Streptococcus mutans sortase catalyzes cell wall anchoring of WapA and FruA

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Abstract

Previous studies using a sortase-deficient mutant (SrtA⁻ mutant) of Streptococcus mutans have demonstrated that the sortase (SrtA) catalyzes cell wall anchoring of the surface protein antigen PAc, a dextranase and a glucan-binding protein C. In this study, cell wall anchoring of a wall-associated protein antigen A (WapA) and an exo-β-D-fructosidase (FruA) in S. mutans was examined by Western blot analysis with a specific antiserum. In the SrtA⁻ mutant, FruA and WapA were not bound to the cell wall but were secreted into the culture supernatant. In contrast, in the wild type, both proteins were associated with the cell wall of S. mutans. Biological properties of the SrtA⁻ mutant were examined by determination of fructan fermentation and adherence to a smooth surface. Both the SrtA⁻ mutant and the wild type retained the ability to ferment levan. In addition, adherence to a smooth surface of the SrtA⁻ mutant was as extensive as that of the wild-type 109c when sucrose was present. However, in the absence of sucrose, the adhesion of the SrtA⁻ mutant remarkably decreased as compared with that of the wild type. These results suggest that SrtA catalyzes anchoring of WapA and FruA to the cell wall in S. mutans and that surface proteins anchored by SrtA are involved in the initial adhesion of S. mutans to smooth surface. In addition, it was shown that both cell wall-anchored and extracellular FruA are related to the degradation of extracellular fructan as a nutrient source.

Introduction

Streptococcus mutans is a gram-positive oral bacterium and the primary etiologic agent of human dental caries. Formation of plaque biofilm on the tooth surface by this organism is an important biological step in the progression of dental caries, and many factors responsible for biofilm formation have been reported. Proteins that S. mutans displays on the cell surface contribute to biofilm formation, and some of these surface proteins possess a C-terminal sorting signal, which consists of a conserved LPXTG motif, a hydrophobic domain, and a positively charged tail.

A series of studies by Schneewind and colleagues identified a transpeptidase designated sortase (SrtA) in Staphylococcus aureus and demonstrated that SrtA is involved in anchoring of some proteins with a sorting signal to the S. aureus cell wall and plays an important role in the virulence of S. aureus. SrtA and surface proteins with the sorting signal have been found in many gram-positive bacteria such as S. aureus, S. mutans, Streptococcus pyogenes, Streptococcus suis, Streptococcus gordonii, Listeria monocytogenes and others, and play important roles in the pathogenesis of gram-positive bacterial infection. In S. mutans, it is known that at least five different proteins possess a C-terminal sorting signal: surface protein antigen (PAc), glucan-binding...
protein C (GbpC), dextranase (Dex), wall-associated protein antigen A (WapA) and exo-β-D-fructosidase (FruA)\textsuperscript{16–19}. In our recent studies, we have determined the nucleotide sequence of the \textit{srtA} gene of \textit{S. mutans} GS5, have demonstrated that \textit{S. mutans} SrtA plays a crucial role in cell wall-anchoring of PAc, GbpC and Dex, and that have shown cariogenic properties mediated by these surface proteins are lost by insertional inactivation of the \textit{srtA} gene of \textit{S. mutans}\textsuperscript{12,13,20}. Sequence analysis of the \textit{wapA} and \textit{fruA} genes revealed the sorting signal at the C terminus of the proteins\textsuperscript{16,17}. This structural feature strongly suggests that WapA and FruA are cell wall-anchored proteins. However, the mechanism of cell wall-anchoring of WapA and FruA has not been determined.

In this study, we examined cell wall anchoring of \textit{S. mutans} WapA and FruA by SrtA as well as the biological properties of the \textit{S. mutans} SrtA\textsuperscript{−} mutant.

### Materials and methods

#### Bacterial strains

\textit{Streptococcus mutans} 109c and mutants were grown in Todd Hewitt broth (TH broth; Difco Laboratories, Detroit, MI)\textsuperscript{12}. When needed, erythromycin was added to the medium at 10\textmu g/ml. \textit{Escherichia coli} JM109 was routinely used as a plasmid host and cultured in Luria-Bertani broth.

#### DNA extraction

Genomic DNA of \textit{S. mutans} was purified by ultracentrifugation in a CsCl-ethidium bromide density gradient as described previously\textsuperscript{4}. Plasmid was extracted by a Wizard miniprep purification kit (Promega, Madison, WI).

#### Construction of \textit{S. mutans} mutants

SrtA\textsuperscript{−} and Dex\textsuperscript{−} mutants of \textit{S. mutans} 109c were constructed previously by insertional inactivation of the \textit{srtA} and \textit{dex} genes\textsuperscript{12,20}. WapA\textsuperscript{−}, FruA\textsuperscript{−}, PAc\textsuperscript{−} and GbpC\textsuperscript{−} mutants of \textit{S. mutans} 109c were constructed by insertional inactivation of the respective genes (Fig. 1). Briefly, internal portion of the respective genes PCR-amplified with the primer pair (shown in Table 1) were cloned into a pUCE vector composed of a pUC19 vector with an erythromycin-resistant gene\textsuperscript{12}. The nucleotide sequences of PCR primers used in this study are listed in Table 1. The recombinant plasmid was introduced into \textit{S. mutans} 109c cells\textsuperscript{21}, and mutants which acquired erythromycin-resistance were generated and selected on TH agar containing erythromycin. Recombination between the internal portion and the homologous
gene resulted in insertional inactivation of the gene. Inactivation of the respective genes of *S. mutans* 109c was confirmed by PCR and Southern hybridization analyses (data not shown). PCR was performed under conditions described previously4).

**Western blotting**

Whole cells (30 mg of the cell pellet) were suspended in 600 µl of 1% sodium dodecyl sulfate-1% 2-mercaptoethanol, heated at 100°C for 5 min and centrifuged as reported previously12,22). The resulting supernatant was used as the cell extract. The culture supernatant was adjusted to a final OD280 of 15. The cell extract (15 µl for each sample) and the culture supernatant (30 µl for each sample) were analyzed by SDS-PAGE and Western blotting with anti-WapA or anti-FruA serum.

**Fructan fermentation**

The ability of levan fermentation of *S. mutans* strains was tested by a modified version of the method described by Colby *et al.*20,23) Briefly, cells from overnight TH cultures were washed twice with sterile saline and added to TH broth with or without 0.5% sucrose. After overnight incubation at 37°C, the pH of the culture supernatant was measured.

**Results**

To examine whether WapA and FruA were anchored to the cell wall of *S. mutans* by SrtA, protein profiles of the culture supernatants and cell extracts of the wild-type 109c and the SrtA− mutant were compared. In protein staining with Coomassie blue, the protein bands corresponding to the expected molecular sizes of WapA (29 kDa) and FruA (150 kDa) were not visualized for any fractions (data not shown).

### Table 1 Plasmids and PCR primers

<table>
<thead>
<tr>
<th>Plasmid or primer</th>
<th>Relevant marker(s) or sequence (5’→3’)</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td>pT7Blue T</td>
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<td>Novagen</td>
</tr>
<tr>
<td>pUCE</td>
<td>pUC19 vector containing Em′</td>
<td>12</td>
</tr>
<tr>
<td>pUE</td>
<td>pUCE carrying fragment amplified with PCR</td>
<td>12</td>
</tr>
<tr>
<td>PCR primers</td>
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<td>12</td>
</tr>
<tr>
<td>srtA 266R</td>
<td>5′-AATTGAATTCTCACTACGTGTTGACGAGCAGC-3′</td>
<td>12</td>
</tr>
<tr>
<td>Dex 1131F</td>
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<td>20</td>
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<tr>
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---: BamHI site; : EcoRI site
However, Western immunoblots with anti-WapA or anti-FruA serum showed that WapA (29 kDa) and the FruA (150 kDa) from the SrtA*/H11502 mutant were present in the culture supernatant (Fig. 2A and B, lane 2). In contrast, the WapA (72 and 29 kDa) and FruA (150 kDa) produced by wild-type 109c were associated with the cell surface, but not detected in the culture supernatant (Fig. 2A and B, lanes 1 and 4). Anti-WapA serum detected a 72 kDa band in the cell extracts of both the wild type and the SrtA*/H11502 mutant (Fig. 2A, lanes 4 and 5). In contrast, the 29 kDa and 72 kDa bands were detected in neither the culture supernatant nor the cell extract of the *S. mutans* WapA* mutant (Fig. 2A, lanes 3 and 6).

The role of SrtA in modulating the cell surface properties of *S. mutans* was investigated in levan fermentation and adherence to a smooth surface. In levan fermentation, acid production from levan was compared among wild-type 109c and the mutants of SrtA* and FruA*. When grown in TH broth containing levan, the SrtA* mutant and wild-type 109c were able to ferment the levan, but the FruA* mutant failed to ferment levan (Fig. 3).

When *S. mutans* cells were assayed for adherence to the surface of a plastic plate in the presence of sucrose, the SrtA* mutant and wild-type 109c adhered to the plate (Fig. 4, upper wells). Mutants such as WapA*, PAc*, FruA*, GbpC* and Dex* behaved similarly to wild-type 109c. However, in the absence of sucrose, a striking difference between the SrtA* mutant and the wild-type 109c was observed. The SrtA* mutant was nonadherent, and markedly less adhesive than the wild-type 109c and mutants such as WapA*, PAc*, FruA*, GbpC* and Dex* (Fig. 4, lower wells).

**Discussion**

In Western blot analysis with anti-FruA serum, the SrtA* mutant resulted in the complete loss of surface attachment of FruA (150 kDa), with appearance in the culture supernatant (Fig. 2B, lanes 2 and 4). The FruA* mutant failed to produce any immunoreactive proteins (Fig. 2B, lanes 3 and 6), confirming that the 150 kDa protein of detected by anti-FruA serum was derived from the *fruA* gene. This finding indicates that the FruA is a surface protein linked to the cell wall by SrtA in *S. mutans*. Burne et al. have reported many findings on cell localization of the *S. mutans* FruA*.

Fig. 2 Detection of cell wall-anchored proteins in *S. mutans* Cell extracts and culture supernatants were prepared from *S. mutans* cells cultured in Todd Hewitt broth and were subjected to SDS-PAGE. Equal amounts of proteins were added to each lane (see Materials and methods). Western blot analysis with (A) anti-WapA serum and (B) anti-FruA serum. Lanes 1 and 4, wild-type strain 109c; lanes 2 and 5, SrtA* mutant; lanes 3 and 6, WapA* mutant or FruA* mutant.

Fig. 3 Levan fermentation of *S. mutans* *S. mutans* 109c, the SrtA* mutant and the FruA* mutant were grown in Todd Hewitt broth (solid bars) or Todd Hewitt broth with 0.5% levan (hashed bars). The pH of the culture was measured after 18h.
over 95%) of the FruA is found in the culture supernatant when S. mutans is grown at a pH of 7.0 in chemostat, whereas approximately half of it is associated with the cell surface when S. mutans is grown at a pH of 5.0. They also demonstrated that release of FruA into culture supernatant is inhibited by copper. From the studies of Burne et al. and the present study, it is speculated that S. mutans FruA is initially linked to the cell wall by SrtA and then released into culture supernatant by proteolytic degradation. Growth pH and copper might affect the enzymatic activity of protease or SrtA, resulting in alteration of FruA localization in S. mutans. Further study is required to clarify this phenomenon.

FruA is known as an enzyme that degrades polysaccharides such as levan and inulin as well as sucrose and raffinose. It is suggested that a role of the S. mutans FruA in dental plaque is to provide a source of nutrient. In this study, both the SrtA− mutant and wild-type 109c retained the ability to ferment levan. In contrast, the FruA− mutant did not ferment this polysaccharide (Fig. 3). These observations suggest that not only an extracellular but also a cell-bound FruA contribute to utilization of levan as a nutrient source in S. mutans. However, the importance of the surface-localized FruA in S. mutans cells has not been clarified.

Western blot analysis with anti-WapA serum detected the 72 and 29 kDa bands in the cell extract of wild-type 109c (Fig. 2A). Although the 29 kDa band was released into the culture supernatant by inactivation of the srtA gene, the 79 kDa band remained on the cell surface. In contrast, the WapA− mutant produced no protein bands reactive with anti-WapA serum, indicating that the 72 and 29 kDa bands were derived from the wapA gene. These results indicate that the WapA (72 and 29 kDa) are cell-associated in S. mutans cells; however, only cell-wall anchoring of the 29 kDa-WapA is catalyzed by SrtA, while that of the 79 kDa-WapA is not. Russell et al. and Ferretti et al. previously detected two bands of WapA with molecular sizes of 72 and 29 kDa in strain Ingritt of S. mutans and showed that the 72 kDa band was the wall-associated form while the 29 kDa band was almost exclusively in the culture supernatant. They reported that 72 kDa-WapA existed as a wall-associated form and was released as 29 kDa-WapA into culture supernatant, which is inconsistent with our present study in cell localization of 29 kDa-WapA. This inconsistency was resolved by our recent study of the srtA gene of S. mutans Ingritt. We found that strain Ingritt has a deletion in the srtA gene. This deletion generates a new termination codon, resulting in production of an incomplete SrtA enzyme protein. Thus, it was clarified that the SrtA of strain Ingritt does not function in the cell wall-anchoring process of surface proteins with a sorting signal. Therefore, 29 kDa-WapA of strain Ingritt was detected in the culture supernatant, as reported by Russell et al. and Ferretti et al. Although it was shown by using the WapA− mutant that the 72 kDa protein was derived from the wapA gene, cell localization of this protein was not controlled by SrtA (Fig. 2A, lanes 4 and 5).
This reason has not been clarified yet, however, we speculate that WapA (29 kDa) might be linked to some other wall component. Additional work is necessary to define how interactions occur between WapA (29 kDa) and other cell wall components.

The results of adherence assay indicated that the SrtA− mutant, as well as the S. mutans wild-type 109c and mutants such as WapA−, PAc−, Frua−, GbpC− and Dex−, adhered to the plastic plate when grown in the presence of sucrose (Fig. 4). A preliminary experiment we performed revealed that there was essentially no difference in expression of polysaccharide synthesis between the SrtA− mutant and wild-type 109c (data not shown), which could support the finding of sucrose-dependent adherence shown in Fig. 4 (upper wells). In contrast, in the absence of sucrose, a significant decrease in adherence to the plastic plate was observed for the SrtA− mutant compared with wild-type 109c and other mutants (Fig. 4, lower wells). It is clear that this phenomenon is due to a change in localization of surface proteins with a sorting signal, in particular WapA and PAc, in the SrtA− mutant, since previous genetic and immunological studies have shown that PAc and WapA play important roles in the initial adhesion of S. mutans to the tooth surfaces\textsuperscript{30–33}. In addition, immunological studies have shown that PAc and WapA could be useful antigens for an anti-caries vaccine, since anti-PAc or anti-WapA antibodies effectively block the initial adhesion of S. mutans to tooth surfaces\textsuperscript{30,31,34,35}. These observations suggest that change in localization of WapA and PAc due to inactivation of the srtA gene has protective effects.

Our recent studies have shown that the S. mutans SrtA is involved in cell wall anchoring of WapA, PAc, GbpC, Dex, and Frua (this study)\textsuperscript{12,13,20} and that the SrtA− mutant results in loss of multiple cariogenic properties mediated by these surface proteins of S. mutans, such as initial adhesion to the tooth surface, dextran-dependent aggregation and modulation of adhesive properties of extracellular glucan (Fig. 4)\textsuperscript{12,13,20}. These phenomena would be occurred not only by inactivation of the srtA gene but also by inhibition of the SrtA enzyme of S. mutans. Therefore, S. mutans SrtA could be a novel and attractive target for prevention of biofilm formation and subsequent dental caries.

References


15) Pallen, M.J., Lam, A.C., Antonio, M. and Dunbar,