Glms and NagB Regulate Amino Sugar Metabolism in Opposing Directions and Affect Streptococcus mutans Virulence

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Abstract

Streptococcus mutans is a cariogenic pathogen that produces an extracellular polysaccharide (glucan) from dietary sugars, which allows it to establish a reproductive niche and secrete acids that degrade tooth enamel. While two enzymes (Glms and NagB) are known to be key factors affecting the entrance of amino sugars into glycolysis and cell wall synthesis in several other bacteria, their roles in S. mutans remain unclear. Therefore, we investigated the roles of Glms and NagB in S. mutans sugar metabolism and determined whether they have an effect on virulence. NagB expression increased in the presence of GlcNAc while Glms expression decreased, suggesting that the regulation of these enzymes, which functionally oppose one another, is dependent on the concentration of environmental GlcNAc. A glms-inactivated mutant could not grow in the absence of GlcNAc, while nagB-inactivated mutant growth was decreased in the presence of GlcNAc. Also, nagB inactivation was found to decrease the expression of virulence factors, including cell-surface protein antigen and glucosyltransferase, and to decrease biofilm formation and saliva-induced S. mutans aggregation, while glms inactivation had the opposite effects on virulence factor expression and bacterial aggregation. Our results suggest that Glms and NagB function in sugar metabolism in opposing directions, increasing and decreasing S. mutans virulence, respectively.

Introduction

Streptococcus mutans is a commensal bacterium present in the oral cavity and one of the first bacteria to colonize the tooth surface. This bacterium can be isolated from humans with or without dental cavities, suggesting that the oral environment of the host plays an important role in the virulence of S. mutans. Growth of this species also changes local environmental conditions, allowing it to attach to the tooth surface by producing an extracellular polysaccharide called glucan, which is involved in the formation of dental plaque [1,2,3]. Dental plaque formation is important for the survival and adhesion of S. mutans to the tooth surface because biofilms allow bacteria to resist immune factors and host-derived antibacterial agents [4]. Sucrose is the most important substrate involved in the synthesis of water-insoluble glucan (mutan), a glucose polysaccharide [5,6]. Streptococcus mutans expresses several glucosyltransferases (GTFs) that produce water-insoluble and/or water-soluble glucan molecules (mutan and dextran, respectively) from sucrose. Mutan and dextran function as major matrix components in biofilms [5]. Other sugar metabolic processes are important for maintaining homeostatic bacterial growth and survival. For example, sucrose and other sugars are substrates that drive various metabolic pathways, including glycolysis, peptidoglycan biosynthesis, and teichoic acid biosynthesis [7,8]. The enzymatic conversion of sugars by S. mutans, accompanied by the extracellular production of cell-surface protein antigen (PAC; also known as SpAP), glucan binding protein, dextranase, and acid tolerance factor (H+ pump), facilitates acid-catalyzed tooth decay and leads to erosion of the hydroxyapatite of the teeth [5,6,9,10,11].

Bacteria can uptake and utilize various sugars, including glucose, sucrose, and amino sugars such as glucosamine (GlcN), N-acetylg glucosamine (GlcNAc), and N-acetylneuraminic acid. Incorporated sugars are primarily utilized in glycolysis and cell wall biosynthesis [7,8]. To utilize sugars for such metabolic functions, they are ultimately processed into fructose-6-phosphate (Fru-6P) and glucosamine-6-phosphate (GlcN-6P), which are the initial substrates of glycolysis and peptidoglycan synthesis, respectively [7,12]. Glutamine-fructose-6-phosphate aminotransferase (GlmS) is involved in the production of GlcN-6P from Fru-6P, and glucosamine-6-phosphate deaminase (NagB) is involved in the production of Fru-6P from GlcN-6P. Specifically, these enzymes possess opposing activities; that is, GlmS catalyzes the conversion of Fru-6P to GlcN-6P, while NagB reverses the catalytic process mediated by GlmS (GlcN-6P to Fru-6P) [7,13]. The roles of GlmS and NagB have been characterized in several organisms, including Escherichia coli, Bacillus subtilis, and Staphylococcus aureus [7,8,12,13,14]. However, the
mechanism by which these enzymes function in S. mutans; sugar metabolism is poorly understood. Therefore, in this study we constructed glmS- and nagB-inactivated mutants to characterize the roles of these two opposing enzymes, which are key factors in glycolysis and cell wall synthesis in S. mutans. We also examined the S. mutans virulent phenotypes associated with sugar metabolism to link glmS and nagB to the production of PAc, surface adhesion, and GTf.

Materials and Methods

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. Streptococcus mutans UA159 and E. coli M15 were grown in tryptonecase broth (TSB) (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) and Luria-Bertani broth, respectively. Erythromycin (10 μg/mL) and spectinomycin (600 μg/mL) for S mutans or ampicillin (100 μg/mL) and erythromycin (300 μg/mL) for E. coli were added when necessary. A chemically defined medium (CDM), which was supplemented with glucose (50 mM) as the sole carbon source, was prepared and used in this study (CDM-G50). CDM-G50, which was initially used to culture S. aureus [8,15], consisted of the following five solutions: Solution 1 (20.1 g L-tryptophan, 3 g of KH2PO4, 150 mg each of L-aspartic acid, L-glutamic acid, L-isoleucine, L-leucine, L-proline, L-threonine, and L-valine; 100 mg each of L-alanine, L-arginine, glycine, L-histidine, L-lysine, L-methionine, L-phenylalanine, L-serine, L-tryptophan, and L-tyrosine; and 50 mg of L-cysteine dissolved in 700 mL of distilled water and adjusted to pH 7.2); Solution 2 (0.1 mg of biotin, 2 mg of nicotinic acid, 2 mg of D-pantothenic acid, 4 mg of pyridoxal, 4 mg of pyridoxamine dihydrochloride, 2 mg of riboflavin, and 2 mg of thiamine hydrochloride dissolved in 100 mL of distilled water); Solution 3 (20 mg of adenine sulfate and 20 mg of guanine hydrochloride dihydrate, 2 mg of riboflavin, and 2 mg of thiamine hydrochloride dissolved in 0.1 M hydrochloric acid [HCl] and made up to 50 mL with distilled water); Solution 4 (10 mg of CaCl2–6H2O, 5 mg of MnSO4, and 3 mg of (NH4)2SO4-FeSO4–6H2O dissolved in 10 mL of 0.1 M HCl); and Solution 5 (50 mL of sucrose (instead of glucose) was added to the mixture of Solutions 1–4 to generate CDM-S50. The mixture of Solutions 1–4 (CDM containing no sugars) was used for some bacterial washes.

Construction of glmS-, nagB-, vicK-, and ccpA-knockout mutants

Streptococcus mutans UA159 knockouts were constructed as described previously [16]; the primers used are listed in Table S1. Bacterial strains and growth conditions

Growth kinetics of wild-type (WT) and mutant S. mutans

Overnight cultures of WT and mutant S. mutans were harvested by centrifugation at 8000 × g for 5 min, and the cells were resuspended in equivalent volumes of TSB, BHI, or CDM-G50 to
adjust the OD₆₆₀ to 1.0. Next, the suspension was diluted with the appropriate medium to 10⁶ cells/mL (1000-fold dilution). Growth was monitored using a SPECTRA max 340PC 384 (Molecular Devices, Sunnyvale, CA, USA) with a 96-well microtiter plate.

Quantitative PCR analysis of gene expression

A small aliquot of *S. mutans* cultured overnight was inoculated into fresh medium and grown at 37°C; bacterial cells at various stages of growth were collected. Total RNA was extracted from the cells using a FastRNA Pro Blue kit (MP Biomedicals, Solon, OH, USA), according to the manufacturer’s protocol. One microgram of total RNA was reverse-transcribed to cDNA using a first-strand cDNA synthesis kit (Roche Diagnostics, Tokyo, Japan). Primers for *gyrA*, *glmS*, *nagB*, *gtfB*, *gtfC*, *spaP* (encoding PAc), *cpM*, and *fruM* were synthesized and used to determine the optimal expression conditions. Primers for the two-component systems (TCSs) were also synthesized. gyrA was used as an internal control. All primers used in this study are shown in Table S1.

Expression of *S. mutans* virulence factors

Virulence factor expression was investigated by quantitative PCR and immunoblotting. Exponential phase *S. mutans* cells (OD₆₆₀ = 0.5) were harvested. For quantitative PCR, total RNA was extracted and cDNA was synthesized as described above. The primers used for *gtfB*, *gtfC*, and *spaP* are listed in Table S1. For immunoblotting, the samples were prepared as described above. Antibodies against *S. mutans* GTF-I and PAc were obtained previously [20]. Because of the strong similarity between GTF-I (*gtfB*) and GTF-SI (*gtfC*) (73% amino acid identity), the antiserum against GTF-I also recognized GTF-SI.

Northern blot analysis

Total RNA from WT and mutant UA159 cells grown in CDM-G50 with or without GlcNAc was extracted as described above, and 10 μg of each sample were used for agarose gel electrophoresis. Electrophoresis and transfer to a nylon membrane were performed as described previously [19]. Hybridization and DIG labeling were performed according to the manufacturer’s protocol (Roche Diagnostics). DIG-labeled PCR fragments of *glmS* or *nagB* were used as probes for hybridization. After pre-hybridization at 42°C for 30 min, hybridization was performed at 42°C for 16 h. The membrane was then washed with 5 X SSC and 0.5% SDS (twice for 5 min each at room temperature) and then with 0.2 X SSC and 0.5% SDS (twice for 15 min each at 42°C). The reacted bands were visualized by the addition of substrate, according to the manufacturer’s protocol.

Table 1. Strains and plasmids used in this study.

| Strains and plasmids | Relevant characteristics |
|----------------------|--------------------------|
| **Streptococcus mutans** |                        |
| UA159                | WT laboratory strain     |
| Δ glmS               | glmS (SMU. 1187) deletion mutant in UA159, Em⁺² |
| Δ nagB               | nagB (SMU. 636) deletion mutant in UA159, Em⁺ |
| Δ vicK               | vicK (SMU. 1516) deletion mutant in UA159, Spc⁻³ |
| Δ glmS+vicK          | glmS and vicK double deletion mutant in UA159, Em⁺, Spc⁻ |
| Δ nagB+vicK          | nagB and vicK double deletion mutant in UA159, Em⁺, Spc⁻ |
| Δ cpA                | cpA(SMU. 1591) deletion mutant in UA159, Em⁺ |
| glmS compl.          | glmS complementation in MM3011, Em⁺, Spc⁻ |
| nagB compl.          | nagB complementation in MM3007, Em⁺, Spc⁻ |
| **Escherichia coli** |                        |
| M15(pREP4)           | Host strain for protein expression (Qiagen) |
| pMM1019              | pMM1019/E. coli M15 for His-tagged GlnS expression, Amp⁺, Km⁻⁵ |
| pMM1020              | pMM1020/E. coli M15 for His-tagged NagB expression, Amp⁺, Km⁻⁵ |
| **Plasmids**         |                        |
| pQE30                | Expression vector for His-tagged protein, Amp⁺ (Qiagen) |
| pMM1019              | glmS PCR fragment/pQE30 |
| pMM1020              | nagB PCR fragment/pQE30 |
| Bluescript SK II (+) | Cloning vector in E. coli, Amp⁺ |
| pMM1001              | Em⁺ gene harboring the flanking region of glmS/pBluescript SK II (+) |
| pMM1002              | Em⁺ gene harboring the flanking region of nagB/pBluescript SK II (+) |

¹GenBank locus tag obtained from the *S. mutans* genome at the Oral Pathogen Sequence Database site.
²Erythromycin resistance.
³Spectinomycin resistance.
⁴Ampicillin resistance.
⁵Kanamycin resistance.

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Evaluation of biofilm formation

Overnight cultures were diluted 1:10 in fresh TSB and grown to the exponential phase (OD₆₆₀ = 0.35). Next, the cells were harvested by centrifugation and resuspended in CDM. Aliquots (20 μL) of exponential stage cells were inoculated into wells containing 200 μL of fresh CDM-G50 with 50 mM sucrose or CDM-S50 in the presence or absence of 1 mM GlcNAc. The plates were incubated at 37°C with 5% CO₂ for 16 h. To monitor bacterial growth, the OD₆₆₀ was measured prior to safranin staining. The medium was then removed, and the wells were washed three times with distilled water. Finally, the biofilm cells were stained with 0.1% safranin for 10 min [21]. After three additional washes with distilled water, biofilm quantification was performed by evaluating the absorbance of each well at 490 nm. All experiments were performed in triplicate.

Electron microscopy

For scanning electron microscopy, WT and mutant UA159 cells were grown on a glass disk in TSB with 2% sucrose in the presence or absence of GlcNAc. After incubating overnight, the biofilm cells were washed twice with PBS. Next, a bacterial cell suspension was mounted on a glass coverslip and fixed with 2.5% glutaraldehyde and 1% osmium tetroxide (OsO₄). After dehydration using a graded ethanol series, drying using the critical-point procedure, and coating with gold-palladium, each specimen was examined under a JEOL JSM-6340F scanning electron microscope at 10 kV.

Saliva-induced aggregation assay

For the saliva-induced aggregation assay, exponential phase S. mutans cells grown in TSB with or without GlcNAc were suspended in aggregation buffer at an OD₆₆₀ of approximately 1.0. Unstimulated whole saliva was collected from a single donor (male, 47 years of age) in an ice-chilled plastic tube and subjected to centrifugation at 12000×g for 15 min. Either whole saliva (100 μL) or 10 μL of salivary agglutinin (0.5 mg/mL) was mixed with 1 mL of the cell suspension. Salivary agglutinin was purified as described previously [22]. CaCl₂ was also added to the salivary agglutinin mixture to a final concentration of 1 mM. Bacterial aggregation was determined by monitoring the change in OD₅₅₀ at 37°C with a spectrophotometer. All experiments were performed in triplicate.

Results

Construction of glmS- and nagB-knockout mutants

We constructed S. mutans glmS- and nagB-knockout mutants using recombination to replace glmS or nagB with the Emr gene (Table 1). The knockouts and their complementation were verified by PCR and immunoblotting using GlmS- or NagB-specific antibodies (Fig. S1).

Since GlcNAc significantly affected the growth of the glmS-knockout mutants in S. aureus [8], we investigated the growth of these S. mutans mutants in the presence or absence of 10 mM GlcNAc (Fig. 1). The doubling time and final OD of the WT and mutant cells grown under various conditions are shown in Table 2. In the absence of GlcNAc in TSB or CDM-G50, the glmS mutant did not grow, but in the presence of 10 mM GlcNAc, the glmS mutant replicated at a rate equal to that of WT. The glmS-knockout mutant strain complemented with glmS grew in the absence of GlcNAc, although its doubling time (60.4 min in TSB and 137.6 min in CDM-G50) was higher than that (45.9 min in TSB and 112.3 min in CDM-G50) of the WT strain. In contrast, the presence of 10 mM GlcNAc inhibited nagB mutant growth. The nagB-knockout mutant strain complemented with nagB grew in the presence of GlcNAc. On the other hand, in the absence of GlcNAc, the uncomplemented nagB mutant grew, but its doubling time (31.8 min in TSB and 132.7 min in CDM-G50) was higher than that of wild type (45.9 min in TSB and 112.3 min in CDM-G50).
Effect of GlcNAc on GlmS and NagB expression

We investigated the expression of NagB and GlmS in the presence of various concentrations of GlcNAc in CDM-G50 (Fig. 2). Since TSB contains several sugars, we used CDM-50 for this assay to control the glucose/GlcNAc ratio. Exponential growth phase S. mutans cells (OD = 0.8) in CDM-G50 containing various concentrations of GlcNAc were prepared. In the absence of GlcNAc, NagB was not detected by immunoblotting, while GlmS was expressed. GlmS expression was not altered by GlcNAc at concentrations below 0.4 mM. However, at a GlcNAc concentration of 0.8 mM or higher, GlmS expression decreased gradually until the GlcNAc concentration reached 12.5 mM, at which point GlmS expression was no longer detected. In contrast, while NagB was not expressed at GlcNAc concentrations of less than 0.8 mM, its expression at a GlcNAc concentration of 1.6 mM or higher was detected, suggesting that protein expression increased gradually under increasing GlcNAc concentrations. Quantitative PCR and immunoblotting corroborated these results, demonstrating that the expression of both GlmS and NagB was altered in the presence of 1.6 mM GlcNAc (Fig. 2B). Northern blotting confirmed these results, showing that significant amounts of glmS transcript were present in CDM-G50 in the absence of GlcNAc, but that the addition of GlcNAc reduced the transcript level (Fig. 2C). In contrast, nagB transcript was detected in CDM-G50 in the presence of GlcNAc, but was abolished in the absence of GlcNAc (Fig. 2C).

We next investigated NagB and GlmS expression over time after the addition of GlcNAc. Ten minutes after GlcNAc addition, the glmS transcript level was significantly decreased, while the nagB transcript level was increased (Fig. 3B). By immunoblotting, NagB was detected after 30 min, while the amount of GlmS did not change up to 60 min after the addition of 25 mM GlcNAc (Fig. 3A). These differential responses can be attributed to the rate of degradation of the protein and mRNA.

Effects of glmS and nagB on virulence factor expression

Since numerous studies have explored the effects of sugars on virulence factors, including glucan synthesis, biofilm formation, and acid production, in S. mutans [5,6,23], we investigated the expression of virulence factors in our glmS and nagB mutants in the exponential growth phase (OD = 0.8) in CDM-G50 with or without 10 mM GlcNAc. GlmS or NagB expression was completely abolished in the glmS and nagB mutants, respectively. However, GlmS expression in the nagB mutant as well as NagB expression in the glmS mutant was similar to that in WT bacteria, indicating that expression of the enzyme targeted for knockout was only abolished by its particular mutation (and not by the mutation of other enzymes) (Fig. 4A). Expression of the virulence proteins GTF-I (involved in water-insoluble glucan synthesis), GTF-IS (involved in water-soluble and -insoluble glucan synthesis), and PAc (a cell-surface antigen that mediates adhesion to hydroxypatite and salivary components) in mutants grown in CDM-G50 with or without GlcNAc was analyzed by immunoblotting and quantitative PCR (Fig. 4A and B). We also investigated the expression of GlmS and NagB early in the exponential growth phase (OD = 0.4) and in the stationary phase (OD = 1.0) and obtained results similar to those obtained in the mid-exponential phase (OD = 0.8) [data not shown]. Immunoblotting revealed that the PAc and GTF concentrations were increased in the glmS mutant and decreased in the nagB mutant compared to wild type. In the WT strain, GTFs and PAc expression was slightly decreased by GlcNAc (Fig. 4A). In quantitative analysis, the expression of the gtfB (GTF-I), gtfC (GTF-IS), and spaP (PAc) in the WT strain were decreased at 2.8-, 1.5- and 1.2-fold, respectively, by addition of GlcNAc. In the nagB mutant, the expression of the gtfB (2.2-fold lower), gtfC (4.8-fold lower), and spaP (4.2-fold lower) were decreased compared to the WT strain. In the glmS mutant, the expression of the gtfB (4.0-fold higher), gtfC (4.2-fold higher), and spaP (7.5-fold higher) were increased compared to the WT strain. In the glmS-complemented strain, the expression of these genes was reduced compared to the glmS mutant, but not fully recovered to those of the WT strain. Also, in the nagB-complemented strain, the expression of these genes was increased compared to the nagB mutant, but the expression of gtfB and gtfC was not fully restored (Fig. 4B).

Association between TCS and virulence factor expression in the glmS and nagB mutants

In S. mutans UA159, 15 sets of TCSs (including one orphan TCS) were identified in the genome, some of which are known to be associated with virulence factor expression, including gifs [16,24-27]. In this study, the glmS and nagB mutants showed altered spaP and gff expression. Therefore, we investigated whether this altered expression was due to the effect of inactivation of glmS and nagB on TCS expression. First, we investigated the expression of all TCSs in the glmS and nagB mutants (Fig. S2) and found that three TCSs (vicR, comE and SMU.1815) expression was increased in the glmS mutant, while only vicR was decreased in the nagB mutant, showing only vicR expression was changed in glmS and nagB mutant. The expression of vicR in the glmS and nagB mutants was restored in the respective complemented strains (Fig. 5A). vicR was previously shown to affect gffB and gffC expression [25,26]. Next, we constructed a vicK mutant to investigate the interaction of vicRR with spaP, gffB, and gffC.

Table 2. Doubling times of UA159, glmS or nagB deletion mutant and its complement strains grown in TSB or CDM-G50.

|            | TSB            | TSB + GlcNAc | CDM-G50 | CDM-G50 + GlcNAc |
|------------|----------------|--------------|----------|------------------|
|            | DT (min)       | final OD     | DT (min) | final OD         |
|            |                |              |          |                  |
| UA159      | 45.9 ± 1.0     | 1.30 ± 0.14  | 39.8 ± 1.3 | 1.46 ± 0.02       |
| glmS       | ND             | 0.09 ± 0.01  | 37.5 ± 1.6 | 1.50 ± 0.02       |
| nagB       | 51.8 ± 0.9     | 1.33 ± 0.01  | ND        | 0.10 ± 0.01       |
| glmS:nagB  | 60.4 ± 2.3     | 0.98 ± 0.01  | 41.8 ± 1.0 | 1.50 ± 0.01       |
| nagB:glmS  | 48.2 ± 2.2     | 1.30 ± 0.02  | 56.9 ± 0.1 | 1.36 ± 0.03       |

1 Doubling time (DT) was calculated based on the formulas ln Z – ln Z0 = g(t – t0), where k is the growth rate, and g = 0.693/k×60, where g is the doubling time (min). Values are the mean ± standard deviation obtained from three independent experiments.

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expression. Since vicR is known to be an essential gene [16], we constructed a vicK mutant and found that the absence of vicK increased vicR expression (Fig. 5A). The vicK mutation in the nagB mutant as well as in the wild type showed the increase of vicR expression. In the case of vicK mutation in the glmS mutant, vicR expression is similar to that of the glmS mutant, although its

Figure 2. Effect of GlcNAc on GlmS and NagB expression. After washing a WT UA159 overnight culture, a small aliquot was inoculated into CDM-G50 containing various concentrations of GlcNAc and then incubated at 37°C with 5% CO2. When the sample reached an OD660 of 0.5, the cells were collected. Next, the samples were prepared for immunoblotting (A), quantitative PCR (B), and Northern blotting (C) as described in the Materials and Methods. Panel B: The diamonds and squares represent glmS and nagB expression, respectively.

Figure 3. Time course of GlmS and NagB expression after the addition of GlcNAc. Streptococcus mutans UA159 was grown in CDM-G50 at 37°C with 5% CO2. When the cells reached an OD660 of 0.5, GlcNAc was added at 2.5 or 25 mM. Cells were collected 10, 30, and 60 min after the addition of GlcNAc. Next, the cells were prepared for immunoblotting (A) and quantitative PCR (B) as described in the Materials and Methods. Panel B: The symbols represent samples without GlcNAc (diamonds), with 3 mM GlcNAc (squares), and with 25 mM GlcNAc (triangles). Normal and dashed lines represent glmS and nagB expression, respectively. doi:10.1371/journal.pone.0033382.g003
expression was increased compared to wild type. In addition, spaP, gtfB, and gtfC expression was increased in the vicK mutant (Fig. 5B). Therefore, vicRK, whose expression was increased in the glmS mutant and decreased in the nagB mutant, appeared to modulate the virulence genes spaP, gtfB, and gtfC. Furthermore, we investigated the expression of virulence factors in the double mutants (vicK combined with glmS or nagB). In the vicK and nagB double mutant, spaP, gtfB, and gtfC expression was increased compared to that in the nagB mutant, although nagB mutation in the vicK mutant reduced the expression of these factors compared to that of the vicK mutant (Fig. 4B and Fig. 5B). The vicK and glmS double mutant showed similar expression to that in the vicK or glmS mutant (Fig. 4B and Fig. 5B).

Effect of CcpA on the expression of glmS and nagB

Since CcpA plays a central role in carbon catabolite repression and affects the virulence of S. mutans [28], we investigated whether CcpA affects the expression of glmS and nagB. In ccppA mutant cells grown in CDM-G50 with or without 10 mM GlcNAc, glmS expression was unchanged compared with that in wild type (Fig. 6). The expression of nagB in the ccppA mutant grown in CDM-G50 was slightly decreased compared with that in wild type; however, its expression increased upon the addition of GlcNAc to a similar level as in wild type. gtfBC and spaP expression was unchanged in the ccppA mutant (data not shown).

Biofilm formation in the nagB and glmS mutants

Since the glmS and nagB mutants showed altered expression of gfs, which plays a pivotal role in bacterial adhesion to the tooth surface via insoluble glucan formation from sucrose, we investigated biofilm formation in these mutants using CDM containing either sucrose (CDM-S50) or glucose and sucrose (CDM-G50 with 50 mM sucrose) (Fig. 7). In both types of media, biofilm formation in the nagB mutant exceeded that of wild type. In CDM containing sucrose or glucose and sucrose plus 10 mM GlcNAc, biofilm formation by the glmS mutant exceeded that of wild type. We also evaluated biofilm formation in TSB and BHI media with or without 10 mM GlcNAc and found patterns similar to those in the CDM-based medium (data not shown).

Next, we investigated the expression of GlmS, NagB, GTFs, and PAc in biofilm cells by immunoblotting and quantitative PCR (Fig. S3). Although the expression level of these factors were different between planktonic and biofilm condition, the expression pattern was almost similar between them. GlmS expression in wild type and the nagB mutant decreased in the presence of GlcNAc, while NagB expression in wild type and the glmS mutant was increased in...
the presence of GlcNAc. The expression of $gffB$, $gffC$ and $spaP$ were decreased in the nagB mutant, showing a similar pattern with that of planktonic condition. However, in the glmS mutant, $gffB$ expression in biofilm cells was decreased compared to that of the WT strain, while its expression in planktonic cells was increased. The expression of $gffC$ and $spaP$ in the glmS mutant under biofilm was increased and showed a similar pattern to that of planktonic condition.

Electron microscopic observation revealed that the amount of extracellular matrix in the nagB mutant was reduced, while the glmS mutant had large amounts of extracellular matrix compared with wild type (Fig. 8).

Saliva-induced aggregation of the nagB and glmS mutants

Since PAc is involved in saliva-induced aggregation in S. mutans [29], we performed a saliva-induced aggregation assay using whole saliva and purified salivary agglutinin with wild type and the nagB and glmS mutants (Fig. 9). Under both conditions, the glmS mutant showed strong aggregation while the nagB mutant showed weak aggregation when compared to wild type. There was no difference in salivary aggregation between the WT strain grown in TSB with or without GlcNAc. We also performed an aggregation assay using whole saliva from three other volunteers and found the same results (data not shown).

Discussion

In this study, we found that GlmS and NagB coordinately regulated the conversion between glucose and GlcNAc and that failure of this regulation affected the expression of virulence factors in S. mutans. Since significant growth inhibition was observed in the glmS mutant grown in the absence of GlcNAc and in the nagB mutant grown in the presence of GlcNAc (Fig. 1), each enzyme is considered essentially under specific conditions (with or without GlcNAc) in S. mutans. In some bacterial species, GlmS and NagB
function to distribute sugar substrates to various metabolic pathways, including glycolysis and peptidoglycan biosynthesis [7,8]. Based on the results of the present study and supported by previous reports [7,8], we propose a similar mechanism for sugar distribution involving two factors in S. mutans (Fig. 10). GlcN-6P is mainly utilized for peptidoglycan biosynthesis, but a high concentration of GlcN-6P in the bacterial cytoplasm is toxic [30,31]. Therefore, the controlled conversion of GlcN-6P to a non-toxic molecule (Fru-6P) is required, resulting in increased NagB expression. Additionally, the production of GlcN-6P from Fru-6P is suppressed by reducing GlmS expression. In the absence of GlcNAc, GlcN-6P is solely synthesized from Fru-6P (mediated by GlmS), resulting in high GlmS and low NagB expression. Thus, in the glmS mutant, the supply of GlcN-6P converted from Fru-6P was abolished, forcing the bacteria to rely on GlcNAc to survive and grow. The nagB mutant, in the presence of high GlcNAc concentrations, was unable to process sufficient levels of toxic GlcN-6P, causing growth inhibition. This suggests that tight regulation of GlmS and NagB is critical for sugar metabolism in S. mutans. Previously, we demonstrated that nagB-knockout S. aureus did not show strong growth inhibition in the presence of GlcNAc [8]. This difference is likely due to differences in GlmM activity, which mediates the conversion of Glc-6P to GlcN-1P, the first substrate in peptidoglycan synthesis. One possibility is that increased GlmM activity in S. aureus reduces the amount of GlcN-6P. Further study will be required to clarify the difference.

Figure 6. glmS and nagB expression in ccpA mutant cells. After washing overnight cultures of WT and ccpA mutant cells, a small aliquot of each
The precise mechanism of GlmS and NagB regulation in *S. mutans* remains unclear. In *B. subtilis*, glmS regulation involves the self-degradation of glmS mRNA [32,33,34,35]. This regulation, known as ribozyme regulation, involves self-cleavage induced by excess GlcN-6P, a product of the GlmS reaction. A core region consensus sequence 200–300 bp upstream of the glmS coding region is required for ribozyme activity. This region of the mRNA can bind to GlcN-6P, inducing self-cleavage and inhibiting the translation of glmS. There is no consensus sequence (homologous to the *B. subtilis* core region of ribozyme) upstream of the glmS coding region in *S. mutans*. Also, the transcriptional start site in glmS, identified by rapid amplification of cDNA ends (RACE) experiments, is 87 bp upstream of the coding region (Fig. S4). This suggests that *S. mutans* glmS has no ribozyme activity. In *Enterobacteriaceae*, *E. coli*, *Salmonella typhimurium*, and *Yersinia pseudotuberculosis*, small RNAs (glmY and glmZ) were found to regulate glmS expression [36,37]. The small RNA glmZ binds to glmS mRNA and inhibits translation of the glmS gene [36,37]. glmY is also a small RNA that regulates glmZ expression by binding to glmZ mRNA directly [36,37]. Although the small RNAs glmZ and glmY can down-regulate the translation of glmS in *Enterobacteriaceae*, it is unknown whether the same system is present in *S. mutans*. In addition, little is known regarding whether the regulation of NagB involves self-degradation or small RNAs in *S. mutans*; thus,
additional studies are required. Furthermore, we searched for possible regulatory protein binding sites in the promoter regions of both genes and found none.

In this study, we investigated several phenotypes using nagB and glmS mutants. Of the phenotypes observed, the most striking was that the nagB mutant produced a reduced biofilm, while the glmS mutant had slightly elevated levels, compared with wild type (Fig. 7). This was caused by decreased amounts of GTFs (GTF-I and -SI) and PAc in the nagB mutant and increased amounts in the glmS mutant. We confirmed that these changes were constant during growth (data not shown). We also investigated the effect of pH on the expression of these genes and found similar pH values of the medium in which the mutants grew compared with wild type (data not shown). These results indicate that glmS or nagB inactivation altered the expression of these virulence factors. In S. mutans, three GTFs have been identified that are known to be involved in sucrose-dependent biofilm formation [5]. Besides these factors, gbpB and atlA were also reported to be associated with biofilm formation [21,25,36], although the major factors for sucrose-dependent biofilm formation are GTF-I and -SI, which synthesize water-insoluble glucan. It was previously reported that gbpB was regulated by VicR and associated with the initiation of biofilm formation [38]. We investigated the expression of these two factors and found that gbpB expression in the glmS and nagB mutants was similar to that of gbfC, while atlA expression was unchanged in both mutants (data not shown). In addition, we found that both mutants had altered salivary-induced aggregation (Fig. 9). PAc is responsible for surface hydrophobicity and sucrose-independent adherence to tooth surfaces and salivary aggregation [11,29,39,40]. Salivary agglutinin, gp340, binds to PAc in S. mutans, resulting in aggregation [22,40]. Therefore, altered salivary aggregation activity may be associated with PAc expression in the mutants. One orphan response regulator (gcrR) and one TCS (vicRK) were shown to alter gbfC and gbfC expression in S. mutans [24,25,26]. We investigated the expression of these regulators in WT and mutant strains, and found that vicRK expression was altered in the glmS and nagB mutants, while gcrR expression was unchanged (data not shown). We also found that the vicK mutants had increased expression of vicR, resulting in increased expression of spaP, gbfC, and gbfC. The mechanism underlying the increased expression of vicR in the vicK mutant is not well understood. A similar result was found in which the knockout of vicH increased vicR expression [27]. Furthermore, in the glmS+vicK double mutant, the expression of gbf and pae was comparable to that in the vicK and glmS single mutants, while their expression in the nagB+vicK double mutant was increased compared to that in the nagB mutant. However, nagB mutation in the vicK mutant reduced gbf and pae expressions, this implies that other factor, which is dependent for nagB, but independent for vicR, is involved in the expression of virulence factors. It was previously shown that the consensus region of the VicR binding site (TGTWAHNNN NNTGTWAH) is upstream of gbfB and gbfC [25]. We also found the consensus region upstream (136 bp) of the spaP transcriptional start site (data not shown). This suggests that the altered expression of gbfB, gbfC, and spaP in the glmS and nagB mutants is caused by VicRK, although the mechanism underlying the altered expression of vicRK in these mutants has not been determined.

Recently, it was shown that CcpA, a transcriptional regulator, affects virulence [28]. CcpA plays a central role in carbon catabolite repression, together with the HPs and PTS system. HPs is activated by HP kinase, which is activated by an enhanced level of glycolytic intermediates, including fructose-1,6-bisphosphatase or glucose-6-P. Next, CcpA and HPs form a complex, resulting in enhanced binding to catabolite responsive elements in the promoter regions of various genes. Although many CcpA-related genes were identified, a relationship between CcpA and gbfBC, spaP, or vicRK was not demonstrated. In addition, we analyzed the expression of glmS and nagB in the ccaA mutant and found that glmS expression was decreased upon the addition of GlcNAc, while nagB expression was increased. These results suggest that CcpA is not involved in the expression of glmS or nagB under the specific growth conditions tested.

In conclusion, we demonstrated that the expression of NagB and GlmS in S. mutans is tightly regulated and modulated by the presence or absence of GlcNAc in the environment. The failure of NagB and GlmS regulation affected virulence-associated factors such as biofilm formation and saliva-induced aggregation, indicating that sugar metabolism is related to the virulence of S. mutans. These observations help explain how S. mutans is able to colonize and form dental caries in the oral cavity.

Supporting Information

Figure S1 NagB and GlmS expression in the knockout mutants and their complementation strains. After washing WT and mutant cells grown overnight in TSB with or without 10 mM GlcNAc, a small aliquot of each was inoculated into CDM-G50 with or without 10 mM GlcNAc and incubated at 37°C with 5% CO2. When the sample reached an OD600 of 0.5, the cells were collected. Samples were prepared for immunoblotting (A) and quantitative PCR (B) as described in the Materials and Methods. *p < 0.05, as determined by Tukey’s HSD; **p < 0.005, as determined by Tukey’s HSD. (TIF)

Figure S2 TCS expression in the glmS and nagB mutants. After washing WT and mutant cells grown overnight in TSB with or without 10 mM GlcNAc, a small aliquot of each was inoculated into CDM-G50 with or without 10 mM GlcNAc and then incubated at 37°C with 5% CO2. When the sample reached an OD600 of 0.5, the cells were collected. Samples were prepared for quantitative PCR as described in the Materials and Methods. HK and RR represent histidine kinase and response regulator, respectively. *p < 0.05, compared to WT as determined by a t-test. (TIF)

Figure S3 Expression of GlmS, NagB, and virulence factors in WT and mutant UA159 cells in a biofilm. Biofilm cells grown in CDM-G50 containing sucrose and/or GlcNAc and planktonic cells in CDM-G50 with or without GlcNAc were collected and prepared for immunoblotting (A) and quantitative PCR (B) as described in the Materials and Methods. Panel B: 1: wild type, 2: ΔnagB, 3: ΔglmS, 4: ΔnagBΔglmS, 5: ΔnagBΔvicRK, 6: ΔglmSΔvicRK, 7: ΔnagBΔglmSΔvicRK. *p < 0.05, as determined by Tukey’s HSD. (TIF)
2: glmS mutant, 3: nagB mutant. *p < 0.05, compared to WT as determined by a t-test; **p < 0.05, compared to WT as determined by a t-test.

Table S1 Primers used in this study.

| Primer | Sequence |
|--------|----------|
| glmS_AF | 5'-CCACTTCAGTGGTTTCTATG-3' |
| glmS_AR | 5'-AGTGCTGAGTGCTGAGGAC-3' |
| nagB_AF | 5'-TCTGCGTCTGGTTGCTTGA-3' |
| nagB_AR | 5'-TCTGCGTCTGGTTGCTTGA-3' |

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Author Contributions

Conceived and designed the experiments: HK MK-M YM TK SM.Performed the experiments: MK-M YM MK YO SY HK. Analyzed the data: MK-M YM MK YO HK. Contributed reagents/materials/analysis tools: MK-M YM TO HK SM. Wrote the paper: MK-M HK TK SM SY TO.

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