Contribution of phosphoglucomamine mutase to the resistance of *Streptococcus gordonii* DL1 to polymorphonuclear leukocyte killing

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Received 9 February 2009; accepted 26 May 2009.
Final version published online 22 June 2009.
DOI:10.1111/j.1574-6968.2009.01673.x

**Editor:** Robert Burne

**Keywords**
*Streptococcus gordonii*; phosphoglucomamine mutase; phagocytosis; lysozyme; peptidoglycan.

**Abstract**

Phosphoglucomamine mutase (GlmM; EC 5.4.2.10) catalyzes the interconversion of glucosamine-6-phosphate to glucosamine-1-phosphate, an essential step in the biosynthetic pathway leading to the formation of the peptidoglycan precursor uridine 5'-diphospho-N-acetylglucosamine. We have recently identified the gene (*glmM*) encoding the enzyme of *S. gordonii*, an early colonizer on the human tooth and an important cause of infective endocarditis, and indicated that the *glmM* mutation in *S. gordonii* appears to influence bacterial cell growth, morphology, and sensitivity to penicillins. In the present study, we assessed whether the *glmM* mutation also affects escape from polymorphonuclear leukocyte (PMN)-dependent killing. Although no differences in attachment to human PMNs were observed between the *glmM* mutant and the wild-type *S. gordonii*, the *glmM* mutation resulted in increased sensitivity to PMN-dependent killing. Compared with the wild type, the *glmM* mutant induced increased superoxide anion production and lysozyme release by PMNs. Moreover, the *glmM* mutant is more sensitive to lysozyme, indicating that the GlmM may be required for synthesis of firm peptidoglycans for resistance to bacterial cell lysis. These findings suggest that the GlmM contributes to the resistance of *S. gordonii* to PMN-dependent killing. Enzymes such as GlmM could be novel drug targets for this organism.

**Introduction**

*Streptococcus gordonii* and other closely related oral bacteria primarily colonize the human tooth surface as members of the biofilm community, commonly referred to as dental plaque (Gibbons, 1984; Hsu et al., 1994). Additionally, these streptococci colonize damaged heart valves and are the most frequently identified bacteria acting as primary etiological agents of infective endocarditis (Baddour et al., 1989; Baddour, 1994; Durack, 1975). The presence of endocardial vegetations composed of a fibrin–platelet matrix, leukocytes, and bacteria is the hallmark of infective endocarditis. The binding of streptococci to human platelets is thought to be a major virulence determinant in the pathogenesis of infective endocarditis (Herzberg et al., 1983). On the other hand, significant differences in the virulence of representative *S. gordonii* strains in the rat model of infective endocarditis do not appear to be correlated with the adhesion of these bacteria to isolated platelets or the fibrin–platelet matrix (Lee et al., 2006). *Streptococcus gordonii* is alive within associated polymorphonuclear leukocytes (PMNs) or monocytes before fixation of vegetations on heart valves containing dense masses of infecting streptococci in localized regions of granulocytosis (Durack, 1975). Thus, the ability of these bacteria to resist or avoid host cellular and humoral defenses may be an important virulence determinant for infective endocarditis. However, the mechanism by which streptococci escape from the immune response, including phagocytosis, during the progression of infective endocarditis is not well understood.

We have recently reported that the *S. gordonii* DL1 phosphoglucomamine mutase (GlmM: EC 5.4.2.10) is involved in bacterial cell growth, morphology, biofilm
formation, and sensitivity to penicillins (Shimazu et al., 2008). GlmM catalyzes the interconversion of glucosamine-6-phosphate to glucosamine-1-phosphate, an essential step in the biosynthetic pathway leading to the formation of peptidoglycan precursor uridine 5'-diphospho-N-acetylglucosamine (Jolly et al., 1999). Although the GlmM is not essential for the growth of S. gordonii, this mutation may result in impaired and/or unusual peptidoglycan synthesis in the glmM mutant, leading to an increase in sensitivity to cell wall inhibitors and morphological changes (Shimazu et al., 2008). These findings prompted us to assess whether the glmM mutation also affects escape from phagocytosis. In the present study, we found significant differences in the survival of S. gordonii between the wild-type strain and the glmM mutant in PMNs. In addition, we show that the difference is probably due to unusual peptidoglycan synthesis in the glmM mutant, resulting in sensitivity to bacterial cell lysis.

**Materials and methods**

**Bacterial strains and growth conditions**

The S. gordonii strains used in this study were DL1 (Challis strain; wild type) and its derivatives, EM230 (DL1 hsa:<ermAM>) and EM231 (DL1 glmM:<ermAM>) (Takahashi et al., 1997, 2002; Shimazu et al., 2008). Streptococci were cultured overnight in brain–heart infusion (BHI) broth or BHI agar plates (Becton Dickinson and Company, Sparks, MD) at 37 °C. The medium was supplemented, as needed, with 10 μg mL⁻¹ erythromycin (Sigma-Aldrich, St. Louis, MO).

**Immunological procedures and lectin blot**

The rabbit antiserum against the sialic acid-binding adhesin (Hsa) and whole cells of S. gordonii DL1 have been described previously (Takahashi et al., 1997, 2002); the latter has been confirmed to detect the wild type and the glmM mutant equally before the experiment. Biotinylated succinyl wheat germ agglutinin (sWGA–biotin) was purchased from EY Laboratories Inc., San Mateo, CA. Dot blotting was performed to detect the Hsa on bacterial cells. Nitrocellulose membranes were spotted with 1 μL of each streptococcal cell suspension (1 × 10⁶ cells mL⁻¹), blocked, incubated with primary antibody or sWGA-biotin, and developed with peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) (Bio-Rad Laboratories, Richmond, CA) or avidin D–horseradish peroxidase (Vector Laboratories Inc., Burlingame, CA), respectively, as described previously (Takahashi et al., 1997). Lipoteichoic acid (LTA) in the preparation of bacterial phenol extracts was assayed by enzyme-linked immunosorbent assay (ELISA) using anti-LTA monoclonal antibody (Clone 55, Hycult Biotechnology, Uden, the Netherlands) and the LTA standard (Sigma-Aldrich) as described previously (Hogg et al., 1997). Binding of S. gordonii strains to extracellular matrix proteins, including fibronectin (Sigma-Aldrich), type I collagen (Sigma-Aldrich), type II collagen (Cosmo Bio, Tokyo, Japan), and elastin (Sigma-Aldrich), adsorbed to microtiter wells (Costar EIA/RIA plate, high-binding, Corning Inc., Corning, NY), was assessed by an ELISA assay (Giomarelli et al., 2006).

**Hemagglutination and platelet aggregation assay**

Bacterium-mediated hemagglutination with human erythrocytes, and bacterium-mediated aggregation of human platelets were assayed as described previously (Takahashi et al., 1997; Yajima et al., 2008).

**Isolation of human PMNs**

Human PMNs were isolated from peripheral blood collected from healthy donors by density gradient centrifugation and dextran sedimentation (Thompson & Wilton, 1991) using Ficoll-Paque PLUS (GE Healthcare Bioscience, Uppsala, Sweden). Residual erythrocytes were eliminated by hypotonic lysis. PMNs were washed three times and resuspended in supplemented Rosewell Park Memorial Institute medium [RPMI-1640 with 1% bovine serum albumin (BSA), 0.2% HEPES, and 0.15 mM CaCl₂].

**Observation of S. gordonii attachment to human PMNs**

Both PMNs (1 × 10⁶) and bacteria (5 × 10⁷) opsonized with normal human serum (Rakita et al., 1999) were suspended in supplemented RPMI, mixed, and incubated in 1.5-mL polypropylene tubes for 3 h at 37 °C. Cells were washed twice with 1 × phosphate-buffered saline (PBS) containing 1% BSA and stained by the Wright–Giemsa method using Protocol HEMA3® (Biochemical Sciences, Swedesboro, NJ).

**Bactericidal assay**

Killing of bacteria by human PMNs was measured by a colony-forming assay (Tsuda et al., 2000; Lee et al., 2006). Briefly, both PMNs (1 × 10⁶) and the opsonized bacteria (1 × 10⁷) were suspended in supplemented RPMI, mixed, and incubated in 1.5-mL tubes for 0, 10, 30, and 60 min at 37 °C with mixing using a rotator (RT-50, Taitec Corp., Koshigaya, Japan) at c. 10 r.p.m. The mixtures without PMNs were included as negative controls. The PMNs were disrupted with sterile water at room temperature, diluted with PBS, and plated on BHI agar. Colonies were counted after incubation of the plates at 37 °C for 3 days.
Fluorescent dye exclusion

Intracellular bacterial viability was observed in situ by examining exclusion of a fluorescent dye using the LIVE/DEAD Bac Light Viability Kit (Molecular Probes, Eugene, OR) (Rakita et al., 1999). A fluorescent dye mixture was added to the PMN-opsonized bacteria mixture to yield a final concentration of 10 μM STYO 9 and 60 μM propidium iodide, and the mixture was incubated for 15 min at room temperature. Then, 5 μL of the mixture was placed on a glass slide, overlaid with a coverslip, and examined using a fluorescence microscope (Leica DMRB/E, Leica Microsystems, Tokyo, Japan). Bacteria with intact cell membranes appeared green due to staining with STYO 9, while bacteria with damaged cell membranes were stained orange–red from propidium iodide.

Superoxide anion production and lysozyme release by PMNs

The extent of PMN superoxide anion production was assayed using WST-1 (Dojindo, Kumamoto, Japan) (Thompson & Wilton, 1991; Tan & Berridge, 2000; Kishikawa et al., 2008). For the microtiter plate WST-1 assay, the superoxide dismutase (SOD)-inhibitable reaction of WST-1 was determined in a total volume of 0.2 mL HBSS containing 5 × 10^5 PMNs, 1mM WST-1, and 20 μg mL^{-1} catalase (Wako) with or without 20 μg mL^{-1} SOD (Sigma-Aldrich). Samples were equilibrated at 37 °C and the reaction was initiated by adding 2 × 10^9 bacteria (Sigma-Aldrich) for 1 h. A_{450 nm} was measured using SpectraMax Plus384 (Molecular Devices Corp., Sunnyvale, CA).

Lysozyme release was assayed using 0.5 mg mL^{-1} lyophilized Micrococcus lysodeikticus (Sigma-Aldrich) as the substrate in 50 mM phosphate buffer (pH 6.2) (Thompson & Wilton, 1991; Kumar et al., 2003). The turbidity was monitored at 620 nm at 1, 5, and 10 min after 50 μL of the PMN–bacterium mixture had been added to 150 μL of the suspension. The total intracellular content of lysozyme obtained by the PMN cell lysis using 0.2% Triton X-100 was also measured, and the release of lysozyme was expressed as a percentage of the total content.

Lysozyme and hydrogen peroxide sensitivity

The minimum inhibitory concentration (MIC) was determined using the microdilution method as described elsewhere (Kristian et al., 2005; Shimazu et al., 2008) with BHI broth containing lysozyme (Sigma-Aldrich) or hydrogen peroxide. Bacterial survival in the lysozyme solution was temporally assessed by a colony-forming assay. Bacterial cells (5 × 10^7) were incubated in the presence or absence of 5 mg mL^{-1} lysozyme in 0.2 mL of 20 mM sodium phosphate buffer (pH 6.8) for 0, 10, 30, and 60 min. The suspension was diluted with PBS, and plated on to BHI agar. Colonies were counted after incubation of the plates at 37 °C for 3 days.

Autolysis assay

Autolysis of S. gordonii cells at 37 and 44 °C was monitored by spectrophotometrical measurement of the bacterial cell suspension every 30 min for 48 h as described previously (Shibata et al., 2005) using the TVS062CA Bio-photorecorder (Advantec, Tokyo, Japan).

Statistical analysis

Statistical differences in the means of the values obtained were evaluated using an unpaired t-test. Differences were considered significant at P < 0.05.

Results and discussion

The glmM mutation does not affect binding of S. gordonii to PMNs

We have reported previously that S. gordonii possesses cell wall-anchored sialic acid-binding adhesin (Hsa), mediating sialic-acid-specific binding of this organism to host cells such as erythrocytes, platelets, and PMNs (Takahashi et al., 2002, 2004; Yajima et al., 2005, 2008; Urano-Tashiro et al., 2008). Therefore, we first considered whether the glmM mutation results in decreased Hsa anchoring to the peptidoglycan. To compare the amount of Hsa on the bacterial cell surface, bacterial cells of the wild-type strain (DL1) and the glmM mutant were dot-blotted, and the Hsa molecules were detected using anti-Hsa antibody and sWGA–biotin, the latter of which specifically detects GlcNAc of Hsa glycoprotein (Takahashi et al., 1997, 2004). As a result, Hsa molecules on the bacterial cell surface of the glmM mutant and the wild type were equally detected (data not shown). In addition, no differences in bacterium-mediated hemagglutination, bacterium-mediated aggregation of platelets (data not shown), and attachment to human PMNs (Fig. 1, upper panel) were observed between the glmM mutant and the wild type. Therefore, the glmM mutation affected neither Hsa anchoring nor sialic acid-specific binding properties. We also observed no differences in the occurrence of LTA, binding to fibronectin, type I collagen, type II collagen, and elastin (data not shown), further suggesting that the glmM mutation does not affect anchoring of cell wall-anchored molecules and binding to the extracellular matrix proteins.

The glmM mutation results in increased sensitivity to PMN-dependent killing

Although binding and phagocytosis of bacteria by PMNs generally results in bacterial killing, a previous study have
reported that no significant killing of *S. gordonii* DL1 cells occurred during a 2-h incubation with human PMNs (Lee et al., 2006). However, as shown in the lower panel of Fig. 1, more glmM mutant cells appeared to be phagocyted and lysed in phagosomes compared with the wild type. To confirm that the glmM mutation results in increased sensitivity to PMN-dependent killing, the susceptibility of *S. gordonii* strains was assessed by a colony-forming assay. As shown in Fig. 2, significant PMN-dependent killing of bacteria was observed in reaction mixtures containing the glmM mutant (*P* < 0.005). Moreover, we observed the integrity of bacterial membranes inside PMNs *in situ* using fluorescent dyes (Fig. 3). Representative fluorescence micrographs of the wild-type bacteria demonstrated that most of the intracellular bacteria were able to exclude propidium iodide and instead stained green, indicating that most of the bacterial cells were alive (Fig. 3a). In contrast, observation of the glmM mutant bacteria demonstrated that the majority of intracellular bacteria stained orange–red from the propidium iodide (Fig. 3b). These data clearly show that the glmM mutant cells are significantly more sensitive to PMN-dependent killing than wild-type cells.

**The glmM mutant induces more superoxide anion production and lysozyme release**

We considered the possibility that the properties of the glmM mutant, such as unusual peptidoglycan synthesis and enlargement of bacterial cell size, cause an increase in the stimulation of superoxide anion production and lysozyme release by PMNs, both of which are related to the PMN-dependent killing. When 5 × 10⁵ PMNs were incubated with 2 × 10⁶ bacteria for 1 h, a significant increase in the production of superoxide anion by PMNs with the *glmM* mutant (5.9 ± 2.1 μM) compared with that with the wild type (3.6 ± 0.9 μM) was observed (*n* = 8, *P* < 0.01); the latter was similar to that without bacterial stimulation (2.9 ± 0.3 μM).

The lysozyme release initiated by mixing PMNs with the *glmM* mutant was significantly increased (84.8 ± 9.1%, relative to no stimulation of intracellular lysozyme)
compared with lysozyme release stimulated by wild-type cells (58.1 ± 5.1%) when 5 × 10^6 PMNs were incubated with 5 × 10^7 bacteria for 1 h (n = 8, P < 0.001). Therefore, peptidoglycan structure and/or bacterial cell size may affect the superoxide anion production and lysozyme release by PMNs.

The glmM mutant is more sensitive to lysozyme

To test whether the glmM mutant strain is more sensitive to oxidizing agents than the wild type, we compared the sensitivity of the mutant to hydrogen peroxide with that of the wild type. The glmM mutant is more sensitive to hydrogen peroxide (MIC = 1.7 mM) than the wild type (MIC = 3.4 mM), although the difference is too slight to describe as significant.

Several streptococci are highly resistant to lysis by lysozyme (Kondo & McKay, 1982). We hypothesized that the peptidoglycan of the glmM mutant is more sensitive than that of the wild type, because unusual peptidoglycan synthesis may occur in the mutant. While 30 mg mL\(^{-1}\) lysozyme was insufficient to abolish the growth of the wild-type bacteria, glmM mutant cells did not grow in the medium containing 15 mg mL\(^{-1}\) lysozyme. The increase in the sensitivity of the glmM mutant to lysozyme was also confirmed by counting CFU after different durations of incubation in the lysozyme solution. As shown in Fig. 4, survival of the glmM mutant was significantly less than that of the wild type in the presence of 5 mg mL\(^{-1}\) lysozyme (P < 0.005). In contrast, no significant differences in autolysis were observed between the glmM mutant and the wild type (data not shown). Consequently, the glmM mutation results in an increase in sensitivity to lysozyme, suggesting that this may be one mechanism by which the mutation affects the resistance of this organism to PMN-dependent killing.
Concluding remarks

In this study, we have shown that GlmM contributes to the resistance of S. gordonii to PMN-dependent killing. The enzyme may be needed to synthesize peptidoglycans firm enough to resist the cell lysis activity of lysozyme. In addition, we have shown that the glmM mutant stimulates increased superoxide anion production and lysozyme release by PMNs compared with the wild type. Our findings are consistent with previous studies that certain strains of S. gordonii are alive within PMNs (Durack, 1975; Lee et al., 2006), and indicate that a certain factor for peptidoglycan synthesis appears to be associated with the resistance of this organism to PMN-dependent killing. Enzymes involved in bacterial cell wall synthesis such as GlmM could be novel potential drug targets for this organism. Further studies of GlmM may provide important insights into the pathogenesis of infective endocarditis induced by the oral viridans group streptococci and may contribute to the development of prevention methods for infective endocarditis.

Acknowledgements

We thank Eizo Takashima and Ikumi Ishiguro for preparation of human PMNs and erythrocytes. We thank Fusako Mitsuhashi for helpful comments. This study was supported by grants-in-aid for scientific research (#18592014 and #19791352) from the Japan Society for the Promotion of Science.

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