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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*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 complementary 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.

*Mutans* is 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* y6224, y6224 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 TnJ0, which is genetically linked to hsdR. An mtlD hsdR transductant was isolated and designated y6157. 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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(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 μg/ml; ampicillin, 100 μg/ml; neomycin, 200 μg/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 μl of a 5,000-U/ml mutanolysin solution. The cell suspension was incubated for 1 h at 37°C.

**TABLE 1. Bacterial strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference(s) or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. mutans</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UA130</td>
<td>mtl*</td>
<td>27</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC118</td>
<td>araD139 Δ(ara-leu)7697 lacX74 phoAΔ20 galE galK recA1 rpsE rpoB argElAm(α) thi</td>
<td>22</td>
</tr>
<tr>
<td>L239</td>
<td>leuB6 lacZ4 supE44 rfbD1 rpsL8 mitC12 mtlD13 thi-1</td>
<td>19, 36</td>
</tr>
<tr>
<td>MOB145</td>
<td>gal-35 relA1 spoT1 thi-1 hsdR4 zij-202 : Tn10</td>
<td>39</td>
</tr>
<tr>
<td>XL1-Blue</td>
<td>F(−proAB lacP2ZΔM15 Tn10) supE44 lac gyrA96 recA1 relA1 endA1 thi-1 hsdR17</td>
<td>2</td>
</tr>
<tr>
<td>x6157</td>
<td>Derivative of L239 which carries hsdR4</td>
<td>This study</td>
</tr>
<tr>
<td>x6224</td>
<td>Derivative of CC118 which contains Tn5seq1</td>
<td>This study</td>
</tr>
</tbody>
</table>

Proteinase (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-lyss 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* x6224. 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 10⁹/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 x6157 was assayed by transferring colonies containing various plasmids with Tn5seq1 inserts within the *PstI* fragment from tryptic soy agar plates to mannitol MacConkey media. Positive transformants were then selected by using the mannitol indicator plate and confirmation of transposition was achieved by the loss of mannitol utilization after selection.
**FIG. 2.** Illustration of the 2.3-kb *PstI* 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 affect *mtlD*-complementing activity.

Indicator plates. Colonies containing plasmids which had retained MtlD activity formed red colonies on this 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), Seqence (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*, x6157. 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 *PstI* 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 *PstI* 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 *PstI* fragment was subjected to transposon mutagenesis with Tn5seq1 (28). Plasmids containing inserts within the cloned *PstI* fragment were used to transform *E. coli* x6157. 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 *PstI* 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 *PstI* fragment in vectors pUC18 and pUC19 (40) were also used for nucleotide sequence determination. The complete nucleotide sequence of the 2.3-kb *PstI* 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 *PstI* 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 *PstI* 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 β-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...
FIG. 3. Nucleotide sequence of the 2.3-kb PstI fragment containing the *S. mutans* orfK, mtf, 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* gene is placed in the presence of mannitol (36). This selective pressure is evident by the isolation of plasmids containing the same 2.3-kb *PstI* fragment from *S. mutans* in two separate cloning experiments using this selection procedure. Cloning of the streptococcal
mannitol-1-phosphate dehydrogenase gene allowed conversion of mannitol-1-phosphate to fructose-6-phosphate, growth of \textit{E. coli} mtlD mutant $\lambda 6157$ 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 manniol complementation assays allowed localization of the streptococcal \textit{mtlD} gene. It should be noted that we isolated transposon insertions on either side of the streptococcal \textit{mtlD} gene which did not effect the \textit{mtlD}-complementing activity in \textit{E. coli}. Transposon insertions 5' to the \textit{mtlD} gene did not have a polar effect, as one might expect. This might suggest that the \textit{mtlD} gene promoter was located on the \textit{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.

\textbf{Nucleotide sequence analysis.} Analysis of the nucleotide sequence for putative transcription control elements similar to those found in \textit{E. coli}, \textit{Bacillus subtilis}, or \textit{Lactococcus} species (9, 20, 26) did not reveal any apparent promoterlike elements preceding the mtlF and mtlD genes. The manniol PTS operon from \textit{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 \textit{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 \textit{E. faecalis}. Additional evidence for the lack of a promoter within this region was provided by the integration of a nonrepicate vector into the streptococcal chromosome at this site to form a partial merodiploid (15). This event resulted in an Mtl$^-$ phenotype and is consistent with an apparent operon structure similar to that found in \textit{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. 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 \textit{PstI} insert.

GAG, respectively, and are similar to those found in \textit{E. coli}, \textit{B. subtilis}, and \textit{Lactococcus} species (9, 20, 26). These sequences are underlined in Fig. 3. Lack of the nucleotide sequence of the 16S rRNA from \textit{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 \textit{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 \textit{E. faecalis} manniol operon. On the basis of a similar gene organization and the homology between the mtlF and mtlD proteins of \textit{S. mutans} and \textit{E. faecalis}, this first ORF should predictably encode a protein analogous to the OrfX protein of \textit{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 on 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 \textit{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 manniol-specific factor III components from \textit{E. faecalis} (12) and \textit{Staphylococcus carnosus} (11). In addition, homology was also demonstrated with the cytoplasmic domain of the \textit{E. coli} Mla protein (18) and the \textit{S. typhimurium} FPr protein (13). The Mla protein is the independent enzyme II component of the manniol PTS from \textit{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 \textit{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 in vitro transcription-translation assays) imply that \textit{S. mutans} utilizes an enzyme II-factor III complex in manniol utiliza-
TABLE 2. Homology index for the proteins encoded by the *S. mutans* mannotol operon

<table>
<thead>
<tr>
<th>Proteins compared</th>
<th>No. of residues</th>
<th>Conserved</th>
<th>Exact</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor III vs <em>E. faecalis</em> factor III</td>
<td>88</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>Factor III vs <em>E. coli</em> MtlA</td>
<td>77</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>Factor III vs <em>S. carnosus</em> factor III</td>
<td>79</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Factor III vs FPr</td>
<td>71</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>MtlD vs <em>E. faecalis</em> MtlD</td>
<td>86</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>MtlD vs <em>E. coli</em> MtlD</td>
<td>85</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>PhNA vs <em>E. coli</em> PhNA</td>
<td>91</td>
<td>73</td>
<td></td>
</tr>
</tbody>
</table>

* Exact, identical residues; conserved, conserved amino acid substitutions as determined by the TFASTA program (30).

The fourth ORF spans 100 bp and encodes a protein 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**

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**REFERENCES**