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Regulated Expression of the *Streptococcus mutans dlt* Genes Correlates with Intracellular Polysaccharide Accumulation

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Intracellular polysaccharides (IPS) are glycogen-like storage polymers which contribute significantly to *Streptococcus mutans*-induced cariogenesis. We previously identified and cloned a locus from the *S. mutans* chromosome which is required for the accumulation of IPS. Sequencing of this locus revealed at least four contiguous open reading frames, all of which are preceded by a common promoter region and are transcribed in the same direction. Analysis of the amino acid sequence deduced from the first of these open reading frames (ORF1) revealed domains which are highly conserved among D-alanine-activating enzymes (DltA) in *Lactobacillus rhamnosus* (formerly *Lactobacillus casei*) and *Bacillus subtilis*. The deduced amino acid sequences derived from ORF2, -3, and -4 also exhibit extensive similarity to DltB, -C, and -D, respectively, in these microorganisms. However, Southern hybridization experiments indicate that this operon maps to a locus on the *S. mutans* chromosome which is separate from the *glgP*, *glgA*, and *glgD* genes, whose products are known mediators of bacterial IPS accumulation. We therefore assigned a new *dlt* designation to the locus which we had formerly called *glg*. We maintain that the *dlt* genes are involved in *S. mutans* IPS accumulation, however, since they complement a mutation in *trans* which otherwise renders *S. mutans* IPS deficient. In this study, we found that expression of the *S. mutans dlt* genes is growth phase dependent and is modulated by carbohydrates internalized via the phosphoenolpyruvate phosphotransferase system (PTS). We demonstrated that the *S. mutans dlt* genes are expressed constitutively when non-PTS sugars are provided as the sole source of carbohydrate. Consistent with a role for the PTS in *dlt* expression is a similar constitutive expression of the *dlt* genes in an *S. mutans* PTS mutant grown in a chemically defined medium supplemented with glucose. In summary, these findings support a novel role for the *dlt* gene products in *S. mutans* IPS accumulation and suggest that *dlt* expression in this oral pathogen is subject to complex mechanisms of control imposed by growth phase, dietary carbohydrate, and other factors present in the plaque environment.

Streptococcus mutans, the chief etiologic agent of dental cavities in humans (13), colonizes the oral cavity shortly after tooth eruption and is found ubiquitously in approximately 98% of the population worldwide (6). The *S. mutans*-induced caries-forming process is complex, beginning with the attachment of the bacterium to the tooth pellicle and culminating in tooth decay owing to the production of lactic acid as a sole metabolic by-product.

Carbohydrates consumed in the diet are internalized by *S. mutans* primarily via the phosphoenolpyruvate phosphotransferase transport system (PTS) (7, 20, 25). More recently, Tao et al. described a multiple-sugar metabolism (*msm*) operon in *S. mutans* which provides a pathway for the uptake of non-PTS sugars, such as raffinose and melibiose (30). Sugars internalized via PTS or non-PTS pathways are subsequently metabolized, and the lactic acid which is produced demineralizes tooth enamel to form carious lesions.

S. mutans can also metabolize intracellular glycogen-like storage polymers (intracellular polysaccharides [IPS]). Tanzer et al. were the first to suggest that IPS can serve as metabolic substrates for acid production during periods of carbohydrate limitation in the oral cavity (29). The utilization of IPS by *S. mutans* can therefore exacerbate tooth decay by prolonging the period of exposure of host tissues to organic acids, such as during the periods between meals.

We previously confirmed a significant role for IPS in the *S. mutans*-induced cariogenic process by constructing genetic mutants altered in their ability to accumulate IPS (27, 28). Specifically, we generated an *S. mutans* transposon library by using the streptococcal transposon Tn*916* (23) and screened it for IPS-altered mutants by iodine staining. Two mutants were identified, and their IPS contents were confirmed by quantitating electron-dense cytosolic glycogen-like granules on transmission electron micrographs (27, 28). One mutant, SMS201, is IPS deficient and significantly less cariogenic than the UA130 wild-type progenitor strain in a germ-free rat model system (28). Southern analysis confirmed a single transposon insertion in SMS201 and revealed that the transposon interrupts a region immediately upstream of an operon on the *S. mutans* chromosome. Importantly, the generation of a knockout mutation at the site of transposon insertion in *S. mutans* UA130 gave rise to a mutant which also proved to be IPS deficient (28), thereby ruling out the possibility that polar effects were imposed by the transposon insertion in SMS201.

Northern hybridization studies conducted in our laboratory support transcription of this locus, which we named *glg*, as a single 6.2-kb polycistronic mRNA (27). However, sequence analysis of this locus revealed at least four contiguous open reading frames (ORFs) which exhibit significant homology to the previously described *dlt* genes from *Lactobacillus rhamnosus* (formerly *Lactobacillus casei*) (9, 11) and *Bacillus subtilis* (17). Moreover, the results of Southern hybridization experi-

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
E. coli strains		
HB101	*supE44 hsdS20 recA13 ara-14 proA2 lacY galK2 rpsL20 xyl-5 mtl-1*	2
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi*Δ(*lac-proAB*)	33
CLEO1	HB101 transformed with pMC1; Tc^r Cm^r	This work
S. mutans strains		
UA130	Serotype c; IPS^+	P. Caufield
GMS100	UA130 with *dlt*::*cat* fusion from pMC1; Tc^r	This work
BM71	PTS^+	3, 16
DC10	*ptsI*::*erm* PTS^-	8
Plasmids		
pAM620	pVA891::pAD1*Eco*RIF′::Tn*916* Tc^r Em^r	32
pMH109	Contains promoterless *cat* gene preceded by pUC13 MCS; Tc^r	12
pNC10	pMH109 derived; contains promoterless *cat* gene; Tc^r ColE1	This work
pNC12	pNC10 derived; ColE1 *tetM*	This work
pMC1	pNC12 derived; ColE1 *dlt*::*cat tetM* Cm^r	This work
pDLT1-2	3.1-kb *S. mutans* amplicon containing *dlt1* and *dlt2* in pGEM-T Easy; Ap^r	This work
pDLT3	0.3-kb *S. mutans* amplicon containing *dlt3* in pGEM-T Easy; Ap^r	This work
pDLT3-4	1.7-kb *S. mutans* amplicon containing *dlt3* and *dlt4* in pGEM-T Easy; Ap^r	This work
pYA3023	6.9-kb *Bgl*II fragment containing *S. mutans dlt* in pUC19; Ap^r	28
pYA3031	8-kb *Xba*I fragment containing *S. mutans dlt* in pUC19; Ap^r	This laboratory

ments indicated that the *S. mutans glgP* gene, cloned by Smith et al. (26), and the *S. mutans glgA* and *glgD* genes, recently cloned in our laboratory, occupy a locus on the *S. mutans* chromosome which is independent of *glg*. Consequently, we assigned a new designation, *dlt*, to the *S. mutans glg* locus. However, we maintain that it is uniquely involved in IPS accumulation, since the cloned *dlt* operon is capable of *trans* complementation in the IPS-deficient mutant SMS201 (28). In the present study, we determined the organization of the *S. mutans dlt* operon and monitored its expression in the presence of various dietary carbohydrates. Indeed, an improved understanding of the mechanism(s) which regulates expression of the *dlt* genes could elucidate how *dlt* affects IPS accumulation and, consequently, how *S. mutans* persists and causes disease in the oral cavity.

MATERIALS AND METHODS

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

Culture conditions. *Escherichia coli* HB101 and JM109 were grown with aeration at 37°C in Luria-Bertani (LB) broth supplemented with 0.1% dextrose. *S. mutans* UA130 (serotype c) and its derivatives were grown in Todd-Hewitt broth (Difco) at 37°C in a 5% CO_2 atmosphere. When propagating the GMS100 fusion strain, 5 μg of tetracycline was added to the medium. *S. mutans* BM71 was grown on Tryptone-yeast extract–0.3% raffinose plates, while the DC10 isogenic mutant was propagated on the same medium supplemented with 8 μg of erythromycin per ml.

For expression studies, *S. mutans* UA130, GMS100, BM71, and DC10 were grown as described above but in a chemically defined medium (CDM) (31) supplemented with various dietary carbohydrates each to a final concentration of 1%. The carbohydrate-supplemented medium was inoculated with 150 μl of a concentrated *S. mutans* frozen stock.

For reporter gene studies, an aliquot of a GMS100 overnight culture was diluted 1:10 in prewarmed CDM supplemented with 1% glucose and grown as described above for an additional 2 h. These cultures were designated as the zero time points. Aliquots from the zero time point cultures were then washed in phosphate-buffered saline, inoculated into prewarmed CDM supplemented with the test carbohydrate(s), and grown as usual before harvesting from early-, mid-, and late-logarithmic-phase and stationary-phase cultures.

For dot blot and Northern hybridization experiments, *S. mutans* was grown, as described above, in CDM supplemented with a PTS sugar (e.g., glucose, fructose, or sucrose) or with a non-PTS sugar (e.g., raffinose or melibiose). Growth phase was determined by periodic measurements of the optical density at 600 nm and comparisons made against previously defined standard growth curves.

For primer extension studies, *S. mutans* UA130 was grown to mid-logarithmic phase, as described above, in CDM supplemented with either 1% glucose or both 0.5% glucose and 1% fructose.

DNA isolation and digestion. *S. mutans* chromosomal DNA was isolated as previously described (14, 28) and purified by ethidium bromide-cesium chloride density gradient centrifugation. Plasmid DNA was isolated from *E. coli* by the alkaline lysis method of Birnboim and Doly (1). Restriction enzyme digestions were performed as recommended by the enzyme supplier (Promega).

Bacterial transformation. Electrocompetent *E. coli* HB101 was transformed with pNC10, pNC12, or pMC1 in an electroporator according to protocols provided by the manufacturer (Bio-Rad). The resulting strains were selected on LB agar supplemented with only tetracycline (15 μg/ml) or with both tetracycline (15 μg/ml) and chloramphenicol (15 μg/ml). Electrocompetent *E. coli* JM109 was transformed with pDLT1-2, pDLT3, or pDLT3-4 as described above, and the resulting transformants were selected on LB agar supplemented with ampicillin (100 μg/ml).

S. mutans UA130 was transformed with pMC1 (10 μg) by electroporation as previously described (28). Transformants were screened on Todd-Hewitt agar supplemented with tetracycline (5 μg/ml). Chromosomal DNA isolated from tetracycline-resistant transformants was analyzed by Southern blotting with a 0.36-kb *Hin*dIII-*Sph*I fragment internal to *dlt1* as a probe. This confirmed integration of the plasmid into the chromosome, and the resulting fusion strain was designated GMS100.

Southern blot analysis. Southern blotting was performed according to standard procedures (21) with a 0.6-kb *Hin*dIII-*Sph*I probe which harbors the *dlt* promoter region or the 0.36-kb *Hin*dIII-*Sph*I fragment which is internal to *dlt1*. Hybridizations were carried out under stringent conditions at 42°C, and filters were washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) at room temperature with gentle agitation for 30 min; this was followed by two 30-min washes in 0.5× SSC–0.1% SDS at 50°C. The filters were air dried and exposed to Kodak X-OMat XAR5 film at −80°C overnight.

RNA isolation. Total RNA was isolated from *S. mutans* cultures grown to early logarithmic, mid-logarithmic, late logarithmic, or stationary phase by a hot phenol extraction method described previously (27).

Northern and dot blot analyses. For Northern hybridization experiments, total RNA was resolved on 0.8% formaldehyde–agarose gels in 20 mM morpholinepropanesulfonic acid (MOPS) at 40 V overnight. Equal loading was confirmed by densitometric scanning of ethidium bromide-stained gels. The RNA was transferred to nylon membranes (Micron Separations, Inc., Westboro, Mass.) as previously described (21) and cross-linked in an FB-UVXL-1000 cross-linker apparatus (Fisher Scientific, Springfield, N.J.). Dot blots were prepared by spotting equal amounts of total RNA, as determined spectrophotometrically at 260 nm and by densitometric scanning of ribosomal subunits on ethidium bromide-stained gels, onto nitrocellulose membranes (Protran) by the use of a microsample filtration manifold (Schleicher and Schuell, Keene, N.H.). The nitrocellulose membranes were baked in a vacuum oven at 80°C for 2 h. Northern and dot blots were probed with the radiolabeled 0.36-kb *Hin*dIII-*Sph*I *dlt* fragment or a 0.62-kb *dlt*-specific PCR product, both of which are internal to the *dlt1* coding region. Hybridization and wash conditions were as described above. Individual dots were then excised from the membrane with a hole punch, and

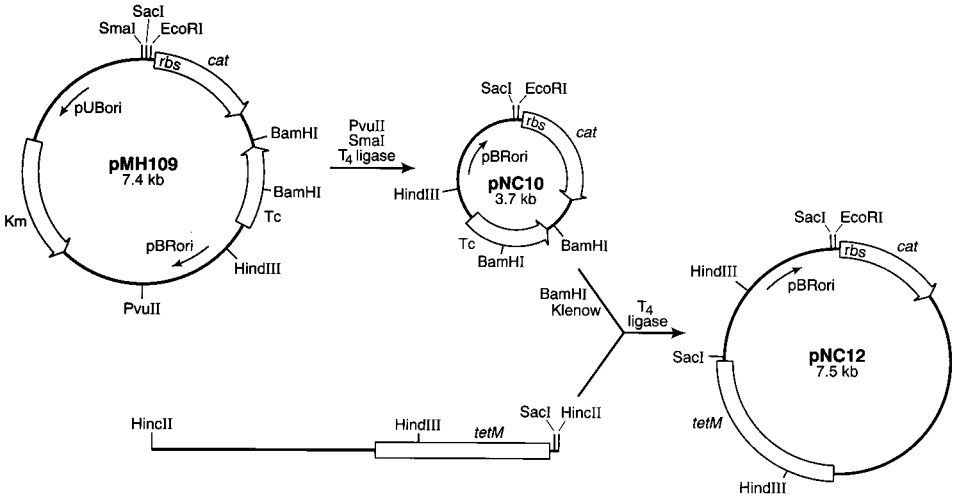

FIG. 1. Construction of integration vector pNC12. Plasmid pMH109 was digested with *Pvu*II and *Sma*I, and the fragment which harbors pBRori, the promoterless *cat* reporter gene, and the tetracycline resistance-encoding gene was recircularized with ligase and transformed into *E. coli* HB101. Transformants were selected on LB agar containing 10 μg of tetracycline per ml, and the recombinant plasmid, pNC10, was isolated and confirmed by restriction enzyme mapping. pNC10 was digested with *Bam*HI to create a deletion in the tetracycline resistance gene, and the ends of the linearized plasmid were filled in with the Klenow fragment of DNA polymerase. The *tetM* gene, contained on a 4.8-kb *Hin*cII fragment purified from plasmid pAM620 (32), was ligated into blunt-ended pNC10, and *E. coli* transformants were selected on LB agar containing 10 μg of tetracycline per ml. Plasmid pNC12 was isolated by an alkaline lysis protocol (1) and confirmed by restriction enzyme and Southern blot analyses. pBRori, origin of replication functional in *E. coli* but not in *S. mutans*; pUBori, origin of replication functional in *S. aureus* and *B. subtilis* but not in *E. coli*; Tc, tetracycline resistance gene selectable in *E. coli* but not in *S. mutans*; *tetM*, gene from Tn*916* which encodes resistance to tetracycline in both *S. mutans* and *E. coli*; Km, kanamycin resistance gene.

radioactivity was counted in a Packard (Meriden, Conn.) Tri-Carb model 1900CA liquid scintillation analyzer.

Preparation of radiolabeled probes. DNA fragments used to probe Southern blots were isolated from 1% low-melting-temperature agarose gels following digestion with *Hin*dIII and *Sph*I (Promega). The 0.62-kb fragment used to probe the Northern and dot blots was derived from plasmid pYA3023 by PCR with 5′-CTCATAGGCCGCAAATGCTG-3′ and 5′-GAGCAAGAGACCTTCATC TGTC-3′ as primers. The DNA fragments were purified by spin column chromatography (Qiagen) and subsequently labeled by nick translation (19). Unincorporated nucleotides were removed from the reaction mixture by Sephadex G-25 chromatography (5 Prime→3 Prime, Inc., Boulder, Colo.).

Construction of an *S. mutans dlt*::*cat* fusion strain (GMS100). A DNA fragment harboring an *E. coli* origin of replication, a promoterless *cat* reporter gene with an appropriate ribosome binding site (RBS), and a gene encoding resistance to tetracycline in *E. coli* was isolated from plasmid pMH109 (12). Following the addition of T4 DNA ligase (Promega), the resulting 3.7-kb plasmid, designated pNC10, was transformed into *E. coli* HB101 (2), and transformants were selected on LB agar supplemented with tetracycline (10 μg/ml). The *tetM* gene, which is expressed in both *E. coli* and *S. mutans*, was isolated from Tn*916* on a 4.8-kb *Hin*cII fragment and ligated into pNC10 as illustrated in Fig. 1. *E. coli* transformants harboring the new construct, pNC12, were selected as described above. This plasmid was subsequently linearized with *Eco*RI and made blunt ended with the Klenow fragment of DNA polymerase (Fig. 2). A 0.6-kb *Hin*dIII-*Sph*I fragment from pYA3023, which harbors the *S. mutans dlt* promoter region, was similarly made blunt ended and ligated to pNC12. The resulting operon fusion construct, pMC1, which harbors the *dlt* promoter region and the *cat* gene cloned in the same orientation, was transformed into *E. coli* as described above, and its presence was confirmed by restriction endonuclease mapping and Southern analysis with the radiolabeled 0.6-kb *Hin*dIII-*Sph*I fragment as the probe (data not shown). Finally, pMC1 was introduced into the *S. mutans* chromosome by electroporation, and the Campbell-type insertion illustrated in Fig. 2 was selected by screening transformants for resistance to tetracycline. The single-crossover event was confirmed by Southern blot analysis with a 0.36-kb *Hin*dIII-*Sph*I *dlt* fragment as the probe (data not shown).

Preparation of *S. mutans* GMS100 cell lysates and determination of CAT specific activity. GMS100 cell pellets grown in carbohydrate-supplemented CDM were resuspended in 1 ml of Tris-EDTA buffer, pH 8.0, and combined with 300 μl of 0.1-mm-diameter zirconium beads. The cells were lysed by mechanical disruption in a Mini-Bead Beater (BioSpec) for two 1.5-min intervals at 4°C with intermittent cooling on ice. The beads were then pelleted by centrifugation, and the supernatant was clarified by an additional low-speed centrifugation. Fifty microliters of the lysate was removed from each sample for protein determination with a bicinchoninic acid protein assay kit (Pierce). The remaining lysate was stored at 0°C for subsequent monitoring of chloramphenicol acetyltransferase (CAT) specific activity by the spectrophotometric assay of Shaw (24). CAT specific activities were expressed as nanomoles of chloramphenicol acetylated per minute per milligram of total protein.

Primer extension studies. Total RNA was isolated from *S. mutans* UA130 as described above and stored at −80°C. For 5′ primer extension, an oligonucleotide with the sequence 5′-GCAAGCGAATCAGAATCAGC-3′ was used. End labeling and primer extension reactions were performed as previously described (10).

In vitro transcription-translation studies. The gene products derived from the *S. mutans dlt* operon were analyzed in a coupled in vitro transcription-translation system (Promega Biotech, Madison, Wis.) according to the recommendations of the supplier. Plasmids pDlt1-2, pDlt3, and pDlt3-4, which were used in this analysis, are described in Table 1. [^{35}S]methionine was used to radiolabel the reaction products, which were subsequently resolved on SDS–12% polyacrylamide gels and visualized by autoradiography.

DNA sequencing and analysis. DNA was sequenced by the dideoxy chain termination method (22). Double-stranded template DNA was isolated by alkaline lysis (1) and purified by a polyethylene glycol precipitation procedure (21) prior to sequencing. Sequence analyses were carried out with the Macintosh program MacDNAsis 3.0 (Hitachi Software Engineering Co., Ltd.) and the programs BLASTP and BLASTX (National Center for Biotechnology Information Laboratories, Los Alamos, N. Mex.). Alignment of the deduced amino acid sequences was performed with DNASTAR software (DNASTAR Inc., Madison, Wis.).

Nucleotide sequence accession number. The *S. mutans dlt* sequence reported herein has been entered into the GenBank database under accession no. AF050517.

RESULTS

Organization and nucleotide sequence of the *S. mutans dlt* genes. Shown in Fig. 3A is the organization of the *S. mutans dlt1*, *dlt2*, *dlt3*, and *dlt4* genes, which are harbored on a 6.9-kb *Bgl*II fragment on plasmid pYA3023 (28) and an 8.3-kb fragment on plasmid pYA3031. Nucleotide sequencing of these constructs revealed a 1,545-bp ORF, *dlt1*, which encodes a putative gene product of 515 amino acids with a predicted molecular mass of 56 kDa. Upstream of *dlt1* is a putative RBS (AGGAAG) which precedes the ATG start codon by 5 nucleotides (nt) (Fig. 3B). The site of the transposon insertion which renders *S. mutans* SMS201 IPS deficient is 94 nt upstream of the RBS. The DNA sequence 3′ of the *dlt1* gene contains another ORF (*dlt2*); the *dlt2* initiation codon overlaps the termination codon of *dlt1* by 1 nt. The *dlt2* ORF is 1,262 nt in length and codes for a predicted protein of 46 kDa with distinct

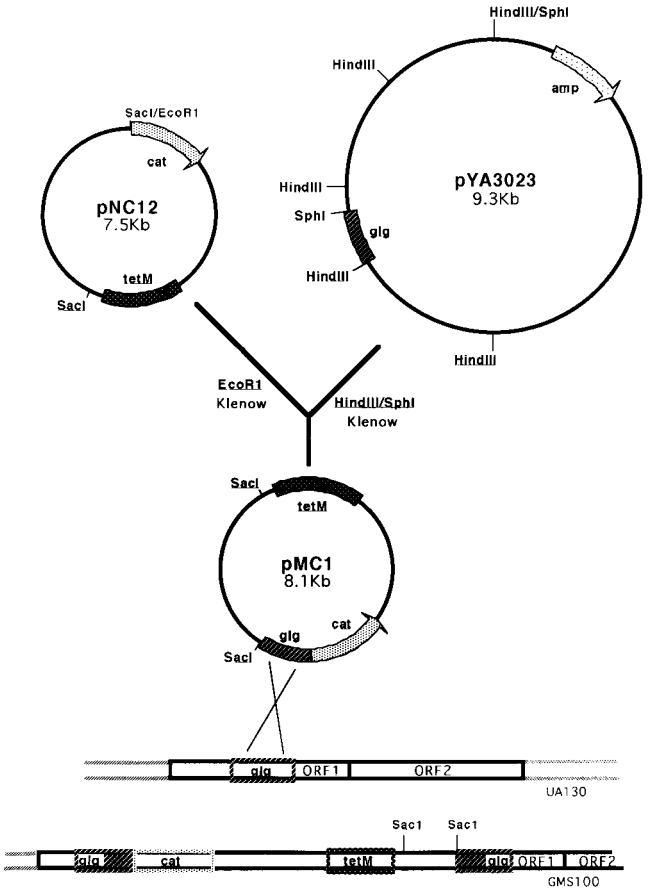

FIG. 2. Cloning strategy used to generate pMC1 for subsequent chromosomal integration. The *S. mutans dlt* promoter region, contained on a 0.6-kb *Hin*dIII-*Sph*I fragment on plasmid pYA3023, and pNC12 digested with *Eco*RI were treated with the Klenow fragment of DNA polymerase prior to blunt-end ligation, which was performed overnight at 20°C. The resulting operon fusion construct, pMC1, was electroporated into *E. coli* DH5α, and transformants were selected on LB agar plates containing tetracycline (15 μg/ml). The operon fusion was confirmed by restriction enzyme mapping and Southern analysis with the *dlt* promoter fragment as a probe. *S. mutans* UA130 was then electrotransformed with pMC1, and transformants that had undergone plasmid integration into the chromosome (GMS100) were identified by selection on Todd-Hewitt agar containing tetracycline.

hydrophobic domains. Another ORF (*dlt3*) is located 15 bp downstream of *dlt2* and is preceded by its own putative RBS. This ORF is 237 bp in size and encodes a predicted protein of 8.7 kDa. Finally, the *dlt4* gene overlaps *dlt3* by 1 nt and, like *dlt2*, is 1.26 kb in size and gives rise to a predicted protein of 46 kDa.

Shown in Fig. 4 are results of primer extension studies, which reveal two possible transcription start sites upstream of the *S. mutans dlt* operon, at nt 156 and 203. Consequently, we localized the *dlt* promoter regions to TATAAT at −10 and TTCCTA at −35 for the former and TAAAAT at −10 and TTGAAT at −35 for the latter (Fig. 3B).

Comparison of the sequence of the deduced Dlt1 protein with those of ATP-utilizing enzymes from other bacteria revealed significant homology, especially with the D-alanine-activating enzymes (DltA) from *L. rhamnosus* (11) and *B. subtilis* (17). These cytosolic enzymes catalyze the biosynthesis of D-alanyl-lipoteichoic acids in *L. rhamnosus* and *B. subtilis*, and each has a conserved phosphate binding loop encoded by the consensus sequence GXXGXPK. Two additional domains conserved among this group of enzymes, GRXDXQXKXXGX RXE and $PX_9PX_5KXDX_3L$, presumably catalyze the transfer of D-alanine to lipoteichoic acids via a thiol ester intermediate. Multiple alignment of the amino acid sequences indicates that all three functional domains are highly conserved in *S. mutans* Dlt1, *L. rhamnosus* DltA, and *B. subtilis* DltA. Comparative analysis of Dlt1 from *S. mutans* with DltA from *L. rhamnosus* revealed 95, 70, and 58% identity at the amino acid level within regions 1, 2, and 3, respectively, and an overall identity of 48.5%. A gene product of approximately 56 kDa is derived from *dlt1*, as determined via in vitro transcription-translation (data not shown).

The *S. mutans* Dlt2 sequence exhibits 48% amino acid identity to the DltB proteins from *L. rhamnosus* (9) and *B. subtilis* (17), respectively. DltB is a putative membrane-associated transporter which presumably mediates the transport and subsequent transfer of cytosolic D-alanine residues to neighboring lipoteichoic acids at the bacterial cell surface. In vitro transcription-translation studies revealed that a protein of approximately 45 kDa is derived from *dlt2* (data not shown).

S. mutans Dlt3 exhibits 44% amino acid identity to the *L. rhamnosus dltC* gene product; the latter encodes a D-alanine carrier protein which exhibits significant similarity to fatty acid acyl carrier proteins of *E. coli* and *Saccharomyces cerevisiae* (17). Interestingly, Dlt3 harbors a serine residue at position 35 which is strictly conserved among acyl carrier proteins. The *S. mutans dlt3* gene encodes a protein in vitro which approximates 8.7 kDa as predicted (data not shown).

Finally, Dlt4 exhibits 30% amino acid identity to the *B. subtilis dltD* gene product. Based on the amino acid composition,

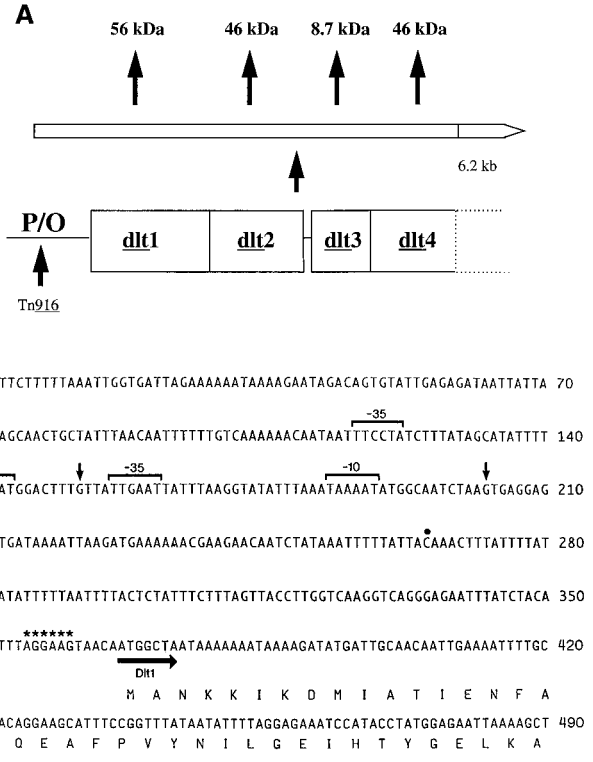

FIG. 3. (A) Operon-like arrangement of the *S. mutans dlt* genes. P/O, promoter-operator. (B) Nucleotide sequence of the *S. mutans dlt* promoter region. The transcription start sites and −10 and −35 promoter regions are indicated by vertical arrows and brackets, respectively. The putative RBS is designated by asterisks. The dot marks the site of transposon insertion into *S. mutans* SMS201, which renders it IPS deficient The horizontal arrow indicates the direction of transcription of the *dlt* operon.

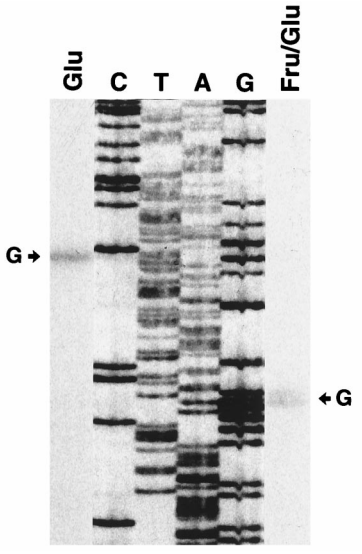

FIG. 4. Primer extension analysis of *dlt* transcripts. A radiolabeled oligonucleotide was annealed to RNA isolated from *S. mutans* cultures grown in CDM supplemented with either glucose alone (Glu) or both fructose and glucose (Fru/Glu). The cDNAs resulting from the addition of avian myeloblastosis virus reverse transcriptase were run alongside the dideoxy sequencing reactions involving plasmid pYA3023. The arrows indicate the transcription start sites at guanine residues corresponding to nt 156 (left) and 203 (right).

the presence of an amino-terminal signal peptide is suggested. Specifically, two positively charged residues are followed by a run of hydrophobic residues (MLKRLWLILGPVFCALVL VF). Thus, Dlt4 is likely to be a secreted protein which may be anchored into the cell membrane at its amino-terminal end (17). By in vitro transcription-translation analysis, it was also revealed (data not shown) that a protein of approximately 45 kDa is derived from *dlt4*.

Expression of *dlt* in *S. mutans* GMS100. CAT specific activities were determined for the *S. mutans* GMS100 *dlt*::*cat* fusion strain grown in CDM supplemented with glucose, fructose, or sucrose, all of which are carbohydrates internalized by the PTS. Interestingly, the *dlt* genes were not expressed in early-logarithmic-phase cultures when glucose was provided as the sole source of carbohydrate; however, expression was induced during mid-logarithmic phase, reaching a maximum of 1.1 nmol/min/mg (Fig. 5A). During later stages of growth, the CAT specific activity returned to near baseline levels. Maximal *dlt* expression (0.5 nmol/min/mg) was also achieved during mid-logarithmic phase for cultures propagated in CDM supplemented with sucrose (Fig. 6A). Other PTS sugars, including fructose as well as glucose and fructose in combination, similarly gave rise to maximal *dlt* expression during mid-logarithmic phase (data not shown). In contrast, different patterns of *dlt* expression were observed when *S. mutans* UA130 was grown in CDM supplemented with a non-PTS sugar, such as raffinose (Fig. 7A) or melibiose (data not shown). These carbohydrates seemed to direct the constitutive expression of *dlt* during all stages of growth, including the early logarithmic phase.

Northern blotting of total RNA isolated from the GMS100 fusion strain grown in CDM supplemented with glucose, fructose, or sucrose revealed that *dlt* expression in GMS100 was identical to that of the UA130 wild-type strain (data not shown). This confirms that *dlt* expression in the fusion strain is not aberrantly affected by integration of pMC1 into the chromosome.

Dot blot analysis of *S. mutans dlt* expression. Dot blotting of total RNA isolated from *S. mutans* UA130 cultures grown in the presence of various PTS sugars confirmed the expression patterns derived from *cat* reporter gene assays. That is, maximal expression of *dlt*-specific mRNA was evident during the mid-logarithmic phase of growth when glucose was provided in the medium (Fig. 5B). This expression declined during the late logarithmic and early stationary phases. The growth of *S. mutans* cultures supplemented with either fructose alone or both fructose and glucose gave rise to the same pattern of *dlt* expression (data not shown). When sucrose was provided as the sole carbohydrate source, expression of the *dlt*-specific transcript was also maximal during mid-logarithmic phase (Fig. 6B). In contrast, we observed constitutive *dlt* expression during all stages of growth when raffinose (Fig. 7B) or melibiose (data not shown) was present in the medium.

***dlt* expression in an *S. mutans* PTS mutant.** The Northern blot shown in Fig. 8 reveals the constitutive *dlt* expression evident in *S. mutans* DC10, a PTS mutant which we propagated in CDM supplemented with glucose. Also noteworthy is the unique expression of a large *dlt*-specific transcript (>10 kb) in *S. mutans* DC10 during early logarithmic phase which is not apparent in the BM71 progenitor strain (16) under the same growth conditions.

DISCUSSION

IPS are high-molecular-weight glucan polymers which provide *S. mutans* with a metabolizable source of carbon during periods of nutrient deprivation in the oral cavity (29). The inactivation of *S. mutans dlt* expression diminishes IPS accumulation (28), resulting in significantly reduced cariogenicity in germ-free rats (15) maintained on a sucrose-containing, caries-promoting diet. Importantly, complementation of the IPS-deficient phenotype in *S. mutans* SMS201 can be achieved by providing the cloned *dlt* locus in *trans* (28). The ability of *dlt* to restore *S. mutans* IPS accumulation to wild-type levels in this mutant suggests at least an ancillary role for the *dlt* locus in IPS biosynthesis, storage, and/or breakdown. In fact, recent reports in the literature reveal novel roles for the streptococcal *dlt* genes, such as in *S. mutans* acid tolerance (3) and in *Streptococcus gordonii* intrageneric coaggregation (5).

In this study, we found that *dlt* expression in *S. mutans* is growth phase dependent and that it is regulated at the level of transcription. Specifically, growth of an *S. mutans dlt*::*cat* fusion strain in CDM supplemented with glucose, fructose, or sucrose gave rise to maximal *cat* specific activity during the mid-logarithmic phase. This activity declined markedly during the late logarithmic and early stationary phases despite the presence of excess carbohydrate in the growth medium. Importantly, an increase in cell mass (measured as milligrams of protein) after peak expression during mid-logarithmic phase is insufficient to account for the subsequent decrease in *dlt* expression. Similar expression of the *dlt* operon was reported in *B. subtilis*, in which transcription occurs maximally during the exponential phase of growth and then decreases during stationary phase and before the onset of sporulation (17). In nonsporulating cultures of *B. subtilis*, however, expression of the *dlt* operon is not regulated, suggesting that the modulation of *dlt* transcription in *B. subtilis* may be stress responsive.

Sugars consumed in the host diet also seem to play a role in the regulation of *S. mutans dlt* expression. We noted two different primer extension products derived from *S. mutans* cultures propagated in CDM supplemented with glucose alone or with both glucose and fructose. This suggests that different promoters may be recognized by *S. mutans* RNA polymerase under these and possibly other carbohydrate conditions. In addition, non-PTS sugars such as raffinose and melibiose gave

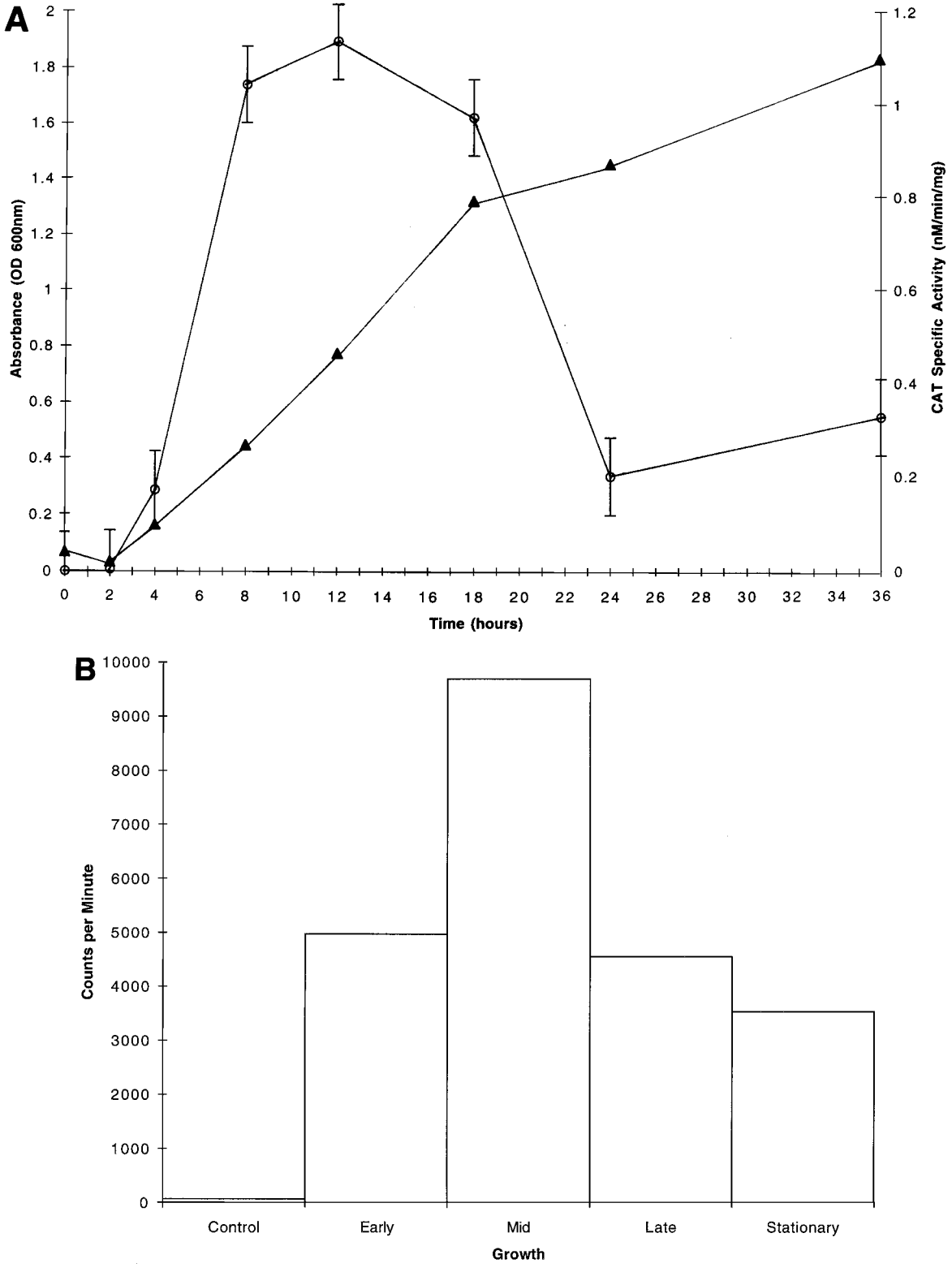

FIG. 5. (A) Growth curve (closed triangles) and CAT specific activities (open circles) for *S. mutans* GMS100 cultures grown in CDM supplemented with 1% glucose. Each CAT specific activity data point represents the mean of values from three independent experiments. Standard deviations are negligible except where indicated by vertical bars. (B) Total RNA (1 μg) isolated from *S. mutans* UA130 grown to early, mid-, or late logarithmic phase or to stationary phase in CDM supplemented with 1% glucose was dot blotted onto a nitrocellulose membrane and probed with a DNA fragment which is internal to *dlt1*. Individual dots were excised, and the radioactivity was counted in a Packard Tri-Carb model 1900CA liquid scintillation analyzer. A dot representing nonspecific hybridization was included as a control. Shown are the results of a single representative experiment. Note the maximal expression of *dlt* during mid-logarithmic phase.

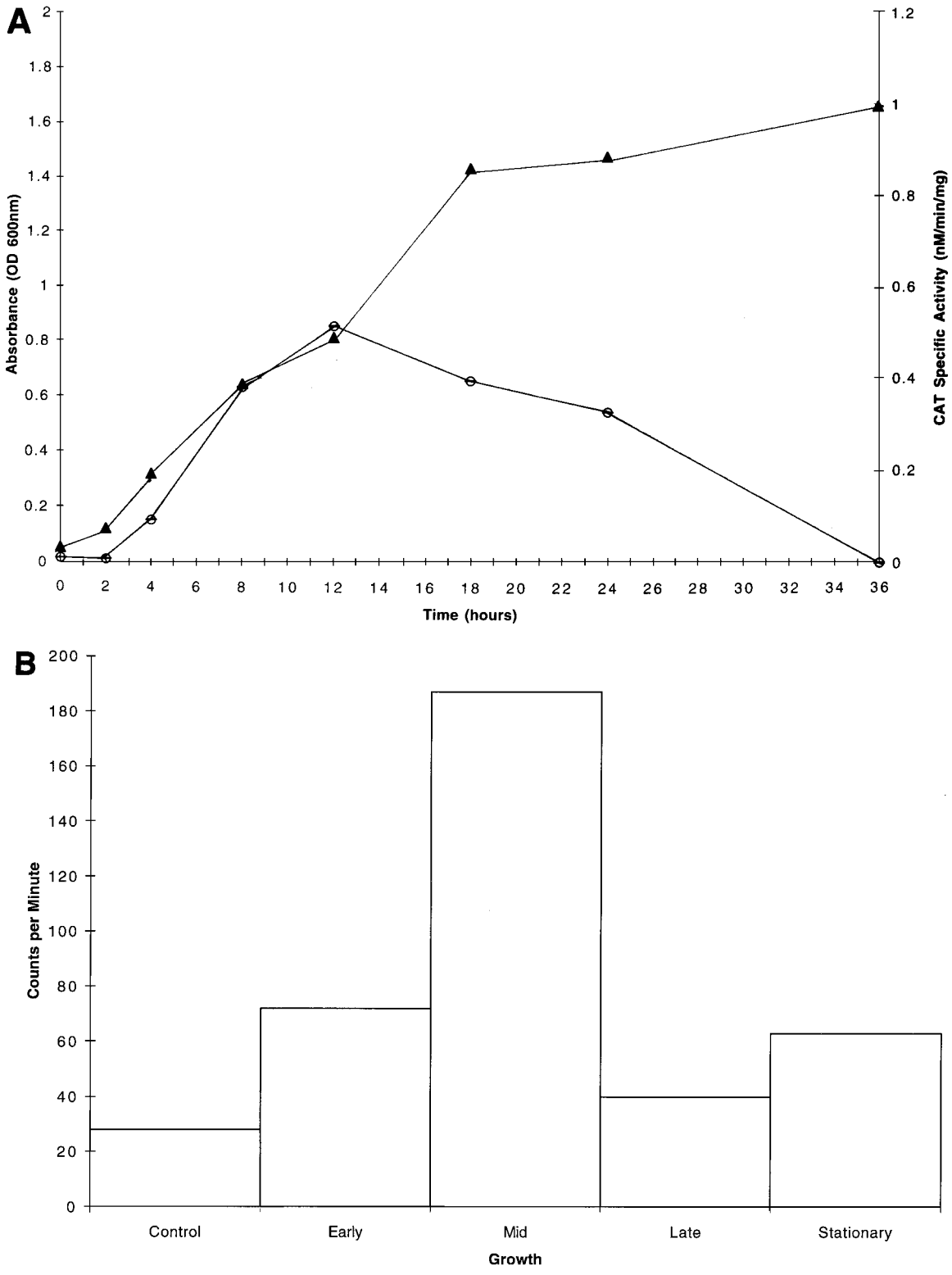

FIG. 6. (A) Growth curve (closed triangles) and CAT specific activities (open circles) for *S. mutans* GMS100 cultures grown in CDM supplemented with 1% sucrose. For each CAT specific activity data point, representing the mean of values from three independent experiments, the standard deviation was negligible. (B) Total RNA (1 μg) isolated from *S. mutans* UA130 grown to early, mid-, or late logarithmic phase or to stationary phase in CDM supplemented with 1% sucrose was dot blotted onto a nitrocellulose membrane and probed with a DNA fragment which is internal to *dlt1*. Individual dots were excised, and the radioactivity was counted in a Packard Tri-Carb model 1900CA liquid scintillation analyzer. A dot representing nonspecific hybridization was included as a control. Shown are the results of a single representative experiment. Note the maximal expression of *dlt* during mid-logarithmic phase.

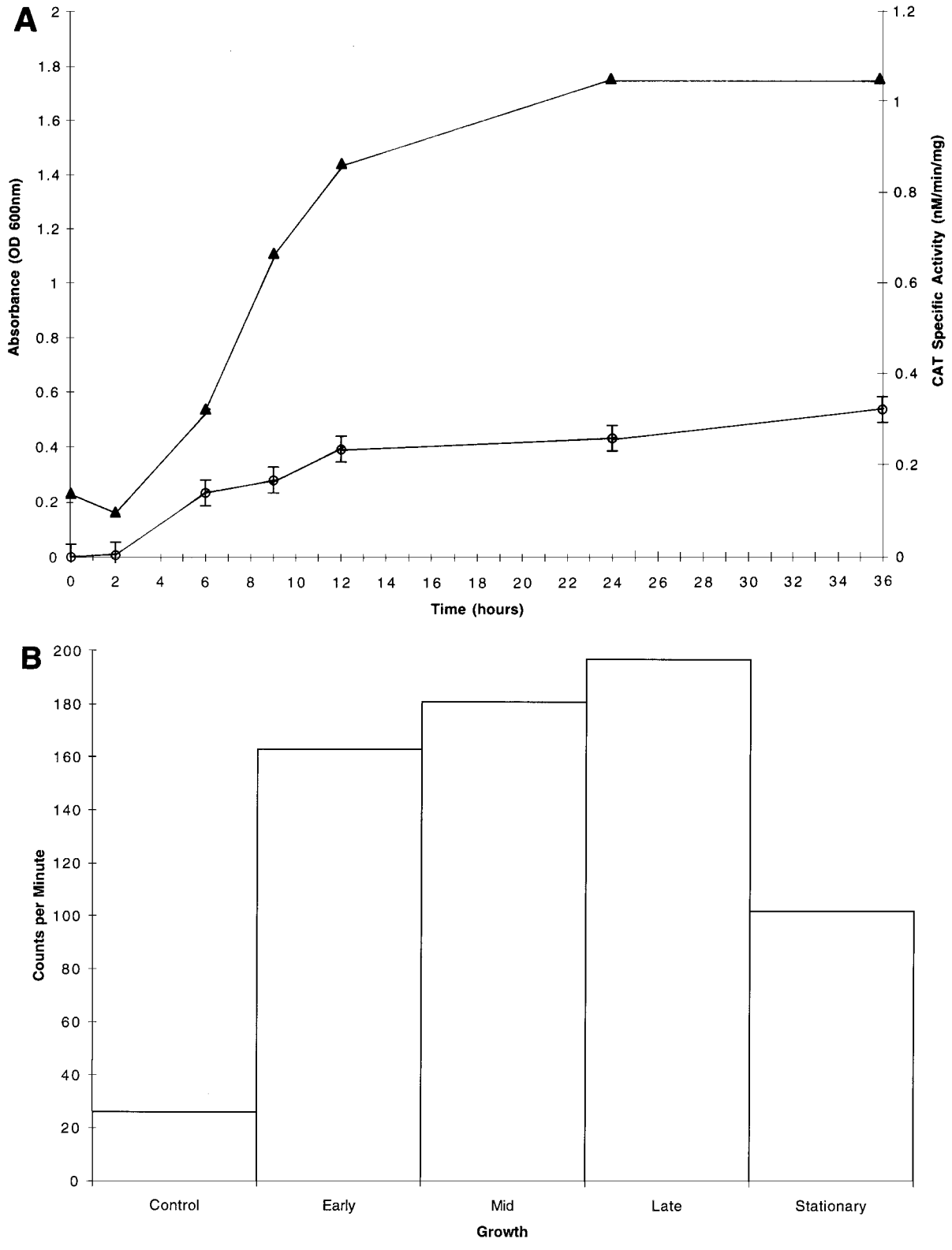

FIG. 7. (A) Growth curve (closed triangles) and CAT specific activities (open circles) for *S. mutans* GMS100 cultures grown in CDM supplemented with 1% raffinose. Except where indicated by the vertical bars, the standard deviation was negligible for each data point, representing the mean of values from three independent experiments. (B) Total RNA (1 μg) isolated from *S. mutans* UA130 grown to early, mid-, or late logarithmic phase or to stationary phase in CDM supplemented with 1% raffinose was dot blotted onto a nitrocellulose membrane and probed with a DNA fragment which is internal to *dlt1*. Individual dots were then excised, and the radioactivity was counted in a Packard Tri-Carb model 1900CA liquid scintillation analyzer. A dot representing nonspecific hybridization was included as a control. Shown are the results of a single representative experiment. Note the constitutive expression of the *S. mutans dlt* genes.

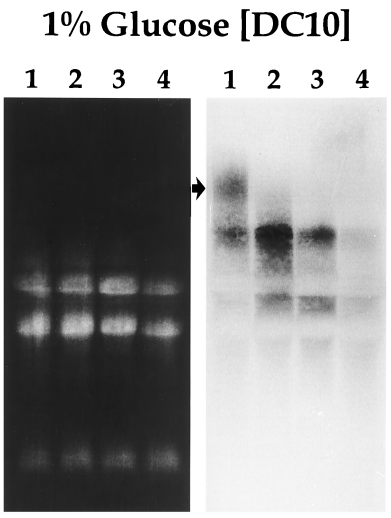

FIG. 8. Ethidium bromide-stained gel (left panel) and corresponding Northern blot (right panel) of total RNA isolated from *S. mutans* DC10, a PTS mutant. Cells were grown in CDM supplemented with glucose to early logarithmic phase (lane 1), mid-logarithmic phase (lane 2), late logarithmic phase (lane 3), and stationary phase (lane 4). Hybridization was carried out with a radiolabeled *dlt1*-specific PCR product as the probe. Note that expression of the *S. mutans dlt* genes is constitutive for all phases of growth. A unique transcript (>10 kb) which is expressed only during early logarithmic phase is designated by the arrow.

rise to a unique pattern of *S. mutans dlt* expression. Unlike the modulated *dlt* expression of cultures grown in the presence of glucose, fructose, or sucrose (carbohydrates internalized by the PTS system), the constitutive expression of *dlt* was directed by non-PTS sugars during the early, mid-, and late logarithmic phases and the stationary phase of growth. This expression pattern is presumably due to induction of the *msm* operon by raffinose and/or melibiose to promote the internalization of these and other sugars via this non-PTS pathway (30).

A similar hybridization pattern was observed on Northern blots of total RNA isolated from *S. mutans* DC10, a PTS mutant which we propagated in CDM supplemented with glucose. This mutant bears a mutation in the enzyme I component of the PTS, such that glucose is prevented from entering bacterial cells via the conventional PTS pathway. The fact that *dlt* expression is altered in this mutant suggests a role for the PTS in *dlt* regulation. Moreover, the absence of a functional PTS system in the DC10 strain necessitates the rerouting of glucose uptake via an alternative pathway. In fact, reports by Cvitkovitch et al. (8) indicate that some glucose uptake in DC10 can occur via a non-PTS pathway that is independent of the *msm* operon. Additional reports suggest that such a transport pathway may involve a transmembrane carrier protein capable of recognizing glucose (4). Interestingly, the expression of a novel *dlt*-specific transcript in *S. mutans* DC10 was evident during early logarithmic phase in cultures grown in the presence of glucose (Fig. 8); this transcript was not expressed, however, by the BM71 progenitor strain. The early onset of expression of this unique transcript in the mutant is consistent with the elaboration of a protein required for glucose recognition so that uptake via a non-PTS route may follow. Studies to determine whether this transcript is absent from *S. mutans* DC10 cultures propagated in the presence of raffinose and/or melibiose are currently under way.

In conclusion, we assigned a novel *dlt* designation to the previously described *S. mutans glg* locus. However, we maintain that this locus is involved in IPS accumulation, which is known to contribute significantly to the *S. mutans*-induced caries-forming process. We report that the regulated expression of the *S. mutans dlt* genes is cell density dependent and subject to regulatory control by PTS sugars. One might propose a role for the membrane-associated D-alanyl lipoteichoic acids in sensing the plaque environment and, as part of a two-component signal transducing system, regulating IPS accumulation and possibly other cellular activities. This is consistent with the temporal expression in *Staphylococcus aureus* of an accessory gene regulator (*agr*) (18) which indirectly modulates the expression of other target genes in response to pH, cell density, and carbohydrate availability. Exactly how cell density and dietary carbohydrate modulate *dlt* expression in *S. mutans* is unclear, and it is not known whether these growth conditions are likely to regulate the coordinate expression of the *S. mutans glg* genes. Investigations aimed at elucidating putative cross-regulation between the *dlt* operon and other genes whose products contribute to carbohydrate metabolism and virulence in *S. mutans* will address this question. Taken collectively, these studies will reveal the regulatory network which is likely to control *S. mutans* IPS accumulation in the plaque environment and the complexity of events which promote cariogenesis in a niche as dynamic as the oral cavity.

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