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# Involvement of Sortase Anchoring of Cell Wall Proteins in Biofilm Formation by *Streptococcus mutans*

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***Streptococcus mutans*** **is one of the best-known biofilm-forming organisms associated with humans. We investigated the role of the sortase gene (*srtA*) in monospecies biofilm formation and observed that inactivation of *srtA* caused a decrease in biofilm formation. Genes encoding three putative sortase-dependent proteins were also found to be up-regulated in biofilms versus planktonic cells and mutations in these genes resulted in reduced biofilm biomass.**

*Streptococcus mutans* is considered a major causative agent of human dental caries, one of the most common infectious diseases that affect humans (32). This bacterium is also among the oral microorganisms that can cause infective endocarditis (9). Its main cariogenic virulence factors are its abilities to promote adhesion and accumulation on teeth, its acidogenicity, and its aciduricity (2, 6). The adherence of bacteria to dental surfaces is the first step in the development of the complex biofilm community that constitutes dental plaque (6). For *S. mutans*, adhesion can be mediated by either sucrose-dependent or sucrose-independent mechanisms. In the absence of sucrose, *S. mutans* expresses several surface adhesins which can bind to salivary components, such as those that form the acquired pellicle on the teeth (22).

In gram-positive bacteria, many surface proteins are covalently linked to the cell wall by a membrane-associated transpeptidase called sortase (24). Proteins for sortase-mediated cell wall anchoring contain several features that are essential for their localization. These features are located at the C terminus of the protein and include an LPXTG motif, followed by a hydrophobic region and a charged tail (7). Using *Staphylococcus aureus* protein A as a model, many steps in sortase-mediated cell wall anchoring have been elucidated. In a two-step transpeptidation reaction, the sortase cleaves the LPXTG motif between the threonine and glycine residues and the newly liberated carboxy terminus of threonine becomes anchored to cell wall peptidoglycan (21, 23). Bacteria frequently encode more than one sortase, and the number varies among organisms (25). Recently, Comfort and Clubb (4) performed a comparative analysis of 72 sequenced microbial genomes and showed that sortases could be divided into five distinct subfamilies based upon their primary sequences.

*S. mutans* possesses a single sortase which belongs to the SrtA subfamily (1, 4). The members of the SrtA subfamily are first distinguished by their genomic proximity to the *gyrA* gene encoding DNA gyrase subunit A. Moreover, genes encoding the SrtA-type enzyme are never proximal to genes encoding potential substrates (4). An analysis of the *S. mutans* UA159 genome indicates that it encodes six proteins containing the LPXTG motif located at the C terminus and followed by a hydrophobic region and a charged tail: the cell surface protein P1 (also known as antigen I/II, SpaP, and Pac), fructanase (FruA), wall-associated protein A (WapA), wall-associated protein E (WapE), glucan-binding protein C (GbpC), and dextranase (DexA) (1). The significance of sortase in the virulence of several gram-positive pathogens has become apparent in *Staphylococcus aureus*, *Listeria monocytogenes*, and *Streptococcus gordonii* and recently in *Streptococcus pneumoniae* (25). In *S. mutans*, a sortase mutant showed a decreased ability to colonize the oral mucosa and the teeth (17).

Many species of oral streptococci are known to form biofilms (14). Among the various streptococcal species, *S. mutans* is one of the best-known biofilm-forming organisms associated with humans. This bacterium has evolved a biofilm lifestyle for survival and persistence in its natural environment, dental plaque (14). To our knowledge, the role of streptococcal sortases in biofilm formation has never been investigated. In this study, we examined whether the *S. mutans* sortase is involved in biofilm formation. We also investigated the differential gene expression of the LPXTG-containing proteins of cells growing in biofilms versus their free-living counterparts.

*S. mutans* wild-type strain UA159 and its mutants were grown in Todd-Hewitt broth supplemented with 0.3% (wt/vol) yeast extract and incubated at 37°C in air with 5% $CO_2$. Genomic DNA was isolated from *S. mutans* using a Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN). An *S. mutans srtA* mutant was constructed by insertion-duplication mutagenesis. An internal 473-bp fragment of *srtA* was amplified from *S. mutans* UA159 genomic DNA by using primers CMT-36 (5′-CTGCAGCTGCAGTCCATGCCTTCTTTTGCAC-3′) and CMT-37R (5′-GAATTCGAATTCTTAAGGACCTTTCTGCCTATCC-3′) and cloned into the suicide vector pVA8912 (20). The plasmid, designated pCMT1, was transformed into *S. mutans* UA159 that had been induced to genetic competence by incubation with competence-stimulating peptide (18). Insertion of pCMT1 in *srtA* of *S. mutans* UA159 was

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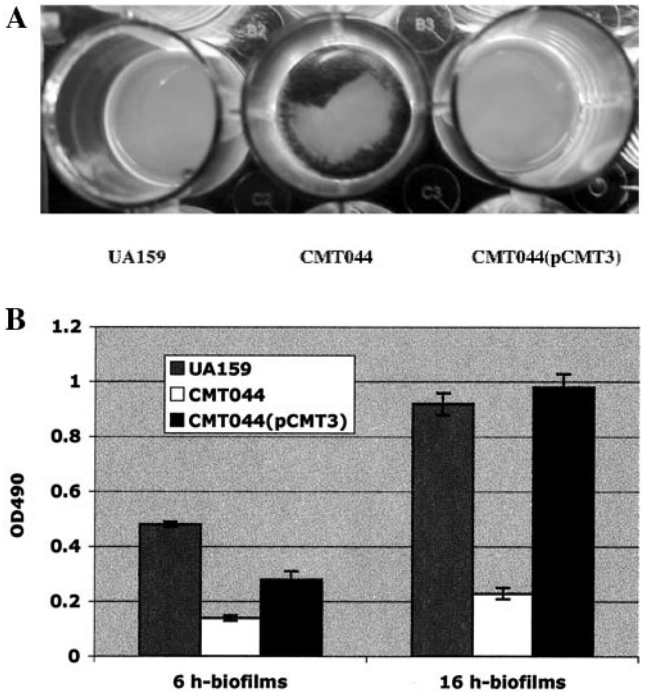


FIG. 1. *S. mutans* biofilm formation. (A) Photograph of typical 16-h-biofilms grown in a 24-well polystyrene microtiter plate. The parent UA159 and its SrtA-deficient mutant grow into biofilms of different architectures. Introduction of a functional copy of the *srtA* gene into the CMT044 mutant restores the wild-type phenotype. (B) Quantification of biofilm mass by optical density of UA159, CMT044, and CMT044(pCMT3) biofilms. Mean optical densities at 490 nm (OD490) ± standard errors are shown. Results represent the average of three independent experiments.

confirmed by Southern blot analysis (data not shown). The *srtA* null mutant was designated CMT044.

To determine if biofilm formation was affected in the CMT044 mutant, we performed a simple biofilm assay. Biofilms were developed in 96-well and 24-well polystyrene microtiter plates. The growth of the biofilm was initiated by inoculating 10 μl of an overnight culture into 300 μl of semidefined minimal medium (19) in the individual wells of a 96-well microtiter plate or by inoculating 50 μl into 1.5 ml of semidefined minimal medium in a 24-well plate. Wells without cells were used as blank controls. The microtiter plates were then incubated at 37°C in air with 5% $CO_2$ for 6 h or 16 h without agitation. After the incubation, the planktonic cells were carefully removed and the plates were air dried overnight. The plates were then stained with 0.01% (wt/vol) safranin for 10 min, rinsed with sterile distilled water, and air dried. Biofilms were quantified by measuring the absorbance of stained biofilms at 490 nm with a microplate reader (model 3550; Bio-Rad Laboratories, Richmond, CA). Biofilms formed in 24-well plates were not stained but photographed immediately after the planktonic cells were removed. The results presented in Fig. 1A show that the CMT044 mutant had a noticeable difference in biofilm architecture on the polystyrene surface compared with the wild-type strain. When the total biomass of the CMT044 mutant biofilm was compared with that of the wild-type biofilm, we noticed that the inactivation of *srtA* caused decreased biofilm mass as illustrated in Fig. 1B. Indeed, mutant CMT044 showed 70.8% ± 2.1% and 75% ± 2.2% decreases in biofilm mass relative to that of the parent strain for 6-h and 16-h biofilms, respectively. The reduction of biofilm mass did not result from a decrease in growth yield, as the CMT044 mutant had the same cell densities as the wild type in a liquid growth kinetics assay (data not shown).

In order to test whether the phenotype was truly the consequence of the *srtA* knockout, we introduced a functional copy of the *srtA* gene into the CMT044 mutant. The full-length coding region of *srtA*, preceded by a potential promoter sequence (TTGTCA-$N_{19}$-TATCAT), was amplified from UA159 genomic DNA by using primers CMT-46 (5′-GGATCCGGATCCTGAATACCCGACTAAAGGACG-3′) and CMT-47R (5′-GAGCTCGAGCTCTCTCACACCATCACACCAGC-3′) and cloned into pDL277 (16) to generate pCMT3. To confirm that the transformants contained the plasmid of desired size, pCMT3 was reisolated from the complemented mutant by the method of Frère (8). The results presented in Fig. 1 clearly show that a functional *srtA* gene in *trans* restored the wild-type phenotype. Introduction of pDL277 alone had no effect (data not shown). However, the results for 6-h biofilms showed that CMT044(pCMT3) had a reduced biomass relative to the parent strain (approximately 60% of the wild-type level) (Fig. 1B). This reduction may have resulted from a decrease in growth yield, since the complemented mutant in liquid culture showed a slower generation time (50.3 ± 2.9 min) compared with that of the parent strain (44.7 ± 0.3 min). Consequently, these results demonstrate that a defect in the *S. mutans* sortase yields a phenotype that generates a decreased biofilm biomass.

In *S. mutans*, six LPXTG-containing proteins have been identified (1). Recent studies demonstrated that the *S. mutans* sortase was involved in the cell wall anchoring of three of them, P1 (11, 17), GbpC (13), and DexA (12). These results suggest that *S. mutans* cells lacking an active sortase may lose the biological function mediated by theses cell surface proteins. Since the sortase is apparently involved in *S. mutans* biofilm formation, we investigated the differential gene expression of the six LPXTG-containing proteins in biofilm versus planktonic wild-type cells. For quantitative real-time PCR (qRT-PCR) experiments, total RNA was extracted from 16-h-old planktonic and biofilm wild-type cells as described previously (10), with the following modification: 20-μg aliquots of each isolated RNA preparation were treated with 30 U of RQ1 DNase (Promega, Madison, WI). DNA-free RNA samples were subjected to reverse transcription using a first-strand cDNA synthesis kit (MBI Fermentas, Burlington, Ontario, Canada) following the manufacturer's protocol. For each RNA sample, the cDNA synthesis reaction was also carried out without reverse transcriptase in order to identify and control for contamination by residual genomic DNA. Single-stranded cDNA synthesized from total RNA was amplified in a QuantiTect SYBR Green PCR Master Mix (QIAGEN) containing HotStarTaq DNA polymerase, deoxynucleoside triphosphates, SYBR Green I, ROX (reference fluorescent dye), and 5 mM $MgCl_2$. PCRs were carried out in a 25-μl volume containing 1× Master Mix, 0.5 μM each primer, and 400 ng of cDNA. Amplification was performed in a Cepheid Smart Cycler system (Cepheid, Sunnyvale, CA) using the following cycling protocol: 15 min of initial denaturation at 95°C, followed by a

TABLE 1. Primer sequences for genes whose expression in biofilm and planktonic cultures was compared[a]

| Gene | Primer sequence (5′ to 3′) | |
|---|---|---|
| | Forward | Reverse |
| *gyrA* | ATTGTTGCTCGGGCTCTTCCAG | ATGCGGCTTGTCAGGAGTAACC |
| *srtA* | GAAGCTTCCTGTAATTGGCG | TTCATCGTTCCAGCACCATA |
| *spaP* | TTTGCCGATGAAACGACCAC | TACTCGCACTCCCTTGAGCCTC |
| *gbpC* | CGCATTTCTCCTTTTCTTGCCG | ACAACTCCTGATGAACCAACGC |
| *dexA* | GCTGACTGCTTCTGGAGTTTGC | AAGTGCCAAGACTGACGCTTTG |
| *fruA* | TGTAGGTCTCGGTTTGTGGGAC | TCTTGAGCCAATGCTTCTGGTG |
| *wapA* | TGACTTTGACTGATGTTGTCGGAG | GAAAAATCCTCAGCATAAGGTCGC |
| *wapE* | CTTCTGATAAAGCAACCGCCTAC | AAGACCTAAGCCCATTCCAGTTC |

[a] Primers were designated and analyzed with MacVector 7.2 software.

three-step profile consisting of 30 s of denaturation at 94°C, 30 s of annealing at 58°C, and 30 s of extension at 72°C for a total of 40 cycles. For each set of primers (Table 1), a standard curve was plotted with cycle threshold values obtained from amplification of known quantities of UA159 genomic DNA. The standard curves were used to transfer cycle threshold values of the experimental samples into the relative number of DNA molecules. The quantity of cDNA for each gene was normalized to the quantity of *gyrA*, a constitutively transcribed control gene whose expression does not vary under the experimental assay conditions used.

The qRT-PCR results presented in Fig. 2 show that three genes encoding LPXTG-containing proteins were significantly expressed at increased levels in the 16-h-old biofilms in comparison to the planktonic phase ($P < 0.05$). The greatest relative increase was observed for *fruA*, with mRNA levels increased 4.49-fold in biofilm cells. The *fruA* gene encodes β-D-fructosidase, an enzyme responsible for the hydrolysis of fructans (3). Fructans are polysaccharides that are commonly synthesized by dental plaque microorganisms (27). Because of their physical properties (large size and viscosity), it is believed that fructans do not diffuse from the dental biofilm and likely serve as energy storage polysaccharides (3). FruA may thus enhance cell survival during periods of nutrient starvation by

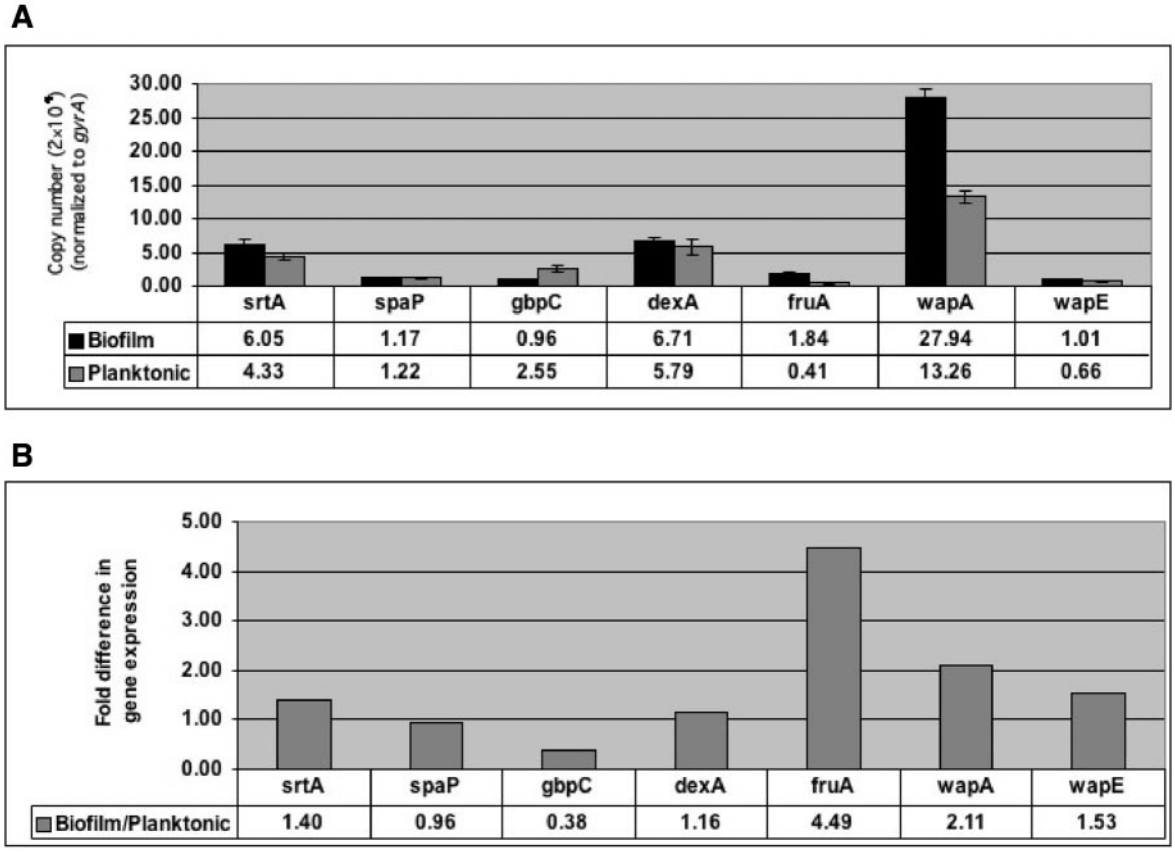


FIG. 2. Gene expression of SrtA and the LPXTG-containing proteins in planktonic and biofilm wild-type cells. (A) RNA level relative to the level of *gyrA*. The means ± standard errors of three different experiments are shown. (B) Differential gene expression in biofilm growth versus planktonic cells.

degrading fructans. For *S. mutans*, FruA may contribute to the extent and duration of the acid challenge at the tooth surface following sucrose exposure. Although FruA may be considered a potential virulence factor, the loss of FruA in an *S. mutans* strain evidently does not alter its cariogenic properties in a rat model (33).

The *wapA* gene was also found to be significantly expressed at an increased level in the biofilm phase ($P < 0.05$) (Fig. 2). Levels of *wapA* mRNA were increased approximately twofold in biofilm-derived cells. The *wapA* gene encodes wall-associated protein A, a major *S. mutans* surface protein, also known as AgIII (29). Studies done by Qian and Dao (26) demonstrated that inactivation of *S. mutans wapA* resulted in a reduction in cell aggregation and adhesion to smooth surfaces. The data suggest that WapA may play a role in the colonization of the tooth surface by *S. mutans* and consequently, in the buildup of dental biofilms. Sucrose-dependent adhesion of *S. mutans* within the dental plaque is primarily responsible for establishing its colonization and accumulation on tooth surfaces. However, the contribution of WapA to sucrose-dependent adhesion is still open to interpretation (2).

The third gene whose expression was shown to be significantly increased in biofilm phase was *wapE* ($P < 0.05$). Levels of *wapE* mRNA were increased 1.53-fold in biofilm cells. The *wapE* gene encodes an uncharacterized secreted protein that is predicted to be wall associated (1). In silico sequence analyses showed that WapE is predicted to have an estimated molecular mass of 55.1 kDa and an isoelectric point of 4.61. BLAST searches of current databases did not reveal significant identity with any other bacterial proteins. However, BLAST searches against the unfinished microbial genomes (http://tigrblast.tigr.org/ufmg/) revealed that an ortholog of this protein was found in the genome of *Streptococcus sobrinus* 6715 (E value, $2.9e^{-77}$). *S. sobrinus*, a member of the mutans streptococcus group, colonizes the smooth surfaces of teeth and is also recognized as a principal etiological agent responsible for initiating caries in humans (28, 32). As *wapE* expression is increased in *S. mutans* biofilm cells, WapE may be involved in the adhesion of this organism to the tooth surface and therefore may play a role in the formation of dental plaque.

Of the genes encoding LPXTG-containing proteins, *gbpC* was the only one with significantly decreased levels of expression in biofilm cells ($P < 0.05$) (Fig. 2). Levels of *gbpC* mRNA were observed to be reduced 2.66-fold in the biofilm phase. The *gbpC* gene encodes a glucan-binding protein that enables the cells to aggregate in the presence of glucans like dextran (31). It has been hypothesized that proteins capable of binding glucan may contribute to sucrose-dependent adhesion and to the coadhesive nature of the dental plaque biofilm (2). Our results were not anticipated; *gbpC* expression was actually reduced in the biofilm state. Previous research had found that GbpC promotes dextran-dependent aggregation only when *S. mutans* cells are stressed by subinhibitory concentration of antibiotics and addition of dextran (30, 31). It is possible that *S. mutans* is so well adapted to a biofilm environment that *gbpC* in the biofilm state is regulated as if under low-stress conditions. In contrast, the planktonic cells may encounter transient stress in the oral environment (e.g., presence of antibiotics, carbohydrate limitation, or changes in physical factors) that may induce *gbpC* expression.

TABLE 2. Primers used to construct the *fruA*, *wapA*, and *wapE* mutants by PCR restriction-ligation mutagenesis[a]

| Primer | Nucleotide sequence[b] (5′ to 3′) | Product size (bp) |
|---|---|---|
| FruA-P1 | TACAAGAATGGGCGTTGGCAGG | 968 |
| FruA-P2 | **GGCGCGCC**ACACAAAAAAGACGGCACAG | |
| FruA-P3 | GGCCGGCCGCTGGCTATTTCAAAAAACGC | 799 |
| FruA-P4 | GGCATTCCACAAGACCTAAGTC | |
| WapA-P1 | TTTGATGCGGCTCCAAGTTGTGCG | 909 |
| WapA-P2 | **GGCGCGCC**TTACTGAAAGAAAGTGGTTCCC | |
| WapA-P3 | GGCCGGCCTTGTTGCTGTGGCAGGTGTC | 648 |
| WapA-P4 | GAAATCCTCCGCTTCTATGG | |
| WapE-P1 | CGCAATGGCAATCAGGAGTATG | 669 |
| WapE-P2 | **GGCGCGCC**TGACGCAAGGGCAACAACTG | |
| WapE-P3 | GGCCGGCCAAAGGCACTTCCCCTGATGC | 966 |
| WapE-P4 | CCAACCATTTTAGCAACAGTCTG | |
| Erm-19 | **GGCGCGCC**CCCGGGCCCAAAATTTGTTTGAT | 876 |
| Erm-20 | GGCCGGCCAGTCGGCAGCGACTCATAGAAT | |

[a] Primers were designated and analyzed with MacVector 7.2 software.
[b] AscI restriction sites are in boldface; FseI restriction sites are underlined.

The present study has demonstrated that inactivation of *S. mutans srtA* caused a decrease in biofilm formation. In addition, we showed that three genes, *fruA*, *wapA*, and *wapE*, were expressed at increased levels in *S. mutans* biofilm cells, providing evidence that sortase-dependent display of FruA, WapA, and/or WapE may be involved in the formation of biofilms. Therefore, in an effort to determine if these LPXTG-containing proteins are involved in the formation of *S. mutans* biofilms, we constructed individual mutants and tested their ability to form biofilms. The *fruA*, *wapA*, and *wapE* mutants were constructed by a PCR-based deletion strategy involving restriction-ligation and allelic replacement as described previously (15). Using this technique, the reading frame of the target gene and that of the erythromycin resistance gene (antibiotic selection marker) are aligned, preserving the original downstream reading frames in order to avoid polar effects (15). The primers used to construct and confirm the mutants are listed in Table 2. For example, to construct the *fruA* mutant, a 968-bp DNA fragment (PCR product A) 5′ from the *fruA* start codon and a 799-bp DNA fragment (PCR product B) 3′ from the *fruA* stop codon were amplified from *S. mutans* UA159 genomic DNA using primer pairs FruA-P1–FruA-P2 and FruA-P3–FruA-P4, respectively. The erythromycin resistance gene (*erm*) was amplified from a synthetic erythromycin resistance cassette (15) using primers Erm-19 and Erm-20. These PCR products were subjected to restriction digestion and ligation. The ligated fragment (product A-Erm-product B) was then transformed directly into *S. mutans* UA159 using the competence-stimulating peptide. Following a double-crossover homologous recombination, the internal region of *fruA* was completely replaced by the *erm* gene as confirmed by DNA sequencing using primers FruA-P1, FruA-P4, Erm-19, and Erm-20. In order to confirm that the downstream reading frame *fruB* was not disrupted by the insertion of the *erm* gene, qRT-PCR was performed using primers FruB-F (5′-CAAGCAGATGCCCAAGTGTC-3′) and FruB-R (5′-TCCTTTTGCCCATTCCAGTG-3′). The qRT-PCR results demonstrated that no significant difference in *fruB* expression was observed between the wild-type strain and the *fruA* mutant (data not shown). The *wapA* and *wapE* mutants were constructed using the same strategy. As for the *fruA* mutant, qRT-PCR was performed for the *wapA* and *wapE*

mutants and compared with the wild-type strain in order to confirm that the insertion of the *erm* gene did not disrupted the downstream genes SMU.988 (forward, 5′-TCACGTACTTTC CGAATGGAC-3′; reverse, 5′-TCACCCCACATCAATCCT TC-3′) and SMU.1093 (forward, 5′-AGACAAATGGATGCT GGTCC-3′; reverse, 5′-TTGCCTGTGATGTGCCCATC-3′), respectively (data not shown). The *fruA*, *wapA*, and *wapE* mutants were then individually tested for 16-h biofilm formation and quantification as described previously. The results demonstrated that all three mutants formed stable and reproducible biofilms typical of wild-type strain UA159. However, a significant difference in total biofilm biomass was observed compared to that of the wild-type strain. Indeed, the *fruA*, *wapA*, and *wapE* mutants had reduced biofilm biomass of 19.9% ± 2.8%, 24.1% ± 1.5%, and 24.7% ± 0.4% of the wild-type level, respectively ($P < 0.05$). Consequently, these results demonstrated that these LPXTG-containing proteins are involved in biofilm formation by *S. mutans*. Moreover, these results suggested that the reduction in biofilm formation in the *srtA* mutant may be due to a change in the cell wall anchoring of FruA, WapA, and WapE.

Cell surface proteins of gram-positive pathogens play various important roles in pathogenicity. Over the past decade, surface proteins from the oral pathogen *S. mutans* have been studied for their role in oral colonization, as well as their potential as vaccine targets (28). The LPXTG-containing proteins are the only surface proteins known to be covalently linked to the bacterial cell wall (5). In *S. mutans*, LPXTG-containing proteins FruA, WapA, and WapE are involved in biofilm formation. Therefore, these proteins are of great interest in terms of understanding the *S. mutans* infection process and could become future targets for the prevention of dental caries.

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