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Streptococcus mutans Serotype c Tagatose 6-Phosphate Pathway Gene Cluster

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DNA cloned into *Escherichia coli* K-12 from a serotype c strain of *Streptococcus mutans* encodes three enzyme activities for galactose utilization via the tagatose 6-phosphate pathway: galactose 6-phosphate isomerase, tagatose 6-phosphate kinase, and tagatose-1,6-bisphosphate aldolase. The genes coding for the tagatose 6-phosphate pathway were located on a 3.28-kb *Hin*dIII DNA fragment. Analysis of the tagatose proteins expressed by recombinant plasmids in minicells was used to determine the sizes of the various gene products. Mutagenesis of these plasmids with transposon Tn*5* was used to determine the order of the tagatose genes. Tagatose 6-phosphate isomerase appears to be composed of 14- and 19-kDa subunits. The sizes of the kinase and aldolase were found to be 34 and 36 kDa, respectively. These values correspond to those reported previously for the tagatose pathway enzymes in *Staphylococcus aureus* and *Lactococcus lactis*.

Streptococcus mutans is the principal etiological agent of dental caries in humans and in animal models. Carbohydrate metabolism leads to polymer synthesis and acid production, which play important roles in the process by which oral streptococci colonize the teeth and cause decay (11, 15). It has previously been demonstrated that a cloned 5.1-kb *Bam*HI fragment of *S. mutans* PS14 (serotype c) chromosomal DNA complements various *Escherichia coli* strains with mutations in or deletion of the *galKTE* genes (35). *E. coli* metabolizes galactose by the Leloir pathway (27), whereas staphylococci (4) and streptococci (16) use the tagatose 6-phosphate pathway to metabolize galactose and lactose. In the latter pathway, galactose or the galactose moiety of lactose is phosphorylated during its transport through the cell membrane. Transported lactose-phosphate is cleaved to form galactose 6-phosphate and glucose. In either process, the resultant galactose 6-phosphate is then converted sequentially to tagatose 6-phosphate by an isomerase, to tagatose-1,6-bisphosphate by a kinase, and to dihydroxyacetone phosphate and glyceraldehyde 3-phosphate by an aldolase (4, 10).

It has been shown that the genes encoding the enzymes of the tagatose 6-phosphate pathway are part of the lactose phosphotransferase system (PTS) operon, which is located either on a plasmid (22, 37) or on the chromosome (31, 33) in gram-positive bacterial species. A high level of similarity has been demonstrated in the organization of the *lac* PTS operons of *S. mutans*, *Staphylococcus aureus*, and *Lactococcus lactis* (31, 33, 37). The genetic organization of the heptacistronic *S. aureus lac* operon is *lacRABCDFEG* (28, 31), while the *L. lactis lac* operon consists of eight genes. Gene *X*, which specifies a 34-kDa protein of unknown function, is located downstream from the *lacG* locus in the *L. lactis* operon (13). Repression of the *lac* operon in *S. aureus* is mediated by the 28-kDa protein specified by the *lacR* gene, which is located just upstream of the first gene of the operon, *lacA* (28, 29). The most extensively studied genes of the gram-positive bacterial *lac* PTS operons are the *lacG* genes, encoding the phospho-β-galactosidases (6, 13, 22, 26), and the *lacE* and *lacF* genes, specifying the components of the lactose-specific PTS (1, 5, 12).

The first four genes of the *lac* PTS operon, *lacABCD*, specify the enzymes for galactose utilization. These genes, which encode the tagatose 6-phosphate pathway enzymes, from *S. aureus* (31, 32) and *L. lactis* (37) have been cloned and sequenced. The products of *lacA* and *lacB* constitute subunits of the tagatose 6-phosphate isomerase, *lacC* encodes the tagatose 6-phosphate kinase, and *lacD* specifies the tagatose-1,6-bisphosphate aldolase.

In this report, we describe our studies on the *S. mutans* galactose utilization gene cluster introduced into *E. coli*. Experiments involving analysis of the proteins expressed by recombinant plasmids in minicells demonstrated several proteins specified by *S. mutans* genes. Analysis of the protein products produced by the transposon Tn*5*-generated mutants, combined with enzyme activity assays, permitted localization of the genes encoding the enzymes of the tagatose 6-phosphate pathway. A companion article (33) reports the sequence of the 5.1-kb *S. mutans* DNA which encodes the galactose utilization enzymes, and those results are totally in accord with those reported in this article.

MATERIALS AND METHODS

Bacterial strains and growth media. The bacterial strains used were *E. coli* K-12 χ1849 [F⁻ *tonA53 dapD8 minA1 purE41 supE42* Δ(*gal-uvrB*)*47* λ⁻ *minB2 his-53 nalA25 metC65 oms-1* T3ʳ Δ(*bioH-asd*)*29 ilv-277 cycB2 cycA1 hsdR2* (17)] and χ2782. χ2782 is an undefined ethyl methanesulfonate mutant of χ1849 which grows faster on galactose minimal medium when it contains plasmid clones which carry the *S. mutans* genes for galactose utilization. *E. coli*

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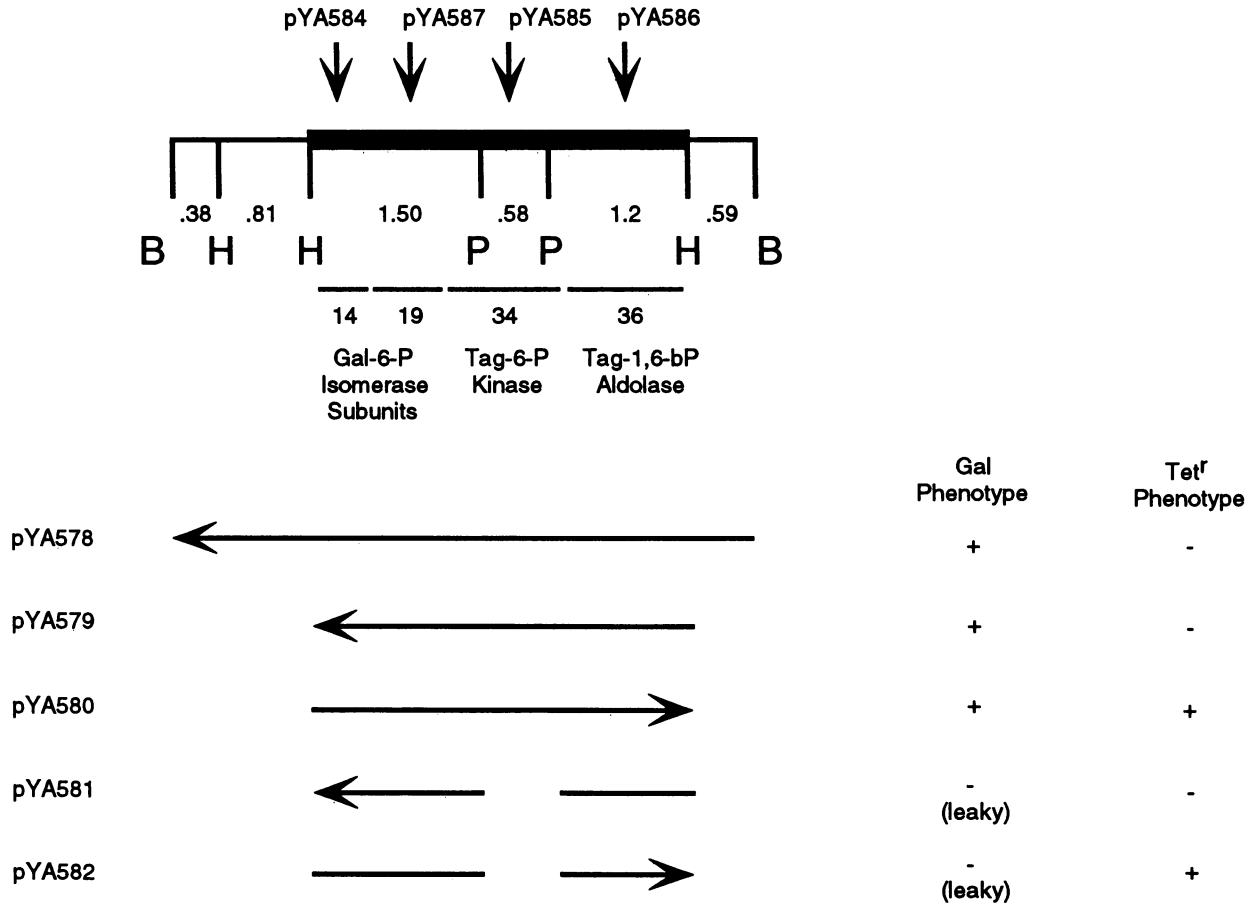

FIG. 1. The 5.1-kb *Bam*HI *S. mutans* DNA fragment cloned in pYA578 is shown, with additional restriction endonuclease sites marked (B, *Bam*HI; H, *Hin*dIII; P, *Pst*I). The sizes (in kilobases) of different restriction fragments are shown between the restriction endonuclease sites. The thick middle line is the 3.28-kb *Hin*dIII fragment that contains the genes necessary for galactose utilization. Below this region are lines which show the lengths of DNA necessary to encode polypeptides of 14, 19, 34, or 36 kDa. These lines are labeled with the size of the polypeptide and the enzymatic function associated with that polypeptide. At the bottom of the figure, bars indicate the region of DNA contained within the various subclones listed at the left. Arrowheads indicate the orientation of the insert relative to the tetracycline resistance gene of the vector pACYC184. Plasmid subclones with the arrowhead toward the right confer tetracycline resistance on the host cells. Subclones with the arrowhead toward the left contain fragments in the opposite orientation and do not confer tetracycline resistance. At the right of the bars, the galactose and tetracycline resistance phenotypes conferred on χ1849 and χ2782 by the various subclones are given. The sites where transposon Tn*5* has inserted into pYA578 to make Gal$^-$ derivative plasmids pYA584 through pYA587 are shown by arrowheads above the figure. Gal-6-P, galactose 6-phosphate; Tag-6-P, tagatose 6-phosphate; Tag-1,6-bP, tagatose 1,6-bisphosphate.

K-12 strain χ2340 [*ΔaraC266 tonA53 dapD8 minA1 Δ(gal-chlD) ΔtrpBC13 minB2 rfb-2 gyrA25 ΔthyA57 endA1 oms-1 asdA4 cycB2 cycA1 hsdR2*] was used for Tn*5* mutagenesis. All strains were grown at 37°C.

Liquid minimal medium (19) contained 0.5% glucose or galactose and the following supplements (per milliliter): 20 μg of L-isoleucine, 80 μg of DL-threonine, 40 μg of adenine, 50 ng of biotin, 2 μg of thiamine HCl, and 50 μg of DL-*meso*-diaminopimelic acid (DAP). To grow strains for minicell harvests, the following amino acids were added to liquid minimal medium (per milliliter): 200 μg of DL-alanine, 22 μg of L-arginine HCl, 100 μg of glycine, 100 μg of L-glutamic acid, 100 μg of L-glutamine, 22 μg of L-histidine HCl, 20 μg of L-leucine, 88 μg of L-lysine HCl, 20 μg of DL-methionine, 20 μg of L-phenylalanine, 30 μg of L-proline, 100 μg of DL-serine, 20 μg of L-tryptophan, 20 μg of L-tyrosine, and 20 μg of L-valine. All supplements for minimal medium were obtained from Sigma. The complex media used were L broth (24) supplemented with DAP and L broth plus DAP solidified by adding 1.2% agar (Difco) and Penassay agar (Difco). The ability to ferment galactose was tested on MacConkey agar (Difco) with 1.0% (wt/vol) D-galactose and the required supplements. Antibiotics to maintain or select plasmid-containing strains were used at the following concentrations: chloramphenicol, 25 μg/ml; kanamycin, 25 μg/ml; and tetracycline, 10 μg/ml (Sigma).

Recombinant plasmids. All recombinant plasmids contained *S. mutans* PS14 (serotype c) tagatose 6-phosphate pathway genes for the metabolism of galactose (35). The vector pACYC184 (7) was used for all recombinant clones. Plasmids pYA578 through pYA582 are described in Fig. 1 and by Smorawinska et al. (35), in which article the plasmids were described but not named. Plasmids pYA584 through pYA587 are derivatives of pYA578 in which Tn*5* has been inserted into the tagatose 6-phosphate pathway gene cluster. The construction and mapping of the Tn*5* derivatives are described in the Results section.

Transposon mutagenesis. Plasmid pYA578 was transferred

into strain χ2340 by transformation by the method of Hansen et al. (17), with selection for chloramphenicol resistance. Transformants were also able to grow with galactose as the sole carbon source. A selected transformant was infected with phage lambda (genotype *c*I857 *b*221 *Pam* Tn*5*) at 30°C and a multiplicity of infection of 0.1. The phage lysate was a gift of Anthony Maurelli (Uniformed Services University of the Health Sciences, Bethesda, Md.). Bacteria and phage were incubated together for 1 h. Cells were then plated on Penassay agar medium that contained DAP (50 μg/ml), thymine (40 μg/ml), chloramphenicol, and kanamycin to select for the plasmid and Tn*5* markers and 2.5 mM sodium PP_i to prevent lambda reabsorption.

Minicell analysis of plasmid gene products. Plasmid-containing minicells of *E. coli* χ1849 were isolated by standard methods (14). Minicells from a late-logarithmic-phase culture in liquid minimal medium supplemented with amino acids were purified by one differential centrifugation and two discontinuous sucrose density gradient sedimentations. Between the two density gradient sedimentations, cells were incubated for 2 h in growth medium that lacked DAP. Purified minicells were suspended in a modified liquid minimal medium. In the modified medium, DAP was absent and the concentration of DL-methionine was 20 μM. The purity and concentration of the minicell preparation were tested by plating a sample on L agar plus DAP to determine the viable-cell number and by measuring the optical density at 620 nm, respectively. Approximately 8×10^8 minicells per ml give an A_{620} of 0.2.

Proteins were labeled by adding 10 μCi of L-[^{35}S]methionine (New England Nuclear; 10 mCi/ml) to the medium and incubating at 37°C for 45 min. The minicells were washed and then lysed by boiling for 5 min in 100 μl of sample buffer (2.3% sodium dodecyl sulfate [SDS], 5% β-mercaptoethanol, 10% glycerol, 62.5 mM Tris-HCl [pH 6.8]). For SDS-polyacrylamide gel electrophoresis, samples containing equal numbers of minicells were used. To analyze proteins of medium molecular weight (see Fig. 2), the SDS-polyacrylamide gel electrophoresis (6% [wt/vol] polyacrylamide stacking gel, 10% [wt/vol] polyacrylamide separating gel) method of Laemmli and Favre was used (21). To separate proteins of lower molecular weight (see Fig. 3), the urea-SDS-polyacrylamide gel electrophoresis method of Shapiro et al. (34) was modified. Both the stacking and separating gels contained 0.1 M sodium phosphate buffer (pH 7.2), 0.1% SDS, and 6 M urea. The polyacrylamide (bisacrylamide-acrylamide, 1:37.5) concentration was 3.5% (wt/vol) in the stacking gel and 15% (wt/vol) in the separating gel. The running buffer contained 0.1 M sodium phosphate buffer (pH 7.2) and 0.1% SDS. Protein samples were adjusted to 6 M urea and 0.1 M sodium phosphate buffer, pH 7.2, before electrophoresis. After electrophoresis, the gels were impregnated with a fluor (En^{3}Hance; New England Nuclear), dried, and fluorographed. Protein molecular mass standards labeled with ^{14}C (BRL) included: insulin, 3.0 kDa; bovine trypsin inhibitor, 6.2 kDa; lysozyme, 14.3 kDa; β-lactoglobulin, 18.4 kDa; α-chymotrypsinogen, 25.7 kDa; and ovalbumin, 43.0 kDa.

Enzyme assays. Cell extracts for enzyme assays were prepared from 24-h cultures grown at 37°C on a shaker with 100 ml of minimal liquid medium and 0.1% carbohydrate in a 500-ml Erlenmeyer flask. Cells were washed in 100 ml of 20 mM $NaKHPO_4$ buffer containing 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol (pH 6.5). Cells were disrupted by shaking them twice for 2-min intervals with 3 ml of glass beads on a Mickle homogenizer. Cell debris was removed by centrifugation (27,000 × *g* for 10 min). The supernatant fluid was used for protein determinations (25) and enzyme assays.

Isomerase assays. The galactose 6-phosphate isomerase activity was assayed by a three-step procedure (9). The first step involved incubating galactose 6-phosphate isomerase in an assay mixture (100 μl) containing 100 mM triethanolamine hydrochloride buffer (pH 7.8) and 10 mM tagatose 6-phosphate for 0, 15, and 30 min. The reaction was stopped by heating in a boiling-water bath for 5 min. In step 2, 30 μl of 1 M glycerol–NaOH buffer (pH 10.5) and 70 μl of alkaline phosphatase (Sigma; 7 U of calf intestine alkaline phosphatase [EC 3.1.3.1]) were added to the stopped reaction mixture and incubated for 60 min at 25°C. Step 3 involved determining galactose levels enzymatically by the procedure of Kurz and Wallenfels (20). One unit of isomerase activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of galactose 6-phosphate from tagatose 6-phosphate per min (initial rate).

Aldolase assays. The standard 1-ml assay mix (9) contained 50 mM triethanolamine hydrochloride buffer (pH 7.8), 0.25 mM NADH, nonlimiting amounts of the coupling enzymes α-glycerolphosphate dehydrogenase (EC 1.1.1.8) (1.2 U) (Sigma; from rabbit muscle), and triosephosphate isomerase (EC 5.3.11) (11.5 U) (Sigma; from rabbit muscle), and limiting amounts of aldolase and 0.16 mM tagatose 1,6-bisphosphate. Rates were corrected for NADH oxidase activity, which was measured prior to the addition of ketohexose bisphosphate. The reaction was monitored at 340 nm (25°C) with a Gilford model 250 spectrophotometer. The rate was proportional to the aldolase concentration and was initially constant with time. One unit of aldolase activity was defined as the amount of enzyme that catalyzed the cleavage of the ketohexose bisphosphate at an initial rate of 1 μmol/min.

6-Phosphokinase assays. The standard 1-ml assay mix (9) contained 50 mM triethanolamine hydrochloride buffer (pH 7.8), 0.25 mM NADH, 2 mM ATP, 7 mM $MgCl_2$, nonlimiting amounts of the coupling enzymes α-glycerolphosphate dehydrogenase (1.2 U), triosephosphate isomerase (11.5 U), tagatose-1,6-bisphosphate aldolase (0.6 U; purified from *Streptococcus cremoris* E8 [10]), limiting amounts of kinase, and either 1 or 0.3 mM tagatose 6-phosphate. The spectrophotometric assay conditions were the same as those for the isomerase assay. Adjustments were made for NADH oxidase and dehydrogenase activities contained in the cell extracts. One unit of kinase activity was defined as the amount of enzyme that catalyzed the phosphorylation of ketohexose phosphate at an initial rate of 1 μmol/min.

RESULTS

Analysis of plasmids containing the *S. mutans* galactose gene cluster. Several recombinant plasmids that contained all or part of the cloned *S. mutans* galactose utilization gene cluster are depicted in Fig. 1. Figure 1 presents a partial restriction map of pYA578, which contains the 5.1-kb *S. mutans* DNA fragment containing the galactose utilization gene cluster (35), and indicates the regions of pYA578 contained in various subclones and the orientation of these fragments in the vector. Plasmid subclones containing the internal 3.28-kb *Hin*dIII fragment allowed *E. coli* strains χ1849 and χ2782, both galactose deletion mutants, to grow on galactose minimal medium. Deletion of a 0.58-kb internal *Pst*I fragment from within the 3.28-kb *Hin*dIII fragment gave

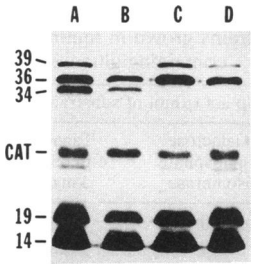

FIG. 2. Fluorograph, after SDS-polyacrylamide gel electrophoresis, of radioactively labeled proteins from *E. coli* minicells containing plasmids with *S. mutans* tagatose 6-phosphate pathway galactose utilization genes. A, χ1849(pYA579); lane B, χ1849 (pYA580); lane C, χ1849(pYA581); lane D, χ1849(pYA582). The sizes of various galactose utilization gene products are marked (in kilodaltons). CAT, chloramphenicol acetyltransferase enzyme encoded by the vector pACYC184.

cells carrying the deleted recombinant plasmids a leaky galactose-negative phenotype (35).

When the 3.28-kb *Hin*dIII insert and its deletion derivatives were cloned into pACYC184 in one orientation, the recombinant plasmids, pYA580 and pYA582, had the ability to confer the phenotype of tetracycline resistance (Tc^r); cells containing recombinant plasmids pYA579 and pYA581, with the insert in the other orientation, were sensitive to tetracycline (Tc^s). In one orientation, the *S. mutans* DNA must provide promoter activity, because the *tet* promoter of pACYC184 is disrupted by *Hin*dIII cloning.

Minicell analysis of cells containing the *S. mutans* galactose genes. The *S. mutans*-specific proteins encoded by the recombinant plasmids pYA579, pYA580, pYA581, and pYA582 are shown in Fig. 2. Five proteins, of 14, 19, 34, 36, and 39 kDa, were encoded by the 3.28-kb insert and were synthesized in minicells when the insert was in either orientation (Fig. 2, lanes A and B). This fact suggests that the *S. mutans* 3.28-kb *Hin*dIII DNA fragment contains at least one functional *S. mutans* promoter recognized by *E. coli* RNA polymerase. Derivative plasmids pYA581 and pYA582, missing the internal *Pst*I fragment (Fig. 1), no longer synthesize the 34-kDa protein (Fig. 2, lanes C and D). Recombinant plasmids pYA579 and pYA581 (Fig. 2, lanes A and C), which had a Tc^s phenotype and had inserts in the same orientation, also appeared to specify synthesis of greater amounts of the *S. mutans*-specific proteins than plasmids pYA580 and pYA582 (Fig. 2, lanes B and D), which had the inserts in the opposite orientation. This was especially evident for the 34-, 36-, and 39-kDa gene products (Fig. 2, compare lane A with lane B and lane C with lane D), although an equal amount of minicells was loaded in each well. The chloramphenicol acetyltransferase enzyme encoded by the vector pACYC184 served as an internal standard when comparing the amount of *S. mutans*-specific proteins made by the different recombinant plasmids.

All of the streptococcal DNA inserts analyzed for their protein production (Fig. 2) are cloned into the *Hin*dIII site of pACYC184. The *Hin*dIII site lies in a region with two promoters, P1 and P2, which function in opposite directions (36). The transcription level and expression of the *S. mutans* genes appear to be dependent on the insert orientation and the influence of the strong P1 promoter of pACYC184, which initiates transcription in the opposite direction from the tetracycline resistance promoter, P2.

Assays for the *S. mutans* tagatose 6-phosphate enzymes. *E. coli* cells that contained the Gal^+ recombinant plasmid pYA579 or pYA580 were assayed for the tagatose 6-phosphate pathway enzymes, and the results are shown in Table 1. Cells containing pYA579 or pYA580 acquired three tagatose 6-phosphate pathway enzyme activities: galactose 6-phosphate isomerase, tagatose 6-phosphate kinase, and tagatose-1,6-bisphosphate aldolase. *E. coli* χ1849 had some endogenous kinase activity for tagatose 6-phosphate; that endogenous activity and the *S. mutans*-encoded tagatose 6-phosphate kinase were better distinguished when a lower tagatose 6-phosphate substrate concentration (0.3 mM) was used (Table 1).

TABLE 1. Specific activities of enzymes in cell extracts prepared from *E. coli* χ1849 containing various plasmid subclones grown in minimal liquid medium containing glucose[a]

Strain	Sp act (μmol of substrate utilized/mg of protein/min)			
	Galactose 6-phosphate isomerase	Tagatose 6-phosphate kinase at tagatose 6-phosphate concn:		Tagatose 1,6-bisphosphate aldolase
		1 mM	0.3 mM	
χ1849	0.0[b]	0.35	0.004	0.0
	0.0	0.38	—[c]	0.0
χ1849(pACYC184)	0.0	0.19	0.003	0.0
	0.0	0.22	—	0.0
χ1849(pYA579)	1.16	0.86	0.58	0.32
	1.23	1.08	—	0.42
χ1849(pYA580)	0.46	0.41	0.21	0.17
	0.53	0.68	—	0.20
χ1849(pYA581)	1.09	0.30	0.003	0.49
	1.48	0.24	—	0.58
χ1849(pYA582)	0.56	0.38	0.005	0.22
	0.62	0.39	—	0.25

[a] Different lines for the same strain represent cell extracts prepared on different days. Each value is the average of duplicate assays. Tagatose 6-phosphate kinase activity was assayed with two different substrate concentrations, as shown. See Materials and Methods for assay conditions.

[b] 0.0, not detectable (less than 0.002 μmol of substrate utilized per mg of protein per min).

[c] —, not determined.

Deletion of the *Pst*I fragment to form pYA581 and pYA582, which resulted in a leaky Gal^- phenotype, correlates with the loss of *S. mutans* tagatose 6-phosphate kinase activity (Table 1). The leaky phenotype was consistent with the endogenous level of *E. coli* kinase activity for tagatose 6-phosphate. Presumably, the 34-kDa gene product missing from the *Pst*I deletion mutants (Fig. 2) is the protein exhibiting the *S. mutans* tagatose 6-phosphate kinase activity (Table 1). Second, the increased amounts of *S. mutans*-specific gene products seen for the recombinant plasmids pYA579 and pYA581 (Fig. 2) correlated with higher tagatose 6-phosphate pathway enzyme activities than were seen with pYA580 and pYA582 (Table 1).

Transposon mutagenesis of the *S. mutans* galactose genes. The *S. mutans* genes for galactose utilization contained on plasmid pYA578 were mutagenized by using lambda phage containing Tn*5*. The frequency of transposition was approximately 3×10^{-5} per infecting phage (data not shown). Plasmid DNA was isolated from approximately 1,500 pooled colonies by the method of Birnboim and Doly (3) and transformed into χ1849, with selection for chloramphenicol resistance alone or combined chloramphenicol and kanamy-

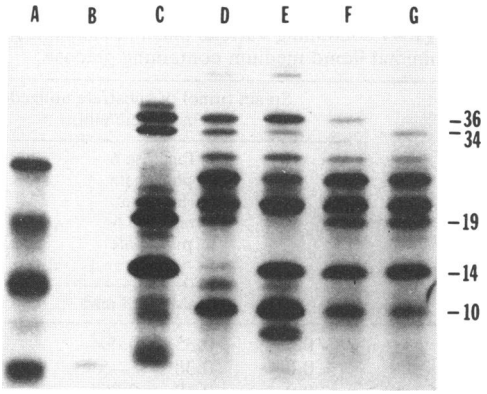

FIG. 3. Fluorograph, after SDS-polyacrylamide gel electrophoresis in the presence of urea, of radioactively labeled proteins from *E. coli* minicells containing *S. mutans* tagatose 6-phosphate pathway galactose utilization genes. Lane A, protein size standards; lane B, χ1849; lane C, χ1849(pYA579); lane D, χ1849(pYA584); lane E, χ1849(pYA587); lane F, χ1849(pYA585); lane G, χ1849(pYA586). The sizes of various galactose utilization gene products are marked (in kilodaltons). The chloramphenicol acetyltransferase of pACYC184 and the kanamycin acetyltransferase of Tn*5* are the two darker bands above the 19-kDa protein in lanes D through G.

cin resistances. The frequency of transformation for double antibiotic resistance was 0.5% of the frequency of transformation for chloramphenicol resistance alone. Such doubly resistant colonies putatively contained pYA578::Tn*5* derivatives. Growth of the doubly resistant clones on MacConkey galactose agar showed that approximately 20% of them were unable to ferment galactose as a carbon source.

The plasmid DNAs from 16 galactose-negative colonies were screened by size, and the results were consistent with the insertion of Tn*5* into pYA578. All 16 plasmids had Tn*5* inserted into the 3.28-kb *Hin*dIII fragment located within the 5.1-kb *S. mutans* DNA fragment contained in pYA578 (data not shown). The locations of the Tn*5* insertions within the 3.28-kb *S. mutans* *Hin*dIII fragment were more precisely mapped in four plasmids by digesting plasmid DNA with *Pst*I plus *Bam*HI or with *Hin*dIII alone. These four plasmids all had insertions in different locations and were designated pYA584 through pYA587. The locations of these insertions into pYA578 are indicated in Fig. 1.

Analysis of transposon insertion mutations in the galactose utilization gene cluster. The gene products of the four plasmids containing Tn*5* insertions into the *S. mutans* galactose genes were analyzed by using minicells. Figure 3 shows which *S. mutans* gene products were affected in the transposon insertion mutants. The plasmid pYA584 (Fig. 3, lane D) lacks the 14-kDa protein, pYA587 (Fig. 3, lane E) lacks the 19-kDa protein, pYA585 (Fig. 3, lane F) lacks the 34-kDa protein, and pYA586 (Fig. 3, lane G) lacks the 36-kDa protein. All four derivatives lacked the 39-kDa protein. Some protein bands seen faintly towards the top of the gel, especially in lanes D and E, were Tn*5* specific (30). The 36-kDa protein in pYA585 and the 34-kDa protein in pYA586 (Fig. 3, lanes F and G, respectively) were both diminished in amount compared with the amounts in pYA584 and pYA587 (Fig. 3, lanes D and E). The 22-kDa protein (Fig. 3, lanes C through G) is the chloramphenicol acetyltransferase enzyme (2), and the 26-kDa protein (Fig. 3, lanes D through G) present in the four Tn*5* derivatives is a product of the kanamycin resistance gene (30).

TABLE 2. Specific activities of enzymes in cell extracts prepared from *E. coli* strains grown in minimal liquid medium containing glucose[a]

Strain	Sp act (μmol of substrate utilized/mg of protein/min)		
	Galactose 6-phosphate isomerase	Tagatose 6-phosphate kinase[b]	Tagatose-1,6-bisphosphate aldolase
χ2782	0.0[c]	0.17	0.0
	0.0	0.36	0.0
	0.0	0.20	0.0
χ2782(pYA579)	1.88	1.01	0.32
	1.79	1.75	0.25
	2.00	1.21	0.28
χ2782(pYA584)	0.0	2.35	3.19
	0.0	3.02	3.14
	0.0	—[d]	1.16
χ2782(pYA585)	0.0	0.45	1.07
	0.0	0.15	0.28
	0.0	0.45	0.27
χ2782(pYA586)	0.0	1.31	0.0
	0.0	1.29	0.0
	0.0	1.40	0.0
χ2782(pYA587)	0.0	0.87	0.22
	0.0	0.86	0.29
	0.0	0.85	0.25

[a] Different lines for the same strain represent cell extracts prepared on different days. Each value is the average of duplicate assays. See Materials and Methods for assay conditions.
[b] Tagatose 6-phosphate present at 1 mM.
[c] 0.0, not detectable (less than 0.005 μmol of substrate utilized per mg of protein per min).
[d] —, not determined.

The four transposon mutants were also screened for their ability to confer galactose utilization on χ2782 and for the presence of the tagatose 6-phosphate pathway enzymes. Cells containing pYA585 had a leaky Gal$^-$ phenotype, while cells containing the other plasmids were tightly Gal$^-$. The tagatose 6-phosphate pathway enzyme activities of χ2782 transformed with plasmids pYA584 through pYA587 are shown in Table 2. Plasmid pYA585 appeared to be negative for tagatose 6-phosphate kinase activity, pYA586 was negative for tagatose-1,6-bisphosphate aldolase activity, and all four lacked galactose 6-phosphate isomerase activity.

DISCUSSION

In this report, we have characterized the *S. mutans* genes for galactose utilization cloned into *E. coli*. Using minicell analysis, transposon mutagenesis, and enzyme activity assays, we have determined the gene order and the sizes of the gene products and have related enzyme activity to specific gene products. The initial analysis of the plasmids pYA579 and pYA580 revealed that the same *S. mutans* chromosomal DNA insert cloned into pACYC184 in both orientations has the ability to confer on the *E. coli* galactose-negative mutants χ1849 and χ2782 the ability to grow on galactose minimal medium. These data indicate that the streptococcal DNA insert contains an active promoter and expression is not dependent on the plasmid vector. This information would also explain why tetracycline resistance is conferred on the host cell when plasmid pYA580 or pYA582 is present in the cell. It would appear that an internal streptococcal promoter is responsible for the transcription of the tetracycline resistance gene when the cloned DNA fragments are in that given orientation. When these fragments are cloned in

the opposite orientation, plasmids pYA579 and pYA581, the host cells are tetracycline sensitive. This conclusion is also consistent with the observation that the amount of streptococcal proteins produced in minicells is orientation dependent (Fig. 2). When the streptococcal promoter is oriented to transcribe toward the tetracycline resistance gene (conferring tetracycline resistance), the P1 promoter is initiating transcription in the opposite direction (36) and decreases the amount of streptococcal proteins produced (Fig. 2). In the plasmids pYA579 and pYA581, both the streptococcal promoter and the P1 promoter initiate transcription in the same direction, and the quantity of streptococcal proteins produced increases (Fig. 2).

Minicell analysis revealed that the streptococcal DNA fragment produced proteins of 14, 19, 34, 36, and 39 kDa (Fig. 2). Production of these proteins was independent of the orientation of the insert within the plasmid vector. The removal of the internal *Pst*I fragment eliminated production of the 34-kDa protein (Fig. 2). This localized the area of the streptococcal insert which encodes this protein (Fig. 1). Enzyme activity assays on cells containing plasmids pYA579 and pYA580 revealed the presence of galactose 6-phosphate isomerase activity, tagatose 6-phosphate kinase activity, and tagatose-1,6-bisphosphate aldolase activity (Table 2). Analysis of cells containing plasmid pYA581 or pYA582 revealed the loss of tagatose 6-phosphate kinase activity. Thus, the 34-kDa protein must be the streptococcal gene product responsible for tagatose 6-phosphate kinase activity. The endogenous tagatose 6-phosphate kinase activity displayed by the *E. coli* strains used may be due to the *pfkA* or *pfkB* gene product, both of which are enzymes used by *E. coli* in galactitol metabolism, which proceeds through the tagatose 6-phosphate pathway (23). Rosey and Stewart (33) were able to complement *E. coli fda* and *pfk* mutations by using a plasmid consisting of the 5.1-kb *Bam*HI fragment from pYA578 cloned into pBR322 (pYA501) (35).

To further analyze the streptococcal galactose utilization genes, plasmid pYA578 was subjected to transposon mutagenesis and subsequent minicell and enzyme analyses. The mutagenized plasmids, which contain Tn*5* at various locations, lost the ability to produce different streptococcal proteins. These results are shown in Fig. 3. The plasmids pYA584, pYA585, pYA586, and pYA587 do not produce the 14-kDa protein, the 34-kDa protein, the 36-kDa protein, and the 19-kDa protein, respectively. This allowed the relative order of the streptococcal tagatose 6-phosphate pathway genes on the *Bam*HI fragment to be determined. The overall order of these genes is shown in Fig. 1. Interestingly, all of the transposon mutants lost the ability to produce the 39-kDa protein. At present, this result is not understood.

Enzyme activity assays for the tagatose 6-phosphate pathway enzymes done with cell lysates from the transposon insertion mutants allowed enzymatic functions to be correlated with various streptococcal gene products. The deletion of the internal *Pst*I fragment had previously indicated that the 34-kDa protein was responsible for tagatose 6-phosphate kinase activity. This conclusion was substantiated with plasmid pYA585. This plasmid also does not produce the 34-kDa protein or possess the corresponding enzyme activity (Fig. 3 and Table 2). Plasmid pYA586 does not produce the 36-kDa protein and does not exhibit tagatose 6-phosphate aldolase activity (Fig. 3 and Table 2). Thus, the 36-kDa protein must be responsible for aldolase activity.

The correlation of the loss of a specific enzyme activity with the loss of a specific protein produced by the streptococcal insert cannot be done for plasmids pYA584 and pYA587. Although the transposon insertions in these plasmids eliminate the production of the 14- and 19-kDa proteins, galactose 6-phosphate isomerase activity is lost in all of the transposon mutants (Table 2). At this time, this result is not explained.

Our results on the overall structure and function of the *S. mutans* galactose utilization genes are consistent with the description of the tagatose 6-phosphate genes from other organisms (31, 37) and agree with the results in a companion article which describes the sequence of the streptococcal DNA fragment contained in pYA578 (33). From the nucleotide sequence homology between the *lacA* and *lacB* genes of *S. mutans* (33), *S. aureus* (31), and *L. lactis* (37) and the similarity of galactose utilization gene organization among these organisms, we concluded that the 14- and 19-kDa proteins are most likely subunits of galactose 6-phosphate isomerase. van Rooijen et al. (37) have demonstrated that expression of both the *lacA* and *lacB* genes of *L. lactis* was required to obtain galactose 6-phosphate isomerase activity, but the data presented here do not explain the loss of isomerase activity in all four transposon mutants.

Minicell analysis of Tn*5* mutants of pYA578 containing the 5.1-kb *Bam*HI fragment (Fig. 3) showed production of a 10-kDa protein and a 28-kDa protein which was not present in the analysis of plasmids containing the smaller *Hin*dIII fragment (Fig. 2). The locations of the DNA encoding these proteins and their potential functions can be predicted from sequencing data (33) and the similarity of the lactose PTS operon organization between *S. mutans*, *S. aureus*, and *L. lactis* (31, 33, 37). They are most likely expressed from *S. mutans* coding sequences outside the 3.28-kb *Hin*dIII fragment, which contains the tagatose 6-phosphate utilization structural genes, but are from within the larger *Bam*HI fragment. The 10-kDa protein is most likely enzyme III-lactose, a component of the lactose-specific PTS, and the 28-kDa protein is potentially the lactose operon repressor, the product of the *lacR* gene. This hypothesis is consistent with the data of Rosey and Stewart (33).

However, the results of Tn*5* transposon mutagenesis were not consistent with an operon structure. Strong polarity from the transposon insertions into the coding sequences for the four different proteins was not seen (Fig. 1 and 3). Transposon insertions 5′ to various intact genes did not eliminate enzyme activity from the downstream locus (Fig. 1 and Table 2). In addition, deletion of a DNA fragment encoding tagatose 6-phosphate kinase had no effect on synthesis of upstream- and downstream-encoded enzymes (Fig. 2 and Table 1). Nevertheless, sequencing data (33) revealed high levels of similarity between the *S. mutans* and *S. aureus* tagatose genes, and the *S. mutans* tagatose 6-phosphate pathway genes are part of the *lac* gene cluster. The sequence data strongly suggest that a *lac* promoter is located upstream of the *lac* structural genes, which are organized in an operonlike array (33). The results of the minicell analysis may have been affected by the *E. coli* background. *S. mutans* DNA is more AT rich (35 to 38 mol% G+C [8]) than *E. coli* DNA, and this may permit false transcription initiation from AT-rich regions and give the appearance of independent gene control. The lack of strong polar effects by transposon insertions into cloned streptococcal DNA has been described before (18). Further investigation is required to fully understand the regulatory mechanisms for expression of the genes for galactose and lactose metabolism in gram-positive bacterial species.

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REFERENCES

1. **Alpert, C. A., and B. M. Chassy.** 1990. Molecular cloning and DNA sequence of *lacE*, the gene encoding the lactose-specific enzyme II of the phosphotransferase system of *Lactobacillus casei*: evidence that a cysteine residue is essential for sugar phosphorylation. J. Biol. Chem. **265**:22561–22568.
2. **Alton, N. K., and D. Vapneck.** 1979. Nucleotide sequence analysis of the chloramphenicol resistance transposon Tn*9*. Nature (London) **282**:864–869.
3. **Birnboim, H. D., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
4. **Bisset, D. L., and R. L. Anderson.** 1973. Lactose and D-galactose metabolism in *Staphylococcus aureus*: pathway of D-galactose 6-phosphate degradation. Biochem. Biophys. Res. Commun. **52**:641–647.
5. **Breidt, F., Jr., W. Hengstenberg, M. Finkeldei, and G. C. Stewart.** 1987. Identification of the genes for the lactose-specific components of the phosphotransferase system in the *lac* operon of *Staphylococcus aureus*. J. Biol. Chem. **262**:1644–1649.
6. **Breidt, F., Jr., and G. C. Stewart.** 1986. Cloning and expression of the phospho-β-galactosidase gene of *Staphylococcus aureus* in *Escherichia coli*. J. Bacteriol. **166**:1061–1066.
7. **Chang, A. C. Y., and S. N. Cohen.** 1987. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134**: 1141–1156.
8. **Coykendall, A. L.** 1977. Proposal to elevate the subspecies of *Streptococcus mutans* to species status, based on the molecular composition. Int. J. Syst. Bacteriol. **27**:26–30.
9. **Crow, V. L., G. P. Davey, L. E. Pearce, and T. D. Thomas.** 1983. Plasmid linkage of the D-tagatose pathway in *Streptococcus lactis*: effect on lactose and galactose metabolism. J. Bacteriol. **153**:76–83.
10. **Crow, V. L., and T. D. Thomas.** 1982. D-Tagatose 1,6-diphosphate aldolase from lactic streptococci: purification, properties, and use in measuring intracellular tagatose 1,6-diphosphate. J. Bacteriol. **151**:600–608.
11. **Curtiss, R., III.** 1985. Genetic analysis of *Streptococcus mutans* virulence. Curr. Top. Microbiol. Immunol. **118**:253–277.
12. **de Vos, W. M., J. Boerrigter, R. J. van Rooijen, B. Reiche, and W. Hengstenberg.** 1990. Characterization of the lactose-specific enzymes of the phosphotransferase system in *Lactococcus lactis*. J. Biol. Chem. **265**:22554–22560.
13. **de Vos, W. M., and J. Gasson.** 1989. Structure and expression of the *Lactococcus lactis* gene for phospho-β-galactosidase (*lacG*) in *Escherichia coli* and *L. lactis*. J. Gen. Microbiol. **135**:1833–1846.
14. **Frazer, A. C., and R. Curtiss III.** 1975. Production, properties and utility of bacterial minicells. Curr. Top. Microbiol. Immunol. **69**:1–84.
15. **Hamada, S., and H. D. Slade.** 1980. Biology, immunology, and cariogenicity of *Streptococcus mutans*. Microbiol. Rev. **44**:331–384.
16. **Hamilton, I. R., and H. Lebtag.** 1979. Lactose metabolism by *Streptococcus mutans*: evidence for induction of the tagatose 6-phosphate pathway. J. Bacteriol. **140**:1102–1104.
17. **Hansen, J. B., Y. Abiko, and R. Curtiss III.** 1981. Characterization of the *Streptococcus mutans* plasmid pVA318 cloned into *Escherichia coli*. Infect. Immun. **31**:1034–1043.
18. **Honeyman, A. L., and R. Curtiss III.** 1992. Isolation, characterization, and nucleotide sequence of the mannitol-phosphate dehydrogenase gene and the mannitol-specific factor III gene of the phosphoenolpyruvate phosphotransferase system. Infect. Immun. **60**:3369–3375.
19. **Jagusztyn-Krynicka, E. K., M. Smorawinska, and R. Curtiss III.** 1982. Expression of *Streptococcus mutans* aspartate-semialdehyde dehydrogenase gene cloned into plasmid pBR322. J. Gen. Microbiol. **127**:1135–1145.
20. **Kurz, G., and K. Wallenfels.** 1974. D-Galactose: UV-assay with galactose dehydrogenase, p. 1279–1282. *In* H. W. Bergmeyer (ed.), Methods of enzymatic analysis, vol. 3. Verlag Chemie, New York.
21. **Laemmli, U. K., and M. Favre.** 1973. Maturation of the head of bacteriophage T4. J. Mol. Biol. **80**:575–599.
22. **Lee, J., J. B. Hansen, E. K. Jagusztyn-Krynicka, and B. M. Chassy.** 1982. Cloning and expression of the β-D-phosphogalactosidase galactohydrolase gene of *Lactobacillus casei* in *Escherichia coli* K-12. J. Bacteriol. **152**:1138–1146.
23. **Lengeler, J.** 1977. Analysis of mutations affecting the dissimilation of galactitol (dulcitol) in *Escherichia coli* K-12. Mol. Gen. Genet. **152**:83–91.
24. **Lennox, E. S.** 1944. Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1**:190–206.
25. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
26. **Maeda, S., and M. Gasson.** 1986. Cloning, expression and location of the *Streptococcus lactis* gene for phospho-β-D-galactosidase. J. Gen. Microbiol. **132**:331–340.
27. **Maxwell, E. S., K. Kurahasi, and H. M. Kalckar.** 1962. Enzymes of the Leloir pathway. Methods Enzymol. **5**:174–189.
28. **Oskouian, B., and G. C. Stewart.** 1987. Cloning and characterization of the repressor gene of the *Staphylococcus aureus* lactose operon. J. Bacteriol. **169**:5459–5465.
29. **Oskouian, B., and G. C. Stewart.** 1990. Repression and catabolite repression of the lactose operon of *Staphylococcus aureus*. J. Bacteriol. **172**:3804–3812.
30. **Rochstein, S. J., R. A. Jorgensen, K. Postle, and W. S. Reznikoff.** 1980. The inverted repeats of Tn*5* are functionally different. Cell **19**:795–805.
31. **Rosey, E. L., B. Oskouian, and G. C. Stewart.** 1991. Lactose metabolism by *Staphylococcus aureus*: characterization of *lacABCD*, the structural genes of the tagatose 6-phosphate pathway. J. Bacteriol. **173**:592–598.
32. **Rosey, E. L., and G. C. Stewart.** 1989. The nucleotide sequence of the *lacC* and *lacD* genes of *Staphylococcus aureus*. Nucleic Acids Res. **17**:3980.
33. **Rosey, E. L., and G. C. Stewart.** 1992. Nucleotide and deduced amino acid sequences of the *lacR*, *lacABCD*, and *lacFE* genes encoding the repressor, tagatose 6-phosphate gene cluster, and sugar-specific phosphotransferase system components of the lactose operon of *Streptococcus mutans*. J. Bacteriol. **174**:6159–6170.
34. **Shapiro, A. L., E. Vinuela, and J. V. Maizel.** 1967. Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. Biochem. Biophys. Res. Commun. **28**:815–820.
35. **Smorawinska, M., J. C. Hsu, J. B. Hansen, E. K. Jagusztyn-Krynicka, and R. Curtiss III.** 1983. Clustered genes for galactose metabolism from *Streptococcus mutans* cloned in *Escherichia coli*. J. Bacteriol. **153**:1095–1097.
36. **Stuber, D., and J. Bujard.** 1981. Organization of transcriptional signals in plasmids pRB322 and pACYC184. Proc. Natl. Acad. Sci. USA **78**:167–171.
37. **van Rooijen, R. J., S. Van Schalkwijk, and W. M. de Vos.** 1991. Molecular cloning, characterization and nucleotide sequence of the tagatose 6-phosphate pathway gene cluster of the lactose operon of *Lactococcus lactis*. J. Biol. Chem. **10**:7176–7181.