulome, and toxic profile of colistin-resistant *C. gallinarum*, isolated, for the first time, from the urine of a female patient diagnosed with UTI in Bhubaneswar city at our university's tertiary care hospital. It indicated that *C. gallinarum* might have the potential to grow in uroepithelial cells. This species was first isolated in 2014 from a pharyngeal scrape of a healthy chicken in Germany and showed keratin degrading activity (Kämpfer et al., 2014). Previously, Park et al. (2015) and Kang et al. (2021) have provided genetic insight into the keratin degradation mechanism in this species. We also explored several unique features, i.e., oxidative-stress response, hemolysis activity, and capsular polysaccharide (CPS) secretion ability. We evaluated the shared conserved genes and their potential role in different habitats, including natural and clinical environments through comparative genomics analysis by incorporating genomes of other *Chryseobacterium* species.

METHODOLOGY

Sample Collection, Identification, and Antimicrobial Susceptibility

During a surveillance study conducted during the period of 2018–2019, a colistin-resistant bacteria MGC42 was recovered in Bhubaneswar city at Central laboratory of our university's tertiary care hospital from a 20-year old Outpatient Department (OPD) patient diagnosed with UTI. We initially identified the organism and subsequently tested its antimicrobial susceptibility with the VITEK 2 automated system (BioMérieux, France) using the ID-GNB and AST-381 cards, respectively, in accordance with the manufacturer's instructions. We interpreted the results of antibiotic susceptibility based on the Clinical and Laboratory Standards Institute (Wayne, 2018) breakpoint recommendations. The identity of the strain MGC42 was further verified by amplification and sequencing its 16S rRNA gene using 16S rRNA universal primers (16S-F, 5′-AGAGTTTGATCATGGCTC-3′; 16S-R, 5′-GGTTAC CTTGTTACGACTT-3′). The 16S rRNA gene sequence was then searched using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and compared with other 16S rRNA sequences available in GeneBank of National Center for Biotechnology Information (NCBI). To determine whether the cells produced flexirubin-type pigments, we flooded a mass of bacterial cells collected on a LB agar plate with 20% (w/v) KOH. The mass instantaneously turns dark red/brown (Reichenbach, 1989) if cells produce flexirubin-type pigments, whereas no color change develops if the yellow color is not due to carotenoid type of pigments. Minimum inhibitory concentrations (MIC) values for colistin and meropenem were determined by the broth micro-dilution method using cation-adjusted Mueller–Hinton broth according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2018). The lowest concentration of antibiotics that completely inhibited microbial growth was considered MIC.

DNA Isolation, Whole-Genome Sequencing, Annotation, and Comparative Genomics

We extracted the genomic DNA of the strain by using a modified ROSE (rapid one-step extraction) method (Dey et al., 2020). Briefly, the bacterial cells were harvested at mid-log phase, and pellet was incubated with 500 µl of ROSE solution for 60 min at 90°C with intermittent shaking. Phenol, chloroform, and isoamyl alcohol were subjected at the ratio of 25:24:1. The aqueous layer was separated upon centrifugation at 12,000 × g for 30 min. Precipitation of the sample was done with 2.5 volumes of ethanol at −20°C. DNA pellet was dissolved in 100 µl of T10E1 (pH 8.0) and treated with RNase (10 gm/ml) at 37°C. The extracted DNA was visualized on 0.8% ethidium bromide-stained agarose gel. We outsourced the sequencing of the genome of *Chryseobacterium gallinarum* (MGC42) to Agrigenome, India, where its genome was sequenced on the Illumina HiSeq platform. The NEBNext Ultra DNA Library Kit was used for library preparation by using 100 ng of total DNA. The sequenced pair-end reads were check for qualitative and quantitative analysis using FastQC v.0.11.5 (Andrews, 2010). Adapters were removed, and low-quality ends were trimmed from the sequences with a sliding window of 4 and a minimum quality of 20 using Trimmomatic v0.36 (Bolger et al., 2014). Then, we *de novo* assembled the quality-filtered fastq reads with Unicycler v0.5.0 assembler (Wick et al., 2017). We further rearranged, reconstructed, and scaffolded the assembled genome into lesser number of contigs using Ragout (Reference-

Assisted Genome Ordering UTility) tool based on reference genome of *C. gallinarum* DSM 27622 (GCA_001021975.1) and FDAARGOS_636 (GCA_012273615.1) (Kolmogorov et al., 2014). Finally, we assessed the quality of the genome with QUAST v**5.1** (Mikheenko et al., 2018) tool and compared our genome with the complete genomes of *C. gallinarum* DSM 27622 (GCA_001021975.1) and FDAARGOS_636 (GCA_012273615.1), respectively.

We predicted the Open Reading Frame (ORFs) and annotated our assembled genome using Prokka (Seemann, 2014) of contigs with length ≥200 bp. We identified the biological pathways and molecular functions of the predicted ORFs using the kofamKOALA (Aramaki et al., 2020). We then identified the antibiotic resistance genes by using the CARD, ARG-ANNOT, ResFinder, and NCBI AMR databases. By implanting k-mer (PATRIC), machine learning, and Hidden Markov models (HMM)-based classifier (PathoFact) (de Nies et al., 2021), we further improved the antibiotic resistance, virulence, and toxin gene annotation. We identified phage sequences in the genome using PHASTER (PHAge Search Tool Enhanced Release) (Arndt et al., 2016). We submitted our genome assembly to the TYGS online server (Meier-Kolthoff and Göker, 2019) for whole-genome–based taxonomic analysis and determination of closest type strain genomes. A cladogram was then inferred on the basis of Genome BLAST Distance Phylogeny approach (GBDP) and visualized in FigTree (https://github.com/rambaut/figtree).

We compared the complete genome of *C. gallinarum* MGC42 to the complete genome of *C. gallinarum* DSM 27622, *C. gallinarum* FDAARGOS_636, *C. contaminans* DSM 27621, *C. oranimense* G311, and *C. indologenes* MARS15 in terms of their shared orthologous, core, and accessory genes using OrthoVenn (Xu et al., 2019) and Roary (Page et al., 2015). We identified and located the O-antigen cluster based on homology with o-antigen cluster of *C. oranimense* G311, *C. gelum* ATCC 35910, and *Chryseobacterium* spp. CF314 (Sharma et al., 2015).

Characterization of Virulence-Associated Phenotypes

We studied the external morphology of *Chryseobacterium gallinarum* MGC42 using transmission electron microscopy (TEM). We incubated the strain MGC42 overnight in Luria Bertini (LB) broth and then diluted this culture to 5×10^6 cells (OD 0.05) in tryptic soy broth. We loaded 10 µl of culture into electron microscopy grids and dried them under light exposure. We soaked the spare culture in tissue paper. We finally added 2% of caesium chloride to this dried culture and dried it again under light exposure. Finally, we performed TEM at an operating voltage of 200 kV and visualized the images at magnification of ×14,500. We used the ethidium bromide (EtBr) cartwheel method as described by Martins et al., 2011 to determine the Efflux pump activity of MGC42. We used *E. coli* ATCC 25922 and *K. pneumoniae* SDL79 (Dey et al., 2020) as reference and positive control, respectively. We poured Tryptic Soy Agar nutrient media with a range of EtBr concentrations (0–5 µg/ml) in Petri plates and streaked the overnight culture of these strains in a cartwheel pattern on the plates. The plates were then incubated overnight at 37°C and observed under UV for fluorescence (Bio-Rad Gel-Doc XR system, Hercules, CA, USA). We validated the presence of functional catalases by bubble test (catalase test) using the slide method (Reiner, 2013; Kataria et al., 2016). We added about three to four drops of 3% medical hydrogen peroxide (H_2O_2) to the fresh culture cell mass and immediately observed the slides for bubble formation, to check for the presence of catalase.

β-hemolysis involves the total lysis of the red blood cells and is marked by the formation of a clear and transparent zone surrounding the colony on the agar plate, whereas α-hemolysis is marked by a distinct greenish zone around the colonies. We inoculated the MGC42 strain on nutrient agar plate supplemented with 5% sheep blood and incubated the plate at 37°C under aerobic conditions for 16–18 h. We observed the results under a bright light. The plates used were ready-made and were procured from HiMedia Laboratories Pvt. Ltd.

RESULT AND DISCUSSION

Identification Anomaly, Antibiogram Profiling, and Diagnostic Prospect

In the present study, we recovered the strain *Chryseobacterium gallinarum* MGC42 from the urine sample of a 20-year-old pregnant outpatient diagnosed with suspected UTI at the tertiary care hospital of our university. This isolate was also characterized by distinct bright yellow–colored colonies. The colonies displayed a drastic shift from their characteristic yellow color to brown when flooded with 20% KOH, suggesting that this coloration may have been imparted by the secretion of flexirubin type of pigment (**Supplementary Figure S1**) (Weeks, 1981).

This isolate was identified by the VITEK-II automated system (BioMérieux, France) as C. Indologenes. However, to our surprise, this strain was later reidentified as C. Gallinarum by amplification and sequencing of the 16S rRNA gene. Lin et al., 2017 detected a very low concordance between automated bacterial identification systems like VITEK-II, VITEK MS and molecular typing methods like 16S rRNA gene sequencing for the identification of Chryseobacterium species. This probably explains the identification anomaly in our study. The inability of such a system to distinguish between C. gallinarum and C. indologenes may create a false impression suggesting that the prevalence of C. gallinarum is low. Hence, there is every possibility that the prevalence of C. gallinarum may have been underestimated. Therefore, we propose the identification of Chryseobacterium species by automated systems like VITEK-II and VITEK MS that are over-reliant on factory default databases and often lack timely amendment must always be supplemented with 16S rRNA gene sequencing to confirm their identity which in turn will help guide proper therapeutic decisions.

The strain MGC42 was also resistant to several classes of antibiotics including penicillin, carbapenems, aminoglycoside, tetracycline, and polypeptide/polymyxin. Nevertheless, *Chryseobacterium* species have also been documented to be susceptible to some antibiotics. In our study, the strain MGC42

TABLE 1 | Antimicrobial susceptibility testing using VITEK 2 system and micro-broth dilution–based MIC and MBC of *C. gallinarum* MGC42.

Group of Antibiotic/Drug Class	Antimicrobial	MIC (VITEK 2)	Interpretation	MIC (MBD)	MBC
Aminoglycoside	Amikacin	≥64	R	NT	NT
	Gentamicin	≥16			
	Netilmicin	≥32			
Carbapenems	Imipenem	≥16	R		
	Meropenem	≥16		≥16	32
Cephalosporins, Third Generation	Ceftazidime	ND		NT	NT
	Cefoperazone-Sulbactam	32	I		
Cephalosporins, Fourth Generation	Cefepime	ND			
Fluoroquinolone	Ciprofloxacin	1	S		
	Levofloxacin	1			
Glycylcycline	Tigecycline	≥8	R		
Penicillins	Ticarcillin-Clavulanic Acid	≥128	R		
	Piperacillin-Tazobactam	≥128			
Phosphonic	Fosfomycin	ND			
Polymyxin/Polypeptide	Colistin	≥16	R	≥1024	>1024
Sulphonamide	Trimethoprim-Sulfamethoxazole	≤20	S	NT	NT
Tetracycline	Minocycline	≤1	S		

**R, resistant; I, intermediate; S, sensitive; ND, not determined; NT, not tested.*

was similarly found to be susceptible to fluoroquinolones, sulphonamides, and tetracycline. To the best of our knowledge, the antibiotic susceptibility pattern of *C. gallinarum* has never been studied before. The antibiotic susceptibility pattern of the MICs of colistin and meropenem was ≥1,024 and ≥16 μg/ml, respectively, which agreed with the antibiotic susceptibility results derived from VITEK 2 (**Table 1**). This is particularly worrying as carbapenems and polymyxins are often the last viable options for the treatment of gram-negative bacterial infections (Armstrong et al., 2021; Mohapatra et al., 2021).

General and Specific Features of the *C. gallinarum* MGC42 Genome

A total of 6,557,593 paired reads were quality-filtered with 37.9% GC content. Furthermore, after assembly and reorientation, we obtained the size of the *C. gallinarum* MGC42 genome to be 4,349,499 bp with 96.67% genome coverage and 37.08% GC content and divided over into 61 contigs. All the contigs belong to the chromosomal DNA as no plasmid was detected (**Supplementary Table S1**). The Type (Strain) Genome Server (TYGS) returned *C. gallinarum* DSM 27622 as the closest type with 98% similarity (**Figure 1A**). The Prokka predicted a total of 3,879 ORFs, which includes 3,799 protein-coding ORFs and 80 RNAs (three rRNAs, 63 tRNAs, one tmRNAs, and 13 misc_RNAs). Of the 3,879 ORFs, 1,911 (49.26%) were assigned a putative function, whereas 1,968 (50.73%) were annotated as hypothetical proteins.

A total of 1,246 unique K numbers were assigned to 1,531 (39.46%) ORFs, which are further mapped to 240 different KEGG pathways based on the scoring criteria (**Supplementary Table S2**). The top mapped pathways were metabolic pathways (462), biosynthesis of secondary metabolites (220), microbial metabolism in diverse environments (120), biosynthesis of cofactors (105), and carbon metabolism (72). The pathways for metabolism in diverse envirnoments were ranked third in genome of MGC42 strain. This finding corroborates with the fact that Chryseobacterium species are ubiquitously distributed in the natural environment (Bernardet et al., 2006).

Using the combination of AMR databases, k-mer, and a machine learning–based classifier–based improved annotation, we identified the 12 antimicrobial resistance (AMR) genes belonging to resistance mechanism like antibiotic efflux (6), inactivation (3), target alteration (3), and target replacement (1) including two putatively novel β-lactamases (**Supplementary Table S3**). Our findings show that the AMR gene identification is consistent with the antibiotic resistance profile of MGC42. We also screened the homologs of the genes commonly associated with colistin resistance (*pho*P, ORF01530; *pho*Q, ORF00881; *pmr*A, ORF00440; *pmr*B, ORF00439; *lpx*A, ORF00011; *lpx*C, ORF00012; *lpx*D, ORF00013) for mutation analysis. Our study revealed that only *pmr*B and *lpx*D harbor single-point mutation. The two-component protein *pmr*B harbors a single-point mutation at position 384 (M384T). Similarly, the third enzyme of the LPS biosynthesis pathway (*lpx*D) harbors a single-point mutation at position 66 (I66V). These mutations could most likely be responsible for colistin resistance in *C. gallinarum* MGC42 (**Supplementary Figure S2**).

Three-hundred forty genes are predicted as a secreted and non-secreted virulence factors (VFs) (**Supplementary Table S4**). Most ORFs belonged to the type I secretion system (T1SS), type VI secretion system (T6SS), bacterial secretion system (Sec), and quorum sensing (QS) categories of VFs (**Table 2**). To the best of our knowledge, virulence profile of *C. gallinarum* has never been studied before. These predicted VFs further need to be classified into different VF categories, which need deep-annotation and further experimental validations.

While understanding the pathogenicity of a bacterium, toxins including neurotoxin play a key role in causing severe human ailments (Mansfield et al., 2019). Therefore, to answer this, we also explored the genome of the strain MGC42 using a state-of-the-art approach as to date, no experimental and sequencing information about the toxicity of this species is available. In this regard, we predicted 28 putative secretory and non-secretory toxins. Apart from this, we predicted 39 uncharacterized ORFs, which could potentially belong to a novel toxin family and might

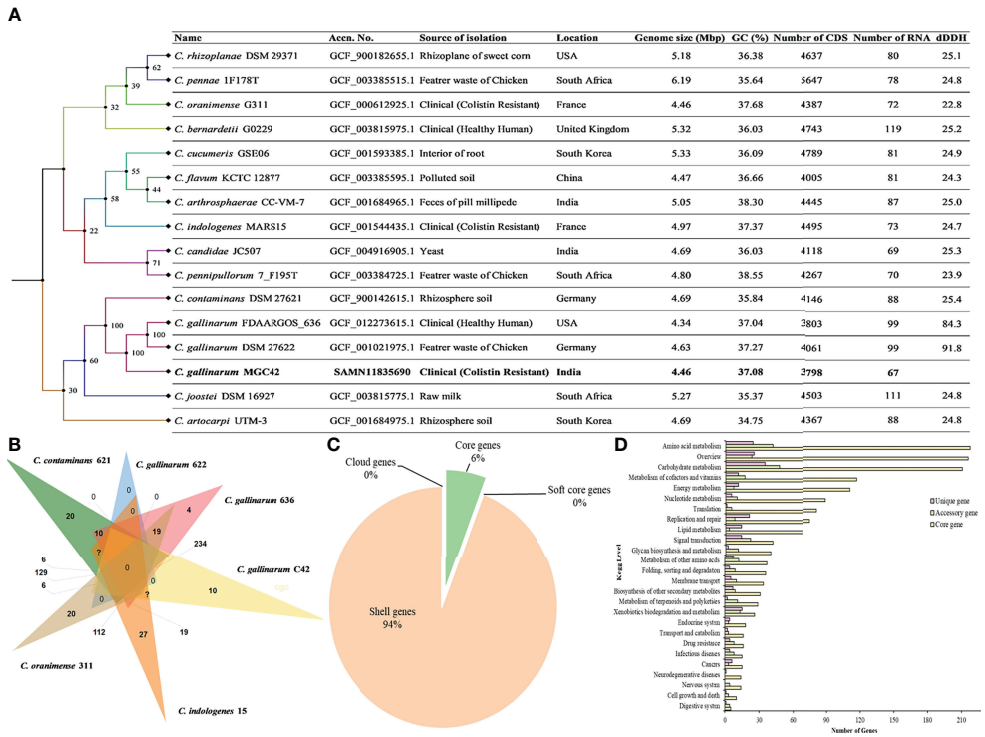

FIGURE 1 | **(A)** Whole-genome–based phylogenetic tree between *C. gallinarum* MGC42 and other *Chryseobacterium* strain. Sequence highlighted the position of *C. gallinarum* MGC42 with their metadata. Phylogenetic inferences were obtained through TYGS tools. The branch color represents the bootstrap value. The branches of the tree are indicated by the genus and species name with type strains followed by other metadata of the respective species. **(B)** Venn diagrams generated by OrthoVenn show the distribution of shared and unique genes among six different sets of *Chryseobacterium* spp. **(C)** Core and accessory genes of *C. gallinarum* strains. **(D)** The numbers of core, cloud, soft, and shell genes were calculated by Roary. The core genes are shared by all the included organisms in the pangenome analysis.

be species-specific, which needs further *in silico* and *in vitro* characterization (**Supplementary Table S5**).

The cluster of orthologous gene of the strain MGC42 was compared with *C. gallinarum* DSM 27622, *C. gallinarum* FDAARGOS_636, *C. contaminans* DSM 27621, *C. oranimense* G311, and *C. indologenes* MARS15 to provide insights into conserved cellular components, biological processes, and molecular functions. It was found that among 4,277 clusters, 3,080 orthologous clusters contain at least three species, 1,368 orthologous clusters contains at least two species, and 1,482 singletons. Of this, 78 essentially *paralogous* genes (*in-paralogs*) were predicted in *C. gallinarum* MGC42 that could be due to the divergence of lineages and duplication within the lineage of *C. gallinarum* species (**Figure 1B**).

The prediction of conserved homologous/orthologous was further improved by performing core-genome analysis in between all six species using Roary. The core-genome analysis genomes revealed that they all shared 683 genes (6%) in the core region of their genomes, whereas 11,120 genes (94%) are found as accessory/shell genes. The majority (~73%) of accessory genes were strain-specific having a specific role to interact with the host or helping in niche adaptation (**Figure 1C**). Among them, 2,427 genes are shared by *C. gallinarum* DSM 27622 (Chicken feathers), *C. gallinarum* FDAARGOS_636 (Healthy Human), and *C. gallinarum* MGC42 (patient with UTI). Moreover, we also identified a novel toxin Ntox23 domain (PF15528) containing proteins (ORF01631 and ORF00315) of polymorphic toxin systems with conserved ND, DxxR motifs, and a histidine residue, which is exported by TcdB/TcaC secretion system in the strain MGC42 (**Supplementary Table S5**) (Zhang et al., 2012). We hypothesize that this additional VF identified only in the strain MGC42 might have a role to play in its ability to colonize and infect human host. At functional level, the core genes were mostly associated with amino acid metabolism, carbohydrate metabolism, metabolism of cofactors and vitamins, energy metabolism, nucleotide metabolism, and translation. However, the unique and accessory genes have higher proportion of carbohydrate metabolism, amino acid metabolism, signal transduction, metabolism of cofactors and vitamins, xenobiotics biodegradation, and metabolism categories (**Figure 1D**, **Supplementary Figure S3**).

Genome mining through antiSMASH revealed distinctive secondary metabolite clusters, like resorcinol/arylpolyene (felxirubin-like), non-ribosomal peptide synthetases (NRPS), lanthipeptide, microviridin, and siderophore (putrebactin/avaroferrin) biosynthetic gene clusters (BGCs). In contrast, we

TABLE 2 | List of predicted virulence factors and their respective VF classes.

VF Class	Subclass	ORF	KO Number	Gene	Description
Type I secretion system	ABC transporters	ORF00074	–	hlyD	Hemolysin secretion protein d
		ORF03531	K02065	MetN	Methionine import atp-binding protein
		ORF00740	K02066	MlaE	Intermembrane phospholipid transport system permease protein
		ORF00727	K02067	hp	Hypothetical protein
		ORF03531	K02071	MetN	Methionine import atp-binding protein
		ORF03532	K02072	MetI	D-methionine transport system permease protein
		ORF03533	K02073	MetQ	D-methionine-binding lipoprotein
		ORF00001	K06861	LptB	Lipopolysaccharide export system atp-binding protein
		ORF02984	K07091	hp	Hypothetical protein
		ORF01293	K09690	hp	Hypothetical protein
		ORF01292	K09691	TagH	Teichoic acids export atp-binding protein
		ORF03745	K09808	LolE	Lipoprotein-releasing system transmembrane protein
		ORF02767	K09810	LolD	Lipoprotein-releasing system atp-binding protein
		ORF02242	K09811	FtsX	Cell division protein
		ORF00530	K09812	FtsE	Cell division atp-binding protein
		ORF01202	K11720	hp	Hypothetical protein
		ORF00500	K18889	YheI	Putative multidrug resistance abc transporter atp-binding/permease protein
		ORF00396	K18890	NA	Putative abc transporter atp-binding protein
		ORF00500	K18889	YheI	Putative multidrug resistance abc transporter atp-binding/permease protein
		ORF00396	K18890	NA	Putative abc transporter atp-binding protein
Type VI secretion system		ORF01931	–	vgrG1a	NA
		ORF02111	–	vgrG1c	NA
		ORF01506	–	Hp	NA
		ORF02959	–	vgrG1a	NA
		ORF03302	–	Hp	NA
Bacterial secretion system (Sec)		ORF00833	K03070	SecA	Preprotein translocase subunit
		ORF00433	K03073	SecE	Preprotein translocase subunit
		ORF01866	K03075	SecG	Preprotein translocase subunit
		ORF01412	K03076	SecY	Preprotein translocase subunit
		ORF03130	K03106	SRP54	Signal recognition particle subunit
		ORF00194	K03110	ftsY	Fused signal recognition particle receptor
		ORF01523, ORF02447	K03116	TatA	Sec-independent protein translocase protein
		ORF02447	K03117	TatB	Sec-independent protein translocase protein
		ORF03209	K03118	TatC	Sec-independent protein translocase protein
		ORF00497	K03210	YajC	Preprotein translocase subunit
		ORF00344	K03217	YidC/Oxa1	Yidc/oxa1 family membrane protein insertase
		ORF01506, ORF01931, ORF02111, ORF02959, ORF03302	K11904	NA	T6ss
		ORF02441	K12257	SecD/SecF	Secd/secf fusion protein
		ORF00982, ORF03199, ORF03646	K12340	NA	Outer membrane protein
Quorum sensing		ORF00084	K20483	nisB	Nisin biosynthesis protein nisb
		ORF00085	K20483	Hp	Hypothetical protein
		ORF00194	K03110	ftsY	Signal recognition particle receptor ftsy
		ORF00227	K18139	oprM	Outer membrane protein oprm
		ORF00344	K03217	yidC2	Membrane protein insertase yidc 2
		ORF00433	K03073	Hp	Hypothetical protein
		ORF00497	K03210	yajC	Sec translocon accessory complex subunit yajc
		ORF00604	K01114	plcN	Non-hemolytic phospholipase c
		ORF00833	K03070	secA	Protein translocase subunit seca
		ORF01052	K18139	oprM	Outer membrane protein oprm
		ORF01412	K03076	secY	Protein translocase subunit secy
		ORF01520	K01497	ribA	Gtp cyclohydrolase-2
		ORF01675	K11752	ribD	Riboflavin biosynthesis protein ribd
		ORF01820	K18139	oprM	Outer membrane protein oprm
		ORF01866	K03075	Hp	Hypothetical protein
		ORF02228	K01897	FadD15	Long-chain-fatty-acid–coa ligase fadd15
		ORF02239	K20276	Hp	Hypothetical protein

(Continued)

TABLE 2 | Continued

VF Class	Subclass	ORF	KO Number	Gene	Description
		ORF02328	K01658	pabA	Aminodeoxychorismate synthase component 2
		ORF02329	K01657	trpE	Anthranilate synthase component 1
		ORF02441	K12257	secDF	Protein translocase subunit secdf
		ORF02582	K15657	srfAD	Surfactin synthase thioesterase subunit
		ORF02628	K13075	NA	Putative metallo-hydrolase
		ORF02964	K18139	oprM	Outer membrane protein oprm
		ORF03078	K20483	Hp	Hypothetical protein
		ORF03079	K20483	nisB	Nisin biosynthesis protein nisb
		ORF03080	K20484	Hp	Hypothetical protein
		ORF03130	K03106	ffh	Signal recognition particle protein
		ORF03458	K06998	yddE	Putative isomerase ydde
		ORF03678	K01897	FadD15	Long-chain-fatty-acid–coa ligase fadd15

further explored the felxirubin-like pigment synthesis genes cluster that shared 75% similarity with known flexirubin biosynthesis cluster (BGC0000838), indicating that it is most likely novel and species-specific (**Figure 2B**). Furthermore, we also located the genes cluster of o-antigen (lipopolysaccharide biosynthesis) as shown in **Figure 2A**, whereas the genes of k-antigen cluster (CPS biosynthesis) as they are not in continues sequence in the genome and could be due the unfinished draft genome (**Supplementary Table S6**). The phage-driven gene flow analysis using the online server PHASTER revealed five different incomplete phage-like regions encoded the Phage-like Protein, Coat Protein, Tail Shaft, Fiber Protein, Terminase, and portal protein as shown in the **Supplementary Figure S4**.

Identification of novel of β-Lactamase Genes

We identified an 879-bp ORF (ORF03368) encoding a protein having 292–amino acid residues sharing 73.29% amino acid identity with $bla_{\text{CIA-4}}$, an extended-spectrum β-lactamase encoded on a 143.85-kb contig (**Supplementary Figure S5A**). A serine active site characteristic of β-Lactamases was found within this mature protein sequence. It has all the four conserved elements of class A β-Lactamases: a Ser-X-X-Lys consensus active site serine residue at position 70 (Fan et al., 2007), an SDN loop at position 130, a conserved Glu166, and a KTG sequence at position 234. This distantly related Ambler class A $bla_{\text{CIA-like}}$ gene has a G+C content of 41% and is most likely responsible for β-lactam antibiotics resistance in this organism. We identified another 723-bp ORF (ORF03015) encoding a 240–amino acid residue protein in a 203.52-kb contig. It shares 82.92% amino acid identity with $bla_{\text{CGB-1}}$, a class B β-lactamase and is most likely responsible for resistance to imipenem (**Supplementary Figure S5B**). Both genes are most likely novel and require further biochemical characterization. These AR genes were also flanked by many hypothetical proteins with no BLAST hit. There might be a possibility that those sequences carry transposases and unknown insertion elements responsible for the mobilization of these genes.

Pathogenic Characterization of MGC42

The TEM of *C. gallinarum* MGC42 strain in presence of negatively stained CsCl revealed the presence of secreted capsular material (**Supplementary Figure S6**). From genome analysis, we located 21 homologous ORFs related to CPS biosynthesis in a different region of the first contigs and could be a putatively novel cluster specific to this species (**Supplementary Table S6**). The capsular material that is anchored on the outermost layer of the cell is often involved in

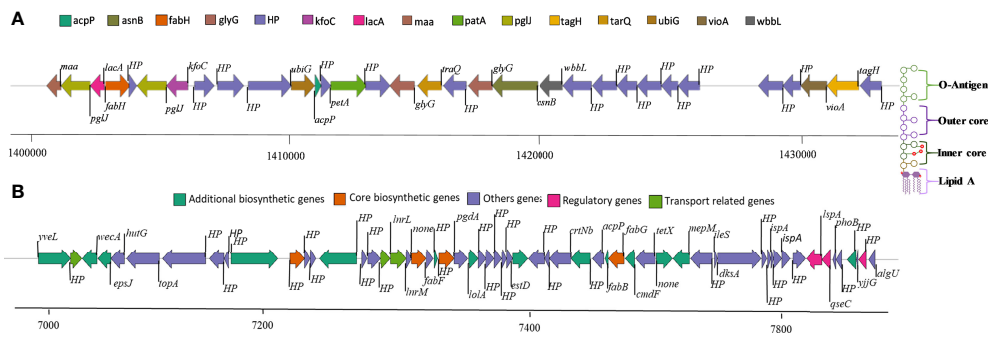

FIGURE 2 | **(A)** O-antigen synthase operon arrangement in *C. gallinarum* MGC42 starting from 1411059 to 1420661 bp. **(B)** Polyketide synthesis operon arrangement in *C. gallinarum* MGC42 at ORF 700929 to 765695. Genes of the same functional classification are shown in the same color.

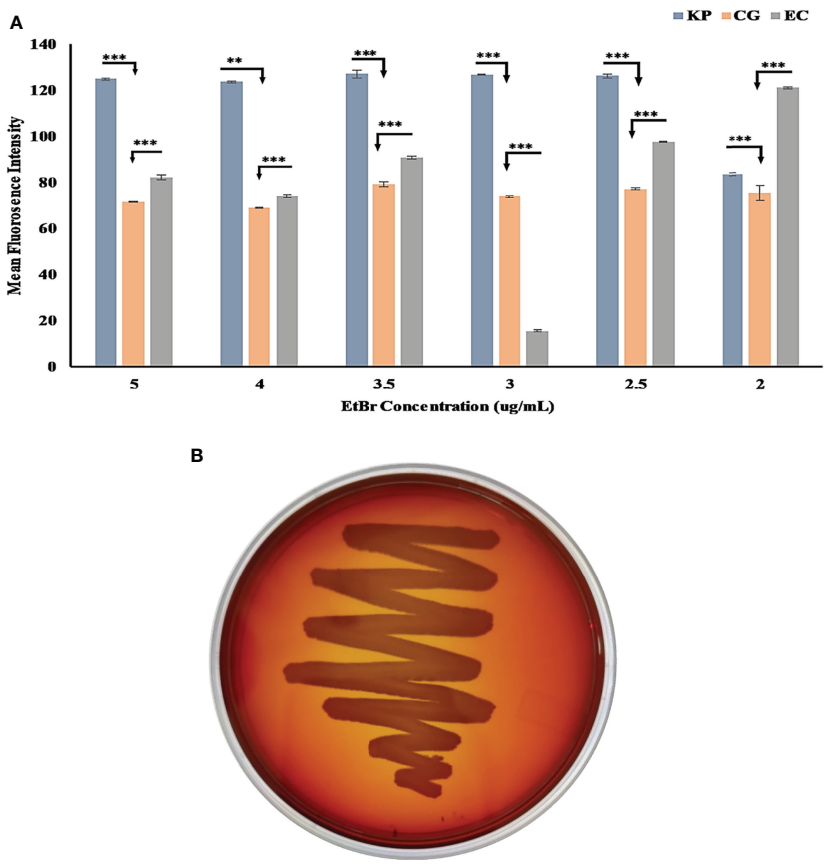

FIGURE 3 | **(A)** Bar graph of *C. gallinarum* C42 and *K. pneumoniae* SDL79 strains along with *E. coli* ATCC 25922 by the EtBr agar method. The error bar represent the mean standard error, whereas the star represents the significant difference in efflux activity between pair of SDL79 - MGC42 and ATCC 25922 - MGC42 at different p-value (* <0.05; ** <0.01; *** <0.001). The x-axis represents the concentration of EtBr, and the y-axis represents the mean fluorescence intensity area value. **(B)** *C. gallinarum* MGC42 showing α-hemolysis in greenish color on 5% sheep blood agar plates. Culture was incubated at 37°C for 18 ± 2 h.

mediating direct interactions between the bacteria and its environment and is therefore considered an important VF for many bacterial pathogens (Taylor and Roberts, 2004). Apart from pathogenicity due to CPS, this could be one of the reasons for contributing toward colistin resistance by MG42 strain that is further supported by the report on colistin resistance mechanism acquired by *K. pneumoniae* due to the presence of secreted capsular material (Campos et al., 2004).

The MGC42 strain showed a moderate efflux activity in all concentrations of ethidium bromide (EtBr) starting from 2.0 to 5.0 μg/ml (**Figure 3A**) in comparison with *E. coli* ATCC 25922 (non-pathogenic) and *K. pneumoniae* SDL79 (pathogenic) (Dey et al., 2020). As this strain has moderate efflux activity, we further improved the annotation of ORFs using the KEGG database. A total of 110 ORFs were assigned with K numbers through KEGG mapping and classified into 14 unique superclasses of transporter genes and might be involved in pathogenicity in many different ways. It includes ABC-2 type, drug aquaporins, organic acid protein small neutral solute, metallic cation, iron-siderophore, vitamin B12, saccharide, polyol, lipid, phosphate, amino acid, and unknown transporter superclasses (**Supplementary Table S7**). In addition to their role in resistance mechanism, it is proven that efflux pumps are key factors involved in the detoxification of intracellular metabolites, exporting VFs, biofilm formation, pH homeostasis, and QS (Martinez et al., 2009; Teelucksingh et al., 2020; Alav et al., 2021). However, to date, no documental evidence is reported that explains the role of efflux pumps in this species. These transporters might have a role to play in this strain's niche adaptation.

Catalase is one of the key antioxidant enzymes, and it appears to be involved in protection against immune infection and oxidative stress. The excessive hydrogen peroxide (H_2O_2) produced by the host defense response is a poisonous compound to bacteria and acts as a second messenger in signal transduction pathways. At the genomic level, the hydrogen peroxide–inducible gene activator (*Oxy*R: ORF00677) and a LysR family transcriptional regulator are involved in the hydrogen peroxide (H_2O_2) defense mechanism through the activation of the catalase enzymes (Parvatiyar et al., 2000). The presence of ORFs for catalase-peroxidase (*kat*G: ORF0165 and *kat*E: ORF2808) enzymes in the genome of the MGC42 strain is known to reduce H_2O_2 to water and oxidize it to molecular oxygen (Zamocky et al., 2008). This was further

supported by glass slide–based catalase test, where upon the addition of three to four drops of 3% medical H_2O_2, the MGC42 strain immediately exhibited bubbling effect due to breakdown of H_2O_2 into water and oxygen (**Supplementary Figure S7**). However, the non-pathogenic *E. coli* ATCC 25922 strain, which is taken as negative control, ensures no production of oxygen bubbles.

Species from the genus *Chryseobacterium* have shown no-hemolysis to strong hemolysis activity (Hsueh et al., 1996; Mehta and Pathak, 2018). When the MGC42 strain was grown on 5% sheep blood agar nutrient media for 16 to 18 h, the hemoglobin of blood cells around and below the colonies was reduced to green methemoglobin, which was confirmed by the strain's α-hemolytic activity (**Figure 3B**). The oxidation of oxy-hemoglobin (Fe+2) to non-oxygen-binding methemoglobin (Fe +3) by hydrogen peroxide causes α-hemolysis (McDevitt et al., 2020). The *hly*D (ORF00074) protein, the second component of the hemolysin secretion system (T1SS), is responsible for this phenomenon; however, we did not find any other component of this system in the genome of the MGC42 strain.

Transition From the Environment to Human

This study provides insight into changes in major genomic features, specifically unique genes, indicating the uncommon potential of a new clinically identified bacterium *C. gallinarum* MGC42 isolated initially from the pharyngeal scrape of a health chicken (Kämpfer et al., 2014).

Unravelling the regulatory systems that govern bacterial pathogens' transition from a free-living non-pathogenic state to a virulent state will be the next critical step toward understanding *C. gallinarum* to establish strategies for regulating its spread. Similarly, the signaling pathways that may have driven the formation of its multicellular communities in actual hosts must be determined in the future. The *C. gallinarum* MGC42 could be used as a model organism to understand their diagnosis, the genetic basis of disease, and treatment that could take a giant leap forward with the creation of networks to connect clinicians with geneticists and their lifestyle switching. In addition, this bacterium could be an opportunistic human pathogen in immunocompromised patients in future.

Uncharacterized VFs, mechanisms of pathogenesis, and the absence of ecological and epidemiological knowledge compounded by existing resistance to several classes of antibiotics could make treatment of *C. gallinarum* infection challenging. Moreover, the latest VITEK-GNI card's failure to distinguish this organism from *C. indologenes* indicates that its prevalence in the hospital may have been significantly undermined. Our finding warrants the implementation of molecular typing to direct appropriate antibiotic regimens without over-reliance on the VITEK 2 system that utilizes the factory default database lacking in the timely amendment. Therefore, we recommend more prolonged periods of laboratory-based surveillance with population-based data to determine the prevalence of *C. gallinarum* infections. To the best of our knowledge, this is the first documented evidence of the emergence of *Chryseobacterium gallinarum* as a human pathogen.*in vitro*

DATA AVAILABILITY STATEMENT

The whole-genome sequence of the C. gallinarum MGC42 has been deposited in NCBI under the GenBank assembly accession: GCA_023604885.1.

AUTHORS CONTRIBUTIONS

MG, SDe, AS and ES: Conceptualization; MG, SDe, AS, SS, and JZ: Investigation; MG, SDe, SDi, and DB: Formal analysis; MG, SDi, and SS: Visualization; MG, SDe, AS, RS, and ES: Writing-original draft; All author: Writing-review and editing; ES: Funding acquisition; M and ES: Project administration and supervision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.933006/full#supplementary-material

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Co-occurrence of dual carbapenemases KPC-2 and OXA-48 with the mobile colistin resistance gene *mcr-9.1* in *Enterobacter xiangfangensis*

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Bacterial infections with the genus *Enterobacter* are notoriously difficult to treat and often associated with resistance to penicillin, aminoglycosides, fluoroquinolones, and third-generation cephalosporins. Also, *Enterobacter* species have emerged as the third most common hosts for carbapenemases worldwide, forcing the use of colistin as a "last-resort" antibiotic for the treatment. Studies on the population structure of the genus *Enterobacter* repeatedly detect *E. xiangfangensis* as a common clinical species present worldwide. Here, we report on the characteristics of an extreme drug-resistant *E. xiangfangensis* isolate va18651 (ST88), obtained from a cervical swab of an expectant mother. The isolate was resistant to almost all the classes of antibiotics tested, including β-lactams (viz., penicillins, carbapenems, cephalosporin, monobactams, and their combinations), quinolone, aminoglycosides, and sulfonamide/dihydrofolate reductase inhibitor, and exhibited heteroresistance towards colistin. Analysis of its complete genome sequence revealed 37 antibiotic resistance genes (ARGs), including *mcr-9.1*, bla_{KPC-2}, and bla_{OXA-48}, encoded on three of the four different plasmids (cumulative plasmidome size 604,632 bp). An unusually high number of plasmid-based heavy metal resistance gene (HRG) clusters towards silver, arsenate, cadmium, copper, mercury, and tellurite were also detected. Virulence genes (VGs) for the lipopolysaccharide and capsular polysaccharide structures, iron acquisition (*iroBCDEN*, *ent/fep/fes*, *sitABCD*, *iut*, and *fur*), and a type VI secretion system, together with motility genes and Type IV pili, were encoded chromosomally. Thus, a unique combination of chromosomally encoded VGs, together with plasmid-encoded ARGs and HRGs, converged to result in an extreme drug-resistant, pathogenic isolate with survival potential in environmental settings. The use of a disinfectant, octenidine, led to its eradication; however, the existence of a highly antibiotic-resistant isolate with significant virulence potential is a matter of concern in public health

settings and warrants further surveillance for extreme drug-resistant *Enterobacter* isolates.

KEYWORDS

***Enterobacter*, *xangfangensis*, extreme-drug resistance, mobile colistin resistance, MCR-9.1, dual carbapenemases KPC-2 and OXA-48, plasmid**

Introduction

The genus *Enterobacter* of the bacterial order *Enterobacterales* comprises environmental and clinical species (Davin-Regli et al., 2019). Clinical isolates of *Enterobacter* spp. are primarily opportunistic pathogens (Sanders and Sanders, 1997), involved mainly in hospital-associated infections of the urinary and respiratory tract as well as bloodstream infections (Kang et al., 2004; Ramirez and Giron, 2021). Due to similarities in the taxonomically relevant characters, isolates of different *Enterobacter* species have been misidentified as "*E. cloacae*" or "*Enterobacter* species" or "*Enterobacter cloacae* complex (ECC)", and remained unrecognized with respect to their true species nomenclature (De Florio et al., 2018; Wu et al., 2020; Godmer et al., 2021). With the introduction of the high-resolution tools based on whole-genome sequencing in bacterial taxonomy, the precise delineation of bacterial species became possible (Hugenholtz et al., 2021). The genomically revised taxonomic structure of *Enterobacter* revealed *Enterobacter xiangfangensis* (also referred to as *Enterobacter hormaechei* subspecies *xiangfangensis*) as a common pathogenic species worldwide (Chavda et al., 2016; Peirano et al., 2018; Sutton et al., 2018; Wu et al., 2020; Cho et al., 2021; Wu et al., 2021).

Enterobacter species are frequently resistant to first-line antibiotics such as third-generation cephalosporin, penicillin, aminoglycosides, and quinolones, and fourth-generation cephalosporin and carbapenems are currently the most attractive therapeutic options (Davin-Regli et al., 2019). However, for the last 15 years, carbapenem resistance has been increasingly reported from *Enterobacterales* (Potter et al., 2016), with isolates of *Enterobacter* ranked among the top three in this group (Cerqueira et al., 2017). The most predominant carbapenemase type found in *E. xiangfangensis* was NDM, followed by VIM, KPC, OXA-48, and IMP (Peirano et al., 2018), while GIM-1, GIM-2, and IMI-9 were observed only sporadically (Wendel and MacKenzie, 2015; Di Luca et al., 2016; Wendel et al., 2018). Mobile genetic elements such as plasmids, particularly insertion sequence (IS) elements, were observed as a major determinant for the spread of these carbapenemase genes (Chavda et al., 2016; Potter et al., 2016).

For the "last-resort antibiotic", such as colistin, *Enterobacter* species have been considered to be susceptible (WHO, 2018; WHO, 2021). Nevertheless, certain phylogenomic groups of this species exhibit colistin heteroresistance often leading to treatment failures as such isolates may initially be classified as being susceptible (Guérin et al., 2016; Mushtaq et al., 2020). A recent study shows that PhoPQ-dependent regulation of the *arnBCADTEF* gene cassette for transfer of 4-amino-4-deoxy-l-arabinose (l-Ara4N) to lipid A underlies colistin heteroresistance and resistance (Kang et al., 2019).

A trend of cumulative mobile resistance genes has been noted among members of *Enterobacterales*, including isolates of *Enterobacter* (Wang et al., 2019; Yang et al., 2022). Isolates carrying multiple resistance genes against different classes of antibiotics are increasingly reported (Chavda et al., 2019; Li et al., 2019). These genes may be juxtaposed within a single cassette, in different combinations, or on different plasmids within a single isolate. Co-existence of multiple resistance genes engenders extreme drug resistance and treatment of these infections are protracted with few therapeutic options remaining. Surveillance of such extensive or extreme drug-resistant pathogens and respective resistance genes harboring genetic elements is important to understand the epidemiology of dissemination and develop control strategies.

The SurvCARE study monitors the incidence of carbapenem-resistant *Enterobacterales* (CRE) in patients admitted in hospitals across the state of Hessen in Germany. During the surveillance (period 2017–2019), we noticed an overall increase in the number of the two-carbapenemase-carrying CRE isolates from 1.3% (1/79) in 2017, to 4.4% (5/113) in 2018, to 5.6% (9/162) in 2019, and detected an extreme drug-resistant (XDR) *E. xiangfangensis* isolate va18651, which was subsequently found, following whole-genome sequence analysis, to carry two carbapenemases, i.e., KPC-2 and OXA-48, as well as the mobile colistin resistance *mcr-9* gene. Further analysis reveals that the extreme drug resistance capability is plasmid-based, attained through the acquisition of four different plasmids, three of which carried 37 different antibiotic resistance genes that included two different carbapenemase genes $bla_{\text{KPC-2}}$ and $bla_{\text{OXA-48}}$ as well as the mobile colistin resistance gene *mcr-9.1*.

Materials and methods

Isolate, identification, and antibiotic resistance testing

During the 3-year surveillance study on carbapenem resistance (SurvCARE Hesse), the isolate va18651 was obtained from a cervical swab of an expectant mother during a routine checkup in November 2018. As a routine process, the swab samples were streaked on the Columbia blood and MacConkey agar plates. Colonies grown after overnight incubation at 37°C were randomly selected and identified by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS) (Vitek MS, bioMérieux, Nürtingen, Germany). The antimicrobial susceptibility testing was performed by using commercial MICRONAUT MIC-Strip (MERLIN Diagnostika GmbH, Bornheim, Germany), as well as by using cation-adjusted Mueller-Hinton broth 2. Results were interpreted based on the criteria of the European Committee on Antimicrobial Susceptibility Testing (Version 12.0). *E. coli* DH10β that exhibits an MIC of 2 against colistin was used as control. Colistin heteroresistance assays were performed by population analysis profiling as described earlier (Guérin et al., 2016).

Whole-genome sequencing and bioinformatics analysis

The genome sequence of va18651 was obtained using PacBio SMRT sequencing technology using PacBio RSII machine (Pacific Biosciences, Menlo Park, CA, USA). The reads generated were assembled using the SMRT-Link Microbial Assembler 10.1.0.

The whole-genome sequence-based identification was carried out by calculating the average nucleotide identity (ANI) by BLAST using the JSpecies v1.2.1 tool (Richter et al., 2009) and by *in silico* DNA–DNA hybridization using the genome-to-genome distance calculator (formula 2) (Meier-Kolthoff et al., 2013) with the type strains of 23 *Enterobacter* species known as of May 2022 (Cho et al., 2021).

The assembled genome was annotated by the bacteria-specific annotation pipeline Bakta (Schwengers et al., 2021) and refined manually by using well-annotated reference genomes. The Multi-Locus Sequence Types (MLST), plasmid incompatibility (Inc) groups, plasmid MLST (pMLST), and acquired antibiotic resistance genes were identified using the Center for Genomic Epidemiology platform (https://cge.cbs.dtu.dk/services/) and the PubMLST database (https://pubmlst.org; https://bigsdb.pasteur.fr/cgi-bin/bigda.pl?db.), as described previously (Yao et al., 2021). Phylogenetic comparative genomics was performed based on single-nucleotide polymorphism (SNP) using Harvest Suite (ParSNP) (Treangen et al., 2014). The virulence genes were predicted by BLASTN against the VFDB database (Chen et al., 2016). The BLAST Ring Image Generator (BRIG) was employed to perform multiple comparisons of complete plasmid sequences available at the National Center for Biotechnology Information (NCBI), and circular maps were generated (Alikhan et al., 2011). To annotate the genetic contexts surrounding bla_{KPC-2}, bla_{OXA-48}, and *mcr-9.1* and mobile elements, Galileo AMR of ARC Bio was used (Partridge and Tsafnat, 2018). Distribution of the virulence genes was depicted on the circular genome using Circos v0.69.

To compare with other ST88 isolates, whole-genome sequences of 3,246 non-repetitive isolates (<1,000 contigs and >3 Mb assembly size) listed under the genus *Enterobacter* were downloaded from NCBI using e-utilities. These isolates were selected after reconfirming them as *bona fide Enterobacter* species using the OGRI tool as mentioned for va18651, and isolates identified to be ST88 were further studied.

Data availability

The complete genome sequences of the *E. xiangfangensis* va18651 is available in public genome sequence databases with the accession numbers CP097342 (Chromosome), CP097343 (plasmid p1-va18651), CP097344 (p2-va18651), CP097345 (p3-va18651), and CP097346 (p4-va18651) within the BioProject PRJNA837392.

Results

Identification of the isolate va18651

Isolate va18651 was initially identified as a member of the "*Enterobacter* cloacae complex" using MALDI-TOF MS. Based on genome sequencing analysis, the average nucleotide identity (ANI) and *in silico* DNA–DNA hybridization scores (isDDH) were >95% (97.02%) and >70% (75.9%) as compared to type strain of *E. xiangfangensis* LMG 27195, respectively, confirming that va18651 is a member of the species *E. xiangfangensis*. The va18651 was sequence-typed to the clonal group ST88. Phylogenomic comparison to publicly available genomes revealed 21 ST88 isolates with comparable features for plasmid and antibiotic resistance genes (detailed below) (Figure 1A).

The antimicrobial phenotype of the va18651

Broth microdilution methods indicated that the isolate va18651 was resistant to a large number of antibiotics (Table 1). The isolate showed the following MICs to the β-

lactams tested: piperacillin (MIC > 32 μg/ml), piperacillin/tazobactam (MIC > 128/4 μg/ml), cefepime (MIC > 8 μg/ml), ceftazidime (MIC > 32 μg/ml), ceftazidime/avibactam (MIC ≤ 1/4 μg/ml), meropenem (MIC = 16 μg/ml), imipenem (MIC = 4 μg/ml), and aztreonam (MIC > 16 μg/ml). These results display that the isolate is resistant towards almost all β-lactams with the exception of ceftazidime-avibactam. The imipenem MIC leads to the breakpoint category susceptible under increased exposure according to EUCAST standards. However, as the MIC is at the upper limit of this category and the presence of carbapenemases was verified by WGS, this substance should not be used as a therapeutic agent for this isolate. This isolate was also phenotypically resistant to fluoroquinolone (ciprofloxacin), aminoglycosides (gentamicin and tobramycin), and sulfonamides (trimethomprim/sulfamethoxazole), but not resistant towards fosfomycin and colistin (Table 1). However, population analysis profiling for colistin showed a heteroresistance frequency (number of isolates grown on LB agar containing colistin compared to number of isolates grown on normal LB agar) of 1.3 ± 0.12% and 0.55 ± 0.04%, in the presence of 8 and 32 μg/ml of colistin, respectively. Given that the isolate may exhibit inherent low-level heteroresistance towards colistin, we repeated the broth-microdilution assay six times. In two of the assays, the isolate exhibited an MIC of 64 μg/ml toward colistin.

Plasmidome and antimicrobial resistance determinants of the va18651

The general genomic features of va18651 are listed in Table 2. Based on long-read data, the genome of va18651 was assembled into five complete (i.e., circularized) contigs, including a 4,785,021-bp chromosome with a GC content of 55.5%, which encoded 4,433 predicted CDSs including a β-lactamase gene bla_{ACT-7} and 86 tRNAs, 25 rRNAs, and 83 ncRNAs (Table 2, Figure 1B) and four distinct plasmids that resulted in a plasmidome with a cumulative length of 604,632 bp (Table 2).

The mobile colistin-resistance-gene *mcr-9.1*-containing plasmid, p1-va18651 was the largest plasmid carried by the *E. xiangfangensis* va18651 with 307,415 bp in length and a GC content of 47.9%. This mega-multi-replicon plasmid resulted from the fusion of an IncHI2(pST1) and the pKC-CAV1321 plasmids and encoded for a total of 351 predicted CDSs. The p1-va18651 also carried antibiotic resistance genes to β-lactams (bla_{SHV-12} and bla_{TEM-1B}), aminoglycosides [*aadA2b, aac(6´)-Ib3, aac(6´)-IIc, strA*, and *strB*], fluoroquinolones [*qnrA1* and *aac(6´)-Ib-cr*], sulfonamides [*sul1*, 3x, and *sul2*], macrolides [*ere (A)*], phenicols [*catA2*], tetracyclines [*tet(D)*], trimethoprim (*dfrA19*), and quaternary ammonium compounds (*qacΔE*; two copies). The *mcr-9.1* in p1-va18651 was flanked upstream by an IS903 and downstream by an IS26, identical to surroundings of previous studies (Li et al., 2020; Tyson et al., 2020; Macesic et al., 2021). An IncHI1-type replication protein, a transfer–conjugation system, and a toxin–antitoxin system were predicted on the plasmid backbone (Figure 2A). Using BLAST searching on GenBank, the p1-va18651 has significant homology to known plasmids of the *E. hormaechei* strain AR_0365 and *Salmonella enterica* strain CVM N23023 with identity >99.98% (Table 3). Remarkably, a homologous sequence (51% query and 99.99% identity) was also present in the chromosome of *Salmonella enterica* subsp. *enterica* serovar Heidelberg strain NY-N14748.

The bla_{KPC-2}-harboring plasmid p2-va18651 belonged to the plasmid incompatibility group IncN and was a member of the type pMLST15. It was 79,326 bp in size and had a GC content of 52.7% with 100 CDSs predicted. The p2-va18651 contained 14 different ARGs of diverse classes including β-lactams (bla_{KPC-2}, bla_{TEM-1B}, and bla_{OXA-1}), aminoglycosides [*aac(II)-3d*, *strA*, and *strB*], fluoroquinolones [*aac(6´)-Ib-cr* and *qnrB2*], sulfonamides (*sul1*, two copies), macrolides [*mph(A)*], phenicols (*catB3*), rifampicin (*ARR-3*), trimethoprim (*dfrA19*), and quaternary ammonium compound resistances (*qacEΔ_1*). The p2-va18651 exhibited a unique genetic environment surrounding the bla_{KPC-2} gene, being identical to that of a persistent and promiscuous IncN[pMLST15] plasmid from different species in previous studies (Yao et al., 2014; Yao et al., 2021). Comparison analysis revealed that p2-va18651 was virtually identical to plasmids obtained from *Enterobacterales* species isolates from human, food, and environment that have been in circulation for long time in healthcare and environmental settings in Germany hospitals (see pCP13069-KPC2, pCF08698-KPC2, pCF13141-KPC2, and pKV30046-KPC2) as well as to plasmids from isolates obtained within the SurvCARE-Project (unpublished data). The plasmid p2-va18651 only differed from them with an IS*5702* insertion in its multidrug cargo region (Table 3 and Figure 2B). The backbone of the p2-va18651 contained a conjugation system (Figure 2B).

The bla_{OXA-48}-carrying plasmid p3-va18651 was an IncL-plasmid with 63,589 bp length and a GC content of 51.2% and exhibited 85 predicted CDSs (Figure 2C). The plasmid p3-va18651 carried the bla_{OXA-48} carbapenemase gene, which is embedded within a Tn*1999* flanked by an IS*1999* transposase at both ends. It contained a typical IncL backbone comprising features of replication (*repA* and *repC*), maintenance, and stabilization (plasmid partition *parA*, *parB*, toxin–antitoxin system *pemL*, *pemK*, *ssb*, anti-restriction *kicA*, *korC*, and *eexA*) as well as transfer–conjugation machinery (*trbABCN* and *traHIJKLMNOPQRUWXY*) and mobility cassette-encoding *mobA* and *mobB* (Figure 2C). There were more than 80 sequence entries of strains from the species *Escherichia coli*, *Klebsiella pneumoniae*, *Citrobacter freundii*, *Serratia marcescens*, and *Raoultella ornithinolytica* in the NCBI database that were

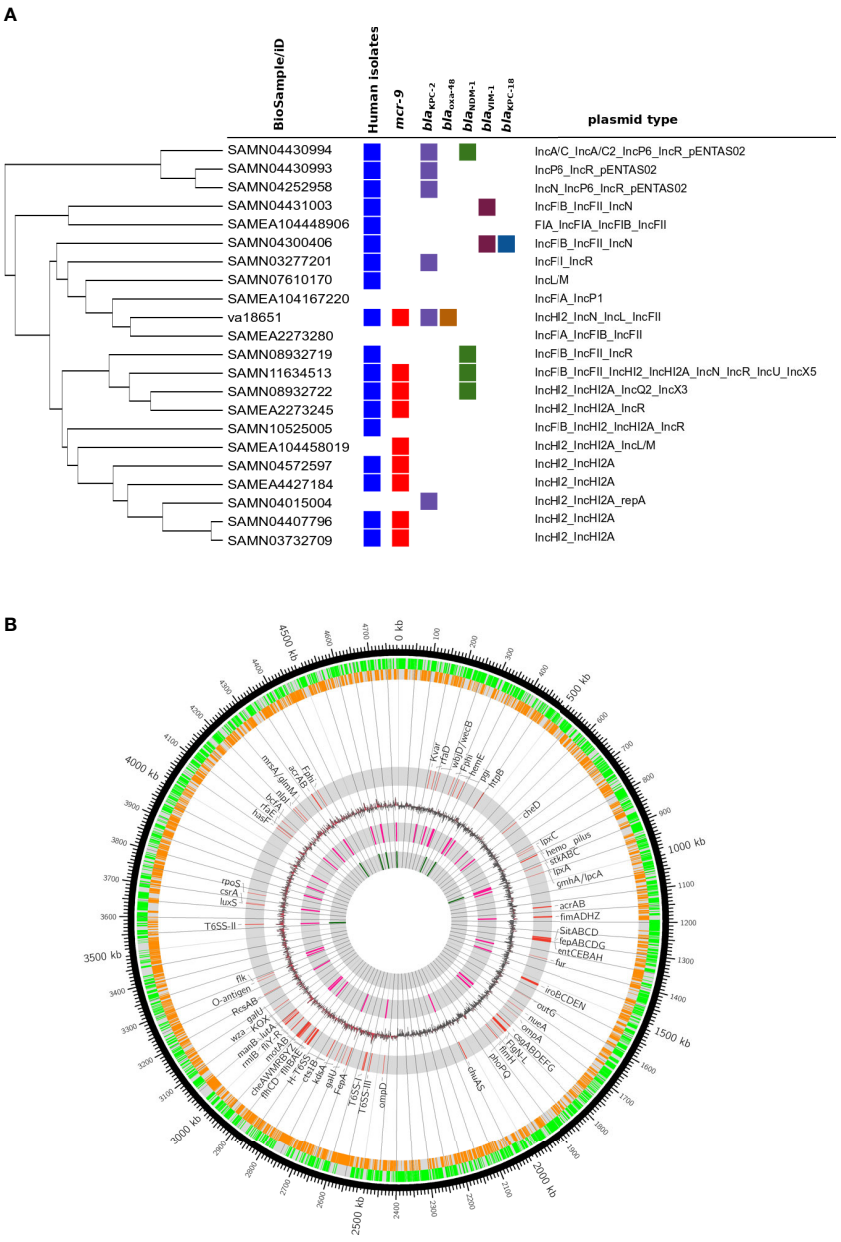

FIGURE 1
Genomic features of extensive drug-resistant *Enterobacter xiangfangensis* va18651. **(A)** Phylogenomic comparison of the va18651 to publicly available genomes of 21 ST88 isolates. The va18651 encoded mcr-9, blaKPC-2, and blaOXA-48 genes on three different plasmids with type IncHI2, IncN, and IncL, respectively. **(B)** Chromosomal features of *Enterobacter xiangfangensis* isolate va18651. The innermost to outermost (gray) circles indicate the respective locations of rRNA, tRNA, GC-skew, virulence genes, forward–reverse genes, and an ideogram indexing base pairs. The isolate va18651 carried all virulence genes on the chromosome while almost all antibiotic resistance genes were found on the plasmid.

100% identical to p3-va1865, suggesting promiscuous transmissibility (Table 3).

The fourth plasmid, p4-va18651, was an IncFII-B(pECLA)-type plasmid of 154,302 bp in size and with a GC content of 51.5%, which encoded 174 predicted CDSs (Figure 2D), but no known antibiotic resistance genes. p4-va18651 harbored a transfer gene cluster, indicating that it was a conjugative plasmid. A similar plasmid with 88% coverage and an identity of 99.99% (166,898 bp) was found from an *E. hormaechei* strain EBp6-L3 (ST88) isolated in the UK 2019 (Accession no. NZ_CP043854.1).

TABLE 1 Antimicrobial susceptibility of *Enterobacter xiangfangensis* va18651.

Antimicrobial	MIC (μg/ml)	Interpretation
Aminoglycosides		
Amikacin	8	S
Gentamicin	32	R
Tobramycin	16	R
β-lactams		
Piperacillin	>32	R
Piperacillin/Tazobactam	>128/4	R
Aztreonam	>16	R
Ceftazidime	>32	R
Cefepime	>8	R
Ceftazidime/Avibactam	≤1/4	S
Imipenem	4	I
Meropenem	16	R
Fluoroquinolones		
Ciprofloxacin	2	R
Levofloxacin	1	I
Fosfomycin		
Fosfomycin	≤16	S
Sulfanilamide		
Trimethomprim/sulfamethoxazole	>8/152	R
Polymyxins		
Colistin	0.25 64*	S R

In the case of colistin, skip-well phenomenon was noted, indicating heteroresistance capability (*). The antimicrobial susceptibility was performed with a broth microdilution assay and results were interpreted according to EUCAST criteria (version 12.0).

Virulence factors and heavy metal and metalloid resistome of va18651

The strain va18651 was classified as *Enterobacter* ST88 based on the *in silico* MLST typing scheme. Diverse virulence genes for the lipopolysaccharide and capsular polysaccharide, iron acquisition (*iroBCDEN*, *ent/fep/fes*, *sitABCD*, *iut*, and *fur*), and a Type VI secretion system, together with motility genes, Type I fimbria, and Type IV pili were encoded on the chromosome (Figure 1). The PhoPQ two-component regulator and its negative inhibitor *mgrB* that plays a global regulatory role in antibiotic susceptibility, physiology, stress adaptation, and virulence were intact. The presence of a number of previously determined virulence determinants indicated that va18651 carried a high pathogenic potential.

Notably, two of the four plasmid harbored heavy metal and metalloid resistance genes. These included resistance gene clusters for tellurium (*terA*, *terB*, *terC*, *terD*, *terE*, *terX*, *terY*,

TABLE 2 Genomic features of *Enterobacter xiangfangensis* va18651.

Structure	Length (bp)	GC (%)	No. of CDS	Antimicrobial resistance genes	MLST/Inc type (pMLST)	Accession no.
Chromosome	4,785,021	55.51	4,433	bla_{ACT-7}, *phoPQ-arnBCDATEF**	ST-88	CP097342
Plasmid						
p1-va18651	307,415	47.89	351	*mcr-9*, bla_{SHV-12}, bla_{TEM-1B}, *ere(A)*, *qacEΔ-1 (2x)*, *catA2*, *tet(D)*, *aadA2b*, *qnrA1*, *aac(6′)-Ib-cr*, *strA*, *strB*, *aac(6′)-Ib3*, *aac(6′)-IIc*, *sul1(3x)*, *sul2*, *dfrA19*	IncHI2(pST1)::pKC-CAV1321	CP097343
p2-va18651	79,326	52.68	100	bla_{KPC-2}, bla_{TEM-1B},bla_{OXA-1}, *aac(3)-IId*, *aac(6′)-Ib-cr*, *strA*, *strB*, *catB3*, *qacEΔ-1*, *ARR-3*, *mph(A)*, *qnrB2*, *sul1 (2x)*, *dfrA19*	IncN (pST15)	CP097344
p3-va18651	63,589	51.23	85	bla_{OXA-48}	IncL	CP097345
p4-va18651	154,302	51.46	174	Not detected	IncFII(pECLA)	CP097346

A total of 36 (25 non-duplicated) genes were observed to be located on the plasmids. * The PhoPQ-dependent 4-amino-4-deoxy-l-arabinose addition to lipid A may result in the colistin heteroresistance in *Enterobacter cloacae* (Kang et al., 2019). The complete genome sequence revealed a chromosome and four plasmids.

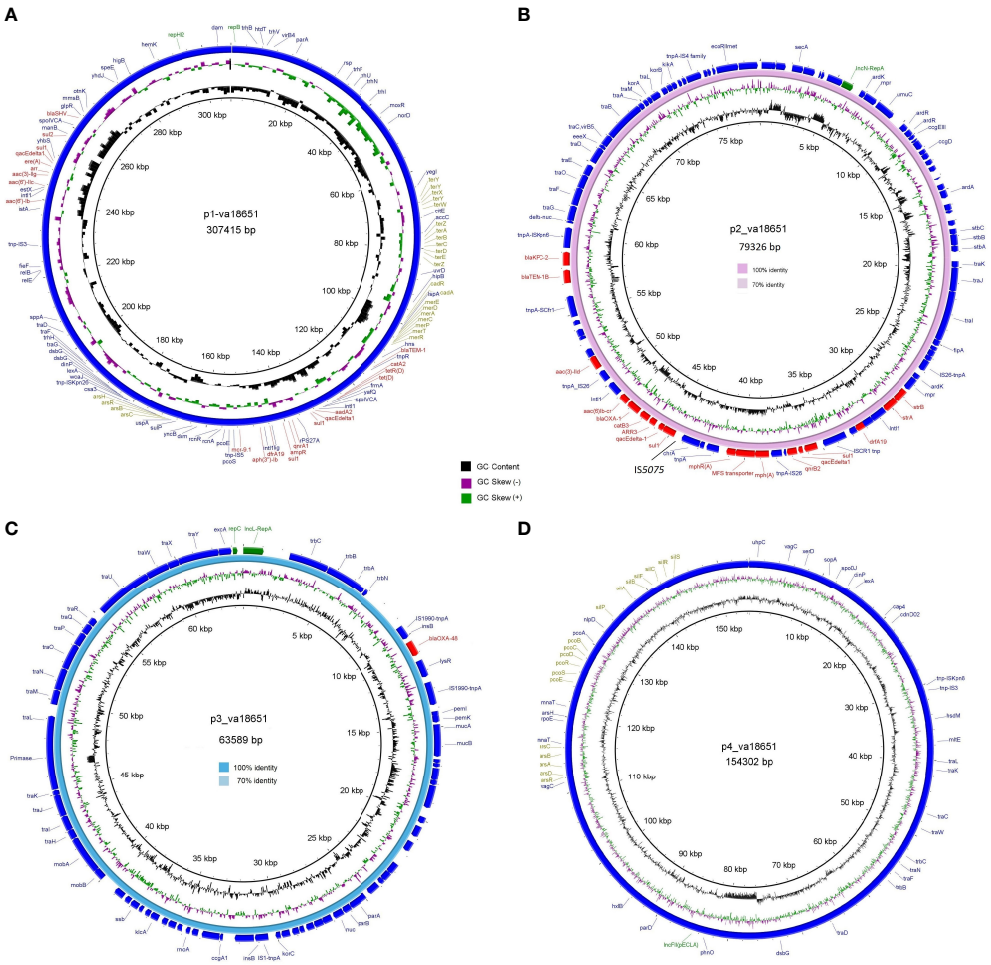

FIGURE 2
Circular genetic maps of the plasmids p1-va18651 **(A)**, p2-va18651 **(B)**, p3-va18651 **(C)**, and p4-va18651 **(D)** from the *E xiangfangensis* isolate va18651. Plasmid replicons, antimicrobial resistance, and heavy metal resistance are marked in green, red, and olive green, respectively. B depicts the BRIG comparison of p2-va18651 with pCP13069KPC2 (VKMZ0100118.1) and C displays the comparison of p3-va18651 with pOXA-48_1639 (LR025105).

terW, and *terZ*), cadmium (*cadA* and *cadR*), mercury (*merA*, *merC*, *merD*, *ere*, *merP*, *merR*, and *merT*), copper (*pcoE* and *pcoS*) and arsenate (*arsB*, *arsC*, *arsH*, and *arsR*) in the p1-va18651, as well as the gene cassettes of silver (*silA*, *silB*, *silF*, *silC*, *silR*, *silS*, and *silP*), copper (*pcoA*, *pcoB*, *pcoC*, *pcoD*, *pcoE*, *pcoS*, and *pcoR*) and arsenate (*arsA*, *arsB*, *arsC*, *arsD*, and *arsR*) in p4-va18651 (Figure 2).

Discussion

In a study between 2017 and 2019, we collected carbapenem-resistant *Enterobacterales* isolates (CRE) obtained from patients covering the entire state of Hessen in Germany. Among these, isolate va18651 was found to harbour two carbapenemase-encoding genes, bla_{KPC-2} and bla_{OXA-48}, and the mobile colistin resistance *mcr-9* gene. This combination was unique and not found in the *Enterobacter* species collection. We also screened 3,246 genomes of *Enterobacter* strains available from the public database to examine if the combination of these genes had been previously reported. The analysis revealed only nine isolates that harbored the combination of two carbapenemases and *mcr-9*; eight of these isolates were classified as members of the species *E. xiangfangensis*. While the OXA-48 is frequently detected in other *Enterobacterales* species, such as *Klebsiella* and *E. coli*, this is the first time we observed the OXA-48 determinant in the genus *Enterobacter*.

The isolate va18651 was initially identified as a member of ECC by MALDI TOF. Due to taxonomic conflicts and the low discriminatory power of MALDI TOF, *Enterobacter* species may not be correctly identified to the species level (Pavlovic et al., 2012; Godmer et al., 2021). Using recommended genome

TABLE 3 Examples of sequences highly related to the plasmids of *Enterobacter xiangfangensis* va18651.

Plasmid	Query Length (bp)	Query Coverage (%)	Identity (%)	Homologies	Acc. length (bp)	Accession no.
p1-va18651 (mcr-9)	307,415	91	100	p-unnamed1 of strain AR_0365	328,871	CP027144
		82	99.98	pN53023 of strain CVM N23023	339,705	CP049311
		51	99.99	Chromosome of strain NY-N14748	4,984,436	CP048926
p2-va18651 (bla_{KPC-2})	79,326	100	100	pCP13069-KPC2	78,021	VKMZ01000118.1
		100	100	pCF08698-KPC2	78,021	VKMD01000077.1
		100	100	pCF13141-KPC2	78,021	VKMY01000050.1
		100	100	pKV30046-KPC2	78,023	JAFHMT000000000
p3-va18651 (bla_{OXA-48})	63,589	100	100	pOXA-48_1639	63,589	LR025105,
		100	100	pACV-OXA-48	63,589	CP045727,
		100	100	p2247421	63,589	CP086451
p4-va18651	154,302	88	99.99	p-unnamed1 of *Enterobacter* strain EB_P6_L3_02.19	166,898	CP043854

sequence-based approaches (Chun et al., 2018), the isolate va18651 was identified as *E. xiangfangensis* (also listed as *E. hormaechei* subspecies *xiangfangensis*) (Sutton et al., 2018; Wu et al., 2020). *Enterobacter* isolates have been reported to be present in the cervix of healthy individuals (Larsen and Monif, 2001; Amabebe and Anumba, 2018) and constitute a risk factor for the urinary tract infection and premature births.

The pathogenicity and virulence gene repertoire of *Enterobacter* are not well understood (Sanders and Sanders, 1997). Very few studies that examined the virulence capabilities of *Enterobacter* species in a mouse model of infection are available (Paauw et al., 2009; Krzymińska et al., 2010; Pati et al., 2018). *Enterobacter* species belonging to MLST sequence type 88 (ST88) have been reported from sporadic human and animal clinical cases as well as from outbreaks (Jia et al., 2018; Börjesson et al., 2019; Tian et al., 2020). To compare va18651 to other ST88 isolates distributed worldwide, we retrieved genomes of 21 ST88 isolates. Interestingly, 8 of 21 (38%) ST88 isolates carried *mcr-9* genes on an identical IncHI2 plasmid (Figure 1A). A total of 10 (47.7%) isolates carried at least one carbapenemase (bla_{KPC-2} and bla_{VIM-1}, bla_{KPC-18} and bla_{NDM-1}). These data indicated that carbapenemases and *mcr-9* are relatively common to ST88. These data urge further systematic studies with the clonal type ST88 in order to validate the common association of carbapenemases and *mcr-9*. The presence of an ST type present in a wide range of hosts and in different environmental settings as reflected by its carriage of carbapenemase-encoding genes of different types such as bla_{KPC-2} and bla_{OXA-48} suggest the emergence of a successful clone transcending species and environmental barriers.

While it is common to observe plasmids of between 10 kb and 500 kb in individual isolates of *Enterobacterales*, the plasmidome of va18651 was, at 604,632 bp, unusually large (Stephens et al., 2020; Darphorn et al., 2021). Highly identical bla_{KPC-2}-harboring IncN plasmids were reported earlier in Germany (Yao et al., 2014; Becker et al., 2018; Yao et al., 2021). The bla_{OXA-48} plasmid observed was identical to the OXA-48-encoding IncL plasmid, which is reported globally. IncL/M plasmids are an emerging threat as they represent a current source of class D carbapenemases and are responsible for the worldwide distribution of bla_{CTX-M}. This bla_{OXA-48} IncL plasmid was reported from diverse *Enterobacterales*, suggesting that the p2-va18651 and p3-va18651 and the respective encoded antimicrobial resistances were acquired by horizontal transmission. The environment surrounding *mcr-9.1* associated with the IS*903B* of p1-va18651 was identical with those identified in both chromosomes and IncHI1 plasmids of previous studies (Tyson et al., 2020), indicating an *mcr-9* acquisition mediated by mobile genetic elements. In total, the isolate va18651 carried 26 different antibiotic resistance genes, each present in one to four copies, and was distributed among 10 classes including β-lactams, aminoglycosides, fluoroquinolone, macrolides, phenicol, rifampicin, tetracycline, trimethoprim, and quaternary ammonium compound resistance gene cassettes (*qacEΔ_1*). These genes mediate resistance to a broad range of antibiotics that correspond to the drug and detergent resistance phenotypes.

There are few reports of co-carriage of two carbapenemase classes such as KPC-2 and OXA-48 in *K. pneumoniae* ST11 in Taiwan and Egypt (Wang et al., 2019; Yang et al., 2022) and KPC-2 and NDM-1 in the *Enterobacter* ST88 strain from Colombia (Accession No. SRR3110109) as well as VIM with OXA-48 in *E. xiangfangensis* isolates (Tyson et al., 2020), but there are currently no reports on the co-existence of KPC-2 and OXA-48 carbapenemases together with a colistin resistance-encoding gene *mcr-9.1*.

Enterobacter species carry an *arnBCADTEF* gene cassette, which has shown to be responsible for the colistin heteroresistance (Kang et al., 2019). The presence of this gene cassette on the chromosome of va18651 encouraged us to

determine the colistin heteroresistance capability. As heteroresistance is an incidental phenomenon, the colistin resistance may or may not be detected in routine laboratories. Nevertheless, such heteroresistance capability is important from a clinical perspective as it results in the failure of the antibiotic therapy (Band and Weiss, 2019; Band et al., 2021). Previous studies indicate that the *mcr-9* gene is not induced naturally, and expressed only from artificial promoters or in presence of colistin (Carroll et al., 2019; Kieffer et al., 2019). Thus, while the *mcr-9* gene is functional, the conditions that induce its activity are not known.

While va18651 carried a high number of different ARGs on plasmids (p1-va18651, p2-va18651, and p3-va18651), the co-occurrence of a higher number of heavy metal resistance genes (HGR) on the plasmids p1-va18651 and p4va18651 was remarkable. Such co-occurrence of ARG and HRG has been recognized for a long time (Foster, 1983), but this coexistence is poorly understood (Li et al., 2017). Nevertheless, it is likely to provide it with additional survival and adaptive properties in ecological niches within hospital settings.

The *E. xiangfangensis* isolate va18651 carries a unique combination of risk factors, i.e., virulence genes on the chromosome together with a large number of antibiotic and heavy metal/metalloid resistance genes on plasmids. The presence of multiple plasmids harboring highly mobilizable genetic platforms also provide a "sink" and reservoir for fueling the accelerated dispersion of multiple ARGs, thereby paving the way in creating antimicrobial resistant (AMR)-hypervirulent vectors that could spread easily, expanding the incidence of hard-to-treat infections with fatal outcomes.

Data availability statement

The data presented in this study are available through the Bioproject PRJNA837392 in the NCBI databases.

Author contributions

YY and TC conceptualized the manuscript. YY and SD performed the bioinformatic analysis and wrote the draft of the manuscript. JF, JS, SD and CI performed the phenotyping and WGS genome sequencing. TC, SD and YY prepared the final manuscript with inputs from all Co-authors. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Human *Erysipelothrix rhusiopathiae* infection *via* bath water – case report and genome announcement

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Erysipelothrix rhusiopathiae is a facultative anaerobic, environmentally stable, Gram-positive rod that causes swine and avian erysipelas as a zoonotic pathogen. In humans, the main manifestations described are circumscribed erysipeloid, generalized erysipeloid, and endocarditis. Here, we report a 46-year-old female patient who presented to the physician because of redness and marked *functio laesa* of the hand, in terms of a pain-related restricted range of motion, and was treated surgically. *E. rhusopathiae* was detected in tissue biopsy. The source of infection was considered to be a pond in which both swine and, later, her dog bathed. The genome of the isolate was completely sequenced and especially the presumptive virulence associated factors as well as the presumptive antimicrobial resistance genes, in particular a predicted homologue to the multiple sugar metabolism regulator (MsmR), several predicted two-component signal transduction systems, three predicted hemolysins, two predicted neuraminidases, three predicted hyaluronate lyases, the surface protective antigen SpaA, a subset of predicted enzymes that potentially confer resistance to reactive oxygen species (ROS), several predicted phospholipases that could play a role in the escape from phagolysosomes into host cell cytoplasm as well as a predicted vancomycin resistance locus (*vex23-vncRS*) and three predicted MATE efflux transporters were investigated in more detail.

KEYWORDS

***Erysipelothrix rhusiopathiae*, case report, genome, Vancomycin Resistance, erysipeloid, swine erysipelas, MSMR, vex23-vncRS**

Introduction

A 46-year-old female patient presented to our clinic with swelling, redness, and pain on the right thumb that had been progressive for two days. The patient reported that she suffered a minor lesion of the thumb pad from cracking walnuts. On inspection, a blister approximately 1 cm in diameter was found on the palmar end phalanx of the right thumb, still covered by intact skin. A marked swelling with semicircular redness extended down to the proximal phalanx. On the extensor surface, a well-demarcated striated lymphangitis reached across the wrist down to the distal forearm. Clinical inspection did not allow a clear distinction between erysipelas and cellulitis. To explore, whether a foreign body may have remained from walnut cracking, an incision was made under local anesthesia, which revealed no pus, no indurated tissue, and no foreign body.

Background

The genus *Erysipelothrix*

E. rhusiopathiae belongs to the Erysipelotrichaceae family and is the only human pathogenic microbial species of the genus *Erysipelothrix*. Further microbial species of the genus have been described more recently and include *Erysipelothrix tonsillarum* (Takahashi et al., 1987), *Erysipelothrix inopinata* (Verbarg et al., 2004), *Erysipelothrix muris* (Chen et al., 2006), *Erysipelothrix larvae* (Bang et al., 2015; Bang et al., 2016), *Erysipelothrix piscisicarius* (Pomaranski et al., 2020), *Erysipelothrix anatis* sp. nov., *Erysipelothrix aquatica* sp. nov., *Erysipelothrix urinaevulpis* (Eisenberg et al., 2022) and the as yet undesignated *Erysipelothrix* species 1, *Erysipelothrix* species 2, and *Erysipelothrix* species 3 (Takahashi et al., 2008).

Historical classification of the species *Erysipelothrix rhusiopathiae*

The species designation changed a number of times. Robert Koch first isolated a bacterium of the genus *Erysipelothrix* in 1876 from a mouse that he had previously inoculated with putrid blood. He designated this pathogen as the bacterium of mouse septicemia, *E. mursiseptica* (Wang et al., 2010). Friedrich Löffler isolated a similar organism, under the name *Bacillus* of swine erysipelas (latinized by Kitt in 1893 as *Bacillus rhusiopathiae suis*), from the skin blood vessels of a pig that had died of swine erysipelas in 1886 and he was the first to describe the pathogen and the disease caused by it in swine (Wang et al., 2010). Friedrich Julius Rosenbach isolated a bacterium similar to Robert Koch's bacterium from a patient with localized skin lesions in 1909, so that *Erysipelothrix* was now established as a human pathogen after initial case reports since 1870. Rosenbach coined the term "erysipeloid" to distinguish between the streptococcal infection "erysipelas" and the efflorescence he had observed. Rosenbach still distinguished three different microbial species: *E. muriseptica*, *E. porci*, and *E. erysiploides*, depending on their origin of isolation from mice, swine, or humans (Wang et al., 2010). Later it was discovered that they were three nearly identical isolates of the same microbial species, which was named *E. insidiosa*, as originally proposed by Trevisan in 1885. This name, as well as all other 36 names circulating for this bacterium at the time, were discarded in 1966 in favor of *E. rhusiopathiae*, a combination of genus and species that had been coined as early as 1918 (Wang et al., 2010). According to comparative genomic analyses, the species *E. rhusiopathiae* can be further subdivided into three clades, as well as a fourth clade phylogenetically intermediate between clades 2 and 3 (Forde et al., 2020). In addition, *Erysipelothrix* spp. can be divided into at least 28 known serovars (Opriessnig et al., 2020). Serovar 1 is considered to be the most virulent and therefore of greatest veterinary importance (Selbitz et al., 2011).

Culture and morphology

The cell wall of *E. rhusiopathiae* consists among others of lysine and glycine, which distinguishes it from *Listeria* and *Corynebacteria*. The negative catalase test differentiates *Erysipelothrix* spp. from *Kurthia* spp. In the Gram stain, Gram-positive rods are recognizable in chain formation, but with a highly variable single cell length, from 0.8 µm to 2.5 µm (Figure 1A). Occasionally, even filaments of up to 60 µm in length can be seen. *E. rhusiopathiae* is readily decolorized during Gram staining, and Gram-labile or even Gram-negative staining behavior may be apparent (Figure 1B) (Carroll et al., 2019).

Regarding culture conditions, *E. rhusiopathiae* is relatively undemanding and can be grown on aerobically incubated sheep blood or chocolate blood agar. Nevertheless, a capnophilic atmosphere favors bacterial growth. When grown on Schaedler KV agar, chocolate agar, or Columbia sheep blood agar, *E. rhusiopathiae* exhibits two colony morphologies. On the one hand, it appears as a smooth (S) colony in the form of clearly delimited pin-points, on the other hand, as a rough (R) colony with irregular boundaries (Figures 1C, D) (Carroll et al., 2019). The S-form is particularly observable in initial culture from acute disease cases, but the R-form is particularly observable in culture from chronic disease cases and after more frequent passaging *in vitro* (Selbitz et al., 2011). Moreover, the facultative anaerobic bacterium can be cultivated in trypticase soy or Schaedler broth. To suppress possible accompanying flora, especially when isolating *E. rhusiopathiae* from

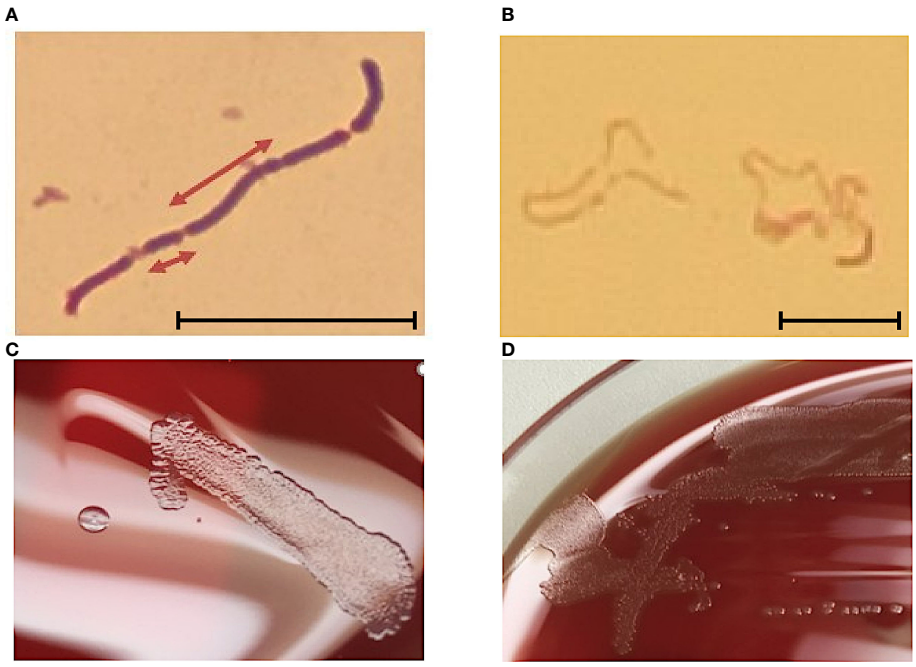

FIGURE 1
(A) *E. rhusopathiae* Gram stain (magnification 1:1000, scale bar = 5 µm). The arrows indicate two Gram-positive rods with distinctly different lengths. **(B)** Gram stained slide with *E. rhusopathiae* (magnification 1:1000, scale bar = 5 µm). A decolorized (pseudo-) Gram-negative sample is shown. This phenomenon occurs regularly when preparing the microscopic specimen with colonies appearing "rough" on agar plates. **(C)** Growth of *E. rhusopathiae* on Schaedler KV agar. On the left, a smooth clearly circumscribed pinpoint colony can be seen, next to a bed of confluent colonies (right). **(D)** smooth and rough colonies on Columbia sheep blood agar.

environmental and marine locations, or from animal waste and products, numerous selective media were developed (Brooke and Riley, 1999; Fidalgo et al., 2000; Wang et al., 2010).

Identification

Recently, species identification of *E. rhusiopathiae* has become a minor issue with availability of MALDI-TOF mass spectrometry and modern biochemical methods such as VITEK® 2 GP ID card (Biomérieux, Nürtingen, Germany), API® Coryne (Biomérieux, Nürtingen, Germany), Rapid ID 32 STREP (Biomérieux, Nürtingen, Germany), or BD Phoenix™ PID Panel (BD Biosciences, Sparks, MD, USA). More reliable identification may have contributed to an observed increase of *E. rhusiopathiae* detection in human specimen since about 2008 (Farfour et al., 2012; Principe et al., 2016). Additionally, several PCR-based assays have been developed to detect *E. rhusopathiae*; to differentiate it from other *Erysipelothrix* species; and to subdifferentiate (serotype) within the microbial species (Fidalgo and Riley, 2004; Yamazaki, 2006; Pal et al., 2010; Shiraiwa et al., 2017; Shimoji et al., 2020). For epidemiological purposes, different subtyping methods have been developed based on multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE) (Janßen et al., 2015) and sequencing of the hypervariable region of the *spaA* gene (Nagai et al., 2008).

Epidemiology

E. rhusiopathiae is widespread among mammals, birds, and fish, but most commonly found in swine and their feces (Funke, 2009). In approximately 50% of healthy swine, *E. rhusiopathiae* can be detected in the tonsils or lymphoid tissue (Spiteri and Taylor-Robinson, 2018). Due to its high tenacity, the pathogen persists for several months in soils and water bodies as well as in decaying animals and fish (Selbitz et al., 2011). Human infection usually occurs in occupational groups exposed to appropriate animal products or excreta, such as farmers, veterinarians, furriers, butchers, fishermen, fishmongers, homemakers, cooks, and grocers.

Disease in swine and other animals

Susceptible to infection with *E. rhusiopathiae* are primarily swine between 3 and 12 months of age. After oral, conjunctival, or percutaneous infection, bacteremia occurs after three to five

days of incubation, later resulting in the characteristic manifestations of the skin (Selbitz et al., 2011). Acute swine erysipelas, most commonly associated with serovar 1 - subtype 1a, manifests with high fever up to 42°C and the typical landmark redness of the skin. In the peracute form of the disease, the animals can also die before the pathognomonic symptoms have developed. This is referred to as "white erysipelas" (Selbitz et al., 2011). The subacute but also the acute course is characterized by pathognomonic raised rhomboid skin lesions of several centimeters in diameter that are called "diamond skin". Chronicity of the symptoms of erysipelas, that this means a disease duration of more than four weeks, is possible. Chronic erysipelas may occur as a result of acute disease but may also be a direct consequence of persistent infection with low virulent strains of *E. rhusiopathiae*. Typical manifestations are skin necrosis, polyarthritides and/or *endocarditis valvularis*, which may be manifested by cauliflower-like thrombotic-ulcerative deposits on the atrioventricular valves (Selbitz et al., 2011). *E. rhusiopathiae* infections are not restricted to swine. It also occurs in sheep, in which the disease frequently manifests as chronic polyarthritis and rarely as septicemia, as well as in turkeys, ducks, chickens, mice, rats, beavers, cattle, rabbits, horses, minks, foxes, and dolphins (Selbitz et al., 2011). For prophylaxis, 17 different commercial vaccines are currently available in veterinary medicine (Opriessnig et al., 2020).

Clinical manifestations in humans

In contrast to swine, serotypes 2, 7, and 16 are the most commonly involved in the pathogenesis of human erysipeloid (Veraldi et al., 2009). If the pathogen enters the skin *via* a wound, there are basically three clinical manifestations: a localized skin lesion (so-called erysipeloid), a generalized skin lesion, and bloodstream infection. Local erysipeloid is characterized by sharply circumscribed, painful, reddish, non-repressible edema often accompanied by vesiculation and erosive lesions without pus formation (Wang et al., 2010). In addition, arthralgia, myalgia, lymphadenitis and mild fever may occur (Rostamian et al., 2022). Frequently, the back of the hand (as in our patient) or the extensor side of the fingers are affected, because the tendons form a row being wrapped around very tight (Veraldi et al., 2009). Usually, the spread of the pathogen is limited to a roundish area at the point of entry. Rarely, the so-called multiple or systemic erysipeloid is reported. This results in the radial spread of multiple erysipeloids from the original focus (Wang et al., 2010) particularly seen in immunocompromised individuals (Veraldi et al., 2009). Bloodstream infection is rare but associated with severe disease, e.g. endocarditis (Drekonja, 2013; Hofseth et al., 2017; Wang et al., 2020). Other complications include meningitis, osteomyelitis, or septic arthritis (Wang et al., 2010; Groeschel et al., 2019). Immunosuppression represents an important causal factor for systemic infection.

Methods

Culture, species identification and susceptibility testing

According to the diagnostic routine at the Department of Medical Microbiology and Hospital Hygiene of the Medical Faculty of Otto-von-Guericke University Magdeburg, Columbia CNA agar with 5% sheep blood (aerobic), Schaedler/Schaedler KV agar (anaerobic) and Schaedler broth (media obtained from Fisher Scientific GmbH, Schwerte, Gemany) were inoculated with the tissue biopsy taken.

Species identification was performed both biochemically, using a VITEK® 2 GP ID card and a VITEK® 2 XL device (Biomérieux, Nürtingen, Germany) as well as by MALDI-TOF MS (Vitek-MS, Biomérieux, Nürtingen, Germany).

Susceptibility testing was carried out using MIC test strips (Liofilchem S.r.l., Roseto degli Abruzzi (Teramo), Italy).

Whole genome sequencing

Prior to DNA isolation the *E. rhusiopathiae* isolate was cultivated on Columbia agar supplemented with 5% sheep blood (Becton Dickinson, Beckton-Dickinson, New Jersey, USA) and incubated at 37°C for 16h. DNA was isolated from agar colony material (ca. 5 C.F.U.) using the CTAB-lysozyme protocol by Larsen and coworkers (Larsen et al., 2007). Cells were harvested (10 min., 3,000 x g), resuspended in 450 µl GTE solution (10 mM EDTA and 50 mM Glucose in 25 mM Tris-CL) and digested for one hour at 37°C with lysozyme (adding 50 µl of 10 mg/ml lysozyme to a final concentration of 1 mg/ml). Then, the cell suspension was incubated after adding 150 µl proteinase K (10 mg/ml) in 10% SDS (30 min, 55°C). Treatment with 4 µl RNase A (Qiagen, Hilden Germany; 700 U/ml, 2 min, room temperature) was followed by addition of 200 µl NaCl (5 M). CTAB (4.1 g NaCl in 90 ml water + 10 g cetrimide, Sigma cat. No. H5882, in NaCl) was preheated to 65°C, 160 µl were added and the solution was incubated (10 min, 65°C). This is followed by 2 extraction steps of a chloroform:isoamyl alcohol extraction. After adding chloroform/isoamylalkohol (24:1, ca. 1 ml) the solution was centrifuged (10,000 x g, 5 min). Then the upper aqueous phase was transferred to a fresh tube and again chloroform/isoamylalkohol (24:1, ca. 0,9 ml) was added. The solution was centrifuged once again (10,000 x g, 5 min). The aqueous layer (800 µl) was transferred to a fresh tube, 560 µl isopropanol were added, and the tube was inverted until DNA precipitates. The suspension was incubated for 5 min at room temperature and subsequently centrifuged (10,000 x g, 10 min).

Afterwards, the pellet was washed twice with ethanol (70%, 10,000 x g, 10 min). 50 µl TE-buffer were added after 15 min of air-drying. Library preparation was performed using the TruePrep DNA Library Prep Kit V2 for Illumina (1 ng) (Vazyme Biotech Co. Ltd., Nanjing, China) and samples were barcoded with the Nextera XT Index Kit (24 indexes, 96 samples, Illumina, San Diego, USA).

Bioinformatics

Data were analyzed with Ridom SeqSphere+ (Ridom™, Münster, Germany) using a custom made core genome with the Fujisawa strain (NC_015601.1) as seed genome. Nine different *E. rhusiopathiae* genomes were used as query genomes (SY1027, NC_021354.1; GXBY-1, NZ_CP014861.1; WH13013, NZ_CP017116.1; ML101, NZ_CP029804.1; KC-Sb-R1; NZ_CP033601.1; NCTC8163; NZ_LR134439.1; G4T10, NZ_CP011860.1; SE38; NZ_CP011861.1; ZJ, NZ_CP041995.1). The samples were analyzed after a *de novo* assembly with the SKESA (version 2.3.0) algorithm (Souvorov et al., 2018).

Screening for the presence of antimicrobial resistance genes and point mutations causing antimicrobial resistance was performed using Resfinder V4.1 (Zankari et al., 2012), PointFinder (Zankari et al., 2017), and ResFinderFG V1.0 (Sommer et al., 2009; Pehrsson et al., 2016).

Results and discussion

Microbiological results and clinical course

E. rhusiopathiae was cultured from the wound biopsy. The microbial species was identified using both VITEK® 2 GP ID card with 98% likelihood and MALDI ToF/Vitek MS (99.9%). Susceptibility testing using MIC test strips revealed the minimum inhibitory concentrations (MIC) listed in Table 1. For rarely isolated species such as *E. rhusiopathiae*, no specific breakpoints currently exist according to the EUCAST (European Committee on Antimicrobial Susceptibility Testing) guidelines, therefore the interpretation of MIC values was according to the non-species-specific EUCAST PK-PD breakpoints. Following the EUCAST guidance document for the use of PK-PD breakpoints, the results of susceptibility testing cannot be reported in a categorical terms, but only in the form of a guidance for treatment. Accordingly, antimicrobials listed as S "may be used for treatment", and substances listes as R "should not be used for therapy". Therefore, due to the non-species-specific EUCAST PK-PD breakpoints, the use of benzylpenicillin, the antibiotic of choice, aminopenicillins cephalosoprines, carbapenems, and fluoroquinolones can be encouraged. In contrast, the U.S. Clinical Laboratory Standards Institute (CLSI) defines *E. rhusiopathiae* specific breakpoints in its document M45 "Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria", according to which benzylpenicillin, ampicillin, ceftriaxone, meropenem, imipenem, ciprofloxacin, and levofloxacin have been tested susceptible (Table 1). Other potential therapy options due to the CLSI breakpoints were macrolides (such as erthromycin) and clindamycin. However, no EUCAST PK-PD breakpoints are defined for these antimicrobials. The (intrinsic) resistance to vancomycin and aminoglycosides described in the literature was confirmed in the measured MIC values.

TABLE 1 Susceptibility of *E. rhusiopathiae* 319078 to various antimicrobials and assessment according to EUCAST PK-PD breakpoints.

Antimicrobial substance	MIC [mg/L]	Susceptibility EUCAST PK-PD	Susceptibility CLSI
benzylpenicillin	0.032	S	S
ampicillin	0.064	S	S
cefuroxime	0.032	S	–
ceftriaxone	0.032	S	S
ceftazidime	0.032	S	–
meropenem	<0.016	S	S
imipenem	0.008	S	S
ciprofloxacin	0.032	S	S
levofloxacin	0.032	S	S
moxifloxacin	0.032	S	–
gentamicin	128.00	R	R
vancomycin	32.00	IE (R)	R
erthromycin	0.25	IE	S
clindamycin	0.125	IE	S

Postoperative therapy was initial administration of cefazolin 2 g intravenously, followed by ampicillin/sulbactam (Unacid) orally (375 mg q6h) for 5 days (Table 2). The follow-up treatment was according to the standard of the "University Clinic for Plastic and Hand Surgery". The intraoperative wound flap was removed on the first

TABLE 2 Disease progression timeline.

Day	Symptoms, signs, medical findings and procedures
1	presentation with swelling, redness, and pain on the right thumb immediate surgical exploration and tissue biopsy perioperative prophylaxis with cefazolin 2 g i.v.
2	removal of the intraoperative wound flap prescription of ampicillin/sulbactam (Unacid) orally (375 mg q6h) for 5 days
4	cultural detection of *E. rhusiopathiae*
5	availability of the results of the susceptibility testing see Table 1
6	complete regression of redness, lymphangitis, and swelling termination of splint immobilization of the thumb ending antibiotic therapy
15	removal of suture material

postoperative day. By the fifth postoperative day, there was complete regression of redness, including lymphangitis and swelling. The skin in the wound area appeared non-irritant with contiguous wound edges and splint immobilization of the thumb was terminated. The suture material was removed on the 14th postoperative day.

The origin of the pathogen could not be clarified with complete certainty. The patient denied any direct contact to pigs, including handling of raw pork. She suspected that the source of infection could be a pond in which pigs usually bathe. The pond had no connection to other waters. It was located near several farms and functioned as a watering and bathing place for a number of animals. Fish that could potentially be another source of *E. rhusiopathiae* were not observed in the water body and it is also very unlikely that this water body was a suitable habitat for fish. Her dog bathed in the pond and she petted and dried him afterwards. We attempted to culture *E. rhusiopathiae* from pond water unsuccessfully.

Genome analysis

SKESA analysis of the Illumina sequences resulted in 50 continuous long reads (CLR) with an average (total) length of 34,401 base pairs. We additionally sequenced long-reads on a MinION (Oxford Nanopore technologies ltd., Oxford, United Kingdom), and performed hybrid assembly with unicycler (v0.4.8, https://github.com/rrwick/Unicycler). This resulted in a single polished contig of 1,780,614 base pairs. The G+C content of the contig was 36.5% and the top species match identity by Ridom Seqsphere+ was *E. rhusiopathiae* by 99%. No extrachromosomal elements were detected.

Application of the NCBI-annotation pipeline resulted in 1,714 genes. Of this total number of genes, 1,621 are protein-coding genes, 13 are pseudo-genes, and 80 are RNA-coding genes. Of the 80 RNA-coding genes, 55 encode tRNAs, 4 encode ncRNAs, 7 encode 5S rRNAs, 7 encode 16S rRNAs, and 7 encode 23S rRNAs. The genome harboured neither prophages nor transposable elements.

Additional application of the RAST-annotation pipeline yielded 1152 predicted coding sequences and 508 predicted hypothetical proteins. Based on sequence identity, motif analysis, and structural homology to proteins of known function (mostly from other microbial species), a functional prediction of protein function is made as part of the annotation process, which usually requires further experimental verification. The functional categorization of the predicted coding sequences is based on a pure *in silico* analysis that would have to be confirmed *in vitro* or *in vivo* to be considered certain. The RAST subsystem coverage was 25% (413 of 1660 genes). "Protein metabolism" (107 of 558 terms, 19.2%), "carbohydrate metabolism" (104 terms, 18.6%), "nuceloside/nucleotide synthesis" (48 terms, 8.6%), "amino acid metabolism" (47 terms, 8.4%), and "Cofactors, Vitamins, Prosthetic Groups, Pigments" (42 terms, 7.5%) form the largest functional categories in terms of number (Figure 2). In order to classify this subsystem category distribution, the distribution of our human isolate was compared to a bovine, a dolphin and a porcine isolate (Table 3). However, it must be taken into account that the porcine isolate was also present as a closed genome (1 contig), the dolphin isolate was present as an incomplete genome consisting of 109 contigs, and the bovine isolate genome consisted of 240 contigs from a bovine ruminal metagenome project. Comparison showed that the subsystem category distributions were nearly identical in the

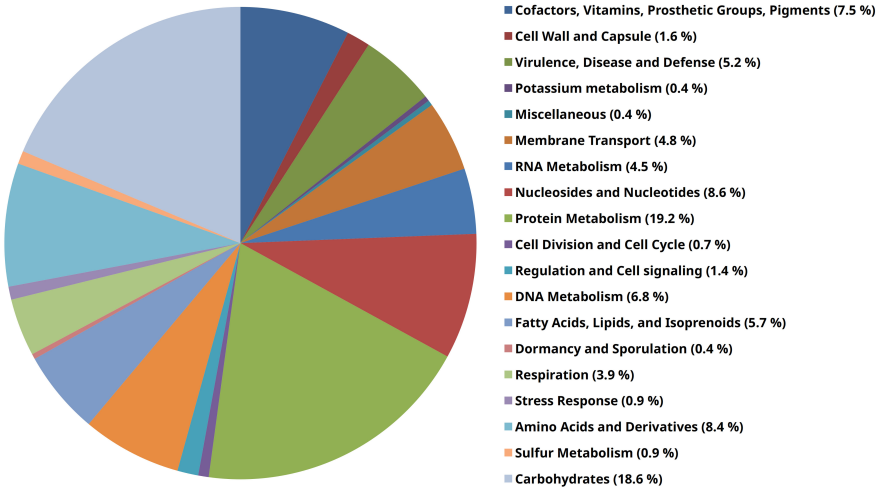

FIGURE 2
Pie chart of RAST subsystems that were identifiable in the genome of *E. rhusiopathiae* 319078. The 19 most abundant subsystems at the "category" level identified by RAST are represented by a specific color in the legend on the right side of the figure.

TABLE 3 Comparison of functional subsystem category distribution of *E. rhusiopathiae* isolates of different origins.

Subsystem Category	319078 human	RUG14096 cattle	19DISL dolphin	NCTC8163 swine
subsystem coverage	25%	26%	23%	25%
cofactors, vitamins, prosthetic groups, pigments	7.5%	8.2%	7.7%	7.6%
cell wall and capsule	1.6%	7.3%	1.6%	1.6%
virulence, disease and defense	5.2%	4.3%	5.2%	5.2%
potassium metabolism	0.4%	0.4%	0.4%	0.4%
miscellaneous	0.4%	0.5%	0.4%	0.4%
membrane transport	4.8%	2.5%	4.8%	4.9%
RNA metabolism	4.5%	4.5%	4.5%	4.5%
nucleosides and nucleotides	8.6%	6.0%	8.6%	8.7%
protein metabolism	19.2%	8.9%	18.9%	19.3%
cell division and cell cycle	0.7%	0.4%	0.7%	0.7%
regulation and cell signaling	1.4%	0.9%	1.4%	1.4%
DNA metabolism	6.8%	5.0%	6.4%	6.5%
fatty acids, lipids, and isoprenoids	5.7%	2.6%	5.7%	5.8%
dormancy and sporulation	0.4%	0.1%	0.4%	0.4%
respiration	3.9%	3.0%	3.9%	4.0%
stress response	0.9%	1.5%	0.9%	0.9%
amino acids and derivatives	8.4%	19.2%	8.4%	8.5%
sulfur metabolism	0.9%	0.4%	0.9%	0.9%
carbohydrates	18.6%	22.4%	18.9%	18.4%
secondary metabolism	0.0%	0.5%	0.0%	0.0%
nitrogen metabolism	0.0%	1.3%	0.0%	0.0%
phages, prophages, transposable elements, plasmids	0.0%	0.0%	0.4%	0.0%

human, the porcine, and the dolphin isolate. In the dolphin isolate, one category stood out in comparison to the human and the porcine isolate: "phages, prophages, transposable elements, plasmids". The genome of the dolphin isolate contained a temperate phage, as indicated by the corresponding genes for a phage terminase, a phage portal protein, a phage-associated type III restriction enzyme, and various bacteriophage hypothetical proteins, among others. The incomplete *E. rhusiopathiae* genome of bovine origin diverged most significantly from the genomes of the other three isolates in subsystem category distribution (Table 3). However, these discrepancies were most likely due to the fact that the contigs were derived from a metagenome analysis.

Antimicrobial resistance genes

Analysis of the genome using Resfinder V4.1, PointFinder, and ResFinderFG V1.0 (Selected %ID threshold 50% & Selected minimum length 40% for both tools) did not reveal any acquired antimicrobial resistance genes. Genes encoding gyrase (*gyrA*/*B*) and topoisomerase IV (*parC*/*parE*) were identified as potential determinants of quinolone resistance, but since no quinolone resistance was detected phenotypically, it was assumed that these genes were present in the wild-type form and did not contain point mutations that cause quinolone resistance. Furthermore, the genome of *E. rhusiopathiae* 319078 contained three genes encoding for proteins with homology to a MATE (Multidrug And Toxic Compound Extrusion) family MDR efflux pump (Table 4). This family of multidrug efflux transporter pumps was associated with fluoroquinolone resistance in *Bacteroides thetaiotaomicron* (Miyamae et al., 2001) as well as with fluoroquinolone, ethidium, and aminoglycoside resistance in *Vibrio parahaemolyticus* (Morita et al., 1998). However, the presence of these genes encoding proteins with a predicted function of MATE family efflux transporters did not confer phenotypic quinolone resistance but could be a factor accounting for the phenotypically observed aminoglycoside resistance in *E. rhusiopathiae* 319078.

A gene cluster homologous to the "*Streptococcus pneumoniae* vancomycin tolerance locus" was identified as a presumptive factor for intrinsic vancomycin resistance/tolerance in *E. rhusiopathiae*. The "*Streptococcus pneumoniae* vancomycin tolerance locus" (*vex123-pep*$_{27}$*-vncRS* locus) consists of an ABC transporter formed by the gene products of *vex1*, *vex2*, and *vex3*, the two-component response regulator VncR and it's associated sensor histidine kinase VncS as well as Pep27, a secreted peptide sensed by VncR/S (Novak et al., 1999; Mitchell and Tuomanen, 2002). In contrast, the homologous gene cluster of *E.*

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TABLE 4 Antimicrobial resistance genes.

Locus tag	Gene	Predicted function
NBX27_04295	*vex3*	ABC transporter membrane-spanning permease
NBX27_04300	*vex2*	ABC transporter, ATP-binding protein
NBX27_05510		MATE family efflux transporter
NBX27_06310		MATE family efflux transporter
NBX27_06335		MATE family efflux transporter
NBX27_00250		beta-lactamase class C-like and penicillin binding proteins (PBPs) superfamily
NBX27_04735		MBL fold metallo-hydrolase, beta-lactamase domain protein
NBX27_07960		MBL fold metallo-hydrolase, Zn-dependent hydrolase (beta-lactamase superfamily)

rhusiopathiae 319078 lacked the homologue of the secreted peptide gene *pep27* and the homologous gene to the Vex1 subunit of the ABC transporter, a transmenbrane protein (Figure 3 and Tables 4, 5). While it has been shown that knock-out of *pep27* has no effect on vancomycin-induced autolysis of *S. pneumoniae* (Robertson et al., 2002; Haas et al., 2004), the role of Vex1 has not yet been investigated in detail, neither in *S. pneumoniae* nor in *E. rhusiopathiae*. Vex1 and Vex3 are proposed to form a transmembrane protein channel while Vex2 is an ATP-binding cassette protein. It may be that in *E. rhusiopathiae* the Vex3 homolgue alone would able to shape a sufficient transmembrane channel. Since vancomycin resistance of *E. rhusiopathiae* is considered a characteristic resistance of this microbial species, further investigation of this four-gene gene cluster provides an interesting starting point for future experiments on vancomycin resistance. On the other hand, this gene cluster may also be completely non-functional, mainly due to the absence of a gene with predicted function of the Vex1 protein. The *vex23-vncRS* locus of *E. rhusiopathiae* is highly conserved in the available genome sequences. Among the 10 *E. rhusiopathiae* genomes deposited at NCBI, *vex2* has 99.84% to 100% sequence identity at a 100% coverage, *vex3* has 99.64% to 100% sequence identity at a 100% coverage, *vncS* has 99.64% to 99.86% sequence identity at a 100% coverage, and *vncR* (*luxR*) has 99.55% to 100% sequence identity at a 99% to 100% coverage.

In addition, one gene encoding a protein with the predicted function of a class C beta-lactamase and two genes each encoding a protein with the predicted function of a metallo-beta-lactamase were also present in the genome of *E. rhusiopathiae* 319078 (Table 4). However, phenotypically, all penicillins, aminopenicillins, cephalosporins, and carbapenems were tested susceptible.

Peptidoglycan biosynthesis

E. rhusiopathiae possesses a complete set of genes encoding enzymes with a predicted function for peptidoglycan biosynthesis (Table 5). These genes are not organized in a cluster but are scattered throughout the whole genome. In previous writings, intrinsic vancomycin resistance of this microbial species was thought to be due to the termination of the peptide stem of the peptidoglycan with D-alanine-D-lactate. This would be similar to what was found in vancomycin-resistant enterococci of the *vanA*/*vanB* phenotype (Nelson, 1999). However, according to our

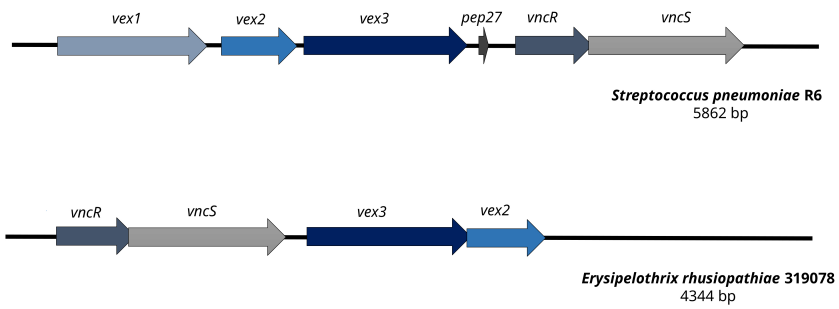

FIGURE 3
Comparison of the vancomycin tolerance locus of *Streptococcus pneumoniae* R6 and *Erysipelothrix rhusiopathiae* 319078. In contrast to the vancomycin tolerance locus of *S. pneumoniae* R6, *E. rhusiopathiae* 319078 lacks the *pep27* and *vex1* genes, yet the microbial isolate has a vancomycin MIC of 32.0 mg/L.

TABLE 5 Enzymes involved in peptidoglycan biosynthesis.

Locus tag	Gene	Predicted function
NBX27_08175	*murA*	UDP-N-acetylglucosamine-1-carboxyvinyltransferase
NBX27_02770	*murB*	UDP-N-acetylmuramate dehydrogenase
NBX27_02935	*murC*	UDP-N-acetylmuramate–L-alanine ligase or UDP-N-acetylmuramate–L-serine ligase?
NBX27_02805	*murD*	UDP-N-acetylmuramoyl-L-alanine-D-glutamate ligase
NBX27_04865	*murE*	UDP-N-acetylmuramoylalanyl-D-glutamate–L-alanine ligase
NBX27_03210	*murF*	UDP-N-acetylmuramoyl-tripeptide–D-alanyl-D-alanine ligase
NBX27_02800	*mraY*	Phospho-N-acetylmuramoyl-pentapeptide-transferase
NBX27_04480	*murG*	Undecaprenyldiphospho-muramoylpentapeptide beta-*N*-acetylglucosaminyltransferase
NBX27_05455	*murI*	glutamate racemase
NBX27_05555	*murJ/mviN*	murein biosynthesis integral membrane protein MurJ
NBX27_00365	*alr*	alanine racemase
NBX27_00660	*glmM*	phosphoglucosamine mutase
NBX27_02455	*glmS*	glutamine–fructose-6-phosphate transaminase
NBX27_02525	*mltG*	endolytic transglycosylase MltG
NBX27_03160	*uppS*	polyprenyl diphosphate synthase
NBX27_08340	*glmU*	bifunctional UDP-*N*-acetylglucosamine diphosphorylase/glucosamine-1-phosphate N-acetyltransferase GlmU
NBX27_02795	*pbp*	penicillin-binding protein

annotation, this does not seem to be the case, since the predicted function of the *murF* gene (NBX27_03210) was a UDP-N-acetylmuramoyl-tripeptide–D-alanyl-D-alanine ligase, and thus the pentapeptide stem ending would be D-Ala-D-Ala. Nevertheless, *E. rhusiopathiae* has some peculiarities concerning the cell wall peptidoglycan structure. There was a discrepancy in the predicted function of the protein encoded by *murC* between the NCBI and RAST annotation piplines. While NCBI predicted here the function of a UDP-N-acetylmuramate–L-alanine ligase the predicted function according to RAST was UDP-N-acetylmuramate-L-serine ligase. Thus, instead of an L-Ala, there could be an L-Ser at position 1 of the pentapeptide as in *Butyribacterium rettgeri* (Vollmer et al., 2008). A second peculiarity exists at position 3 of the pentapeptide. Instead of an L-Lys, as found in most Gram-positive bacteria, *E. rhusiopathiae* has a D-Ala at this position (Vollmer et al., 2008), which was also in agreement with the predicted function of the *murE* gene product as a UDP-N-acetylmuramoylalanyl-D-glutamate-L-alanine ligase. The pentapeptide stem of *E. rhusiopathiae* therefore should have an amino acid sequence of D-Ala-D-Glu-D-Ala-D-Ala-D-Ala or possibly L-Ser-D-Glu-D-Ala-D-Ala-D-Ala. To what extent this specific structure of the pentapeptide is related to the intrinsic vancomycin resistance of the bacterium requires further investigation.

Virulence factors

The virulence factors of *E. rhusiopathiae* were systematically characterized as part of the first complete genome announcement by Ogawa and colleagues (Ogawa et al., 2011). After more than a decade of improvements in the annotation pipelines and since our isolate was of human origin we decided to reassess the virulence factors in the genome of our isolate.

Two-component signal transduction systems

Bacteria regulate the expression of a variety of genes, including those encoding virulence-associated factors, with two-component signal transduction systems integrating external signals. Ogawa and coworkers were able to identify a total of 15 genes that presumptively encode response regulators, and for 14 of them they were able to identify the corresponding presumptive sensor histidine kinase upstream or downstream. But unfortunately, they could assign a predicted function only for 4 two-component signal transduction systems (Ogawa et al., 2011). With the help of the annotation pipelines we employed (NCBI & RAST), we were able to assign a predicted function to all 14 two-component signal transduction systems (Table 6). As shown previously by Ogawa and coworkers, the two-component system NBX27_00670/NBX27_00665 exhibited homology to CssS & CssR and therefore its presumptive function could be the control of cellular responses to protein secretion stress (Hyyryläinen et al., 2001; Ogawa et al., 2011). Similarly, the two-component system, for which a function in the regulation of the phosphate regulon responsible for uptake of inorganic phosphate was predicted, had also been localized in the genome of *E. rhusiopathiae* 319078 (NBX27_04520/NBX27_04525) (Ogawa et al., 2011; Santos-Beneit, 2015). Due to their sequence identity, we could assign two further two-component systems (NBX27_01470/NBX27_01465 &

TABLE 6 Two-component signal transduction systems.

Kinase	Response regulator	Predicted function
NBX27_00670	NBX27_00665	CssS, HAMP domain-containing histidine kinase & CssR, response regulator transcription factor (control of cellular responses to protein secretion stress)
NBX27_01190	NBX27_01185	HAMP domain-containing histidine kinase & LuxR family, response regulator transcription factor (quorum-sensing)
NBX27_01280	NBX27_01285	sensor histidine kinase, YesM & response regulator transcription factor, TrxR (thioredoxin reductase)
NBX27_01470	NBX27_01465	GHKL domain-containing protein & LytTR family DNA-binding domain-containing protein (virulence factors, e.g. extracellular polysaccharides, toxins and bacteriocins)
NBX27_01630	NBX27_01625	ATP-binding protein & response regulator transcription factor, OmpR family (adaptation to osmolality in *E. coli*; invasin expression in *Yersinia enterocolitica* (Brzóstkowska et al., 2012))
NBX27_01645	NBX27_01640	HAMP domain-containing histidine kinase & response regulator transcription factor, YrkP (Ogura et al., 2008)
NBX27_03955	NBX27_03960	histidine kinase of the competence regulon ComD & response regulator of the competence regulon ComE (capsular polysaccharide, CPS)
NBX27_04290	NBX27_04285	VncS, HAMP domain-containing sensor histidine kinase & VncR-homologue, two-component transcriptional response regulator (vancomycin tolerance)
NBX27_04520	NBX27_04525	two-component system sensor histidine kinase & phosphate regulon transcriptional regulatory protein PhoB (SphR, Pi uptake)
NBX27_04825	NBX27_04830	osmosensitive K^+ channel histidine kinase KdpD & two-component transcriptional response regulator, OmpR family (adaptation to osmolality)
NBX27_05995	NBX27_06000	ABC transporter-like sensor linked histidine kinase & ABC transporter-like sensor linked response regulator (magnesium and cobalt transport)
NBX27_06020	NBX27_06025	GHKL domain-containing protein & LytTR family DNA-binding domain-containing protein (virulence factors, e.g. extracellular polysaccharides, toxins and bacteriocins)
–	NBX27_06540	orphan response regulator
NBX27_07145	NBX27_07140	sensor histidine kinase & two-component transcriptional response regulator, LuxR family (quorum-sensing)
NBX27_07455	NBX27_07460	ABC transporter-coupled two-component system, signal transduction histidine kinase & ABC transporter-coupled two-component system, LuxR family response regulator (quorum-sensing)

NBX27_06020/NBX27_06025) to the LytTR family, which are potentially involved in regulating the expression of many virulence factors, e.g. extracellular polysaccharides, toxins and bacteriocins (Nikolskaya and Galperin, 2002; Ogawa et al., 2011). Three of the two-component systems (NBX27_01190/NBX27_01185, NBX27_07145/NBX27_07140, NBX27_07455/NBX27_07460) had response regulators with homologues to the LuxR family and could presumptively be involved in the regulation of quorum-sensing factors. The ComD/ComE system was shown to function as a negative transcriptional regulator of the capsular polysaccharide (*cps*) locus of *S. pneumoniae* (Zheng et al., 2017). A pair of homologous genes (NBX27_03955/NBX27_03960) was detected in *E. rhusiopathiae* 319078. The two-component signal transduction system NBX27_04290/NBX27_04285, which was homologous to *vncS*/*vncR* of *S. pneumoniae*, has already been discussed in the subsection "Antimicrobial resistance genes", as it may presumptively play a role in tolerance to vancomycin *via* regulation of *vex2* & *vex3* expression. Furthermore, we could localize two-component signal transduction systems in the genome of *E. rhusiopathiae* 319078 for which a function in the regulation of thioredoxin reductase expression, adaptation to osmolality, invasin expression, as well as magnesium and cobalt transport was predictable (Table 5). In the vicinity of the response regulator localized at locus tag NBX27_06540, no sensor histidine kinase was found in the genome of *E. rhusiopathiae* 319078, so that this remains an orphan response regulator gene (Table 6).

Capsular polysaccharide synthesis

Another important virulence-associated factor is the ability of a bacterium to form a capsule, or capsular polysaccharide synthesis. A seven-gene capsular polysaccharide synthesis locus was identified in the genome of *E. rhusiopathiae* Fujisawa (Ogawa et al., 2011), which was also found in *E. rhusiopathiae* 319078 at 100% coverage and 99.24% sequence identity.

Surface-associated proteins

In the genome of *E. rhusiopathiae* Fujisawa, a total of 21 proteins was detected containing an LPTXTG-motif. Based on this motif, these proteins are predicted to be covalently linked to peptidoglycan chains by a specific sortase, and based on this pepdidoglycan linkage, it is assumed that these were surface-associated proteins (Ogawa et al., 2011). Both the sortase (NBX27_00075) and its potential 21 substrates were localized by us in the genome of *E. rhusiopathiae* 319078. Updates in the annotation are provided in Table 7. Of particular note is the surface protective antigen adhesin SpaA (NBX27_00545), which also functions as antigen in many subunit vaccines (Opriessnig et al., 2020). SpaA itself belongs to three surface proteins that bind to choline residues of teichoic acid and by this become

TABLE 7 Bacterial surface proteins.

Locus tag	Gene	Predicted function
NBX27_00075		(sortase A, LPXTG specific)
NBX27_00445		shaft pilin (SpaA) isopeptide-forming pilin-related protein
NBX27_00545	*spaA*	surface protective antigen adhesin SpaA (choline-binding protein)
NBX27_00835	*hylA*	hyaluronate lyase precursor, polysaccharide lyase, family 8
NBX27_00890		LPXTG cell wall anchor domain-containing protein, peptidase M14
NBX27_01135		InlB B-repeat-containing protein
NBX27_01240		family 16 glycosylhydrolase, sialidase
NBX27_01430		DUF4573 domain-containing protein, cell-envelope associated proteinase, subtilase family
NBX27_01495		LPXTG cell wall anchor domain-containing protein
NBX27_01575	*nanH.1*	exo-alpha-sialidase (neuraminidase)
NBX27_02145	*cbpA*	glucosaminidase domain-containing protein, Choline binding protein A
NBX27_02955		discoidin domain-containing protein
NBX27_03280		Cna B-type domain-containing protein
NBX27_03285		Cna B-type domain-containing protein
NBX27_03565		leucine-rich repeat domain-containing protein, possible surface protein responsible for cell interaction; contains cell adhesion domain and ChW-repeats
NBX27_03750	*hylB*	hyaluronate lyase precursor, polysaccharide lyase, family 8
NBX27_03765	*cbpB*	choline-binding protein
NBX27_03810		C69 family dipeptidase
NBX27_05710	*ushA*	5'-nucleotidase C-terminal domain-containing protein
NBX27_06110	*hylC*	hyaluronate lyase precursor, polysaccharide lyase, family 8
NBX27_06345		cell wall anchor protein
NBX27_07175		(shaft pilin) SpaA isopeptide-forming pilin-related protein
NBX27_07275		putative peptidoglycan bound protein (LPXTG motif) Lmo2179 homolog, peptidase
NBX27_07355		protein phosphatase 1 regulatory subunit 42
NBX27_08485		Cna B-type domain-containing protein, LPXTG-motif cell wall anchor domain

membrane anchored (Ogawa et al., 2011; Borrathybay et al., 2015). It plays a significant role in virulence, adhesion to host cells, and serum resistance of *E. rhusiopathiae* (Borrathybay et al., 2015). In addition, two homologues to the *Streptococcus pyogenes* shaft pilin SpaA (Ramirez et al., 2020) were found in the genome (NBX27_00445 & NBX27_07175), which should not be confused with the surface protective antigen adhesin SpaA of *E. rhusiopathiae* and which possess an LPXTG motif and are therefore predicted to be membrane-anchored *via* the sortase already described. Hyaluronate lyases are considered to be a significant virulence factor, especially with regard to spreading in relatively hyaluronic acid-rich tissues such as the skin. Three coding sequences (CDSs) encoding proteins for which a hyaluronate lyase activity was predicted (NBX27_00835, NBX27_03750, & NBX27_06110) were found in the genome of *E. rhusiopathiae* 319078, representing potentially important factors in the pathogenesis of erysipeloid. Major virulence factors with complex action are bacterial neuraminidases (Soong et al., 2006). Both the *E. rhusiopathiae* Fujisawa (Ogawa et al., 2011) and *E. rhusiopathiae* 319078 genomes have two CDSs encoding for proteins for which a neuraminidase function was predicted. One carries the LPXTG motif and therefore should potentially be cell surface associated (NBX27_01575), the second apparently could act potentially as an extracellular enzyme (NBX27_03725).

Inactivation of reactive oxygen species

Other significant virulence-associated factors are those that enable intracellular survival of the bacterium. Bacteria must protect themselves from reactive oxygen species (ROS) after the formation of the phagolysosome. Analysis of the *E. rhusiopathiae* Fujisawa genome identified 9 genes encoding enzymes with a predicted function indicating that they potentially play a role in the neutralization of ROS: a predicted superoxide dismutase, two predicted thioredoxins, two predicted thioredoxin-disulfide reductases, a predicted thiol peroxidase, a predicted glutaredoxin, and two predicted alkylhydroperoxide reductases (Ogawa et al., 2011). We were able to add three more CDSs to this funcional subgroup (Table 8): a predicted third thioredoxin gene (NBX27_00960), a predicted peptide methionine (S)-S-oxide reductase MsrA (NBX27_00585) that presumably reduces ROS-generated methionine sulfoxide in proteins back to methionine (Weissbach et al., 2002), and a predicted peroxide stress protein YaaA-homologue (NBX27_07905). YaaA was shown to reduce hydrogen peroxide induced damage by decreasing the fraction of intracellular unincorporated iron (Liu et al., 2011).

TABLE 8 Antioxidant factors.

Locus tag	Gene	Predicted function
NBX27_00585	*msrA*	peptide-methionine (S)-S-oxide reductase MsrA
NBX27_00895	*tpx*	thiol peroxidase
NBX27_00955	*ahpC*	peroxiredoxin, Bcp-type
NBX27_00960		thioredoxin
NBX27_01880	*nrdH*	glutaredoxin
NBX27_01975	*trxA.1*	thioredoxin
NBX27_05285	*sodA*	superoxide dismutase
NBX27_06495	*trxB.1*	thioredoxin reductase, NAD(P)/FAD-dependent oxidoreductase
NBX27_06660	*ahpD*	carboxymuconolactone decarboxylase family protein
NBX27_07535	*trxA.2*	thioredoxin
NBX27_07755	*trxB.2*	thioredoxin reductase, FAD-dependent oxidoreductase
NBX27_07905	*yaaA*	peroxide stress protein YaaA

Phospholipases

Another group of enzymes that play a role in the intracellular life cycle of some bacteria are phospholipases. For example, it has been shown that patatin phospholipases of *Rickettsia typhi* contribute to open the phagosome or phagolysosome membrane and allow the bacterium to escape into the cytoplasm (Rahman et al., 2013; Smith and May, 2013). More recent studies demonstrated that phospholipases aid in the escape from vacuoles and phagosomes for *Listeria monocytogenes*, *Shigella* spp., *Plasmodium berghei*, *Salmonella* spp., and *Legionella pneumophila* (Bianchi and van den Bogaart, 2020; Petrišič et al., 2021; Srivastava and Mishra, 2022). Additionally, it was experimentally proven that *Mycobacterium tuberculosis* recruits the cytoplasmic phospholipase A_2 to permeabilize the endosomal membrane in infected macrophages and to translocate to the cytosol (Jamwal et al., 2016). On the other it was shown that *E. rhusiopathiae* predominantly replicates in the cytoplasm of macrophages in the spatial vicinity of the entry site (Shimoji et al., 1996; Shimoji, 2000). Therefore, Ogawa and colleagues postulated that phospholipases also play a pivotal role in intracellular translocation of *E. rhusiopathiae* during phagosome opening. However, experimental evidence of this role remains to be provided for this microbial species. Ogawa and coworkers identified a total of 9 CDSs with homology to phospholipases in the genome of *E. rhusiopathiae* Fujisawa genome (Ogawa et al., 2011), which we also found in the genome of *E. rhusiopathiae* 319078 (Table 9).

Further virulence associated factors

One of the predicted virulence-associated factors additionally detected by RAST subsystem analysis in the genome of *E. rhusiopathiae* 319078 was a homologue to the multiple sugar metabolism regulator, (MsmR, NBX27_01505, Table 10). MsmR, an AraC/XylS type transcriptional regulator,

TABLE 9 Phospholipase genes.

Locus tag	Gene	Predicted function
NBX27_00385		patatin-like phospholipase family protein
NBX27_00485		dienelactone hydrolase family protein
NBX27_00825	*pldB*	lysophospholipase, monoglyceride lipase
NBX27_01735	*cls*	cardiolipin synthase
NBX27_01740		patatin family protein
NBX27_01835		dienelactone hydrolase family protein
NBX27_02035		phospholipase D family protein
NBX27_06130		lysophospholipase, monoglyceride lipase alpha/beta hydrolase
NBX27_07160		lysophospholipase

is part of the *Streptococcus pyogenes* recombinatorial zone. In *S. pyogenes* this highly recombinatorial zone consists of genes encoding chaperonin, Hsp33; sortase, Spy0135; serum opacity factor, SOF; transcriptional regulator, RofA; negative transcriptional regulator, Nra; fibronectin-binding protein, PrtF; fibronectin-binding protein 2, PrtF2; collagen-binding adhesin, Cpa; multiple sugar metabolism regulator, MsmR; electron transfer flavoprotein 1A, EtfLS; and signal peptidase I, LepL. The gene products include several MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) and play a crucial role in pili-production and mediate adhesion to human cells and tissues (Podbielski et al., 1999; Kreikemeyer et al., 2007). Transcriptome analysis in *S. pyogenes* serotype M49 showed that the MsmR regulon contains 24 genes under positive MsmR control and 36 genes repressed by MsmR (Nakata et al., 2005). It was demonstrated by electrophoretic mobility shift assay (EMSA) that MsmR binds directly to the promoter regions of the genes encoding fibronectin-binding protein 2 (*prtF2*), negative regulator of group A Streptococci (*nra*), collagen-binding protein (*cpa*), NAD-glycohydrolase (*nga*), and streptolysin O (*slo*) (Nakata et al., 2005). In Gram-positive bacteria such as group A streptococci, a cytolysin-mediated translocation (CMT) system

TABLE 10 Virulence assocated factors (incl. hemolysins, adhesins, etc.).

Locus tag	Gene	Predicted function
NBX27_03725	*nanH.2*	glycoside hydrolase (neuraminidase)
NBX27_02485		hemolysin family protein (containing CBS domains)
NBX27_03180		hemolysin III family protein
NBX27_08265		thermostable hemolysin delta-VPH
NBX27_01505		multiple sugar metabolism regulator (MsmR), AraC family transcriptional regulator
NBX27_05085		fibronectin/fibrinogen-binding (NFACT family) protein
NBX27_06720	*znuA*	zinc ABC transporter substrate-binding protein
NBX27_07335		Ig-like domain-containing protein

replaces the "type III secretion machinery" commonly found in Gram-negative bacteria. Transcription of this CMT system, which plays an important role in host cell interaction, is regulated in particular by MsmR (Madden et al., 2001). However, only a MsmR homologue of this gene cluster is present in the genome of *E. rhusiopathiae* 319078. Whether a similar role in host cell adherence, internalization, and cytotoxicity exists in *E. rhusiopathiae* analogous to the role in *S. pyogenes* remains to be confirmed experimentally. The predicted MsmR was detectable in all of the 10 *E. rhusiopathiae* genomes deposited at NCBI with 99.84-99.92% sequence identity at a 100% coverage. Therefore, the MsmR homologue appears to be ubiquitous in *E. rhusiopathiae*. In addition, genes for a predicted type III hemolysin (NBX27_03180) and a predicted hemolysin-related protein (NBX27_02485) with a cystathionine-beta-synthase (CBS) domain exist in the *E. rhusiopathiae* 319078 genome that could be part of a potential CMT system. Another predicted hemolysin not previously described for *E. rhusiopathiae* is a homologue of the thermostable hemolysin delta-VPH (NBX27_08265), which was first described in *Vibrio parahaemolyticus* (Taniguchi et al., 1990).

Conclusions

In summary, we presented a patient with erysipeloid and lymphangitis. The site of entry was probably a minor trauma of the thumb. The source of infection could not be identified with certainty. A body of water in which pigs and the patient's dog bathed consecutively was considered as potential reservoir. The genome of the *E. rhusiopathiae* isolate causative for the infection was whole-genome sequenced. A predicted *vex23-vncRS* locus homologous to the "*S. pneumoniae* vancomycin tolerance locus" and three predicted MATE family efflux transporters were identified as potential antimicrobial resistance determinants. Several genes encoding proteins with predicted functions that qualify them as potential virulence-associated factors have been identified, including a predicted homologue to the multiple sugar metabolism regulator, MsmR, which cloud play a role in host cell interaction, three predicted hemolysins, two predicted neuraminidases, three predicted hyaluronate lyases, the surface protective antigen adhesin SpaA, a subset of predicted enzymes that potentially play a role in intracellular survival, several predicted two-component signal transduction systems, two dozen predicted surface-associated proteins and a homologue to the capsular polysaccharide synthesis locus.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, CP098031.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

Conceptualization, AK, AZ; methodology, AK, AZ; writing-original draft preparation, AZ, C-JS; writing-review and editing, AT, C-JS, PM, MR, MĐ, AD, DM, AK, AZ; visualization, AZ, MĐ; investigation, MĐ, DM; funding acquisition AK, whole genome sequencing and genome assembly, AT, PM, MR; data curation, annotation, patient treatment, AD; All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary Material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.981477/full#supplementary-material

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Shewanella putrefaciens, a rare human pathogen: A review from a clinical perspective

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Shewanella putrefaciens is a gramnegative, facultatively anaerobic, rod shaped bacterium. It belongs to the class of the *Gammaproteobacteria* and was first described in 1931. *S. putrefaciens* is part of the marine microflora and especially present in moderate and warm climates. The bacterium is a rare oppurtonistic human pathogen associated mainly with intra-abdominal as well as skin and soft tissue infections. However, it has also been reported in association with more severe diseases such as pneumonia, intracerebral and ocular infections and endocarditis. In these cases the clinical courses are often associated with underlying, predisposing diseases and risk factors. For successful treatment of *S. putrefaciens*, a combination of appropriate local therapy, e.g. surgical treatment or drainage, and antibiotic therapy should be performed. Since multiple resistances to antibiotics are described, the results of the antimicrobial susceptibility testing must be considered for effective therapy as well. Furthermore, a main challenge in clinical practice is the accurate microbiological identification, and especially the correct differentiation between *S. putrefaciens* and *S. algae*. Under certain circumstances, *Shewanella*-infections can have severe, sometimes even fatal consequences. Therefore, we decided to present the current state of knowledge as well as further aspects with regard to future diagnostics, therapy and research.

KEYWORDS

***Shewanella putrefaciens*, human infection, rare bacterial pathogen, Shewanella, rare human pathogen**

Introduction

The species *Shewanella putrefaciens* is a gram-negative bacterium first described in 1931 (Derby and Hammer, 1931). It is part of the marine microflora and especially present in moderate and warm climates (Holt et al., 2005; Vignier et al., 2013; López Aperador et al., 2016; Yu et al., 2022). In addition, it is an important spoilage agent of protein-rich refrigerated foods (Brink et al., 1995; Vogel et al., 1997).

S. putrefaciens is a rare oppurtonistic human pathogen (Tsai et al., 2008). The bacterium is mainly associated with skin- and soft-tissue and intra-abdominal infections, especially biliary tract infections and peritonitis (Chen et al., 1997; Holt et al., 2005; Vignier et al., 2013). *S. putrefaciens* can also lead to bacteremia with septic and possibly even lethal courses (Brink et al., 1995; Chen et al., 1997; Tang et al., 2016). *S. putrefaciens* infections are often polymicrobial, so the human pathogenic role of the bacterium requires further clarification (Brink et al., 1995; Chen et al., 1997; Yu et al., 2022).

According to the "List of Prokaryotic names with Standing in Nomenclature" (LSPN) there are currently 80 species of the genus Shewanella validly described (Parte et al., 2020). To the best of our knowledge, only 3 human pathogenic Shewanella spp. have been described so far. Thus, *S. putrefaciens*, *S. algae* and *S. xiamenesis* have been cultured from clinical isolates (Zong, 2011; Yu et al., 2022). *S. haliotis* has been described in the past as another human pathogenic Shewanella spp. (Poovorawan et al., 2013; Byun et al., 2017). However, Szeinbaum et al., 2018 stated that *S. haliotis* must be identified as *S. algae* (Szeinbaum et al., 2018). Previously, *S. algae* was considered a subspecies of *S. putrefaciens* (Khashe and Janda, 1998; Tsai et al., 2008). It was not until 1990 that *S. algae* was described by Simidu et al., 1990 as a new species of the genus Shewanella (Simidu et al., 1990; Nozue et al., 1992). In the following years, subsequent studies and investigations revealed that probably more than 80% of human infections previously thought to be caused by *S. putrefaciens* are actually caused by *S. algae* (Holt et al., 2005; Tsai et al., 2008). This is due to the fact that biochemical and phenotypic characterization tests, respectively, and conventional bacterial identification systems are hardly able to correctly distinguish between these two clinically relevant Shewanella species (Tsai et al., 2008; Sharma and Kalawat, 2010; Vignier et al., 2013; Benaissa et al., 2021). Therefore, currently correct pathogen identification requires analyses beyond routine daily diagnostics.

In this review, we summarize the presently available knowledge of *S. putrefaciens* infections and describe the microbiological aspects of the species, the difficulties of species identification, pathogenicity, clinical features, the antimicrobial susceptibility, options for antimicrobial treatment and aspects of further research.

Search strategy and selection of articles

A literature search in PubMed was performed using the following keywords: `*Shewanella putrefaciens* AND Infection´. All studies published in PubMed since the initial description up to March 31st 2022 were included. All available manuscripts related to *S. putrefaciens* and references cited in the relevant articles were evaluated for their relevance for the topic of this review. Only case reports of human infections caused or associated with *S. putrefaciens* were included. *S. algae* was frequently misclassified as *S. putrefaciens* in the past and was first described only in 1990, so published case reports attributed to *Achromobacter putrefaciens* or *Pseudomonas putrefaciens* were excluded (Simidu et al., 1990; Nozue et al., 1992; Vogel et al., 1997; Holt et al., 2005; Tsai et al., 2008; Vignier et al., 2013).

History and taxonomy

S. putrefaciens was first described by Derby and Hammer in 1931 (Derby and Hammer, 1931). They isolated a strain from putrified butter, which was initially identified as *Achromobacter putrefaciens*. Further studies have shown that it is a species that has not yet been described (Nozue et al., 1992; Holt et al., 2005). In 1941, it was assigned to the genus *Pseudomonas* and subsequently named *Pseudomonas putrefaciens* (Long and Hammer, 1941). According to Shewan et al., 1960 in the following decades this organism was classified in Pseudomonas group IV (Shewan et al., 1960). Based on the G+C content, the assignment to the genus of *Alteromonas* was made in 1972 (Baumann et al., 1972). However, based on phylogenetic studies, these organisms were reclassified in 1985 to the new genus *Shewanella*, named after the scotish bacteriologist James M. Shewan (Chaudhary et al., 2022), and included in the family *Vibrionaceae* (MacDonell and Colwell, 1985; Holt et al., 2005). In 1990, Simidu et al. isolated a new mesophilic Shewanella species from red alga and described it as *S. alga* (Simidu et al., 1990; Nozue et al., 1992; Vogel et al., 1997). Nozue et al. concluded in 1992 that the vast majority of strains previously identified as *S. putrefaciens* should be categorized as *S. alga* (Nozue et al., 1992). Finally, in 2004, Ivanova et al. introduced the familiy *Shewanellaceae* with *Shewanella* as the type genus (Ivanova et al., 2004). Today, according to the "List of Prokaryotic names with Standing in Nomenclature" (LPSN), *S. putrefaciens* belongs to the family *Shewanellaceae* within the class of *Gammaproteobacteria* (Ivanova et al., 2004; Parte et al., 2020). As far as we know, *S. putrefaciens* is one of 3 human pathogenic Shewanella spp. known to date (Zong, 2011; Yu et al., 2022).

Phenotypic characteristics of *Shewanella putrefaciens*

S. putrefaciens is a gramnegative, facultatively anaerobic, rod shaped, oxidase and catalase positive, motile bacterium with a single polar flagellum (Nozue et al., 1992; Héritier et al., 2004; Holt et al., 2005; Sharma and Kalawat, 2010; Yu et al., 2022). The bacterium has a G+C content between 45-48 mol% and grows in 1-2mm large, yellowish-brown colonies (Nozue et al., 1992; Holt et al., 2005; Jayalekshmi et al., 2022). Hydrogen sulfide generation is the main phenotypic feature (Holt et al., 2005). According to Vogel et al., 1997 various electron acceptors such as trimethylamine N-oxide (TMAO), elemental sulfur, nitrate, iron, thiosulfate, manganese or fumarate can be dissimilatory reduced by this bacterium (Vogel et al., 1997).

As far as we know, *S. algae* and *S. putrefaciens* are two of three potentially human pathogenic Shewanella-species isolated from clinical specimens to date (Zong, 2011; Vignier et al., 2013; Benaissa et al., 2021; Yu et al., 2022). After the initial description of the species *S. putrefaciens*, *S. algae* were often misidentified as *S. putrefaciens* (Sharma and Kalawat, 2010). A strain that parasitizes in red algae was first described as *S. alga* in 1990 by Simidu et al. (Simidu et al., 1990; Nozue et al., 1992; Vogel et al., 1997; Pagniez and Berche, 2005). Compared to *S. algae*, *S. putrefaciens* has stronger saccharolytic activity (Holt et al., 2005). According to Holt et al., 2005, the bacterium is able to produce acid from maltose, glucose, partially also from sucrose and arabinose, while *S. algae* usually metabolizes only ribose (Holt et al., 2005). In addition, unlike *S. putrefaciens*, *S. algae* also grows at 42°C, on Salmonella-Shigella agar, has a tolerance to 6% NaCl and forms beta-hemolytic, mucoid colonies on sheep blood agar (Nozue et al., 1992; Holt et al., 2005). However, Vogel et al., 1997 recommended the use of 10% NaCl to distinguish between the two species (Vogel et al., 1997).

Occurrence and natural habitat

S. putrefaciens was first isolated from water supplies of dairies and putrified butter (Derby and Hammer, 1931; Holt et al., 2005).

Their natural occurrence includes all types of water including fresh, marine, river and sewage all over the world (Brink et al., 1995; Chen et al., 1997; Khashe and Janda, 1998; Oh et al., 2008; Vignier et al., 2013; Yu et al., 2022). The bacterium is a component of the marine microflora (Vignier et al., 2013). Geographically, they are mainly found in moderate and warm climates (Holt et al., 2005; López Aperador et al., 2016). In addition, they have already been detected in natural energy reserves such as petroleum brines or natural gas (Chen et al., 1997; López Aperador et al., 2016).

They have also been isolated from a variety of foods including milk, cream, butter, eggs, poultry, (raw) fish or seafood and beef products (Chen et al., 1997; Khashe and Janda, 1998; Bulut et al., 2004; Oh et al., 2008). *S. putrefaciens* is a biofilm former and able to reduce TMAO to trimethylamine (Vogel et al., 1997; Bagge et al., 2001; Holt et al., 2005; Jayalekshmi et al., 2022). This seems to be a relevant reason why this bacterium is an important spoilage agent of protein-rich refrigerated foods, especially for frozen white-fleshed fish from temperate waters (Brink et al., 1995; Vogel et al., 1997; Jayalekshmi et al., 2022).

Identification of *Shewanella putrefaciens* in routine diagnostics

Proper differentiation between *S. putrefaciens* and *S. algae* in daily routine diagnostics is challenging. Both Shewanella spp. grow after an incubation period of 18-24 hours on conventional solid culture media, *S. putrefaciens* e.g. on Luria-Bertani (LB) broth, and on media commonly used in microbiological diagnostics, such as Mac-Conkey agar (Holt et al., 2005; Jayalekshmi et al., 2022).

In a study published in 1992, Nozue et al. found that *S. putrefaciens* strains with a high G+C content of 52 to 54 mol% did not belong to the type strain of *S. putrefaciens* (ATCC 8071) but to that of *S. alga*, later referred to as *S. algae* (Nozue et al., 1992; Trüper and De'Clari, 1997; Khashe and Janda, 1998). Recent data indicate that more than 80% of clinical *S. putrefaciens* isolates have been misidentified in the past and probably need to be assigned to *S. algae* (Holt et al., 2005; Tsai et al., 2008; Vignier et al., 2013). This is due to the fact that both conventional bacterial identification systems and biochemical testing methods cannot clearly distinguish between the both (Tsai et al., 2008; Sharma and Kalawat, 2010; Vignier et al., 2013; Benaissa et al., 2021). For example, the databases of semi-automatic and automatic systems such as Vitek 2, API ID 32 GN, API 20E and 20 NE contain *S. putrefaciens* but not *S. algae*, which may lead to misidentification as *S. putrefaciens* due to the high similarity of both species (Holt et al., 2005; Vignier et al., 2013; Yu et al., 2022). By performing 16S rRNA analyses on three isolates, previously identified as *S. putrefaciens* by biochemical assays, Vignier et al., 2013 were able to correctly identify all three isolates as *S. algae* by molecular analyses (Vignier et al., 2013). To our knowledge, MALDI-TOF mass spectrometry also appears to be a good method for identifying Shewanella spp. but requires further analysis (Byun et al., 2017; Yu et al., 2021; Yu et al., 2022). Therefore, analysis beyond routine daily diagnostics by 16S rRNA sequencing, ribotyping, or whole-cell protein profiling is required to correctly distinguish between the two Shewanella spp. (Vogel et al.,

1997). In our experience, whole genome sequencing followed by digital DNA-DNA hybridisation (dDHH) has proven to be very useful in determining the actual species present (Kopf et al., 2021). This procedure is nowadays regarded as the gold standard of molecular species identification. It can therefore be assumed that *S. putrefaciens* and *S. algae* can also be sufficiently identified using this method (Richter and Rosselló-Móra, 2009). Futhermore, a more advanced method to distinguish between individual Shewanella spp. is multilocus sequence analysis (MLSA) of different protein-coding genes (Fang et al., 2019).

Pathogenicity and potential virulence factors

S. algae seems to cause more human infections than *S. putrefaciens* (Nozue et al., 1992; Vogel et al., 1997; Khashe and Janda, 1998; Sharma and Kalawat, 2010). The causative pathomechanisms have not been fully clarified. Based on current knowledge, in human pathogenic Shewanella-subspecies appear to colonize appropriate tissues and subsequently cause local and eventually invasive infection in patients with predisposition (Yohe et al., 1997; Sharma and Kalawat, 2010).

Hepatobiliary disorders, such as cholelithiasis or liver cirrhosis, are important risk factors for *S. putrefaciens* infections (Chen et al., 1997; Yu et al., 2022). In addition, numerous infections of the biliary tract system by this pathogen have been described (Chen et al., 1997). The species has also been isolated in the past from oil, petroleum or fatty foods, so its lipophilia seems to be a possible explanation for its biliary affinity (Chen et al., 1997; Oh et al., 2008). Another important virulence factor appears to be the production of extracellular enzymes such as lecithinase, lipase and DNase (Papanaoum et al., 1998). Local enzyme production favors the development of necrosis of the skin and subcutaneous tissue and thus the development of skin and soft tissue infections (Papanaoum et al., 1998). In addition, *S. putrefaciens* is a biofilm former (Bagge et al., 2001; Holt et al., 2005; Jayalekshmi et al., 2022). This ability could play an important role in causing catheter-associated infections as described by Shrishrimal in 2012 (Shrishrimal, 2012). In addition, *S. putrefaciens* is able to attach to and invade human intestinal epithel cells (Dias et al., 2019). In our view, the causative virulence factors of the bacterium that lead to infections in humans have not yet been fully elucidated.

Clinical features and risc factors for *Shewanella putrefaciens* infections

Case reports deemed clinically relevant to this review have been compiled in Supplementary Table 1.

We identified a total of 87 relevant, published cases of *S. putrefaciens* infections. In 27 cases, it was not possible to make a statement about the sex due to missing information in the individual case reports. The remaining 60 cases were 73.3% male and 26.7% female. Considering only the infections in adults, it was not possible to make a statement about the sex of a total of 7 cases. The remaining 58 cases were 75,9% male and 24,1% female.

Predominantly, the bacterium is associated with skin and soft-tissue infections (Brink et al., 1995; Chen et al., 1997; Yohe et al., 1997; Papanaoum et al., 1998; Pagani et al., 2003; Bulut et al., 2004; Otsuka et al., 2007; Sharma and Kalawat, 2010; Prinja et al., 2013; Mohr et al., 2016; Ryan et al., 2018; Latif et al., 2019; Patel et al., 2020). Under certain circumstances, these can take fatal courses up to necrotizing fasciitis (Yim et al., 2010; Giroux et al., 2017) and the development of a Fournier's gangrene (Tang et al., 2016). In addition, *S. putrefaciens* can cause arthritides and osteomyelitides (Levy and Tessier, 1998; Carlson and Dux, 2013; Guinetti-Ortiz et al., 2016). Potential entry ports and a typical predisposing factors for corresponding infections are chronic ulcers, especially of the lower extremities, and/or traumatic injuries associated with (sea) water or fish (water) exposure (Oh et al., 2008; Carlson and Dux, 2013; Vignier et al., 2013; Guinetti-Ortiz et al., 2016).

Another major risk factor for infection with this pathogen seems to be an end-stage renal disease. Several cases, particularly CAPD-associated *S. putrefaciens* infections with concomitant peritonitis and, in some cases, associated bloodstream infections, have been published (Chen et al., 1997; Bhandari et al., 2000; Chang et al., 2005; Yim et al., 2010; Shrishrimal, 2012; Lee et al., 2016; López Aperador et al., 2016). *S. putrefaciens* is a biofilm former (Bagge et al., 2001; Holt et al., 2005; Vickers and Ullian, 2011; Jayalekshmi et al., 2022). Therefore, we conclude that dialysis catheters placed in the body, i.e., peritoneal or hemodialysis catheters, are important risk factors and potential entry ports.

Due to the lipophilia of the bacterium, diseases of the bile ducts and also the use of external hepatobiliary drainage catheters are another predisposing factors for *S. putrefaciens* infections (Chen et al., 1997; Oh et al., 2008). Biliary tract infections caused by this Shewanella-species have sometimes been described in association with liver abscess formation (Chen et al., 1997).

Due to traumatic lesions, the pathogen can also lead to severe infections of the eye (Butt et al., 1997; Mohan et al., 2014). Chronic otitis media also appears to be a possible entry port for intracerebral infections (Süzüku et al., 2004; Yilmaz et al., 2007). In the rare case of colonization of the upper and possibly lower respiratory tract by this bacterium, it can also cause severe pneumonia, sometimes accompanied by respiratory failure and the need for ventilation (Holt et al., 2005; Basir et al., 2012; Durdu et al., 2012; Patel et al., 2012).

In the case series published by Brink et al. in 1995, numerous neonatal and pediatric *S. putrefaciens* infections were reported.

In particular, low birth weight in combination with poor living standards, especially in premature infants, appears to be a relevant risk factor for bacteremia and, especially, septic or even lethal courses at this age (Brink et al., 1995). Bloodstream infections are not unique to childhood. After all 65.33% (n=75, in 11 cases not reported, in 1 case no blood cultures performed) of the cases in this review were bacteremic. In fact, 2 publications even reported infective endocarditis due to this pathogen, one each with poly- and monomicrobial bacteremia (Dhawan et al., 1998; Constant et al., 2014).

S. putrefaciens infections can also cause severe septic courses up to the development of septic shock with multiple organ failure, especially in predisposed individuals. The overall mortality rate in our review was 20% (n=85), but only 18.82% of patients died due to infection. It should be noted that, in addition to the diseases already mentioned, diseases such as diabetes mellitus, peripheral vascular disease, and malignant neoplasms, as well as (drug-induced) immunosuppression, are important risk factors for infection with the described bacterium (Holt et al., 2005; Yilmaz et al., 2007; Basir et al., 2012; Carlson and Dux, 2013; Benaissa et al., 2021). Low socioeconomic status, poor personal hygiene, private or occupational exposure to (sea) water, and consumption of contaminated seafood or fish meat also appear to be predisposing factors for development of *S. putrefaciens* infections (Otsuka et al., 2007; Yilmaz et al., 2007; Oh et al., 2008; Carlson and Dux, 2013; Muñoz et al., 2015).

Most of the Shewanella-infections listed in Supplementary Table 1 are community-aquired infections. In 2008, Oh et al. reported an in-hospital Shewanella outbreak at a tertiary acute care hospital (Oh et al., 2008). A reused measuring cup for emptying catheter bags was retrospectively identified as the source of the outbreak (Oh et al., 2008). After changing of measuring cups after each use, and adherence to strict hygiene procedures, the local epidemic was contained and controlled (Oh et al., 2008). Shewanella-infections can thus also be spread by contact transmission (Oh et al., 2008).

Antibiotic susceptibility and treatment

Susceptibility testing is essential, especially regarding targeted anti-infective treatment. Currently, there are no defined criteria for interpreting antibiotic resistance in Shewanella spp. (Yu et al., 2022). Different methods have been used for antimicrobial susceptibility testing. For example, Chen et al., 1997 and Brink et al., 1995 used the disc diffusion method; Otsuka et al., 2007 and Benaissa et al., 2021 used the microdilution method to determine the minimum inhibitory concentration (MIC) (Brink et al., 1995; Chen et al., 1997; Otsuka et al., 2007; Benaissa et al., 2021). Due to the expression of β-lactamases, they often show resistance to penicillin, which is frequently used for the treatment of soft tissue infections (Héritier et al., 2004; Vignier et al., 2013; Ryan et al., 2018). *S. putrefaciens* is usually susceptible to piperacillin, fluoroquinolones, aminoglycosides and carbapenems (Vogel et al., 1997; Holt et al., 2005; Vignier et al., 2013; Muñoz et al., 2015; Ryan et al., 2018; Benaissa et al., 2021). It must be kept in mind that Shewanella spp. may exhibit resistance to imipenem due to possible oxacillinase secretion (Héritier et al., 2004). According to the results of Héritier et al., 2004, *S. algae* KB-1 owns a chromosome-encoded ß-lactamase gene encoding the Ambler class D enzyme OXA-55 (Héritier et al., 2004). OXA-55 usually leads to development of a narrow-spectrum ß-lactam resistance phenotype (Héritier et al., 2004). This oxacillinase has carbapenem-hydrolyzing activity, which explains the lower susceptibility of *S. algae* KB-1 to imipenem (Héritier et al., 2004). There are already reports of infections caused by carbapenem-resistant bacteria (Brink et al., 1995; Otsuka et al., 2007; Baruah and Grover, 2014). So, the use of carbapenems should be avoided.

Variable susceptibility is seen to ampicillin and cephalosporins, with the majority of clinical isolates testing susceptible to third and fourth generation cephalosporins (Chen et al., 1997; Holt et al., 2005; Benaissa et al., 2021; Yu et al., 2022). Interestingly, *S. putrefaciens* is usually susceptible to erythromycin, a macrolide antibiotic, which is particularly effective in the gram-positive range (Chen et al., 1997). An important distinction feature between *S. algae* and *S. putrefaciens* is the susceptibility to polymixin (Holt et al., 2005; Benaissa et al., 2021). Holt et al., 2005 reported that *S. algae* were resistant to colistin, whereas *S. putrefaciens* isolates tested susceptible (Holt et al., 2005).

According to the current literature, infections with *S. putrefaciens* should be treated, also depending on the primary focus, by antibiotic therapy in combination with a sufficient local therapy (Yohe et al., 1997; Bulut et al., 2004; Süzüku et al., 2004; Holt et al., 2005). As part of empiric anti-infective therapy, intravenous treatment with a fluoroquinolone or a betalactam may be considered initially (Holt et al., 2005). Because of the potential for beta-lactamase expression, a combination with a beta-lactam inhibitor should be considered if a beta-lactam is used (Héritier et al., 2004). In severe courses, especially with existing sepsis, intravenous combination treatment using, for example, an aminoglycoside antibiotic should be considered (Brink et al., 1995). Depending on resistance testing and the clinical course, oral sequential antibiotic therapy may be appropriate in special cases (Guinetti-Ortiz et al., 2016; Patel et al., 2020).

Summary

S. putrefaciens is a rare human pathogenic bacterium whose infections can lead to serious clinical or even fatal consequences, especially in predisposed individuals. Microbiological identification and especially correct differentiation between *S. putrefaciens* and *S. algae* is difficult because biochemical and

phenotypic characterization tests, respectively, provide insufficient discriminatory criteria and *S. algae* is not included in the databases of most commercial identification systems. Following the change in nomenclature and the distinction between *S. putrefaciens* and *S. algae*, numerous case reports were reexamined in relation to the human pathogen. Recent studies and data suggest that there should be the majority of clinical *S. putrefaciens* isolates assigned to the species *S. algae* (Vogel et al., 1997; Khashe and Janda, 1998; Holt et al., 2005; Tsai et al., 2008; Vignier et al., 2013). Therefore, 16S rRNA sequencing, ribotyping, or whole-cell protein profiling is required to correctly distinguish the two Shewanella spp. (Vogel et al., 1997). On this basis, it can be assumed that the majority of *S. putrefaciens* infections listed in this review are indeed infections caused by *S. algae*. Only in one case report, 16S rDNA amplification assay was performed (Duan et al., 2015). In some case reports, no information was provided on the identification method (Supplementary Table 1). Therefore, the present review should be considered under the above limitations.

S. putrefaciens can lead to skin and soft tissue infections, arthritides and osteomyelitides, intracerebral, ocular, respiratory and intra-abdominal infections as well as severe bloodstream infections with septic courses and rarely even endocarditis. Severe courses are often associated with underlying, predisposing diseases and other risk factors. *S. putrefaciens* may exhibit intrinisic resistance to penicillins and possibly also to carbepenems. For successful treatment of *S. putrefaciens*, a combination of appropriate local therapy and antibiotic therapy should be performed, taking into account current susceptibility testing.

To prevent nosocomial infections through contact transmission, according to Oh et al., 2008, the following points should be observed in addition to the usual rules of hospital hygiene: wearing gloves during direct patient contact and contact with potentially infectious fluids, washing hands before and after patient care, following strict "no-touch" techniques when draining body fluids, and providing single-use products whenever possible (Oh et al., 2008).

Author contributions

PS had the idea for this mini-review. PS and SM worked together to develop the concept. SM, PS and MK performed the literature research. SM performed the data analysis and wrote the first draft of the manuscript. All authors reviewed and improved the manuscript and approved the submission.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

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Bacterial pathogens in pediatric appendicitis: a comprehensive retrospective study

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Background: Appendicitis is a frequent condition, with peak incidences in the second decade of life. Its pathogenesis is under debate, but bacterial infections are crucial, and antibiotic treatment remains essential. Rare bacteria are accused of causing complications, and various calculated antibiotics are propagated, yet there is no comprehensive microbiological analysis of pediatric appendicitis. Here we review different pre-analytic pathways, identify rare and common bacterial pathogens and their antibiotic resistances, correlate clinical courses, and evaluate standard calculated antibiotics in a large pediatric cohort.

Method: We reviewed 579 patient records and microbiological results of intraoperative swabs in standard Amies agar media or fluid samples after appendectomies for appendicitis between May 2011 and April 2019. Bacteria were cultured and identified *via* VITEK 2 or MALDI-TOF MS. Minimal inhibitory concentrations were reevaluated according to EUCAST 2022. Results were correlated to clinical courses.

Results: Of 579 analyzed patients, in 372 patients we got 1330 bacterial growths with resistograms. 1259 times, bacteria could be identified to species level. 102 different bacteria could be cultivated. 49% of catarrhal and 52% of phlegmonous appendices resulted in bacterial growth. In gangrenous appendicitis, only 38% remained sterile, while this number reduced to 4% after perforation. Many fluid samples remained sterile even when unsterile swabs had been taken simultaneously. 40 common enteral genera were responsible for 76.5% of bacterial identifications in 96.8% of patients. However, 69 rare bacteria were found in 187 patients without specifically elevated risk for complications.

Conclusion: Amies agar gel swabs performed superior to fluid samples and should be a standard in appendectomies. Even catarrhal appendices were only sterile in 51%, which is interesting in view of a possible viral cause. According to our resistograms, the best *in vitro* antibiotic was imipenem with 88.4% susceptible strains, followed by piperacillin-tazobactam, cefuroxime with metronidazole, and ampicillin-sulbactam to which only 21.6% of bacteria were susceptible. Bacterial growths and higher resistances correlate to an elevated risk

of complications. Rare bacteria are found in many patients, but there is no specific consequence regarding antibiotic susceptibility, clinical course, or complications. Prospective, comprehensive studies are needed to further elicit pediatric appendicitis microbiology and antibiotic treatment.

KEYWORDS

pediatric appendicitis, rare bacteria, anti-infective treatment, complications, outcome

Background

Appendicitis is among the most frequently treated surgical conditions, with peak incidences in the second decade of life (Bhangu et al., 2015). The disease occurs with a global incidence of 100 per 100.000 people while reaching even 151 per 100.000 in Germany (Körner et al., 1997). Due to its specifically high incidence from 10 to 14 years in boys and 10 to 19 years in girls (Andersen et al., 2009; Ohmann et al., 2014; Jaya Kumar et al., 2017), there is a need for a detailed analysis of this disease in a pediatric cohort.

For many decades, bacterial transmigration and invasive infections were thought to be critical in the development and progression of appendicitis. Consequently, perioperatively administered antibiotic prophylaxis and treatment remain essential in its management. Thus, different calculated antibiotics and antibiotic combinations have been discussed in the past, and still, different guidelines exist on this matter. Most importantly, no specific up-to-date guidelines on pediatric appendicitis exist, which makes an analysis of bacterial pathogens and their antibiotic resistance in a pediatric cohort even more valuable. Therefore, in this study, we test four different commonly used antibiotic agents as they have been advocated in the past and used in our department: ampicillin-sulbactam (Kambaroudis et al., 2010; Kronman et al., 2016), cefuroxime with metronidazole (Sauerland et al., 2010; Rollins et al., 2016), piperacillin-tazobactam (Fallon et al., 2013; Mazuski et al., 2017; Sartelli et al., 2017; Roque et al., 2019b), and imipenem (Kambaroudis et al., 2010; Mazuski et al., 2017; Sartelli et al., 2017). In addition, a comprehensive analysis of bacterial growths in pediatric appendicitis is fundamental, given the recent debate on treating uncomplicated appendicitis conservatively.

Recently many authors propagated antibiotic management without initial surgery for uncomplicated appendicitis (Varadhan et al., 2012; Rollins et al., 2016), and various evidence points to a different pathomechanism for uncomplicated and complicated appendicitis (Livingston et al., 2007; Rawolle et al., 2019). For the former, a viral cause is debated (Andersson et al., 1995; Alder et al., 2010; Richardsen et al., 2016), which might leave transmural migration of bacteria to gangrenous and perforated appendicitis. Because of these arguments, the microbiological analysis of catarrhal and phlegmonous appendicitis is also intriguing.

Studies have advocated blood culture bottles for sample collection (Jiménez et al., 2019) when others propagate routine swabs (Davies et al., 2010; Son et al., 2020). Given the importance of good coverage of possibly causative bacteria in appendicitis, comparing different modes of sample collection will add to the discussion.

Some rare bacteria have been accused of causing complications in appendicitis: peritonitis caused by *Actinomyces odontolyticus* (Lopes et al., 2017), suspected bowel perforation (Legaria et al., 2020) and abscess formation by *Clostridium ramosum* (Forrester and Spain, 2014) and *Eikenella corrodens* (Paul and Patel, 2001), free abdominal fluid (in perforated appendicitis) and psoas abscess by *Comamonas kerstersii* (Almuzara et al., 2013; Almuzara et al., 2017), gangrenous appendicitis by *Eggerthella lenta* (Gardiner et al., 2015). However, since case presentations usually arise from complications, the pathogenicity of rare bacteria can easily be overestimated. Therefore, a comprehensive approach might help to get a hold of uncomplicated clinical courses despite rare bacteria.

Finally, various calculated antibiotics or combinations are propagated for appendicitis, yet there is no comprehensive microbiological analysis of pediatric appendicitis. Here we review different pre-analytic pathways, identify bacteria, rare pathogens, and their resistances, correlate clinical courses, and evaluate standard calculated antibiotic managements in a large pediatric cohort.

Materials and methods

We reviewed patient records and microbiological results of all appendectomies due to appendicitis between May 2011 and April 2019. During this period, two types of samples were sent for microbiological analysis: either an intraoperative swab was wiped by the surgeon along the serosa of the appendix and sent in a conventional Amies gel transport medium (Sarstedt AG & Co. KG, Nümbrecht, Germany) (Van Horn et al., 2008; Reinisch et al., 2017) or intraabdominal fluid was aspirated and directly sent natively to microbiology (12 ml PS Tube, sterile, greiner bio-one GmbH, Frickenhausen, Germany). All samples were directly analyzed within routine microbiological diagnostics. In 110 cases, both swabs and native material were sent.

Both, swabs and fluids, were processed according to the standard routine procedures of the microbiology laboratory (Supplementary Figure 1). Bacteria were identified *via* VITEK 2 or MALDI-TOF MS and minimal inhibitory concentrations were determined with routine methods and evaluated according to EUCAST 2022 (The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of

MICs and zone diameters. Version 12.0, 2022. http://www.eucast.org.). For this, the minimal inhibitory concentrations (MIC) were individually retrieved from our laboratory reports and re-evaluated with current EUCAST breakpoints. We reviewed 619 children 2 to 17 years of age with postoperatively confirmed appendicitis. All found bacteria and resistances were evaluated and correlated to clinical courses. In addition, we estimated the specific incidence and performed literature research on each identified species to identify rare pathogenic bacteria.

Definition of complications

When evaluating the clinical course, we defined complications as unplanned outpatient visits after appendectomy for pain, wound healing problems, or GI-symptoms. Further complications included readmissions for gastrointestinal problems and re-operations for abdominal problems within one year after appendectomy. However, when patient records revealed unusual pain, fever, delayed enteral nutrition, or constipation during initial inpatient treatment, this was noted as a complication only if inpatient treatment lasted longer than the average hospital stay of 7 days.

Definition of rare bacterial pathogens

There is no commonly agreed definition for rare bacterial pathogens. Like in rare diseases, accepted definitions include the low number of affected patients and the little knowledge on this disease. Commonly used definitions for rare diseases are based on prevalence which works well for chronic conditions but risks omitting short-lasting illnesses. To overcome this problem, the RARECARE project chose an incidence-based definition for rare cancers as those with an annual incidence of less than six per 100,000 people (Gatta et al., 2017). For this study, we deducted our definition for rare bacterial pathogens from the above-mentioned criteria: since, in Germany, appendicitis has an overall incidence of 151/100 000 (Ferris et al., 2017), any bacteria found in less than 4% of our appendicitis patients or less than 23 of 579 cases, was considered to have a "rare incidence".

However, to be termed a "rare pathogen", a bacteria should have little published evidence in regard to human infections. To accomplish this discrimination, we searched MEDLINE *via* PubMed on Juli 15th, 2022 for the name of the bacteria AND "human" AND "infection". For rarely published bacteria, we set an arbitrary threshold at any bacteria with less than 0.3% of publications on human infections with *E. coli*, the most common bacteria in human appendicitis (Wilms et al., 2011; Fallon et al., 2013; Kenig and Richter, 2013; Bhangu et al., 2015; Tartar et al., 2018; Son et al., 2020; Plattner et al., 2021).

Statistics

In this exploratory analysis, continuous data were described by mean and standard deviation or median and interquartile range, as appropriate. Categorical data were presented by absolute and relative frequencies. Data observed in different groups were tested for differences by t-test for independent groups, paired t-test, chi-square test, Fisher's exact test or McNemar test, as appropriate. When comparing more than two groups, the Kruskal-Wallis-Test was used. Multiple logistic regression was performed to investigate the influence of independent risk factors on complications.

Significance level was set to 5 percent. As the analyses focused on description and hypotheses generation, no adjustment of type-one-error for multiple testing was applied. Statistical analyses were performed by Microsoft Excel version 2016 and SAS version 9.4.

Results

Descriptive statistics of patients and management

In the analyzed 8-year period, we screened 710 appendectomies (OPS 5-470.x). We excluded 34 patients who were opportunistically appendectomized during other operations. Another 32 patients above 16 years of age were not treated in the department of pediatric surgery and therefore excluded from further analysis. From the remaining 644 patients, we excluded 25 patients in whom no appendicitis could be confirmed intraoperatively (false positive = 3.9%). Another 40 patients had to be excluded because they did not have microbiological results in their records for various reasons, e.g., sample not taken, sample lost, no valid results due to long transportation, or irretrievable results. This left 579 patients for our analysis (Figure 1).

Our patients were on average 10.3 years old (range 2-17) with a ratio of 308 (53.2%) boys to 271 girls. Open appendectomy was performed in 4.8% of patients. The remaining 95.2% were operated endoscopically, either as conventional laparoscopy in three-port-technique or with single-incision or single-port technique. In 7.6% of endoscopic procedures, there was an intraoperative conversion to a laparotomy.

Intraoperatively the vermiform appendix was evaluated macroscopically by the operating surgeon. Thus we classified the appendicitis according to the operation report into simplex (n=25/644 = 3.9%, excluded from further analysis), catarrhal (n=102/579 = 17.6%), phlegmonous (n=215/579 = 37.1%), gangrenous (n=120/579 = 20.7%), and perforated (n=142/579 = 24.5%). Across all patients, the length of inpatient treatment was analyzed after excluding 6 oncological patients who stayed in the hospital due to their underlying disease. The median length of admission for the remaining 573 patients was 6 days with an interquartile range (IQR) of 5 to 10 (Supplementary Figure 2).

In the postoperative course, we found minor complications or adverse events in 21.2% (n=123/579). However, only 35/579 = 6% required a surgical re-intervention such as percutaneous or transrectal drainage of an abscess or re-operation. The most frequent minor complication was unusual pain (n = 32) and prolonged wound healing (n = 19) due to dehiscence or minor local infections, followed by fever and gastrointestinal symptoms such as constipation, vomitus, or diarrhea.

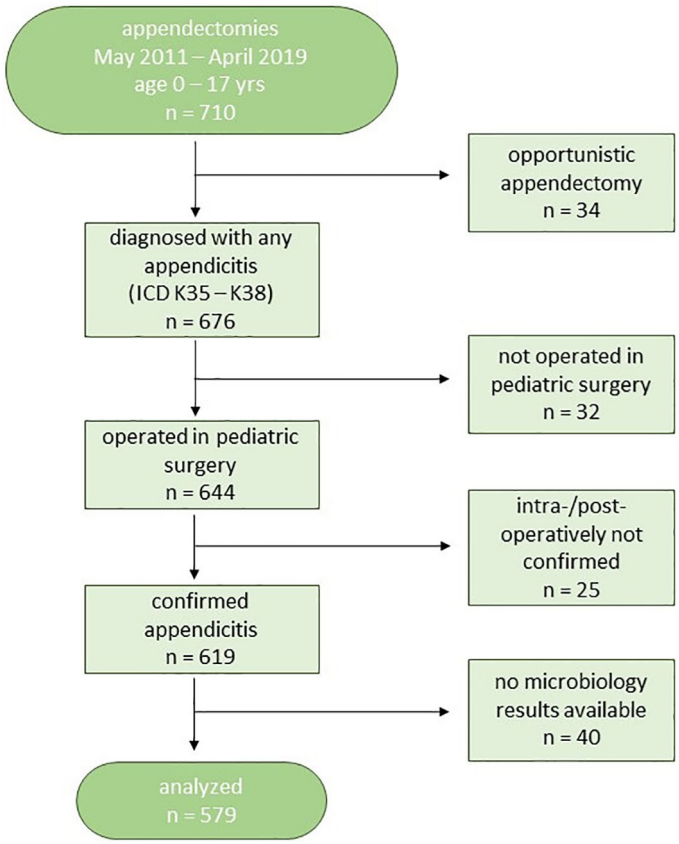

FIGURE 1
Patient flow-chart.

Statistics of pre-analytics

An intraoperative swab was taken and sent in a conventional Amies agar gel transport medium in 85.8% of cases (n = 497). Intraabdominal fluid was sent natively to microbiology in 33.2% (n = 192). In 19.0% (110 patients), both swabs and native material were sent. In 387 patients, only a swab was taken, while in 82 cases, we only analyzed intraabdominal fluid samples. All three groups (swabs, fluid, and both) were comparable in terms of operative access, mode of ligation, the intraoperative status of the appendix, and the rate of complications (Supplementary Table 1).

Additionally, both swabs and fluids generated comparable top-20-profiles of identified bacteria (Supplementary Figure 3).

Only 156/497 = 31.4% of all swabs turned out to be sterile as no bacteria could be cultivated. This contrasts with 138/192 = 71.9% of all native fluid samples that did not result in bacterial growth in our laboratory. When excluding the patients with both swab and fluid sample, these proportions do not change to a relevant extend (Table 1). Even when comparing only patients with both types of samples taken simultaneously, we saw sterile results in 54/110 (49.1%) fluid samples when bacterial growth was detected from the corresponding swabs. Only 4/110 (3.6%) of fluid samples resulted in bacterial growth when the corresponding swab remained sterile. This is in good accordance with the number of identified species: on average, 2.2 species could be identified from bacterial swabs when fluid samples delivered only 1.3 different species (Tables 1–3). Around 85.7% of all identified bacteria (n = 1330) were found due to bacterial swabs when only 14.2% of identified bacteria originated in the analysis of fluid samples.

Statistics of bacterial growths

We identified bacteria in 136/142 = 95.8% of perforated appendicitis. Even in catarrhal appendicitis, bacteria were found in 50/102 = 49% of patients. The different rates of sterile results in the four forms of appendicitis were statistically significant ($p<0.001$) (Table 4). We only had 25 false positives without signs of inflammation. In these patients, microbiological material was mostly not taken or other pathologies than appendicitis were present.

When on average, 1.43 different species were found in all catarrhal appendicitis, we found 1.5 in phlegmonous, 2.03 in gangrenous, and 4.35 in perforated appendicitis. When excluding sterile samples from the analysis, catarrhal appendicitis delivered on average 2.92, phlegmonous 2.88, gangrenous 3.3, and perforated appendicitis delivered 4.54 different species (Table 4). The proportion of rare bacteria in different forms of appendicitis did not differ significantly (Table 4). In all stages, *E. coli* was the

TABLE 1 Comparison of swaps and fluid samples.

Total (n = 469)	Swab only (n = 387)		Fluid only (n = 82)	
sum of identified bacteria	870		103	
samples with bacterial growth	264	68.2%	27	32.9%
samples that remained sterile	123	31.8%	55	67.1%
mean value of species identified	2.24		1.25	
SD of species identified"	2.24		2.38	
variance of species identified	5.04		5.70	
range of species identified	13		15	
p-value	<0.001			

dominating species, followed by different members of the genus *Bacteroides* and *Pseudomonas* (Supplementary Figures 4–7)

Primarily open (10.7%) and converted (4.8%) appendectomies had by far the lowest rates of sterile results, while the rate of sterile samples was highest in laparoscopically operated patients (39.7%) (Supplementary Table 2).

All analyzed patients with bacterial growths had, on average, 3.58 different bacteria (range 1 to 15, SD=2.27). When excluding the sterile samples, almost 90% of patients had 6 or less different bacteria (Supplementary Figure 8).

Statistics of pathogens

Since many patients had a polymicrobial spectrum, the frequency of detected bacteria does not fully correspond to the rate of patients positive for certain bacteria. The most frequently detected genus among all detected bacteria was *Bacteroides* spp. (367/1330) followed by *Escherichia* spp. (315/1330), *Streptococcus* spp. (139/1330), *Pseudomonas* spp. (75/1330), *Bilophila* spp. (54/1330), and *Enterococcus* spp. (49/1330) (Supplementary Figure 9).

However, *Escherichia* spp. was detected in the samples of most patients (282/579), followed by *Bacteroides* spp. (252/579), *Streptococcus* spp. (114/579), *Pseudomonas* spp. (70/579), *Bilophila* spp. (53/579), and *Enterococcus* spp. which was only present in 43/579 (Supplementary Figure 10).

We identified bacteria down to species level 1259 times, thus totaling 102 different bacterial species. Without surprise, *E. coli* was the most found species, followed by *B. fragilis*, *P.aeruginosa*, *S. anginosus* and *B. wadsworthia*. We evaluated all bacteria found according to our definition of rare bacterial pathogens: a specific annual incidence below 6/100000 and less than 0.3% of publications compared to the most frequently published pathogen. The bacteria with the highest incidence in human pediatric appendicitis was *E. coli*. This bacteria also generated the most hits on MEDLINE, which is 66,199. Therefore, any bacteria with less than 199 publications related to human infections were considered to have little publications. Combining both criteria, we defined rare bacterial pathogens in pediatric appendicitis (Table 5).

If bacteria were detectable or not impacted the patient's chance for complications: patients without detectable bacteria suffered complications in only 13.5% (28/207), while patients with bacterial growth had complications in 25.5% (95/372) ($p<0.001$).

To analyze the impact of rare pathogens on pediatric appendicitis, we compared patients with rare pathogens and those without rare pathogens with regards to complications: when no rare bacteria were present, patients suffered complications in 20.5% (38/185). This rate increased to 30.5% (57/187) when rare pathogens were identified ($p<0.05$). However, the impact of different bacteria varies greatly. Many rare bacteria have been detected less than 5 times in total. Many more have been detected together with complications only once. When we had more than 5 patients with a certain rare species that coincided in more than 20.5% with complications, we marked this species as "rare bacteria with elevated risk for complications". In total, 8 different rare bacteria

TABLE 2 Comparison of swabs and fluid samples that were taken simultaneously.

	Simultaneuous swabs (n = 110)		Simultaneuous fluids (n = 110)	
sum of identified bacteria	270		87	
number of sterile samples	33	30.00%	83	75.45%
number of samples with bacteriel growth	77	70.00%	27	24.54%
mean value of species identified	2.45		0.79	
SD of species identified	2.34		1.60	
variance of species identified	5.52		2.56	
p-value (paired t-test)	<0.001			

TABLE 3 Cross-table of simultaneously taken samples.

Total (n = 110)	Sterile swab	Swab with bacteria	Total
sterile fluid	29	54	83
fluid with bacteria	4	23	27
total	33	77	110
p-value	<0.001		

fulfilled these criteria (Table 5). They will be discussed later. There was no stringent correlation of rare pathogens to certain forms of appendicitis except for perforated appendicitis, where rare pathogens were found in 63.2%.

Following various publications, we identified standard calculated antibiotic regimes: ampicillin-sulbactam (Kambaroudis et al., 2010; Kronman et al., 2016), cefuroxime-metronidazole (Sauerland et al., 2010; Rollins et al., 2016), piperacillin-tazobactam (Fallon et al., 2013; Mazuski et al., 2017; Sartelli et al., 2017; Roque et al., 2019b) and imipenem (Kambaroudis et al., 2010; Mazuski et al., 2017; Sartelli et al., 2017; Roque et al., 2019b).

For 1330 different bacterial pathogens, we were able to obtain a resistogram. When testing resistances globally across all identified bacteria in all analyzed patients, only 21.6% were sensitive to ampicillin-sulbactam. The overall susceptibility was much higher against the combination of cefuroxime-metronidazole and piperacillin-tazobactam, reaching 72.3 and 78.9%. Finally, 88.4% of all found bacteria were susceptible to imipenem (Supplementary Table 3).

When considering all bacteria found in one specific patient, 91.4% of patients had at least one bacterium resistant to ampicillin-sulbactam, while only 30.9% of patients carried at least one bacterium resistant to imipenem. However, these proportions change when we include the patients without bacterial growth. In the total cohort of 579 patients, only 58.7% had bacteria resistant to ampicillin-sulbactam, 37.3% to cefuroxime + metronidazole, 31.4% to piperacillin-tazobactam, and 19.9% to imipenem (Supplementary Table 4).

We also examined rare and common bacteria separately concerning their resistances to ampicillin-sulbactam, cefuroxime with metronidazole, piperacillin-tazobactam, and imipenem: rare bacteria were more often resistant to ampicillin-sulbactam, but less often resistant to all other tested antibiotics. (Supplementary Table 5).

Correlations of resistances with forms of appendicitis

To test the impact of antibiotic resistances on the course of pediatric appendicitis, we compared the rate of resistant bacteria in patients with different forms of appendicitis (Table 6). It is remarkable that patients with perforated appendicitis have higher chances of resistant bacterial growth against all tested antibiotics.

Correlation of resistances with complications

We also analyzed the presence of resistant bacteria in patients with and without complications. It is noteworthy that patients with complicated clinical courses have more frequently at least one resistant bacterium and consistently higher rates of resistant bacteria than those without complications (Table 7).

Correlation of bacterial growths with complications

The risk of complications was only 13.5% when no bacterial growth was seen. However, when the microbiological samples were unsterile, this rate increased to 25.6% (Supplementary Table 6).

TABLE 4 Number and rarity of bacteria found in different forms of appendicitis.

579 patients analyzed	Catarrhal (n = 102)		Phlegmonous (n = 215)		Gangrenous (n = 120)		Perforated (n = 142)	
207 patients without bacterial growth	52	51.0%	103	47.9%	46	38.3%	6	4.2%
372 patients with bacterial growth	50	49.0%	112	52.1%	74	61.7%	136	95.8%
of these 372, patients with rare pathogens	27	7.3%	41	11.0%	33	8.9%	86	23.1%
1330 bacteria detected in total	146		322		244		618	
thereof number rare bacteria	47	32.2%	72	22.4%	54	22.1%	139	22.5%
bacteria per patient (n = 579)	1.43		1.5		2.03		4.35	
bacteria per patient (n = 372), steriles excluded	2.92		2.88		3.3		4.54	
number different species	52		69		57		81	
thereof rare species	20	38.5%	28	40.6%	24	42.1%	37	45.6%

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TABLE 5 Identified bacteria and associated complication rates, rarity marked with background color.

Species (n = 102)	Number of publications	Study cohort		
		Detected frequency	Complications	Complication rate
Escherichia coli	66199	312	74/312	23.7%
Staphylococcus aureus	63663	27	7/27	25.9%
Helicobacter pylori	35634	1	0/1	0.0%
Pseudomonas aeruginosa	30423	75	22/75	29.3%
Streptococcus pneumoniae	23255	5	1/5	20.0%
Klebsiella pneumoniae	14915	14	2/14	14.3%
Haemophilus influenzae	14351	2	1/2	50.0%
Streptococcus pyogenes	11029	4	1/4	25.0%
Staphylococcus epidermidis	7717	6	0/6	0.0%
Enterococcus faecalis	6434	12	3/12	25.0%
Enterococcus faecium	3728	7	1/7	14.3%
Yersinia enterocolitica	3094	1	0/1	0.0%
Proteus mirabilis	2997	7	5/7	71.4%
Bacteroides fragilis	2583	207	57/207	27.5%
Clostridium perfringens	2567	1	0/1	0.0%
Stenotrophomonas maltophilia	1587	1	1/1	100.0%
Fusobacterium nucleatum	1469	27	6/27	22.2%
Streptococcus sanguinis	1062	3	0/3	0.0%
Klebsiella oxytoca	923	16	3/16	18.8%
Prevotella intermedia	855	5	4/5	80.0%
Citrobacter freundii	797	10	2/10	20.0%
Fusobacterium necrophorum	750	4	0/4	0.0%
Staph.haemolyticus	733	1	0/1	0.0%
Eikenella corrodens	633	2	1/2	50.0%
Streptococcus anginosus	583	66	23/66	34.8%
Streptococcus intermedius	494	11	7/11	63.6%
Streptococcus salivarius	493	3	0/3	0.0%
Staphylococcus lugdunensis	485	2	0/2	0.0%
Staphylococcus hominis	463	2	2/2	100.0%
Morganella morganii	440	2	0/2	0.0%
Streptococcus gordonii	422	2	0/2	0.0%
Streptococcus constellatus	310	38	13/38	34.2%
Staphylococcus capitis	262	1	0/1	0.0%
Aeromonas veronii	253	2	1/2	50.0%
Bacteroides thetaiotaomicron	231	53	15/53	28.3%
Prevotella nigrescens	228	7	4/7	57.1%
Bifidobacterium longum	210	4	0/4	0.0%
Streptococcus mitis/oralis	202	1	0/1	0.0%

(Continued)

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TABLE 5 Continued

Species (n = 102)	Number of publications	Study cohort		
		Detected frequency	Complications	Complication rate
Enterobacter cloacae	201	4	1/4	25.0%
Citrobacter koseri	196	4	1/4	25.0%
**Parvimonas micra*	179	12	4/12	33.3%
Cutibacterium acnes	167	4	1/4	25.0%
**Gemella morbillorum*	157	7	3/7	42.9%
Enterococcus gallinarum	151	2	1/2	50.0%
Peptostrep. anaerobius	123	1	0/1	0.0%
Actinomyces odontolyticus	122	2	1/2	50.0%
Providencia rettgeri	118	3	1/3	33.3%
Bacteroides vulgatus	95	45	9/45	20.0%
Finegoldia magna	94	1	1/1	100.0%
Neisseria sicca	84	1	0/1	0.0%
Enterococcus avium	83	28	5/28	17.9%
Raoultella planticola	79	1	0/1	0.0%
Aggregatibacter aphrophilus	72	1	0/1	0.0%
Granulicatella adiacens	72	1	0/1	0.0%
**Bilophila wadsworthia*	66	54	16/54	29.6%
Listeria ivanovii	59	1	0/1	0.0%
Bifidobacterium adolescentis	57	1	0/1	0.0%
Salmon. enter. ser. Typhimurium	48	1	0/1	0.0%
**Bacteroides ovatus*	47	30	7/30	23.3%
**Eggerthella lenta*	47	6	2/6	33.3%
**Bacteroides uniformis*	38	11	4/11	36.4%
Clostridium ramosum	36	2	1/2	50.0%
Streptococcus parasanguinis	31	3	0/3	0.0%
Comamonas testosteroni	30	3	0/3	0.0%
Actinomyces turicensis	28	2	1/2	50.0%
Prevotella buccae	28	1	1/1	100.0%
Clostridium innocuum	27	1	0/1	0.0%
Porphyrom. asaccharolytica	27	1	1/1	100.0%
Escherichia fergusonii	25	1	0/1	0.0%
Prevotella oris	25	1	1/1	100.0%
Parabacteroides distasonis	24	21	4/21	19.0%
Bacillus circulans	23	1	0/1	0.0%
Bacteroides thetaiotaomicron	22	4	0/4	0.0%
Citrobacter braakii	22	1	1/1	100.0%
Slackia exigua	21	1	0/1	0.0%
Eubacterium aerofaciens	20	1	0/1	0.0%

(Continued)

TABLE 5 Continued

Species (n = 102)	Number of publications	Study cohort		
		Detected frequency	Complications	Complication rate
**Solobacterium moorei*	20	6	3/6	50.0%
Hungatella hathewayi	18	4	0/4	0.0%
Bacteroides caccae	16	4	1/4	25.0%
Streptococcus pluranimalium	12	1	0/1	0.0%
Bacteroides stercoris	10	1	0/1	0.0%
Comamonas kerstersii	10	2	1/2	50.0%
Fusobact. gonidiaformans	10	1	1/1	100.0%
Eggerthia catenaformis	9	1	1/1	100.0%
Fusobacterium naviforme	9	1	1/1	100.0%
**Eubacterium limosum*	8	7	2/7	28.6%
Collinsella aerofaciens	7	4	1/4	25.0%
Paeniclostridium sordellii	6	1	0/1	0.0%
Citrobacter youngae	5	1	0/1	0.0%
Bacteroides intestinalis	4	2	0/2	0.0%
Clostridium aldenense	4	1	0/1	0.0%
Porphyromonas somerae	4	1	0/1	0.0%
Bacteroides nordii	3	2	0/2	0.0%
Fusobacterium canifelinum	3	1	0/1	0.0%
Streptococcus massiliensis	3	1	1/1	100.0%
Bacillus simplex	2	1	0/1	0.0%
Clostridium citroniae	2	1	1/1	100.0%
Bacteroides cellulosilyticus	1	1	0/1	0.0%
Bacteroides salyersiae	1	1	0/1	0.0%
Bacteroides eggerthii	1	1	0/1	0.0%
Prevotella maculosa	1	1	0/1	0.0%
Escherichia coli (mucous)	0	2	0/2	0.0%

Rare bacteria with elevated risk for complications marked with *.

Moreover, the rate of rare bacteria among all detected bacteria did not differ between patients suffering complications (23% rare bacteria) and those who did not suffer any complications (24% rare bacteria).

Correlation of bacterial growth with forms of appendicitis and hospital stay

However, 46% of samples with at least one rare pathogen originated from perforated appendicitis but only 2.8% of sterile samples were taken in patients with perforation. Surprisingly, 25.1% of sterile samples were taken in catarrhal appendicitis when this rate was highest with 49.8% in phlegmonous appendicitis (Supplementary Table 7).

When comparing patients with sterile samples, to those with only common bacteria and those with rare bacteria, the latter two groups spent significantly more days in hospital. Patients with rare bacteria had the longest hospital stay (Supplementary Figure 11).

Forms of appendicitis and bacterial growths as prognostic factors for complications

In a logistic regression model, only perforation was identified as statistically significant prognostic factor for complications with an odds ratio of 2.6 (95% CI 1.3 to 4.9) compared to catarrhal appendicitis. The elevated risk of rare pathogens for complications diminished after adjusting for kind of appendicitis (odds ratio 1.36; 95% CI 0.83 to 2.23, Supplementary Table 8).

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TABLE 6 Resistant bacteria in different forms of appendicitis.

Number of patients with at least 1 resistant pathogen against antibiotic	Catarrhal (n = 50)		Phlegmonous (n = 112)		Gangrenous (n = 74)		Perforated (n = 136)	
Ampicillin/Sulbactam	43	86.0%	92	82.1%	70	94.6%	135	99.3%
Cefuroxime/Metronidazole	25	50.0%	50	44.6%	44	59.5%	97	71.3%
Piperacillin/Tazobactam	22	44.0%	43	38.4%	34	45.9%	83	61.0%
Imipenem	11	22.0%	24	21.4%	26	35.1%	54	39.7%

Discussion

Strengths and weaknesses of this study

This study is the most comprehensive research on bacterial growths in different forms of pediatric appendicitis that gives insides on the role of common and rare bacteria as well as antibiotic susceptibility with regard to common calculated antibiotics, hospital stay and complications. However, it is a retrospective study. Furthermore, all included patients were treated in a single center reflecting the local situation. Still, antibiotic managements have to be prospectively evaluated and results might differ depending on local aspects. In addition, the exact culture methods for swabs and fluids were comparable but not 100% equal. Some methods were used less frequently on fluid samples. Therefore, the superiority of swabs over native fluid samples might be slightly overrated. However, the minimal difference on the frequency of culture techniques used cannot be held responsible for the tremendously better results of swabs.

There is no commonly agreed definition for "rare pathogens". We here provide an approach based on the rarity of a clinical condition (appendicitis) in the presence of certain bacteria together with the scarcity of literature on this individual pathogen. However, we encourage and welcome future debate on this definition.

How does our population compare to the literature?

This study investigated 619 patients of one center who underwent appendectomy. The 579 patients who met the inclusion criteria with an intraoperatively inflammatorily altered appendix represent the largest cohort considered for the study of appendectomies in this age group that we are aware of in the current literature. Regarding age and sex ratio, our population is consistent with the literature (Omling et al., 2019). The clinically suspected appendicitis was not confirmed intraoperatively in only 25 patients (n=25/579 = 3.9%). This low rate of false positive appendectomies is far below the 15% to 35% reported in the literature (Ohle et al., 2011; Brockman et al., 2013; Garcia et al., 2018). One reason may be that the initial conservative therapy, supportive measures in case of unclear findings, and repeated reevaluations by experienced surgeons are highly prioritized in our center. Intriguingly, this approach did not increase the rate of perforated appendicitis (24.5%) above numbers published in the literature for other tertiary centers (Smink et al., 2005). This finding could support reports suggesting a different entity of uncomplicated and complicated appendicitis compared to the classic progressive disease hypothesis.

The preferred surgical method in our clinic is the primary laparoscopic approach, either as a classic three port laparoscopy or as a single port approach (SILS). Only 4.8% of patients required a primary laparotomy, 95.2% were operated on laparoscopically. In 7.6%, an initial endoscopic procedure was converted intraoperatively to a laparotomy. Thus, a high rate of laparoscopic appendectomies (87.6%) is present in our population. Currently, the standard surgical method in Germany is still heterogeneous, and laparoscopic surgery is not yet established as the primary standard procedure in all hospitals because about 25% of appendectomies in Germany are still performed *via* laparotomy (Téoule et al., 2020) while worldwide, this rate is reported to be as high as 42% (Sartelli et al., 2018).

TABLE 7 Resistant bacteria and complications.

Number of patients with at least 1 resistant pathogen	Complication (n = 95)		No complication (n = 277)	
Ampicillin/Sulbactam	92	96.8%	248	89.5%
Cefuroxime/Metronidazole	77	81.1%	139	50.2%
Piperacillin/Tazobactam	59	62.1%	123	44.4%
Imipenem	38	40.0%	77	27.8%
Average rate of resistant bacteria in patients w...	with complications (n = 95)		without complications (n = 277)	
Ampicillin/Sulbactam	80.7%		71.9%	
Cefuroxime/Metronidazole	35.4%		22.9%	
Piperacillin/Tazobactam	24.0%		19.7%	
Imipenem	12.8%		10.2%	

The average hospital stay of our patients was 7.9 days (2-41 days), and the median length of stay was six days. These numbers are not entirely due to medical needs but also reflect organizational standards and family needs in a center that serves a large rural area. In addition, no mortality occurred in our population when the literature still reports overall mortality of 0.09% up to 0.28% (Bhangu et al., 2015; Sartelli et al., 2018).

What role do bacteria play in appendicitis?

In the majority of our cases, we were able to detect bacteria. Even in catarrhal appendicitis, bacteria were detectable in 49%. That underlines the role of bacterial migration in acute appendicitis, although it is still unclear if the bacterial infection is the reason for appendicitis or a secondary appearance. However, what we were able to show in the patient population studied, is the correlation of bacterial infection and the occurrence of complications in the further course. This is corroborated by the fact that the severity of clinical findings is positively related to the probability of positive bacterial detection. As the severity of the inflammatory change increases, so does the number of bacterial species detected. This is well explained by the further increasing permeability of the appendiceal wall, up to perforation. Also understandable is the increase in the frequency of complications with the detection of more bacterial pathogens. When patients with sterile swabs suffer complications in only 13.5%, they do so in 25.5% of cases with unsterile swabs ($p<0.001$). Another fact that supports the significance of bacterial infection in appendicitis is the different resistance patterns in patients with postoperative complications compared to those with an uneventful postoperative course.

Do we really see transmigrated bacteria or are the bacteria in our samples due to iatrogenic contamination during laparoscopic handling?

We could not detect significant contamination with skin flora. Even in catarrhal appendicitis, the skin flora did not play a role in our population. However, the fact that more fluid samples that are commonly taken at the beginning of an operation prior to the excision of the appendix remained sterile supports the theory of intraoperative contamination of samples with intraluminal bacteria that might have been freed upon excision of the appendix.

However, the fact that the stage of the appendix inflammation directly correlates with the number of bacteria found argues against the contamination theory. Another fact that makes contamination of swabs in catarrhal and phlegmonous appendicitis unlikely is the high rate of unsterile swabs in open appendectomy since during open appendectomy, iatrogenic contamination of a swab with intraluminal bacteria is hardly imaginable. Future prospective studies should take intraoperative swabs at the beginning of the operation prior to the appendix excision with swabs suitable for laparoscopic approaches.

Is it better to take swabs or to send in intraabdominal fluid?

Due to our retrospective analysis, we recommend Amies agar gel transported swabs. The tremendous rate of sterile fluid samples supports this recommendation. Swabs gave more unsterile results and a greater amount of different identified bacteria. Even when directly comparing fluid samples and swabs in patients who received both simultaneously, the swab outperformed the fluid sample. Finally, we would recommend a standardized procedure suitable for all patients. This standard can only be the swab since intraabdominal fluid is not always present in appendicitis.

Are specific rare bacteria predictive of clinical complications?

Although in most appendicitis common bacterial pathogens can be found, one has to keep in mind that we detected rare bacteria in 32.3% (187/579). As mentioned above, authors repeatedly describe complications in appendicitis caused by rare bacteria (Paul and Patel, 2001; Almuzara et al., 2013; Forrester and Spain, 2014; Gardiner et al., 2015; Almuzara et al., 2017; Lopes et al., 2017; Legaria et al., 2020). This is most likely an example of a reporting bias since publications often arise from unusual complications while uncomplicated clinical courses remain underreported. The pathogenicity of rare bacteria is thus often overestimated. In our comprehensive approach, we could demonstrate an overrepresentation of rare bacteria in appendicitis with complications. Eight rare species were found to be associated to an above-average risk for complications with more than 2 patients affected.

Solobacterium moorei was detected in 6 patients of whom 3 suffered complications. This rare bacterium had thus the highest rate of complications in our population. It is an obligate anaerobic Gram-positive bacillus described mostly within the human oral cavity and human intestinal flora (Barrak et al., 2020). Recently several studies point to its role in oral infections. Being part of the tongue microbiota with beta-galactosidase activity potentially also producing volatile sulfur compounds, it is accused to cause halitosis (Barrak et al., 2020). Even though it is known to be an opportunistic pathogen in bloodstream and surgical site infections with excellent susceptibility to most antibiotics, there are some reports of *Solobacterium moorei* as being the only recovered bacteria in complicated infection (Alauzet et al., 2021). To our knowledge, our 6 patients of whom 3 suffered from complications, are the first appendicitis patients with *Solobacterium moorei* reported in literature.

Gemella morbillorum was detected in 7 patients of whom 3 suffered complications. It has been first described in 1917 as *Streptococcum morbillorum* and is part of the normal flora of human oropharynx, genitourinary system, and gastrointestinal system (Romero-Velez et al., 2020). There are case reports of *G. morbillorum* causing necrositing fasciitis of the torso, thoracic aortic aneurysm, and endocarditis (Ural et al., 2014; Romero-Velez et al.,

2020; Said and Tirthani, 2021). To our knowledge, our 7 patients of whom 3 suffered from complications, are the first appendicitis patients with *G.morbillorum* reported in literature.

Bacteroides uniformis was detected in 11 patients of whom 4 suffered complications. It is part of the human gut microbia (Grondin et al., 2022) and is thought to have anti-obesity effects. Although being described as a pathogen in human appendices more than 40 years ago, *B. uniformis* is very rarely mentioned in literature in regards of appendicitis (Elhag et al., 1986).

Eggerthella lenta was detected in 6 patients of whom 2 suffered complications. It is anaerobic, non-sporulating, Gram positive and part of the normal human microflora (Jiang et al., 2021). *E. lenta* has been described to cause appendicitis (Jiang et al., 2020; Jiang et al., 2021).

Parvimonas micra was detected in 12 patients of whom 4 had complications. It is a fastidious, anaerobic, Gram−positive coccus that is found in healthy human oral and gastrointestinal flora (Xu et al., 2020). It is described as a rare cause of spondylodiscitis (Yoo et al., 2019). Changes in the abundance of *P. micra* have been described in children with complex appendicitis (Durovic et al., 2020). However, 8 of our 12 patients with *P. micra* had an uneventful clinical course without complications.

Eubacterium limosum was detected in 7 patients of whom 4 had complications. It is a Gram-positive, methanol-utilizing aceto-gen (Flaiz et al., 2021). *E. limosum* is a human gut symbiont (Ellenbogen et al., 2021). To our knowledge, our 7 patients of whom 3 suffered from complications, are the first appendicitis patients with *E. limosum* reported in literature.

Bacteroides ovatus was detected in 30 patients of whom 7 had complications. It is a gram-negative human gut bacteria able to suppress inflammation in the gastrointestinal tract (Fultz et al., 2021). There are few reports for *B. ovatus* being isolated in patients after appendectomy (Tocchioni et al., 2016; Fuse et al., 2022; Ward et al., 2022).

Bilophila wadsworthia was detected in 54 patients of whom 16 had complications, thus having the highest total number of associated complications. It is a Gram-negative sulfite-reducing human gut bacillus (Natividad et al., 2018). Recently several studies point to its role in the human gut microbiome (David et al., 2014). Though it is well known to be associated with appendicitis and colitis (Burrichter et al., 2021), many other infections like scrotal abscess, mandibular osteomyelitis or bacteremia have been described in relation to *B. wadsworthia* (Finegold et al., 1992; Kasten et al., 1992).

However, of 63 rare bacteria in our study, 32 were detected in patients who recovered without any problems. 6 rare pathogens were detected in our population two or more times without any associated complications. Among them were *Bacteroides thetaiotaomicron*, that could be found in 4 patient of whom no one suffered complications. This bacteria is seldom reported in literature. It is a Gram-negative, anaerobic gut bacteria, which is considered a high efficient degrader of polysaccharides and a potential probiotic. We were able to find 2 reports of wound- (Agarwal et al., 2014) and knee joint infection (Brandariz-Núñez and Gálvez-López, 2021) caused by this species. The wound infection occurred in a chronically ill patient with disseminated myeloma, and the knee infection occurred in a previously healthy young man after several surgical procedures on the knee. However, we consider this pathogen to be opportunistic and, according to our data, without great clinical relevance.

Hungatella hathewayi was also associated with no complications and could be found in 4 patients. This bacterium is Gram-negative, anaerobic species is reported in connection with the development of eczema in early childhood (Chan et al., 2021). We could find two reports of septicemia in the setting of perforated appendicitis with this pathogen (Woo et al., 2004; Randazzo et al., 2015). Thus, this bacterium appears to have clinical relevance. Based on the antibiotic therapy administered, this bacterium seems to have been adequately treated. Maybe, we could not find any complications in our patient population, due to the low complication rate and the small size of our sample.

Streptococcus parasanguinis was detected in 3 patients without any reported complications. This Gram-positive bacterium is usually found in the mouth, where it is a plaque-forming agent. It also plays a role in subacute endocarditis, especially after dental surgery, and causes bone infection of the periodontium (Chen et al., 2020). Additionally, we could find two reports of osteomyelitis of the spine or skull base with *Strep. parasanguinis* (Valanejad and Hill, 2020; Kim et al., 2021), in both cases as combined infection with other pathogens. According to the current state of the literature, complications with this pathogen appear to be limited to older, previously ill or immunocompromised patients.

Comamonas testosteronii was found in 3 patients of whom no one suffered complications. That is a very interesting, because this Gram-negative, wide spread environmental bacteria is often reported in association with human infection and appendicitis (Gul et al., 2007; Khalki et al., 2016). Remarkable is the fact of the high reported frequency of this species in perforated appendicitis in rather young patients (Tiwari and Nanda, 2019; Miloudi et al., 2020). Infections with this bacterium must be considered in view of the current literature and the now numerous reports in younger patients. However, this pathogen has shown a good response to standard antibiotics. Also, the possibility of a broad resistance to antibiotics has already been discussed and should be considered (Miloudi et al., 2020).

Bacteroides intestinalis was found in 2 patients without complications. This bacteria has not been reported with pathological findings, yet. It is considered as a useful commensal of the human gut with the ability to degrade dietary fiber with even health benefits (Yasuma et al., 2021). Of course, this bacteria has only been detected in our population together with other bacteria.

Bacteroides nordii was found in 2 patients without any complications. This Gram negative, anaerobic bacterium is a naturally occurring component of the microbiome. It has been isolated previously from abdominal swabs, e.g. in perforated appendicitis, but always in mixed cultures (Song et al., 2004). There has been no evidence of manifest infections by this bacterium to date, nor has there been any evidence of it as a pathogen in blood culture. Therefore a low virulence is considered.

Since complications are not frequent overall, they are even less often observed with rare bacteria. The elevated risk of rare

pathogens on complications found in univariate analysis became insignificant after adjusting for the different forms of appendicitis. Additionally, the rare bacteria detected in our study population were always found together with other bacterial growth. Therefore it is not possible to attribute the eventual complications to the rare bacteria when other, common bacteria were also present. In summary, it was not possible to get statistically significant data on the relative risk profile of certain rare bacteria in pediatric appendicitis in our study population. Maybe if more comprehensive reports on bacteria in appendicitis were published, meta-analyses could elicit specific risk profiles of different bacteria in the future.

Is perioperative antibiotic prophylaxis useful?

Although our retrospective study did not intend to evaluate the benefit of perioperative antibiotics clinically, our data support its routine use. Many authors would argue that no antibiotics are needed in appendectomy when dealing with uncomplicated appendicitis (Kizilcan et al., 1992). On the other hand, authors argue that uncomplicated appendicitis can be treated with antibiotics only (Di Saverio et al., 2020). Thus, bacterial infections seem to play a role even in uncomplicated appendicitis. Our data show that intraperitoneal bacteria is present in even more than 50% of catarrhal and phlegmonous appendicitis. Since past research has demonstrated that surgeons tend to underestimate the degree of inflammation in laparoscopic appendectomy (Holloway et al., 2020), it does not make much sense to spare antibiotic prophylaxis for intraoperatively diagnosed gangrenous appendicitis. It seems more sensible to administer perioperative antibiotics 30 minutes prior to incision while reserving prolonged therapy for complicated disease (Daskalakis et al., 2014; Gorter et al., 2016; Di Saverio et al., 2020).

Which calculated antibiotics should be used?

Given our findings, imipenem would undoubtedly be the best calculated antibiotic. However, since imipenem is considered a reserve antibiotic (Roque et al., 2019a), piperacillin-tazobactam should be the calculated substance of choice. Since most severe complications were noticed in patients whose rate of bacteria resistant to piperacillin-tazobactam was higher, imipenem remains a good choice for calculated escalation of antibiotic management. Only 11.6% of all bacteria found in this study were resistant to imipenem. Even in perforated appendicitis, imipenem-resistant bacteria were only found in 12.0%. When looking at patients with severe complications, only 13.0% of their intraoperatively found bacteria were resistant to imipenem. This data only reflects the situation in our region and is subject to changes with time. Also, different antibiotics should be evaluated in a prospective trial.

Conclusion

Bacteria play an important role in all forms of appendicitis and, most of all, in its complications. Therefore, standard bacterial swabs should be taken intraoperatively from the appendix before its excision. Based on the detected microbiomes in this study, for pre-operative prophylaxis and, if needed, for antibiotic treatment, piperacillin-tazobactam would be a reasonable first choice. Imipenem can cover up to 88% of expected bacteria when calculated escalation of antibiotic treatment is needed. This strategy should be evaluated in larger, prospective studies. Future studies are also required to elicit certain rare bacteria's roles and pathomechanisms when their pathogenicity was overrated due to the apparent publication bias in case reports that can be overcome by comprehensive approaches as we have presented here.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: Anonymized extracted data is available upon reasonable request at the corresponding author. Requests to access these datasets should be directed to jurek.schultz@uniklinikum-dresden.de.

Ethics statement

The studies involving human participants were reviewed and approved by Institutional Review Board Technical University Dresden Medical Faculty Fetscherstraße 74 01307 Dresden IORG0001076, IRB00001473. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

JF: literature research, data extraction, data analysis, statistics, preparation of figures, preparation of manuscript, selection of references, writing of manuscript, reviewing and proof reading. BG: data analysis, statistics, preparation of figures, literature research and selection of references, reviewing and proof reading, submitting the manuscript. AR: data extraction, data analysis, preparation of figures, preparation of manuscript, literature research and selection of references, writing of manuscript, reviewing and proof reading. EW: data analysis, preparation of figures, literature research and selection of references, preparation of manuscript, writing of manuscript, English language editing, reviewing and proof reading. ET: data analysis, statistics, preparation of figures, reviewing and proof reading. SP: conceptualization of study, planning of study, data analysis, reviewing and proof reading. GF: conceptualization of study, planning of study, literature research and selection of references, reviewing and proof reading. JS: conceptualization of study, planning

of study, data extraction, data analysis, statistics, preparation of figures, preparation of manuscript, literature research and selection of references, writing of manuscript, English language editing, reviewing and proof reading, submitting the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1027769/full#supplementary-material

SUPPLEMENTARY TABLE 1
Comparison of Patients with swab only, fluid sample only, and both.

SUPPLEMENTARY TABLE 2
sterile and unsterile results with regards to mode of appendectomy.

SUPPLEMENTARY TABLE 3
Antibiotics susceptibility of identified bacteria.

SUPPLEMENTARY TABLE 4
Patients with resistant bacteria.

SUPPLEMENTARY TABLE 5
Resistances of rare and common bacteria to standard antibiotics.

SUPPLEMENTARY TABLE 6
Sterile, unsterile, and unsterile samples with rare pathogens in patients with and without complications.

SUPPLEMENTARY TABLE 7
Different forms of appendicitis when no, only common or rare bacteria were found.

SUPPLEMENTARY TABLE 8
Logistic regression for complications in different forms of appendicitis and with only common bacteria, no bacteria, or rare bacteria, when adjusted for form of appendicitis (CI = confidence interval).

SUPPLEMENTARY FIGURE 1
Laboratory processing of swabs and fluids.

SUPPLEMENTARY FIGURE 2
Length of in-patient treatment for appendicitis with appendectomy.

SUPPLEMENTARY FIGURE 3
Top-20-profiles of bacteria identified in fluids **(A)** and swabs **(B)**.

SUPPLEMENTARY FIGURE 4
Top 10 bacteria in catarrhal appendicitis.

SUPPLEMENTARY FIGURE 5
Top 10 bacterial pathogens in phlegmonous appendicitis.

SUPPLEMENTARY FIGURE 6
Top 10 bacterial pathogens in gangrenous appendicitis.

SUPPLEMENTARY FIGURE 7
Top 10 bacterial pathogens in perforated appendicitis.

SUPPLEMENTARY FIGURE 8
Number of identified bacteria per patient (sterile samples excluded).

SUPPLEMENTARY FIGURE 9
Top-6 genus of all identified bacteria.

SUPPLEMENTARY FIGURE 10
Amount of patients in whom certain genera were identified.

SUPPLEMENTARY FIGURE 11
hospital stay in the presence of sterile, only common bacteria or at least one rare pathogen ($p<0.001$).

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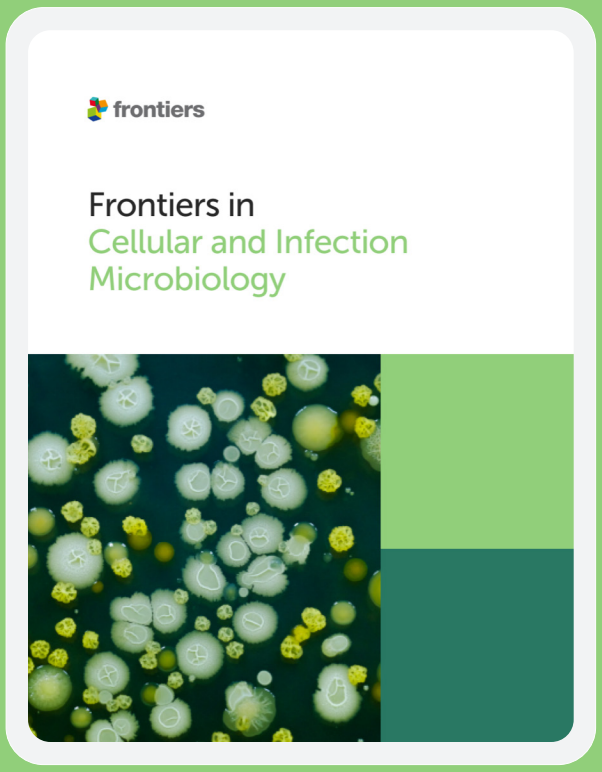

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