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Effects of *N*-Acetylglucosamine on Carbohydrate Fermentation by *Streptococcus mutans* NCTC 10449 and *Streptococcus sobrinus* SL-1

KAREN A. HOMER,* RUPAL PATEL, AND DAVID BEIGHTON

Hunterian Dental Research Unit, London Hospital Medical College, Turner Street, Whitechapel, London, E1 2AD, England

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We have investigated the ability of two species of streptococci isolated from the human oral cavity (*Streptococcus mutans* NCTC 10449 and *Streptococcus sobrinus* SL-1) to metabolize *N*-acetylglucosamine (GlcNAc), a naturally occurring amino sugar present in saliva and human glycoproteins, when provided as the sole fermentable carbohydrate and determined the effects of the presence of GlcNAc on the fermentation of other carbohydrates. *S. mutans* used GlcNAc at concentrations of up to 10 mM to increase cell numbers, but *S. sobrinus* was unable to ferment the amino sugar alone and its uptake only occurred in the presence of a fermentable carbohydrate. GlcNAc had a marked inhibitory effect on the ability of *S. sobrinus* to produce lactic acid from glucose, sucrose, and fructose, at the same time increasing the lag period and doubling time of batch-grown cells. Such patterns of inhibition were found with *S. mutans*, but the effects were less than those seen in *S. sobrinus*. In mixed culture studies of the two species, *S. sobrinus* became the predominant organism when 10 mM glucose was supplied as the sole fermentable carbohydrate, with a concomitant decrease in the numbers of *S. mutans* cells, but supplementation of the broth with 10 mM glucose and 10 mM GlcNAc resulted in the emergence of *S. mutans* as the predominant organism. *S. mutans* and *S. sobrinus* grown in media containing glucose possessed the ability to transport glucose and GlcNAc, probably via the same glucose-phosphotransferase system at similar rates. However, intracellular levels of *N*-acetylglucosamine-6-phosphate deacetylase and glucosamine-6-phosphate deaminase were markedly higher in *S. mutans* grown on glucose and GlcNAc than in *S. sobrinus*: 34 and 398 and 8 and 17 nmol of NADPH formed per min per mg of protein for *S. mutans* and *S. sobrinus*, respectively. We propose that GlcNAc inhibited growth of *S. sobrinus* in media containing glucose and GlcNAc by competing with glucose for the glucose phosphotransferase, depleting intracellular levels of phosphoenolpyruvate, and possessing, in contrast to *S. mutans*, low levels of *N*-acetylglucosamine-6-phosphate deacetylase and glucosamine-6-phosphate deaminase activity. Together, these data suggest that in dental plaque, *S. sobrinus* when exposed to GlcNAc will have a reduced ability to compete with *S. mutans* for dietary carbohydrates, contributing to the greater frequency of isolation of *S. mutans* from human populations.

The mutans streptococci are a biochemically and genetically diverse group of organisms of which *Streptococcus mutans* and, to a lesser extent, *Streptococcus sobrinus* have been implicated as the principal etiologic agents of dental caries, including nursing bottle caries, coronal caries, and root surface caries (25). The sequence of events which allows these cariogenic bacteria to establish on the dentition has been investigated in numerous studies (10, 15).

S. mutans and *S. sobrinus* differ in properties which should influence their survival and persistence in vivo and which are believed to potentiate the initiation and progression of dental caries. Thus, *S. sobrinus* strains are more acidogenic, more aciduric, produce more water-insoluble polymer from sucrose, and exhibit a greater degree of cariogenicity in gnotobiotic animals than *S. mutans* strains (4, 11, 12, 14, 18, 35). Despite possessing these properties, *S. sobrinus* is isolated from human populations far less frequently than *S. mutans* and, when isolated, is almost invariably present in lower numbers than *S. mutans* (25). The mechanisms suppressing the emergence of *S. sobrinus* in human dental plaque are not known.

In the absence of host diet, bacterial growth in the oral cavity is primarily dependent upon the bacterial degradation of host-derived macromolecules, including mucins, transferrin, and immunoglobulins, which enter the oral cavity in salivary secretions and from gingival crevicular fluid exudate (8, 9). The oligosaccharide side chains of these macromolecules are rich in the amino sugar *N*-acetylglucosamine (GlcNAc), which is fermented by *S. mutans* but not by *S. sobrinus* (5, 21). Extracellular *N*-acetyl-β-D-glucosaminidase activity, necessary for the release of GlcNAc from the oligosaccharide portion of glycoproteins, is elaborated in many species of the plaque microflora (3, 20, 32, 33, 36), and GlcNAc is found free in saliva (1). It is likely that oral bacteria utilize GlcNAc, a potential source of both carbon and nitrogen, once it is released by the action of bacterial enzymes, as occurs in continuous cultures of mixed populations of dental plaque bacteria (6). Indeed, the complementary action of enzymes has been suggested as a mechanism by which mutans streptococci may interact to degrade glycoproteins synergistically (19, 34).

We have studied the ability of both these species of mutans streptococci to utilize GlcNAc as the sole fermentable carbohydrate and determined the effects of GlcNAc on the metabolism of other carbohydrates, because this might provide an insight into their very different abilities to thrive and persist in vivo.

* Corresponding author.

MATERIALS AND METHODS

Growth and maintenance of bacterial strains. *S. mutans* NCTC 10449 and *S. sobrinus* SL-1 (both type strains) were stored at −20°C in brain heart infusion broth (Oxoid Ltd., Basingstoke, Hampshire, England) containing 10% (vol/vol) glycerol. Strains were routinely subcultured onto fastidious anaerobe agar (FAA; LabM Ltd., Bury, Lancashire, England) containing 5% (vol/vol) horse blood and incubated at 37°C under microaerophilic conditions. Single colonies of each strain were subcultured into 20 ml of brain heart infusion broth containing 0.2% (wt/vol) glucose (Oxoid), incubated aerobically without shaking at 37°C for 18 h, and used to inoculate semidefined media.

Utilization of carbohydrates and growth in semidefined media. A modification of a semidefined medium (29) was used in all studies of growth and carbohydrate utilization. Neither *S. mutans* nor *S. sobrinus* was capable of growth on this medium in the absence of supplementary carbohydrate sources. The medium contained 30 g of sodium acetate, 25 g of casein hydrolysate (Oxoid Ltd.), 15.75 g of anhydrous Na_2HPO_4, 10.25 g of anhydrous NaH_2PO_4, 3 g of ammonium sulfate, 2.2 g of KH_2PO_4, 1.5 g of K_2HPO_4, 1.125 g of trisodium citrate, 0.15 g of adenine, 0.15 g of uracil, 0.1 g of guanine, 0.05 g of ferrous sulfate, 0.05 g of manganous sulfate, and 0.05 g of NaCl in a total volume of 5.0 liters of distilled water. The medium was sterilized by autoclaving at 121°C for 15 min and cooled. Immediately prior to use, filter-sterilized solutions (0.2-μm pore size, Millipore Ltd., Watford, England) of $MgSO_4$, cysteine-HCl, and Na_2CO_3 were added to final concentrations of 0.25, 2.2, and 0.4 g/liter, respectively, along with 5.0 ml of a filter-sterilized vitamin solution. The vitamin solution contained 20 mg of nicotinamide, 8 mg of pantothenate, 8 mg of pyridoxamine, 4 mg of riboflavin, 4 mg of thiamine, 0.5 mg of biotin, 0.1 mg of folic acid, and 0.1 mg of *p*-aminobenzoate in 20 ml of distilled water.

The medium was dispensed into sterile Universal containers (Sterilin Ltd., Stone, Staffordshire, United Kingdom) in 18-ml aliquots. Filter-sterilized stock solutions of glucose, sucrose, fructose, GlcNAc and glucosamine (GlcN) were prepared at a 0.1 M concentration. Single sugars or mixtures were added to the basal medium to give 1, 5, or 10 mM concentrations. The final volume of the media was maintained at 20 ml by the addition of sterile distilled water where appropriate. Control media contained no added carbohydrate, and sterile distilled water replaced the carbohydrate solutions.

Prior to inoculation, 1-ml aliquots of medium were removed for subsequent substrate analyses and stored at −20°C. Media were inoculated with 1 ml of an 18-h culture of *S. mutans* or *S. sobrinus*, mixed, and incubated aerobically without agitation at 37°C. Growth was monitored by removing 200-μl aliquots of each culture at intervals, after gentle inversion of the Universal containers, and the increase in optical density was monitored at 620 nm in clear 96-well microtiter trays by using a microtiter plate reader (MCC-340; ICN-Flow Laboratories Ltd., Hertsfordshire, England). Samples (1 ml) were removed from cultures throughout the period of growth and placed into microcentrifuge tubes, and cells were pelleted by centrifugation (13,000 × *g*, 5 min, MSE Microfuge). Culture supernatants were stored at −20°C for the subsequent analysis of carbohydrate and lactate concentrations (as described below) and determination of pH, which was carried out with a Corning 240 pH meter.

Estimation of carbohydrate and lactate concentrations. Carbohydrate and lactate concentrations were determined in growth media and in culture supernatants appropriately diluted with distilled water. All absorbance values were determined in 96-well microtiter trays by using a microtiter plate reader. Where kits were used in determinations, manufacturers' instructions were followed, except that volumes of reagents were reduced.

Glucose concentrations were determined by using Sigma Chemical Co. Ltd. (Poole, Dorset, United Kingdom) kit no. 510. Glucose was converted to gluconic acid and hydrogen peroxide in the presence of glucose oxidase. *o*-Dianisidine reacted with the hydrogen peroxide product in the presence of peroxidase to yield an oxidized form of the *o*-dianisidine, which is brown in color. The A_{450} at the end point of the reaction was used to estimate glucose concentrations by comparison with a series of standard glucose concentrations (linear to 1 mM) made up in distilled water.

GlcNAc concentrations were estimated by using a modification of the method of Levvy and McAllan (22). The reagent for this assay was made up of 11 ml of concentrated HCl, 1.5 ml of distilled water, 87.5 ml of glacial acetic acid, and 10 g of 4-(*N*,*N*-dimethylamino)-benzaldehyde (Sigma). Immediately prior to use, 10 ml of this reagent was diluted to 100 ml with glacial acetic acid. Samples (125 μl) were added to 25 μl of 0.2 M dipotassium tetraborate (pH 9.0) in a microcentrifuge tube and placed in a boiling water bath for 3 min. Samples were removed and cooled to room temperature, 750 μl of the diluted reagent was added, and the samples were incubated at 37°C for 20 min. Treated samples were cooled to room temperature, the A_{540} was determined, and GlcNAc concentrations were calculated by comparison with reference to a standard curve obtained with GlcNAc in distilled water at concentrations up to 0.5 mM. GlcN was measured with the GlcNAc assay described above, except that 800 μl of the diluted culture supernatant was treated with 100 μl of a 1.5% (vol/vol) solution of acetic anhydride in acetone for 5 min at room temperature to bring about *N*-acetylation prior to addition of the borate buffer.

Lactic acid concentrations in culture supernatants were determined by using Sigma kit no. 826. Lactate dehydrogenase acted upon lactic acid in the presence of NAD to form pyruvic acid and NADH. The A_{340}, because of the formation of NADH, at the end point of the reaction was used as a measure of the initial concentration of lactic acid. Concentrations of lactic acid in distilled water up to 10 mM were treated in the same manner and used as standards, giving a linear response over the concentration range described.

The bacteria were grown under each culture condition in duplicate, and each assay was also performed in duplicate. The mean values of the four estimates for each parameter are presented. From these data, the doubling time (26), lag period (time taken for an increase of 0.1 in optical density to occur), and maximum rate of substrate utilization (micromoles per hour) of each culture were calculated.

Growth of *S. mutans* and *S. sobrinus* in mixed culture. *S. mutans* and *S. sobrinus* were grown for 18 h in brain heart infusion broth, and these cultures were used to inoculate 100-ml volumes of semidefined medium supplemented with 10 mM glucose, 10 mM GlcNAc, 10 mM glucose, and 10 mM GlcNAc as well as a control broth with no added supplementation. All cultures were set up in duplicate, and the initial bacterial concentrations were 4.8×10^7 CFU/ml for *S. mutans* and 1.2×10^7 CFU/ml for *S. sobrinus*. The cultures were incubated at 37°C, aliquots were withdrawn at intervals, cultures were diluted in phosphate-buffered saline, and

the total numbers of *S. mutans* and *S. sobrinus* in each culture were determined by plating 100-μl aliquots of appropriate dilutions in triplicate onto TYC medium (LabM; 13) and incubating these samples anaerobically for 3 days. The two species can be differentiated because *S. sobrinus* colonies were surrounded by a white halo, and *S. mutans* formed small, slightly embedded colonies; fewer than 0.2% of the *S. mutans* colonies exhibited white halos similar to those produced by *S. sobrinus*. From these colony counts, the relative proportions of the two species of mutans streptococci were calculated.

Phosphoenolpyruvate-carbohydrate-PTS assays. These assays were carried out after the method of Slee and Tanzer (31). *S. mutans* and *S. sobrinus* cells were grown in semidefined medium containing either glucose or GlcNAc at a final concentration of 10 mM. After two transfers in these media, cultures were allowed to reach the late exponential phase or the early stationary phase and were harvested by centrifugation. Cells were washed with a 0.5% volume of 50 mM Tris-HCl, pH 7.0, and harvested. Cell pellets were resuspended in the same buffer, the A_{600} was adjusted to approximately 2.0, and suspensions were stored at −20°C for periods of up to 1 week.

The membrane integrity of cells was perturbed by the addition of toluene-acetone (1:4) to a final concentration of 1% (vol/vol) to thawed cell suspensions. Portions of the cell suspensions in glass test tubes were agitated vigorously at room temperature for 90 s with a Vortex mixer, and the decryptified cells were stored on ice. The protein concentrations of decryptified cell suspensions were determined by using the Coomassie blue dye-binding assay (7) and by comparison with standard concentrations of bovine serum albumin (Sigma).

The phosphotransferase (PTS) assay contained the following components in a final volume of 1.0 ml: 1.0 mM phosphoenolpyruvate (Sigma), 0.1 mM NADH (Sigma), 2.0 U of lactate dehydrogenase (Sigma), 1 mM $MgCl_2$, 10 mM sodium fluoride, 25 mM potassium phosphate buffer (pH 7.0), decryptified cells, and 10 mM glucose, GlcNAc, or mannitol. Mannitol was included as a negative control since it is transported via a specific PTS distinct from that for glucose (27). All assays were incubated at 37°C for 10 min prior to the addition of the carbohydrate solution. The decrease in A_{340} due to the oxidation of NADH was monitored with a Shimadzu UV-160A recording spectrophotometer. Control assays were set up in which the carbohydrate solution was omitted, with the total assay volume maintained at 1.0 ml, to take into account the endogenous rate of NADH oxidation. All assays were carried out in triplicate. Concentrations of NADH in assay systems were calculated by comparing assay absorbance values with those obtained for standard concentrations of NADH (linear to at least 0.1 mM). PTS activities of cells are expressed as nanomoles of NADH oxidized per minute per milligram of bacterial protein.

***N*-acetylglucosamine-6-phosphate deacetylase and glucosamine-6-phosphate deaminase assays.** *S. mutans* and *S. sobrinus* were inoculated into semidefined medium (20 ml) containing either 10 mM glucose, 10 mM GlcNAc, 10 mM GlcN, and 5 mM glucose plus 5 mM GlcNAc or 5 mM glucose plus 5 mM GlcN. All cultures were set up in triplicate and were incubated at 37°C until growth reached the midexponential phase. Cells were harvested by centrifugation (4,000 × *g*, 10 min), washed in 5 ml of Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid buffer (TES buffer; 0.1 M, pH 7.5), pelleted by centrifugation, and resuspended in 2 ml of TES buffer. Cells were disrupted by shaking with glass beads in a Mickle disintegrator for 10 min at 4°C. Cell debris was removed by centrifugation (13,000 × *g*, 10 min) at 4°C, and supernatants were used as a source of *N*-acetylglucosamine-6-phosphate deacetylase and glucosamine-6-phosphate deaminase activities.

Preliminary studies on *N*-acetylglucosamine-6-phosphate deacetylase activity in crude cell extracts were carried out by the method of Bates and Pasternak (2). Assays contained 200 μl of 0.2 M sodium phosphate buffer (pH 7.5), 50 μl of 10 mM *N*-acetylglucosamine-6-phosphate, and an appropriate amount of crude cell extract in a final volume of 500 μl. Assays were incubated at 37°C, and reactions were terminated, while the rate of substrate utilization was linear, by heating to 100°C for 3 min. Concentrations of *N*-acetylglucosamine-6-phosphate remaining were estimated by using a modification of the Levvy and McAllan assay described above (22) and by comparison with a standard curve of *N*-acetylglucosamine-6-phosphate. White and Pasternak (38) noted that *N*-acetylglucosamine-6-phosphate deacetylase activity could be measured with an assay coupled through glucosamine-6-phosphate deaminase if a sufficient amount of this enzyme was present and was not found to be rate limiting. Activity levels of glucosamine-6-phosphate deaminase were always found to be higher than those of *N*-acetylglucosamine-6-phosphate deacetylase in crude cell extracts of the mutans streptococci. Assays for the deacetylase and deaminase activities were therefore routinely carried out by the method of White and Pasternak (38). Assays contained 25 μl of 10 mM *N*-acetylglucosamine-6-phosphate, 50 μl of 0.2 M sodium phosphate buffer (pH 7.5), 25 μl of 2 mM NADP, 4 U of phosphoglucose isomerase, 1.5 U of glucose-6-phosphate dehydrogenase, and crude cell extract in a total volume of 250 μl in 96-well microtiter trays. Glucosamine-6-phosphate deaminase activity was measured in an assay system identical to that described for *N*-acetylglucosamine-6-phosphate deacetylase, except that glucosamine-6-phosphate replaced *N*-acetylglucosamine-6-phosphate. All assays were performed in duplicate on each culture, and the assays were incubated at 37°C. Control assays contained no cell extract, but TES buffer (0.1 M, pH 7.5) was added to maintain the volume at 250 μl. The formation of NADPH was followed by monitoring the increase in A_{340}. Rates of formation of NADPH were calculated from the extinction of standard concentrations of NADPH in the same buffer system. Protein concentrations of crude cell extracts were determined using the Coomassie blue dye-binding assay (7) and by comparison with standard concentrations of bovine serum albumin (Sigma). Rates of enzyme activity are given as units per milligram of protein, where 1 U is the amount of enzyme required to catalyze the production of 1 μmol of NADPH per min.

RESULTS

Utilization of glucose, GlcNAc, and GlcN. *S. mutans* NCTC 10449 utilized the three single carbohydrates or combinations of these to increase cell numbers, with little difference in the doubling time, irrespective of the carbon source (Table 1). However, the lag period was increased when either GlcNAc or GlcN was incorporated into the culture medium. In all cases, the increase in lactic acid measured in culture supernatants paralleled the increase in optical density of cultures (data not shown). *S. sobrinus* SL-1 when grown on glucose exhibited a shorter doubling time, a shorter lag period, and a correspondingly higher rate of substrate utilization than *S. mutans* grown on the same substrate (Table

TABLE 1. Comparison of the growth of *S. mutans* NCTC 10449 with that of *S. sobrinus* SL-1 in media supplemented with glucose, GlcNAc, and GlcN

CHO[a] added	*S. mutans* 10449			*S. sobrinus* SL-1		
	Doubling time (h)	Lag time (h)	Maximum rate of CHO utilization (μmol/h)	Doubling time (h)	Lag time (h)	Maximum rate of CHO utilization (μmol/h)
Glucose	2.0	2.8	57.5	1.0	1.6	59.6
GlcNAc	2.1	3.8	45.5	ND[b]	ND	ND
GlcN	2.1	3.4	36.9	1.6	4.0	52.2
Glucose + GlcNAc	2.2	3.2	21.9 + 15.9	2.9	4.0	14.6 + 12.2
Glucose + GlcN	2.2	2.6	39.1 + 7.2	1.0	1.6	43.9 + 32.8

[a] CHO, carbohydrate.
[b] ND, no growth detected.

1). However, *S. sobrinus* did not grow in media supplemented with GlcNAc over an 8-h incubation period. Growth of *S. sobrinus* on media containing glucose and GlcNAc was characterized by a marked increase in doubling time, a longer lag period, and a decreased rate of glucose utilization (Table 1).

A more detailed comparison of the growth of the two strains of mutans streptococci on combinations of glucose and GlcNAc is shown in Fig. 1 and 2. *S. mutans* utilized combinations of glucose and GlcNAc to increase cell numbers with a concomitant production of lactic acid (Fig. 1); glucose uptake preceded that of GlcNAc. Both sugars were completely utilized during the incubation period, with no obvious evidence of a diauxic effect. Combinations of glucose and GlcNAc increased the doubling time and reduced the rate of glucose uptake by *S. sobrinus* (Fig. 2 and Table 1). Despite the inability of *S. sobrinus* to grow at the expense of GlcNAc as the sole fermentable carbohydrate, GlcNAc was utilized when supplied in combination with glucose, albeit at a relatively slow rate, and lactic acid was the major end product. In a manner similar to that during the growth of *S. mutans*, no diauxic effect was observed during the growth of *S. sobrinus*.

Effect of varying concentrations of GlcNAc on glucose utilization. The inhibitory effect that equimolar concentrations of GlcNAc had on glucose utilization by *S. sobrinus* led to an investigation of the effect of different concentrations of GlcNAc on growth and glucose utilization by *S. mutans* and *S. sobrinus*. Figure 3A shows that the doubling time of *S. mutans* on 10 mM glucose was relatively unaffected by the addition of GlcNAc at concentrations of up to 10 mM. However, the doubling time for *S. sobrinus* was increased from 1.0 h with 10 mM glucose and no added GlcNAc to 2.5 h with 10 mM glucose and 10 mM GlcNAc. Increasing concentrations of GlcNAc resulted in an increased lag period for both *S. mutans* and *S. sobrinus* (Fig. 3B), but while that of *S. mutans* increased from 2.6 to 3.6 h with 0 and 10 mM GlcNAc added to media containing 10 mM glucose, respectively, the increase in lag period for *S. sobrinus* was more pronounced, from 2.5 to 5.2 h. With glucose alone, the rates of glucose utilization by *S. mutans* and *S. sobrinus* were 46.7 and 68.2 μmol/h, respectively, but in media with 10 mM glucose and 10 mM GlcNAc, this rate had decreased by 15% to 39.7 μmol/h with *S. mutans* and by 75% to 17.0 μmol/h with *S. sobrinus*.

Effect of GlcNAc on the metabolism of glucose, sucrose, and fructose. Growth of *S. mutans* and *S. sobrinus* on 10 mM glucose was not affected by the presence of 1 mM GlcNAc but was reduced in the presence of this compound at a

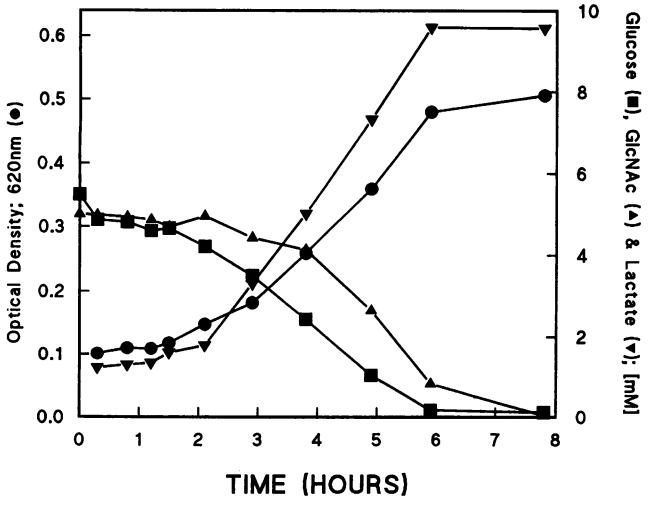

FIG. 1. Growth characteristics of *S. mutans* NCTC 10449 in semidefined media supplemented with 5 mM glucose and 5 mM GlcNAc incubated at 37°C in static culture, increase in optical density, utilization of glucose and GlcNAc, and production of lactate.

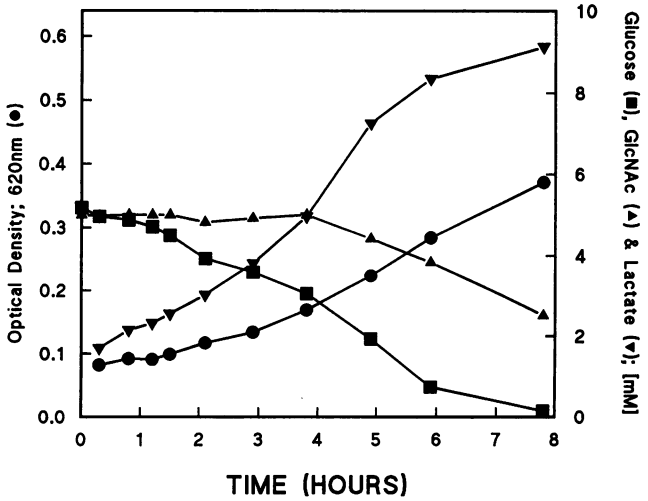

FIG. 2. Growth characteristics of *S. sobrinus* SL-1 in semidefined media supplemented with 5 mM glucose and 5 mM GlcNAc incubated at 37°C in static culture, increase in optical density, utilization of glucose and GlcNAc, and production of lactate.

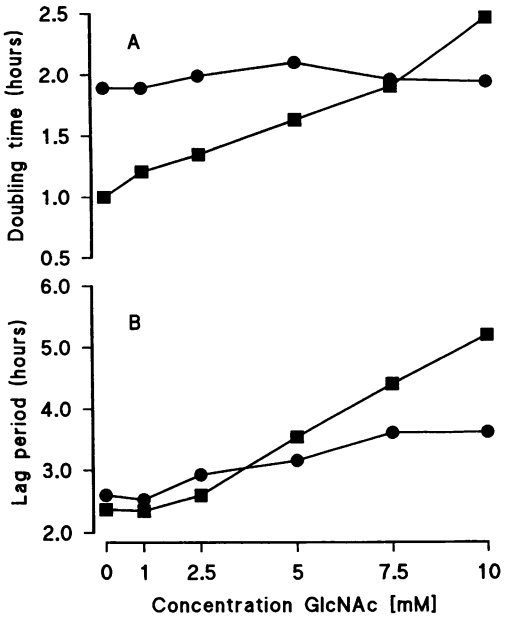

FIG. 3. Effect of increasing GlcNAc concentration on doubling time (A) and lag period (B) of *S. mutans* NCTC 10449 (●) and *S. sobrinus* SL-1 (■) grown in semidefined media containing 10 mM glucose at 37°C in static culture.

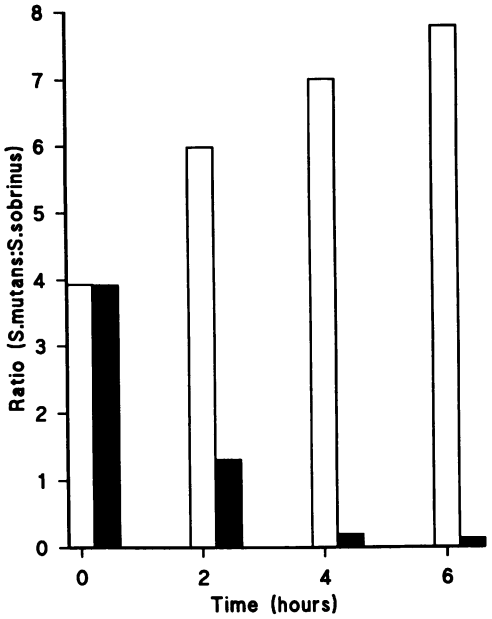

FIG. 4. Influence of GlcNAc on the growth of *S. mutans* NCTC 10449 and *S. sobrinus* SL-1 in mixed culture in semidefined media supplemented with 10 mM glucose alone (solid bars) or 10 mM glucose and 10 mM GlcNAc (open bars). Initial bacterial concentrations were 4.8×10^7 and 1.2×10^7 CFU/ml for *S. mutans* and *S. sobrinus*, respectively.

concentration of 10 mM (Table 2). This effect was most pronounced with *S. sobrinus*, as reflected in the lower increase in optical density and the reduced fall in the pH of culture supernatants than those of *S. mutans*. These effects were more dramatic with *S. sobrinus* grown in media supplemented with 10 mM sucrose or 10 mM fructose and 1 mM GlcNAc. For *S. sobrinus*, the addition of 10 mM GlcNAc reduced growth on sucrose and fructose to almost undetectable levels. The metabolism of sucrose and fructose by *S. mutans* in the presence of GlcNAc was also reduced, but the inhibitory effects of GlcNAc were considerably less than those found with *S. sobrinus*.

Effect of GlcNAc on the growth of combinations of *S. mutans* and *S. sobrinus*. Additional studies were undertaken in order to ascertain the effect that GlcNAc had on the growth of mixtures of *S. mutans* and *S. sobrinus* supplied with glucose as a fermentable carbohydrate source. In the presence of 10 mM glucose alone, the ratio of *S. mutans* cells to *S. sobrinus* cells decreased rapidly from 4 at the start of the incubation period to 0.135 after 6 h of incubation at 37°C (Fig. 4), indicating more efficient utilization of glucose by *S. sobrinus* than by *S. mutans*. When the two species were grown in media supplemented with both 10 mM glucose and 10 mM GlcNAc, the ratio of *S. mutans* cells to *S. sobrinus* cells steadily increased over the incubation period.

Glucose and GlcNAc PTS activities of *S. mutans* and *S.*

TABLE 2. Comparison of the effects of added GlcNAc on growth and culture pH of *S. mutans* NCTC 10449 and *S. sobrinus* SL-1 in media supplemented with glucose, sucrose, and fructose

CHO[a]	*S. mutans* NCTC 10449		*S. sobrinus* SL-1	
	Increase in optical density at 620 nm	Decrease in pH	Increase in optical density at 620 nm	Decrease in pH
10 mM glucose				
+0 mM GlcNAc	0.23	0.55	0.35	0.80
+1 mM GlcNAc	0.24	0.62	0.39	0.96
+10 mM GlcNAc	0.17	0.60	0.11	0.47
10 mM sucrose				
+0 mM GlcNAc	0.31	0.75	0.33	1.04
+1 mM GlcNAc	0.20	0.66	0.01	0.18
+10 mM GlcNAc	0.17	0.54	0.01	0.11
10 mM fructose				
+0 mM GlcNAc	0.20	0.70	0.33	0.91
+1 mM GlcNAc	0.10	0.31	0.01	0.03
+10 mM GlcNAc	0.11	0.43	0.01	0.00

[a] CHO, carbohydrate.

TABLE 3. Glucose and GlcNAc PTS activities of *S. mutans* NCTC 10449 and *S. sobrinus* SL-1

Culture and growth conditions	Carbohydrate challenge	PTS activity (nmol of NADH oxidized/min/mg of protein)
Glucose-adapted *S. sobrinus* SL-1	Glucose	121
	GlcNAc	104
	Mannitol	<5
Glucose-adapted *S. mutans* NCTC 10449	Glucose	95
	GlcNAc	101
	Mannitol	<5
GlcNAc-adapted *S. mutans* NCTC 10449	Glucose	123
	GlcNAc	115
	Mannitol	<5

sobrinus. Glucose-adapted *S. mutans* cells transported glucose and GlcNAc at comparable rates. Similar levels of PTS activity were observed when GlcNAc-adapted *S. mutans* cells were challenged with either of the two carbohydrates (Table 3). Glucose-adapted *S. sobrinus* cells also transported glucose and GlcNAc at rates similar to those observed in *S. mutans*. None of the decryptified cells exhibited significant PTS activity for mannitol.

Production of *N*-acetylglucosamine-6-phosphate deacetylase and glucosamine-6-phosphate deaminase. No detectable *N*-acetylglucosamine-6-phosphate deacetylase and glucosamine-6-phosphate deaminase activity was present in glucose-grown *S. mutans* and *S. sobrinus* cells (Table 4). Significantly higher levels of these enzymes were found in both GlcNAc- and GlcN-grown *S. mutans* cells than in *S. sobrinus* cells, while combinations of glucose with either of these amino sugars reduced the levels of activity of both enzymes. The induction of these enzymes by GlcNAc in *S. sobrinus* could not be studied because of its inability to grow in media with GlcNAc as a sole source of fermentable carbohydrate. Both *N*-acetylglucosamine-6-phosphate deacetylase and glucosamine-6-phosphate deaminase were detected in *S. sobrinus* cells grown in media containing either GlcN, glucose, and GlcN or glucose and GlcNAc, but their levels were markedly lower than those found in *S. mutans* (Table 4).

TABLE 4. Production of *N*-acetylglucosamine-6-phosphate deacetylase and glucosamine-6-phosphate deaminase by *S. mutans* NCTC 10449 and *S. sobrinus* SL-1 grown in the presence of different carbohydrates

CHO[a]	Production[b] (nmol of NADPH formed/min/mg of protein) of:			
	S. mutans NCTC 10449		*S. sobrinus* SL-1	
	Deacetylase	Deaminase	Deacetylase	Deaminase
Glucose	<5	<5	<5	<5
GlcNAc	64	408	ND[c]	ND
GlcN	66	499	12	65
Glucose + GlcNAc	34	398	8	17
Glucose + GlcN	38	289	13	40

[a] CHO, carbohydrate.
[b] Measured in cell extracts.
[c] ND, no growth detected.

DISCUSSION

S. sobrinus utilized glucose more efficiently than *S. mutans*, producing lactic acid at a more rapid rate than *S. mutans*. However, *S. sobrinus* did not metabolize the naturally occurring amino sugar GlcNAc. *S. mutans* fermented GlcNAc but at a rate slower than it was able to ferment glucose. Furthermore, GlcNAc inhibited the utilization of fermentable carbohydrates by *S. sobrinus* to a considerably greater extent than was observed with *S. mutans*, resulting in a markedly decreased cell yield for *S. sobrinus*. The preferential GlcNAc-mediated inhibition of carbohydrate utilization by *S. sobrinus* was studied by using mixed cultures and clearly demonstrated a reduced ability of *S. sobrinus* to grow in the presence of GlcNAc compared with *S. mutans*.

The inability of *S. sobrinus* to utilize GlcNAc as a sole source of carbon for growth initially suggested that either this organism was incapable of transporting GlcNAc or that it did not possess the requisite enzymes for its catabolism. GlcNAc was removed from cultures of *S. sobrinus* when provided in combination with glucose; however, under these conditions, the removal of glucose occurred before that of GlcNAc.

In *Escherichia coli*, GlcNAc can enter the cell via at least two pathways initiated by the phosphoenolpyruvate-sugar-PTS system (27). GlcNAc is phosphorylated either by a specific enzyme II or via the major enzyme II for glucose, the former being induced by GlcNAc in *E. coli* but not the latter (23). PTS systems have been demonstrated in oral streptococci for several sugars, including glucose, sucrose, and fructose (17). Alternative carbohydrate transport systems, namely those driven by proton motive force (17) and employing specific, non-PTS carrier proteins (30), have also been proposed for the transport of carbohydrates by oral streptococci. In the present study, only transport of sugars via the PTS system was investigated. Our data indicate that GlcNAc was transported by glucose-adapted *S. mutans* and *S. sobrinus* cells, possibly via an enzyme II induced by glucose, as occurs in *E. coli* (23). However, *S. mutans* may also possess an enzyme II, specifically induced by GlcNAc but lacking in *S. sobrinus*. Our investigations were unable to differentiate between PTS specific for glucose or GlcNAc.

Enzymatic studies on the production of *N*-acetylglucosamine-6-phosphate deacetylase and glucosamine-6-phosphate deaminase activities by mutans streptococci revealed that both species could produce these enzymes under the appropriate growth conditions, although the specific activities of these enzymes were consistently lower in *S. sobrinus* than in *S. mutans*. These activities were, in *S. mutans*, inducible by both GlcNAc and GlcN, indicating that they were coordinately induced. This is contrary to the situation observed in *E. coli* (28, 37) and *Bacillus subtilis* (2), where GlcNAc induces both the deacetylase and the deaminase but GlcN induces only the deaminase. Strong repression of the deacetylase and deaminase activities was observed in *S. mutans* and *S. sobrinus* cell extracts which had been derived from glucose-grown cells.

Taken together, the transport data and enzymic studies suggest that glucose-adapted *S. sobrinus* cells are unable to grow in the medium with GlcNAc as the sole carbon source because *N*-acetylglucosamine-6-phosphate deacetylase and glucosamine-6-phosphate deaminase are repressed in glucose-adapted cells. Furthermore, the inhibitory effect of GlcNAc on *S. sobrinus* growing on mixtures of glucose and GlcNAc may be due to a combination of effects, including

competition for the glucose-PTS enzyme II and depletion of intracellular phosphoenolpyruvate reserves as *N*-acetylglucosamine-6-phosphate is formed. The further metabolism of *N*-acetylglucosamine-6-phosphate by such cells is likely to be slow in *S. sobrinus* because of the low levels of intracellular enzymes associated with its catabolism compared with the consistently higher levels of intracellular enzymes found in *S. mutans*.

The present findings might provide an explanation for the lower frequency of isolation of *S. sobrinus* from human populations than would be expected from its in vitro growth characteristics. Many in vitro studies have indicated that *S. sobrinus* possesses a number of potential virulence determinants which should enable it to be the most successful species of human mutans streptococci and that it should be the species most closely related to the initiation and progression of dental caries. Thus *S. sobrinus* is more acidogenic and more aciduric, produces greater amounts of water-insoluble polysaccharide, and induces more caries in gnotobiotic animals than *S. mutans* (4, 11, 12, 14, 18, 35). Despite these properties, *S. sobrinus* is less frequently isolated from human populations than *S. mutans*, and when isolated, it is usually found in combination with *S. mutans* and is almost invariably in lower numbers than *S. mutans* (25).

Other investigators have suggested that the differential distribution and colonization of teeth by these streptococci may be influenced by a number of factors, including competition for essential nutrients, the production of bactericidal agents such as bacteriocins (24), and attachment to different pellicle receptors (16). In this report, we have shown that GlcNAc significantly reduces carbohydrate fermentation by *S. sobrinus* and therefore suggest that GlcNAc may also exert this effect in vivo and so influence the relative proportions of *S. mutans* and *S. sobrinus* in the human oral cavity. There are number of different sources of GlcNAc; it is found free in saliva (1), is a product of the action of *N*-acetyl-β-D-glucosaminidases on oligosaccharide side chains of glycoproteins, and is present in cell walls and extracellular polymers of bacteria. Thus, GlcNAc may be liberated from glycoproteins derived from salivary and crevicular secretions and from macromolecules released during the lysis of bacterial cell walls. Although the concentration of GlcNAc in dental plaque has not been determined and no doubt will vary with plaque age and bacterial composition, the present study raises the possibility that the in vivo growth and competitiveness of *S. sobrinus* may be influenced by GlcNAc.

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