Functional characterization of cell-wall-associated protein WapA in *Streptococcus mutans*

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Streptococcus mutans is known as a primary pathogen responsible for dental caries. One of the virulence factors of *S. mutans* in cariogenicity is its ability to attach to the tooth surface and form a biofilm. Several surface proteins have been shown to be involved in this process. A 29 kDa surface protein named wall-associated protein A (WapA, antigen A or antigen III), was previously used as a vaccine in animal studies for immunization against dental caries. However, the function of WapA in *S. mutans* is still not clear. This study characterized the function of WapA in cell surface structure and biofilm formation. Compared to the wild-type, the *wapA* mutant had much-reduced cell chain length, diminished cell–cell aggregation, altered cell surface ultrastructure, and unstructured biofilm architecture. Furthermore, *in vivo* force spectroscopy revealed that the cell surface of the *wapA* mutant was less sticky than that of the wild-type cells. More interestingly, these phenotypic differences diminished as sucrose concentration in the medium was increased to 0.5%. Real-time RT-PCR analysis demonstrated that sucrose strongly repressed *wapA* gene expression in both planktonic and biofilm cells. These results suggest that the WapA protein plays an important structural role on the cell surface, which ultimately affects sucrose-independent cell–cell aggregation and biofilm architecture.

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INTRODUCTION

Streptococcus mutans is considered as a principal pathogen responsible for dental caries (Hamada & Slade, 1980). Biofilm formation is one of the well-recognized virulence factors of *S. mutans*, which involves a sucrose-independent initial attachment, cell–cell aggregation, a sucrose-dependent stabilization, and eventual biofilm maturation (Kuramitsu, 2000).

The mechanism of sucrose-dependent biofilm formation is well understood. In the presence of sucrose, *S. mutans* produces extracellular polysaccharides named glucans from the glucose moiety of sucrose, through the enzymic activity of three glucosyltransferases (GtfB, -C and -D). Glucan formation allows *S. mutans* to firmly stick to the smooth tooth surface (Kuramitsu, 1993; Yamashita *et al.*, 1993). Another group of proteins called glucan-binding proteins (i.e. GbpA, -B, -C and -D) are thought to play important roles in subsequent cell–cell aggregation and biofilm development (Douglas & Russell, 1982; Sato *et al.*, 2002b; Shah & Russell, 2004; Smith & Taubman, 1996).

Compared with sucrose-dependent biofilm formation, the mechanism of sucrose-independent surface attachment is less well understood. The surface-associated protein antigen I/II (Pac), which binds to the salivary glycoproteins, was shown to be required for the initial attachment of *S. mutans* to the saliva-coated tooth surface (Bowen *et al.*, 1991). Recently, LytR, a homologue of a regulator of autolysin activity in *Bacillus subtilis*, was shown to play an important role in sucrose-independent attachment to polystyrene surfaces in *S. mutans* (Wen & Burne, 2002; Yoshida & Kuramitsu, 2002). Another surface-associated protein, wall-associated protein A (WapA), is a well-studied human vaccine candidate (Russell & Johnson, 1987; Russell *et al.*, 1995); however, the function of this protein remains controversial. It has been reported that antibodies to this antigen do not interfere with aggregation induced by sucrose or dextran (Douglas & Russell, 1982), or with adherence to saliva-coated hydroxyapatite (Douglas & Russell, 1984). However, Qian & Dao (1993) reported that inactivation of *wapA* in *S. mutans* strain GS-5 results in a significant decrease in sucrose-dependent adherence to surfaces. Later, Harrington & Russell (1993) constructed the same mutant in a different strain and found no such effect. More recently, Levesque *et al.* (2005) reported that, in the absence of sucrose, a *wapA* knockout mutant derived from strain UA159 forms stable and reproducible biofilms with a reduced biomass of 24.1%

Abbreviations: AFM, atomic force microscopy; Gbp, glucan-binding protein; Gtf, glucosyltransferase; WapA, wall-associated protein A.

of that of the wild-type. To obtain a clear answer on the function of WapA, we used different analytical methods, such as confocal and atomic force microscopy (AFM; Binnig *et al.*, 1986), to elucidate the structural and biological functions of WapA in *S. mutans*. Our results demonstrate that WapA is involved in sucrose-independent cell–cell aggregation and biofilm formation.

METHODS

Strains and growth conditions. *S. mutans* strain UA140 (Qi *et al.*, 2001) and its derivative were grown in Todd–Hewitt (TH; Difco Laboratories) medium or on brain heart infusion (BHI; Difco Laboratories) agar plates. For selection of antibiotic-resistant colonies, BHI plates were supplemented with 800 µg spectinomycin ml⁻¹ (Sigma). All *S. mutans* strains were grown anaerobically (80 % N₂, 10 % CO₂ and 10 % H₂) at 37 °C. *Escherichia coli* cells were grown in Luria–Bertani (LB; Fisher) medium with aeration at 37 °C. *E. coli* strains carrying plasmids were grown in LB medium supplemented with 200 µg spectinomycin ml⁻¹.

Gene inactivation of *wapA*. The inactivation of *wapA* was achieved via single crossover homologous recombination. A 330 bp fragment of SMU.987 (*wapA*) was amplified from chromosomal DNA of strain UA140 with primers WapA-F (5'-CTATTACTTCCAGATGAAG-3'), corresponding to +202 to +223 of the coding region, and WapA-R (5'-GTTAACATCTGGACTTATTGG-3'), corresponding to +527 to +548 of the coding region (GenBank accession number, AE014133; *wapA* locus tag, SMU.987). The PCR product was cloned into the pCR 2.1 TOPO TA vector (Invitrogen), and the correct fragment insertion was confirmed by sequencing. To construct the isogenic mutant in *wapA*, the *wapA* fragment was excised from pCR 2.1 TOPO with *SalI* and *HindIII* (New England Biolabs), following DNA extraction from agarose gel (1 %, w/v) with the Qiagen QIAquick Gel Extraction kit. The backbone vector for the single-crossover inactivation was pFW5, which contains a spectinomycin-resistance marker (gene *aad9*) that works in both Gram-negative and Gram-positive bacteria (Podbielski *et al.*, 1996). pFW5 was also digested with *SalI* and *HindIII*, and ligated with the *wapA* fragment to generate pFW5::*wapA*, using a standard ligation procedure. The resulting plasmid containing the *wapA* gene fragment was confirmed by PCR and sequencing. The plasmid was transformed into *S. mutans* UA140 and transformants were selected on TH plates with 800 µg spectinomycin ml⁻¹. Insertion of the plasmid into the *S. mutans* chromosome via single-crossover integration was further confirmed by PCR.

Confocal laser scanning microscopy (CLSM). *S. mutans* cells were cultured in TH medium at 37 °C under anaerobic conditions. For biofilm formation, cells were grown anaerobically in TH medium overnight, and diluted 1:100 into TH medium with 0, 0.01 and 0.5 % sucrose in the Lab-TekII Chamber Slide System (Nalge Nunc International) for confocal imaging. Biofilm cells were stained with CellTracker Orange CMTMR [5-(and-6)-((4-chloromethyl)-benzoyl) amino]tetramethylrhodamine] according to the manufacturer's instructions (Molecular Probes). After 24 h incubation, CLSM was performed using an LSM 5 PASCAL with LSM 5 PASCAL software (Carl Zeiss). Images were obtained with a 10 × 0.3 Plan-Neofluar and a 40 × 1.4 Plan-Neofluar oil objective. Images were further processed using CorelDraw 10.

Immobilization of *S. mutans* cells for AFM analysis

(i) Dry immobilization. A volume (0.5 ml) of each overnight culture was washed twice with nanopure water and resuspended in the same

volume. Serial dilutions in water (10⁻¹, 10⁻² and 10⁻³) were prepared and 8 µl was transferred onto a 10-well microscope slide (Erie Scientific). The slide was air-dried and imaged directly with AFM.

(ii) Wet immobilization. Two millilitres of an overnight culture in TH medium (5 × 10⁸ bacteria ml⁻¹) were filtered through an isopore polycarbonate membrane (Millipore) with a pore size of 0.6 µm (i.e. slightly smaller than the diameter of streptococcal cells) to immobilize the bacteria through mechanical trapping, a very common technique used to gather AFM images and force measurements of molecular interactions at microbial surfaces (Pelling *et al.*, 2004; van der Aa *et al.*, 2002; Kasas & Ikai, 1995). After filtering, the filter was carefully removed from the filter device and fixed with double-sided sticky tape onto a small Petri dish. Five millilitres of 20 % (v/v) BHI broth was added to the sample prior to AFM imaging.

AFM methodology. All imaging in air and fluid was conducted using a Nanoscope IV Bioscope (Veeco Digital Instruments). AFM images were collected in contact mode using sharpened silicon nitride cantilevers with experimentally determined spring constants of 0.02 N m⁻¹ and a tip radius of <20 nm (Levy & Maaloum, 2002). Fluid imaging and mechanical measurements were obtained at room temperature (~20 °C), with force measurements recorded at a pulling rate of 1 Hz. Height and deflection images were simultaneously acquired, and are both of importance with regard to microbial cell surface characterization as they yield complementary information. Deflection-mode images consistently reveal cellular ultrastructure with higher sensitivity, although these images are not representative of the true sample surface height variations; however, height-mode images provide quantitative height measurements, yielding accurate surface-roughness and surface-feature measurements (van der Aa *et al.*, 2002; Paige *et al.*, 1998; Pelling *et al.*, 2005). The images presented in this study are deflection-mode images; however, all quantitative cellular measurements were taken from the corresponding sample-height data.

Roughness analysis with AFM. Surface-roughness values (Pelling *et al.*, 2005) for the extracellular ultrastructure of the cells were calculated from high-resolution AFM height-mode images using Nanoscope software (Veeco Digital Instruments). For both the wild-type UA140 and the *wapA* mutant, the mean *R*_{rms} value was calculated by collecting 100 *R*_{rms} values over areas of 250 nm² on ~10–20 cells for each strain (giving a mean 5–10 measurements per cell), and calculating the mean (Pelling *et al.*, 2005). For a particular scan line over the cell surface, the calculated roughness, *R*, was defined as the SD (where *R*_{rms} noise is the SD for large datasets) in the *i*th height value, *h*_{*i*}, from the mean height, <*h*> (Pelling *et al.*, 2005). The mean rms height divergence (<*R*_{rms}>) was calculated from high-resolution AFM height-mode images according to the following equation:

$$R_{\text{rms}} = \sqrt{\frac{\sum_i (h_i - \langle h \rangle)^2}{n}}$$

(where *n* is the number of data points), and taking the mean. All images used to gather *R*_{rms} values were no more than 4 × 4 µm in scan size to provide local nanoscale roughness measurements, and all tips were from the same wafer batch to alleviate variations in tip geometry.

RNA extraction and real-time RT-PCR. Strain UA140 cultures were grown in 100 ml TH medium with 0, 0.01 and 0.5 % sucrose. Cells were harvested at OD₆₀₀ 0.3 by centrifugation (4700 r.p.m. for 15 min at 4 °C), washed three times with PBS, pelleted and stored at -80 °C. For RNA extraction, frozen cell pellets were ground three times with liquid nitrogen to break up the cells. Subsequently, 0.5 ml chloroform, 2 ml TRIzol (Invitrogen) and 2 ml Tris/EDTA

buffer were added to the cell pellets for extraction. RNA was precipitated with one-tenth vol. 3 M sodium acetate (pH 5.2) and 0.7 vols. 2-propanol (100%). Total RNA (3 µg) was used for cDNA synthesis using Stratascript RT (Stratagene), according to the manufacturer's protocol. For real-time RT-PCR, primers were designed according to sequence data provided by GenBank (accession no. AE014133), and SYBR green (Bio-Rad) was used for fluorescence detection with the iCycler (Bio-Rad) real-time PCR system, according to the manufacturer's protocol. Real-time RT-PCR primer sequences were as follows: wapRTf 5'-TCAAACGAATGTTCCGACAA-3' and wapRTr 5'-CAAAAGCCCTGCTTGTCAC-3'. Total cDNA abundance between test samples was normalized using the 16S rRNA gene as a housekeeping control.

RESULTS

Construction and initial phenotypic characterization of the *wapA* mutant

WapA, encoded by SMU.987 from GenBank (<http://www.ncbi.nlm.nih.gov/>), has a typical Gram-positive signal peptide and a sorting signal for protein export, which includes the LPXTG motif, a hydrophobic domain and a charged tail (Ferretti *et al.*, 1989; Fischetti *et al.*, 1990; Levesque *et al.*, 2005). Previous studies have demonstrated a ubiquitous localization of WapA on the cell surface (Moro & Russell, 1983). To further study the role of WapA in cell surface structures and related functions, we constructed an isogenic mutant of *wapA* by insertional inactivation. The cell morphology of the *wapA* mutant was then examined by optical microscopy. As shown in Fig. 1(a), the cell morphology of the *wapA* mutant differed dramatically from that of the wild-type when grown in TH medium without sucrose. While the wild-type displayed long cell chains (mean 10.2 ± 5.7 cells per chain), the *wapA* mutant showed only short cell chains (mean 2.1 ± 1.2 cells per chain). Interestingly, this difference diminished as the sucrose concentration in the medium was increased. At 0.01 % sucrose, the mean chain length of *wapA* became a little longer (2.7 ± 1.6 cells per chain), but much less cell-cell aggregation was observed at this sucrose concentration compared with the large cell aggregates formed in the wild-type culture (Fig. 1b). At 0.5 % sucrose, the morphology of *wapA* was nearly indistinguishable from that of the wild-type, either by chain length (wild-type 9.8 ± 5.6 cells per chain, *wapA* 8.3 ± 4.0 cells per chain) or by sucrose-dependent aggregation (Fig. 1c). These results suggest that WapA may play a role in cell-cell attachment between sister cells in the absence of sucrose.

Effects of *wapA* mutation on biofilm architecture

To establish whether the morphological change of the *wapA* mutant also affects biofilm architecture, cells of wild-type and mutant strains were labelled with a cell tracker dye, and the biofilm architecture was analysed using CLSM. Previous studies have shown that *S. mutans* can form a biofilm in the absence of sucrose (Levesque *et al.*, 2005; Wen *et al.*, 2005; Yoshida & Kuramitsu, 2002), but under our experimental

conditions, strain UA140 did not form a biofilm in the absence of sucrose. The biofilms were first observed with a 10×0.3 Plan-Neofluar objective to get an overview of the entire field, and then a 40×1.4 Plan-Neofluar oil objective was used to take seven random snapshots from different positions in the confocal field of each chamber. A representative image of the biofilm of each strain at 0.01 and 0.5 % sucrose is shown in Fig. 2. It is apparent that, at 0.01 % sucrose, the wild-type cells formed a biofilm with large but sparsely distributed microcolonies and large areas occupied by unstructured or single-cell chains (Fig. 2a). This is typical of the biofilm morphology at 0.01 % sucrose displayed by strains UA140 and UA159 (Kreth *et al.*, 2004). In contrast, the *wapA* mutant attached to the surface mostly as unstructured cell layers and formed a thin biofilm. It was conspicuous that these biofilms were quantitatively different. However, at 0.5 % sucrose, both the wild-type and *wapA* mutant formed thick, confluent biofilms consisting of dense microcolonies with small, water-channel-like void areas (Fig. 2b). These results are consistent with the morphological difference between the wild-type and mutant cells presented in Fig. 1, suggesting that WapA also plays a role in determining *S. mutans* biofilm architecture at lower sucrose concentrations.

Cell surface ultrastructure of the *wapA* mutant

To further study the contribution of WapA protein in maintaining the cell surface properties of *S. mutans*, AFM was used to detect the difference in cell surface ultrastructure between the wild-type and the *wapA* mutant. When air-dried wild-type and *wapA* mutant cells were probed using AFM, the extracellular ultrastructure was realized on the nanoscale, revealing distinct morphological differences, as shown in Fig. 3. In general, the wild-type cells displayed distinct surface structures, most prominently, protrusive equatorial septa, as previously observed by Cross *et al.* (2006) (Fig. 3a). Moreover, single-cell surface cross-sections of wild-type cells showed the presence of protrusive septa in the nanoscale cell surface topology (data not shown). In contrast, the *wapA* mutant cells revealed a more amorphous cellular ultrastructure in both the AFM deflection-mode image (Fig. 3b) and the single-cell surface cross-section (data not shown).

To analyse the effect of sucrose on the nanoscale morphology of wild-type and *wapA* mutant cells, both strains were grown overnight in TH medium with 0.5 % sucrose, and immediately air-dried prior to imaging with AFM (see Methods). With the addition of sucrose, the distinction in nanoscale cell surface ultrastructure between the wild-type and *wapA* mutant cells became less visible (Fig. 3c, d); both cells showed septum topology, as seen in the AFM deflection-mode images (Fig. 3c, d), and a more homogeneous overall cell surface morphology, as detected in the single-cell surface cross-sections (data not shown). This was probably due to the presence of glucan layers on the cell surface, which are produced during growth in the presence of sucrose.

(a) Wild-type ***wapA*** **(b) Wild-type** ***wapA*** **(c) Wild-type** ***wapA***

Fig. 1. Cell morphology of *S. mutans* UA140 wild-type (left column) and *wapA* mutant (right column) grown overnight with different sucrose concentrations in static TH liquid medium. (a) Cells grown in TH without sucrose; (b) cells grown in TH with 0.01 % sucrose; (c) cells grown in TH with 0.5 % sucrose. Images were obtained at a magnification of $\times 400$. Bar, 35 μm .

Fig. 2. CLSM images of *S. mutans* wild-type (left column) and *wapA* mutant (right column) biofilms grown in TH with (a) 0.01 % and (b) 0.5 % sucrose. Cells were labelled with CellTracker Orange CMTMR. Pictures were taken with an overall magnification of $\times 400$. Bar, 40 μm .

To quantify the difference in nanoscale surface morphology between the wild-type and *wapA* mutant cells before and after sucrose treatment, a surface roughness analysis was conducted. The roughness of the cellular surfaces was calculated from one hundred 250 nm² areas on AFM height images no larger than 4 $\times$ 4 μm in scan size ($n=100$ in each case; wild-type and *wapA* mutant before and after sucrose). Using the equation described in Methods, mean R_{rms} values were obtained (Pelling *et al.*, 2005) for the wild-type and *wapA* mutant cells before and after sucrose addition (Fig. 4). The corresponding R_{rms} values for the wild-type and *wapA* mutant cells before sucrose were

determined to be 7.20 ± 1.31 nm and 5.43 ± 0.74 nm, respectively. After the addition of sucrose, the mean surface roughness values were found to be 9.76 ± 0.95 nm for the wild-type cells and 9.63 ± 0.93 nm for the *wapA* mutant cells. A two-sample independent *t* test was conducted on the mean R_{rms} values before and after treatment with sucrose. At the 95 % confidence level, it was found that the population means before sucrose addition were significantly different between the wild-type and mutant cells ($P < 0.0005$; Fig. 4a), whereas, after sucrose addition, the population means for the wild-type and mutant cells were not significantly different (Fig. 4b).

Fig. 3. AFM deflection-mode images of *S. mutans* UA140 wild-type and *wapA* mutant cell morphology in air. (a) *S. mutans* UA140 wild-type cells and (b) *S. mutans* *wapA* mutant cells grown without sucrose, with the highlighted area showing individual streptococcal cells in each case. (c) *S. mutans* UA140 wild-type cells and (d) *wapA* mutant cells grown with 0.5% sucrose. Bars, 400 nm.

The effect of *wapA* mutation on cell surface stickiness of *S. mutans*

Biofilm formation is largely reliant on intercellular adhesion involving intra- and inter-species cell–cell and cell–matrix interactions. Since the biofilm architecture is qualitatively different between the wild-type and *wapA* mutant cells, we were interested in establishing whether these two cell types were also different in terms of surface adhesive properties. We applied *in vivo* force spectroscopy to living *S. mutans* wild-type and *wapA* mutant cells, thus measuring the variation in cell adhesion as a function of the nanomechanical properties of the existing surface adhesive molecules.

The wild-type and *wapA* mutant cells were immobilized on a filter, as previously described by Dufrene *et al.* (1999). Force–displacement curves were then collected on the cells by positioning the AFM cantilever tip over a cell and pressing the tip against the cell surface, while recording cantilever deflection on approach and subsequent retraction of the tip from the cell. For these experiments, the applied force of the tip against the cell was ~ 5 nN. During tip retraction, numerous rupture events occurred, forming sawtooth-like patterns (Fisher *et al.*, 2000; Sen *et al.*, 2005), and revealing the adhesive interactions between the tip and cell surface proteins. Most likely, the observed rupture events were due to breakage of multiple adhesive bonds formed between the tip and cell surface adhesive molecules, as suggested in similar studies involving surface-adherent

substances in various other cell systems (Chen & Moy, 2000; Pelling *et al.*, 2005; van der Aa *et al.*, 2002). Force spectroscopy experiments involving tip–cell interactions are commonly used to quantitatively probe the mechanical properties of macromolecules at the microbial surface (van der Aa *et al.*, 2001). The transient and local interactions between the cell surface and the AFM tip are a reasonable model for probing adhesive properties at the surface of microbial cells (Pelling *et al.*, 2005; van der Aa *et al.*, 2001). Moreover, force curves taken on the bare substrate, before and after those performed on the cell, revealed force curves lacking rupture events, with little to no variation between the approach and subsequent retraction curves, thus indicating null adhesion.

Adhesion forces for living *S. mutans* wild-type UA140 and *wapA* mutant cells were obtained, revealing mean rupture forces of 84 ± 156 and 42 ± 13 pN, respectively. Probed under analogous conditions, the wild-type cells exhibited considerably more adhesion events than the mutant cells. In particular, wild-type (Fig. 5b inset) cells revealed sawtooth-like patterns in the retraction traces of the force–displacement curves indicative of multiple tip–cell interactions, whereas *wapA* mutant cells (Fig. 5a inset), in general, only revealed single tip–cell adhesion events. As shown by the histogram in Fig. 5(a), the rupture forces observed due to tip–cell adhesion fell within a much narrower range of ~ 20 to 80 pN for the *wapA* mutant cells, as compared to the wild-type cells, whose rupture events ranged from ~ 20 to

Fig. 4. Surface roughness analysis of *S. mutans* wild-type UA140 and *wapA* mutant cells showing the mean surface roughness, $\langle R_{rms} \rangle$, in nanometres, as calculated from high-resolution AFM height-mode images according to the equation described in Methods. For both the UA140 wild-type and the *wapA* mutant, the mean R_{rms} value was calculated by collecting 100 R_{rms} values over 250 nm^2 areas on ~ 10 – 20 cells for each strain (giving a mean 5–10 measurements per cell), and taking the mean. For a particular scan line over the cell surface, the calculated roughness, R , was defined as the SD (where R_{rms} noise is the SD for large datasets) in the i th height value, h_i , from the mean height, $\langle h \rangle$. (a) Histogram with Gaussian fits showing $\langle R_{rms} \rangle$ in nanometres for the wild-type (black bars) and *wapA* mutant (grey bars) cells grown without sucrose ($n=100$ in each case). The corresponding $\langle R_{rms} \rangle$ values for the wild-type and *wapA* mutant cells were determined to be 7.20 ± 1.31 and 5.43 ± 0.74 nm, respectively ($P < 0.0005$). (b) Histogram with Gaussian fits showing $\langle R_{rms} \rangle$ in nanometers for wild-type (black bars) and *wapA* mutant (grey bars) cells grown with 0.5% sucrose ($n=100$ in each case). The corresponding $\langle R_{rms} \rangle$ values were calculated to be 9.76 ± 0.95 nm for the UA140 wild-type cells and 9.63 ± 0.93 nm for the *wapA* mutant cells.

330 pN (Fig. 5b). Moreover, the number of rupture events observed for the wild-type cells was significantly more than that exhibited by the mutant cells. Since these analyses were conducted for the wild-type and mutant cells under the

same conditions, these results suggest that the wild-type cells are more sticky than the *wapA* mutant cells.

Effect of sucrose on *wapA* gene expression

The wild-type and *wapA* mutant phenotypes presented above indicate that glucans synthesized from sucrose play a dominant role in cell morphology and biofilm architecture. Additionally, sucrose may also affect the gene expression of *wapA*. To test this hypothesis, we measured *wapA* gene expression by real-time RT-PCR in the wild-type cells grown in TH medium supplemented with different concentrations of sucrose. In the presence of sucrose, *wapA* gene expression was strongly repressed. In cells grown with 0.01 % sucrose, the *wapA* gene transcription was reduced by 23 % compared to cells grown without sucrose. As the sucrose concentration increased to 0.5 %, *wapA* gene expression was down to 3 % of the level without sucrose. RNA samples were also obtained from biofilm cells grown with 0.01 and 0.5 % sucrose, and similar results were obtained (data not shown). Taken together, these results demonstrate that *wapA* gene expression is repressed by sucrose in both planktonic and biofilm cells.

DISCUSSION

The WapA protein of *S. mutans* has attracted some interest from the dental research community because of its potential utilization in the development of a dental caries vaccine. Despite its presence on the cell surface, its biological function remains elusive. In this study, we used a combination of image analysis tools to elucidate the biological functions of WapA in *S. mutans*. We showed that, in the absence of sucrose, the *wapA* mutant has a much shorter cell-chain length than the wild-type. Further detailed analysis using AFM demonstrated that the mutant cell surface morphology revealed a less distinct cellular ultrastructure, as signified by the absence of an equatorial septum. According to Harrington & Russell (1993), these cellular ultrastructural changes might relate to the significantly reduced wall-associated lipoteichoic acid level in *wapA* mutants. Moreover, the mutant cell surface appeared to be less sticky when probed using *in vivo* force spectroscopy. Consequently, the *wapA* mutant formed less structured biofilms compared to the wild-type. Interestingly, all the observed phenotypes of *wapA* mutation disappeared when cells were grown in 0.5 % sucrose. Taken together, these results suggest that WapA is more likely to be involved in sucrose-independent cell–cell aggregation than in sucrose-dependent adherence. This result is consistent with the findings of Russell's group (Douglas & Russell, 1982, 1984; Harrington & Russell, 1993), but contradictory to those of Qian & Dao (1993). Recently, at least two mutations have been identified in strain GS-5, the strain studied by Qian & Dao. The two mutations are localized on two surface-protein genes, *gbpC* and *pac* (Murakami *et al.*, 1997; Sato *et al.*, 2002a). Based on our own experience, strain GS-5 has cell morphology that is very distinct from that of most

Fig. 5. Histograms of rupture force between the AFM tip and living *S. mutans* *wapA* mutant (a) and wild-type (b) cells. Insets show typical force-displacement curves measured on mechanically trapped, living *wapA* mutant (a) and wild-type (b) cells in each case. Force-displacement approach and retraction curves are shown in light grey and dark grey, respectively. Each histogram represents the rupture force observed due to tip-cell adhesion for three individual cells in both the wild-type and mutant strains. In the case of *S. mutans* wild-type cells (b), adhesion forces for the observed rupture events ranged from ~20 to 330 pN. Those observed for the *wapA* mutant cells (a) had a range between ~20 and 80 pN. The mean rupture forces for the *wapA* mutant and UA140 wild-type cells were 43 ± 13 and 84 ± 156 pN, respectively. Typical force-displacement curves recorded for *S. mutans* *wapA* mutant (a, inset) and wild-type (b, inset) cells reveal adhesive interactions between the cantilever tip and cell surface in the retract trace. Wild-type cells (b) revealed sawtooth-like patterns in the retraction traces, indicative of multiple tip-cell interactions, whereas mutant cells (a) generally only revealed single tip-cell adhesion events. The force spectroscopy analysis of these cells consisted of 100 measurements made on three individual cells for both *S. mutans* wild-type and *wapA* mutant strains.

S. mutans clinical isolates, and forms biofilms with architecture different from that of strains UA159 and UA140. The *wapA* phenotype reported by Qian & Dao (1993) may well be caused by multiple surface-protein gene mutations.

The optical and confocal microscopy studies showed shorter cell chains in the *wapA* mutant when grown in the absence of sucrose. This phenotype may be due to the distribution of WapA during cell division. It has been proposed that the chain formation is due to incomplete separation of peptidoglycan between sister cells, which permits an easy orientation of cell division sites at the contact points between cells (Cole & Hahn, 1962). Newly synthesized surface proteins first appeared at the contact sites between cells, and then extended slowly over the entire surface of the cell. This suggests that WapA may be required for maintaining cell-cell attachment during cell replication or may prevent complete separation of peptidoglycan between cells.

As a surface structural protein, WapA may also be involved in protecting the bacteria from some forms of environmental

stress. To test this hypothesis, we compared the resistance of the wild-type and *wapA* mutant to antibiotics (ampicillin, kanamycin, tetracycline, chloramphenicol and ofloxacin), antimicrobial peptides, triclosan and chlorhexidine. The MIC data did not show any difference between these two strains. We also measured growth rates of the wild-type and mutant strains under different stress conditions, such as oxidative stress, low pH and high temperature, but no difference was observed between the two strains (data not shown). Therefore, we conclude that WapA is probably not involved in functions other than those associated with cell surface structure and properties.

Real-time RT-PCR demonstrated repression of *wapA* gene expression by sucrose. Why does *S. mutans* modulate *wapA* expression according to the sucrose concentration in the environment? From the point of view of the bacterial cell, we speculate that it is related to the bio-economics of the cell. Since both WapA and sucrose are involved in cell-cell aggregation (through cell-cell contact for the former, and glucan formation for the latter), and glucan-mediated attachment appears to be stronger than WapA-mediated

cell-cell aggregation, the production of glucan in the presence of sucrose makes biosynthesis of WapA unnecessary. Thus, it makes more bio-economical sense to shut down *wapA* gene expression when sucrose is present. On the other hand, in the absence of sucrose, the production of WapA becomes important for maintaining cell-cell contact and aggregation, which is probably pivotal for successful colonization of *S. mutans* in the oral cavity.

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