Amino Sugars Enhance the Competitiveness of Beneficial Commensals with *Streptococcus mutans* through Multiple Mechanisms

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ABSTRACT

Biochemical and genetic aspects of the metabolism of the amino sugars *N*-acetylglucosamine (GlcNAc) and glucosamine (GlcN) by commensal oral streptococci and the effects of these sugars on interspecies competition with the dental caries pathogen *Streptococcus mutans* were explored. Multiple *S. mutans* wild-type isolates displayed long lag phases when transferred from glucose-containing medium to medium with GlcNAc as the primary carbohydrate source, but commensal streptococci did not. Competition in liquid coculture or dual-species biofilms between *S. mutans* and *Streptococcus gordonii* showed that *S. gordonii* was particularly dominant when the primary carbohydrate was GlcN or GlcNAc. Transcriptional and enzymatic assays showed that the catabolic pathway for GlcNAc was less highly induced in *S. mutans* than in *S. gordonii*. Exposure to H₂O₂, which is produced by *S. gordonii* and antagonizes the growth of *S. mutans*, led to reduced mRNA levels of *nagA* and *nagB* in *S. mutans*. When the gene for the transcriptional regulatory NagR was deleted in *S. gordonii*, the strain produced constitutively high levels of *nagA* (GlcNAc-6-P deacetylase), *nagB* (GlcN-6-P deaminase), and *glmS* (GlcN-6-P synthase) mRNA. Similar to NagR of *S. mutans* (NagR_{Sm}), the *S. gordonii* NagR protein (NagR_{Sg}) could bind to consensus binding sites (*dre*) in the *nagA*, *nagB*, and *glmS* promoter regions of *S. gordonii*. Notably, NagR_{Sg} binding was inhibited by GlcN-6-P, but G-6-P had no effect, unlike for NagR_{Sm}. This study expands the understanding of amino sugar metabolism and NagR-dependent gene regulation in streptococci and highlights the potential for therapeutic applications of amino sugars to prevent dental caries.

IMPORTANCE

Amino sugars are abundant in the biosphere, so the relative efficiency of particular bacteria in a given microbiota to metabolize these sources of carbon and nitrogen might have a profound impact on the ecology of the community. Our investigation reveals that several oral commensal bacteria have a much greater capacity to utilize amino sugars than the dental pathogen *Streptococcus mutans* and that the ability of the model commensal *Streptococcus gordonii* to compete against *S. mutans* is substantively enhanced by the presence of amino sugars commonly found in the oral cavity. The mechanisms underlying the greater capacity and competitive enhancements of the commensal are shown to depend on how the genes for the catabolic enzymes are regulated, the role of the allosteric modulators affecting such regulation, and the ability of amino sugars to enhance certain activities of the commensal that are antagonistic to *S. mutans*.

The oral microbiome of humans consists of hundreds of bacterial taxa, and the activity and composition of the microbiota have a potent influence on the health of the host. Dental caries has been dubbed an “ecological catastrophe” (1) that is characterized by an increase in the relative abundance of pathogenic bacteria and reductions in the proportions of species that are associated with oral health (2). Many of these health-associated bacteria are considered commensal, but many have distinctly beneficial properties that may impede the development of caries (3–5). Host factors and dietary habits also have a profound influence on the initiation and progression of caries; for example, frequent ingestion of carbohydrate-rich foodstuffs leads to the production of organic acids through fermentation by the microbiome. The low pH created by these by-products directly effects demineralization of the tooth, and repetitive and prolonged exposure to low pH exerts a selective pressure on the microbial communities that leads to the emergence of an aciduric (acid-tolerant) flora enriched with organisms, like *Streptococcus mutans* and lactobacilli (1, 6). Conversely, increased proportions of another group of lactic acid bacteria, including *Streptococcus gordonii* and *Streptococcus sanguinis*, have been associated with dental health. In fact, certain studies found an inverse correlation between these particular bacteria and *S. mutans* in the microbiomes of healthy humans and subjects

with dental caries (7, 8). At the molecular level, recent studies with some of these commensal bacteria have identified alkali-producing mechanisms, e.g., the arginine deiminase (AD) system in *S. gordonii* and urease in *Streptococcus salivarius* are capable of releasing ammonia (pK_a = 9.2) from arginine and urea, respectively (9, 10). Perhaps as important is the ability of *S. gordonii*, *S. sanguinis*, and related bacteria to metabolize oxygen to produce significant quantities of hydrogen peroxide (H₂O₂), which is particularly effective at suppressing the growth and persistence of *S. mutans* at physiologically relevant (low-millimolar) concentrations (11, 12).

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The amino sugars glucosamine (GlcN) and *N*-acetylglucosamine (GlcNAc) are among the most prevalent sugars in the biosphere. For example, amino sugars are the major components of the exoskeletons of crustaceans and other arthropods and the cell envelopes of fungi and bacteria, and they are critical constituents of glycoproteins across all domains of life. Consistent with the ubiquity of these sugars, the pathways for the metabolism of amino sugars for energy production and anabolic processes are well conserved in bacteria (13). Notably, GlcNAc is a preferred sugar for many bacteria, providing efficiently utilized sources of carbon (glucose) and nitrogen (ammonium ion) (14, 15). After internalization of amino sugars via the sugar:phosphotransferase system (PTS) (16, 17) or ABC transporters (18), bacteria depend on GlcNAc-6-P deacetylase (NagA) for converting GlcNAc-6-P into GlcN-6-P, and then GlcN-6-P deaminase (NagB) for the concurrent deamination and isomerization of GlcN-6-P into fructose-6-P (F-6-P), which can enter the Embden-Meyerhof-Parnas pathway for energy production (16, 19). GlcN-6-P also supplies bacteria with a critical precursor for synthesis of the polysaccharide backbone of peptidoglycan. Conversely, when exogenous sources of GlcNAc or GlcN are insufficient, bacteria generate GlcN-6-P using F-6-P and glutamine as the substrates for GlcN-6-P synthase, the product of the *glmS* gene (20). Regulation of the balance between catabolism and biosynthesis of GlcN and GlcNAc is essential for bacterial growth, so it has been studied somewhat intensively, especially in the model bacterial systems *Escherichia coli* and *Bacillus subtilis* (17, 21–23).

Recently, we characterized the metabolic pathways for amino sugars in the dental pathogen *S. mutans* and demonstrated that regulation of the expression of the genes for NagA, NagB, and GlmS by the GntR/HutC-type regulator NagR was direct (24, 25), unlike the regulatory systems for these genes in many other bacteria. We also showed that the glucose/mannose-PTS (EII^{Man}) is the primary transporter for GlcNAc in *S. mutans*, that significant amounts of ammonia are released by *S. mutans* when catabolizing these amino sugars, and that growth on these compounds affected acid tolerance and the acidogenic potential of the organism (24). NagB and GlmS have also been implicated in the manifestation of virulence-related traits by *S. mutans*, including biofilm formation (26). As the homologues of the *nagA*, *nagB*, and *glmS* genes have been identified in numerous other bacteria, it is conceivable that competition for limited quantities of these amino sugars exists between various members of the oral microbiota. Here, we compare the capacity of a collection of *S. mutans* isolates with a group of commensal oral streptococci to utilize GlcN and GlcNAc for growth and investigate the impact of amino sugars on the competition between *S. mutans* and the model commensal *S. gordonii*.

MATERIALS AND METHODS

Bacterial strains, reagents, and culture conditions. *Escherichia coli* strain DH10B was grown in Luria-Bertani medium (L broth) and on agar plates at 37°C in air. Streptococcal bacterial strains, including several wild-type isolates of *S. mutans* (UA159, OMZ175, 11SSST2, 3SN1, U2A, SM6, and ST1) (27), and several commensal streptococci, including *Streptococcus gordonii* DL1, *Streptococcus salivarius* 57.I, *Streptococcus sanguinis* SK150, *Streptococcus cristatus* A55 (4), *Streptococcus intermedius* ATCC 31412, *Streptococcus mitis* UF28, *Streptococcus australis* A13, and a recently characterized close relative of *Streptococcus australis* designated *Streptococcus* sp. A12 (4), were maintained on brain heart infusion agar (BHI) (Difco Laboratories, Detroit, MI). These media were supplemented with antibiotics (Sigma, St. Louis, MO) when required at the following concentra-

tions: for streptococci, 1 mg/ml kanamycin (Km) and 10 µg/ml erythromycin (Em); and for *E. coli*, 100 µg/ml ampicillin.

For enzymatic assays and preparation of RNA, streptococcal cells were cultured using a semisynthetic TV base medium (28) or a chemically defined FMC medium (24) that was modified to contain particular carbohydrates (Thermo Fisher Scientific, Waltham, MA; and Sigma), as specified. Plates and liquid bacterial cultures were maintained at 37°C in aerobic incubators with 5% CO₂. For monitoring growth, bacterial strains were first cultured overnight in BHI, subcultured in BHI until the optical density at 600 nm (OD₆₀₀) reached 0.4 to 0.5, and then diluted 1:150 into FMC medium containing the specified carbohydrate(s). The growth (OD₆₀₀) of the bacterial cultures was monitored using a Bioscreen-C (Oy Growth Curves AB, Helsinki, Finland) for 24 to 48 h, with doubling times (*T_d*) calculated using data collected during the exponential phase of growth, according to the formula $(t_2 - t_1) \times \log(2)/\log(OD_2/OD_1)$, where *t*₁ and *t*₂ are two time points and OD₁ and OD₂ are the optical densities (600 nm) measured at these respective time points. All restriction enzymes were purchased from New England BioLabs (Beverly, MA) and DNA oligonucleotides (see Table S1 in the supplemental material) from Integrated DNA Technologies, Inc. (Coralville, IA).

Creation of *S. gordonii* mutant strains and recombinant NagR proteins. A mutant of *S. gordonii* lacking an apparent *nagR* homologue (SGO_1121) was created using a ligation-transformation approach detailed elsewhere (29). Briefly, a pair of primers (SGOnagR-1 and SGOnagR-2Bm; see Table S1 in the supplemental material) was used to PCR amplify a DNA fragment located upstream of the coding sequence of SGO_1121, and another pair (SGOnagR-3Bm and SGOnagR-4) was used for a fragment downstream of SGO_1121. The two PCR products were digested with BamHI, purified, and ligated with a kanamycin resistance cassette that was released with the same restriction enzyme (30). The ligation mixture was used to transform competent *S. gordonii* strain DL1 induced with competence-stimulating peptide (31). Kanamycin-resistant colonies obtained in this assay were further analyzed by PCRs coupled with DNA sequencing that confirmed the intended allelic replacement and lack of undesired mutations in the flanking regions. Similarly, a strain defective in the PTS enzyme I (*ptsI*, SGO_1555) was created in essentially the same way in the genetic background of strain DL1 using the kanamycin marker and the following primer sets: SGO_1555-1 and SGO_1555-2Bm for the upstream arm, and SGO_1555-3Bm and SGO_1555-4 for the downstream arm.

To overexpress and purify the NagR protein of *S. gordonii* (NagR_{sg}) in the form of a maltose-binding protein (MBP) fusion protein, the *nagR* gene of strain DL1 was cloned as described previously for *nagR* of *S. mutans* (24). Briefly, a pair of primers, SGOnagR-5'Bm and SGOnagR-3'HIII, was used to amplify the coding sequence of SGO_1121, with BamHI and HindIII sites engineered into the 5' and 3' ends of the DNA fragment, respectively. After restriction enzyme digestion, the DNA fragment of SGO_1121 was inserted into the expression vector pMal-p2X (New England BioLabs) that had been digested with the same enzymes, followed by transformation of *E. coli* DH10B. Induction and purification of the recombinant MBP-NagR were carried out according to protocols provided by New England BioLabs. Subsequently, the purified MBP-NagR protein was treated with a factor Xa in a solution containing 100 mM NaCl. Similar to recombinant NagR from *S. mutans* (NagR_{sm}), cleavage of the MBP fusion protein resulted in precipitation of NagR_{sg}. The precipitated protein was harvested by centrifugation, washed, and redissolved in a buffer that contained 300 mM NaCl, 50 mM KCl, 1 mM EDTA, 5 mM dithiothreitol, 10 mM HEPES (pH 7.9), and 10% glycerol (25).

Liquid coculture competition. To enumerate the CFU of *S. gordonii* and *S. mutans* after cocultivation, a gene for a β-fructosidase (*fruA*) that hydrolyzes homopolymers of fructose (32, 33), but is not required for growth under the conditions tested, was replaced by a kanamycin and erythromycin resistance cassette in strains UA159 and DL1, respectively (34). These two strains, UA159-Km and DL1-Em, were cultured in triplicate in TV with glucose (TV-Glc) or TV-GlcNAc overnight, diluted 1:50

into 2 ml of the same fresh medium, and cultured to early exponential phase (OD_{600} , 0.3). Based on cell density, *S. gordonii* and *S. mutans* were combined at a 1:1 ratio and diluted 1:100 into 5 ml of fresh TV base medium containing Glc (20 mM), GlcN (20 mM), or GlcNAc (20 mM). This resulted in two competition assays (each in triplicate) for each condition: (i) DL1-Em in Glc plus UA159-Km in Glc, and (ii) DL1-Em in GlcNAc plus UA159-Km in GlcNAc. All samples were incubated overnight in the presence of 5% CO_2 . After 20 h of incubation, each culture was dispersed using an external sonicator (FB120; Fisher Scientific) at 100% power for two 30-s intervals, and aliquots (100 μ l) were then removed, serially diluted, and plated onto BHI-Em and BHI-Km agar for CFU enumeration. All plates were incubated for 2 days at 37°C in a 5% CO_2 atmosphere before the colonies were counted.

For competition on plates, similarly mixed cultures of DL1-Em and UA159-Km were diluted 1:100 in 3.5% tryptone broth, and 10 μ l of the admixture was spotted onto TV agar plates containing 20 mM Glc, GlcN, or GlcNAc. After a 20-h incubation, an agar plug containing the cells was excised using the top of a sterile 1-ml pipette tip and transferred to 3.5% tryptone broth. The cells were dispersed by sonication, diluted, and plated on selective agar medium.

Dual-species biofilms and confocal laser scanning microscopy. To investigate the impact of various carbohydrates on the interaction between UA159 and DL1 in biofilms, a previously described dual-species biofilm model was utilized, with minor modifications (35). Similar to the procedure described above, UA159-Km and DL1-Em strains that were grown exponentially in BHI broth (OD_{600} , 0.5) were mixed at a 1:1 ratio based on optical density, diluted 1:100 into 3 ml of biofilm base medium (BM) supplemented with 18 mM glucose (Glc) and 2 mM sucrose (BMGS) (35), and the cell suspension was pipetted into the wells of a 6-well tissue culture plate, each of which contained a sterile glass coverslip. After 24 h of incubation at 37°C in a 5% CO_2 aerobic atmosphere to allow substantial biofilm development on the glass coverslip, the original BMGS medium and planktonic cells were removed, the biofilm was gently washed once with 3 ml of BM base medium and resupplied with 3 ml of BMGS or BM constituted with 20 mM Glc, GlcN, or GlcNAc. After another 24 h of incubation, the biofilm was gently washed with 3 ml of sterile phosphate-buffered saline (PBS), and 1 ml of PBS was added to each well. For analysis via confocal laser scanning microscopy, biofilms were stained with a LIVE/DEAD BacLight bacterial viability kit (Thermo Fisher), and the images were acquired using a spinning disk confocal system connected to a Leica DM IRB inverted fluorescence microscope that was equipped with a Photometrics cascade-cooled EMCCD camera. Syto9 fluorescence was detected by excitation at 488 nm, and emission was collected using a 525-nm (± 25 nm, green) band-pass filter. Detection of propidium iodide (PI) fluorescence was performed using a 642-nm excitation laser and a 695-nm (± 53 nm, red) band-pass filter. All z-sections were collected at 1- μ m intervals using a 63 $\times$ /1.40 oil objective lens. Image acquisition and processing were performed using VoxCell (VisiTech International, Sunderland, United Kingdom). Biofilm stacks were also rendered in three dimensions using Imaris (Bitplane, Belfast, United Kingdom). For CFU enumeration, the biofilm was scraped off the glass coverslip, sonicated, and subjected to serial dilution and plating on selective agar plates.

RNA preparation and quantitative reverse transcription-PCR. *S. gordonii* wild-type and *nagR* mutant strains were cultured to exponential phase (OD_{600} , 0.5) in modified FMC broth containing Glc, GlcN, or GlcNAc as the sole carbohydrate. Cells were harvested by centrifugation, and RNA extraction was carried out using the RNeasy kit (Qiagen, Germantown, MD), as detailed elsewhere (24). Reverse transcription was performed using random hexamers and the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA), according to a protocol provided by the supplier. Subsequently, real-time quantitative PCR was carried out using gene-specific primers: SGOnagA-S and SGOnagA-AS (*nagA*), SGOnagB-S and SGOnagB-AS (*nagB*), SGOglmS-S and SGOglmS-AS (*glmS*), and arcA-S and arcA-AS (*arcA*). The 16S rRNA levels measured (with primers 16S-S and 16S-AS) in each sample were used as internal

controls to normalize the results. To measure mRNA levels for *nagA*, *nagB*, and *glmS* in *S. mutans* samples, previously published primers (24) were used by performing the same procedure described here. To monitor *dpr* mRNA levels, primers *dpr*-S and *dpr*-AS were used.

Enzymatic assays. Activities of NagA and NagB were measured according to established procedures (36). Briefly, cultures of *S. mutans* UA159 or *S. gordonii* DL1 were grown to mid-exponential phase (OD_{600} , 0.5) in TV base medium containing 20 mM Glc, GlcN, or GlcNAc before being harvested for assays. Clarified cell lysates were added to reactions in which the enzymatic activities of NagA and NagB were coupled to the formation of NADPH, which was tracked using a spectrophotometer (at OD_{340}). The protein concentration in each cell lysate was determined by a bicinchoninic acid (BCA) assay, and the NagA and NagB activities (nanomoles of NADPH created/[milligrams of protein $\times$ minute]) were calculated according to the following formula: $10^6 \times \text{rate}/[\text{protein concentration (in milligrams per milliliter)} \times \text{volume of cell lysate (in microliters)} \times 6.22]$.

PTS assays measuring phosphoenolpyruvate (PEP)-dependent GlcNAc:phosphotransferase activities were performed as detailed elsewhere (24, 37). The wild-type strain UA159 and a derivative of *S. gordonii* DL1 deficient in NADH oxidase activity (*nox* mutant) (33) were each grown to mid-exponential phase in TV medium supplemented with 0.5% of Glc or GlcNAc, prior to harvesting for PTS assays. A *nox* mutant was used to eliminate the high background NADH oxidase activities in wild-type DL1 cells, since the assay monitors the decrease in NADH levels by coupling it to phosphorylation of the sugar substrates. Arginine deiminase (AD) activity was measured for *S. gordonii* according to an established protocol described elsewhere (9, 10). Cells were grown to mid-exponential phase in TV base medium supported only with 0.5% Glc, GlcN, or GlcNAc, with or without the addition of 10 mM arginine, before harvesting for assays.

Protein-DNA interactions. Recombinant NagR_{SG} protein was used in fluorescence polarization (FP) and electrophoretic mobility shift assays (EMSA), as detailed in previous reports (24, 25). For FP, a 6-carboxyfluorescein (FAM)-labeled, 45-nucleotide (nt) probe (25) that contained the consensus NagR-binding sequence (*dre*) deduced from a number of streptococcal genomes and that was functionally validated (25) was employed, and analysis of the FP data allowed for calculation of the equilibrium dissociation constant (K_D). EMSA used three 0.3-kbp biotin-labeled DNA fragments that contained the intergenic regions upstream of the coding sequence of *nagA*, *nagB*, and *glmS* in *S. gordonii*.

RESULTS

Comparison of growth on amino sugars by *S. mutans* and commensal bacteria. We began this study by investigating the growth phenotypes of a number of common oral commensals and isolates of a dental caries pathogen in FMC broth containing GlcNAc or GlcN as the sole carbohydrate source. We reported previously that *S. mutans* wild-type strain UA159 exhibits a long lag phase (>10 h) when transferred to FMC containing GlcNAc from BHI broth with glucose, but then the cells grow at a rate comparable to that in FMC containing glucose (Fig. 1A) (24). To investigate whether this behavior was strain specific or a phenotype that is common in *S. mutans* isolates, six additional wild-type strains (OMZ175, 11SSST2, 3SN1, U2A, SM6, and ST1) encompassing the three most prevalent Brattthall serotypes of *S. mutans* (*c*, *e*, and *f*) (27) were selected and tested under the same conditions. The results indicated that most isolates displayed similarly long lag phases (4 to 11.5 h) before significant growth was detected (Table 1; see also Fig. S1 in the supplemental material). In contrast, no apparent delay in growth was noted for any of the *S. mutans* strains in FMC containing the same concentrations of glucose (Glc) or GlcN. In contrast, when we tested two common commensal bacteria, *Streptococcus gordonii* strain DL1 and *Streptococcus salivarius* strain

FIG 1 Growth curves of *S. gordonii* DL1, *S. salivarius* 57.I, and *S. mutans* UA159 on modified FMC medium containing 20 mM GlcNAc (A) or GlcN (B). Cultures were inoculated using exponentially growing cells supported by BHI, and the optical density (OD₆₀₀) was monitored using a Bioscreen C machine maintained at 37°C, with readings taken every 30 min.

57.I, a prolonged lag phase was not observed on FMC-GlcNAc (Fig. 1). In fact, both DL1 and 57.I grew better on GlcNAc than on GlcN, with shorter lag phases and doubling times and higher final yields (Table 1 and Fig. 1). Closer examination of the growth characteristics of all *S. mutans* isolates indicated that despite the long lag phases, cells invariably displayed shorter doubling times and higher yields when grown exponentially on GlcNAc than on GlcN (Table 1). Interestingly, when *S. mutans* UA159 was first cultured in FMC containing GlcN instead of BHI and then inoculated into fresh FMC-GlcNAc, the long lag phase was no longer observed (data not shown). These results provide evidence that *S. mutans* is capable of rapid growth on GlcNAc if the catabolic system has been sufficiently induced.

To further explore the preference for amino sugars by other oral bacteria, we examined additional streptococci, including one isolate each of *Streptococcus sanguinis* (SK150), *Streptococcus cristatus* (A55), *Streptococcus intermedius* (ATCC 31412), *Streptococcus australis* (A13), *Streptococcus* sp. A12 (3), and *Streptococcus mitis* (UF28), bacteria that are generally considered to be commensal or associated with oral health. Most of these species were able to grow on FMC medium with GlcNAc as the primary carbo-

hydrate source with considerably shorter lag phases than those of the *S. mutans* strains (Table 1; see also Fig. S2 in the supplemental material). Notably, for *S. mitis* UF28, GlcNAc was the only carbohydrate of those tested that supported significant growth in the medium tested. Therefore, it appears that many commensal streptococci may have a significant advantage over the dental pathogen *S. mutans* in their ability to transition to growth on, and rapidly catabolize, GlcNAc in the oral cavity.

Competition between *S. mutans* and *S. gordonii*. (i) **Liquid coculture assay.** A mixed-culture competition model was adopted (34) to investigate the effects of GlcN or GlcNAc on competition between *S. mutans* UA159 and *S. gordonii* DL1. Both strains were genetically modified to contain an antibiotic resistance marker, Em (erythromycin) for DL1 and Km (kanamycin) for UA159, which was used to replace the *fruA* gene of each organism. FruA is an exo-hydrolase that attacks homopolymers of fructose but is not essential for growth on any carbohydrates other than inulins and levans (32, 33). The two bacteria were each cultured on either TV-Glc or TV-GlcNAc to mid-exponential phase and then mixed at a 1:1 ratio, with each species having been grown on the same carbohydrate. The cell mixtures were then inoculated

TABLE 1 Growth characteristics of *Streptococcus mutans* and oral commensal strains^a

Strain mixed with FMC	Glucose			GlcN			GlcNAc		
	<i>T</i> _d (avg ± SD) (min)	OD ₆₀₀	<i>T</i> _{lag} (min)	<i>T</i> _d (avg ± SD) (min)	OD ₆₀₀	<i>T</i> _{lag} (min)	<i>T</i> _d (avg ± SD) (min)	OD ₆₀₀	<i>T</i> _{lag} (min)
<i>S. mutans</i>									
UA159	55.4 ± 2.7	1.14	<60	162.5 ± 18.5	0.78	<180	105.4 ± 6.6	1.06	<570
OMZ175	56.9 ± 2.7	1.31	<60	290.9 ± 20.1	0.70	<210	269.0 ± 7.2	1.03	<690
11SSST2	58.0 ± 3.7	1.17	<90	132.5 ± 15.3	0.77	<90	91.1 ± 6.9	1.17	<480
3SN1	67.9 ± 8.6	0.91	<90	162.8 ± 14.8	0.75	<60	100.3 ± 6.2	0.96	<510
U2A	73.4 ± 5.1	0.97	<60	313.4 ± 21.4	0.64	<60	167.5 ± 11.4	0.99	<720
SM6	115.2 ± 13.7	0.77	<90	520.4 ± 15.9	0.64	<90	117.3 ± 10.8	1.01	<270
ST1	73.8 ± 7.2	1.15	<30	230.5 ± 21.1	0.73	<150	143.4 ± 4.3	1.19	<240
<i>S. gordonii</i> DL1	46.1 ± 8.9	1.01	<90	129.7 ± 21.0	0.54	<90	52.3 ± 11.0	0.86	<60
<i>S. salivarius</i> 57.I	42.3 ± 6.3	1.30	<60	86.7 ± 9.9	0.76	<60	52.7 ± 4.2	1.13	<30
<i>S. intermedius</i> ATCC 31412	193.9 ± 15.1	0.56	<210	592.8 ± 24.6	0.47	<270	128.1 ± 11.8	0.71	<540
<i>S. sanguinis</i> SK150	92.0 ± 9.8	0.94	<120	335.7 ± 33.5	0.51	<120	85.4 ± 11.0	0.92	<120
<i>S. australis</i> A13	60.1 ± 10.1	0.95	<90	ND	0.06	<240	ND	0.28	<330
<i>Streptococcus</i> sp. A12	150.0 ± 14.4	0.55	<120	ND	0.10	<300	155.1 ± 9.2	0.64	<420
<i>S. mitis</i> UF28	ND	0.37	<150	ND	0.10	<180	134.8 ± 4.0	0.99	<180
<i>S. cristatus</i> A55	96.8 ± 19.3	0.54	<90	165.5 ± 28.7	0.32	<120	102.5 ± 19.0	0.48	<150

^a Doubling time (*T*_d), maximal optical density (OD₆₀₀), and length of the lag phase (*T*_{lag}) of various streptococcal strains growing in modified FMC containing specified carbohydrates (each at 20 mM). The end of the lag phase was defined as when the OD₆₀₀ reaches 0.01. ND, not determined.

FIG 2 Liquid coculture competition between *S. mutans* (S. m.) (UA159 Km-resistant derivative) and *S. gordonii* (S. g.) (DL1 Em-resistant derivative). The strains were each cultivated in TV supplemented with Glc or GlcNAc until exponential phase before being inoculated at a 1:1 ratio into fresh TV medium containing 20 mM Glc, GlcNAc, or GlcN. After 20 h of incubation, aliquots of the cultures were diluted and plated onto BHI agar plates containing antibiotics. The relative abundance of both strains at the start of the competition was similarly monitored (data not shown). Values within the bars show the proportions of the cultures constituted by *S. mutans* or *S. gordonii*, as indicated by dark or light shading, respectively. Error bars represent standard deviations.

using a 1:100 dilution into TV base medium supplemented with 20 mM Glc, GlcN, or GlcNAc. After 20 h of incubation, the dual-species cultures were serially diluted and plated on selective agar plates to enumerate the two species. The relative abundance of these bacteria at the start of the competition was also monitored by plating to confirm that the use of optical density to normalize the cell number was appropriate (data not shown) (34). As presented in Fig. 2, when both bacteria were provided with glucose in both the preculture and the competition, 69% ± 17% (mean ± standard deviation) of the cells were identified as *S. gordonii* and 31% ± 17% as *S. mutans*. When GlcNAc was used in the preculture but Glc in the competition, DL1 showed a slight increase in its dominance over UA159, constituting 81% ± 14% of the population. When the competitions were conducted using amino sugars, however, further dominance by the commensal was apparent, with *S. gordonii* constituting >93% of the mixed population in TV-GlcN and >98% in TV-GlcNAc. There were no significant differences whether the precultures were prepared using Glc or GlcNAc. pH measurements at the end of the competition indicated that GlcN-based cultures had the highest pH values, followed by GlcNAc and Glc (Fig. 2). Thus, in a planktonic environment where amino sugars are the sole carbohydrate source, commensal *S. gordonii* can dominate the pathogen *S. mutans*, presumably because of more-efficient utilization of the sugar coupled with moderation of environmental acidification due to the release of ammonia from GlcN or GlcNAc.

(ii) Dual-species biofilm. Although *S. gordonii* grows better on amino sugars than *S. mutans* in planktonic cultures, the question remains whether this is the case when cells are living in a biofilm growth mode. To answer this question, UA159-Km and DL1-Em strains were each grown to mid-exponential phase in BHI medium, mixed in equal optical densities, and then inoculated into a synthetic medium (38) for development of biofilms. As detailed in Materials and Methods, the medium for biofilm growth included 2 mM sucrose during the first 24 h of growth, which is favorable for biofilm development due to the synthesis of glucans that act as adhesive polymers for oral biofilm formation (39). After 1 day of incubation, the original sucrose-containing medium (BMGS) was replaced with BMGS medium or base medium (BM) supple-

mented with 20 mM Glc, GlcN, or GlcNAc, and the biofilms were incubated for another 24 h. Subsequently, the biofilms were harvested for enumeration of CFU or prepared for confocal laser scanning microscopy (CLSM) to investigate structural differences in the dual-species biofilms. As shown in Fig. 3A, *S. mutans* constituted the majority of viable cells in the biofilms grown with BMGS and BM-Glc, at 79% and 72% of the population, respectively. These results are consistent with the fact that *S. mutans* is more acid resistant than *S. gordonii* and can outcompete the commensal in prolonged cultures supported solely by Glc, largely due to the low-pH environment created under these conditions (34). However, when the biofilms were formed in medium supported by GlcN or GlcNAc, the commensal prevailed over *S. mutans* by a considerable margin, with DL1 accounting for 73% ± 4% and 62% ± 2% of the total population, respectively, which was statistically different from the BMG and BMGS cultures (each $P < 0.005$).

When the biofilms were imaged using CLSM, GlcNAc supported the most robust biofilm formation (Fig. 3B), as evidenced by the strikingly thick mushroom-like structures that were almost completely absent in biofilms formed on Glc or GlcN. In other experiments in which an additional round of medium replacement and incubation (24 h) were incorporated, improved biofilm formation was noted on BM-GlcN medium (data not shown) compared to biofilms formed with glucose. Considering that *S. gordonii* outcompetes *S. mutans* on both GlcN and GlcNAc in terms of viable counts, these observations highlight the ability of GlcNAc to remodel both the composition and architecture of this dual-species biofilm community.

Expression of amino sugar metabolic genes in *S. mutans* and *S. gordonii*. The *S. gordonii* DL1 genome includes open reading frames (ORFs) SGO_0549 and SGO_1586, which encode proteins that are highly conserved with known NagA and NagB enzymes, respectively, and share 64% amino acid sequence identity each with NagA and NagB of *S. mutans*. Also present in *S. gordonii* DL1 is an apparent homologue of a GntR/HutC-type regulator (SGO_1121, referred to here as NagR_{sg}) that shares 75% identity with NagR of *S. mutans* (NagR_{sm}), with the sequence divergence being greatest in the C-terminal effector-binding domains (see

FIG 3 Dual-species biofilms formed using *S. mutans* (*S. m.*) and *S. gordonii* (*S. g.*). Equal amounts of UA159-Km and DL1-Em cells were used to inoculate biofilm medium (BM) supported by 18 mM Glc and 2 mM sucrose (BMGS) and allowed to form a biofilm on a glass coverslip. After 24 h, the medium was replaced by BM supplemented with 20 mM Glc, GlcN, or GlcNAc, or fresh BMGS. After another 24 h of incubation, biofilms were harvested for CFU counting (A) and imaging by confocal laser scanning microscopy (B). Values within the bars in panel A show the percentage of the populations constituted by *S. mutans* or *S. gordonii*, as indicated by dark or light shading, respectively. Error bars represent standard deviations.

Fig. S3 in the supplemental material). Similar to in *S. mutans*, the *nagA* and *nagB* genes are distant from one another in the genome of *S. gordonii* DL1, with putative promoter elements and NagR-binding sites (*dre*) predicted upstream of their coding sequences (see Fig. S4 in the supplemental material) (25). Different from *S. mutans*, though, is the fact that a three-gene locus, including another GntR-type regulator (*busR*) that is situated immediately downstream of *nagR* (24) in *S. mutans*, is not present anywhere in the genome of DL1.

To begin to understand the basis for the considerable differences in the growth phenotypes of *S. mutans* and *S. gordonii* on amino sugars, *in vitro* assays were performed to compare the rates of PTS-dependent transport of GlcNAc. When UA159 was cultivated in Glc or GlcNAc as the primary carbohydrate in TV medium, similar levels of PTS activity (152.7 and 154.7 units, respectively) were measured with GlcNAc as the substrate (see Fig. S5 in the supplemental material). This finding is consistent with the fact that EII^{Man} is the primary transporter of GlcNAc in *S. mutans*, and the expression of the genes for this permease (*manLMN*) is comparable to that in cells grown on Glc or GlcNAc (24). Interestingly, despite the apparently faster growth on GlcNAc by DL1 than that by UA159 (Fig. 1), the *S. gordonii* strain grown on Glc produced much lower levels of GlcNAc PTS activity (16.6 units), and only a small increase in activity was noted in cells that were cultured on GlcNAc (up to 26.6 units; see Fig. S5), compared to the same activity in *S. mutans*. While EII^{Man} is a primary transporter for GlcNAc in *S. mutans*, it is possible that these results reflect that PTS-independent uptake of amino sugars is a major contributor to the catabolism of these compounds in *S. gordonii*. To test this hypothesis, we constructed an allelic exchange mutant of the gene for enzyme I (EI) of the PTS, which is absolutely required for

PTS-dependent sugar transport, in the DL1 genetic background. When tested on a GlcNAc-based TV medium, the EI mutant of DL1 was still able to grow, albeit more slowly than the wild-type strain (Fig. 4). Therefore, *S. gordonii* possesses redundant PTS and

FIG 4 Growth phenotypes of the EI (*ptsI*) mutant of *S. gordonii* on TV-based agar plates containing Glc, GlcN, or GlcNAc. Also included are the wild-type parental strain DL1 and strains of *S. mutans*: wild-type UA159 and *ptsI*, *nagA*, and *nagB* mutants.

FIG 5 Measurements of NagA (A) and NagB (B) specific (sp) activities in cell lysates of *S. mutans* (wild-type UA159 and its *nagA* and *nagB* mutants) and *S. gordonii* DL1. The *nagA* and *nagB* mutants were cultivated only on Glc. Error bars represent standard deviations.

non-PTS pathways for internalization of GlcNAc. Interestingly, the EI mutant of DL1 failed to grow on GlcN-based medium (Fig. 4), indicating that the catabolism of GlcN, but not GlcNAc, appears to require internalization by the PTS under the conditions tested.

Next, we measured GlcNAc-6-P deacetylase (NagA) and GlcN-6-P deaminase (NagB) activity in the lysates of exponentially growing cells of *S. mutans* UA159 and *S. gordonii* DL1 using an assay that couples enzymatic activity to the reduction of NADP. Each strain was grown to exponential phase in TV base medium supplemented solely with Glc, GlcN, or GlcNAc before the cells were harvested for enzyme assays. UA159 derivatives deficient in *nagA* and *nagB* were grown in TV-Glc and used as negative controls. While it was clear that both strains produced increased levels of NagA and NagB activity when grown on GlcNAc, and more so when grown on GlcN, relative to the same cells grown on Glc, there was a significant difference in these activities between *S. mutans* and *S. gordonii*. First, *S. gordonii* showed higher NagA activity than *S. mutans* when grown on either amino sugar (Fig. 5A), a result consistent with their growth characteristics (Table 1). On the other hand, *S. mutans* showed higher NagB activity than *S. gordonii* under all three growth conditions (Fig. 5B). As NagB activity is overwhelmingly higher than NagA activity in *S. mutans*

(Fig. 5) (36), the conversion of GlcNAc-6-P to GlcN-6-P by NagA is likely the rate-limiting step in GlcNAc catabolism by *S. mutans*. Further, since *S. mutans* actually presented with slightly higher NagA activity than *S. gordonii* when grown on glucose, it appears that the long lag preceding the growth of UA159 on GlcNAc is not due to low levels of NagA activity in the cultures grown in BHI, which contains Glc.

To examine if the differences in NagA activity between *S. mutans* and *S. gordonii* were determined at the transcriptional level, quantitative real-time PCR (qRT-PCR) was performed using RNA samples extracted from UA159 and DL1 cultures grown on Glc, GlcN, or GlcNAc. The levels of mRNA for *nagA* in cells grown on GlcNAc-based medium were 14.6-fold higher in *S. gordonii* but only 9.1-fold higher in *S. mutans* ($P = 0.038$) (Fig. 6; see also Table S2 in the supplemental material) (24) than those with cells grown on glucose. Similar results were obtained in a comparison of the increases in *nagB* mRNA levels in cells grown on GlcNAc (29.0-fold for DL1 versus 11.7-fold for UA159), relative to Glc-grown cells. When the results from GlcN-grown cells were compared to Glc-grown cells, the contrast between DL1 and UA159 was even greater (Fig. 6; see also Table S2); that is, the fold induction in GlcN- versus Glc-grown cells in DL1 was much greater in *S. gordonii* than in *S. mutans*. Compared to *S. gordonii*, *S. mutans* pro-

FIG 6 Quantitative real-time RT-PCR measurements of the message levels of *nagA* (A), *nagB* (B), and *glmS* (C) genes in *S. gordonii* wild-type DL1 and its *nagR*-null mutant. Error bars represent standard deviations.

duces slightly higher NagA activity in Glc-based medium but lower NagA activity when grown on GlcNAc; this is consistent with the less-efficient induction of *nag* genes in *S. mutans* seen here. We therefore posit that differences in regulation of the transcription of *nagA* and *nagB* in these two bacteria explain in large part their distinct growth phenotypes on GlcNAc.

H₂O₂ impacts expression of *nagA* and *nagB* by *S. mutans*. There are multiple mechanisms by which *S. gordonii* and *S. mutans* can antagonize the growth of one another (11). For example, the low-pH environment produced by *S. mutans* when excess carbohydrates are present inhibits the growth and persistence of acid-sensitive commensal bacteria. Most *S. mutans* strains also produce a suite of bacteriocins (mutacins), many of which are effective against commensal streptococci. Conversely, many commensal streptococci secrete relatively high concentrations (in the millimolar range) of H₂O₂ (11), which is strongly inhibitory to the growth of *S. mutans*. Given the advantage that amino sugars appear to confer to the commensals, we examined the possibility that H₂O₂ influences amino sugar metabolism by *S. mutans* by quantifying mRNA for *nagA*, *nagB*, and *glmS* (GlcN-6-P synthase) using qRT-PCR. RNA samples were prepared using UA159 cells grown exponentially on GlcN in the absence or presence of 0.005% (wt/wt) (1.5 mM) H₂O₂ for 30 min. As a positive control, we also measured the transcript levels of *dpr*, which encodes a protein that contributes to oxidative stress tolerance and is inducible by H₂O₂ treatment (40). A 3-fold decrease (see Fig. S6A in the supplemental material) in mRNA levels for *nagA* and a nearly 2-fold decrease for *nagB* was evident following H₂O₂ treatment. The levels of *glmS* mRNA were slightly elevated in the H₂O₂-treated samples, but the increase was not statistically significant. Thus, it appears that physiologically relevant levels of H₂O₂, i.e., comparable to the levels produced by *S. gordonii* in aerobic static cultures (12), are sufficient to negatively impact the expression of the *nag* genes by *S. mutans*.

As the production of H₂O₂ by *S. gordonii* is stimulated by oxygen (12), we modified our dual-species liquid competition test (see above) by placing the mixed inoculum of UA159 and DL1 strains directly on the surface of an agar plate prepared using TV medium constituted with Glc, GlcN, or GlcNAc. The plates were then placed in an aerobic environment containing 5% CO₂ for 20 h before the entire patch of growth was excised, the cells were dispersed, and the suspension was diluted and plated. Enumeration of CFU revealed that there were about 3-log-greater viable *S. gordonii* cells than *S. mutans* cells in the suspension, but there was no significant difference as a function of the growth of carbohydrate (see Fig. S6B in the supplemental material). Since it is safe to assume that *S. gordonii* produces higher levels of H₂O₂ on the plates than in static liquid cultures (12), the results obtained are most likely associated with severe growth inhibition of *S. mutans* by H₂O₂ rather than any specific effect on *nag* gene expression.

S. gordonii is also able to express comparatively high levels of the enzymes of the arginine deiminase (AD) pathway, in which arginine is metabolized for the production of ATP and acid-neutralizing ammonia. Regulation of the AD operon in *S. gordonii* is subject to catabolite repression by glucose, as AD activity and gene expression are much greater in cells grown on the poorly catabolite-repressing sugar galactose (9). AD gene expression in exponentially growing cells is also inducible by arginine and low pH and repressible by oxygen (41, 42). To examine the potential impact of amino sugars on AD pathway expression, we performed

qRT-PCR and AD enzymatic assays on strain DL1 grown in TV with Glc, GlcN, or GlcNAc. Compared to cells grown with Glc, *S. gordonii* expressed significantly higher levels of the transcript of the arginine deiminase gene *arcA* (16.0-fold $\pm$ 3.5-fold higher in GlcN and 1.7-fold $\pm$ 0.2-fold higher in GlcNAc) and $\sim$ 2-fold-higher AD activity when grown on either amino sugar (see Fig. S7 in the supplemental material). Since the AD system is absent in all *S. mutans* strains examined to date, the amino sugars can uniquely promote the survival of the commensal by enhancing arginine metabolism to moderate acidification of the environment.

NagR_{sg} regulates *nagA*, *nagB*, and *glmS* in *S. gordonii*. To explore the molecular mechanisms governing differential expression of the genes for amino sugar metabolism in *S. gordonii*, an allelic exchange mutant of the gene for NagR_{sg} was created using DL1 as the parental strain. Compared to DL1, the *nagR* mutant showed minor growth defects in a number of carbohydrates, including Glc, fructose, mannose, galactose, lactose, cellobiose, and sucrose, as well as GlcN and GlcNAc (see Fig. S8 in the supplemental material and data not shown). This is in contrast to the behavior of a *nagR* deletion mutant of *S. mutans* UA159, which showed drastically improved growth in GlcNAc, with complete elimination of the long lag phase seen in the parental strain (24). The growth phenotypes on GlcNAc are consistent with the observation that the addition of GlcNAc to DL1, but not to UA159, results in complete alleviation of NagR-mediated repression of the *nagA* gene (Fig. 6) (24).

Besides the genes needed for the catabolism of internalized amino sugars, *nagA* and *nagB*, *S. gordonii* also harbors a gene for GlcN-6-P synthase (*glmS*, SGO_1757). Similar to *S. mutans*, computer algorithms predict at least one putative binding site (*dre*) for NagR upstream of ORF SGO_1757 in *S. gordonii* (see Fig. S4 in the supplemental material) (25). To assess the impact of the deletion of *nagR* on *glmS* transcription in *S. gordonii*, qRT-PCR was performed on RNA samples from the *nagR*-null and wild-type strains grown in Glc, GlcN, or GlcNAc. As expected, the loss of NagR_{sg} resulted in markedly increased mRNA levels for *nagA* and *nagB* when cells were grown in Glc-based medium, whereas for cultures grown with GlcN or GlcNAc, *nagA* and *nagB* transcript levels in the *nagR* mutant were comparable to the derepressed levels observed in the wild-type genetic background (Fig. 6). We also measured *glmS* mRNA in DL1 and its *nagR*-null mutant under these conditions. In the wild-type background, *glmS* transcripts decreased in abundance by approximately 4- and 5-fold in cells grown on GlcN and GlcNAc, respectively, compared to those grown in glucose. However, in the *nagR* mutant, *glmS* mRNA levels were markedly higher than those in the wild-type strain and showed no response to changes in the growth of carbohydrate (Fig. 6). Collectively, then, NagR_{sg} plays a role similar to that of NagR of *S. mutans*, regulating the expression of *nagA*, *nagB*, and *glmS* in *S. gordonii*.

In vitro interactions between NagR_{sg} and its cognate binding sites (*dre*). Our current model for the regulation of amino sugar metabolism in *S. mutans* posits that the differential expression of *nagAB* and *glmS* by NagR depends on changes in the ability of NagR_{sm} to interact with the promoters of these genes in response to the abundance of certain metabolites, including GlcN-6-P and glucose-6-phosphate (G-6-P) (25). To determine if NagR_{sg} interacted with its putative target regions in a manner similar to NagR_{sm}, recombinant NagR_{sg} was overexpressed in *E. coli* as an

FIG 7 Protein-DNA interactions assessed by EMSA and FP. A recombinant NagR (sg) was overexpressed as MBP-NagR in *E. coli*, purified and released by protease cleavage, and then used in an EMSA with biotin-labeled DNA fragments (2 fmol per reaction) containing promoter regions upstream of *glmS*, *nagB*, and *nagA* from *S. gordonii* (A), and a fluorescent polarization (FP) assay (B) against a 6-FAM-labeled probe containing consensus binding sequence of *dre*. CHO, carbohydrate.

MBP-fusion protein, purified, released from MBP by factor Xa treatment, and used in binding assays with DNA probes.

Using three biotin-labeled DNA probes containing the promoter regions of *nagA*, *nagB*, and *glmS* of *S. gordonii*, an EMSA was performed to study the interactions between NagR_{sg} and these putative target genes. In some cases, GlcN-6-P and G-6-P were added to these reactions to examine their potential roles as effector molecules that modulate the DNA binding activity of NagR_{sg}. As shown in Fig. 7A, NagR_{sg} bound to the *nagA* and *nagB* probes with comparable affinity but displayed considerably lower affinity for the *glmS* probe. Further, the interaction of both the *nagA* and *nagB* probes with NagR_{sg} at lower protein concentrations (e.g., 0.1 μM) resulted in a single shifted species, whereas at higher concentrations of protein (1 μM), complexes formed that did not readily enter the gel, similar to what is commonly seen with other NagR proteins (24, 43). For the *glmS* probe, however, titration of NagR_{sg} resulted in multiple bands at lower concentrations (0.1 to ~0.2 μM) and smearing or failure to enter the gel at higher concentrations (1 μM). When 20 mM GlcN-6-P was included in the binding reaction, both *glmS* and *nagB* probes showed a near-complete loss of interaction with the protein, whereas the *nagA* probe still complexed with NagR_{sg} but at an increased migration rate compared to the reaction using the same amount of protein absent of any GlcN-6-P. Finally, when 20 mM G-6-P was included in the reactions, regardless of the levels of NagR_{sg}, no impact was noted on the migration rates of these probes (Fig. 7A). Therefore, it appears that GlcN-6-P is able to inhibit or completely block the interaction of NagR_{sg} with its target promoters, a result similar to that observed with *S. mutans* and other NagR-like proteins. However, unlike for NagR of *S. mutans*, G-6-P did not have any effect on the binding activities of NagR_{sg} (25).

To further study the response of NagR_{sg} to key metabolic intermediates, a fluorescence polarization (FP) assay was conducted

using a 45-nt 6-FAM-labeled DNA probe that contains only the consensus sequence of a NagR-binding site (*dre*) (25). Kinetic analysis of the FP data yielded the equilibrium dissociation constant (K_D), which reflects the affinity of the NagR_{sg} for *dre*. As shown in Fig. 7B, NagR_{sg} alone bound to the probe with a K_D value of 23.3 ± 2.9 nM. The addition of 5 mM GlcN-6-P increased the K_D to 78.6 ± 29.8 nM, indicative of a significant reduction in binding affinity of NagR_{sg}. In contrast, the presence of 5 mM G-6-P did not cause significant changes in the affinity of NagR_{sg} for *dre*, yielding a K_D value of 28.8 ± 7.7 nM. These results further support that NagR_{sg} responds to metabolic effectors in a manner that is different from the *S. mutans* NagR protein.

DISCUSSION

The initiation and progression of dental caries are associated with perturbations in the composition and biochemical activities of the microbial communities at particular sites on the tooth surface, involving hundreds of bacteria and other microorganisms. Each bacterial species displays distinct behaviors that determine its ecological niche. Understanding the interactions between these microorganisms and the mechanisms by which they compete and gain advantages in particular environments is critical to understanding the basis for this disease and for success in developing therapeutic strategies to impede the onset of caries.

GlcNAc is naturally present in the oral cavity as a constituent of many biological polymers, including bacterial peptidoglycan, fungal cell walls, and glycoconjugates decorating mucins and other salivary glycoproteins (44). It is estimated that as much as 80% of the total mass of a salivary mucin molecule can be composed of carbohydrates, and the most common components include sialic acid, galactose, fucose, and acetyl-hexosamines, including GlcNAc (45). Bacteria frequently harbor genes encoding NagA and NagB for the purpose of metabolizing GlcN and GlcNAc. As discussed above, the *nag* system in *S. gordonii* is similar to that in *S.*

mutans, both in terms of genetic organization and conservation in the primary sequence of the gene products. However, important differences in amino sugar metabolism in *S. mutans* and *S. gordonii* were disclosed in this study. For example, when the enzymatic activities of NagA and NagB were measured, *S. gordonii* expressed higher NagA activity than *S. mutans*, while the opposite was the case for NagB. Although these enzymatic assays were carried out at a pH value that is optimal for most of these enzymes (36), one would predict the intracellular pH of streptococci in mid-exponential phase to be near neutral, as is the pH of resting dental plaque when host glycoproteins are a primary source of energy. The activities are also consistent with the mRNA levels measured in cells.

All data thus far support that GlcN-6-P, rather than GlcNAc-6-P, serves as the allosteric effector of NagR that derepresses the transcription of both *nagA* and *nagB* promoters in the bacteria studied here. Deacetylation of GlcNAc-6-P by NagA may be part of a positive feedback loop to derepress *nagA* and *nagB* expression through the production of GlcN-6-P. Notably, the conversion of GlcNAc-6-P to GlcN-6-P appears to be rate limiting and appears to explain the different characteristics of growth of *S. mutans* and *S. gordonii* on GlcNAc. Further, for reasons that are not yet completely understood, there appears to be a less-efficient induction of *nag* gene expression in *S. mutans*, resulting in generally lower *nagA* mRNA levels and NagA enzyme activities. Another factor that may contribute to the difference in the catabolic capacity of GlcNAc by the commensal and pathogen is the finding that *S. mutans* depends entirely on the PTS for GlcNAc uptake, whereas *S. gordonii* has both PTS and non-PTS (presumably ABC transporter) activities for internalization of GlcNAc. Since *S. mutans* possesses constitutive and relatively high levels of GlcNAc PTS activity, without similarly high levels of the catabolic enzyme, the accumulation of toxic levels of GlcNAc-6-P in cells that have not been preadapted to growth on GlcNAc may explain the prolonged lag phase of *S. mutans* when transferred from glucose to GlcNAc as the primary carbohydrate source. In support of this hypothesis, prior induction of the system, by either GlcNAc or GlcN, allows for increases in NagA activity and completely eliminates the delay of growth on GlcNAc (data not shown). A similar scenario is unlikely to exist in *S. gordonii* because it has low GlcNAc PTS activity and rapid induction of NagA activity, so GlcNAc-6-P or other inhibitory compounds should not accumulate. If this hypothesis is correct, one would predict that *S. mutans* might fare better when grown on lower levels of GlcNAc. In fact, we have observed that compared to growth on GlcN alone, the addition of GlcNAc at 0.01% (about 0.45 mM) promoted the growth of *S. mutans* on GlcN, although GlcNAc at levels of >0.05% caused a notable lag phase (see Fig. S9A in the supplemental material). For *S. gordonii*, however, titration of GlcNAc showed a concentration-dependent augmentation of the growth rate on GlcN-containing medium (see Fig. S9B in the supplemental material). Further study is needed to understand the nature of the non-PTS transporter for GlcNAc and subsequent metabolism of the carbohydrate by *S. gordonii*.

Another significant finding of this study lies in the elucidation of the molecular mechanisms governing the expression of the *nagA*, *nagB*, and *glmS* genes in *S. gordonii*. *S. gordonii* is the second streptococcal species for which we have confirmed that NagR directly controls the promoter activity of *glmS* via binding to its cognate *dre* site. As reported previously, mechanisms used by the

Gram-negative bacterium *E. coli* and the Gram-positive bacterium *B. subtilis* in regulating *glmS* message levels and expression are apparently not relevant to *S. mutans* (13, 25). Furthermore, the fact that NagR_{sg} appears to respond only to GlcN-6-P, unlike NagR_{sm}, which also responds to G-6-P, points to different capacities of these regulators to sense diverse metabolic signals that in turn affect the way they regulate various target genes. For example, *S. mutans* shows slightly higher NagA activity than *S. gordonii* when grown on Glc, but NagA enzyme levels on GlcNAc-grown cells are significantly lower than those in the commensal. Multiple reasons might contribute to the lower induction of the *nagA* gene in *S. mutans*, but one possibility is the influence of metabolic intermediates that affect NagR function: G-6-P alone or in combination with other factors might somehow reduce the efficiency of GlcN-6-P-mediated derepression of the *nagA* promoter. Similarly, despite the sequence conservation between NagR_{sg} and NagR_{sm}, significant divergence exists in their C-terminal effector-binding domains (see Fig. S3 in the supplemental material), a region that has been shown to be critical for NagR_{sm} to recognize GlcN-6-P and G-6-P (L. Zeng, unpublished data). These findings provide important evidence supporting our hypothesis that interactions between NagR and various metabolic signals are key to the ability of NagR to regulate not only the catabolic genes but also the *glmS* gene that is required for the biosynthesis of amino sugars.

Metagenomic studies and the clearly antagonistic relationship that exists between *S. mutans* and *S. gordonii* support the notion that the two organisms likely occupy distinct ecological niches in humans. As an early colonizer, *S. gordonii* must first adhere to the surface of tooth or soft tissue and be able to extract energy from host macromolecules, which are presented largely in saliva and sloughed epithelial cells. On the other hand, *S. mutans* thrives in a biofilm environment when there is an ample supply of dietary carbohydrates. While *S. gordonii* has been known to metabolize sugars released from mucins, many *S. mutans* strains are not particularly good at utilizing mucins or saliva as a primary energy source (see Fig. S1 in the supplemental material) (27, 34, 46). The high PTS activity of *S. mutans* may suggest that it anticipates only small amounts of GlcNAc, whereas the redundancy in GlcNAc uptake systems by *S. gordonii* may reflect the importance of this carbohydrate for its establishment and persistence. Consistent with this notion, the PTS-negative EI mutant of *S. gordonii* retains the ability to grow not only on GlcNAc but also on galactose and mannose (data not shown), two additional sugars frequently found decorating the side chains of mucins. It is also important to note that the high PTS activity possessed by *S. mutans* for amino sugars may simply be due to the fact that the permease (EI^{Man}) that internalizes these carbohydrates is also the primary route by which *S. mutans* internalizes glucose and certain other abundant carbohydrates.

Besides these inherent differences in growth abilities between *S. mutans* and commensals on amino sugars, additional factors could be considered in support of the antagonism of *S. mutans* by *S. gordonii* in the context of amino sugar metabolism. First, the release of H₂O₂ by *S. gordonii* and similar bacteria not only inhibits the growth of *S. mutans*, but it also negatively affects the levels of NagA and NagB activity produced by *S. mutans*. Second, we have consistently noted higher steady-state pH values in the mixed-species cultures, both batch culture and biofilms, as a result of the metabolism of GlcNAc or GlcN, than those of Glc. It is

understood that amino sugar metabolism by both types of bacterial species, pathogen and commensal, leads to the release of ammonia, which has the direct consequence of increasing pH. Moreover, our data clearly show that *S. gordonii* expresses significantly higher AD activity when grown on amino sugars, presumably as a result of reduced catabolite repression of the AD operon relative to glucose. Collectively, these conditions clearly favor the less-acid-tolerant species *S. gordonii* and other commensals over the caries pathogens that derive their primary ecological advantage from being better able to grow in an acidic pH environment than commensals that are considered to be health associated or beneficial.

One intriguing finding of this study is the fact that the NagA enzyme, as well as *nagA* mRNA, is highly inducible by GlcN, even though NagA is unlikely to be needed for GlcN metabolism. In our protein-DNA binding assays, we found no interactions between NagR_{SG} and unphosphorylated GlcN or GlcNAc (data not shown). Thus, if GlcN-6-P is the allosteric effector for NagR, GlcN is likely a more efficient inducer than GlcNAc, since GlcNAc requires the action of NagA to become GlcN-6-P. In fact, this is what was observed (Fig. 5 and 6): higher levels of NagA activity were detected in cells grown on GlcN than on GlcNAc. However, most bacteria showed higher growth rates on GlcNAc than on GlcN, despite the fact that GlcN-based cultures often had higher final pH values (Fig. 2) and released greater quantities of ammonia (24). Together, these observations point to one plausible explanation for this enigmatic finding: perhaps GlcNAc is the only substrate that is commonly or naturally available to oral bacteria. Indeed, the largest known source of GlcN is via manufacturing, i.e., through acid hydrolysis of chitin to break down the GlcNAc polymer with concomitant deacetylation of GlcNAc to GlcN. Most of the naturally present GlcN in the environment and oral cavity likely originates from the deacetylation of GlcNAc. For example, deacetylation of an extracellular polymer of GlcNAc [poly-β-(1,6) N-acetyl-D-glucosamine (PNAG)] has been shown to be important for biofilm development in staphylococci (47). However, this source of GlcN is likely limited in comparison to GlcNAc. It is also of significance that GlcN is relatively unstable under neutral or alkaline pH conditions (24).

In conclusion, by contrasting the capacity of amino sugar metabolism in *S. mutans* and commensal streptococci, our study presented evidence of the potential of amino sugars to affect bacterial ecology in the oral environment. These effects, mediated in part by the release of ammonia that neutralizes acidic pH and in part by the ability of these compounds to foster better growth of beneficial bacteria, allow us to envision an amino sugar-containing therapeutic that may inhibit the development or progression of dental caries, either as a standalone prebiotic treatment or as part of a probiotic regimen aimed at reestablishing a healthy microbiota. At the molecular level, we have made important discoveries that could help foster a better understanding of how lactic acid bacteria effect NagR-dependent gene regulation. More research is needed in order to expand our understanding of amino sugar metabolism into other caries-related bacteria, and as importantly, its impact on the virulence potential of the oral microbiome in a clinical setting.

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