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Comparison of glucan-binding proteins in cariogenicity of *Streptococcus mutans*

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Streptococcus mutans has been implicated as a primary causative agent of dental caries in humans. Bacterial components associated with the adhesion phase of *S. mutans* include cell-associated and cell-free glucosyltransferases (GTFs), as well as protein antigen c and proteins that bind glucan. At least four types of *S. mutans* glucan-binding protein (Gbp) have been identified; GbpA, GbpB, GbpC and GbpD. In the present study, GbpA-, GbpB- and GbpC-deficient mutants (AD1, BD1 and CD1, respectively) were constructed, and their cariogenic properties were evaluated by comparing them to those of their parent strain MT8148. All of the Gbp mutants showed lower levels of dextran binding, while the sucrose-dependent adhesion levels of AD1 and CD1 were lower than in the parental strain. The expression of each GTF was detected in the Gbp mutants, however, they had lower levels of cell-free-GTF activity than the parental strain. On the other hand, in acid tolerance assays, BD1 was the most sensitive among all of the tested strains. These results suggest that GbpA and GbpC in *S. mutans* have strong relationships with cariogenicity, while GbpB may have another biological function.

Key words: cariogenicity; glucan-binding protein; *Streptococcus mutans*

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Streptococcus mutans has been implicated as a primary causative agent of dental caries in humans (7, 14). Several proteins involved in its pathogenicity are located on the cell surface and some glucan-binding proteins (Gbps) have been shown to mediate the binding of the synthesized glucans from sucrose by the action of cell-associated (CA) and cell-free (CF) glucosyltransferases (GTFs). Among them, GbpA protein has been purified (26) and shown to share a homology with the putative glucan-binding domain of GTFs, while its gene, *gbpA*, was found to encode a constitutively expressed secreted protein (23). In our previous study, GbpA was shown to participate in cellular adherence to the tooth surface and to contribute to the cariogenicity of *S. mutans* (17). In another study, GbpC was shown to be a cell-wall anchoring protein that belongs to the Spa family of streptococcal proteins (24), while we found that GbpC plays an important role in sucrose-dependent adhesion by binding to soluble glucan synthesized by GTFD (16). In addition, GbpD was recently sequenced and found to have a high homology with GbpA, as well as having a role in interspecies competition during biofilm formation (25).

GbpB has been purified and shown to be immunologically distinct from other Gbps expressed by *S. mutans* and *Streptococcus sobrinus*. GbpB was also shown to possess a homology to peptidoglycan hydrolases of other gram-positive microorganisms, while results of a comparative genomic analysis of the *gbpB* region suggested a functional relationship between the genes involved in cell shape and cell wall maintenance (18). GbpB is considered to play some roles in the cariogenicity of *S. mutans*, as mucosal immunization has been found to induce protective immune responses against experimental dental caries (27, 28). In the present study, we attempted to identify the function of each Gbp using *gbp* isogenic mutants.

Materials and methods

Bacterial strains

The *S. mutans* MT8148 (serotype *c*), along with GbpA-, GbpB-, and GbpC-deficient mutant strains, was used in the present study. All strains were grown in brain–heart infusion (BHI) broth, Todd Hewitt (TH) broth (Difco Laboratories, Detroit, MI), and mitis-salivarius agar (Difco), as required, with the appropriate antibiotics

(erythromycin 10 μg/ml, kanamycin 500 μg/ml, and spectinomycin 1 mg/ml) used for selection.

Generation of recombinant Gbps and anti-Gbp sera

Recombinant GbpC and anti-GbpC serum samples were generated as reported previously (16). The polymerase chain reaction (PCR) primers utilized for the generation of recombinant GbpA and GbpB in this study are shown in Table 1. The coding regions of *gbpA* (1) (GenBank accession no. M30945) and *gbpB* (18) (GenBank accession no. AY046410) were amplified separately using genomic DNA from strain MT8148 by PCR using AmpliTaq Gold (Applied Biosystems, Foster City, CA). PCR primers (GbpA: GbpA-*Bam*HI and GbpA-*Xho*I, GbpB: GbpB-*Sal*I and GbpB-*Not*I) were constructed based on sequences that added a restriction enzyme site at the 5′ and 3′ ends (*Bam*HI and *Xho*I for *gbpA*, *Sal*I and *Not*I for *gbpB*). The amplified fragments of *gbpA* were subcloned separately into the expression vectors pGEX6p-1 and pET-42a(+) (Novagen, Darmstadt, Germany), with the resultant plasmids being named pMM335 and pMM336, respectively, after which they were transformed into *Escherichia coli* BL21(DE3). The *E. coli* BL21(DE3) organisms carrying pMM335 or pMM336 were grown in Luria–Bertani broth containing ampicillin (100 μg/ml) and tetracycline (7.5 μg/ml) at 37°C to the mid-exponential phase. Isopropylthio-β-D-galactoside (IPTG, Wako Chemical Industries, Osaka, Japan) was then added to give a final concentration of 1.0 mM, and the cultures were incubated for an additional 3 h to induce GST-GbpA or GST-GbpB protein synthesis, after which the cells were harvested by centrifugation. Pelleted cells were suspended in 10 mM phosphate buffer (10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 0.5 M NaCl, 20 mM imidazole, pH 7.4) and ultrasonicated on ice. Supernatants were obtained by centrifugation and purified using a glutathione Sepharose™ 4B column (Amersham Biosciences, Uppsala, Sweden). After the GST fusion proteins were treated with thrombin (Itoham Food Inc., Hyogo, Japan) at 4°C, rGbpA and rGbpB were purified separately using the same column. The purified rGbpA and rGbpB samples were then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Antisera for GbpA and GbpB were prepared by repeated intramuscular injections of rabbits with purified rGbpA and rGbpB emulsified with Freund's complete adjuvant. The antibody titer of each antiserum sample was then confirmed by Western blot analysis using rGbpA and rGbpB.

Construction of Gbp-defective mutants

Recombinant plasmids carrying fragments of the erythromycin resistance gene (*erm*) from pVA838 (15), the spectinomycin resistance gene (*aad9*) from pSF152 (12), and the kanamycin resistance gene (*aphA*) from transposon Tn1545 (3) were used. *gbpA* was amplified by PCR and ligated into a pGEM-T Vector (Promega, Madison, WI) to generate pMMN25, and the primers GbpA-F and GbpA-R were synthesized based on that sequence. The plasmid pMMN25 was digested with *Hin*dIII to become linear at a unique site, then blunted and ligated with an *erm* cassette to yield pMMN26. The *gbpC* gene (24) (GenBank accession no. D85031) was amplified and ligated into a pGEM-T Vector to generate pMMN28, which was restricted with *Eco*RI to become linear at a unique site, after which primers for GbpC-F and GbpC-R were synthesized based on the sequence. pMMN28 was then blunted and ligated with an *aphA* cassette to yield pKF6. After being digested to become linear at a unique restriction site, the plasmids pMMN25 and pKF6 were introduced into *S. mutans* MT8148 by transformation to allow allelic exchange, using the method described by Tobian and Macrina (29). Then, the plasmid pSF152 containing an *aad9* cassette was digested with *Eco*RI and *Bam*HI, while *gbpB* was amplified by PCR and ligated to generate pKF8. The primers GbpB-F and GbpB-R were synthesized based on that sequence, and the plasmid was introduced by transformation as described above. The transformants were screened on mitis-salivarius agar plates containing the appropriate antibiotics. Appropriate insertional inactivation into the mutants (AD1, BD1 and CD1) was confirmed by determining the nucleotide sequences of *erm*, *aphA* and *aad9* by PCR, as well as Western blotting of the whole cells of the mutant strains with GbpA- or GbpB-specific rabbit antiserum, as described above, or with GbpC-specific rabbit antiserum, which was generated in our previous study (16).

Table 1. PCR primers used in this study

Primer	Sequence (5′ → 3′)
GbpA-F	CCCGGATCCAAAATGATGAAAGAAAAGACACG
GbpA-R	GCTTCTCGAGTGCCATGGGATTTACCAACTA
GbpC-F	GAACTGGAGCTCAAGCCTTCAACAGTTCAG
GbpC-R	GCGGACTGTTAAAGGCTCGAGTCTAATCTA
GbpB-F (*Eco*RI)	GAGTGGTGGAATTCTTAGTTCTGCGACAAC
GbpB-R (*Bam*HI)	AGCTGGATCCTGCGTTACTTTTTTGAGCAC
GbpA-*Bam*HI	AAGGAGGTAAAGGATCCAAAGAAAAGACAC
GbpA-*Xho*I	TTTACCAACTAGTCTCGAGTGATAACTTCA
GbpB-*Sal*I	AGTCTTATCGTCGACGGAGTTATATTTGTA
GbpB-*Not*I	AAAAAGCGGCCGCTAAGATAAATCTTACCT

Underlining indicates the alignment of the restriction enzyme.

Dextran-binding assay

Dextran-binding assays of MT8148, AD1, BD1 and CD1 were performed using the method of Lis *et al.* (13), with some modifications. Cells from the tested strains were cultured in BHI broth for 18 h, then harvested by centrifugation and resuspended in phosphate-buffered saline to an optical density at 630 nm of 0.2, before being coated onto an enzyme-linked immunosorbent assay plate (200 μl per well) at 4°C for 16 h. The wells were washed three times with distilled water, then blocking buffer (0.5% bovine serum albumin in 10 mM sodium acetate buffer, pH 6.0) was added and the plates were incubated at 37°C for 1–2 h. After washing again with distilled water, the wells were filled with blocking buffer supplemented with 0.5 μg/ml biotin–dextran (molecular weight 70,000; Sigma, St Louis, MO) for 10 min at room temperature. After another washing with distilled water, streptavidin–horseradish peroxidase conjugate (GIBCO-BRL, Gaithesburg, MD) was added to all of the wells and the mixtures were incubated for 5 min at room temperature. After a final washing with distilled water, a color detection solution was applied, as recommended by the supplier, and the samples were incubated for various time periods depending on the glucan-binding abilities of the samples. The subsequent A_{490} results were determined using a microplate reader. All assays were carried out three times, with the results presented as the mean and standard deviation.

Sucrose-dependent adhesion to glass surface

Sucrose-dependent cellular adhesion to a glass surface was analysed using a procedure described by Kawabata and Hamada (10). Briefly, the test strains were grown in BHI broth (Difco Laboratories) containing 1% sucrose at 37°C for 18 h at an angle of 30°. After incubation, the culture tubes were vigorously vibrated with a Vortex mixer for 3 s and the non-adhering cells were transferred to fresh tubes. Cells remaining on the glass surface (adhesive cells) were removed using a rubber scraper and suspended in 3 ml of water. Both adhesive and non-adhesive cells were dispersed by ultrasonication, and their masses were determined by reading the optical density at 550 nm (OD_{550}). Total cells were defined as OD_{550} of adhesive cells plus OD_{550} of non-adhesive cells and the per cent adherence was calculated by the formula $100 \times (OD_{550}$ of adhesive cells)/(OD_{550} of total cells). All assays were carried out three times, with the mean and standard deviation presented.

The sucrose-dependent adherence of resting cells was determined according to the method presented by Ooshima et al. (20). The test strains were grown in BHI broth with the appropriate antibiotics, as required, at 37°C for 18 h. Cells were collected by centrifugation and washed with 0.1 M potassium phosphate buffer (pH 6.0) containing 0.05% NaN_3 at 4°C. The cells were then re-suspended in the same buffer containing 1% sucrose and adjusted to an OD_{550} of 1.0. Aliquots (3 ml) were incubated at 37°C for 18 h at a 30° angle. The percentage of resting cell adherence was determined as described above.

Expression of GTFs

Immunoblotting of GTFs was performed to assess the amount of expressed protein using CA-GTF and CF-GTF antisera, which were generated in a previous study (kindly provided by Professor Hamada) (6). The tested organisms were grown in BHI broth at 37°C to an OD_{550} of 1.0, then the bacterial cells were re-suspended with phosphate-buffered saline and added to make a final dilution of 1× in SDS gel loading buffer, while the supernatants were dissolved in the same buffer, after being concentrated by 50% ammonium sulfate precipitation. An equal amount of each protein was separated by 7% SDS–PAGE and then transferred onto polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, MA). The transferred protein bands were reacted with anti-rabbit antibodies against CA-GTF or CF-GTF, and then visualized using the alkaline phosphatase-conjugated anti-rabbit immunoglobulin G antibody (New England Biolabs, Beverly, MA) and 5-bromo-4-chloro-3-indolylphosphate-nitro-blue tetrazolium substrate (Moss Inc., Pasadena, MD). The intensities of GTFB, GTFC, and GTFD in all tested strains were then compared visually.

Measurement of CA-GTF and CF-GTF activities

CA-GTF and CF-GTF activities were estimated using ^{14}C-labeled glucose sucrose as described previously (10). The tested organisms were grown in BHI broth at 37°C to an OD_{550} of 1.0, after which 10 ml of the culture was centrifuged, and the cells and supernatant were separated. The cells were washed with 50 mM potassium phosphate buffer (pH 6.0) containing 0.05% NaN_3 at 4°C and resuspended in 50 µl of the same buffer. Next, 10 µl of the cell suspension or supernatant was mixed with 10 µl 20 mM ^{14}C-labeled glucose sucrose (1.85 GBq/ml) in 50 mM potassium phosphate buffer, and incubated at 37°C for 60 min. The reaction mixture was spotted on a square filter paper, then the filter was washed with methanol and immersed in scintillation fluid to estimate the amount of ^{14}C-labeled glucan. One unit of GTF activity was defined as the amount of enzyme needed to incorporate 1.0 µmol of glucose residue from sucrose into glucan per minute (6). The data are expressed as CA-GTF activity (mU) per cell in 1 ml culture and CF-GTF activity (mU) per 1 ml cellular supernatant.

Cell surface hydrophobicity

Cell surface hydrophobicity of the oral streptococci was measured as described by Rosenberg et al. (22). The organisms were cultured in 300 ml BHI broth and collected by centrifugation, then the cells were washed three times and suspended in PUM buffer (potassium phosphate buffer containing 30 mM urea and 0.8 mM $MgSO_4$; pH 6.5) to an OD_{550} of 0.6. Cell suspensions (3.0 ml) were placed in test tubes (13 × 100 mm) and, after being left to stand for 10 min at room temperature, 0.2 ml *n*-hexadecane was added and the tubes were agitated uniformly on a vortex mixer for 1 min. After the *n*-hexadecane phase had separated from the aqueous phase, the OD_{550} of the aqueous phase was determined. Hydrophobicity is expressed as the percentage of absorbance of the hexadecane phase, which was calculated by subtracting the absorbance of the aqueous phase from the absorbance of the initial cell suspension before mixing with hexadecane.

Acid tolerance assay

The bacterial survival of MT8148, AD1, BD1 and CD1 in acid tolerance assays was analysed using the method described by Hanna et al. (8), with some modifications. The tested strains were cultured overnight in TH broth containing 0.3% yeast extract (THYE), then diluted 10-fold in fresh THYE (pH 7.5) and incubated at 37°C until the mid-log phase was reached. Cells were harvested by centrifugation and resuspended in THYE adjusted to pH 7.5 and 5.0 by HCl for unadapted and adapted conditions, respectively, then incubated at 37°C for 2 h. Detection of the acid tolerance response was considered valid when the cells were able to withstand 3 h of incubation at 37°C at a killing pH of 3.5, and the findings were quantitatively confirmed in triplicate by plating cells before and after incubation at killing pH on THYE plates. The results are shown as percent survival rate, which was calculated as follows: (number of cells following incubation at pH 3.5/number of cells before incubation at pH 3.5) × 100 (%).

Statistical analysis

Intergroup differences of various factors were estimated by a statistical analysis of variance for factorial models. Fisher's protected least-significant difference test was used to compare individual groups. Statistical computations were performed using STAT-VIEW II (Macintosh computer application).

Results

Western blotting of anti-Gbp serum with a cellular extract of MT8148 showed single bands for the GbpA protein (approximately 75 000 molecular weight) and GbpB protein (approximately 90 000 molecular weight), while that with anti-GbpA and anti-GbpB antisera revealed no positive bands for GbpA- (AD1) or GbpB- (BD1) (Fig. 1A,B). On the other hand, cellular extracts of MT8148, AD1 and BD1 showed several bands with anti-GbpC serum, while CD1 showed none (Fig. 1C). The maximum size of those bands was approximately 70 000, which is

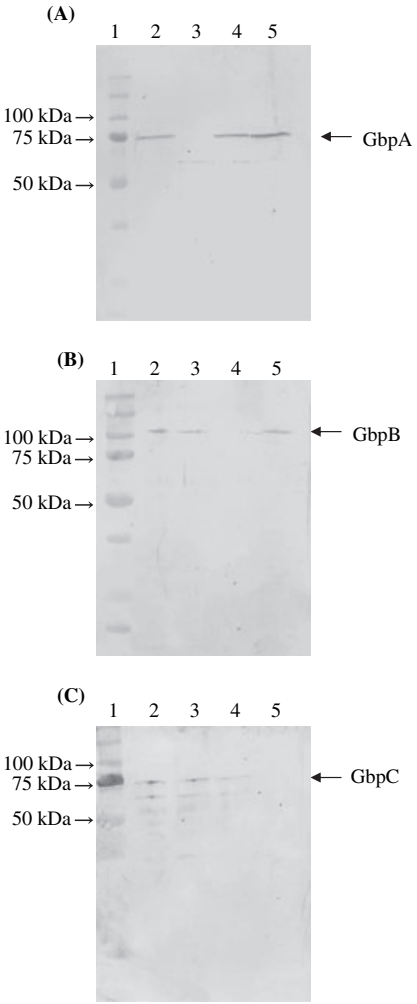

Fig. 1. Western-blotting analysis of *Streptococcus mutans* MT8148 and the Gbp-deficient mutant strains. (A) Anti-GbpA serum. (B) Anti-GbpB serum. (C) Anti-GbpC serum. Lane 1, marker; lane 2, *S. mutans* MT 8148; lane 3, GbpA-deficient mutant strain AD1; lane 4, GbpB-deficient mutant strain BD1, lane 5, GbpC-deficient mutant strain CD1.

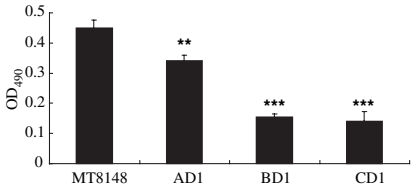

Fig. 2. Dextran-binding assay of *Streptococcus mutans* MT8148 and Gbp-deficient mutant strains. There were statistically significant differences between *S. mutans* MT8148 and the Gbp-deficient mutant strains, as shown by Fisher's PLSD analysis (**$P < 0.01$, ***$P < 0.001$).

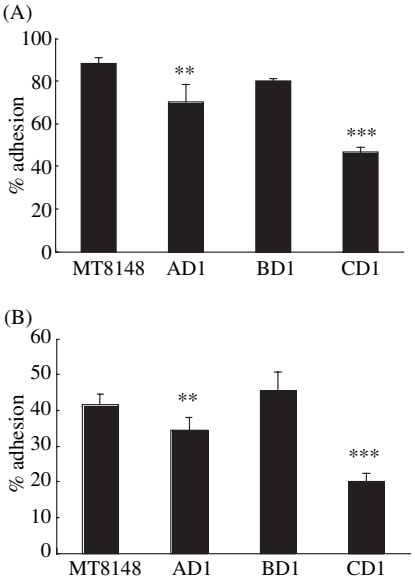

Fig. 3. Sucrose-dependent adhesion of *Streptococcus mutans* MT8148 and Gbp-deficient mutant strains. (A) Sucrose-dependent adhesion by growing cells. (B) Sucrose-dependent adhesion by resting cells. There were statistically significant differences between *S. mutans* MT8148 and the Gbp-deficient mutant strains, as shown by Fisher's PLSD analysis (**$P < 0.01$, ***$P < 0.001$).

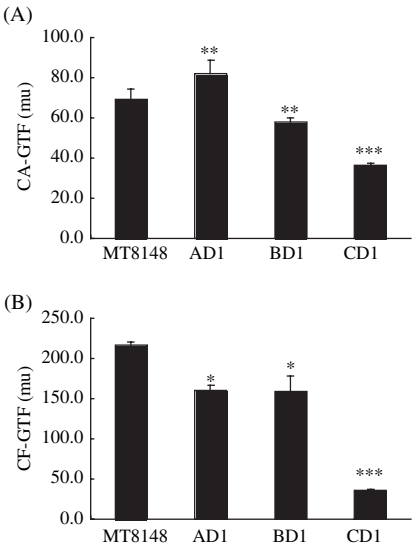

Fig. 4. GTF activities of *Streptococcus mutans* MT8148 and Gbp-deficient mutants. (A) CA-GTF activity. (B) CF-GTF activity. There were statistically significant differences between *S. mutans* MT8148 and the Gbp-deficient mutant strains, as shown by Fisher's PLSD analysis (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

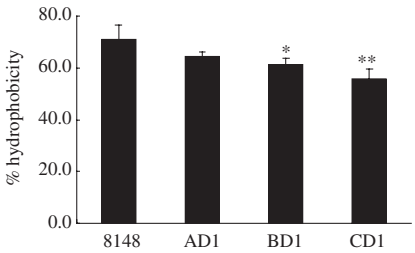

Fig. 5. Hydrophobicity of *Streptococcus mutans* MT8148 and Gbp-deficient mutant strains. There were statistically significant differences between *S. mutans* MT8148 and the Gbp-deficient mutant strains, as shown by Fisher's PLSD analysis (*$P < 0.05$, **$P < 0.01$).

the size of the GbpC protein. The anti-GbpC serum did not react with *S. mutans* GTF or the other Gbp proteins (data not shown).

The dextran-binding activities of all three Gbp mutant strains were significantly lower than that of MT8148 (Fig. 2), with the reduction rates of AD1, BD1 and CD1 determined to be 24%, 66% and 69%, respectively. AD1 and CD1 had significantly lower rates of sucrose-dependent adhesion by both growing and resting cells, whereas there were no significant differences in sucrose-dependent adhesion of either growing or resting cells between MT8148 and BD1 (Fig. 3).

GTFB, GTFC and GTFD were found to be expressed in all of the tested strains, however, the amounts of expression varied. The expression of CA-GTF in MT8148, AD1, BD1 was similar, while its expression in CD1 was significantly different because the GTFB band in CD1 was weak compared to those in the other strains. On the other hand, the expression of CF-GTF in MT8148, BD1 and CD1 was nearly the same, while that in AD1 was degraded. Furthermore, the activities of the Gbp-deficient mutants varied and were significantly different from those of MT8148 (Fig. 4). The CA-GTF activity in AD1 was higher than that in MT8148, whereas the activities in BD1 and CD1 were significantly lower than in MT8148. As for the activity of CF-GTF, all of the Gbp-deficient mutant strains showed significantly lower levels than in MT8148.

In the hydrophobicity assays, the values for all of the Gbp-deficient mutants were lower than for MT8148, with those of BD1 and CD1 significantly different from MT8148 (Fig. 5). Furthermore, the acid tolerance assays revealed that all of the Gbp-deficient mutants were more sensitive to low pH conditions, with BD1 showing a significantly lower survival rate in an acid environment than MT8148, AD1 and CD1 (Fig. 6, $P < 0.05$).

Discussion

The role of Gbps in the virulence of *S. mutans* is thought to be related to cohesive

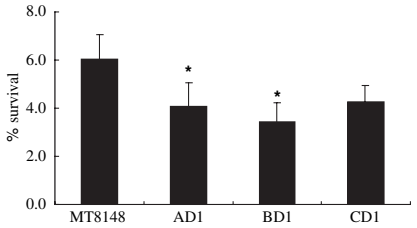

Fig. 6. Survival rates in acid tolerance assays of *Streptococcus mutans* MT8148 and Gbp-deficient mutant strains. There were statistically significant differences between *S. mutans* MT8148 and the Gbp-deficient mutant strains, as shown by Fisher's PLSD analysis (*$P < 0.01$).

plaque formation (2). In the present study, the GbpA-deficient mutant AD1 showed lower activities for dextran-binding (Fig. 2), sucrose-dependent adhesion (Fig. 3), acid tolerance (Fig. 6) and CF-GTF activity (Fig. 4B) as compared to MT8148, while hydrophobicity was similar to that of MT8148. Although AD1 demonstrated a higher level of CA-GTF, the sucrose-dependent activity of AD1 was lower than that of the parent strain. This may have been caused by the low level of activity of CF-GTF, as an optimum ratio of the three GTFs is required for the adhesion of *S. mutans* to a hard surface (20). Also, it has been demonstrated that the structure of a biofilm is changed by GbpA deficiency (9). Although enzyme activities have not been shown in glucan-binding proteins (26), the role of enzymes in the virulence of *S. mutans* has been implicated in assays of cohesive plaque formation, cellular adherence and accumulation in plaque (1). Furthermore, our results suggest that GbpA has a relationship with GTF expression and may regulate the sucrose-dependent adhesion of *S. mutans*. On the other hand, the GbpC-deficient mutant strain CD1 showed the lowest levels of dextran-binding (Fig. 2), sucrose-dependent adhesion (Fig. 3), GTF activity (Fig. 4) and hydrophobicity (Fig. 5). Although GbpC is expressed in the stressed condition of growth and has no sequence similarities to the glucan-binding domains of GTFs (24), it is involved in α-1,6-glucan-dependent cellular aggregation and is considered to be a major cell-surface glucan receptor; it was also shown to preferably bind to the water-insoluble α-1,6-glucan synthesized by GTFD in our previous study (16). Together, these results indicate that among the three Gbps, GbpC is most closely related to the cariogenicity of *S. mutans*. In addition, we previously showed that the caries-inducing activity of a GbpC-deficient mutant was significantly lower than that of a wild strain in rats (17).

The GbpB-deficient mutant BD1 showed the lowest levels of dextran-binding (Fig. 2), GTF (Fig. 4), hydrophobicity (Fig. 5), and acid tolerance (Fig. 6) among the tested strains. However, surprisingly, BD1 showed a sucrose-dependent adhesion activity similar to the wild strain MT8148 (Fig. 3). These findings suggest that GbpB has a function that is different from that of the other Gbps. In addition, BD1 was the most sensitive to acid among all of the strains in the acid tolerance assays. It is widely accepted that bacteria living in biofilms are more resistant to mechanical, physical and chemical stresses (4, 11, 19), and the ability of *S. mutans* to survive at low pH is an important factor in biofilm formation. GbpB has been shown to have good homology with polypeptide protein required for cell separation of group B streptococcus (PcsB) (21). Both GbpB and PcsB are secreted, cell-associated and contain a conserved cysteine residue in the C-terminal domain that is necessary for peptidoglycan hydrolytic activity (5, 18). Thus, GbpB may have an important role in cell construction and may play some important roles in biofilm formation.

Taken together, our results show that GbpB in *S. mutans* may have other biological functions. Additional research is necessary to elucidate those functions to clearly understand the role of Gbps in the cariogenicity of *S. mutans*.

Acknowledgments

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