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Pili of oral *Streptococcus sanguinis* bind to salivary amylase and promote the biofilm formation

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ABSTRACT

Streptococcus sanguinis is a member of oral streptococci and one of the most abundant species found in oral biofilm called dental plaque. Colonization of the oral streptococci on the tooth surface depends on the adhesion of bacteria to salivary components adsorbed to the tooth surface. Recently, we identified unique cell surface long filamentous structures named pili in this species. Herein, we investigated the role of *S. sanguinis* pili in biofilm formation. We found that pili-deficient mutant, in which the genes encoding the three pilus proteins PilA, PilB and PilC have been deleted, showed an impaired bacterial accumulation on saliva-coated surfaces. Confocal microscopic observations suggested that the mutant was incapable of producing typical three-dimensional layer of biofilm. Ligand blot analysis showed that the ancillary pilus proteins PilB and PilC bound to human whole saliva. Additional analysis demonstrated that PilC bound to multiple salivary components, and one of which was found to be salivary α-amylase. These results indicate that pilus proteins are members of saliva-binding proteins of oral *S. sanguinis*, and suggest the involvement of pili in its colonization on saliva-coated tooth surfaces and in the human oral cavity.

1. Introduction

Dental plaque is a complex biofilm formed on saliva-coated tooth surfaces. Its development is dependent on adhesion of bacteria to salivary components adsorbed to the tooth surface [1–3]. This process is dominated by oral streptococci, and among these, *Streptococcus sanguinis*, a primary colonizer in the human oral cavity, has been shown to play an important role in the formation of dental plaque [2,4,5]. Therefore, the mechanism of attachment of *S. sanguinis* to saliva-coated surfaces has generated considerable interest. In early investigations, some salivary components have been examined for specific binding to oral bacteria, and salivary proline-rich proteins, agglutinins and α-amylase were shown to bind to oral streptococci [2,6–8]. Regarding to α-amylase, the binding is reported to be associated with its maltose oligosaccharides-binding activity [8]. Further investigations using gene cloning have revealed that multiple surface proteins of oral streptococci contribute to bacterial adherence and binding to salivary components [2,4,9–12].

Recently, various pathogenic streptococci such as *Streptococcus pyogenes*, *Streptococcus agalactiae*, and *Streptococcus pneumoniae* were reported to possess long filamentous pilus-like structures [13–15], which consist of a major backbone subunit together with one or more minor subunits that are covalently assembled on the cell surface [16,17]. The pili of these pathogenic streptococci have been found to contribute to adherence to human cells [15,16]. Concerning oral streptococci, we recently reported that *S. sanguinis* possesses pilus-like structures similar to those of pathogenic streptococci, which are also involved in bacterial binding to fibronectin [18].

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In the present study, we found that pili of *S. sanguinis* bind to salivary α-amylase, and contribute to the formation of biofilm on saliva-coated surfaces.

2. Materials and methods

2.1. Bacterial strains and cell cultures

S. sanguinis strain SK36 [19] was provided by Dr. M. Kilian (Institute of Medical Microbiology and Immunology, Aarhus University, Denmark). Construction of pilus-deficient mutant, SK36Δ*ssa1632–1634* (hereafter Δpili), was performed as described previously [18]. Bacteria were cultured in Todd Hewitt broth (Becton Dickinson, Sparks, MD, USA) supplemented with 0.2% Yeast Extract (Becton Dickinson) (THY broth). *Escherichia coli* strain XL10-Gold (Stratagene, La Jolla, CA, USA) was grown in Luria-Bertani broth.

2.2. Biofilm formation and confocal microscopy

Biofilm formation by *S. sanguinis* was determined using an adherence assay in 96-well culture plates. Briefly, an overnight culture of *S. sanguinis* grown in THY broth was diluted to 1:100, and then 200 μl of the bacterial suspension was transferred to flat-bottomed 96-well tissue culture plates (Asahi Glass, Tokyo, Japan) coated with or without saliva. The clarified human whole saliva used in this study was prepared as described previously [20]. The bacteria were grown at 37 °C for 24 h in 5% CO_2, the medium was then removed, and adherent bacteria were stained with 1% (w/v) crystal violet for 15 min. After rinsing with water, bound dye was recovered with 95% ethanol and the biomass was quantitated by measuring optical density at 550 nm (OD_{550}). Wells without bacteria were used as a negative control.

For confocal microscopic observations, bacteria were allowed to grow in saliva-coated Lab Tek II slide glass chambers (Nalge Nunc, Rochester, NY, USA) at 37 °C in 5% CO_2 for 48 h. The resulting biofilms were stained with a Live/Dead BacLight staining kit (Invitrogen, Carlsbad, CA, USA), which showed all bacteria as green (SYTO-9) whereas dead bacteria as red (propidium iodide). SYTO-9 images were obtained using an LSM 510 confocal laser microscope (Carl Zeiss, Oberkochen, Germany), and analyzed with IMARIS software (Bitplane, Zurich, Switzerland).

2.3. Antibodies, ELISA and western blotting

Three pilus genes, *pilA* (*ssa1632*), *pilB* (*ssa1633*), and *pilC* (*ssa1634*), were identified from the entire genome sequence of *S. sanguinis* SK36 (accession No. NC_009009) [21]. DNA fragments without a putative signal sequence and cell-anchoring region were amplified by PCR and cloned into *Bam*HI/*Kpn*I sites of a pQE30 vector (QIAGEN, Hilden, Germany), then the expressed recombinant proteins were purified using Ni-NTA column chromatography (His-Accept gel; Nacalai Tesque, Kyoto, Japan), as previously reported [18]. DNA fragments encoding PilC (27–190) (PilC fragment of amino acids 27–190), PilC (161–310) (PilC fragment of amino acids 161–310) and PilC (291–428) (PilC fragment of amino acids 291–428) were amplified by PCR, cloned into a pQE30 vector, and expressed in *E. coli* XL10-Gold. The PCR primers used are listed in Table 1. DNA fragment (*ssa0907*) encoding a putative fibronectin binding protein (FnBP) of *S. sanguinis* was also amplified by PCR and cloned into *Bam*HI site of a pQE30 vector. The recombinant FnBP was expressed in *E. coli* and purified as described above.

Anti-PilA, -PilB, and -PilC sera were prepared by immunizing ddY mice (Japan SLC, Shizuoka, Japan) with recombinant pilus proteins mixed with Freund's complete adjuvant (Thermo Scientific, Rockford, IL, USA) [18]. Specificity of antiserum was examined using an enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well flat-bottomed ELISA plates (Sumitomo Bakelite, Tokyo, Japan) were coated with purified pilus proteins (1 μg/well) in carbonate buffer (pH 9.6) overnight at 4 °C. After three washes with phosphate buffered saline (pH 7.4) containing 0.1% Tween 20 (PBST), 5% skimmed milk in PBST was added and incubated for 1 h. Then, the antisera (1:1000 dilution in PBST) were added and incubated for overnight at 4 °C. Antibodies bound to the immobilized proteins were detected using horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin G (IgG) (GE Healthcare, Uppsala, Sweden) and 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (ELISA POD substrate TMB kit, Nakalai Tesque). The OD_{450} values were determined after incubation for 30 min at 37 °C.

Table 1
PCR primers used in this study.

Designation	Sequence (5′ to 3′)
PilC(27–190)-F (BamHI)	ATGGATCC-GACACGACCTATACCATTCAGC
PilC(27–190)-R (KpnI)	ATGGTACC-GGAATAATCCGTTATAGTCGTTTCC
PilC(161–310)-F (BamHI)	ATGGATCC-ACAGTAGCAATCAAGTCTGATGTTC
PilC(161–319)-R (KpnI)	ATGGTACC-AGGGTTATTTGAAAAGCTTAGACTAG
PilC(291–428)-F (BamHI)	ATGGATCC-ACACATGCCAGTGCCAATACTAAC
PilC(291–428)-R (KpnI)	ATGGTACC-GCTCTGTCTGAAGTTGCTTGAATAG
FnBP-F (BamHI)	ATGGTACC-AACCGCCAAAGCACAAGCTGC
FnBP-R (BmaHI)	ATGGTACC-ACTTGGACCAGATTGGAGAGTC

Whole cell mutanolysin lysates of *S. sanguinis* SK36 containing high molecular weight native pili were prepared as described previously [18]. The mutanolysin lysates were separated using 5–20% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Atto, Tokyo, Japan), transferred to polyvinylidene difluoride (PVDF) membranes (Atto), and reacted with mouse antiserum against PilA, PilB or PilC in 50 mM Tris–HCl buffer (pH 7.5) containing 150 mM NaCl and 0.05% Tween 20 (TBST). Antibodies bound to the immobilized proteins were detected using HRP-conjugated anti-mouse IgG and TMB development reagent (EzWest; Atto) [18].

2.4. Binding assay and ligand blotting

Human salivary α-amylase was purchased from Sigma Aldrich (St. Louis, MO, USA), and biotinylated using an enhanced chemiluminescence (ECL) protein biotinylation kit (GE Healthcare) [18,22]. Formalin-fixed whole cells of *S. sanguinis* (1×10^9 colony forming units) were blocked with 10% ECL blocking reagent (GE Healthcare) in TBST for 1 h, then washed, and incubated with biotinylated amylase (10 μg/ml in TBST) at 4 °C for overnight. Bound amylase was detected using HRP-streptavidin (GE Healthcare) and TMB substrate. After the reaction, the bacterial suspensions were centrifuged and the OD_{450} values of the supernatants were determined.

Ligand blotting using human whole saliva and PilC was also performed. For this purpose, recombinant PilC and human whole saliva were biotinylated as described above. Then, the target proteins were separated by SDS-PAGE, and transferred to PVDF membranes. Bovine serum albumin (BSA) (Sigma Aldrich) was used as a negative control in some experiments. The membranes were blocked with 10% ECL blocking reagent in TBST, and incubated with biotinylated ligands (10 μg/ml in TBST) at 4 °C for 18 h. Bound ligands were detected using HRP-streptavidin and TMB reagent [18].

2.5. Immunogold labeling and transmission electron microscopy

S. sanguinis SK36 and Δpili mutant were grown to the exponential phase ($OD_{600} = 0.6$), then washed twice with phosphate