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論　文　内　容　の　要　旨

氏　名　（　李 怡萱 Li Yixuan　）	
論文題名	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）

論文内容の要旨

Background and Objectives

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 plaques. 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 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.

Materials and Methods

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 isogenic mutant strains were cultivated at 37° C in ambient air in Todd-Hewitt broth (Becton Dickinson) supplemented with 0.2% yeast extract (Becton Dickinson).
2. **RT-PCR.** Total RNA was isolated from the wild-type strain in the late exponential phase of growth ($OD_{600\ nm}$ = 0.9). The synthesis of cDNA from total RNA was performed using the Transcriptor High-Fidelity cDNA Synthesis Kit (Roche), whereas the contrast group was not treated with reverse transcriptase (non-RT group). RT-PCR amplifications were performed using primers designed to amplify DNA fragments that overlapped between two adjacent genes from *ssa1630* to *ssa1636*.
3. **Construction of gene deletion mutants.** By allelic exchange through double crossover events, *piliX* (*ssa1635*) and *srtC* (*ssa1631*) were deleted separately using the temperature-sensitive suicide vector pSET6s. In addition, a clone carrying the wild-type allele was used in this study as a revertant strain.
4. **Preparation of antisera against pilin subunits.** To express recombinant pilus subunit proteins in *Escherichia coli* cells, the coding regions of the putative pilus proteins (*piliA*, *piliB*, *piliC* and *piliX*) were cloned into the expression vector pQE30 (Qiagen) and then purified from *E. coli* lysates using a QIAexpress protein purification system (Qiagen). Protein eluates were dialyzed against PBS. Mouse antisera against pilus proteins were raised by immunizing BALB/c mice with purified recombinant proteins. Rabbit antisera against pilus recombinant proteins were raised by immunizing male New Zealand white rabbits with purified recombinant proteins.
5. **Immunoprecipitation.** Cell wall fractions of *S. sanguinis* SK36 grown in exponential phase ($OD_{600\ nm}$ = 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 PBST and resuspended in a sample buffer containing SDS. Immunoblot analysis was performed on the immunoprecipitated samples using rabbit sera against pilus proteins.

6. **Biofilm analysis.** The ability of wild-type, *piliX* deletion mutant, and revertant strains to form biofilms was determined using a static assay with saliva-coated polystyrene plates 12 or 24 hours after inoculation.

Results

1. **The PiliX protein is a *S. sanguinis* pilus subunit.**

   a. **Detection of co-transcription of *piliX* with *piliABC* by RT-PCR.** As demonstrated by the electrophoresis results, bands were detected between *piliX*, *piliC*, *piliB*, *piliA*, and *srtC* (the gene encoding Sortase C), confirming the co-transcription of *piliX* with *piliABC* and *srtC* as an operon. DNA contamination was ruled out using PCR analysis on samples from the previous non-RT group.

   b. **Detection of PiliX in both cell wall and culture supernatant fractions.** Separate immunoblots 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. The high molecular weight ladder bands were clearly recognizable in both fractions of wild-type and revertant strains, indicating the presence of a pilus structure similar to that of other Gram-positive bacteria.

   c. **Detection of PiliX in the cell wall fraction of Δ*srtC* mutants.** Dedicated immunoblots using anti-PiliX serum were performed on cell wall extracts from wild-type, *srtC* deletion mutants and revertant strains. Those ladder bands were undetectable in those from the mutant strain lacking *srtC*, the gene that encodes the transpeptidase Sortase C involved in the pilus subunit linkage.

   d. **Effects of piliX deletion on the pilin detection pattern.** Immunoblots using anti-PiliA, B, and C and cell wall extracts from *piliX* deletion mutants, as well as wild-type and revertant strains, revealed that deletion of *piliX* altered the intensity and migration pattern of ladder bands, adding to the evidence that PiliX is a pilus subunit.

   e. **Detection of PiliX in cell wall fractions immunoprecipitated with anti-PiliA serum.** Immunoblots were performed on the fraction immunoprecipitated with anti-PiliA using antisera against PiliB, PiliC, or PiliX. The high molecular weight ladder bands were visible in all IP groups, but not in the non-IP control groups.

2. **The PiliX protein has an effect on the formation of biofilms.** Furthermore, *piliX* deletion compromised the ability of *S. sanguinis* to form biofilm. PiliX deletion mutants were less capable of forming biofilms in the presence of saliva and glucose, implying that PiliX protein promoted biofilm formation.

Conclusions and Discussion

Our findings indicate that PiliX is a novel pilus subunit of the *S. sanguinis* pili. Additionally, PiliX has an effect on biofilm formation, particularly when saliva and glucose are present, suggesting that PiliX may potentiate the biofilm formation activity of *S. sanguinis* in the oral cavity.

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論文審査の結果の要旨及び担当者

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論文審査の結果の要旨

本研究は，感染性心内膜炎の起因菌である *Streptococcus sanguinis* の新規線毛サブユニットタンパク質 PiliX を明らかにし，PiliX が線毛形成とバイオフィルム形成に果たす役割を解析したものである．

野生株の細胞壁画分を用いたウエスタンブロット解析により，PiliX は高分子ラダーバンドとして検出され，線毛特異的に機能するトランスペプチダーゼの遺伝子欠失により高分子ラダーバンドは消失した．RT-PCR 解析において，*piliX* は線毛関連遺伝子群とともに転写されることを確認した．野生株の細胞壁画分を抗 PiliA 抗血清により免疫沈降した検体では，抗 PiliB 抗血清，抗 PiliC 抗血清，および抗 PiliX 抗血清と反応する高分子ラダーバンドを認めた．これらの結果から，PiliX は既知の線毛サブユニットと連結する新規の線毛サブユニットであることが示された．また，*S. sanguinis* のバイオフィルム形成能は *piliX* 欠失により低下したことから，PiliX はバイオフィルム形成を促進することが示唆された．

以上より，本研究は博士（歯学）の学位に値するものと認める．