

A *Streptococcus mutans* Mutant That Synthesizes Elevated Levels of Intracellular Polysaccharide Is Hypercariogenic In Vivo

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Received 19 September 1994/Returned for modification 9 November 1994/Accepted 25 April 1995

We used the streptococcal transposon, Tn916 to identify and isolate mutants of *Streptococcus mutans* with altered intracellular polysaccharide (IPS) accumulation. We report on the isolation and characterization of *S. mutans* SMS202, a transposon mutant which accumulated the glycogen-like IPS in excess of wild-type levels. Southern blot analysis confirmed a single Tn916 insertion into the SMS202 chromosome. Moreover, quantitative ultrastructural analysis revealed significantly increased concentrations of IPS in SMS202 relative to those of the wild-type progenitor strain, UA130. The activities of ADPglucose pyrophosphorylase (GlgC) and glycogen synthase (GlgA), enzymes required for the biosynthesis of bacterial IPS, were also elevated in the IPS excess mutant. Furthermore, SMS202 was significantly more cariogenic on the molar surfaces of germ-free rats than the wild type ($P < 0.01$), thus confirming a central role for IPS in *S. mutans*-induced caries formation. We propose that the increased cariogenic potential of SMS202 is due to constitutive expression of genes which encode glycogen biosynthesis in this oral pathogen. The coordinate expression of GlgC and GlgA along with the results of ongoing nucleotide sequence analysis and Northern hybridization experiments support an operon-like arrangement for the *glg* genes of this oral pathogen.

Streptococcus mutans, the principal etiologic agent of dental caries in humans, infects more than 95% of the population worldwide, often colonizing the oral cavity during the first year of life (18). Prominent among the attributes which contribute to *S. mutans*-induced caries formation is the ability of the organism to metabolize exogenous dietary carbohydrates and to produce lactic acid as a by-product; this acid production in the oral cavity leads to the demineralization of tooth enamel and the onset of dental decay.

Exogenous carbohydrates as substrates for *S. mutans* metabolism have received widespread attention (4, 18); however, they represent only one source of *S. mutans* acid production in the oral cavity. Interestingly, *S. mutans* may also produce acid by metabolizing intracellular polysaccharides (IPS), glycogen-like storage polymers containing α 1,4- and α 1,6-glucosyl linkages (10). In fact, early reports indicate that streptococci isolated from carious lesions were predominantly synthesizers of IPS, while those isolated from caries-inactive plaque were polysaccharide-negative variants (9). Tanzer et al. later demonstrated that the cariogenic potential of nitrosoguanidine-generated mutants of *S. mutans* with defects related to IPS differed from that of the wild-type strain in vivo (33). It may be inferred from these studies that *S. mutans* IPS promote the formation of dental caries by prolonging the exposure of tooth surfaces to organic acids, especially at non-meal times when exogenous carbohydrates are lacking from the oral cavity. However, the mutations generated in these early investigations were uncharacterized; that is, the lesions were chemically induced and were never subsequently genetically or biochemically defined. The construction of precise, well-defined mutants is therefore necessary if our currently limited knowledge of *S. mutans* IPS accumulation and the mechanism(s) which may regulate IPS expression in the plaque environment is to be extended.

In contrast to what is known of IPS biosynthesis in gram-

positive microorganisms, IPS accumulation has been well studied in the members of the family *Enterobacteriaceae*, notably *Escherichia coli* (22) and *Salmonella typhimurium* (17, 31). In these gram-negative microbes, the *glgC* gene product is an ADPglucose pyrophosphorylase (EC 2.7.7.27) which catalyzes the formation of ADPglucose. In a subsequent reaction, glycogen synthase (EC 2.4.1.21), the gene product of *glgA*, catalyzes the transfer of a glucosyl unit from ADPglucose to a preexisting maltodextrin primer or α 1,4-glucan (23). Finally, a *glgB* branching enzyme (EC 2.4.1.18) introduces α 1,6 branch points which constitute nearly 10% of the total linkages present in bacterial glycogen (23). Interestingly, the *glgC*, *glgA*, and *glgB* genes constitute an operon on the *E. coli* and *S. typhimurium* chromosomes; however, the structural organization of the genes which code for activities analogous to ADPglucose pyrophosphorylase, glycogen synthase, and branching enzyme in gram-positive microorganisms remains unexplored. The characterization of genes involved in *S. mutans* IPS accumulation and an investigation of the role of IPS in *S. mutans*-induced caries formation are therefore significant.

We recently used a streptococcal transposon, Tn916, to generate isogenic mutants of *S. mutans* altered in IPS accumulation. We demonstrated that *S. mutans* SMS201, an IPS-deficient mutant bearing a single transposon insertion, is significantly less cariogenic than its wild-type progenitor in a germfree rat model system (30). From these studies, we concluded that *S. mutans* IPS are significant contributors to the caries-forming process. The *glg* locus which flanks the transposon insertion site on the SMS201 chromosome has since been cloned, and its characterization is ongoing. In the present study, we report on the isolation of *S. mutans* SMS202, a novel transposon mutant which produces IPS in excess of wild-type levels. We demonstrate that SMS202 is significantly hypercariogenic ($P < 0.01$) in germfree rats, lending further support to the possibility of a central role for IPS in *S. mutans*-induced cariogenesis. Moreover, we propose that the increased cariogenic potential of this mutant in vivo is the likely result of constitutive expression of a streptococcal *glg* operon. Indeed,

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and phenotype ^a	Source and/or reference
<i>E. coli</i>		
K-12	F ⁻ <i>endA1 hsdR17</i> (r _K ⁻ m _K ⁺)	11
DH5α	<i>supE44 thi-1 recA1 gyrA96 relA1D</i> (<i>lacZYA-argF</i>)U169φ80d (<i>lacZ</i>)DM15	11
<i>S. mutans</i>		
UA130	<i>S. mutans</i> serotype c Glg ⁺	Caufield (21)
SMS201	UA130::Tn916 Glg ⁻	30
SMS202	UA130::Tn916 Glg ⁺⁺	This work
pAM620	pVA891::pAD1 <i>EcoRI</i> F ['] ::Tn916 Em ^r Tc ^r	36

^a Em, erythromycin; Tc, tetracycline; Glg⁺⁺, excess glycogen.

the coordinate expression of GlgC and GlgA activities in *S. mutans* strongly supports the idea of an operon-like arrangement for the *glgC* and *glgA* genes on the chromosome. In addition, ongoing nucleotide sequence analysis of the *glg* locus and the results of Northern (RNA) hybridization experiments further support the argument for a *glg* operon in this oral pathogen.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in the present study are described in Table 1.

Culture conditions. *S. mutans* UA130 (serotype c) and its isogenic mutant SMS202 were routinely grown anaerobically at 37°C in Todd-Hewitt (TH) broth (Difco Laboratories, Grand Island, N.Y.). The mutant was selected on TH agar containing tetracycline (5 µg/ml). In the preparation of solid media, agar (Difco) was added to a final concentration of 1.5%. Colony morphologies were examined periodically on mitis salivarius agar (Difco), and streptococci were subsequently checked for purity by microscopic examination.

To screen for mutants with altered IPS accumulation, *S. mutans* transposon mutants were grown anaerobically for 5 days on Jordan's medium (12) supplemented with 2% glucose and tetracycline. For quantitative determinations of the IPS content, *S. mutans* cultures were grown anaerobically at 37°C in 10 ml of chemically defined medium (CDM) (34) for 1, 3, and 5 days prior to harvesting by centrifugation and cell disruption.

For growth rate and acid production determinations, *S. mutans* UA130 and SMS202 were cultivated in TH broth either in the presence or absence of 2% exogenous glucose, with selective pressure when appropriate. Samples were withdrawn at various time intervals and measured for cell optical density at 560 nm (OD₅₆₀) with a Milton Roy spectrophotometer or for pH with an Orion digital pH meter.

In the preparation of crude enzyme extracts, 500 ml of TH broth supplemented with 2% glucose was inoculated with an overnight culture of UA130 or its isogenic mutant. The mutant was grown in the presence of tetracycline as described above. Cultures were grown standing at 37°C and were maintained at pH 7.0 with 5 M NaOH for up to 12 h. Cells were subsequently harvested by centrifugation, and extracts were prepared as described previously (1).

For ultrastructural analyses, cells were grown in glucose-enriched TH broth to late logarithmic, early stationary, or stationary phase and harvested by centrifugation. Cells were then pretreated with periodic acid and sodium chlorite to enhance the affinity of IPS for subsequent staining with uranyl and lead acetate salts (6).

For Northern blot analyses, cells were grown as standing overnight cultures at 37°C in TH broth or CDM supplemented with 1% glucose. The cells were harvested by centrifugation, and total RNA was isolated as described below.

E. coli DH5α was grown at 37°C in Lennox broth with gentle aeration.

Mutagenesis of *S. mutans*. We used the *E. coli* plasmid pAM620 (27) which harbors the streptococcal transposon Tn916 (Tc^r) to deliver the transposon to the *S. mutans* genome. The resulting transposon library was plated onto TH agar containing tetracycline. Tetracycline-resistant colonies were subsequently replica plated onto Jordan's medium supplemented with tetracycline and screened for alterations in glycogen content by iodine staining as described previously (9).

Determination of IPS content. *S. mutans* IPS was measured qualitatively by staining colonies grown on Jordan's medium with a 0.2% I₂, 2% KI solution (9). After a 15-min incubation in the dark, the color of the stained colonies was recorded. Colonies with phenotypes deviating from the wild type were selected

for further analysis. Quantitative cytological determinations of *S. mutans* IPS were conducted by hydrolyzing streptococcal whole cells with hot KOH and quantitating spectrophotometrically the amount of iodine-polysaccharide complex formed (6).

Electron microscopy. Cells were prepared for electron microscopy as described by DiPersio et al. (6). Specimens were examined on a Hitachi H-600 electron microscope at magnifications ranging from ×40,000 to ×150,000.

Virulence testing of *S. mutans* in germfree rats. The cariogenic potentials of *S. mutans* UA130 and SMS202 were determined in gnotobiotic Fischer rats (20). Specifically, weanling rats (aged 19 days) were challenged orally with saturated swabs (2 × 10⁸ CFU/ml) of UA130 or SMS202, and the animals were maintained subsequently on caries-promoting diet 305 (containing 5% sucrose) provided either ad libitum or at restricted feeding times (6 h/day). Colonization was assessed at 2 days postchallenge and then weekly for the duration of the experiment by collecting oral swab samples and culturing them on mitis salivarius agar with or without tetracycline. The animals were sacrificed at 45 days postchallenge, and plaque microbiology and caries scores were determined by the method of Keyes (13). Postmortem cultures from infected rats were streaked onto mitis salivarius agar plates with selective pressure when appropriate and then were screened for glycogen content on glucose-enriched Jordan's medium (12), as previously described.

Isolation and purification of DNA. Chromosomal DNA from *S. mutans* was isolated by using a modification of the method of Marmur (19); cell lysis at 37°C was accomplished in TE buffer (10 mM Tris hydrochloride, 1 mM EDTA [pH 7.5]) containing 40 mg of lysozyme per ml and 200 U of mutanolysin per ml. Plasmid DNA was extracted from *E. coli* by the alkaline lysis method of Birnboim and Doly (2). DNA was digested with restriction enzymes (Promega Biotech, Madison, Wis.) in accordance with the recommendations of the supplier.

Isolation of total RNA. An overnight culture of *S. mutans* UA130 was diluted 1:50 in TH broth or CDM plus 1% glucose and grown anaerobically in the presence of 1% glycine to an OD₅₆₀ of 0.7. The cells were then combined with an equal volume of cold 100 mM Tris–2 mM EDTA, pH 8.0, and harvested by centrifugation. Cells were resuspended in 2 ml of the same buffer containing lysozyme (15 mg/ml) and incubated for 20 min at 37°C. Mutanolysin (2,500 U) was added, and the cell suspension was incubated for an additional 20 min at 60°C prior to the addition of 0.3 ml of lysis buffer (0.5 M Tris [pH 8.0], 20 mM EDTA, 10% sodium dodecyl sulfate [SDS]). Cell lysis was evident after the cell suspension was placed in a boiling water bath for 2 min. Hot acid-saturated phenol (Sigma) was added immediately, and the mixture was incubated at 65°C for 4 min. The suspension was then frozen in a dry ice-ethanol bath and allowed to thaw at room temperature before centrifugation at 5,800 × g for 5 min. The aqueous layer was transferred to a fresh tube, and the phenol extraction and freeze-thaw steps were repeated. The aqueous layer was removed and extracted first with phenol-chloroform-isoamyl alcohol (125:24:1) and then with chloroform-isoamyl alcohol (24:1). The RNA was precipitated overnight with isopropanol, and the pellet was resuspended in 100 µl of DNase buffer (0.1 M sodium acetate, 5 mM MgSO₄ [pH 5.0]). Ten units of DNase (Gibco-BRL) was then added, and the mixture was incubated for 15 min at room temperature. The phenol-chloroform-isoamyl alcohol extraction was repeated, and the RNA was collected by ethanol precipitation. The dried pellet was resuspended in sterile water to a final concentration of 1 µg/µl and stored at –80°C.

Electroporation of *S. mutans* UA130. *S. mutans* UA130 was prepared for electroporation by dilution of overnight cultures into fresh TH broth (1:20) and growth as a standing culture at 37°C for 3.5 h. The cells were harvested by centrifugation, washed twice in succession with 1 ml of cold 300 mM raffinose, and then resuspended in 120 µl of the same solution. A 40-µl aliquot of cells was mixed with 10 µg of DNA suspended in sterile water and subsequently electroporated in chilled 0.2-cm-gap cuvettes (BioRad). The electroporation parameters applied to the cell-DNA mixture were 25 µF, 1.25 kV, and 400 Ω. Subsequent to the pulse (10 ms), the cells were allowed to recover in prewarmed TH broth for 90 min. Electrotransformants were plated onto TH agar containing 5 µg of tetracycline per ml.

Preparation of radiolabeled probes. ³²P-labeled pAM620 was prepared in vitro by nick translation based on the procedure of Rigby et al. (25). The unincorporated label was separated from radiolabeled DNA by chromatography on Sephadex G-100 columns. [³²P]dCTP (3,000 Ci/mmol) was purchased from New England Nuclear.

Southern and Northern blot analyses. DNA was resolved on 0.8% agarose gels submerged in Tris-borate-EDTA buffer at 40 V overnight. Resolved DNA was transferred to nitrocellulose filters (Schleicher and Schuell, Inc., Keene, N.H.) by the method of Southern (29). Hybridizations were performed with 50% formamide at 42°C for 16 h in the presence of radiolabeled pAM620. Filters were washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0)–0.1% SDS for 30 min with gentle agitation prior to two standing washes at 50°C in 0.5× SSC–0.1% SDS. The filters were air dried and exposed to film (Kodak X-Omat XAR-5) for at least 24 h at –80°C.

Total RNA isolated from *S. mutans* UA130 and RNA markers (Sigma) were fractionated on 0.8% agarose gels (8 µg per lane) containing 8% formaldehyde. RNA was quantitated spectrophotometrically and by ethidium bromide staining of rRNA subunits. Following electrophoresis at 40 V overnight, the RNA was transferred directly onto nitrocellulose membranes and subsequently hybridized with a DNA probe corresponding to an internal fragment from open reading

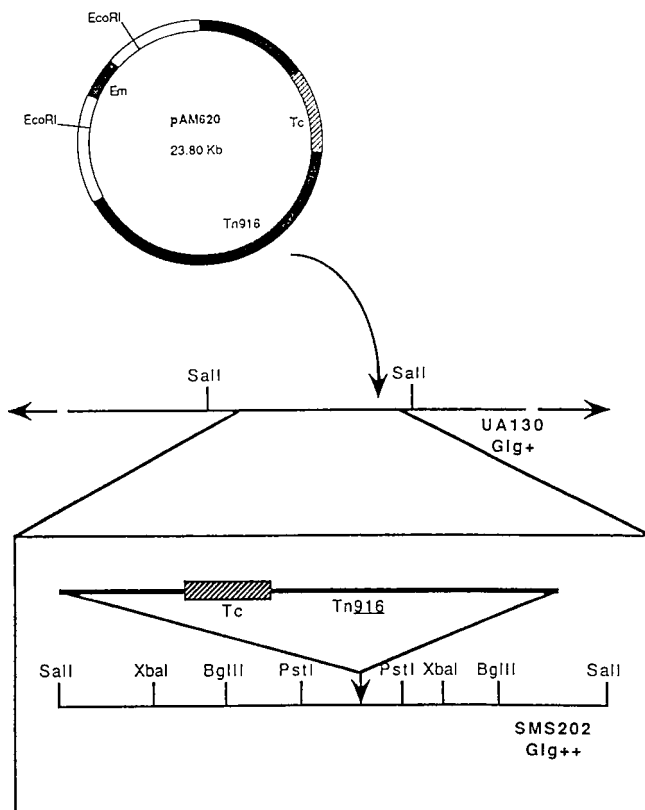


FIG. 1. Generation of *S. mutans* SMS202. Transposon Tn916, harbored on the *E. coli* plasmid pAM620, was used to generate mutants of *S. mutans* with altered IPS accumulation. The insertion illustrated gave rise to SMS202, which produces IPS in excess of wild-type levels. Tc, tetracycline; Em, erythromycin.

frame 2 (ORF2), a putative *S. mutans* *glgB* analog. The probe was isolated and purified from low-melting-temperature agarose gels (UltraPure BRL) and radiolabeled as described above. Hybridizations, filter washings, and autoradiography were also conducted as described above.

Assay for *S. mutans* ADPglucose pyrophosphorylase and glycogen synthase activities. Crude protein extracts were prepared from *S. mutans* cultures as described previously (1), with the following modifications. Cells were disrupted in a Braun (Melsungen, Germany) homogenizer for 3 min in the presence of glass beads and cooled with intermittent bursts of liquid CO₂. The disrupted cell suspension was centrifuged at 37,000 × g for 20 min at 0°C, and the supernatant was standardized with a bicinchoninic acid protein assay reagent (Pierce). The extracts were assayed for ADPglucose pyrophosphorylase (GlgC) and glycogen synthase (GlgA) activities by the spectrophotometric measurement of ATP (ADPglucose pyrophosphorylase)- or ADP (glycogen synthase)-linked reduction of nitroblue tetrazolium (8). Measurements were recorded at an OD₅₄₀ with an LKB enzyme-linked immunosorbent assay reader.

Statistical analysis. All data presented are expressed as the mean plus or minus the standard error of the mean. Electron micrographs were used to quantitate IPS, and the data were analyzed by the Student *t* test. Caries scores were evaluated by analysis of variance and multiple mean comparisons by the Duncan test. Differences were considered to be significant when a value of $P \leq 0.05$ was obtained.

RESULTS

Identification and characterization of *S. mutans* SMS202.

Transposon Tn916 was used to generate glycogen-altered mutants of *S. mutans* as well as to facilitate cloning of the structural genes involved in glycogen accumulation. As illustrated in Fig. 1, the *E. coli* plasmid pAM620 (Tn916) was introduced into the glycogen-proficient *S. mutans* strain UA130 by electroporation. Since the plasmid functioned as a suicide vector in the gram-positive background, the tetracycline resistance

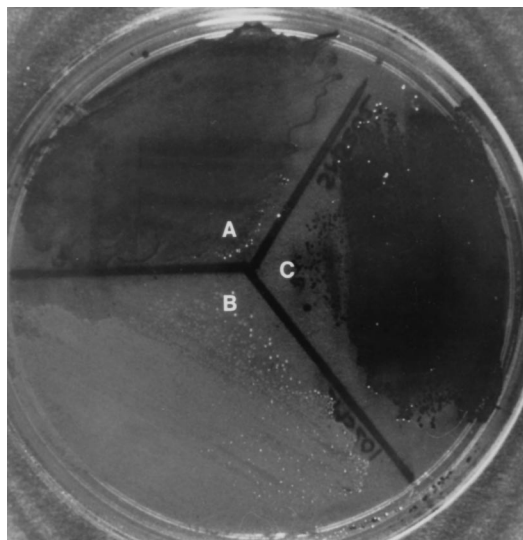


FIG. 2. Analysis of *S. mutans* IPS content by iodine staining. The IPS-proficient *S. mutans* strain UA130 and isogenic mutants SMS201 and SMS202 were grown anaerobically on Jordan's medium and stained with an iodine solution as previously described (9). *S. mutans* UA130 stains brown (A), indicating that it is IPS proficient. The SMS201 IPS-deficient mutant stains yellow (B), while the SMS202 IPS excess mutant demonstrates a superbrown phenotype (C).

marker resident on the transposon could only be rescued upon integration of Tn916 into the *S. mutans* genome.

From the nearly 3,000 tetracycline-resistant electroporants screened for alterations in IPS accumulation by I₂ staining, two mutants were identified. We reported previously on the glycogen-deficient transposon mutant, SMS201, which stained yellow in the presence of iodine (30); this was in contrast to wild-type *S. mutans*, which stained light brown. In the present study, we report on a single glycogen excess mutant, SMS202, which demonstrated a superbrown phenotype upon iodine staining (Fig. 2).

Specifically, ultrastructural analyses of *S. mutans* SMS202 thin sections revealed IPS as cytoplasmic electron-dense granules which were present at significantly higher levels ($P < 0.05$) in the excess mutant (14 granules per cm²) than in the wild-type strain (9 granules per cm²) and the IPS-deficient mutant (5 granules per cm²) (Fig. 3). Cytological determinations of *S. mutans* IPS also indicated that these storage polymers were present in SMS202 at concentrations considerably higher than those of the wild-type progenitor and the IPS-deficient mutant strains (Fig. 4). Importantly, a single transposon insertion in SMS202 was confirmed by Southern analysis, as revealed by two hybridizing *Hind*III junction fragments when pAM620 was used as a probe (data not shown); this result was expected, since *Hind*III cuts only once within the transposon. Moreover, infrequent reversion (rate, 10⁻⁷) of the IPS excess mutant to wild-type glycogen proficiency correlated at all times with the loss of tetracycline resistance; this correlation strongly suggests that the hyperaccumulation of IPS in SMS202 occurred as a direct result of transposon insertion.

SMS202 was further characterized prior to its analysis in vivo. Firstly, the growth rates of SMS202 and its wild-type progenitor were equivalent (data not shown), suggesting that the mutant was not altered in carbohydrate uptake or transport. Secondly, acid production levels in the presence and absence of exogenous glucose appeared to be similar for the SMS202 and the wild-type strains (data not shown); that is, both *S. mutans* strains released acid into the surrounding me-

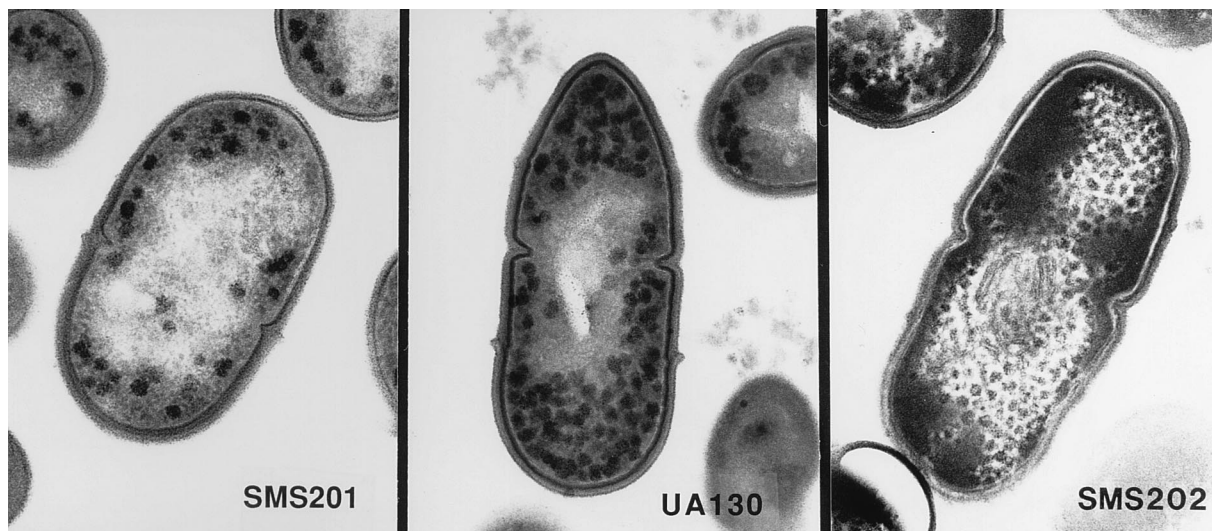


FIG. 3. Ultrastructural analysis of *S. mutans* IPS. *S. mutans* was grown to early stationary phase in TH broth, and thin sections were prepared for electron microscopy. IPS appear as cytoplasmic electron-dense granules which are present in SMS202 at significantly higher concentrations than in the wild-type UA130 and IPS-deficient SMS201 strains ($P < 0.05$). Scale: 1 μm = 167 nm.

dium during the late logarithmic phase of growth and both strains reached and maintained a pH of 5 during stationary phase (after 20 h). Thirdly, levels of adherence of the mutant and wild-type strains to borosilicate glass tubes were indistinguishable; this result was consistent with our observations *in vivo* in that the abilities of the strains to adhere to the surfaces of rat molars were not significantly different (Table 2). Finally, SMS202 proved to be a stable transposon mutant in that it remained tetracycline resistant and IPS abundant for more than 100 generations when passaged *in vitro*, even in the absence of antibiotic selection.

Cariogenic potential of SMS202 in germfree rats. The stability of *S. mutans* SMS202 facilitated subsequent *in vivo* in-

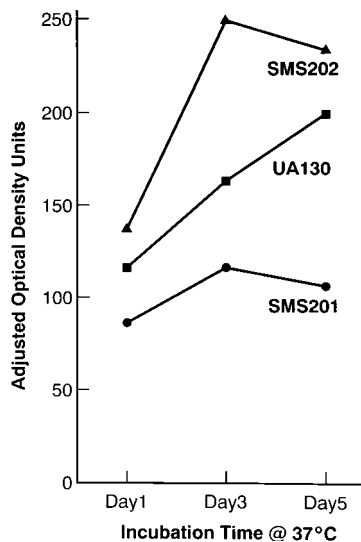


FIG. 4. Cytochemical determination of IPS content in *S. mutans* UA130 and isogenic mutants SMS201 and SMS202 grown in CDM (34). To represent bacterial concentrations as a linear function of photometric turbidity, IPS content is reported in adjusted optical density units (35). The experiment for the data shown is a representative one. The differences between the three strains are significant ($P < 0.05$) on days 3 and 5.

vestigations of IPS and their role in the cariogenic process. The virulence levels of *S. mutans* UA130 and SMS202 as determined with gnotobiotic Fischer rats are presented in Table 2. The results reveal a significantly greater cariogenic potential for the IPS excess mutant than for the wild type on the buccal and sulcal surfaces of rat molars. This trend was significant for animals maintained on a caries-promoting diet provided *ad libitum* as well as for animals maintained on the same diet provided for only 6 h/day; differences in cariogenicity were slightly more pronounced for the restricted dietary regimen than for the unrestricted diet.

Coordinate expression of GlgC and GlgA supports the presence of a *glg* operon in *S. mutans*. In previous reports, we revealed activity levels for both ADPglucose pyrophosphorylase (GlgC) and glycogen synthase (GlgA) in the IPS-deficient mutant SMS201 lower than those observed for the UA130 progenitor (30). In the present work, GlgC and GlgA activities were both increased in SMS202 relative to those of the same UA130 progenitor (Fig. 5). The parallel activities of these enzymes in mutants bearing single-transposon insertions suggest that the *glgC* and *glgA* genes are coordinately regulated in *S. mutans* and that they are therefore likely to constitute an operon on the streptococcal chromosome.

Nucleotide sequence analysis further supports the presence of a *glg* operon in *S. mutans*. Ongoing nucleotide sequence analysis of the *S. mutans glg* locus revealed two contiguous ORFs. As previously reported, ORF1 shares no nucleotide sequence homology with other sequences in the GenBank database (30) while a second ORF, located immediately downstream, shares some sequence homology with the *glgB* genes from *Synechococcus* sp. (15) and *Bacillus stearothermophilus* (14). The latter observation provided the first evidence to suggest the presence of a *glgB* analog in *S. mutans*. The nucleotide sequence of the putative *glg* promoter region from *S. mutans* is represented in Fig. 6. Importantly, the *glg* promoter, when cloned as a 0.57-kb *HindIII-SphI* fusion to a promoterless chloramphenicol acetyltransferase (*cat*) reporter gene, directed the expression of *cat* in *E. coli* (7) as well as in the *S. mutans* fusion strain, GMS100 (unpublished observations).

TABLE 2. Virulence of *S. mutans* UA130 and SMS202 in monoinfected germfree rats^a

Strain	Diet ^b	Cell recovery (CFU/ml) ^c	Mean caries score ± SE ^d											
			Buccal				Sulcal				Proximal			
			E	Ds	Dm	Dx	E	Ds	Dm	Dx	E	Ds	Dm	Dx
SMS202	AL	8.8 × 10 ⁷	17.0 ^e ± 0.6	15.3 ^e ± 1.0	11.3 ± 1.1	7.8 ± 1.1	19.0 ± 0.8	16.2 ^e ± 0.7	10.0 ± 1.1	5.2 ± 0.9	6.0 ^e ± 0.7	1.3 ± 0.4	0	0
UA130	AL	6.0 × 10 ⁷	15.0 ± 0.0	12.25 ± 0.7	9.5 ± 0.8	7.0 ± 0.5	17.0 ± 1.2	13.7 ± 0.9	8.3 ± 0.8	3.7 ± 0.6	1.7 ± 0.3	0.3 ± 0.3	0	0
SMS202	R	3.7 × 10 ⁶	15.1 ^e ± 1.2	12.0 ^e ± 0.5	7.7 ± 0.6	5.1 ± 0.6	18.3 ^e ± 0.9	14.7 ^e ± 0.8	7.3 ^e ± 0.4	2.7 ± 0.8	1.1 ± 0.6	0.6 ± 0.4	0	0
UA130	R	1.5 × 10 ⁶	10.7 ± 0.5	8.7 ± 0.3	6.3 ± 0.3	3.5 ± 0.5	14.2 ± 0.5	11.0 ± 0.6	4.7 ± 0.7	1.0 ± 0.7	1.0 ± 0.7	0.3 ± 0.3	0	0

^a Groups of six rats were tested for all strains. The IPS excess mutant was significantly more cariogenic than its wild-type progenitor on buccal and sulcal surfaces.

^b AL, ad libitum diet; R, diet restricted to 6 h/day.

^c *S. mutans* recovered from rat molar surfaces 2 weeks postchallenge.

^d Caries scores were determined by the method of Keyes (13). Abbreviations: E, enamel involvement; Ds, slight dentinal involvement; Dm, moderate dentinal involvement; Dx, extensive dentinal involvement.

^e Significant difference ($P < 0.01$) between experimental strain score and UA130 score.

A single 6.2-kb mRNA species suggests that the *S. mutans* *glg* genes are cotranscribed. Interestingly, the results of Northern hybridization experiments revealed a single mRNA transcript which hybridizes with an *S. mutans* *glg*-specific probe. Specifically, equivalent amounts of total RNA isolated from *S. mutans* UA130 grown in various carbohydrate environments were fractionated on formaldehyde-agarose gels; the RNA appeared intact, since the 23S, 16S, and 9S rRNA subunits were clearly visible on ethidium bromide-stained gels. An internal 0.36-kb *Hind*III-*Sph*I fragment derived from ORF2 (a *glgB* analog in *S. mutans*) was subsequently radiolabeled and hybridized to the RNAs which had been immobilized on nitrocellulose membranes. Consistent with the presence of a polycistronic mRNA was a single 6.2-kb *glgB*-specific transcript on Northern blots (Fig. 7). The expression of this transcript appeared to be induced by exogenous glucose (Fig. 7), suggesting that *S. mutans* *glg* expression is subject to regulatory control.

DISCUSSION

While early reports suggest that IPS may play a significant role in *S. mutans*-induced cariogenesis (33), the present studies confirm a central role for IPS in the *S. mutans* caries-forming process. We previously reported that an IPS-deficient mutant was significantly less cariogenic in germfree rats than its wild-

type progenitor (30). We now demonstrate that an IPS excess mutant, SMS202, is significantly hypercariogenic in this animal model. Specifically, germfree rats monoinfected with *S. mutans* SMS202 proved to be significantly more cariogenic than those infected with the wild-type UA130 strain on the buccal and sulcal surfaces of their molars ($P < 0.01$); since both strains of *S. mutans* colonized rat molars with equal efficiency, the increased cariogenicity observed in SMS202-infected rats reflects a true increase in the cariogenic potential of the mutant and not a difference which may be accounted for by variations in colonization. Interestingly, differences in cariogenicity were more pronounced when a restricted feeding regimen was followed than when the caries-promoting diet was provided ad libitum (Table 2). Thus, it is likely that the restricted dietary regimen gives rise to an environment in which exogenous fermentable substrates are depleted, such that *S. mutans* is forced to resort to endogenous IPS to satisfy its metabolic needs. Moreover, since we demonstrated that the amounts of acid production in SMS202 and UA130 are equivalent, it is likely that the prolonged exposure of host tissues to organic acids, and not the rate at which acid is released into the oral cavity by the mutant, is ultimately what contributes to the increased cariogenic potential of SMS202. That is, as IPS are metabolized, acid production by SMS202 and UA130 is extended to

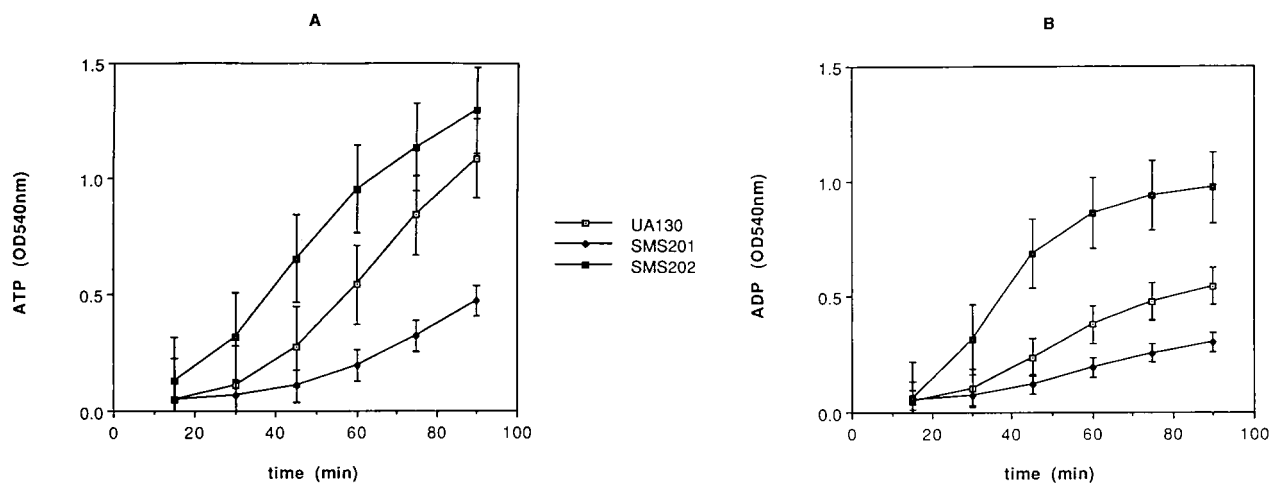


FIG. 5. Biochemical analysis of IPS accumulation in *S. mutans*. Crude protein extracts prepared from *S. mutans* UA130 and SMS202 were standardized for protein content and assayed for ADPglucose pyrophosphorylase (GlgC) (A) and glycogen synthase (GlgB) (B) activities. Both enzyme activities are repressed in SMS201 and elevated in SMS202 relative to the wild-type UA130 strain. Such coordinate regulation supports the hypothesis of an operon-like arrangement for the *glg* genes on the *S. mutans* chromosome. Standard errors of the means are indicated by the error bars.

10 20 30 40 50 60
 ATTAATCATT AAAAGAAAAG AATAAAAGAA AGTGAAGTT TATTTTGTTA TGCTACTTTT
 70 80 90 100 110 120
 TCAAGTTTGG TGAAGTTAAT AAGTCAGTCA GCTTATCCAA TTAATAATA GTCTGTGTTG
 130 140 150 160 170 180
 GAAACAATTT AATCTTTTAA TGGCTTTTTT TAAGTAGGAA GAGCGCTAAG CTCATTATGG
 190 200 210 220 230 240
 TGACGTGTGC ATCTTAGACC TATCCGTCTA CAAAATAAAA GCGATGGGAA TACTGACCAG
 250 260 270 280 290 300
 TTGCTGTGCT GTCACAGTAT CAATACCTTT GGTAACAATA CTGGCAAATT AGAGATTACA
 310 320 330 340 350 360
 AGTTTCGATT TTAGCAATAA AATGACGGAG TCGGTTGCTC GGGTGTGAT CCTCTAAAGC
 370 380 390 400 410 420
 TGACTGGCAA AAAAAGTCAA TCAATAGATG CAACAGGCTT CTAAACAAT CTAAGAAAAA
 430 440 450 460 470 480
 GCTTGAAAGT TGCTTTTAGA AAGAGCTGTT TCAAGCTTTT TCTTTTAAA TTGTGATTAG
 490 500 510 520 530 540
 AAAAAATAA AGAATAGACA GTGTATTGAG AGATAATTAT TAGAGCAGAG CAACTGCTAT
 550 560 570 580 590 600
 TTAACAATTT TTTGTCAAAA AACAATAAAT TTCTATCTT TATAGCATAT TTTGATATAA
 610 620 630 640 650 660
 TGGACTTTGT TATTGAATTA TTTAAGCTAT ATTTAAATAA AATATGGCAA TCTAAGTGAG
 670 680 690 700 710 720
 GAGGGCTTAA AGTTGCTTTT AGAAAGTCTT GTTCAAGCT TTTTCTTTTT AAATTGGTGA
 730 740 750 760 770 780
 TTAGAAAAAA TAAAGAATA GACAGTGTAT TGAGAGATAA TTATTAGAGC AGAGCAACTG
 790 800 810 820 830 840
 CTATTTAAACA ATTTTGTGTC AAAAACAAT AATTTCTAT CTTTATAGCA TATTTTGATA
 850 860 870 880 890 900
 TAATGGAATT GTTATTGAAT TATTTAAGGT ATATTAAAT AAAATATGGC AATCTAAGTG
 910
 ORF1
 AGGAGGATG TTGATAAAT TAAGATGAAA AAACGAAGAA CAATCTATAA ATTTTATTATA
 970 980 990 1000 1010 1020
 CAAACTTTAT TTTATTCCTG TATATTTTTT ATTTTACTCT ATTTCTTTAG TTACTTTGGT
 1030 1040 1050 1060 1070 1080
 CAAGTCCAGG GAGAATTTAT CTACAACGAA TTTTAGGAAG TAACAATGGC TAATAAAAAA
 1090 1100 1110 1120 1130 1140
 ATAAAAGATA TGATTGCAAC AATTGAAAT TTTGCTCAAG AACAGGAAGC ATTTCCGGTT
 1150 1160 1170 1180 1190 1200
 TATAATATTT TAGGAGAAAT CCATACCTAT GGAGAAATAA AAGCTGATTC TGATTGCGCT
 1210 1220 1230 1240 1250 1260
 GCAGCTCATC TTGATCAGTT AGATTAAACA GCAAAATCAC CAGTAGTTGT CTTTGGAGGA
 1270 1280 1290 1300 1310 1320
 CAGGAATATG CCATGCTGGC TAGTTTTGTT GCTCTGACAA AATCAGGGCA TGCCTATATT
 1330 1340 1350 1360 1370 1380
 CCTATTGATC ATCATTGAGC CTTAGAAGT ATTGAGGCTA TTTTAGAGGT AGCAGAGCCA
 1390 1400 1410 1420 1430 1440
 AGTTTAGTTA TTGCTGTTGA TGATTCCCA ATTGACAATC TTCAAGTCCC AGTAATTCAG
 1450 1460 1470 1480 1490 1500
 TATAGTCAAT TAGAAGAAAT TTTTAAACAA AAGCTATCTT ATCAAATCAA TCATGCGGTT
 1510 1520 1530 1540 1550 1560
 AAGGGGGATG ATACCTACTA TATCATCTTT ACTTCAGGGA CAACGTGTAA ACCTAAAGGA
 1570 1580 1590 1600 1610 1620
 GTACAGATT CACATGACAA TCTGCTTAGT TTTACTAATT GGATGATTAA TGCAGAAGCT
 1630 1640 1650 1660 1670 1680
 TTTGCAATAC CTCAAGGCC GCAAATGCTG GCACAACCGC CTTACTCTTT TGATTGTCA
 1690 1700 1710 1720 1730 1740
 GTGATGATT GGGCGCCAC ATTTGGCTTTA GGTGGAACCC TTTTGTCTCT TCCTAAAGAA
 1750 1760 1770 1780 1790 1800
 ATAAGTCCAG ATTTCAAACA ATTTATTACA ACTATAACCA ATTACCATTG GTGTGTGGAC
 1810 1820 1830 1840 1850 1860
 ATCAACACCT TCTTTGTTGA TATGGCTATG CTGTGAGATG ACTTTAATGC ACAGCAATTT
 1870 1880 1890 1900 1910 1920
 CCTCAATCTA ACTCAATCTA TTTTGACGGA GAAGAGTTGA CGGTTAAGAC GGCTAAAAAT
 1930 1940 1950 1960 1970 1980
 TGGCTCAGCG TTTTCCGCAA GCAAGAATTG TCAACGCTTA TGGGCCAACA GAAGCAACTG
 1990 2000 2010 2020 2030 2040
 TTGCTTTATC AGCTTTGGCT GTCAGTATA AAATGCTTGA AACATGCAAA CGTCTGCCAA
 2050 2060 2070 2080 2090 2100
 TTGGCTATAC AAAACCAGAT TCGCCAACCT TTATTATTGA TGAGTCAGGT CATAAATGG
 2110 2120 2130 2140 2150 2160
 CAAATGGTCA GCAAGGAGAG ATTTATTGTT CCGGTCCCGC AGTCTCTAAG GGGTATCTCA
 2170 2180 2190 2200 2210 2220
 ATAATCCTGA ACGAACAGCA GCAGCTTTCT TTGAATTTGA AGGTTTGCCA GCTTATCATA
 2230 2240 2250 2260 2270 2280
 CTGGTGATTT GGCCAAGTAT GACAGATGAA GGTCTCTTGC TCTATGGGGG TCGTATGGAT
 2290 2300 2310 2320 2330 2340
 TTTGAGATTA AATTCAATGG CTATCGTATT GAGTTGGAAG AAGTCTCTCA AAATCTTAAC
 2350 2360 2370 2380 2390 2400
 AAATCGCAAT ATATCGCATC TGCTGTAGCT GTTCCCGGTT ATAAATAAGA CCATAAGGTT

FIG. 6. Nucleotide sequence of the *glg* promoter region of *S. mutans*. Shown is the *S. mutans glg* locus with its two contiguous ORFs, which are transcribed in the same direction (as indicated by the arrows). The ORFs are preceded by a common functional promoter region which directs the expression of a promoterless *cat* reporter gene in *E. coli*. The putative Shine-Dalgarno sequences are underlined. Nucleotide position numbers are shown above the sequence.

include between-meal times when exogenous carbohydrates are absent from the oral cavity; thus, SMS202 is significantly more cariogenic than the wild-type strain since the latter harbors significantly more IPS than the former. Along the same lines, the IPS excess mutant may be particularly well suited for promoting caries formation between meals since it is during these times that the acid-neutralizing effects of saliva are considerably reduced. Finally, we previously reported that *S. mutans* SMS201 harbored significantly fewer IPS than the wild type and that its cariogenic potential was thus significantly reduced in vivo (30). Taken collectively, these observations confirm IPS as significant contributors to *S. mutans* virulence; however, the mechanism(s) which may regulate IPS accumulation still remains unclear.

Interestingly, the insertion in the *S. mutans* SMS201 glyco- gen-deficient mutant decreased but did not eliminate the activities of ADPglucose pyrophosphorylase and glycogen syn- thase. The repression of both of these activities by a single insertion suggests the disruption of a positive regulatory element, possibly a promoter which directs the expression of both *glgC* and *glgA*. The insertion in the SMS202 IPS excess mutant increased the activities of both ADPglucose pyrophosphory- lase and glycogen synthase relative to that of the wild type. This constitutive expression suggests insertional inactivation of an operator-like sequence on the *S. mutans* chromosome which prevents the binding of a putative repressor protein. Alternately, a potential outward-reading promoter on the end of the

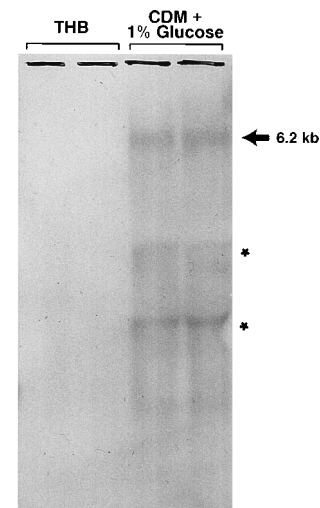


FIG. 7. Northern blot analysis reveals a single *glg*-specific transcript in *S. mutans*. Total RNA isolated from *S. mutans* cultures grown in TH broth (THB) or CDM supplemented with 1% glucose were resolved on formaldehyde-agarose gels (8 µg per lane) and transferred to nitrocellulose membranes. Hybridization was carried out with a DNA probe derived from *S. mutans* ORF2, a *glgB* analog in *S. mutans*. A 6.2-kb *glg*-specific transcript (arrow) is evident when cells are grown in the presence of exogenous glucose. Because the *glg*-specific transcript has undergone some degradation, its degradation products are likely to become nonspecifically trapped by rRNA; we presume this phenomenon to be responsible for the hybridization which is associated with bands corresponding in size to the 23S and 16S rRNA subunits (*).

transposon and adjacent to the chromosome-borne *glg* locus may be responsible for driving the constitutive expression observed in *S. mutans* SMS202. Indeed, a putative promoter site was identified on the right end of Tn916 by Clewell et al. and was shown to be responsible for the hyperexpression of *Enterococcus faecalis* hemolysin (5). Transposon-mapping studies along with sequence analysis across the transposon-chromosome junctions of *S. mutans* are planned to precisely determine the orientation and location of the transposon insertions in SMS201 and SMS202. In any event, the disruption of a putative operator sequence and constitutive expression from a transposon-borne promoter are both scenarios which are consistent with an operon-like arrangement for the *glg* genes on the *S. mutans* chromosome; the idea of such an arrangement is further supported by the presence of the glycogen gene operons previously described for the *Enterobacteriaceae* (26, 31). In fact, nucleotide sequence analysis which has revealed at least two ORFs at the *S. mutans glg* locus along with the identification of a functional upstream promoter lends further support to the likelihood of a *glg* operon in this oral pathogen. Finally, the 6.2-kb *glgB*-specific transcript observed on Northern blots exceeds the *glgB* coding regions reported for *Synechococcus* sp., *B. stearothermophilus*, and *E. coli* by nearly 4 kb (14); this difference suggests that the *S. mutans glg* genes are likely to be cotranscribed and so further supports the idea of an operon-like arrangement for the *glg* genes on the *S. mutans* chromosome.

The continued characterization of the *S. mutans glg* locus will facilitate subsequent studies of regulatory control aimed at understanding *glg* expression in the plaque environment. Preliminary evidence suggests that IPS accumulation in *S. mutans* is inducible, since the 6.2-kb *glg*-specific mRNA is expressed in the presence of exogenous glucose but not in its absence (Fig. 7). It can be reasoned that the biosynthesis of an α 1,4- or α 1,6-glucan polymer would be induced by exogenous glucose. In addition, preliminary evidence derived from Northern analysis indicates that the *glg* genes are also induced by fructose (data not shown). Overall, the inducible expression of the *S. mutans glg* transcript implies that the genes at the *glg* locus are subject to regulatory control.

Interestingly, *glg* expression in the *Enterobacteriaceae* is catabolite repressible (24, 26). Whether the *glg* genes in *S. mutans* are subject to similar regulatory control remains unknown. Lane and coworkers recently described three glucose-repressible promoters in *S. mutans* (16); the mechanism(s) responsible for this catabolite-repressible regulation cannot involve the conventional cyclic AMP (cAMP)-cAMP receptor protein dimer found in gram-negative bacteria, however, since cAMP and adenylate cyclase activities have never been detected in gram-positive microorganisms (3, 28). More recently, Stewart reported that inverted repeat sequences in staphylococci may serve as targets for proteins involved in catabolite repression (32). We can only speculate, on the basis of nucleotide sequence analysis of the *S. mutans glg* promoter region, that inverted-repeat-like sequences centered at 83 and 34 bp upstream of ORF1 are functional in catabolite-repressible regulation (Fig. 6).

Indeed, an operon-like arrangement for the *S. mutans glg* genes reveals possible mechanisms of control for IPS accumulation in the plaque environment; however, additional studies are necessary to extend our understanding of the events which contribute to acid production in the oral cavity. Overall, these investigations will further our currently limited knowledge of metabolic regulation in *S. mutans* and in gram-positive microorganisms in general.

ACKNOWLEDGMENTS

We thank Roy Curtiss III for the helpful discussions which stimulated this work. We also appreciate the assistance of M. Vieth for the preparation of *S. mutans* thin sections for electron microscopy and J. Kiel for providing the *glgB* probes. C. Harmon and G. Richardson are kindly acknowledged for their participation in animal experimentation, as are M. Hudson and R. Landgren for their critical reviews of the manuscript.

This work was supported by Middlebury College and Public Health Service grant F32DE05999 to G.S. and grants DE09081 and DE08182 to S.M. from the National Institute of Dental Research.

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