

# Isolation, Characterization, and Nucleotide Sequence of the *Streptococcus mutans* Mannitol-Phosphate Dehydrogenase Gene and the Mannitol-Specific Factor III Gene of the Phosphoenolpyruvate Phosphotransferase System

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Received 9 January 1992/Accepted 26 May 1992

*Streptococcus mutans*, the causative agent of dental caries, utilizes carbohydrates by means of the phosphoenolpyruvate-dependent phosphotransferase system (PTS). The PTS facilitates vectorial translocation of metabolizable carbohydrates to form the corresponding sugar-phosphates, which are subsequently converted to glycolytic intermediates. The PTS consists of both sugar-specific and sugar-independent components. Complementation of an *Escherichia coli* *mtlD* mutation with a streptococcal recombinant DNA library allowed isolation of the mannitol-1-phosphate dehydrogenase gene (*mtlD*) and the adjacent sugar-specific mannitol factor III gene (*mtlF*) from *S. mutans*. Subsequent transposon mutagenesis of the complementing DNA fragment with Tn5seq1 defined the region that encodes the *mtlD*-complementing activity, the streptococcal *mtlD* gene. Nucleotide sequence analysis of this region revealed two complete open reading frames (ORFs) from within the streptococcal mannitol PTS operon. One ORF encodes the *mtlD* gene product, a 43.0-kDa protein which exhibits similarity to the *E. coli* and *Enterococcus faecalis* mannitol-1-phosphate dehydrogenases. The second ORF encodes a 15.8-kDa protein which exhibits similarity to mannitol factor III proteins from several bacterial species. In vitro transcription-translation assays were used to produce proteins of the sizes predicted by the streptococcal ORFs. These data indicate that the *S. mutans* mannitol PTS utilizes an enzyme II-factor III complex similar to the mannitol system found in other gram-positive organisms, as opposed to that of *E. coli*, which utilizes an independent enzyme II system.

*Streptococcus mutans*, the principal etiologic agent of dental caries (14, 21), infects more than 95% of the world's population and may be the most common infectious agent in developed countries. Health care costs in the United States due to dental caries amounted to over 33 billion dollars in 1987 (37). The pathologic change responsible for this immense expenditure is demineralization of tooth enamel induced by a localized decrease in pH (21). This pH decrease is a result of carbohydrate utilization by *S. mutans*, which ferments dietary carbohydrates via the phosphoenolpyruvate-dependent phosphotransferase system (PTS) to produce large amounts of lactic acid (10, 35). This production of lactic acid results in formation of *S. mutans*-induced dental caries.

Figure 1 depicts the PTS found in *Escherichia coli* (31). Enzyme I and HPr are PTS components which are used by all PTS sugars (except fructose, which utilizes FPr). Phospho-HPr interacts with either a sugar-specific enzyme II or an enzyme II-factor III complex. This interaction results in vectorial transport and concurrent phosphorylation of the metabolizable sugar. In systems which utilize an enzyme II-factor III complex, the sum of the component molecular weights is approximately equal to the molecular weight of an independent enzyme II molecule (33). This implies, and it has been suggested, that either gene fusions or gene separations have occurred in the evolution of the PTS. This system of carbohydrate transport has been well characterized in *E. coli*, *Salmonella typhimurium*, and *Staphylococcus aureus* (31) but has not been characterized at the genetic level in *S. mutans*. For this reason, and to better understand the

molecular process by which utilization of dietary carbohydrates by *S. mutans* leads to dental caries, experiments were initiated to explore the PTS of *S. mutans*.

In this report, we describe the isolation, characterization, and nucleotide sequence of the mannitol-1-phosphate dehydrogenase and mannitol factor III genes from *S. mutans*. The putative gene products of the streptococcal genes were produced by in vitro transcription-translation assays. The proteins deduced from these loci exhibit extensive homology to the gene products specified by these loci in other organisms.

## MATERIALS AND METHODS

**Bacterial strains and media.** The bacterial strains used are shown in Table 1. *S. mutans* serotype c strain UA130 (27) was used as the source of streptococcal DNA. The *E. coli* host strains used for recombinant DNA manipulations were CC118 (22) and XL1-Blue (2). Transposon mutagenesis was done with *E. coli*  $\chi$ 6224.  $\chi$ 6224 is a CC118 derivative that contains transposon Tn5seq1 (28) inserted into the chromosome at an unknown position. A derivative of L239 (19, 36) was used for mannitol complementation assays. L239 is an *mtlD* mutant and was transduced to an *hsdR* mutant by using a P1L4 lysate (6) propagated on MOB145 *hsdR* (39) by selection for tetracycline resistance. MOB145 contains Tn10, which is genetically linked to *hsdR*. An *mtlD* *hsdR* transductant was isolated and designated  $\chi$ 6157. This strain was used as the host strain for the mannitol complementation assays.

*S. mutans* UA130 was grown in brain heart infusion broth (Difco). *E. coli* strains were grown in ML minimal medium with the appropriate nutritional supplements (5), 2YT broth

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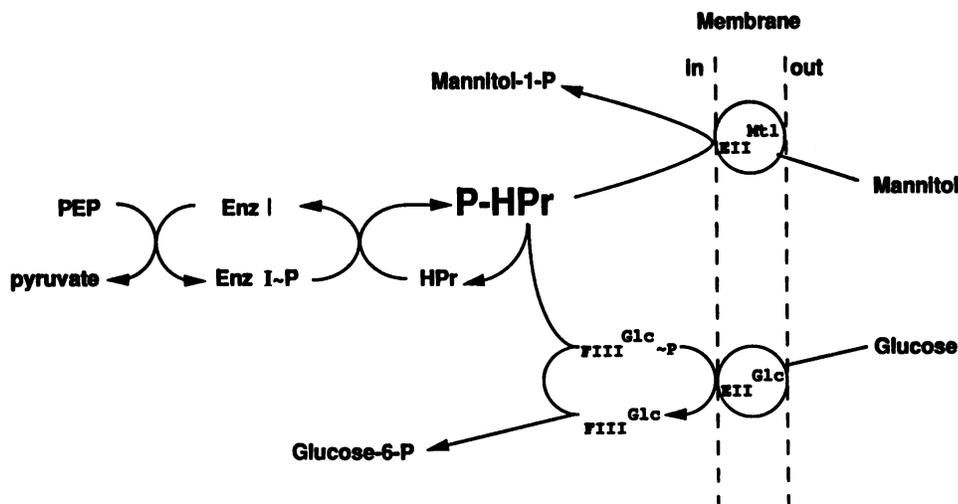


FIG. 1. The PTS found in *E. coli*. Abbreviations: PEP, phosphoenolpyruvate; Enz I, enzyme I; HPr, heat-stable phospho-carrier protein; FIII, factor III; EII, enzyme II; mtl, mannitol; glc, glucose.

(25), on tryptic soy broth or on tryptic soy agar (Difco) plates with appropriate antibiotic selection. Mannitol MacConkey indicator media were made by using MacConkey base agar (Difco) to which 1% mannitol and either ampicillin or kanamycin was added. Antibiotics were used at the following concentrations: kanamycin, 30  $\mu\text{g/ml}$ ; ampicillin, 100  $\mu\text{g/ml}$ ; neomycin, 200  $\mu\text{g/ml}$ .

**DNA isolation and manipulation.** Streptococcal DNA was isolated from UA130 in the following manner. A 5-ml starter culture was grown overnight in brain heart infusion broth in a 15-ml plastic-screw-cap tube. This was used to inoculate 500 ml of prewarmed brain heart infusion broth in a 500-ml flask. This culture was grown stationary without aeration overnight at 37°C. The cells were harvested by centrifugation and washed in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Following the wash step, the cells were resuspended in 6 ml of TE buffer and incubated for 1 h at 65°C. The cells were then cooled on ice prior to addition of 1 ml of 10 mg of lysozyme per ml and 200  $\mu\text{l}$  of a 5,000-U/ml mutanolysin solution. The cell suspension was incubated for 1 h at 37°C.

Pronase (1 ml of a 10-mg/ml stock solution) was added, and incubation was continued for 1 h. The cells were lysed by addition of 1 ml of 20% Sarkosyl. An additional 1 ml of Pronase was added prior to 1 h of incubation at 37°C. The cell lysate was brought to a volume of 17.6 ml, and 19.4 g of cesium chloride and 3.0 ml of 10-mg/ml ethidium bromide were added. The DNA solution was placed in heat-sealable tubes and centrifuged at 50,000 rpm for 20 h in a Sorvall TFT65.13 rotor. The DNA band was removed from the tube with a syringe, extracted with water-saturated butanol, and dialyzed overnight with TE buffer.

Plasmid DNA isolations for either large-scale or miniprep protocols were done as described by Sambrook et al. (34), by using the polyethylene glycol precipitation method. Quick-lysis preparations of plasmid DNA for initial screening procedures were done by a method provided by Stratagene with their mung bean nuclease protocol. All DNA manipulations and enzyme procedures were done in accordance with manufacturer instructions and NIH recombinant DNA guidelines.

**Transposon mutagenesis.** Plasmids to be subjected to transposon mutagenesis were used to transform *E. coli*  $\chi$ 6224. Transformants were screened by a quick-lysis procedure to determine that Tn5seq1 had not transposed to the target plasmid. A transformant containing a plasmid of the correct size was chosen and grown with high aeration without kanamycin selection until a cell density of approximately  $10^8/\text{ml}$  was reached. The cells were concentrated 10-fold, and 200 ml of the cell suspension was spread onto tryptic soy agar plates containing neomycin. After overnight incubation at 37°C, the lawn of bacterial cells was harvested and the plasmid DNA was isolated. This DNA preparation was used to transform *E. coli*, either CC118 or XL1-Blue, with selection for kanamycin resistance. Plasmid DNA was obtained from independent transformants and screened by restriction endonuclease mapping to determine the site of the transposon insertion.

**Mannitol utilization assay.** To localize the streptococcal *mtlD* gene, complementation of the *E. coli mtlD* allele of strain  $\chi$ 6157 was assayed by transferring colonies containing various plasmids with Tn5seq1 inserts within the *PstI* fragment from tryptic soy agar plates to mannitol MacConkey

TABLE 1. Bacterial strains

Strain	Genotype	Reference(s) or source
<i>S. mutans</i> UA130	<i>mtl</i> <sup>+</sup>	27
<i>E. coli</i> CC118	<i>araD139</i> $\Delta$ ( <i>ara-leu</i> )7697 <i><math>\Delta</math>lacX74</i> <i>phoA</i> $\Delta$ 20 <i>galE</i> <i>galK</i> <i>recA1</i> <i>rpsE</i> <i>rpoB</i> <i>argE</i> (Am) <i>thi</i>	22
L239	<i>leuB6</i> <i>lacZ4</i> <i>supE44</i> <i>rfbD1</i> <i>rpsL8</i> <i>mtlC12</i> <i>mtlD13</i> <i>thi-1</i>	19, 36
MOB145	<i>gal-55</i> <i>relA1</i> <i>spoT1</i> <i>thi-1</i> <i>hsdR4</i> <i>zjj-202::Tn10</i>	39
XL1-Blue	F'( <i>proAB</i> <i>lacI</i> <sup>q</sup> <i>ZAM15</i> Tn10) <i>supE44</i> <i>lac</i> <i>gyrA96</i> <i>recA1</i> <i>relA1</i> <i>endA1</i> <i>thi-1</i> <i>hsdR17</i>	2
$\chi$ 6157	Derivative of L239 which carries <i>hsdR4</i>	This study
$\chi$ 6224	Derivative of CC118 which contains Tn5seq1	This study

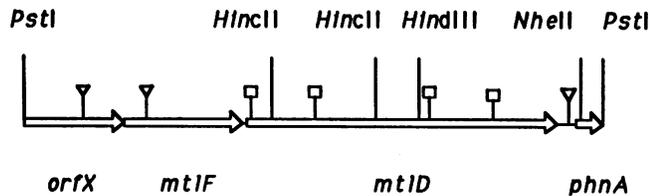


FIG. 2. Illustration of the 2.3-kb *Pst*I fragment which contains *mtlD*-complementing activity. Open arrows indicate ORFs. Open squares indicate transposon insertions which eliminate *mtlD*-complementing activity. Open triangles indicate transposon insertions which do not effect *mtlD*-complementing activity.

indicator plates. Colonies containing plasmids which had retained MtlD activity formed red colonies on the indicator medium and did not have transposon inserts within the streptococcal *mtlD* allele. Colonies whose plasmids had lost *mtlD*-complementing activity did not grow on the mannitol MacConkey medium and contained inserts within the streptococcal *mtlD* gene.

**DNA sequence analysis.** Sequenase-modified T7 DNA polymerase (38) from USB was used in the double-stranded sequencing technique of Chen and Seeburg (4). Nucleotide sequence analysis was done by using the computer programs Genepro (Riverside Scientific), Seqnce (Access Biosystems, Inc.), GCG (University of Wisconsin) (8), and TFASTA (30).

**Transcription-translation assays.** Transcription-translation assays were done on selected plasmid constructs by using the transcription-translation reaction kit obtained from New England Nuclear. Aliquots of the labeled proteins were analyzed by using sodium dodecyl sulfate-denaturing polyacrylamide gel electrophoresis (17) on a 10% polyacrylamide gel. The dried gel was subjected to autoradiography.

**Nucleotide sequence accession number.** The nucleotide sequence reported here has been submitted to GenBank and given accession number M94225.

## RESULTS

**Isolation of the *S. mutans* mannitol PTS genes.** In *mtlD* mutants, growth in the presence of mannitol results in accumulation within the cell of mannitol-1-phosphate, which is growth inhibitory (36). This provided a strong selective mechanism to isolate the streptococcal genes involved with mannitol utilization. DNA was isolated from *S. mutans* UA130, digested with various restriction endonucleases, and ligated to vector pGEM4Z (Promega). The ligation mixture was used to transform an *mtlD* mutant strain of *E. coli*,  $\chi$ 6157. Selection for the streptococcal *mtlD* gene was obtained by growth in either minimal medium containing mannitol as the sole energy source or 2YT containing 1% mannitol. Plasmids containing a 2.3-kb *Pst*I restriction fragment were isolated from both medium cultures. This streptococcal DNA insert allowed the *E. coli* *mtlD* mutant to grow in the presence of mannitol and to use mannitol as the sole energy source. Figure 2 depicts this *mtlD*-complementing streptococcal DNA fragment. Following the initial isolation of the *Pst*I fragment in pGEM4Z, it was cloned into vectors pUC18 and pUC19 (40) for all subsequent manipulations.

**Transposon mutagenesis.** To localize the region of the fragment responsible for the *mtlD*-complementing activity, the 2.3-kb *Pst*I fragment was subjected to transposon mutagenesis with Tn5seq1 (28). Plasmids containing inserts within the cloned *Pst*I fragment were used to transform *E.*

*coli*  $\chi$ 6157. Transformants were tested for mannitol utilization on mannitol MacConkey indicator plates. The positions of selected inserts are shown in Fig. 2. The open squares above the center line indicate transposon insertions which eliminated complementing activity in *E. coli*. The open triangles indicate transposon insertions which did not affect the complementing activity. This procedure localized the putative streptococcal *mtlD* gene to a 1.6-kb region within the *Pst*I fragment.

**Determination of the nucleotide sequence.** Selected Tn5seq1 insertions were used for primer sites as described by Nag et al. (28). Tn5seq1 contains the T7 and SP6 promoter regions at opposite ends of the transposon so that a single transposon insertion can serve as a primer site in both directions from the insert. In addition, various subclones of the *Pst*I fragment in vectors pUC18 and pUC19 (40) were also used for nucleotide sequence determination. The complete nucleotide sequence of the 2.3-kb *Pst*I fragment was determined on both DNA strands and is shown in Fig. 3. The nucleotide sequence analysis revealed the presence of two complete and two partial open reading frames (ORFs). The first partial ORF extends from nucleotides 3 to 383 and would encode the carboxyl-terminal 126 amino acids of an unknown protein and contribute 14.8 kDa toward the total molecular size of that protein. This ORF is immediately followed by the second ORF, which spans nucleotides 385 to 822. This would encode a protein of 145 amino acids with a molecular size of 15.8 kDa. The deduced protein that would be produced by this ORF exhibits homology to various mannitol factor III molecules and has been named the *mtlF* gene. This nomenclature is in agreement with that of other factor III genes (12). The third ORF extends from nucleotides 838 to 1986. A 43.0-kDa protein consisting of 382 amino acids would be produced from this ORF. All of the transposon insertions which eliminate *mtlD*-complementing activity are located within this ORF, defining it as the streptococcal *mtlD* gene. In addition, the deduced protein product is homologous to the mannitol-1-phosphate dehydrogenases from *E. coli* (7, 16) and *E. faecalis* (12), verifying that this is the *mtlD* gene from *S. mutans*.

The fourth ORF extends from nucleotide 2161 to the end of the *Pst*I fragment (nucleotide 2260). This would produce the amino-terminal 33 amino acids of the putative streptococcal PhnA protein (see Discussion).

**Transcription-translation assays.** To confirm the presence of the streptococcal genes, the 2.3-kb *Pst*I fragment cloned into pGEM4Z was subjected to in vitro transcription-translation assays. The results are shown in Fig. 4. The expected 43.0- and 15.8-kDa proteins from the streptococcal *mtlD* and *mtlF* genes were produced. The other two streptococcal ORFs in the construct tested are fused to the alpha peptide of the  $\beta$ -galactosidase gene of the vector out of the correct translational frame so that those protein products are not seen. The vector alone produces a band which has a slightly lower molecular weight than the streptococcal MtlF protein. This is the alpha peptide from pGEM4Z.

## DISCUSSION

**Isolation and transposon mutagenesis of the streptococcal *mtlD* gene.** Isolation of the streptococcal *mtlD* gene was accomplished by complementing an *E. coli* *mtlD* mutation. Mannitol is translocated by the PTS to form mannitol-1-phosphate, which is oxidized to form fructose-6-phosphate by the *mtlD* gene product, mannitol-1-phosphate dehydrogenase (1, 23, 31). Fructose-6-phosphate is then metabolized

12 24 36 48 60  
 CTGCAGTTTCAGATAAACTTATCTGTCAGTAAGTATTAAACGTTTGTCTGCATGTC  
 A V S K D K T Y L S S K L L K R F A V S

72 84 96 108 120  
 CCTTGGCTATTCCAGAGACTCGTTGGCTCTTTGTCATACACAGTCAAGTAAAGTTATAA  
 P L A I P E T R L A L L H T Q S S K V I

132 144 156 168 180  
 CGTCTTGTTTTAAAATTTATGATTTAAAAAGACCTGTAAACAGCTCTGCCATGAATTACG  
 T S C F K I Y D L K R P V T A L S M N Y

192 204 216 228 240  
 AAAAAGACAGTTTAAAGAAATTTGGTTATGTCGACCGGTTAGATGAAAACAAAAGAA  
 E K E T V T R I L V M L T R L D E T K E

252 264 276 288 300  
 TGAGAGATTAAAGACCGCATCAGTCAATCTATCATCGAAAATCATCCTTATACAGAAA  
 M R D L M T A I S Q S I I E N H P Y T E

312 324 336 348 360  
 TTTCAAGACAGGTAATAAAGATAATTTATCAATTTGAATCAATATTTACAGAAA  
 I Y K T G N K D I I Y Q L L N Q I F T E

372 384 396 408 420  
 AAATAAGAAATTTGGAGACCTAAGATGGAATTTCAAAGATTTAATTAATTAAGAACCAA  
 K I K K L E T #

432 444 456 468 480  
 CATTTTCCCGATAAGGAAGCTGCTATTGTTTTGGGACAGCTCTTAGCTGATGGTGGT  
 H F P D K E A A I R F C G Q L L A D G G

492 504 516 528 540  
 TACGTAGAACCAGCTTATTTGACGCCATGATTCAGCGGATAAAGAATCTCTGTTTAT  
 Y V E P A Y I D A M I Q R D K E L S V Y

552 564 576 588 600  
 ATGGGAAATTCATGCGCATCTCCTCGGAAGCGGACCGCTAAAGGATGTTCTCAA  
 H G N F I A I P H G T D D A K K D V L K

612 624 636 648 660  
 TCAGGTATCAGTGTGTTGAGTCCAGACCGTGTAAATTTGGAACAGAAAGCGATCCG  
 S G I T V V Q V P D G V N F G T E D D P

672 684 696 708 720  
 CAAGTGGCAGCAGTTTATTTGGCATCGCTGGAATTTGGTATGAACATTTGCAAAATCATT  
 Q V A T V L F P G I A G I G D E H L Q I I

732 744 756 768 780  
 CAAATATTTGATTTTGGTGGCAGATGTTGATAATGTTGTTAAGTTAGCTGATGCTCAA  
 Q N I S I F C A D V D N V V K L A D A Q

792 804 816 828 840  
 ACAGAGATGAAAGTGTCAAACCTTTAAGTCAAGTCAATTAAGGAGTTTTTAAATCATG  
 T E D E V V K L L S Q V N #

852 864 876 888 900  
 AAAAAAGCAGTACATTTGGAGCAGCAATATGGCCGCTTTATTTGGACAAATTTTA  
 K K A V H F G A G N I G R G F I G Q I L

912 924 936 948 960  
 TTTGAAAATGGCTTTGCTATTGATTTTGTGATGTTAACGATAAAATTAATGCTCTTT  
 F E N G F A I D F V D V N D K I I N A L

972 984 996 1008 1020  
 AATGAACGTCATCTTATGATTTGAAATTCGAAAGATGGGAACGCTCAGATTACAGATT  
 N E R H S Y D I E I A E D G K R H I T V

1032 1044 1056 1068 1080  
 TCTAATGTTGCTGGCATTAAACAAGGAAAATCTCAAGCAGTCTGATGCTGTGGCT  
 S N V A G I N N K E N P Q A V I D A V A

1092 1104 1116 1128 1140  
 GAGACAGATTGATTACAACCGCTATTTGGCCCTAACATTTTGCCTTTTATTTGCCCAATG  
 E T E L I T T A I G P N I L P F I A Q L

1152 1164 1176 1188 1200  
 ATTGCCAAAGTATTGAAAACGTCGTGAAAGTCAGAATCAAAGCCACTTGATATCAAT  
 I A K G I E K R R E S Q N Q T P L D I I

1212 1224 1236 1248 1260  
 GCCTGTGAAATATGATTTGGTCTGCTCTGCTGCGGAGGTCAGAAATACCTC  
 A C E N M I G G S A F L W Q E V Q K Y L

1272 1284 1296 1308 1320  
 AGCGCTGATGCTTTGCTAAAGATTATTTGGCTTTCCAAATGCTGCTGTTGAC  
 S A D G L A F A K D Y I G F P N A V D

1332 1344 1356 1368 1380  
 CGTATTGTTCCAGCTCAGGTTTCATAAAGATCCCCCTTTGTTGGTGGAGCCATTTTCA  
 R I V P A Q V H K D P L F V V E P F S

1392 1404 1416 1428 1440  
 GAATGGTAGTCGAAACCTGCTCTATGAAAATCCTGATTTAAAATTTGCTCAGTTCAC  
 E W V V E T A A M K N P D L K L S S V H

1452 1464 1476 1488 1500  
 TATGAAGAAAATTTGGAACCATTCATTGAAAAGAAAGCTTTTCTCTGTTAATTCAGGTCAT  
 Y E E N L E P F I E R K L F S V N S G H

1512 1524 1536 1548 1560  
 GCAACAACAGCTTATACAGTGTCTATTTTGGTAAAGACCGTCTAGAACGCAATGATAA  
 A T T A Y T G A Y F G A K T V L E A L K

1572 1584 1596 1608 1620  
 GACCAACAAGTTAAAGAGCAAGTAAAGGCAATTTAGGTGAAATTCGCCAGCTTTTAAAG  
 D Q Q V K E Q V K A V L G E I R Q L L M

1632 1644 1656 1668 1680  
 GCTAAATGGCAATTCAAAGAGATGATCTGAAAGTTTATCATGATATTAATTCAGTCGT  
 A K W Q F K E N D L K V Y H D I I S R

1692 1704 1716 1728 1740  
 TTTGAAAATCTTATATTTGATGATGATTTACTCGTGTGCCAGAACCAATTCGTAAAG  
 F E N P Y I V D D V T R V A R T P I R K

1752 1764 1776 1788 1800  
 TTAGGTATGATGAAAGCTTTATCCGTCCTTGGTGGAGCTGAAAGCTGTTGTTATCT  
 L G Y D E R F I R P I R E L K D R G L S

1812 1824 1836 1848 1860  
 TAAGATATCTTTTCAAAGCAGTGGCTATGTTTCCATTCAGGATTCAAATGATGAA  
 Y E Y L L Q T V A Y V F H Y K D S N D E

1872 1884 1896 1908 1920  
 CAAAGTGTCAACTTAAATGCTTCTACAAGAAAAGTCTTTAAAAGCAGTTGTTAAAGAA  
 Q S V Q L K M L L Q E K S L K A V V K E

1932 1944 1956 1968 1980  
 GTAAACAGGATGACAGATGCGCTTTGATGAAAGAAATTTGTTACCGATGTTGATGCTTTA  
 V T G L T D A A L I E E I V T S V E S L

1992 2004 2016 2028 2040  
 GATTAAGGAAAATCTTATAATTAAGAAGACTAGCTCTGTTCTGCGTTTTCTATAAGTT  
 D #

2052 2064 2076 2088 2100  
 AGACAGTACTTAACTCATAGACTCAGTACAGCAGCTAGCTTTTGTGTGATAAGAC

2112 2124 2136 2148 2160  
 TTGCCAATGATAAAAATCAGGTAACCTGTTAATGGTAATCAAATAAAAAGGATTTTAT

2172 2184 2196 2208 2220  
 ATGCTTTTACCTAATTTGCTCAAAATGTCAGTCAAGATGATGCTGATGAAAGTGTATTCTA  
 H S L P N C P K C Q S E Y V Y E D G I L

2232 2244 2256  
 TTGGTTGTCAGAAATGCTTATGAAATGGAATCCGTCAG  
 L V C P E C A Y E W N P A

FIG. 3. Nucleotide sequence of the 2.3-kb *Pst*I fragment containing the *S. mutans* *orfX*, *mtlF*, *mtlD*, and *phnA* genes. The deduced translation products for the four ORFs are shown below the nucleotide sequence. The symbol # indicates termination codons. The putative ribosome-binding sites are underlined.

by the glycolytic pathway. Strong selective pressure for isolation of the streptococcal gene was possible because of accumulation of toxic mannitol-1-phosphate, which is formed when an *mtlD* mutant is placed in the presence of

mannitol (36). This selective pressure is evident by the isolation of plasmids containing the same 2.3-kb *Pst*I fragment from *S. mutans* in two separate cloning experiments using this selection procedure. Cloning of the streptococcal

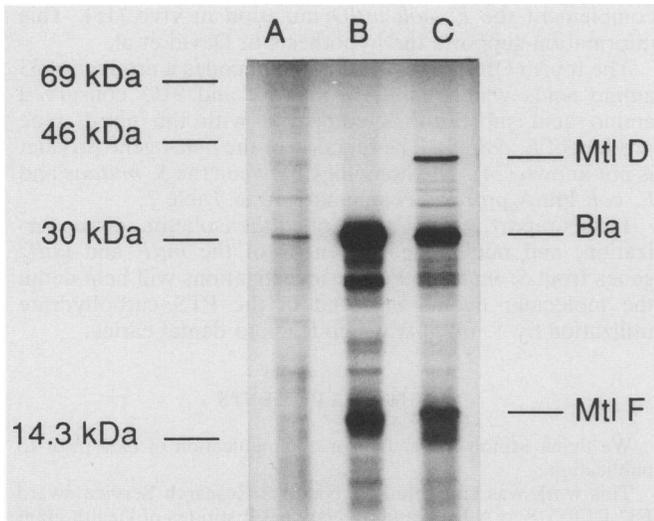


FIG. 4. Autoradiograph of the in vitro transcription-translation assays. Lanes: A, negative control containing no DNA; B, assay for vector pGEM4Z; C, assay for pGEM4Z with the 2.3-kb *PstI* insert.

mannitol-1-phosphate dehydrogenase gene allowed conversion of mannitol-1-phosphate to fructose-6-phosphate, growth of *E. coli mtlD* mutant  $\chi 6157$  in the presence of mannitol, and use of mannitol as a carbon source in minimal medium.

Transposon mutagenesis of the cloned fragment and the subsequent mannitol complementation assays allowed localization of the streptococcal *mtlD* gene. It should be noted that we isolated transposon insertions on either side of the streptococcal *mtlD* gene which did not effect the *mtlD*-complementing activity in *E. coli*. Transposon insertions 5' to the *mtlD* gene did not have a polar effect, as one might expect. This might suggest that the *mtlD* gene promoter was located on the *PstI* fragment. We do not believe that this was the case (see next section) but believe that no polar effects were seen owing to the low GC content of the streptococcal DNA (35.6%), which resulted in nonspecific transcription initiation within the cloned fragment.

**Nucleotide sequence analysis.** Analysis of the nucleotide sequence for putative transcription control elements similar to those found in *E. coli*, *Bacillus subtilis*, or *Lactococcus* species (9, 20, 26) did not reveal any apparent promoterlike elements preceding the *mtlF* and *mtlD* genes. The mannitol PTS operon from *E. faecalis* (12), which shows significant homology to the deduced products from the second and third ORFs, contains genes in the order *mtlA orfX mtlF mtlD*. If the gene order in *S. mutans* is similar, at least two genes would precede the *mtlF* gene and no promoter structure would be located in this region. No promoter structure was evident in this region, and we believe that the gene order is identical to that of *E. faecalis*. Additional evidence for the lack of a promoter within this region was provided by the integration of a nonreplicate vector into the streptococcal chromosome at this site to form a partial merodiploid (15). This event resulted in an *Mtl*<sup>-</sup> phenotype and is consistent with an apparent operon structure similar to that found in *E. faecalis*.

Putative ribosome-binding sites are located immediately 5' to the second, third, and fourth ORFs. The nucleotide sequences of these sites are GGAGA, AAGGAG, and AAG

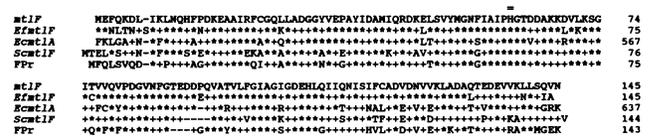


FIG. 5. Homology alignment of the MtlF-like proteins. Abbreviations: *mtlF*, *S. mutans mtlF*; *Efm1F*, *E. faecalis mtlF*; *Ecm1A*, *E. coli mtlA*; *Scmt1F*, *S. carnosus mtlF*; *FPr*, *S. typhimurium FPr*. Symbols: \*, identical residues; +, conserved substitutions; -, gaps in the sequences. Letters indicate replacement residues. The equal sign above His-61 indicates the potential site of phosphorylation in the *S. mutans* MtlF protein. Each number at the right indicates the protein residue number of the last given amino acid for each protein.

GAG, respectively, and are similar to those found in *E. coli*, *B. subtilis*, and *Lactococcus* species (9, 20, 26). These sequences are underlined in Fig. 3. Lack of the nucleotide sequence of the 16S rRNA from *S. mutans* which interacts with the ribosome-binding site inhibits analysis of the binding energies of these putative ribosome-binding sites.

There is an intergenic space of 174 nucleotides between the third and fourth ORFs. Analysis of the nucleotide sequence did not reveal the presence of any transcriptional control structure. It is plausible that a transcription terminator and a promoter element do exist in this region but are not apparent owing to the paucity of information about transcription control in *S. mutans*.

To determine the potential function of the four ORFs on the basis of protein homology, the deduced proteins were used as the target sequence for the TFasta protein homology program (30) by using the GenBank data base. The first ORF spans 381 nucleotides and would encode 126 amino acids of the carboxyl-terminal end of a protein. This deduced protein did not exhibit significant homology to any GenBank entry, including the OrfX protein from the *E. faecalis* mannitol operon. On the basis of a similar gene organization and the homology between the *mtlF* and *mtlD* proteins of *S. mutans* and *E. faecalis*, this first ORF should predictably encode a protein analogous to the OrfX protein of *E. faecalis*. However, no significant homology exists between these proteins. Fischer et al. have postulated that OrfX is a regulatory protein (12). On the basis of the high degree of conservation between the PTS genes of various bacterial species, including the *mtlF* and *mtlD* genes, we find it interesting that no significant homology exists between the two OrfX proteins. The function of the *S. mutans* OrfX protein is unknown.

The second ORF spans 438 bp to encode a protein of 145 amino acids with a molecular size of 15.8 kDa. This deduced protein is highly homologous to the mannitol-specific factor III components from *E. faecalis* (12) and *Staphylococcus carnosus* (11). In addition, homology was also demonstrated with the cytoplasmic domain of the *E. coli* MtlA protein (18) and the *S. typhimurium* FPr protein (13). The MtlA protein is the independent enzyme II component of the mannitol PTS from *E. coli* which does not have a factor III component but has a factor III-like domain located within the enzyme II molecule. FPr is an HPr-like analog which is utilized in fructose transport and appears to be the result of a gene duplication and subsequent fusion between the *mtlA* and HPr genes of *S. typhimurium*. These homologies are shown in Fig. 5 and summarized in Table 2. These data and the apparent size of the deduced protein product (verified by in vitro transcription-translation assays) imply that *S. mutans* utilizes an enzyme II-factor III complex in mannitol utiliza-

TABLE 2. Homology index for the proteins encoded by the *S. mutans* mannitol operon<sup>a</sup>

Proteins compared	No. of residues	
	Conserved	Exact
Factor III vs <i>E. faecalis</i> factor III	88	61
Factor III vs <i>E. coli</i> MtlA	77	41
Factor III vs <i>S. carnosus</i> factor III	79	40
Factor III vs FPr	71	37
MtlD vs <i>E. faecalis</i> MtlD	86	54
MtlD vs <i>E. coli</i> MtlD	85	39
PhnA vs <i>E. coli</i> PhnA	91	73

<sup>a</sup> Exact, identical residues; conserved, conserved amino acid substitutions as determined by the TFASTA program (30).

tion like the systems described in *E. faecalis* and *S. carnosus* rather than an independent enzyme II system like that described in *E. coli*. Thus, we have named the second ORF the streptococcal *mtlF* gene, which is consistent with the genetic nomenclature of other PTS systems.

Of particular interest is the high degree of conservation between the proteins around histidine residue 61 of the *S. mutans* factor III protein. The analogous residue in the *E. coli* MtlA protein and the *S. carnosus* factor III protein has been shown to be the site of phosphorylation during the enzymatic reaction with phospho-HPr (29, 32). This implicates His-61 as the site of phosphorylation in the streptococcal MtlF protein.

The third ORF spans 1,149 bp and encodes a protein of 382 amino acids with a molecular size of 43.0 kDa. This ORF contained all of the transposon insertions which eliminate *mtlD*-complementing activity in *E. coli*. This suggests that this ORF encodes the streptococcal *mtlD* gene product. Analysis of this deduced protein revealed 85 and 86% conserved amino acids compared with the *E. coli* and *E. faecalis* MtlD proteins, respectively. These homologies are shown in Fig. 6 and summarized in Table 2. These data also indicate that the third ORF encodes the streptococcal *mtlD* gene product. Thus, we have named this ORF the *mtlD* gene.

The homology among the three dehydrogenases is highly conserved in the 18 amino-terminal residues of these proteins. Davis et al. (7) have suggested that this region of the molecule is involved with binding of the NAD<sup>+</sup> cofactor required for conversion of mannitol-1-phosphate to fructose-6-phosphate. Removal of the 11 amino-terminal residues from the *S. mutans* *mtlD* gene product and their replacement with the 11 amino-terminal residues of the *E. coli* β-galactosidase gene results in a fusion protein which does not

complement the *E. coli* *mtlD* mutation in vivo (15). This information supports the hypothesis of David et al.

The fourth ORF spans 100 bp and encodes a product of 33 amino acids which has 73% identity and 91% conserved amino acid substitutions compared with the *phnA* gene product of *E. coli* (3). The function of the *phnA* gene product is not known (24). The homology between the *S. mutans* and *E. coli* PhnA proteins is summarized in Table 2.

In this report, we have described the isolation, characterization, and nucleotide sequences of the *mtlF* and *mtlD* genes from *S. mutans*. Future investigations will help detail the molecular events involved in the PTS carbohydrate utilization by *S. mutans* which leads to dental caries.

ACKNOWLEDGMENTS

We thank Milton Saier, Jr., for communication of data prior to publication.

This work was supported by National Research Service award F32-DE05518 to A.L.H. and by National Institutes of Health grant DE06673.

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FIG. 6. Homology alignment of the mannitol-1-phosphate dehydrogenases from *S. mutans*, *E. faecalis*, and *E. coli*. The symbols used are defined in the legend to Fig. 5.

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