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Role of phosphoglucosamine mutase on virulence properties of *Streptococcus mutans*

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Introduction: *Streptococcus mutans* has been strongly implicated as the principal etiological agent in dental caries. As a gram-positive bacterium, *S. mutans* has a thick and compact cell wall to maintain the cell shape and protect the cells against mechanical or osmotic damage. Previous studies have proved that peptidoglycan is the main component of the cell wall involved in the autolysis or biofilm formation processes.
Methods: In this study, we investigated the gene *SMU.1426c* in the amino-sugar metabolism pathway of *S. mutans* UA159, which encodes phosphoglucosamine mutase (GlmM). The *glmM* gene that functions in the biosynthesis of peptidoglycan has been well investigated in *Escherichia coli*. Here a *glmM* mutant strain of *S. mutans* UA159 was constructed and several virulence properties were investigated.
Results: The mutant devoid of the *glmM* gene displayed long chains, reduced growth rate and increased autolysis. Biofilm formation by the mutant was found to be attenuated.
Conclusion: These results proved that peptidoglycan biosynthesis plays an important part in a series of bacterial morphologies. The *glmM* gene may have a constructive role in the virulence properties of *S. mutans*.

Key words: gene inactivation; phosphoglucosamine mutase gene; *Streptococcus mutans*; virulence properties

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Streptococcus mutans is the primary etiological agent of dental caries in humans (8). It has a thick and compact cell wall which surrounds the cytoplasmic membrane and gives the cell shape. This rigid protective structure plays an important role in bacterial survival. It maintains a high solute concentration inside the bacterial cell and supports a great pressure from the membrane to protect the bacterium from osmotic lysis.

In gram-positive cells, the peptidoglycan layer makes up as much as 90% of the cell wall. It is a heavily cross-linked woven structure that wraps around the cell. Cell walls without intact peptidoglycan are structurally weak. Phosphoglucosamine mutase (GlmM) catalyses the interconversion of glucosamine-1-phosphate (GlcNAc-1P) and glucosamine-6-phosphate (GlcNAc-6P) isomers. It is the first step in the biosynthetic pathway leading to UDP-*N*-acetylglucosamine (UDP-GlcNAc), which is one of the main cytoplasmic precursors of bacterial cell wall peptidoglycan (21). When grown at 43°C for 5 h the *Escherichia coli* thermosensitive *glmM* mutant strain GPM83 revealed a decreased UDP-GlcNAc and peptidoglycan content compared with the wild-type strain JM83. After 5–6 h at 43°C, the growth rate of GPM83 was abruptly reduced and the cells entered a stationary phase at a lower cell density. In addition, GPM83 cells progressively changed from rods to greatly enlarged ovoids. If peptidoglycan content of the cell decreases to a critical value (about 60%), the cell will lyse (16). Loo et al. isolated a total of 18 *Streptococcus gordonii* biofilm-defective mutant strains among 25,000 transposon mutants generated by *Tn916* transposon mutagenesis. Biofilm formation was assayed and categorized based on the absorbance at 575 nm of the crystal-violet-stained biofilm, performed on polystyrene plates. One of the biofilm-defective mutants had a gene homologous to *glmM* (14).

We speculated that in *S. mutans*, if GlmM could also affect peptidoglycan synthesis indirectly, the changes to the cell wall might influence the growth kinetics of the bacteria, the accumulation of cells or, maybe, the development of biofilm. Changes to the phenotypes would surely affect the critical virulence of *S. mutans.*

We found GlmM (EC 5.4.2.10), which catalyses D-GlcNAc-1P from D-GlcNAc-6P in the kyoto encyclopedia of genes and

genomes (KEGG) amino-sugars metabolism pathway of *S. mutans*. The accession number of this pathway in the KEGG pathway database is smu00530. The corresponding gene on the chromosome of *S. mutans* was *glmM* (SMU.1426c). Guo et al. (6) compared two clinical isolates of *S. mutans* by suppression subtractive hybridization and the gene fragment of *glmM* in *S. mutans* UA159 was found only in the strain with suspected virulence traits. So, this *GlmM* may play a part in the virulence of *S. mutans*. The purpose of this investigation was therefore to construct a *glmM* mutant strain of *S. mutans* and compare it with the wild-type strain. The phenotypic differences displayed in wild-type and *glmM* mutant strains will help to investigate the gene function.

Materials and methods

Bacterial strains and culture conditions

E. coli DH5α was grown in Luria medium with aeration at 37°C, and *S. mutans* strain UA159, UA159 *glmM*-deficient strain and UA159 *glmM*-complement strain were grown in brain–heart infusion medium (BHI medium; Becton Dickinson and Company, Sparks, MD) with 5% CO_2 at 37°C. For selection of antibiotic-resistant colonies after genetic transformation, spectinomycin (100 μg/ml for *E. coli* or 1 mg/ml for *S. mutans*) or kanamycin (35 μg/ml for *E. coli* or 1 mg/ml for *S. mutans*) was added to the medium. To grow biofilms, a biofilm medium was prepared by the modification of a method described previously (14). This medium contained 58 mM K_2HPO_4, 15 mM KH_2PO_4, 10 mM $(NH_4)_2SO_4$, 35 mM NaCl, filter-sterilized vitamins (0.04 mM nicotinic acid, 0.1 mM pyridoxine HCl, 0.01 mM pantothenic acid, 1 μM riboflavin, 0.3 μM thiamin HCl, 0.05 μM D-biotin), amino acids (4 mM L-glutamic acid, 1 mM L-arginine HCl, 1.3 mM L-cysteine HCl, 0.1 mM L-tryptophan), 0.2% (weight/volume) Casamino acids, and supplemented with glucose or sucrose at a final concentration of 20 mM. Biofilms of *S. mutans* were developed in biofilm medium with proper antibiotics at 37°C with 5% CO_2.

Construction of mutant strains

An open reading frame for the *glmM* gene region was identified in the *S. mutans* UA159 database at the University of Oklahoma Advanced Center for Genomic Technology (http://www.genome.ou.edu/smutans.html); its GenBank accession no. is AAN59090. BLASTn and BLASTp sequence homology analyses were performed by using the BLAST network service of the National Center for Biotechnology Information (NCBI), Bethesda, MD.

The pFW5 ($Spec^r$) *E. coli*–streptococcus shuttle plasmid was used to create the mutant *glmM* gene. The up and down fragments located in the upstream and downstream of the *glmM* were amplified from the *S. mutans* UA159 genomic DNA template using the primers upF (5′-GC GCGGATCCCTCTTCTGTACTAGGTGC TT) and upR (5′-GCGCAAGCTTACG TTCGGGCTATAATCT) as well as downF (5′-GCGCGCATGCTAAAGCCATCACC ACCAA) and downR (5′-GCGCACTA GTGAGGCAAATGTAGAACTAACAC) (The restriction enzyme sites are underlined). The fragments were digested by *Bam*HI–*Hin*dIII and *Sph*I–*Spe*I (New England Biolabs, Ipswich, MA) respectively and inserted into pFW5, which was digested with the same enzymes. The resulting plasmid pFW5-*glmM* with up and down fragments in its multiple cloning sites I and II was then checked by restriction analysis, polymerase chain reaction (PCR) and sequencing. Then pFW5-*glmM* was linearized with a unique *Nhe*I site on the vector and *S. mutans* UA159 was transformed with the resultant linear plasmid by competence-stimulating peptide-induced natural transformation (13, 17) (The vector pFW5 and competence-stimulating peptide were presented by Dr Shi Wenyuan from the Department of Oral Biology and Medicine, UCLA School of Dentistry, Los Angeles, CA). Transformants were selected on BHI agar containing 1 mg/ml spectinomycin. Confirmation of DNA recombination was performed by PCR and nucleotide sequence analysis.

Growth kinetics

Overnight cultures of *S. mutans* UA159 and the *glmM*-deficient strain were adjusted to the same optical density at 630 nm (OD_{630}) and diluted 100-fold in fresh BHI medium. The OD_{630} of both cultures were monitored every 30 min. Each sample was assayed in triplicate, and uninoculated medium was used as blank controls during the 24-h incubation.

Autolysis assay

Autolysis assays were performed as described by Wen et al. (22). *S. mutans* UA159 and the *glmM*-deficient strain were grown in BHI medium and cell samples (30 ml) were collected at late-exponential-stage (OD_{630} = 0.6). After two washes with ice-cold water, bacteria were suspended in 30 ml 50 mM Tris–HCl (pH 8.0) buffer containing 0.2% (volume/volume) Triton X-100. The cells were then incubated at 37°C with 300 r.p.m. agitation (Thermomixer comfort, Eppendorf), and the absorbance at 630 nm was measured at 30-min intervals.

Construction of complemented strains

A fragment of 1650 bp containing the entire *glmM* coding sequence plus 300 bp upstream of the start codon were amplified using primers psmF (5′-GCGCGGGC CCAGTACCGATTATTGTTGAGTTAACA) and psmR (5′-GCGCGGGCCCTTAAATG CCAATTTCTGTTTTGACA) (The *Sma*I restriction enzyme sites are underlined). To complement the *glmM*-deficient mutant strain, the PCR product was directly cloned into the shuttle vector pDL289 (2). The resulting plasmid pDL289-*glmM* was verified by PCR and restriction analysis, and the amplification of the insert was confirmed by DNA sequencing. pDL289-*glmM* which contains the intact *glmM* with its apparent cognate promoter, was used to transform the *S. mutans* UA159 *glmM*-deficient strain. The resulting complementing strain *S. mutans* UA159 *glmM*-complement has double-antibiotic resistance to spectinomycin and kanamycin. The possession of pDL289-*glmM* in this complementing strain was further verified by PCR.

Biofilm formation assay

Biofilm formation was quantified as previously described (1). *S. mutans* UA159, the *glmM*-deficient strain and the *glmM*-complement strain were grown in BHI medium. When the cultures reached an OD_{630} of 0.4, these cultures were diluted 1 : 100 in biofilm medium with glucose or sucrose (20 mM). Every 200 μl diluted culture was distributed in the well of a 96-well flat-bottomed polystyrene microtiter plate (Corning, New York, NY). After incubation for 21 h, the plates were rinsed with distilled water to remove planktonic and loosely bound cells and air-dried. Then, 50 μl 0.1% (W/V) of crystal violet was added to each well and the plates were incubated for 15 min at room temperature. After removing the crystal violet, the wells were rinsed twice with distilled water and air-dried. Then the crystal violet remaining was solubilized in 99% ethanol, and the OD_{490} was determined.

Scanning electron microscopy

S. mutans UA159, UA159 *glmM*-deficient and the *glmM*-complement strains were grown in BHI medium to an OD_{630} of 0.5 and 100 μl of this culture was dropped onto sterile coverglasses. These glasses were placed in six-well polystyrene microtiter plates containing 10 ml biofilm medium with 20 mM sucrose. After incubation for 21 h, the medium with planktonic cells was aspirated and the glass was carefully rinsed twice with 1 ml sterile physiological saline. The biofilm was fixed with 2.5% glutaric dialdehyde at 4°C. After another wash with phosphate buffer, the sample was fixed with 1% osmic acid. Following dehydration through a graded series of ethanol rinses and drying at critical point with liquid CO_2, the glass slides were sputter-coated with gold and analysed by scanning electron microscopy (JSM-5600LV; JEOL, Tokyo, Japan).

Results

Construction of *S. mutans glmM* mutant

Nucleotide homology analyses in the NCBI database showed that the *S. mutans glmM* gene had 78% homology to that of *Streptococcus agalactiae*, 70% homology to *Streptococcus thermophilus*, 69% homology to *Streptococcus pneumoniae* and 69% homology to *Streptococcus sanguinis*. The deduced amino acid sequences of the *glmM* gene showed a homology of about 82–86% to the GlmM among the above streptococci. *S. mutans* GlmM is similar (41% identity) to the GlmM protein of *E. coli*.

The *glmM* gene of *S. mutans* UA159 was disrupted via double-crossover homologous recombination. An internal portion of the gene was replaced by a spectinomycin-resistant cassette of pFW5. The double-crossover event was confirmed by various PCRs. Nucleotide sequence analysis also approved the proper construction of the mutant.

Altered general phenotype of *glmM* mutant

In contrast to the parent strain, the colonies formed by *glmM*-deficient bacteria on BHI agar plates were soft and mucoid. After a 24-h culture, the *glmM*-deficient strain formed clumps and cells accumulated at the bottom of the glass tubes while the UA159 wild-type strain displayed a uniformly turbid appearance in BHI medium. Observations on a light microscope showed that the mutant strain formed significantly longer chains [mean ± standard error, 11.4 ± 13.7] compared with those formed by the parent strain (4.4 ± 2.1) (Fig. 1).

Reduced growth rate and increased autolysis of *glmM* mutant

Growth curve analysis of the mutant strain revealed that when grown in liquid medium, *glmM*-deficient strains grew more slowly than the UA159 wild-type strain during exponential phase. The doubling time of the *glmM*-deficient strain was [mean (min/generation) ± standard error, 117.9 ± 7.6] relative to the wild-type (62.3 ± 0.5). While in the stationary phase, the *glmM*-deficient strain was able to reach a final OD_{630} similar to that of the wild-type strain (Fig. 2).

After 7-h incubation of *S. mutans* with 0.2% (V/V) Triton X-100, the *glmM*-deficient strain underwent a higher rate of autolysis (47%) than the wild-type strain (22%) (Fig. 3). A standard Student's *t*-test ($P < 0.001$) indicated that lysis in the *glmM*-deficient strain was significantly greater than in the wild-type.

Mutation of *glmM* results in defect in biofilm formation

To counteract the influence of the growth yields on biofilm formation, both mutant and wild-type strains were incubated for 21 h, therefore both of them reached stationary phase and had the same number of cells. Biofilm formation of the mutant on *in vitro* surfaces such as polystyrene and glass were remarkably different from the parent strain. When grown in 96-well microtiter plates, biofilms formed in biofilm medium with sucrose by both mutant and wild-type strains were smooth. The whole bottom of the well was evenly covered with cells. In contrast, both strains formed rough and clumpy biofilms when grown with glucose, but the biofilm formed by the mutant strain was obviously thinner than the wild-type biofilm. As assessed by the optical density readings after crystal violet staining of the biofilms, disruption of *glmM* caused a biomass reduction of 40.8% in the biofilm medium with sucrose and a 90.8% decrease in the biofilm medium with glucose relative to the biomass of the parent strain (Fig. 4).

Scanning electron microscopy analysis of biofilms grown on the surfaces of glass in biofilm medium with sucrose revealed that the architecture of biofilms formed by the *glmM*-deficient strain appeared to be aberrant. Wild-type biofilms were very uniform with thick, compact layers of cells. The biofilm revealed a typical mushroom-shaped multicellular structure. There were noticeable large gaps in the

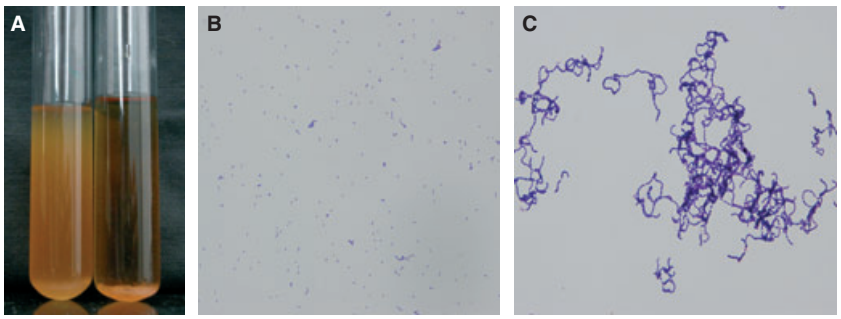

Fig. 1. Morphological characteristics in brain–heart infusion medium. (A) 24-h cultures of *Streptococcus mutans* UA159 (left) and the *glmM*-deficient strain (right). (B) Light microscopic observation of the 24-h cultures of *S. mutans* UA159 (magnification, ×1000). (C) Light microscopic observation of the 24-h cultures of the *glmM*-deficient strain (magnification, ×1000).

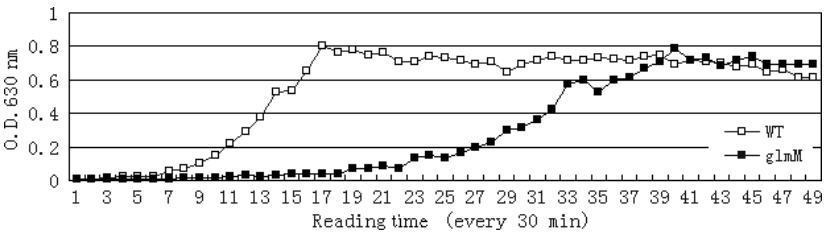

Fig. 2. Growth curves of *Streptococcus mutans* UA159 [wild-type; (WT)] and the *glmM*-deficient strain (glmM). Cultures were grown in brain–heart infusion medium at 37°C with 5% CO_2, and growth was monitored by measuring the absorbance of the cultures at 630 nm. Data points are averages of triplicate samples.

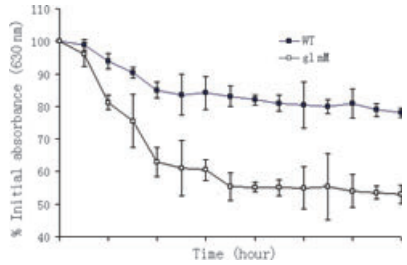

Fig. 3. Autolysis of the whole cells of *Streptococcus mutans* UA159 [wild-type (WT)] and the *glmM*-deficient strain (glmM). Data show the percentage of survival to the initial absorbance at 630 nm. Each datum point is the average of triplicate samples, and the error bars indicate standard deviations.

biofilm matrix. The *glmM* mutant cells accumulated on the glass surface. There was no typical architecture of biofilms but some microcolonies. The number of cells was visibly less than for the wild-type strain (Fig. 5A–D).

The *glmM*-complement strain form similar biofilm to the wild-type

The ability of the *glmM*-deficient strain to form biofilm was mostly complemented by the plasmid pDL289-*glmM*. The *glmM*-complement strain was shown to form similar amounts of biofilm to *S. mutans* UA159 (data not shown) but the appearances of the biofilm formed by the *glmM*-complement and the wild-type strains have some slight differences. Compared to the wild-type, *glmM*-complement displayed a typical three-dimensional structure with multilayer cells (like the wild-type strain) but it presented a sieve-like appearance. There were more gaps visible than the in wild-type and cells were not so tightly accumulated (Fig. 5E,F).

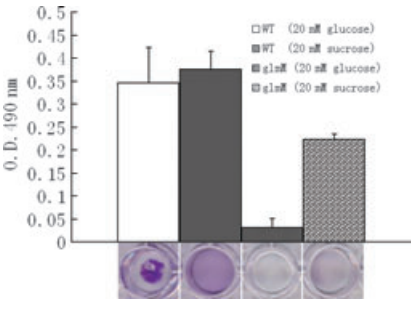

Fig. 4. Biofilm formation of *Streptococcus mutans* UA159 [wild-type (WT)] and the *glmM*-deficient strain (glmM) in biofilm medium supplemented with glucose or sucrose. Each datum point is the average of triplicate samples, and the error bars indicate standard deviations.

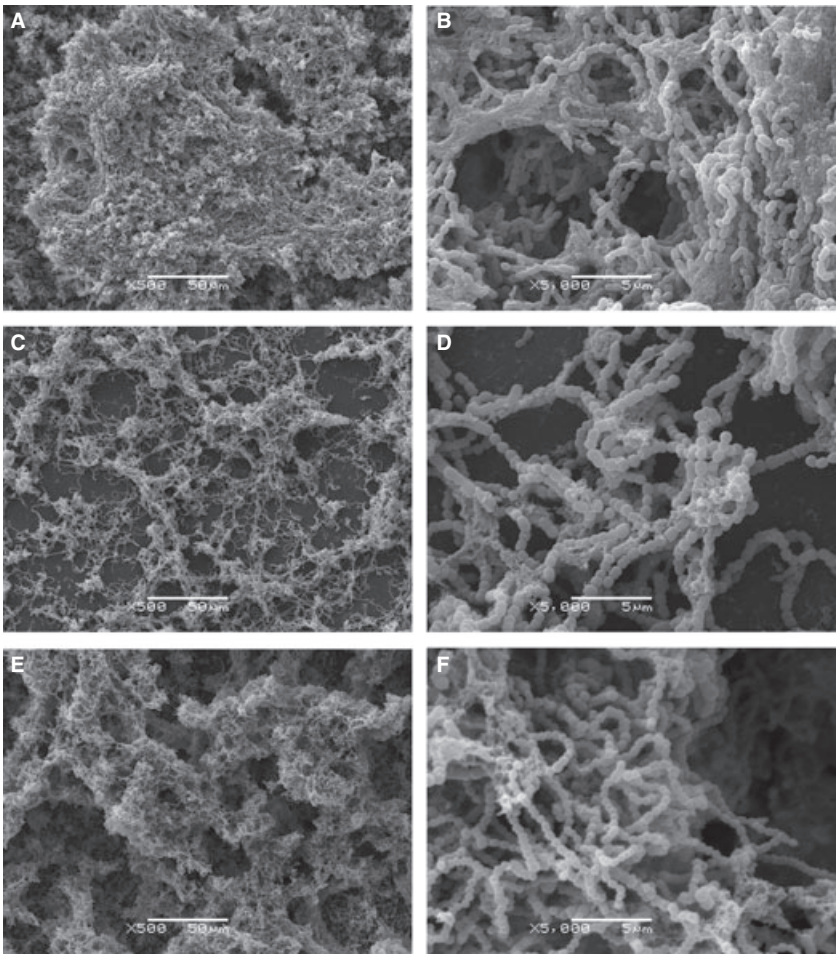

Fig. 5. Scanning electron micrographs of *Streptococcus mutans* biofilms formed on glass surfaces. *Streptococcus mutans* UA159 wild-type biofilms (A,B); *glmM*-deficient strain biofilms (C,D); *glmM*-complement strain biofilms (E,F). Magnifications, ×500 (A,C,E) and ×5000 (B,D,F).

Discussion

Peptidoglycan biosynthesis and bacterial division

The bacterial cell wall is a highly dynamic structure that can expand or rebuild as the cells grow or divide. The cell wall contains a semi-rigid, tight-knit molecular complex called peptidoglycan. As the main component of the cell wall, the continual synthesis of peptidoglycan provides the basic material for bacterial growth and binary fission. Previous studies proved that the synthesis of peptidoglycan affects the bacterial division and growth rate. For example, the synthesis of the *Staphylococcus aureus* peptidoglycan pentaglycine interpeptide bridge is catalysed by the non-ribosomal peptidyl transferases FemX, FemA and FemB. Hübscher et al. (9) found that inactivation of the *femAB* operon in *S. aureus* leads to a poorly cross-linked peptidoglycan and the *femAB* mutants show a reduced growth rate. Penicillin-binding proteins (PBPs) are enzymes that catalyse the polymerization and cross-linking of peptidoglycan precursors in bacterial cell wall biosynthesis (5, 15). Haenni et al. (7) reported that the PBP-deleted mutant strain of *S. gordonii* grew significantly slower than the wild-type strain. In this study, the *glmM*-deficient strain had an obviously slow growth rate. The doubling time of the *glmM*-deficient strain was almost twice as long as that of the wild-type strain. It is conceivable that this change of growth kinetics is linked to the abnormal peptidoglycan biosynthesis.

The biosynthetic pathway leading to UDP-GlcNAc was affected in the *glmM*-deficient strain. The limiting of the biosynthetic rate of UDP-GlcNAc will surely result in the retardation of peptidoglycan biosynthesis and the decreased biosynthetic rate of peptidoglycan in the *glmM*-deficient strain could affect the growth and division of the bacteria.

Peptidoglycan biosynthesis and bacterial autolysis

Bacteria produce a variety of enzymes involved in the modification and degradation of peptidoglycan. The enzymes with peptidoglycan hydrolysing activity will break the links of the peptidoglycan web and help to insert new monomers at specific sites. Some of the peptidoglycan hydrolases are known as autolysins because they digest the cell wall when cells are exposed to unfavorable conditions (19). In the *glmM*-deficient strain, because of the abnormal peptidoglycan produced by the interrupted peptidoglycan biosynthesis pathway, the binding sites of the peptidoglycan hydrolase on the cell wall or the sensitivity to these enzymes may be changed. Then the cell wall may not sense the peptidoglycan hydrolase effectively and the growth and division of the mutant will be slowed. The poor reaction to peptidoglycan hydrolase may contribute to the decreased growth of the *glmM*-deficient strain. In addition, autolysins are involved in the processes of de-chaining and related terminal stages in cell division and separation. The chain length of the chain-forming bacteria is regulated by cell wall-associated autolysins (20). In this study, the long chains formed by the *glmM*-deficient strain also reflect the fact that the cell wall may display a bad reaction to autolysins.

Previous studies have proved that the different activity of autolysins will result in a change in bacterial autolysis. For example, Shibata et al. identified an autolysin-encoding gene, *atlA*. The *atlA* mutant strain of *S. mutans* Xc exhibited a lower autolysis rate than the wild-type Xc strain. Consequently, the inactivation of *atlA* resulted in a remarkable decrease in autolytic activity (18). In this study, incubation of *S. mutans* with 0.2% Triton X-100 induced more lysis of the *glmM*-deficient strain than the wild-type strain. Triton X-100 is a non-ionic surfactant which could induces bacterial lysis through the action of the cellular autolysin enzymes (4). Under incubation conditions with the same concentration of Triton X-100, the *glmM*-deficient strain revealed an increase in autolytic activity. It was hypothesized that the observed increase in cell autolysis could be caused by increased sensitivity to autolysin enzymes. Interestingly, this result seems to contradict the long-chain formation of the *glmM*-deficient strain, where the cell wall seems to display a decreased susceptibility to autolysins. Some studies have showed the same results. LytR of *S. mutans* has 39% homology to *Bacillus subtilis* LytR, a protein that attenuates the expression and activity of autolysin enzymes (12). The LytR (BrpA) mutant strains of *S. mutans* grew in long chains and showed increased autolytic activity (3, 22). The cause of this paradox is still unclear. May be the species of activated autolysins or the susceptibility of the cell wall to autolysins in the *glmM*-deficient strain differentiated along with the incubation conditions (with or without surfactant), or perhaps there are other regulatory mechanisms in these processes that inhibit cell separation and promote lytic events.

Peptidoglycan biosynthesis and biofilm formation

One of the important virulence properties of *S. mutans* is the ability to form biofilms known as dental plaque on tooth surfaces (10, 23). The biofilm growth mode helps *S. mutans* to deal with rapid and often substantial changes in nutrient availability, pH, oxygen tension, redox environment and other stressors. In this study, the *glmM*-defective strain of *S. mutans* could not form a regular biofilm like the parent strain; a finding that is homologous with that of Loo et al. (14). We can conclude that the gene involved in catalysing the interconversion of GlcNAc-1P and GlcNAc-6P isomers affects biofilm formation in *S. mutans* as it does in *S. gordonii*. In addition to *glmM*, in the report by Loo et al., several genes associated with biofilm formation are related to peptidoglycan biosynthesis. For example, the genes encoding PBPs, putative cation-transporting ATPase and undecaprenyl kinase are involved in lipid phosphorylation (14). These investigations suggest that peptidoglycan biosynthesis might play an important role in biofilm formation by bacteria. A bacterial cell without a natural, rigid cell envelope component will have a changed phenotype of biofilm formation. Loo et al. (14) speculated that the decreased ability to form biofilm by peptidoglycan biosynthesis-defective mutants may be the result of a change in the osmotic adaptation system.

In this study, the *glmM*-deficient strain showed alterations in both sucrose-dependent and sucrose-independent (11) biofilm formation. However, sucrose-independent biofilm formation was more notably reduced compared with sucrose-dependent biofilm formation. Yoshida and Kuramitsu isolated 27 sucrose-independent, biofilm-defective mutants of *S. mutans*. One of the mutants was defective in the homologue of the *B. subtilis lytR* gene (24), which is involved in the control of peptidoglycan hydrolase activity in *S. aureus*. So peptidoglycan formation may play a role in sucrose-independent attachment in *S. mutans*. The peptidoglycan biosynthesis-defective strain with an abnormal cell envelope may not sense the molecules functioning in sucrose-independent conditions.

It has been reported that much biofilm-specific physiology is devoted to the dynamic changes that both stimulate an increase in biomass and limit or stabilize accumulation according to environmental constraints. Therefore, multiple bacterial factors are thought to be required to regulate appropriate biofilm formations. In addition, it has been suggested that biofilm structure formation depends on both physical and biological factors, such as hydrodynamic conditions, nutrition diffusion and utilization, growth rate, cell advection and detachment. From these standpoints, a complemented strain was constructed. The transformant *glmM*-complement strain almost recovered the capability of the mutant strain to form biofilm. These results confirm that the change in phenotype observed in the *glmM*-deficient strain was the result of GlmM deficiency. The small difference in biofilm appearance between the *glmM*-complement and wild-type strains may be because the backbone of the plasmid pFW5 remained in the chromosome.

In summary, we showed that GlmM plays an important role in growth, autolysis and biofilm formation and therefore intimately regulates the virulence of *S. mutans*. However, the regulation mechanism of the *glmM* gene needs to be further investigated. These phenotypic differences may result from microstructural changes in the cell wall or perhaps the inactivation of the *glmM* gene influences gene expression in other regulatory pathways.

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