学　位　論　文

Component analysis of cell-wall anchored pili of *Streptococcus sanguinis*

Streptococcus sanguinis が産生する細胞壁架橋型線毛の構成因子

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Abstract

Streptococcus sanguinis has garnered considerable attention in recent years due to its prevalence in the healthy oral environment and its crucial role as a pioneer colonizer of dental plaque. As previously reported, *S. sanguinis* exhibits a strain-specific pilus structure on the cell surface. These pili, which are composed of PiliA, PiliB, and PiliC proteins, assist bacteria in adhering to and internalizing epithelial cells, as well as forming biofilms. However, analysis of the genomic region containing the pilus genes and pilus assembly mechanism has raised concerns about the possibility of an unidentified pilus component in addition to PiliA, PiliB, and PiliC. The objective of this study was to determine whether PiliX, a putative cell surface protein encoded by a gene located adjacent to *piliABC*, is a pilus subunit and whether it has any effect on biofilm formation. Our findings indicate that PiliX is a novel pilus subunit of the *S. sanguinis* pili. Additionally, PiliX affects biofilm formation, particularly when saliva and glucose are present, suggesting that PiliX may potentiate the biofilm forming activity of *S. sanguinis* in the oral cavity.

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1 Introduction

Streptococcus sanguinis is a Gram-positive and facultative anaerobic bacteria that can be abundantly found in the oral cavity of healthy individuals. This bacterium is widely present in various sites of the human oral cavity, such as the tooth surface [1], mucosa [2], and saliva [3].

S. sanguinis, as a pioneer colonizer, subsequently enhances the adhesion of other species and is critical for the formation of oral biofilms, i.e., dental plaque in the oral cavity [4]. The salivary pellicle is a thin organic film containing numerous saliva proteins which forms on any type of surface when exposed to saliva, including the tooth surface [5]. Bacteria adhere to tooth surfaces by various mechanisms, including specific binding to pellicle components [6, 7]. *S. sanguinis* recognizes salivary α-amylase, a primary component of the salivary pellicle, by its pilus subunits [8]. Additionally, another saliva-binding protein, SsaB, is responsible for the attachment of saliva-coated hydroxyapatite to an unknown pH-sensitive receptor [9]. This kind of specific interaction between pioneer bacterial invaders and the salivary surface is essential for biofilm formation to begin [7]. Following the initial colonization, secondary colonizing species adhere to the pellicle-bound species. By biofilm mass developing, additional bacterial species are then coaggregated and subsequently adsorbed [10].

Consequently, *S. sanguinis* works as an early colonizer, while also altering the environment to make it more favorable for the establishment of subsequent bacteria. *S. sanguinis* and *Streptococcus gordonii* have the ability to use argi-

nine deiminase to decompose peptides into ammonia, ornithine, and carbon dioxide [11]. *Fusobacterium nucleatum* can bind these early colonizing organisms and provide subsequent additions to the biofilm, enabling the growth of low PH sensitive species such as *Porphyromonas gingivalis* through ammonia generation during glutamate and aspartate fermentation [12].

Additionally, extracellular DNA (eDNA) plays a vital role in the production of biofilms in a variety of species. In *S. sanguinis*, Streptococcal pyruvate oxidase (SpxB), an enzyme that generates hydrogen peroxide, is essential in interspecies interactions. SpxB-produced H_2O_2 is capable of causing DNA release, resulting in cell aggregation [13].

S. sanguinis could also cause systemic human infection if it disseminated to the bloodstream. Infective endocarditis is the most widely recognized systemic complication of oral streptococcal bacteremia [14]. It is a multisystem disease that is caused by infection of the endocardial surface of the heart, which typically involves the heart valves and surrounding structures..

Usually, infective endocarditis develops as a result of pathogens invading the bloodstream, such as from invasive dental treatments. During transient bacteremia, pathogens adhere to the cardiac valve surface. Attached monocytes release cytokines and tissue factors in response to bacteria's attachment and colonization. Thus, additional platelets are recruited and activated, which leads to the formation of vegetation. Emboli, which are formed from detached vegetation particles, may cause valve blockage, thrombi spreading to other organs, or even death [15, 16]. The mortality rate of endocarditis has been reported to range between 12 and 46 percent in previous retrospective studies.

Oral streptococci are responsible for around 20% of all infective endocarditis

cases, with a higher prevalence in underdeveloped and resource-limited regions [17, 18]. *Streptococcus sanguinis* is one of the most frequent causative organisms for infective endocarditis, occurring at a rate of 34.6% (26.6-43.3%) [19].

Pathogenic mechanisms are usually determined by the surface characteristics of pathogens. In the early 1950s, researchers discovered pili, one of the important bacterial surface structures. These are long proteinaceous structures and initially detected in Gram-negative bacteria using electron microscopy [20]. Since Gram-positive pili have relatively thinner structures, they were discovered much later in *Corynebacterium renale* in 1968 [21]. Since then, pili have been identified in several major *Streptococcus* species, such as *Streptococcus pneumoniae*, *Streptococcus pneumoniae* and *Streptococcus agalactiae*, additionally in a wide range of Gram-positive bacteria [22].

These pili are significantly dissimilar from the pili in Gram-negative bacteria, which form by multiprotein machineries located on the outer membrane, as well as spanning both inner and outer membranes [23]. Pilins are protein subunits that are assembled into these pili structures. Typically, the pili of Gram-positive bacteria are composed of covalently bonded pilins, which are formed into a structure with a diameter of around 3 nm and a length of between 0.1 and 5 micrometers [24]. Pilus genes are located within pilus genomic islands, which were identified by their discovery in publicly available genome sequences [22].

The most extensive research on pilus assembly in Gram-positive bacteria has been conducted on *Corynebacterium diphtheriae* [25]. This model states that the pilus components are secreted in a Sec-dependent manner and remain anchored to the cell membrane via membrane-spanning domains at the C terminus. Each of these pilus components contains an LPXTG motif, which is cleaved between the threonine (T) and glycine (G) residues by pilus-specific Sortases on the mem-

brane, resulting in the formation of acyl-enzyme intermediates containing covalent thioesters between the Sortase enzyme and the pilus component. The intermediate is relieved by nucleophilic attack by an amino group of a lysine residue in the adjacent pilin, resulting in the formation of an intermolecular isopeptide bond between the subunits. The same repeated reaction resulted in oligomerization of pilins. In addition, the pilin located exclusively at the base is recognized and cleaved by the housekeeping sortase SrtA. Specifically, the amino group on the lysine of the pentaglycine cross-peptide of lipid II, a precursor of peptidoglycan, attacks the resulting acyl-enzyme intermediate. The enzyme is released as a result of the nucleophilic attack, and the oligomerized pilus structure is covalently bonded to lipid II. At last, the pilus structure is anchored to the peptidoglycan.

Okahashi *et al.* previously discovered pilus structures on the surface of *S. sanguinis* SK36 and identified three pilus proteins: PiliA, PiliB, and PiliC [26]. Confocal microscopy analysis revealed that the pili-deficient mutant (Δ*piliABC* mutant) was unable to form a typical three-dimensional biofilm layer, and the authors demonstrated impaired bacterial adherence to human malignant epithelial cells, HeLa, and human oral epithelial cells, HSC-2. Additionally, ligand blot using whole human saliva analysis indicated that all pilins bind to salivary proteins. PiliC binds to fibronectin [26] and multiple salivary components [8], and one of which was found to be salivary α-amylase. Furthermore, pili-deficient mutants have a reduced capacity to accumulate on saliva-coated surfaces [8]. This indicates that pili are involved in the colonization of saliva-coated tooth surfaces and the human oral cavity, which is compatible with its role as an oral pioneer colonizer.

However, research of the pilus assembly mechanism has raised concerns about the possibility of an additional pilus component besides PiliA, PiliB, and PiliC.

We found an potential cell surface protein, PiliX (SSA1635) expressed by a gene next to *piliABC*. This protein also has the characteristic structure of the other *Streptococcus* pilus subunits, and is widely shared in clinical isolates of *S. sanguinis*. The purpose of this study was to establish whether PiliX (SSA1635) is a pilus subunit and whether it plays a role in the common pathogenic mechanisms of *S. sanguinis*, i.e. biofilm formation and bacterial survival in human blood.

2 Materials and methods

2.1 Bacterial strains and culture conditions.

The *Streptococcus sanguinis* SK36 strain used in this experiment was generously provided by Dr. M. Kilian (Institute of Medical Microbiology and Immunology, Aarhus University, Denmark). *S. sanguinis* SK36 wild-type and mutant strains were grown at 37°C under ambient air in Todd-Hewitt broth (Becton Dickinson) supplemented with 0.2% yeast extract (Becton Dickinson).

2.2 Construction of gene deletion mutants.

PCR was used to amplify DNA fragments corresponding to areas upstream and downstream of the target genes from genomic DNAusing the primer sets listed in Table 1 (ssa1631ko_F1 and ssa1631ko_R1, ssa1631ko_F2 and ssa1631ko_R2 for deletion of *srtC*; ssa1635ko_F1 and ssa1635ko_R1, ssa1635ko_F2 and ssa1635ko_R2 for deletion of *piliX*). Each of the two fragments of each gene was then mixed and used as a template for overlapping PCR. Purification of the amplified region, digestion with the appropriate restriction endonuclease such as *BamHI*, *Sal I*, or *EcoR I* (NEB), and ligation into the restriction endonuclease cleavage sites of the temperature-sensitive suicide pSET6s vector [27] were performed. Electroporation was used to transform the resulting plasmid into mutanolysin-treated *S.*

sanguinis SK36. Following that, *piliX* (*ssa1635*) and *srtC* (*ssa1631*) were deleted separately by allelic exchange via the double crossover events described before [28] (The strategy is illustrated in Figure 1). Additionally, a clone with the wild-type allele was used as a revertant strain in this study .

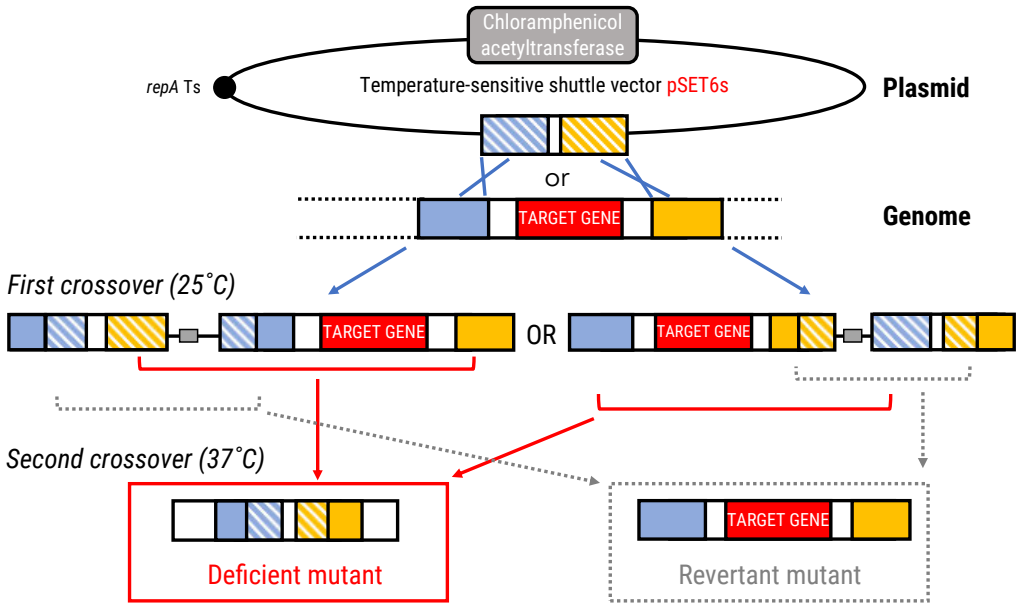


Figure 1: **Construction of a targeted deletion mutant.** PCR was used to amplify DNA fragments corresponding to areas upstream and downstream of the target genes from genomic DNA. Each of the two fragments of each gene was then mixed and used as a template for overlapping PCR. Purification of the amplified region, digestion with the appropriate restriction endonuclease and ligation into the restriction endonuclease cleavage sites of the temperature-sensitive suicide pSET6s vector were performed. Electroporation was used to transform the resulting plasmid into mutanolysin-treated *S. sanguinis* SK36. Following that, target genes were deleted separately by allelic exchange via the double crossover events. Additionally, a clone with the wild-type allele was used as a revertant strain in this study.

Table 1: PCR primers used in this study.

Designation	Sequence (5′ to 3′)	Notes
RT-PCR primers		
SKpilRT_F1	TCTCCTCATGAACCGAGAAAA	*ssa1636 - ssa1635*
SKpilRT_R1	GCCTTGACTTAGGACCTCCAT	
SKpilRT_F2	TTATACCTCTGGGGTGGCTCT	*ssa1635 - ssa1634*
SKpilRT_R2	GTCCCATTTTGGCCTGATACT	
SKpilRT_F3	CTTGTGCTAGGTGCAGGAATC	*ssa1634 - ssa1633*
SKpilRT_R3	AACGGTATCCCAAATTGCATAG	
SKpilRT_F4	ATGACATCTACATCCGCAACAG	*ssa1633 - ssa1632*
SKpilRT_R4	AGCCCACTCGATATTGGTTAAG	
SKpilRT_F5	CAAATCTGACAGCAACTTCAGC	*ssa1632 - ssa1631*
SKpilRT_R5	CCATAATGCCAGTCCCTGTAAC	
SKpilRT_F6	TTATCGCAAACAGTGCAAAAAG	*ssa1631 - ssa1630*
SKpilRT_R6	AAAAACGTAGGACAGACCCAAA	
Primers for *srtC* deletion		
ssa1631ko_F1	GCCGGATCCGATAACTCCAGGTGATCTTAA	
ssa1631ko_R1	CTGTTCCTATTTCCATAAATCACATAATATTTATTCAGTCGCA	
ssa1631ko_F2	TGCGACTGAATAAATATTATGTGATTTATGGAAATAGGAACAG	
ssa1631ko_R2	GCCGTCGACGGTGCCCTATCCATGGCTAAT	
ssa1631ko_checkF	CAATCAAGGATACAATTCCTGCTG	
ssa1631ko_checkR	GCAAAGACAAGTCTAAAGACTC	
Primers for *piliX* deletion		
re1635ko_F1	GCCGGATCCGATTGGCTACGACCGACCGCTAA	
re1635ko_R1	TAAAAGGGTTCCATTCCCACCCTCCGTAACAGCATAGACTGC	
re1635ko_F2	GCAGTCTATGCTGTTACGGAGGGTGGGAATGGAACCCTTTTA	
re1635ko_R2	GCCGAATTCCTCATACTCAGAATAATCATCCA	
ssa1635ko_checkF	CTCAGAATAATCATCCAAGTTC	
ssa1635ko_checkR	TAAGATGGACATTATTGAGCTGC	

2.3 RT-PCR.

Total RNA was isolated from the wild-type strain at the late exponential phase of growth (OD_{600} = 0.9) using the RNeasy Protect Bacteria Mini Kit (Qiagen), as previously described [29]. The synthesis of cDNA from total RNA was performed using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche), whereas the control group was not treated with reverse transcriptase (non-RT group). The RT-PCR amplifications were conducted using primers designed to amplify DNA fragments that overlapped between two adjacent genes from *ssa1630* to *ssa1636* (Table 1).

2.4 Preparation of antisera against pilin subunits.

The method was described before [30]. To express recombinant pilus subunit proteins in *Escherichia coli* cells, the coding regions of the pilus proteins (*piliA*, *piliB*, *piliC*, and *piliX*) without signal sequence and C-terminal hydrophobic regions were cloned into the expression vector pQE30 (Qiagen) and then purified from *E. coli* lysates using a QIAexpress protein purification system (Qiagen). The protein eluates were dialyzed against phosphate buffered saline (PBS). Mouse antisera were obtained by immunizing BALB/c mice (♀, 5 w) with recombinant proteins. An emulsion containing 100 μg of recombinant proteins and TiterMax Gold adjuvant (CytRx) was intradermally administered to mice. After 2 weeks, the emulsion was repeatedly administered 5 times at 1-week intervals. The antiserum was prepared from whole blood collected from the orbital sinus.

2.5 Immunoprecipitation.

The method of extraction of cell fractions was described before [30]. Cells were washed with PBS, and suspended in protoplasting buffer containing 0.1 M KPO_4 (pH 6.2), 40% sucrose, 10 mM $MgCl_2$, Complete EDTA-free protease inhibitors (Roche), and 10 units/ml mutanolysin (SIGMA). The suspension was incubated at 37°C for 3 h and protoplasts were sedimented by centrifugation at 20,000 × *g* for 20 min. Proteins in the supernatant were separated in a 12% acrylamide gel by sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE) and blotted onto polyvinylidene difluoride (PVDF) membranes. The membrane was blocked overnight using a casein-based blocking reagent (Megumilk Snow Brand) at 4°C and incubated at room temperature (RT) for 1 h with mouse

antisera diluted 1:2,000 in Tris-buffered saline (TBS) containing 0.2% Tween 20 (TBST). After washing it thrice with TBST, the membrane was incubated at RT with horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (Cell signaling), diluted 1:2,000 in TBST for 1 h. The membrane was washed three times and developed using the Pierce western blotting substrate (Thermo Scientific). Cell wall fractions of *S. sanguinis* SK36 grown in exponential phase (OD_{600} = 0.5) were dialyzed against PBS and incubated overnight at 4°C with anti-PiliA (SSA1632) mouse serum or nonimmune mouse serum. Subsequently, the mixture was incubated with protein G-coated magnetic beads (10%, v/v) at 4°C for 5 hours. The beads were thoroughly washed five times with PBS containing 0.02% Tween 20 and resuspended in the sample buffer for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE).

Then the membrane was blocked overnight using a casein-based blocking reagent (Megumilk Snow Brand) at 4C and incubated at room temperature (RT) for 1 h with mouse antisera diluted 1:2,000 in Tris-buffered saline (TBS) containing 0.2% Tween 20 (TBST). After washing it thrice with TBST, the membrane was incubated at RT with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (Cell signaling), diluted 1:2,000 in TBST for 1 h. The membrane was washed three times and developed using the Pierce western blotting substrate (Thermo Scientific).

2.6 Biofilm analysis.

A static adherence experiment using saliva-coated 12-well polystyrene plates was used to assess the capacity of wild-type, *piliX* deletion mutant, and revertant strains to produce biofilms. To summarize, a diluted (1:100) overnight culture of

S. sanguinis in THY broth (OD_{600} = 1.0) was transferred to flat-bottomed 12-well tissue culture plates (1000 μl for each well) pretreated with or without saliva for 1 hour. In the process of sterilization, the entire human saliva was mixed from multiple healthy donors, diluted to 1:10 in PBS, incubated at 65°C for 1 hour, and then sterilized through a Millex-GP Filter, 0.22 μm (Merck) according to the previously stated procedures [31]. After 24 hours of growth at 37°C under 5% CO_2, The medium was discarded and the bacteria attached were stained for 15 minutes with 1% (w/v) crystal violet. After two PBS rinses, the stain was recovered with 95% ethanol and the biomass was assessed by measuring an absorbance at 550 nm. Wells only containing THY broth were used as a negative control.

2.7 Survival rate analysis.

The survivability of *S. sanguinis* wild-type, *piliX* deletion mutant, and revertant strains in human whole blood was investigated using a bactericidal assay *in vitro*. *S. sanguinis* strains were grown in THY broth and collected at the time point when OD_{600} reached 0.4, then the cells were washed twice with PBS and resuspended to adjust OD_{600} to 0.4. The suspension was diluted to the optimal concentration and a 10 μl aliquot was combined with 190 μl fresh human whole blood. Then the mixture was incubated at 37°C for 1 to 3 hours. After incubation, the mixture was diluted and inoculated onto THY agar plates. The plates were then incubated overnight at 37°C for colony counting to determine the survival rate.

2.8 Statistical analysis.

All data were obtained from at least three independent experiments with three biological replicates. Ordinary one-way ANOVA was applied to analyze data using Tukey test on biofilm analysis and survival rate analysis. A p-value less than 0.05 was considered statistically significant. All statistical analysis was performed using GraphPad Prism version 9.3.0 for macOS (GraphPad Software).

2.9 Ethics statement.

The animal experiments were approved by the Animal Care and Use Committee of Osaka University Graduate School of Dentistry (approval no. 29-014-0). The sampling protocol of human blood was approved by the institutional review board of Osaka University Graduate School of Dentistry (approval no. H26-E43).

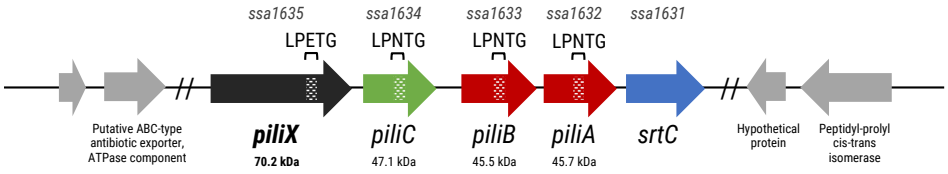
3 Results

3.1 Gene context of the SK36 pilus island and functional analysis of *piliX*

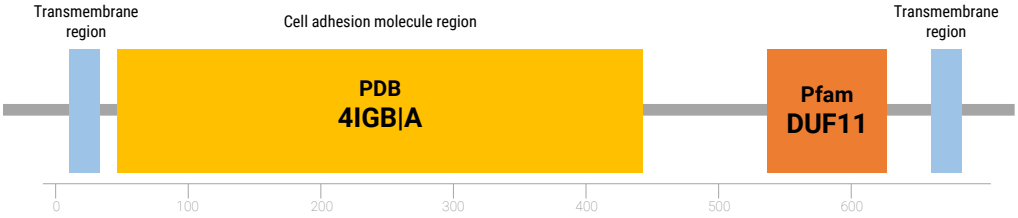
The *piliX* gene (also known as *ssa1635*) is adjacent to *ssa1632* - *ssa1634* and *ssa1631* previously reported pilus subunits and class C Sortase, respectively [32]. The *piliX* gene encodes a protein with a predicted size of 70.2 kDa (Figure 2a). As with the three known pilus subunits, PiliX includes the LPXTG pentapeptide motif, which is assumed to be vital for the mechanism of Gram-positive bacteria protein secretion and the covalent attachment to the cell wall peptidoglycan [33].

The domain structure of PiliX was analyzed by the Simple Modular Architecture Research Tool [34] (Figure 2b), and it was revealed that PiliX has an N-terminal transmembrane domain, indicating that the protein is released onto the cell membrane. It also includes a cell adhesion molecule region, which was previously found in *S. gordonii*'s adhesin and considered to have an adhesion function to type-1 collagen and oral keratinocytes [35].

These data suggested that PiliX (Ssa1635) works as the *S. sanguinis* pilus subunit.



(a) Genetic context of *S. sanguinis* pilus genes



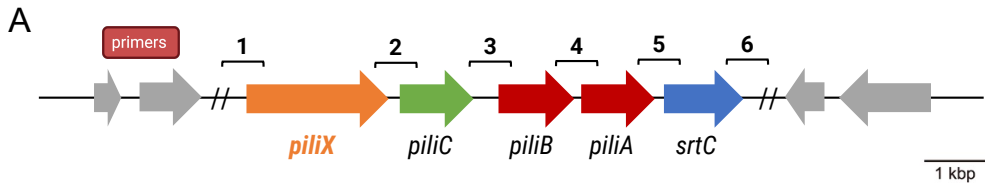
(b) Potential function analysis of PiliX

Figure 2: **Gene context and potential function analysis of *piliX*.** (a) Genetic context of *S. sanguinis* pilus genes. *piliX* (*ssa1635*) is located upstream of previously identified *S. sanguinis* pilus-related genes. The dotted regions: LPXTG pentapeptide motif. (b) Potential function analysis of PiliX by SMART [34].

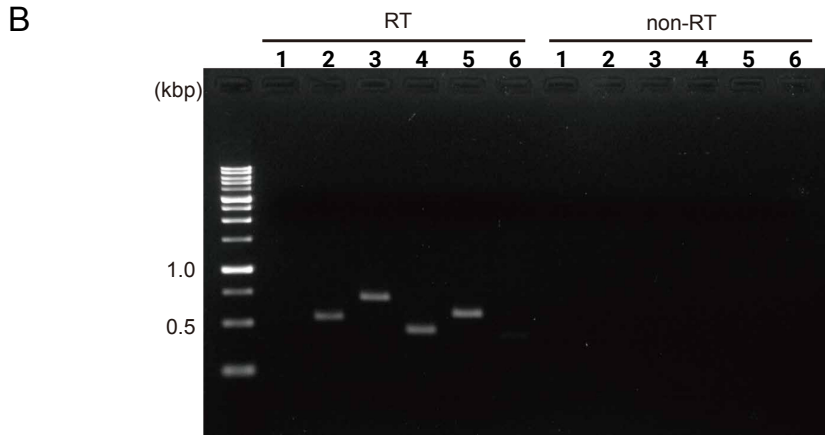
3.2 Detection of co-transcription of *piliX* with *piliABC* by RT-PCR

Following that, we used RT-PCR to determine if the *piliX* gene was co-transcribed with previously discovered pilus-related genes. The total RNA was extracted and then amplified using PCR with six pairs of primers (Table 1) that overlapped each of the two neighboring genes (Figure 3a). As demonstrated by the electrophoresis result in Figure 3b, bands corresponding to the intergenic regions between *piliX*, *piliC*, *piliB*, *piliA*, and *srtC* were detected, confirming co-transcription of *piliX* with *piliABC* and *srtC* as an operon. DNA contamination was ruled out using

PCR analysis on samples from the non-RT group. As a result, we may deduce that *piliX* is co-transcribed with *piliABC* and *srtC*.



(a) Diagram of primers in RT-PCR



(b) Electrophoresis results of RT- PCR

Figure 3: **Detection of co-transcription of *piliX* with *piliABC* by RT-PCR.**(a) Diagram of primers in RT-PCR. The primers that overlapped each of the two neighboring genes. (b) Electrophoresis results of RT- PCR. As a control, the non-RT group was not treated with reverse transcriptase.

3.3 Detection of PiliX in both cell wall and culture supernatant fractions

To test whether PiliX is expressed as a component of pili, immunoblot analyses with anti-PiliX serum were performed on the cell wall and culture supernatant

fractions of *S. sanguinis* wild-type, *piliX* deletion mutant and revertant strains. In Figure 4, in both fractions, a band of approximately 70 kDa, corresponding to the deduced size of the previously proposed protein product of PiliX, was detected. Furthermore, in addition to the band reflecting the monomer, the high molecular weight ladder bands were clearly recognizable in both fractions of wild-type and revertant strains, indicating the incorporation of PiliX into pili.

3.4 Detection of PiliX in the cell wall fraction of Δ*srtC* mutants

Then, dedicated immunoblots using anti-PiliX serum were performed on cell wall fractions from wild-type, *srtC* deletion mutant, and revertant strains. In Figure 5, the high molecular weight ladder bands were undetectable in fractions from the *srtC* deletion mutant strain. The *srtC* gene that encodes the transpeptidase involved in the pilus subunit linkage. In contrast to the wild type and revertant strains, the bands presumed to be monomeric PiliX were also significantly elevated in Δ*srtC* mutants. Given that Sortase C is responsible for the assembly and linkage of pilus subunits, this result implies that PiliX is a pilus subunit.

3.5 Effects of *piliX* deletion on pilus detection pattern

To demonstrate the expression of additional pilin subunits in the PiliX deletion strain, immunoblots using anti-PiliA, B and C and cell wall extracts from piliX

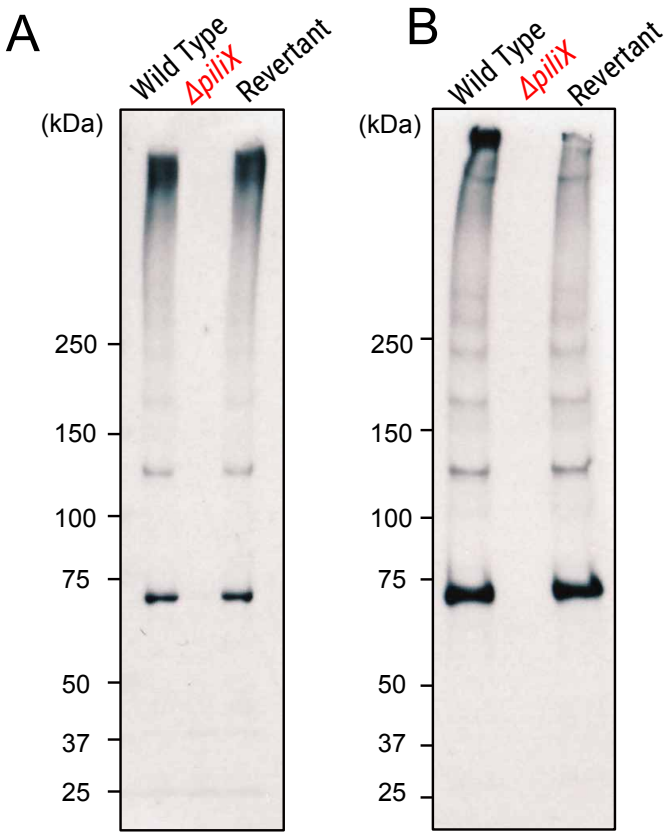


Figure 4: **Detection of PiliX in both cell wall and culture supernatant fractions.** SDS-PAGE with 12% acrylamide gels and immunoblots with anti-PiliX serum prepared from mice and rabbits were performed on equivalent samples collected from (a) culture supernatant and (b) cell wall of *S. sanguinis* SK36 wild type, *piliX* deletion mutant and revertant strain.

deletion mutants, as well as wild-type and revertant strains were performed. Deletion of *piliX* altered the intensity and migration pattern of ladder bands, adding to the evidence that PiliX is a pilus subunit (Figure 6).

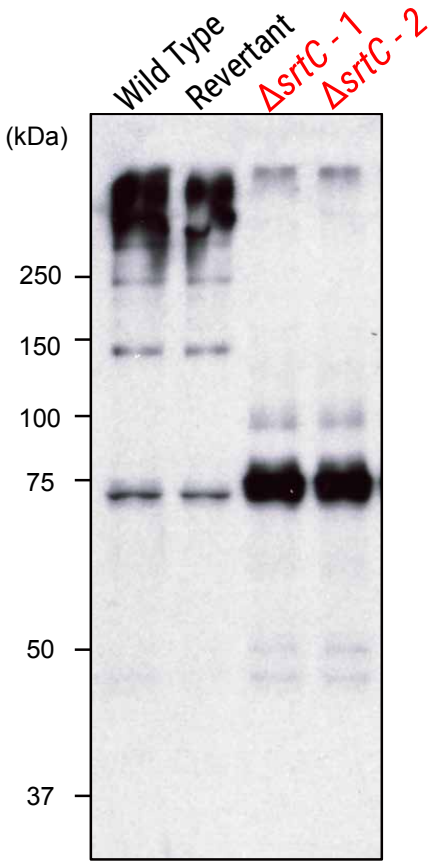


Figure 5: **Detection of PiliX in cell wall fraction of Δ*srtC* mutants.** SDS-PAGE with 12% acrylamide gels and immunoblots using anti-PiliX serum were performed on cell wall extracts from wild-type, *srtC* deletion mutant, and revertant strains.

3.6 Detection of PiliX in cell wall fractions immunoprecipitated with anti-PiliA serum

To further investigate whether PiliX is a pilus subunit, immunoblot analyses using antisera PiliB, PiliC, and PiliX were performed on cell wall fractions immunoprecipitated with anti-PiliA. The high molecular weight ladder bands were

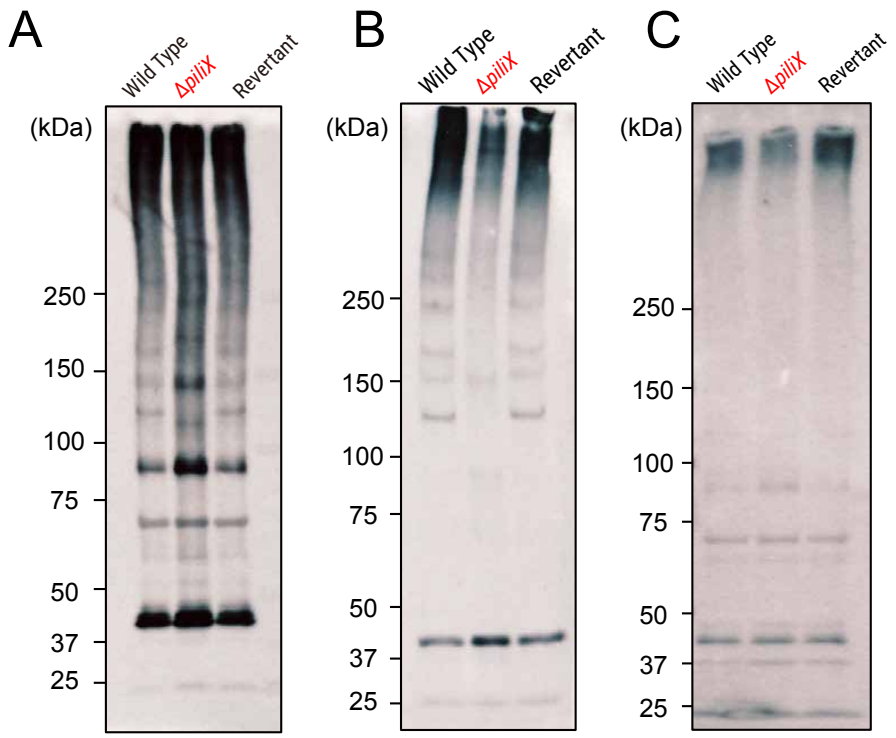


Figure 6: **Detection of PiliX in cell wall fractions.** SDS-PAGE with 12% acrylamide gels and immunoblots using (a) Anti-PiliA serum, (b) Anti-PiliB serum and (c) Anti-PiliC serum were performed on cell wall extracts from wild-type, *piliX* deletion mutant, and revertant strains.

visible in all immunoprecipitated samples, but not in the non-immunoprecipitated control samples.

These data strongly indicate that PiliX is a pilus component.

3.7 The PiliX protein affects the formation of biofilms but not on the survival rate

In previous studies, pilus was found to be involved in biofilm formation [33]. Regarding *S. sanguinis* pili, deletion of *piliABC* greatly reduced ability to form biofilms. Thus, a one-way between subjects ANOVA was conducted to compare the potential involvement of PiliX in biofilm formation. There was a significant effect of the deletion of *piliX* gene on the amount of biomass stained by crystal violet assay, [F (2, 57) = 12.18, $p < 0.0001$]. Tukey's HSD Test for multiple comparisons found that the mean value of amount of biomass was significantly different between wild type and $\triangle$*piliX* (p = 0.0001, 95% C.I. = [0.02861, 0.09654]), $\triangle$*piliX* and revertant strains (p = 0.0004, 95% C.I. = [0.02380, 0.09172]). There was no statistically significant difference between wild type and revertant strain (p = 0.9379). The *piliX* deletion mutant strain was less capable of forming biofilms in the presence of saliva and glucose, implying that PiliX protein promoted biofilm formation (Figure 8a).

Furthermore, because *S. sanguinis* is a major pathogen responsible for infective endocarditis, we examined whether PiliX contributes to bacterial survival in human blood. Wild type, *piliX* deletion mutant, and revertant strains were incubated for three hours in human whole blood and determined their survival rates were determined (Figure 8b). A one-way between subjects ANOVA was conducted to compare the potential involvement of PiliX in human whole blood survival rates. There was not a significant effect of the deletion of *piliX* gene on human whole blood survival rates. The results indicate that PiliX has no effect on *S. sanguinis* blood survival in human blood.

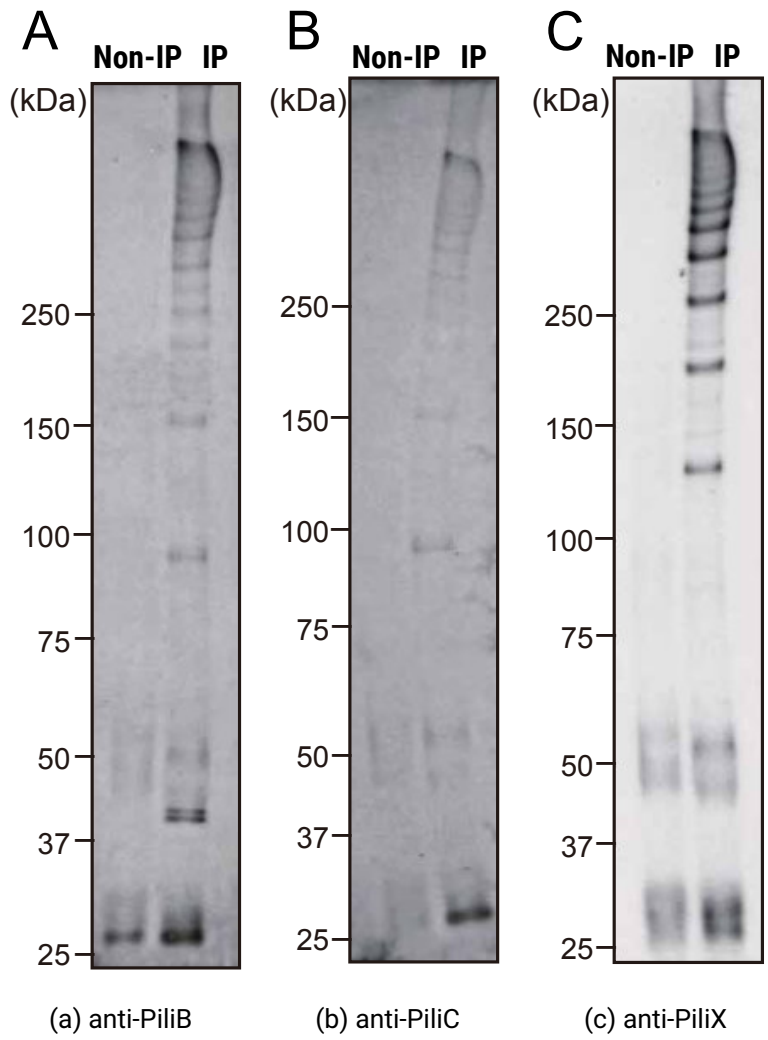
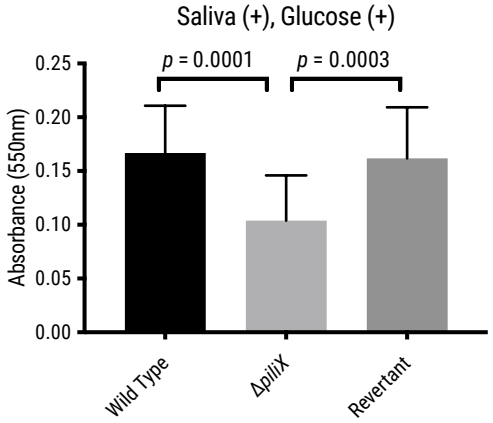
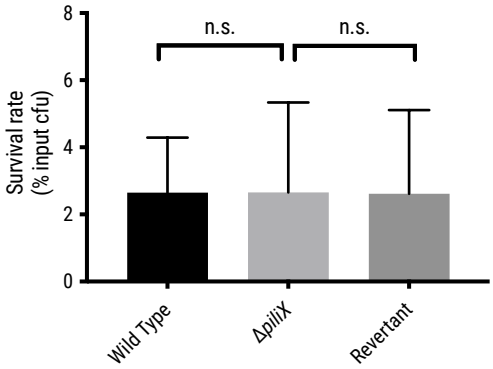


Figure 7: **Detection of PiliX in cell wall fractions immunoprecipitated with anti-PiliA serum.** SDS-PAGE with 12% acrylamide gels and immunoblots using (a) Anti-PiliB antiserum, (b) Anti-PiliC antiserum, (c) Anti-PiliX antiserum were performed on cell wall fractions of *S. sanguinis* SK36 immunoprecipitated with anti-PiliA serum.



(a)



(b)

Figure 8: **The PiliX protein has an effect on biofilm formation, but not on survival rates.** (a) The biofilm formation of *S. sanguinis* SK36 wild type, *piliX* deficient and revertant strains. A static biofilm assay using saliva-coated 12-well polystyrene plates was conducted to assess the capacity to produce biofilms after 24 hours. (b) The survival rates of *S. sanguinis* SK36 wild type, *piliX* deletion mutant and revertant strains in human whole blood. The survival in human whole blood was investigated using a bactericidal assay *in vitro*. All data were obtained from three independent experiments with three biological replicates. Ordinary one-way ANOVA was applied to analyze data of biofilm and bacteriocidal assays. n.s. shows $p > 0.05$.

4 Discussion

Oral streptococci cause disease when they enter the bloodstream from the mouth. One of the most severe consequences is infective endocarditis, characterized by an infection of the heart valves, or an infection of the endocardium. While relatively rare, infective endocarditis is associated with increasing morbidity and mortality. The number of cases are on the rise, with it ranking third or fourth among all life-threatening infections, after sepsis, pneumonia, and intra-abdominal abscess [36]. Among the oral streptococci, *S. sanguinis* is particularly significant since it is one of the most frequent causative agents of infective endocarditis, accounting for between 18 and 30% of cases [37].

S. sanguinis SK36 strain utilized in this work was isolated from human dental plaque. It is also the first *S. sanguinis* strain whose complete genome sequence was published [32]. Therefore, the strain has been used as a standard strain in studies of a variety of oral diseases and infective endocarditis. According to a genomic study published in 2019 [38], 20 clinical isolates of *S. sanguinis* (including 9 oral isolates and 10 infective endocarditis isolates in addition to SK36) were used to establish an open pangenome. A total of 1499 core gene clusters (defined as clusters shared by all strains) were established, comprising 68 percent of the SK36 gene. The SK36 strain was ranked at 7/17 in the virulence model of infectious endocarditis in rabbits, which indicates moderate virulence. However, the virulence genes unique to infective endocarditis isolate strains remain unknown, and it is believed that *S. sanguinis* virulence is highly diverse and is mediated by

a variety of genetic factors.

According to BlastP by the BLAST Sequence Analysis Tool [39], the *piliX* (*ssa1635*) is shared by all of these 20 isolates, while *srtC* is shared by 9 of 20 (5 of 10 oral isolates and 3 of 9 infective endocarditis isolates), *piliA* is shared by 19 of 20 (10 of 11 oral isolates and all 9 infective endocarditis isolates), *piliB* is shared by 16 of 20 (8 of 11 oral isolates and 8 of 9 infective endocarditis isolates), *piliC* is shared by 19 of 20 (10 of 11 oral isolates and all 9 infective endocarditis isolates), which showed that the pilus genes are relatively conserved among strains. Therefore, the majority of *S. sanguinis* strains have the potential to express pilus. However, some strains are unable to produce pilus structures adequately due to the absence of pilus specific Sortase SrtC. Despite this, two other Sortases, SrtA and SrtB, are shared by all these clinical isolates. Further research is required to determine the role of different Sortases during the pilus assembly process of *S. sanguinis*.

According to Figure 8a, when saliva and glucose are present, which mimics the living environment in the mouth, we discovered that PiliX may help *S. sanguinis* form biofilms in the oral cavity. In the absence of either saliva or especially glucose, the amount of biofilm formed by *S. sanguinis* SK36 was relatively low and too fragile for quantitative investigations (Figure 9). Previous studies [8] established that PiliA, PiliB, and PiliC can bind salivary components, yet only PiliC is able to bind α-amylase. However, after deletion of the *piliABC* genes, SK36 still retains a certain degree of ability to bind salivary components [8], implying the presence of additional saliva-binding components in *S. sanguinis*, which could possibly be the newly discovered subunit PiliX. The role of PiliX and saliva requires further investigation.

Prior research indicated that *S. sanguinis* pilus is composed of three subunits,

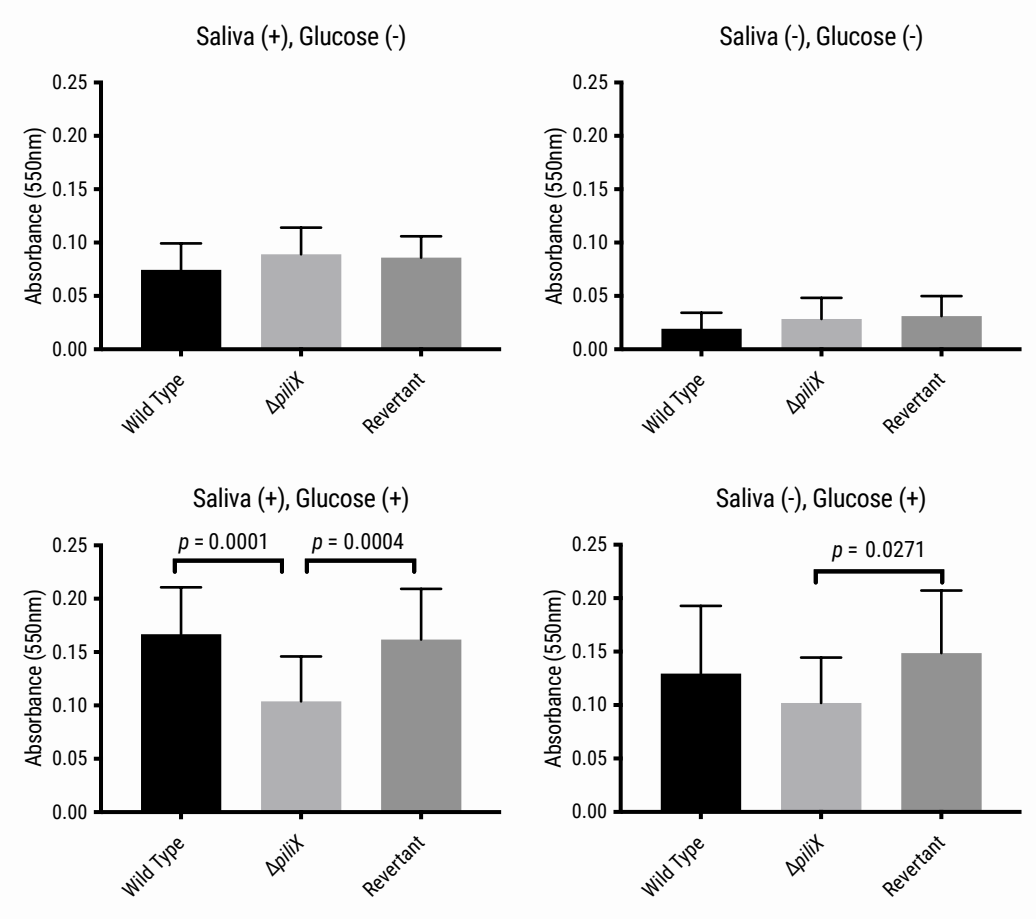


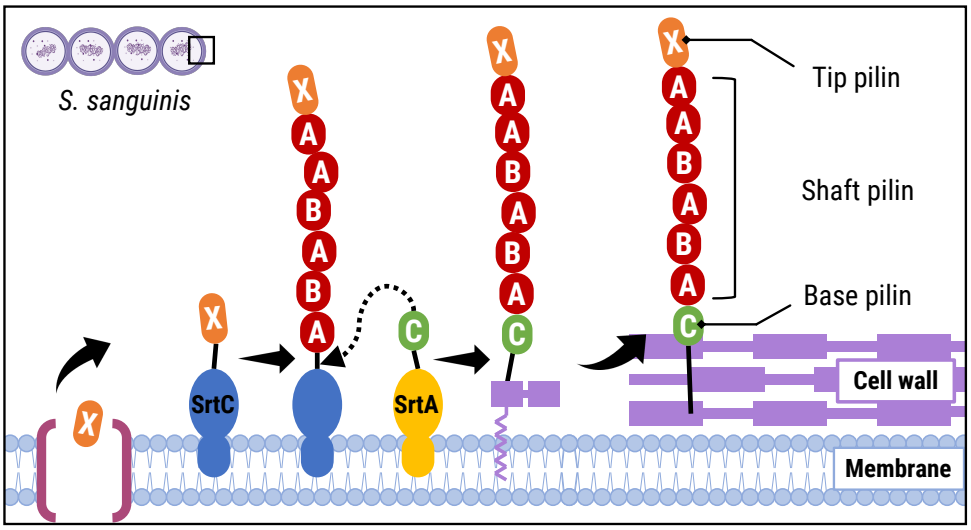
Figure 9: **Both glucose and saliva increased the amount of biofilm formed by *S. sanguinis* SK36.** A static biofilm assay of *S. sanguinis* SK36 wild type, *piliX* deficient and revertant strains using saliva-coated (or not) 12-well polystyrene plates was conducted to assess the capacity to produce biofilms with (or without) glucose after 24 hours. All data were obtained from three independent experiments with three biological replicates. Ordinary one-way ANOVA was applied to analyze data of biofilm and bacteriocidal assays.

including PiliA, PiliB, and PiliC. According to TEM immunogold staining image, PiliA forms the backbone pilin of *S. sanguinis* pilus [8]. PiliA and PiliB, contrary to PiliC, possess a high degree of homology, thus we hypothesize that they are

both backbone subunits. According to the PiliX potential function analysis in Figure 2b, the 4IGB|A region was previously found as a component of adhesin in *S. gordonii* 0707 and has an effect on adhesion to type-1 collagen and oral keratinocytes. As a result, we hypothesize that PiliX is the tip pilin. The detection level of *piliC* in the *piliX* deletion mutant is significantly lower than that of any other pilin subunit, indicating that it is a base protein. Using the information above, we created the schematic diagram in Figure 10.

The overall aim of this work is to understand the pilus structure of *S. sanguinis*, an important oral pioneer colonizer and a major pathogen of infective endocarditis. Based on the results of this study, PiliX appears to be a novel pilus subunit of *S. sanguinis*. Furthermore, PiliX inhibits biofilm formation, particularly when saliva and glucose are present, suggesting that PiliX may enhance the biofilm formation activity, even having a potential role in the colonization and pathogenesis.

(a) Genetic context of *S. sanguinis* pilus genes



(b) Schematic illustration of the structure and assembly of the pilus of *S. sanguinis* SK36

Figure 10: **Diagram of the genetic context, structure, and assembly of the pilus of *S. sanguinis* SK36.** (a) Genetic context of *S. sanguinis* pilus genes. (b) Schematic illustration of the structure and assembly of the pilus of *S. sanguinis* SK36. PiliX and other pilin subunits are secreted to the outer surface of the cell membrane. The LPXTG motif on the pilin subunit is recognized by SortaseC (SrtC), which catalyzes the covalent binding of each pilus subunit. Housekeeping SortaseA (SrtA) is responsible for linkage between assembled pili and a free-amino group of peptidoglycan. Based on previous data and present findings, it was assumed that PiliA and PiliB constitute pilus shaft with PiliC and PiliX located at the pilus base and tip, respectively.

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