

Original article

Role of *Streptococcus sanguinis* sortase A in bacterial colonization

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Received 3 March 2006; accepted 23 August 2006

Available online 14 September 2006

Abstract

Streptococcus sanguinis, a normal inhabitant of the human oral cavity, has low cariogenicity, though colonization on tooth surfaces by this bacterium initiates aggregation by other oral bacteria and maturation of dental plaque. Additionally, *S. sanguinis* is frequently isolated from infective endocarditis patients. We investigated the functions of sortase A (SrtA), which cleaves LPXTG-containing proteins and anchors them to the bacterial cell wall, as a possible virulence factor of *S. sanguinis*.

We identified the *srtA* gene of *S. sanguinis* by searching a homologous gene of *Streptococcus mutans* in genome databases. Next, we constructed an *srtA*-deficient mutant strain of *S. sanguinis* by insertional inactivation and compared it to the wild type strain.

In the case of the mutant strain, some surface proteins could not anchor to the cell wall and were partially released into the culture supernatant. Furthermore, adherence to saliva-coated hydroxyapatite beads and polystyrene plates, as well as adherence to and invasion of human epithelial cells were reduced significantly in the *srtA*-deficient strain when compared to the wild type. In addition, antiopsonization levels and bacterial survival of the *srtA*-deficient mutant were decreased in human whole blood.

This is the first known study to report that SrtA contributes to antiopsonization in streptococci. Our results suggest that SrtA anchors surface adhesins as well as some proteins that function as antiopsonic molecules as a means of evading the human immune system. Furthermore, they demonstrate that SrtA of *S. sanguinis* plays important roles in bacterial colonization.

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Keywords: *Streptococcus sanguinis*; LPXTG motif; Sortase; Surface protein; Endocarditis

1. Introduction

Streptococcus sanguinis (formerly *S. sanguis*) is a member of the viridans group of streptococci [1]. It is the first bacterium to colonize tooth surfaces, where it functions as a 'pioneer' by forming dental plaque, which leads to dental caries, periodontal diseases, and alterations of dental restorations [2]. *S. sanguinis* has been reported to be closely related to infective endocarditis [3,4], which is frequently caused by oral bacteria entering the bloodstream following trauma.

Some bacterial surface proteins play important roles in the adherence to and invasion of human tissues. Surface proteins of Gram-positive bacteria are digested with sortase A (SrtA) at the recognized sequence, LPXTG, and become anchored to the cell wall [5]. SrtA is a broad-range enzyme essential for anchoring the majority of LPXTG-containing proteins of Gram-positive bacteria [6], and inactivation of the *srtA* gene in *Streptococcus gordonii* and *Streptococcus pneumoniae* has been reported to cause multiple defects in their pathogenesis [6,7].

In the present study, we investigated the roles of SrtA in the pathogenesis of *S. sanguinis* by comparing the biological characteristics of an *srtA*-deficient mutant strain with those of the wild type in regard to colonization.

Abbreviation: SrtA, Sortase A.

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2. Materials and methods

2.1. Bacterial strains, eukaryotic cells, and growth conditions

S. sanguinis strain ATCC 10556 (wild type) was purchased from American Type Culture Collection and *Streptococcus mutans* strain MT8148 was isolated at Osaka University Dental Hospital. Both strains were cultured in Todd-Hewitt (Becton Dickinson) broth supplemented with 0.2% yeast extract (THY). HEp-2 cells (ATCC CCL23) were cultured in Dulbecco's modified Eagle medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (Invitrogen).

2.2. Identification of *srtA* gene in *S. sanguinis* genome database

The complete genome sequence of *S. sanguinis* strain SK23 was obtained from the Virginia Commonwealth University Genome database (<http://www.sanguinis.mic.vcu.edu/>), while the nucleotide sequence of the *srtA* gene of *S. mutans* strain UA159 was downloaded from the Streptococcal Genome Sequencing Project at the University of Oklahoma (<http://www.genome.ou.edu/smutans.html>). The open reading frame (ORF) was identified using GeneWorks software ver. 2.4 (IntelliGenetics Inc.) and analyzed with a BLAST search.

2.3. Construction of *srtA*-deficient mutant strain

The sequences (5' to 3') of the oligonucleotides used for construction of an *srtA*-deficient mutant strain were as follows: *srtA*_{fw}, GCGCAAATCCGAAACATGATTATGG; and *srtA*_{rv}, CCTCAATCAGATCCGTTTCGATCTGG. The amplified PCR product was ligated into the pSF151 vector [8] and the resultant plasmid, pTO7, was transformed into strain ATCC 10556 by electroporation, as described previously [9]. The *srtA*-deficient strain, TR-56, was selected by plating on THY agar media containing kanamycin and confirmed by colony-directed PCR assays, as described previously [10]. In addition, the glucosyltransferase (*gtfP*) and IgA1 protease (*iga*) genes of *S. sanguinis* were used as positive controls. The *gtfP* gene specific primer pairs were as follows: *gtfP*_{fw}, GCTAACTCAGCTTCTCAGTTGGAAG; and *gtfP*_{rv}, CAACCAAGGCTCCGGTCTCTGCATCG. The *iga* gene specific primer pairs were as follows: *IgA*_{fw}, GCCTTCACCAGGCGAAATCTCAGG; and *IgA*_{rv}, GGCTTAGCTACTCGGCCAGATAAG.

2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Cell-associated proteins from *S. sanguinis* were extracted using an 8 M urea solution [11]. The culture supernatant was precipitated with saturated ammonium sulfate and dialyzed against phosphate buffered saline (PBS), and the samples were separated using electrophoresis on a 10% SDS-PAGE gel and visualized with a silver staining kit (Amersham Biosciences).

2.5. Determination of cell-surface hydrophobicity

Surface hydrophobicity was determined as described previously [12]. Briefly, lyophilized bacterial cells were suspended in PUM buffer [100 mM sodium phosphate buffer, pH 7.1; 30 mM urea; 0.8 mM MgSO₄] at a final concentration of 0.6 mg/ml and mixed with *n*-hexadecane. Surface hydrophobicity was determined by adding *n*-hexadecane. Following vigorous shaking, optical densities of the aqueous phases were measured at 550 nm (*A*₅₅₀).

2.6. Preparation of saliva-coated hydroxyapatite beads

We prepared saliva-coated hydroxyapatite beads for bacterial adhesion assays as follows. Human saliva samples were collected from 2 healthy volunteers and incubated at 65 °C for 30 min, then centrifuged for 5 min at 5000 × *g* at 4 °C. The resultant supernatants were passed through 0.45-µm filters and incubated with hydroxyapatite beads (Bio Rad), after which saliva-coated hydroxyapatite adhesion assays were performed as described previously [13]. *S. sanguinis* was incubated with saliva-coated hydroxyapatite beads for 1 h. After washing with PBS, each mixture was diluted and plated to count the numbers of adhered bacteria.

2.7. Assay of adhesion to polystyrene plate

Bacterial adhesion to a polystyrene plate was determined as described previously [14]. *S. sanguinis* was incubated in a microtiter plate for 0 to 180 min. After washing with PBS, adhered bacteria were stained with phenol gentian violet and optical density was measured at *A*₆₀₀.

2.8. Bacterial cell adhesion and invasion

Adhesion to and invasion of human epithelial cells were quantified using standard procedures described previously [10,15]. Briefly, cells were infected at a multiplicity of infection of 50 bacteria per cell and incubated for 3 h. To determine the number of adhered *S. sanguinis* organisms, the cells were lysed with distilled water and plated. To count the number of invaded organisms, epithelial cells were treated with a gentamicin and penicillin solution to kill the non-invaded bacteria prior to lysing the cells. Ligand blotting with human fibronectin (Fn) was performed as described previously [10,16].

2.9. Opsonophagocytosis and bactericidal assays

Opsonization and bactericidal assays were performed as described previously [17]. Heparinized whole blood was mixed and incubated with the bacteria, then the percentage of bacteria-associated neutrophils was determined under a microscope after staining the neutrophils with Giemsa solution. To determine the numbers of colony forming units (CFU) of bacteria that had evaded phagocytosis, a mixture of blood with *S. sanguinis* was plated after incubation for 1 h.

2.10. Statistics

A Mann–Whitney *U*-test was used to compare the differences between the *S. sanguinis* wild type and *srtA*-deficient mutant strains. Each assay was repeated at least 3 times. A *P* value of less than 0.05 was considered statistically significant.

3. Results

3.1. Nucleotide sequence of the *srtA* gene of *S. sanguinis*

We obtained a sortase (Srt) homologue of *S. mutans* UA159 using a BLAST search of *S. sanguinis* strain SK36 in the Virginia Commonwealth University *Streptococcus sanguinis* Genome Sequencing Project database. Next, we determined the complete *srtA* sequence of strain ATCC 10556 and deposited it in GenBank (accession no. AB104652.2). The ORF starts with an ATG codon and terminates with a TAA codon. The *srtA* gene was found to consist of 759 nucleotides and encode a protein of 252 amino acids, with a calculated molecular mass of 28.0 k. The homologue of *S. sanguinis* had a 55% identity and 73% positive correlation with the SrtA amino acid sequence of *S. mutans* UA159. In addition, the C-terminal TLXTC motif [18] was found to be conserved among *S. mutans* UA159, *S. sanguinis* SK36, and *S. sanguinis* ATCC 10556.

3.2. Differential analysis of surface proteins between *srtA*-deficient mutant and wild type strains

To investigate the role of SrtA in the virulence of *S. sanguinis*, we constructed an *srtA*-deficient mutant strain, TR-56, by insertional inactivation (Figs. 1A,B) and used a differential display approach to demonstrate the enzymatic activity of SrtA against cell-associated proteins. In a comparison of strains ATCC 10556 and TR-56, at least 6 proteins were released from the 8 M urea extracted fraction of the *srtA*-deficient mutant into the culture supernatant (Fig. 1C). These findings suggested that a lack of SrtA causes *S. sanguinis* cells to lose virulence, which is mediated by cell surface proteins. As a result, we investigated the differences in biological functions between the wild type and *srtA*-deficient strains.

S. sanguinis ATCC 10556 was found to be highly hydrophobic, as it showed 72% adhesion to *n*-hexadecane (Table 1), while strain TR-56 showed a hydrophobicity of 53%, which was significantly different ($P < 0.05$). The hydrophobic strain *S. mutans* MT8148, used as a positive control, showed a hydrophobicity of 61%.

3.3. *SrtA* contributes to various pathogenesises of *S. sanguinis*

We attempted to determine whether there was a defect in the ability of the *srtA*-deficient mutant to adhere to saliva-coated hydroxyapatite beads and the surface of a polystyrene plate. As shown in Fig. 2A, the adherence to saliva-coated

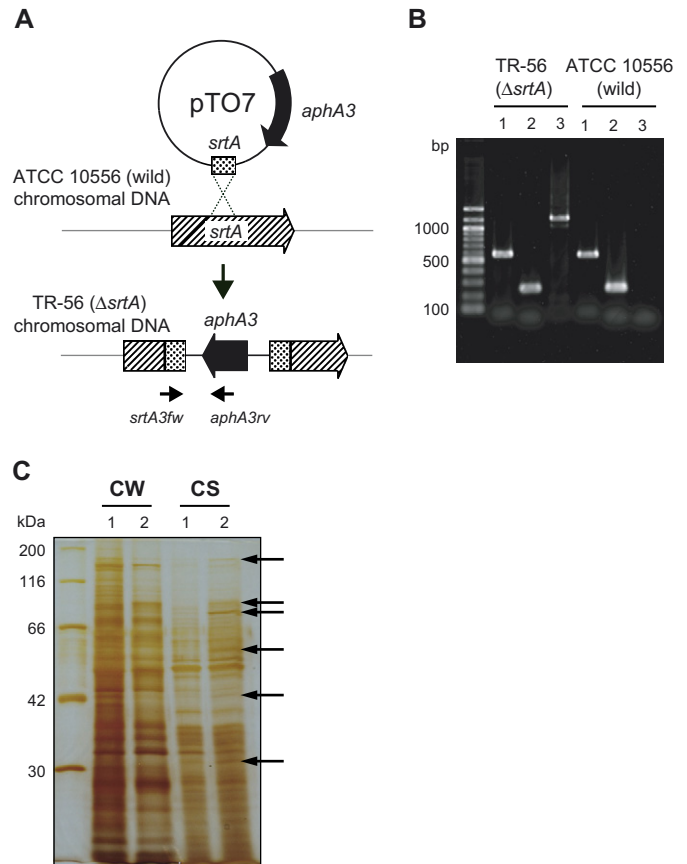


Fig. 1. Differential analysis between wild type *S. sanguinis* and the *srtA*-deficient mutant. A) Targeted mutagenesis of the *srtA* gene in *S. sanguinis* strain ATCC 10556. pTO7 harbored an internal fragment of the *srtA* and *aphA3* genes. B) To confirm mutagenesis, strains ATCC 10556 and TR-56 were subjected to PCR with the following primers. Lane 1: *gtfP* gene specific primer pairs. Lane 2: *iga* gene specific primer pairs used as a positive control. Lane 3: *srtA* gene-specific forward primer and *aphA3* gene specific reverse primer. C) Comparison of protein expressions of the wild type and *srtA*-deficient mutant. Streptococcal cell wall proteins (CW) extracted with 8 M urea and culture supernatant proteins (CS) were separated on SDS-PAGE gels. Lane 1: wild type strain ATCC 10556. Lane 2: *srtA*-deficient strain TR-56. Following electrophoresis, proteins were detected with silver staining. Arrows indicate cell surface differences between strains ATCC 10556 and TR-56. The indicated proteins were detected in the culture supernatants.

hydroxyapatite beads by strain TR-56 was reduced significantly as compared to that by the wild type strain. Further, the wild type strain showed a time-dependent increase in adherence to the polystyrene plate, as detected by crystal violet

Table 1
Hydrophobicity of bacterial strains used in this study

Strains	Relevant characteristics	Hydrophobicity (%)
<i>S. sanguinis</i>		
ATCC 10556	wild type	71.6 ± 3.3
TR-56	$\Delta srtA::aphA3$	52.7 ± 4.0*
<i>S. mutans</i>		
MT8148	wild type	60.6 ± 2.2

Percent hydrophobicity was calculated as follows: $[(A_{550} \text{ without } n\text{-hexadecane}) - (A_{550} \text{ with } n\text{-hexadecane})] / (A_{550} \text{ without } n\text{-hexadecane}) \times 100$. The data are presented as the means ± SE of 6 tubes from 1 representative experiment.

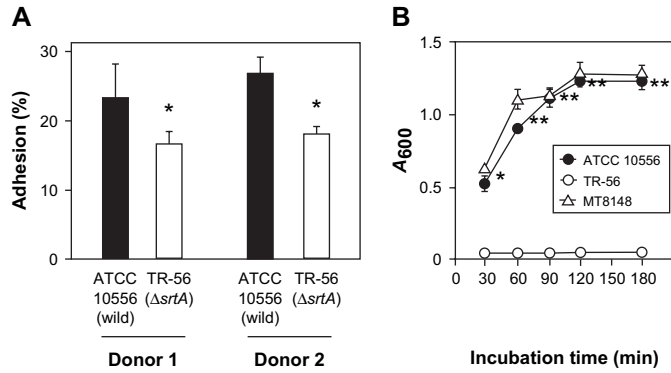


Fig. 2. Adhesion of *S. sanguinis* wild type strain ATCC 10556 and *srtA*-deficient strain TR-56. A) Adhesion to saliva coated hydroxyapatite beads. Percent adhesion to saliva coated hydroxyapatite beads was calculated as (CFU adhered/CFU in the inoculum) \times 100. B) Bacterial adhesion to and growth on polystyrene plates. Growth was quantified at A_{600} with phenol gentian violet staining. Data are presented as the means \pm SE of 6 wells from a representative experiment. * $P < 0.01$, ** $P < 0.005$ (wild type strain ATCC 10556 vs. *srtA*-deficient strain TR-56).

staining after 120 min. In contrast, the TR-56 strain showed a significantly decreased ability to adhere to the polystyrene plate and the titer of TR-56 expressed relative to the baseline of wild-type throughout the experiment (Fig. 2B). Further, to determine the role of SrtA in the interaction with human epithelial HEP-2 cells, *S. sanguinis* adhesion and invasion assays were performed with the *srtA*-deficient mutant strain TR-56. The abilities of strain TR-56 to adhere to HEP-2 cells was reduced by approximately 5.6% as compared to the wild type strain ATCC 10556 ($P < 0.005$) (Fig. 3A) and the invasion efficiency of HEP-2 cells was reduced by approximately 54.6% ($P < 0.01$) (Fig. 3B). These findings suggest that SrtA in *S. sanguinis* participates in anchoring adhesin proteins on the bacterial surface, though the process of bacterial invasion is due to other surface proteins. Since most adhesins and invasins of Gram-positive cocci possess Fn-binding activities, we also examined whether *S. sanguinis* strain ATCC 10556 expressed Fn-binding proteins. A ligand blot analysis using biotinylated-Fn demonstrated that the wild type strain did not express an Fn-binding protein on the surface (Fig. 3C). From these results, we speculated that SrtA mainly has an effect on the adhesion of *S. sanguinis* to epithelial cells and that adhesin molecules do not possess Fn-dependent invasion activities.

Finally, we examined whether SrtA helps *S. sanguinis* escape from phagocytosis, following bacterial internalization by the host. The role of SrtA in phagocytosis resistance was investigated using a bactericidal test, and the wild type and *srtA*-deficient mutant strains were analyzed for their abilities to grow in human whole blood. The antiopsonization and bacterial growth activities of the *srtA* mutant strain TR-56 were found to be reduced in human whole blood as compared to the wild type strain ATCC 10556 (Fig. 4), indicating that the phagocytic capacity of human whole blood enables a rapid clearance of infection by the *srtA*-deficient mutant.

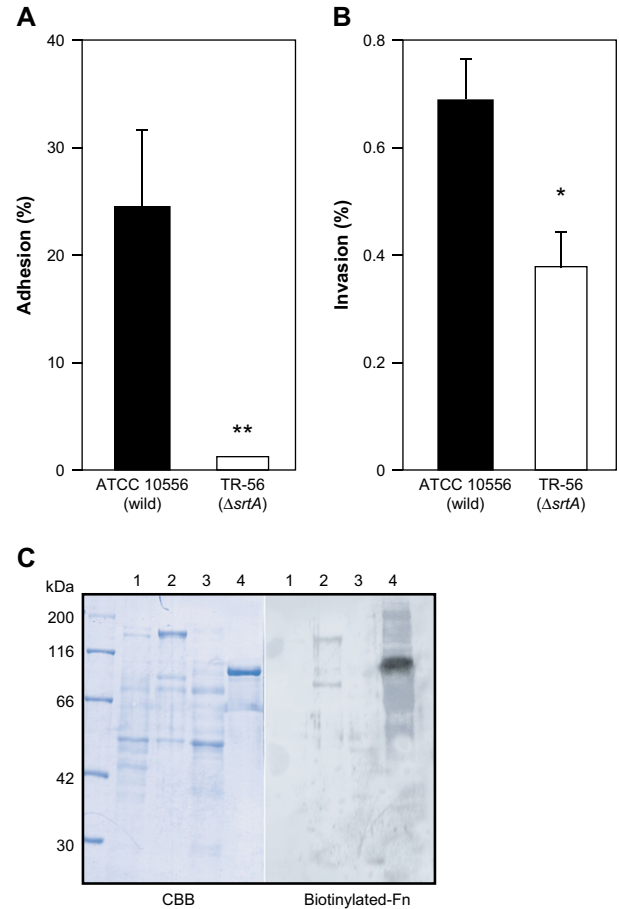


Fig. 3. Bacterial adhesion to and invasion of HEP-2 cells. Closed and open bars indicate the wild type strain ATCC 10556 and *srtA*-deficient strain TR-56, respectively. A) Adhesion assay. B) Invasion assay. Percentages in each assay were calculated as (CFU of adhered or invaded/CFU in the inoculum) \times 100. The experiments were performed 3 times independently. Data are presented as the means \pm SE of 12 wells from a representative experiment. * $P < 0.01$, ** $P < 0.005$ (wild strain ATCC 10556 vs. *srtA*-deficient strain TR-56). C) Fn-binding analysis of streptococcal surface proteins. Surface proteins from streptococci were extracted with 8 M urea, separated by SDS-PAGE, and transferred to a membrane. Biotinylated Fn-reactive proteins were detected with ECL reagents. Lane 1: *S. sanguinis* strain ATCC 10556. Lane 2: *Streptococcus gordonii* strain ATCC 10558. Lane 3: *Streptococcus oralis* strain ATCC 10557. Lane 4: *S. pyogenes* strain SSI-1. Strain SSI-1 is known to express the Fn-binding protein FbaB [16].

4. Discussion

The original sortase gene was identified in *Staphylococcus aureus* as *srtA* [19], after which several *srtA* genes were reported in studies of Gram-positive cocci [6,7]. More than one sortase gene (*srtB*, *srtC*, or *srtD*) has been found in the genome of nearly all streptococcal and staphylococcal bacteria [7], however, *S. sanguinis* possesses only a single sortase gene in its genome. Thus, we considered that this bacterium may be a simple and clear target for investigation of the functions of sortase in bacterial pathogenesis by using a sortase-deficient mutant strain.

An 8 M urea extraction method was originally used to isolate the cell-associated glucosyltransferase of *S. mutans*, and

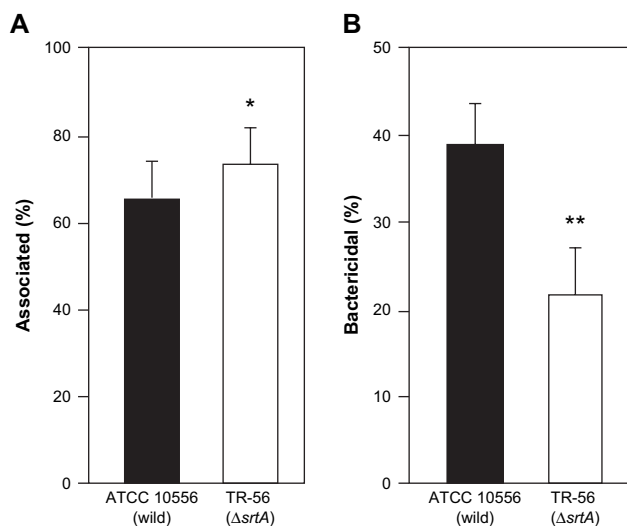


Fig. 4. Effects of *S. sanguinis* SrtA in opsonization and bactericidal assays. Closed and open bars indicate the wild type strain ATCC 10556 and *srtA*-deficient strain TR-56, respectively. A) Percentage of phagocytosed neutrophils observed under a microscope. B) Percentage of CFU following 3 h of incubation at 37 °C in heparinized whole blood. The experiments were performed 3 times independently. The data are presented as the means \pm SE of 6 tubes from a representative experiment. * $P < 0.05$, ** $P < 0.01$ (wild strain ATCC 10556 vs. the *srtA*-deficient strain TR-56).

has also been shown to be a simple and reliable means of extracting surface-associated proteins from various streptococci, such as FbaA, Lbp, and FbaB of *Streptococcus pyogenes* [10,16,20]. The present findings showed that SrtA of *S. sanguinis* was catalyzed to anchor more than 6 surface proteins in the cell wall, suggesting that *S. sanguinis* cells lacking SrtA activity are associated with virulence loss, which is mediated by cell surface proteins.

A significant correlation between bacterial adhesion and cell surface hydrophobicity has been shown [21]. Oral streptococci colonize the smooth surfaces of teeth using hydrophobic interactions, with such hydrophobic bonding recognized as a principal etiological process associated with the formation of dental plaque. Since a deficiency of the *srtA* gene results in a decrease in *S. sanguinis* hydrophobicity, SrtA might be involved in bacterial adhesion to teeth, restorative dental materials, and epithelial cells in the oral cavity. It was previously reported that the *srtA*-deficient *S. mutans* showed a decreased ability of colonization on oral mucosa and teeth in rats [22]. Ke Gong et al. demonstrated that *S. sanguinis* expresses 100/130/170-kDa homologues of P1/Pac [23], which is a major adhesin protein of *S. mutans* [21]. In addition, anti-sera to 100/130/170-kDa proteins inhibited *S. sanguinis* adhesion to saliva-coated hydroxyapatite beads in a dose-dependent manner [23]. Furthermore, P1/Pac of *S. mutans* contains the LPXTG motif and is anchored to the cell wall along with SrtA [22]. Those findings correlate well with our results showing that the *srtA*-deficient mutant lacked the 100/130/170-kDa proteins on the surface (Fig. 1C). Therefore, it is strongly suggested that cell wall anchoring by SrtA-dependent proteins containing

the LPXTG motif is required for colonization of *S. sanguinis*.

Many different types of bacteria adhere to and invade host epithelial cells through their surface proteins, including FbaA, Lbp, and FbaB of *S. pyogenes* [10,16,20]. Our data suggest that SrtA of *S. sanguinis* anchors one or more surface proteins associated with adhesion to and invasion of epithelial cells. Additionally, it is speculated that the missing proteins have a greater relationship with bacterial adhesion than bacterial invasion (Figs. 3A,B). In *S. pneumoniae*, inactivation of *srtA* reduced both adhesion to and invasion of epithelial cells [24]. The difference regarding bacterial invasion might be due to the Fn-binding proteins, as they function as both adhesion and invasion factors [10,16,25]. *S. pneumoniae* possesses an Fn-binding protein on the surface that acts as an adhesin and an invasin [25], whereas *S. sanguinis* strain ATCC 10556 did not express Fn-binding proteins in the present study (Fig. 3C). SrtA of *S. agalactiae* is also involved in anchoring the surface protein ScpB, which is required for binding Fn and fibrinogen [26]. Our findings suggest that *S. sanguinis* possesses at least one adhesin that works through an Fn-independent pathway and is anchored to the cell surface along with SrtA. In addition, we speculated that the loss of some proteins, catalyzed by SrtA, from the surface of *S. sanguinis* led to exposure of invasin molecules not anchored by SrtA on the bacterial surface, thereby increasing the invasion activity of strain TR-56 (Fig. 3B).

The results of the present *in vitro* opsonization and bactericidal assays suggest that SrtA of *S. sanguinis* may link with one or more surface proteins related to antiopsonization, while it also plays an essential role in evasion from the human immune system by the organism. We concluded that LPXTG-containing surface proteins, which are anchored with SrtA, play multiple roles in bacterial colonization and survival.

In summary, SrtA of *S. sanguinis* was shown to have an influence on the expression of various cell surface virulence factors closely related to bacterial endocarditis, including adhesins and antiopsonic proteins. The surface proteins of *S. sanguinis* have not been sufficiently studied in comparison to those of other cariogenic oral streptococci, thus we were unable to select a candidate vaccine against the organism. However, our results demonstrated that SrtA of *S. sanguinis* is an important molecule for infection and colonization, and may be a reasonable target to prevent bacterial infection and disease progression.

Acknowledgements

This research was supported in part by a Grant from the 21st century COE program, Grants-in-Aid for Scientific Research on Priority Areas and Young Scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology, a grant from Grants-in-Aid for Scientific Research (B) from the Japan Society for the Promotion of Science, and the Research Grant for Longevity Sciences (17C-5) from the Ministry of Health, Labour and Welfare.

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