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Characterization of *Streptococcus tigurinus* Small-Colony Variants Causing Prosthetic Joint Infection by Comparative Whole-Genome Analyses

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Small-colony variants (SCVs) of bacteria are associated with recurrent and persistent infections. We describe for the first time SCVs of *Streptococcus tigurinus* in a patient with a prosthetic joint infection. *S. tigurinus* is a novel pathogen of the *Streptococcus mitis* group and causes invasive infections. We sought to characterize *S. tigurinus* SCVs using experimental methods and find possible genetic explanations for their phenotypes. The *S. tigurinus* SCVs were compared with the wild-type (WT) isolate using phenotypic methods, including growth under different conditions, autolysis, and visualization of the cell ultrastructure by use of transmission electron microscopy (TEM). Furthermore, comparative genome analyses were performed. The *S. tigurinus* SCVs displayed reduced growth compared to the WT and showed either a very stable or a fluctuating SCV phenotype. TEM analyses revealed major alterations in cell separation and morphological abnormalities, which were partially explained by impaired autolytic behavior. Intriguingly, the SCVs were more resistant to induced autolysis. Whole-genome sequencing revealed mutations in the genes involved in general cell metabolism, cell division, stringent response, and virulence. Clinically, the patient recovered after a 2-stage exchange of the prosthesis. Comparative whole-genome sequencing in clinical strains is a useful tool for identifying novel genetic signatures leading to the most persistent bacterial forms. The detection of viridans streptococcal SCVs is challenging in a clinical laboratory due to the small colony size. Thus, it is of major clinical importance for microbiologists and clinicians to be aware of viridans streptococcal SCVs, such as those of *S. tigurinus*, which lead to difficult-to-treat infections.

Small-colony variants (SCVs) of bacteria are frequently associated with foreign-body material, such as cardiac devices (1, 2) and prosthetic joints (3), and cause recurrent and persistent infections, which makes definite eradication very difficult (3–5). SCVs are characterized by reduced growth, small colony size, and atypical colony morphology, and they are often linked to a deficiency in electron transport or thymidine biosynthesis resulting in auxotrophy for hemin, menadione, or thymidine (1, 6). The morphological and biochemical characteristics of SCVs have been extensively studied in staphylococci (1). However, SCVs are found in various genera and species, e.g., enterococci (7), *Escherichia coli* (8), and *Pseudomonas aeruginosa* (1).

Viridans streptococci are usually commensals of the oral cavity, but when entering the bloodstream, they can cause severe invasive infections (9). To date, the prevalence of SCVs in viridans streptococci has been rather unknown. A few reports described *Streptococcus pneumoniae* mucoid variants and SCVs in biofilms (10, 11). Considering the small colony size of viridans streptococci, the SCV phenotype might be easily overlooked due to overgrowth by the wild-type (WT) phenotype, when present. However, the accurate detection of these variants has a major clinical impact on patient management as well as antibiotic therapy.

We present for the first time a clinical case of a prosthetic joint infection (PJI) caused by SCVs of *Streptococcus tigurinus*, a novel species belonging to the *Streptococcus mitis* group. *S. tigurinus* causes severe invasive infections, such as infective endocarditis, spondylodiscitis, and meningitis (12, 13), and it is highly virulent in experimental animal models (14). *S. tigurinus* forms alpha-hemolytic, smooth, white-to-grayish colonies with a diameter of 0.5 to 1 mm after incubation at 37°C with CO_2 for 24 h on sheep blood agar (13). The accurate identification of *S. tigurinus* by conventional phenotypic methods is limited because of the morphological resemblance to its closest related species, i.e., *S. mitis*, *Streptococcus oralis*, *S. pneumoniae*, *Streptococcus pseudopneumoniae*, and *Streptococcus infantis*. However, analyses of the 5′ end of the 16S rRNA gene allow for accurate identification of *S. tigurinus*, since a significant sequence demarcation to the most closely related species was demonstrated previously (13).

We characterized the SCV phenotype of *S. tigurinus* by experimental methods and applied whole-genome comparison to unravel the genetic changes associated with this most adapted and persistent form of *S. tigurinus*.

MATERIALS AND METHODS

Bacterial strains. The clinical *S. tigurinus* SCV strains 2425 and 2426 were compared with their parental strain *S. tigurinus* 1366. As a reference

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strain, the type strain *S. tigurinus* AZ_3a^T (CCOS 600; Culture Collection of Switzerland, Wädenswil, Switzerland) was included. The strains were taken from −80°C and grown on Columbia agar plates containing 5% defibrinated sheep blood (bioMérieux, Marcy l'Etoile, France) (COS) at 37°C with CO_2 for 24 h.

Analysis of 16S rRNA gene. An 800-bp fragment of the 16S rRNA gene was obtained as described previously (12). A 16S rRNA gene BLAST analysis was performed using the SmartGene software (SmartGene, Zug, Switzerland).

Antibiotic susceptibility testing. MICs were determined using Etest strips (AB bioMérieux). Susceptibility testing was performed on Mueller-Hinton agar supplemented with 5% sheep blood, using overnight cultures at a 0.5 McFarland standard, followed by incubation at 35 ± 2°C with 5% CO_2 for 20 to 24 h. In addition, the MICs were read at 48 h to take into account the slow growth of the SCVs. Interpretation was done according to the CLSI 2012 guidelines, if available (15).

Effects of serial passages and auxotrophic testing. At least 8 passages were performed on COS by picking single colonies for incubation at 37°C with CO_2 for 24 h. Auxotrophy for hemin, thymidine, and menadione was tested by the disk diffusion method. Commercially available standard disks of hemin (X-factor) were used (Sigma-Aldrich, Buchs, Switzerland), and blank disks were impregnated with 15 μl of menadione at 10 μg/ml, 25 μg/ml, and 125 μg/ml and of thymidine (Sigma-Aldrich) at 100 μg/ml, respectively. To determine auxotrophy, 0.5 McFarland standards of overnight cultures were swabbed on Mueller-Hinton agar, and the disks were placed on the agar surface. The isolate was considered an auxotroph if it showed normal-sized colonies or increased growth surrounding the disks compared to the periphery after 24 h to 48 h of incubation at 37°C with CO_2 (16). *Staphylococcus aureus* MS17 with a *hemB* mutation (17) was used as a hemin auxotroph control, and the *S. aureus* strain A22616/3 (18) was used as a menadione auxotroph control. The experiments were repeated twice independently.

Growth curves. Bacterial growth was monitored in brain heart infusion (BHI) broth (Becton, Dickinson, Germany) at 37°C using a microplate spectrophotometer (PowerWave XS; Bio-Tek, Winooski, VT, USA). Overnight cultures were diluted and adjusted to the same optical density at 600 nm (OD_{600}), and 100 μl was transferred into a clear 96-well flat-bottom plate (BD Biosciences, Franklin Lakes, NJ, USA). To ensure homogeneous turbidity, the plate was shaken every 20 min for 10 s, and the optical density was measured hourly. The experiment was performed with six technical and three biological replicates.

Transmission electron microscopy. The bacterial strains were grown until the exponential phase and centrifuged for 5 min at 5,000 rpm. Transmission electron microscopy (TEM) was performed by the Center of Microscopy and Image Analysis, University of Zurich, Zurich, Switzerland.

Autolysis experiments. The bacterial strains were grown to exponential phase, harvested by centrifugation, and washed with phosphate-buffered saline (PBS) (pH 7.4). The OD_{600} was adjusted to 0.3 in 0.01 M sodium phosphate buffer (pH 7), and the cultures were split. Added to the culture was 0.01% Triton X-100 (TX) (Sigma-Aldrich) or an equal amount of phosphate buffer. Autolysis was monitored every hour at 37°C using a microplate spectrophotometer, with vigorous shaking every 20 min for 10 s. The experiment was performed with three technical and three biological replicates. Statistical analysis was done by Student's *t* test.

Whole-genome and comparative genomic analyses. Purified bacterial genomic DNA was obtained from colonies on COS following cell disruption in lysis medium containing 150 U of mutanolysin, 500 U of achromopeptidase, and 40,000 U of lysozyme (Sigma-Aldrich) for 1 h. After lysis, the DNA was purified using a DNeasy kit (Qiagen AG, Hombrechtikon, Switzerland), according to the manufacturer's recommendations. Genomic DNA was subjected to whole-genome shotgun sequencing using a HiSeq 2000 system (Illumina, Inc.). A comparison of the genome content of *S. tigurinus* AZ_3a^T and *S. tigurinus* 1366 has been described (19). Following fragmentation, end reparation, and sample tagging, the sequencer produced 1.27 and 0.85 million of reads for *S. tigurinus* 2425 and 2426, respectively.

Nucleotide sequence accession numbers. This whole-genome shotgun project was deposited at DDBJ/EMBL/GenBank under the accession numbers ASWZ00000000 and ASXA00000000 for *S. tigurinus* SCV strains 2425 and 2426, respectively. The versions described in this paper are accession numbers ASWZ00000000.1 and ASXA00000000.1. Partial 16S rRNA gene sequences of the *S. tigurinus* strains 1366, 2425, and 2426 were deposited in GenBank under accession numbers KC598122, KC598123, and KC598124, respectively.

RESULTS

Case report. One year after a cemented total knee arthroplasty (in which the cement contained gentamicin), an 83-year-old woman complained of gradually increasing pain leading to limited joint motion. On examination, the knee was hot and swollen with moderate effusion. No sinus tract was observed. Loosening of the tibial prosthesis was evident by conventional X-ray, and a PJI was suspected. Aspiration of the knee revealed purulent joint fluid with a leukocyte count of 17,700 cells/μl, with 94% neutrophils. Viridans streptococci grew in the culture obtained from the aspirate, displaying a normal phenotype (i.e., that of parental strain 1366), and it was subsequently identified as *S. tigurinus* by 16S rRNA gene analyses. The strain was susceptible to penicillin (MIC, 0.012 μg/ml). The dental status of the patient was checked, but no possible bacterial entry source was detected. A transesophageal echocardiogram showed no cardiac vegetation and there were no signs of respiratory infection. No antibiotic treatment had been started.

The patient was referred to a 2-stage exchange of a total knee prosthesis 4 weeks later. During surgery, pus surrounding the prosthesis was observed. Open debridement was performed and a cement spacer was implanted. Six biopsy samples from periprosthetic tissue were obtained before the intravenous administration of amoxicillin-clavulanate. In 5 out of 6 samples, viridans streptococci exhibiting slow growth and small and pinpointed colonies were cultured (SCV strains 2425 and 2426). They were confirmed to be *S. tigurinus* by 16S rRNA gene analyses, as they showed identical sequences to the rapidly growing parental strain 1366. Histopathologic examination showed signs of chronic inflammation of the synovial tissue. A late PJI was diagnosed according to the standard criteria (20). Penicillin was administered intravenously for 3 weeks until reimplantation. Three biopsy specimens from the periprosthetic tissue taken during reimplantation revealed no bacterial growth. Oral amoxicillin was administered for a total duration of 6 months. At the last follow-up examination 16 weeks after reimplantation, the patient showed no clinical or laboratory findings suggestive of infection.

Antibiotic susceptibility testing. SCVs are frequently selected by antibiotic pressure (1). We did not observe any antibiotic susceptibility differences in the *S. tigurinus* SCVs compared to the parental strain. All strains displayed full susceptibility to penicillin (MIC range, 0.012 to 0.047 μg/ml), cefoxitin (MIC range, 1.0 to 2.0 μg/ml), ceftriaxone (MIC range, 0.032 to 0.125 μg/ml), and trimethoprim-sulfamethoxazole (MIC range, 1.9 to 7.6 μg/ml), and no high-level gentamicin resistance was observed (MIC range, 12 to 24 μg/ml). Similar results (≤1 dilution difference) were obtained when the MICs were reread at 48 h.

Colony morphology, effect of serial passages, and auxotrophic testing. *S. tigurinus* 1366 and the AZ_3a^T (data not shown) displayed normal colony morphologies, with a diameter of 0.4 to 0.5 mm (Fig. 1A). The SCV strain 2425 showed very small pin-

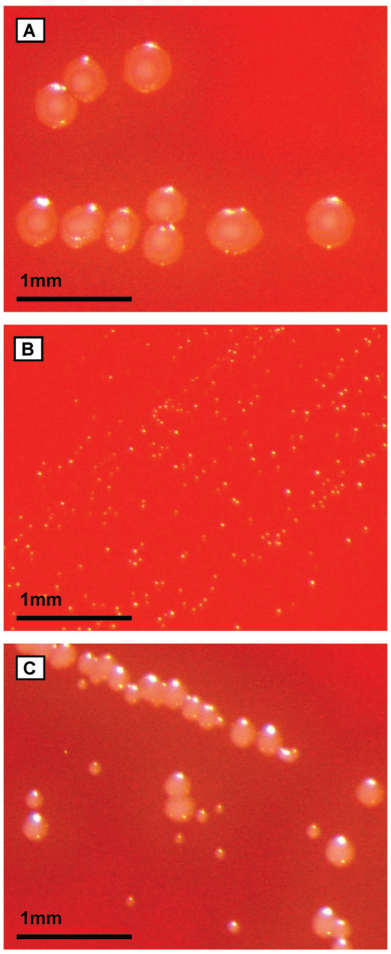

FIG 1 Colony morphologies of the *S. tigurinus* parental strain 1366 (A) and SCV strains 2425 (B) and 2426 (C) on sheep blood agar after 24 h of incubation.

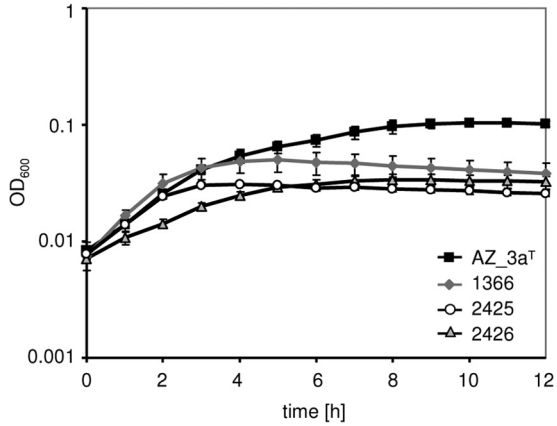

FIG 2 Growth of *S. tigurinus* strains AZ_3a^T, 1366, 2425, and 2426 in BHI broth at 37°C. The mean values of three independent experiments performed in six replicates are shown. The error bars are standard deviations.

point colonies, with a diameter of 0.03 to 0.05 mm (Fig. 1B), whereas SCV strain 2426 produced colonies of various sizes, from very small to almost normal size, with a diameter ranging from 0.03 to 0.3 mm (Fig. 1C). Compared to the WT strains, alpha-hemolysis of the SCV strains was reduced.

The *S. tigurinus* SCV strains showed different phenotypes after serial passages. Strain 2425 retained a very stable SCV phenotype for >8 passages. Conversely, strain 2426 was unstable and showed a "fluctuating phenotype," a phenomenon previously observed in *S. aureus* SCVs (2): following the initial reversion after 1 or 2 passages to their normal size, further passage of the normal colonies resulted in the reappearance of the SCV phenotype. Interestingly, for both *S. tigurinus* SCV strains, no auxotrophy for hemin, menadione, or thymidine was detected, even under prolonged incubation of 72 h (data not shown), suggesting that their SCV phenotypes were caused neither by a deficiency in electron transport nor in thymidine synthesis.

Growth curves. In general, SCVs are characterized by slow growth (1); thus, we monitored the growth of *S. tigurinus* strains. The SCV strain 2425 displayed a slightly slower exponential growth and reached a lower final OD_{600}, whereas SCV strain 2426 showed a considerably reduced exponential growth but reached similar OD_{600} levels after 12 h in comparison to the parental strain 1366 (Fig. 2). The *S. tigurinus* WT strains seem to have characteristic growth profiles, as strain 1366 grew faster in the exponential phase but reached the stationary phase earlier and at a lower OD_{600} than AZ_3a^T (Fig. 2).

TEM analyses. In addition to deficient growth, SCVs have been shown to display an increased cell size (6). The ultrastructure of *S. tigurinus* strains was therefore determined to assess whether cell division differed between the WT and SCV strains. *S. tigurinus* AZ_3a^T (data not shown) and the parental strain 1366 displayed a normal phenotype, exhibiting regular cell separation with single cross walls (Fig. 3A and D). In contrast, the cells of SCVs were heterogeneous, frequently enlarged, and displayed aberrant cell separation with multiple cross walls, resulting in the formation of clustered cells (Fig. 3B, C, and F). Additionally, the SCV cells demonstrated aberrant morphological characteristics, such as mesosome-like structures (Fig. 3E and F).

Autolysis experiments. To assess whether the impaired cell separation was due to aberrant autolytic behavior, both spontaneous and induced autolytic conditions were tested. A considerable difference was observed in the extent of spontaneous autolysis between the WT strains AZ_3a^T and 1366 (Fig. 4A), indicating that WT *S. tigurinus* strains can differ in several characteristics, including growth (see above) and autolysis. Spontaneous autolysis of both SCVs was significantly increased compared to the parental strain but in the range of the type strain AZ_3a^T (Fig. 4A). Triton X-100-induced autolysis was significantly reduced in the SCV strain 2425 compared to the parental and the type strain (Fig. 4B), whereas the SCV strain 2426 showed comparable levels of autolysis to the WT strains. However, both SCV strains showed increased resistance to Triton X-100-induced autolysis, which was contrary to the WT strains, as seen in Fig. 4C to F, which showed strain-specific autolytic patterns.

Whole-genome sequencing of *S. tigurinus* SCVs. To identify any possible genetic reason for the observed differences between the *S. tigurinus* SCVs and the parental strain, the whole genomes of *S. tigurinus* SCV strains 2425 and 2426 were sequenced. *De novo* assembly was performed using Edena version 3.130110 (21) and resulted in core genomes of 1.87 Mb, with a G+C content of 40.9%, and 1,877 genes encoding a minimum of 1,823 proteins for

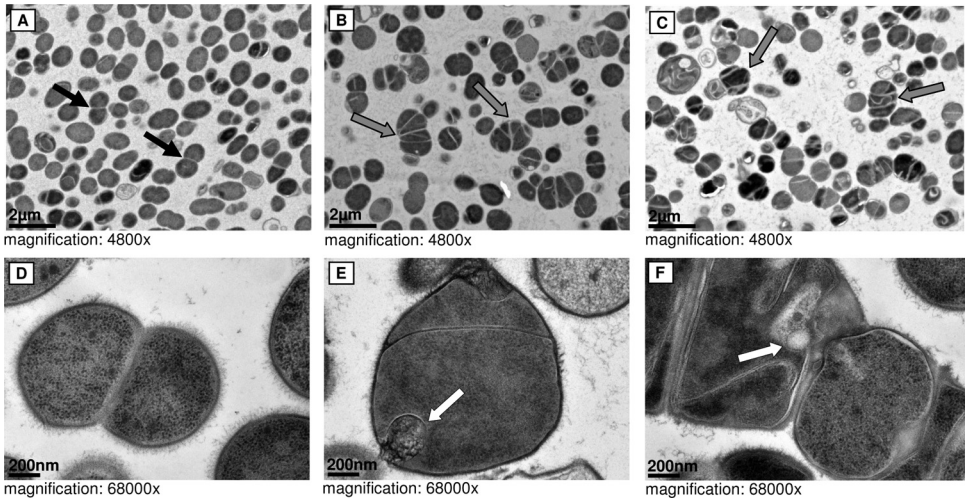

FIG 3 Cell morphology. TEM pictures of the *S. tigurinus* parental strain 1366 (A and D) and SCV strains 2425 (B and E) and 2426 (C and F). Black arrows, regular cell separation; gray arrows, atypical and enlarged SCV cells with multiple cross walls; white arrows, irregularly shaped cells with mesosome-like structures.

S. tigurinus SCV strains 2425 and 2426. These two genomes were assembled in 15 and 25 contigs, respectively, and comparison at the single nucleotide polymorphism (SNP) level was performed using the MUMmer software package (22). The analysis revealed that the larger contigs were 850 kbp for strain 2425 and 739 kbp for strain 2426. The overall assembly values were satisfactory (for strain 2425, sum, 1.87 Mbp and N_{50}, 672 kbp; for strain 2426, sum, 1.88 Mbp and N_{50}, 520 kbp). For SCV strains 2425 and 2426, we detected the same number of genes as in the parental strain 1366 (19) and did not find any large deletions. However, several differences were found between strains 1366, 2425, and 2426. Strain 2426 contains two plasmids (contig GenBank accession no. ASXA01000015, 7,309 bp, and ASXA01000016, 2,491 bp) that are absent from the two other strains. After detailed sequence comparisons, 45 mutations were detected in the core genome (Tables 1 to 3), most of them being SNPs. Overall, one-half of the mutations (23/45) were identified in both SCV mutants at the same positions, suggesting that SCV strains 2425 and 2426 might have evolved from a common SCV precursor clone (Table 1). Only 7 mutations were identified in noncoding regions (Tables 1 to 3). Selected genes carrying mutations are described below regarding their possible relevance for the SCV phenotypes.

Mutated genes in *S. tigurinus* SCVs are involved in stringent response and virulence. Almost all genes of *S. tigurinus* SCVs found to be altered in their nucleotide sequence (Tables 1 to 3) have been identified as differentially regulated either in *S. aureus* SCVs (23) or under conditions inducing a stringent response (24). Alterations in the stringent response that confer growth defects were previously described for *S. aureus* SCVs (25). *thrB* (which encodes the enzyme homoserine kinase) (Table 1) was found to be changed in *S. aureus* SCVs and under stringent response conditions (23, 24). Homoserine kinase is a key element in threonine metabolism, a pathway that contributes to virulence in different microorganisms (26–28). The 7,8-dihydro-8-oxoguanine triphosphatase MutT of the Nudix superfamily is downregulated under stringent response conditions (Table 3) (24). MutT is proposed to protect cells from mutagenic nucleotides (29). Two other genes more directly related to virulence expression were identified in our study: the gene encoding the Zn^{2+}-responsive transcriptional repressor AdcR was found to be truncated in both SCVs (Table 1). In *Streptococcus suis*, deletion of the *adcR* gene leads to a growth defect (30); thus, truncation of this regulator might have contributed to the SCV phenotypes of *S. tigurinus* strains 2425 and 2426. Furthermore, Zn^{2+}, being an important trace metal ion, has been shown to regulate the expression of several virulence genes in streptococci (31); AdcR truncation might therefore have played a role in causing the infection reported here. The second factor likely affecting virulence, the iron uptake ABC transporter ATP-binding protein (Table 2), was found to be mutated in SCV strain 2425. It is reported to be involved in various stress responses and is essential for the expression of virulence in animal models of acute pneumonia caused by *S. pneumoniae* (32).

Mutated genes in *S. tigurinus* SCVs affecting cell metabolism. For both *S. tigurinus* 2425 and 2426, we identified a mutation in the *acpS* gene encoding the 4′-phosphopantetheinyl transferase AcpS, which is an important enzyme in the type II fatty acid biosynthesis pathway (Table 1). Fatty acids are essential components of bacterial membrane lipids and lipopolysaccharides (33).

SCV mutants carrying a defect in the electron transport system have a reduced membrane potential, which is the driving force for the ATP synthetic machinery, and therefore, they produce less ATP (1). Reduced amounts of ATP, which furnishes energy for numerous biological processes, are assumed to contribute to the growth defect in these SCVs. Interestingly, we have identified a mutation in the *atpA* gene, which encodes the F_oF_1-ATP synthase alpha subunit (Table 1). Yet, whether the mutation we found in the *S. tigurinus* SCV strains contributes to the observed changes in growth remains to be determined.

Mutated genes in *S. tigurinus* SCVs affecting autolysis and cell division. In *S. tigurinus* SCV 2425, we found a mutation of the autolytic *N*-acetylmuramoyl-L-alanine amidase, Atl (Table 2) (34). Autolysins are peptidoglycan hydrolases that play important roles in cellular processes, such as lysis of the bacterial septum after cell division, cell wall growth, cell wall turnover, and recycling of muropeptides (34). Since strain 2425 did not show a generally decreased level of autolysis, further work is required to determine how the mutation might have contributed to a higher resistance only to Triton X-100-induced autolysis.

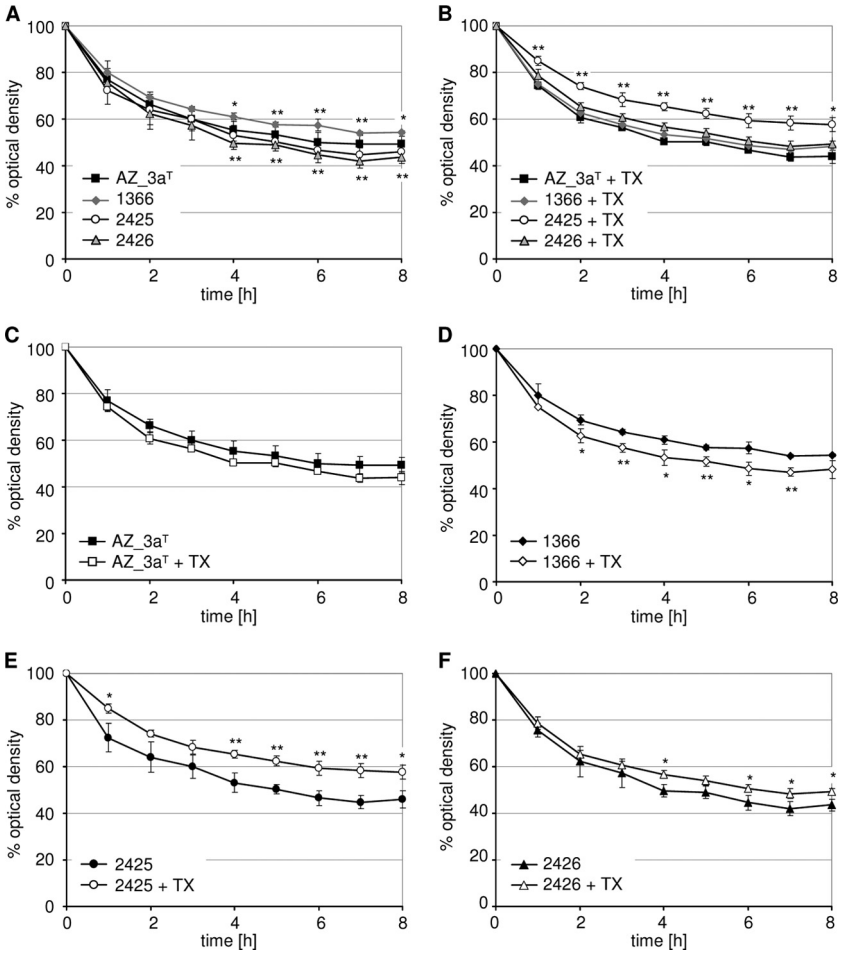

FIG 4 Spontaneous (A) and Triton X-100 (TX)-induced (B) autolysis of *S. tigurinus* strains AZ_3a^T (C), 1366 (D), 2425 (E), and 2426 (F). The mean values of three independent experiments performed in triplicate are shown. The error bars are standard deviations. The statistical analyses shown in panels A and B were performed between the parental strain 1366 and the SCVs 2425 (asterisks above curve) and 2426 (asterisks below curve), respectively. *, $P < 0.05$; **, $P < 0.01$.

Other mutations affecting cell division were found in the cell division protein FtsH of both *S. tigurinus* SCVs (Tables 1 and 2); however, the importance of these SNPs remains to be assessed in future experiments.

DISCUSSION

We present here the first clinical case of *S. tigurinus* SCVs in a patient with PJI. Although PJIs caused by viridans streptococci are less frequently encountered and are associated with a better outcome than those caused by other microorganisms (35, 36), caution must be exercised not only in view of the pathogenic potential of species, such as *S. tigurinus*, but also because the development of SCVs may lead to difficult-to-treat infections. By applying the newest technologies, i.e., comparative complete genome analyses, we found possible genetic reasons for the SCV phenotype observed in *S. tigurinus*. A number of SNPs were identified in the *S. tigurinus* SCV strains evolving from an infective *S. tigurinus* parental strain in genes likely to be associated with cell division and growth, autolytic behavior, metabolic key reactions, virulence, and stringent response.

The *S. tigurinus* SCVs showed typical SCV characteristics, such as slow growth, whereas other phenotypic traits frequently found in SCVs were missing. We did not observe any auxotrophy nor did we detect any difference in the antimicrobial susceptibility patterns of the SCVs compared to those of their parental strain. No long-term antibiotic therapy and thus no antibiotic pressures were documented in our patient, which might explain the selection of such bacterial forms. Nevertheless, the cement of the primary arthroplasty contained gentamicin, which can select for SCVs in staphylococci (37). Serial passages revealed different *S. tigurinus* SCV phenotypes: strain 2425 retained a very stable SCV phenotype, whereas strain 2426 showed a fluctuating phenotype, with reversion to normal-sized colonies and back to SCVs after further passages. The simplest explanation for this difference between the two SCVs might be the presence of an SNP in the *mutT* gene. The deletion of *mutT* has previously been shown to increase mutation rates in *E. coli* (38). Furthermore, higher mutation rates due to oxidative stress, producing damaged nucleotides that are mutagenic and normally degraded by MutT, have been associated with the emergence of SCVs in *S. pneumoniae* (11). However, it remains to be confirmed whether the specific SNP found in the SCV strain 2426 increases the mutation rate.

To the best of our knowledge, only a few SCVs have been analyzed by whole-genome sequencing to date, and a maximum of 4

TABLE 1 Mutations and their locations identified in *S. tigurinus* SCV strains 2425 and 2426 relative to parental strain 1366

Strain 1366 contig GenBank accession no.	Position	Mutation	Effect	Locus tag(s)	Gene product(s)
AORX01000001	20263 to 23836	Inversion[a]	Unknown	H353_00090 and H353_00095	Serine hydroxymethyltransferase and predicted ATPase
AORX01000001	27927	G→A	Synonymous	H353_00130	Hypothetical protein
AORX01000001	64202	●→T	Frameshift	H353_00315	Homoserine kinase ThrB
AORX01000001	138651	A→C	104 F→L	H353_00685	Amino acid ABC transporter ATP-binding protein
AORX01000001	159403	T→C	Synonymous	H353_00795	Uracil phosphoribosyltransferase (Upp)
AORX01000001	210055	C→T	162 A→V	H353_01045	Pyridine nucleotide-disulfide oxidoreductase
AORX01000001	494595	C→A	98 C→F	H353_02420	Acetyltransferase, GNAT family protein
AORX01000001	615189	T→C	Intergenic		
AORX01000001	665652	G→A	102 R→STOP	H353_03270	Zn^{2+}-responsive transcriptional repressor AdcR
AORX01000001	754073	A→C	85 S→R	H353_03690	β-Lactamase
AORX01000001	758016	C→T	364 A→V	H353_03705	Cell division protein FtsH
AORX01000002	34504	C→T	Intergenic		
AORX01000002	92064	C→T	61 S→F	H353_04258	4′-Phosphopantetheinyl transferase AcpS
AORX01000002	235844	A→T	58 I→F	H353_04923	Hypothetical protein
AORX01000002	454207	A→C	182 D→A	H353_06053	F_0F_1- ATP synthase alpha subunit
AORX01000002	501697	C→G	530 S→STOP	H353_06288	Phosphoenolpyruvate-protein phosphotransferase
AORX01000002	610271	●→T	Frameshift	H353_06833	ATPase component of ABC transporter
AORX01000002	670375	T→C	Synonymous	H353_07143	3-Isopropylmalate dehydrogenase
AORX01000002	708850	A→●	Intergenic		
AORX01000003	16071	●→T	Frameshift	H353_07674	Glutamine synthetase
AORX01000003	55915	C→T	Synonymous	H353_07859	Hypothetical protein
AORX01000003	75333	G→A	243 A→V	H353_07954	Peptide ABC transporter permease
AORX01000005	31194	A→G	297 R→G	H353_08704	Maltodextrin ABC transporter permease

[a] Full 3.5-kbp inversion; both H353_00090 and H353_00095 are complete, and the next gene (H353_00100) is annotated as tyrosine recombinase. ●, deleted position.

mutations were found in these cases (25, 39). The *S. tigurinus* SCVs carry relatively high numbers of SNPs: 33 in strain 2425 and 35 in strain 2426. It is possible that numerous mutations usually accumulate in clinical SCVs, a phenomenon that has possibly not yet been reported due to the high costs and limited availability of whole-genome sequencing approaches. Should the SNP in the *mutT* gene in SCV strain 2426 prove to increase mutation rates, one might speculate that a common ancestor of the SCVs 2425 and 2426 existed that had a mutator phenotype. This would explain the numerous mutations and why approximately 50% of the mutations were found in both *S. tigurinus* SCV strains. In that assumptive model, further SNPs were individually accumulated by the two SCVs before *mutT* changed back to the WT allele in strain 2425.

Alterations in the cell wall structure leading to abnormal bacterial growth and irregular cell shapes were previously described for *S. oralis*, a species closely related to *S. tigurinus*. Horne et al. (40) observed typical SCV cell morphologies, such as heterogeneous cell sizes, irregular septa, cell clusters, and slow growth in *S. oralis* bacteria with choline-deprived wall teichoic acids. We found abnormal cell division and autolytic behavior in *S. tigurinus* SCVs, possibly influenced by alterations of the cell envelope components and autolysins. For instance, we detected a mutation in the *acpS* gene in both *S. tigurinus* SCVs. AcpS not only affects the biosynthesis of fatty acids but also of the D-alanylated lipoteichoic acids (LTAs) of bacterial cell walls (41). Modulations in the D-alanyl content of the cell wall directly influence the autolytic mechanism (41). We hypothesize that AcpS function was affected by the mutation, leading to deficiencies of D-alanylated LTAs, which are inhibitors of cell autolysis (42). In agreement with these findings, we observed a significant increase in spontaneous autolysis of the *S. tigurinus* SCVs. In contrast, Triton X-100-induced autolysis was

TABLE 2 Mutations and their locations identified only in *S. tigurinus* SCV strain 2425 relative to parental strain 1366

Strain 1366 contig GenBank accession no.	Position	Mutation[a]	Effect	Locus tag	Gene product
AORX01000001	189337	G→C	171 S→T	H353_00940	Ribosomal small subunit pseudouridine synthase A
AORX01000001	192466	A→G	292 E→G	H353_00965	*N*-Acetylmuramoyl-L-alanine amidase (Atl)
AORX01000001	238421	●→T	Intergenic		
AORX01000001	575645	●→A	Intergenic		
AORX01000001	682287	G→A	Synonymous	H353_03350	Hypothetical protein
AORX01000001	758211	C→T	429 A→V	H353_03705	Cell division protein FtsH
AORX01000002	169627	●→"TATA"	Intergenic		
AORX01000002	258156	G→T	22 S→I	H353_05018	Glycyl-tRNA ligase beta subunit (GlyS)
AORX01000003	31503	A→T	18 D→V	H353_07734	Iron uptake ABC transporter ATP-binding protein
AORX01000007	22400 to 22392	"TGTGATGAG"→●	73 "CDE"→●	H353_09253	Hypothetical protein

[a] ●, deleted position.

TABLE 3 Mutations and their locations identified only in *S. tigurinus* SCV strain 2426 relative to parental strain 1366

Strain 1366 contig accession no.	Position	Mutation[a]	Effect	Locus tag	Gene product
AORX01000001	159120	C→G	26 R→P	H353_00790	ATP-dependent Clp protease ClpP, proteolytic subunit
AORX01000001	217597	A→C	25 S→R	H353_01080	Transporter, major facilitator family protein
AORX01000001	272919	G→A	182 M→I	H353_01325	Manganese ABC transporter permease
AORX01000001	323922	G→T	424 T→K	H353_01545	Hypothetical protein-type I RM system
AORX01000001	475131	●→"CTCA"	Frameshift	H353_02310	Hypothetical protein
AORX01000001	686770	G→A	158 L→F	H353_03365	ATP-dependent Clp protease ClpP, ATP-binding subunit
AORX01000001	717575	C→T	53 A→T	H353_03520	30S ribosomal protein S2 RpsB
AORX01000002	63127	G→T	132 G→V	H353_04073	7,8-Dihydro-8-oxoguanine triphosphatase MutT
AORX01000002	180239	G→T	292 D→Y	H353_04668	Endoglucanase
AORX01000004	21522	G→T	280 G→V	H353_08358	Phosphoribosylformylglycinamidine synthase
AORX01000005	48072	C→T	31 G→E	H353_08809	Hypothetical protein
AORX01000007	38264	A→G	Intergenic		

[a] ●, deleted position.

reduced. Triton X-100 is known to be a potent inducer of cell autolysis (43); therefore, one would expect increased autolysis. The opposite autolytic behavior caused by the presence of Triton X-100 in the *S. tigurinus* SCVs must be due to additional alterations in the cell envelope properties or autolysin regulation compared to the autolytic behavior that might be caused by the mutated *acpS* and might not be reflected at the genome level.

Comparative genomic analysis is a useful tool to enlighten putative pathogenic mechanisms in clinical strains. Future investigations will assess the correlation between the mutations found and the SCV phenotype in *S. tigurinus*. The accumulation of these mutations is probably not the result of random events but rather is from the emergence of adapted variants under selective antibiotic pressure conditions that survive in a hostile environment. Clinicians and microbiologists should be aware of this most adapted and persistent form of *S. tigurinus* leading to difficult-to-treat infections, as it has a major clinical impact on appropriate patient management.

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