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Biological functions of glucan-binding protein B of *Streptococcus mutans*

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Introduction: *Streptococcus mutans* has been implicated as a major causative agent of dental caries in humans. Bacterial components associated with the adhesion phase of *S. mutans* include glucosyltransferases, protein antigen C and proteins that bind glucan. At least four glucan-binding proteins (Gbp) have been identified; GbpA, GbpB, GbpC and GbpD.
Methods: In our previous study, the contributions of GbpA and GbpC to the virulence of *S. mutans* were investigated; however, the biological function of GbpB and its role in the virulence of *S. mutans* remain to be elucidated. Using a GbpB-deficient mutant strain (BD1), we demonstrated in the present study that GbpB has a role in the biology of *S. mutans*.
Results: The growth rate of BD1 was lower than that of other strains, while it was also shown to be less susceptible to phagocytosis and to form longer chains than the parental strain MT8148. In addition, electron microscope observations of the cell surfaces of BD1 showed that the cell-wall layers were obscure.
Conclusion: These results suggest that GbpB may have an important role in cell-wall construction and be involved in cell separation and cell maintenance.

Key words: cell wall; glucan-binding protein B; *Streptococcus mutans*

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Streptococcus mutans has been implicated as a primary causative agent of dental caries in humans (5, 6). One of the important virulence properties of the organisms is their ability to form a biofilm, known as dental plaque, on tooth surfaces. *S. mutans* synthesizes at least four glucan-binding proteins (GbpA, GbpB, GbpC and GbpD). Among them, GbpB has been purified and shown to be immunologically distinct from other Gbps expressed by *S. mutans* and *Streptococcus sobrinus* (18). Primary sequence analysis of the *gbpB* gene revealed the presence of a leucine zipper domain, whereas no sequence homologous to the glucan-binding domain of any glucosyltransferases or GbpA was found (9). Nevertheless, GbpB is considered to play some role in the cariogenicity of *S. mutans* because mucosal immunization with this protein has been shown to induce protective immune responses against experimental dental caries (17). Clinical studies have shown that GbpB is the antigen most commonly recognized by antibodies present in the saliva of young children (13). GbpB was also shown to possess a homology to peptidoglycan hydrolases of other Gram-positive microorganisms, and the results of a comparative genomic analysis of the *gbpB* region suggested a functional relationship between the genes involved in cell shape and those involved in cell-wall maintenance (9), while GbpB may also have a role in peptidoglycan synthesis and cell division (2) because it was recently shown to interact with the ribosomal protein involved in those activities (10). Furthermore, we previously reported that GbpB in *S. mutans* might have another biological function in addition to caries-inducing activity, while GbpA and GbpC were each found to have a strong relationship with cariogenicity (8). Since the biological functions of GbpB remain to be elucidated, we examined a GbpB-deficient mutant to determine its biological properties in the present study.

Materials and methods

Bacterial strains

All the bacterial strains used in this study are listed in Table 1. The construction of Gbp-deficient mutant strains has been described previously (8). In brief, GbpA-deficient, GbpB-deficient and GbpC-deficient mutant strains were constructed by insertional inactivation of the *gbpA*, *gbpB* and *gbpC*

Table 1. Bacterial strains used in the present study

Strain	Relevant characteristics	References
MT8148	Serotype *c* human isolate	14
AD1	GbpA-deficient mutant strain of MT8148, Em^r	8
BD1	GbpB-deficient mutant strain of MT8148, Spe^r	8
CD1	GbpC-deficient mutant strain of MT8148, Km^r	8

Em^r, erythromycin-resistant; Spe^r, spectinomycin-resistant; Km^r, kanamycin-resistant.

genes. The appropriate insertional inactivation into the mutants AD1, BD1 and CD1 was confirmed by determining the nucleotide sequence of the antibiotics cassette inserted into each gene, as well as by Western blotting of whole cells from mutant strains by GbpA-, GbpB-, or GbpC-specific rabbit antiserum (7, 8). Each was grown in Todd Hewitt (TH) broth (Becton Dickinson Co., Franklin Lakes, NJ) or in TH broth containing 0.3% yeast extract (THYE), as required, with the appropriate antibiotics (erythromycin 10 μg/ml, kanamycin 500 μg/ml and spectinomycin 1 mg/ml) used for selection.

Bacterial growth rates

Following overnight culture of strains MT8148, AD1, BD1 and CD1, 1 ml of each was inoculated into 50 ml TH broth at 37°C, from which 1 ml of each culture was collected at hourly intervals. Absorbance at 600 nm was determined using a spectrophotometer (Shimadzu, Kyoto, Japan), and the growth rate was calculated by measuring the mean doubling time between optical densities at 600 nm (OD_{600}) of 0.2 and 0.6, as described previously (15).

Bacterial chain length

The bacterial chain lengths of MT8148, AD1, BD1 and CD1 were determined in THYE cultures at pH 7.5 as described previously (11) using NATIONAL INSTITUTES OF HEALTH IMAGE (Version 1.61, Macintosh computer application, Scion, MD), with the results expressed as the mean ± SE of 50 chains examined for each.

Determination of antimicrobial susceptibility

Antibiotic susceptibility was determined using a solid-medium dilution method in accordance with Clinical and Laboratory Standards Institute recommendations for streptococci (3). Vancomycin, erythromycin, tetracycline and kanamycin were purchased from Wako Chemical Co. (Osaka, Japan), while gentamicin was obtained from Sigma Chemical Co. (St Louis, MO), ofloxacin was from LKT Laboratories, Inc. (St Paul, MN) and penicillin was from MEIJI SEIKA Ltd. (Tokyo, Japan). The antibiotics were used at concentrations ranging from 0.003 to 1 μg/ml.

Phagocytosis assay

Susceptibility to phagocytosis by human polymorphonuclear leukocytes (PMNs) was evaluated according to a method described previously (11). Briefly, MT8148, AD1, BD1 and CD1 were cultured separately in TH broth for 18 h at 37°C. After washing the bacterial cells, the cell concentrations were adjusted with phosphate-buffered saline (PBS; 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 140 mM NaCl, 2.7 mM KCl, pH 7.3) to 1.0×10^8 colony-forming units (CFU)/ml. Human peripheral blood (500 μl) was then collected from a healthy volunteer and incubated with 500 μl (5.0×10^7 CFU) of the tested bacteria for 10 min at 37°C. Interactions between PMNs and the bacteria were observed under a light microscope (magnification ×100; Nikon Co. Ltd., Tokyo, Japan) following Giemsa staining (Wako Chemical Co.). The results are expressed as the mean ratio of phagocytosed PMNs per 100 PMNs, with 500 PMNs examined.

Cell surface observation

Observations of MT8148, AD1, BD1 and CD1 were carried out using a transmission electron microscope to examine the spatial distribution and architecture of the cells. The tested strains were cultured overnight in TH broth at 37°C, then the cells were washed with 0.1 M sodium phosphate buffer (pH 7.0) and fixed with 2.5% glutaraldehyde. After dehydration, the cells were embedded in Epon, then cut into ultrathin sections and used for transmission electron microscopy on an H-300 electron microscope (Hitachi Ltd., Tokyo, Japan).

Statistical analysis

Intergroup differences of various factors were estimated by a statistical analysis of variance (ANOVA) for factorial models. Fisher's protected least-significant differences test was used to compare individual groups.

Results

Doubling time of Gbp-deficient mutants

Evaluations of bacterial growth showed that BD1 grew more slowly than MT8148, AD1 and CD1, because the doubling times of BD1 were 173.2 ± 1.7, 103.5 ± 1.0 and 83.6 ± 1.0 min under initial conditions of pH 5.5, pH 6.5 and pH 7.5, respectively, which were significantly longer than those for MT8148, AD1 and CD1 at the same pH levels ($P < 0.001$) (Table 2). Further, the doubling times of CD1 were shorter than those of the parental strain MT8148 at all of the pH conditions examined ($P < 0.01$).

Properties related to cell-surface structure

The phagocytosis rates for strains AD1, BD1 and CD1 after 10 min of incubation with PMNs were 75.2 ± 4.4%, 71.6 ± 4.3% and 62.4 ± 6.7%, respectively, which were significantly lower than that of MT8148 (88.4 ± 2.2%) (Table 3). In addition, optical microscopy showed extremely long chains in BD1 (Fig. 1) because the BD1 chain length was approximately three times longer than that of any of the other strains at pH 7.5 (Fig. 2). The antibiotic susceptibility assay revealed that BD1 had a tendency of increasing sensitivity to penicillin, erythromycin and tetracycline (Table 4). Further, observation by transmission electron microscopy showed two layers on the cell surfaces of MT8148, AD1 and CD1, while the cell surface of BD1 was unclear and it was difficult to distinguish any layers (Fig. 3).

Discussion

GbpB was first purified and characterized in 1994 (16), while the *gbpB* gene

Table 2. Doubling time of MT8148 and its Gbp-deficient mutants

	Doubling time		
Strain	Initial pH 5.5	Initial pH 6.5	Initial pH 7.5
MT8148	134.4 ± 2.6	71.2 ± 2.4	70.7 ± 1.7
AD1	135.8 ± 6.0	68.1 ± 0.7*	70.9 ± 1.2
BD1	173.2 ± 1.7***	103.5 ± 1.0***	83.6 ± 1.0***
CD1	115.2 ± 7.4**	63.0 ± 0.8**	66.2 ± 2.8*

There were statistically significant difference between MT8148 and its Gbp-deficient mutants: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 3. Phagocytosis rates of MT8148 and its Gbp-deficient mutants

Strain	Phagocytosis rate (%)
MT8148	88.40 ± 2.19
AD1	75.20 ± 4.38***
BD1	71.60 ± 4.34***
CD1	62.40 ± 6.69***

There were statistically significant difference between MT8148 and its Gbp-deficient mutants: ***$P < 0.001$.

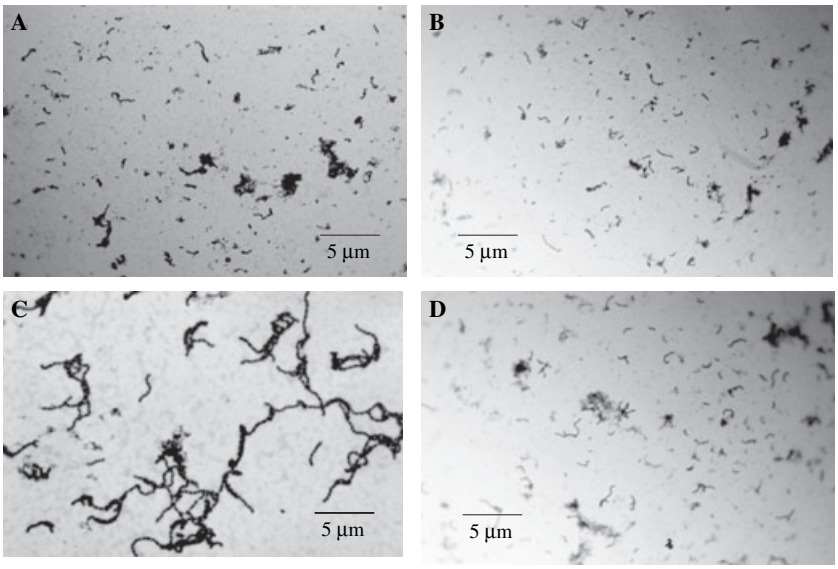

Fig. 1. Light microscopic images of MT8148 and its Gbp-deficient mutants at initial pH 7.5. (A) MT8148, (B) GbpA-deficient mutant strain AD1, (C) GbpB-deficient mutant strain BD1 and (D) GbpC-deficient mutant strain CD1.

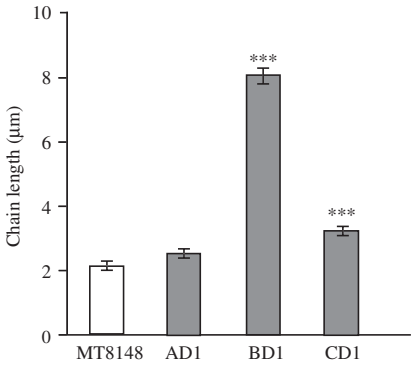

Fig. 2. Chain lengths of MT8148 and its Gbp-deficient mutants at pH 7.5. There were statistically significant differences between MT8148 and the Gbp-deficient mutant strains (***$P < 0.001$).

encoding GbpB was cloned and sequenced in 2001 (9). The predicted amino acid sequence has been shown to share an extensive homology with a putative peptidoglycan hydrolase from group B streptococcus (9). On the other hand, the biological function of GbpB and its role in the virulence of *S. mutans* remain to be elucidated. Mattos-Graner et al. (10) reported that they could not obtain a GbpB-deficient mutant strain and speculated that GbpB was essential for growth. However, Chia et al. (2) reported construction of a mutant strain that was deficient for IDG-60 (GbpB) and found that GbpB was associated with cell maintenance. We successfully constructed a GbpB-deficient mutant strain in our previous study and reported that GbpB possessed a glucan-binding property (8), though the level of binding was lower compared to other Gbps. In the present study, we investigated the biological functions of GbpB using GbpA-, GbpB- and GbpC-deficient mutant strains (AD1, BD1 and CD1, respectively). BD1 grew more slowly than the parental strain MT8148 as well as the GbpA- and GbpC-deficient mutant strains, based on its lower doubling time (Table 2). The instability of the GbpB mutant suggests that the protein may be essential for bacterial growth, while its homology to peptide glycan hydrolase implicates GbpB as a crucial component of cell-wall cycling and synthesis (1).

The protein antigen c (PAc) of *S. mutans* was shown to be correlated with the susceptibility to phagocytosis by human PMNs (11), though the association of glucan-binding proteins and phagocytosis susceptibility has not been clarified. All of the Gbp-deficient mutants used in the present study showed a lower phagocytosis rate than their parental strain MT8148 (Table 3). Recently, analysis of the phagocytosis rate of MT8148 strains with varying chain lengths produced by culturing in media with different initial pH levels showed that there was a statistically significant negative correlation between phagocytosis rate and chain length, suggesting that strains with an ability to produce a longer chain might be less susceptible to phagocytosis (11). It should be noted that the present GbpB-deficient mutant strain BD1 formed longer chains compared to those of the parental strain and the other Gbp-deficient mutant strains (Figs. 1, 2). Therefore, the mechanism of lower susceptibility to phagocytosis of BD1 may be a result of its long chain formation caused by the deficiency of GbpB, which is presumably different from the mechanisms of the GbpA- and GbpC-deficient mutants.

We also found that strain BD1 was approximately twice as susceptible to penicillin, erythromycin and tetracycline compared to MT8148 (Table 4). *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Streptococcus pyogenes* have each been shown to have defects in cell-wall biosynthesis and turnover, resulting in an altered susceptibility to antibiotics (4). Most gram-positive bacteria are surrounded by a thick peptidoglycan cell wall, which appears to offer little resistance to the diffusion of small molecules, such as those of antibiotics, because of its coarse

Table 4. Minimum inhibitory concentrations for seven antibiotics in MT8148 and its Gbp-deficient mutants

	MICs (μg/ml)			
Antibiotic	MT8148	AD1	BD1	CD1
Vancomcin	0.5	0.5	0.5	0.5
Gentamicin	1	1	1	1
Ofloxacin	1	1	1	1
Penicillin	0.064	0.064	0.032	0.064
Erythromycin	0.24	ND	0.12	0.24
Tetracycline	0.25	0.25	0.125	0.25
Kanamycin	25	25	25	ND

ND, not done.

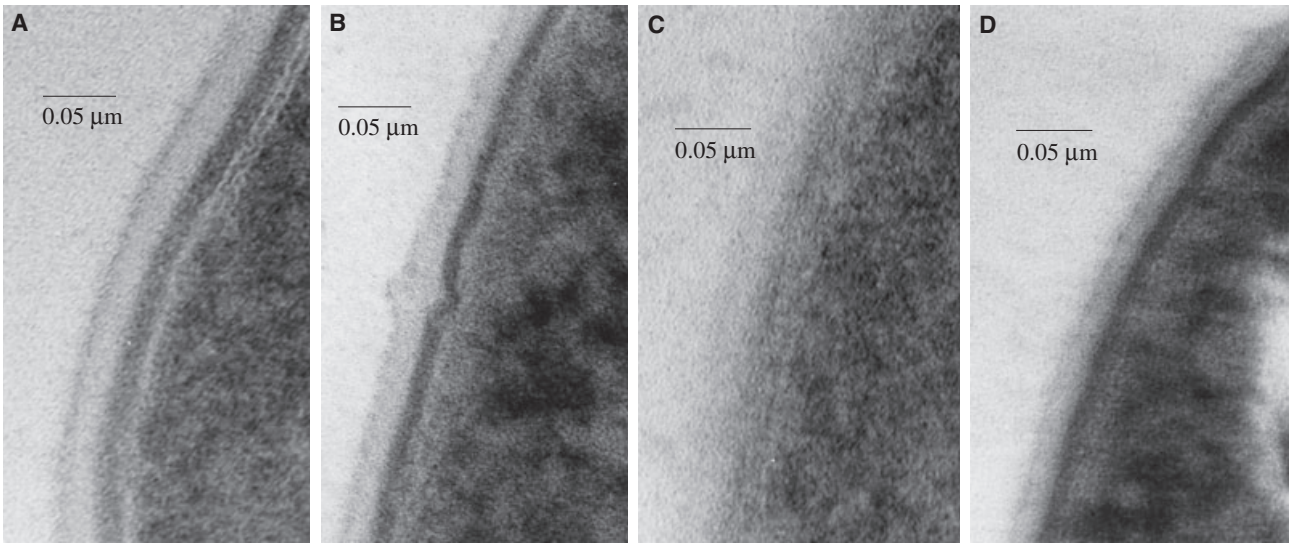

Fig. 3. Transmission electron microscopic images of MT8148 and its Gbp-deficient mutants. (A) MT8148, (B) GbpA-deficient mutant strain AD1, (C) GbpB-deficient mutant strain BD1 and (D) GbpC-deficient mutant strain CD1.

meshwork structure (12). Our results suggest that the defect linked to GbpB caused an alteration of the cell-surface structure.

Electron microscopic observations of the cell surfaces of strain MT8148 and its Gbp-deficient mutants revealed that BD1 alone had unclear layers in the cell wall region (Fig. 3). Gram-positive bacteria such as *S. mutans* have a thick cell wall, which is mainly composed of peptidoglycan and lipoteichoic acid (19); however, the peptidoglycan layer was obscure in BD1. Therefore, we speculated that the primary ligand for glucan-binding in GbpB might be located within the cell wall. Further, GbpB may be an enzyme of which the glucan-binding properties help keep it associated with the cell, though its putative amino acid sequence does not contain a cell-wall anchor motif. Taken together, we consider that GbpB has important biological properties regarding cell separation and cell wall maintenance.

In summary, the present results suggest that GbpB in *S. mutans* may have an important role in cell-wall construction, as well as having relationships with cell separation and cell-wall maintenance.

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