The mannitol-specific enzyme II (mtlA) gene and the mtlR gene of the PTS of Streptococcus mutans

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The phosphoenolpyruvate-dependent phosphotransferase system (PTS) is widely found among Gram-positive bacteria. It is the major source of carbohydrate transport in the dental pathogen Streptococcus mutans. The transported carbohydrates are fermented to produce large amounts of lactic acid which initiates dental caries. The authors have isolated the S. mutans gene for the mannitol-specific Enzyme II (EII) component of the PTS, mtlA, and the adjacent mtlR gene, which is located in the same operon. The mtlR gene is located between mtlA and the genes mtlF and mtlD. The nucleotide sequence of the mtlA and mtlR loci has been determined. The deduced mtlA gene product of S. mutans consists of 589 amino acids with a molecular mass of 62.0 kDa. It exhibits similarity with the mtlA gene products from other organisms. However, the similarity between these proteins is generally restricted to the 470 amino-terminal residues of the S. mutans protein. This region would correspond to the EIICB domains of the PTS. The authors have previously shown that the S. mutans mtlF gene product exhibits 76.6% similarity to the carboxyl-terminal 143 amino acids of the Escherichia coli mtlA product and that the mtlF gene encodes the EIIA domain of the PTS. Thus, the genes that encode the EIICB and the EIIA domains are separated by approximately 2250 bp. In many organisms, all of the EI domains may be fused together to form one molecule. The fact that these domains are separated by this distance in S. mutans supports the hypothesis that various functional domains of the PTS have been rearranged during evolution. The sequence of the 119 carboxyl-terminal amino acids of the S. mutans mtlA gene product also displays homology to the carboxyl-terminal end of the EIIB domain of various mannitol PTSs. Thus, this domain may have been duplicated in S. mutans during evolution of the operon. The mtlR gene is located in the same operon structure as mtlA but these loci are separated by an intragenic space. The precise 5′ end of the mtlR locus cannot be determined either by in vitro transcription–translation assays or based upon nucleotide sequence analysis because of the apparent lack of a ribosome-binding site preceding the gene. The deduced mtlR gene product, which consists of approximately 650 amino acids with a molecular mass of 75.3 kDa, exhibits limited similarity to several potential transcriptional regulators. However, the exact function of this locus is currently unknown.

Keywords: Streptococcus mutans, phosphoenolpyruvate-dependent phosphotransferase system, mannitol

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Abbreviations: EII, Enzyme II; PTS, phosphoenolpyruvate-dependent phosphotransferase system.

The GenBank accession number for the sequence reported in this paper is AF210133.

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INTRODUCTION

The phosphoenolpyruvate-dependent phosphotransferase system (PTS) is the main mechanism by which Streptococcus mutans transports carbohydrates (Schachtele & Mayo, 1973). This system has been described in great detail in Escherichia coli (Postma & Lengeler, 1985; Saier & Reizer, 1992) but a limited description at the molecular level has been done in S. mutans (Boyd et al., 1994; Hiratsuka et al., 1998; Sato et al., 1991). S. mutans uses this type of vectorial transport to utilize various dietary carbohydrates (Schachtele & Mayo, 1973) that are subsequently converted by the glycolytic pathway to lactic acid, which initiates the formation of dental caries (Drucker & Melville, 1968).

The PTS consists of two non-specific components, the Enzyme I and the HPr proteins, which are involved in the initial steps of carbohydrate transport by the PTS (Saier & Reizer, 1992). After the formation of phospho-HPr, sugar-specific components of the PTS, the Enzyme IIs (EIIs), vectorially transport and phosphorylate the metabolizable carbohydrate. The EII components of the PTS consist of three highly conserved functional domains, EIIA, EIIB and EIIC (Saier & Reizer, 1992). These domains may exist on one single molecule or as separate domains on different molecules but all act together to transport the carbohydrate of interest. The mannitol PTS in E. coli utilizes a single sugar-specific Enzyme IICBA molecule. In other organisms, mannitol transport may utilize an EICB-EIIA pair as depicted for mannitol PTSs found in other Gram-positive organisms (Fischer et al., 1989, 1991; Henstra et al., 1996).

In this report, we describe the isolation, characterization and nucleotide sequence of the mannitol-specific EICB gene (mtlA) and an adjacent gene, mtlR. The mtlR gene is located within the same operon as mtlA, and the mtlR gene product is not necessary for mannitol utilization. The mtlR gene product may be involved with regulation of this operon, but the exact function of this locus is unknown.

METHODS

Bacterial strains and media. S. mutans serotype c strain UA130 was used as the strain for DNA isolation and as the recipient strain for streptococcal DNA transformations (Murchison et al., 1986). E. coli host strains CC118 [A(arabinose)7697 araD139 lacX74 galE galKΔphoA20 thi-1 rpsE rpoB argE(Am) recA1] (Manoil & Beckwith, 1985) and XL-1 Blue [F'::Tn10 proA–B– lacI Δ(lacZ)M15] recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 ΔlacI15 (Bullock et al., 1987) were used for recombinant DNA manipulations.

S. mutans strains were grown in Brain-Heart Infusion (BHI) broth or on BHI agar plates (Difco). E. coli strains were grown in Tryptic Soy Broth (TSB) or on Tryptic Soy Agar (TSA) plates (Difco). Antibiotics used for selection, as necessary, with E. coli were ampicillin, 100 µg ml⁻¹, and chloramphenicol, 20 µg ml⁻¹, and for selection with S. mutans, erythromycin, 10 µg ml⁻¹ and kanamycin, 500 µg ml⁻¹. All chemicals were reagent grade and were obtained from Sigma.

DNA isolation and manipulation. Streptococcal chromosomal DNA was isolated in the following manner. A 5 ml starter culture was grown overnight in BHI broth in a 15 ml plastic screw-cap tube. This was used to inoculate 45 ml pre-warmed BHI broth in a 50 ml screw-cap tube. This culture was grown stationary 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 TE buffer and incubated for 1 h at 65 °C. The cells were then cooled on ice prior to the addition of 1 ml of 10 mg lysosome ml⁻¹ and 200 µl of 5000 units mutanolysin (Sigma) ml⁻¹. The cell suspension was incubated for 1 h at 37 °C. Pronase (1 ml of 10 mg ml⁻¹ stock solution) was added and incubation continued for 1 h. The cells were lysed by the addition of 1 ml of 20% Sarkosyl and 6.8 g guanidine hydrochloride was added and dissolved thoroughly. The DNA solution was incubated at 55 °C for 1.5 h prior to being layered onto a two-step cesium chloride gradient. The top layer (2 ml) of the gradient was 38% w/v cesium chloride while the bottom layer (2 ml) was 60%. Each layer contained 20 µl of 10 mg ml⁻¹ ethidium bromide. The gradient was centrifuged at 30000 r.p.m. for 24 h at 4 °C in a Beckman SW41 rotor. The DNA band which formed at the interface between the two layers was removed with a Pasteur pipette. The ethidium bromide was extracted from the sample with an equal volume of water-saturated butanol and the sample dialysed overnight against TE buffer prior to use.

Plasmid DNA isolations for either large-scale or miniprep protocols were done as described by Sambrook et al. (1989) using the alkaline lysis procedure with a PEG precipitation step. Quick-lysis preparations of plasmid DNA for initial screening procedures were done according to a protocol provided by Stratagene with their mung bean nuclease digestion procedure. This involves pelleting 0.1 ml of bacterial cells from an overnight culture in a microcentrifuge tube. The pellets are resuspended in 30 µl of 10 mM EDTA (10 mM Tris, 1 mM EDTA, 150 mM NaCl, pH 8.0). An equal amount of water-saturated phenol is then added to the cells and the tube vortexed vigorously. The emulsion was allowed to set at room temperature for 10 min prior to centrifugation at 13000 r.p.m. for 10 min. Twenty microlitres of the aqueous phase was removed to a new microfuge tube and 1 µl RNase (200 µg ml⁻¹) added. After incubation at room temperature for 5 min, 5 µl gel loading dye was added and the entire sample electrophoresed on a gel with supercoiled DNA standards. Plasmid DNA was subsequently isolated as described by Sambrook et al. (1989) from the cultures of subclones that displayed the appropriate size of plasmid and was screened further by restriction endonuclease digestion. All DNA manipulations and enzyme procedures were done according to the manufacturer’s instructions and under NIH recombinant DNA guidelines.

Isolation of the S. mutans mtlA gene. Isolation of the mtlA gene was accomplished by using a chromosomal walking technique using the integrative suicide vector pVA891 (Macrina et al., 1993). This plasmid will not replicate in S. mutans and streptococcal DNA fragments cloned into pVA891 can facilitate recombination with the homologous site on the streptococcal chromosome. Selection for erythromycin-resistant S. mutans transformants allows the isolation of colonies in which Campbell insertions into the chromosome have occurred.
The 1476 bp PstI–HindIII fragment located in the PstI fragment contained in pYA3121 was cloned into pVA891 as a HindIII fragment and the resultant plasmid was designated pYA3122 (Fig. 1). This plasmid was transformed into S. mutans strain UA130 (Murchison et al., 1986) and cells containing chromosomal integrations of pYA3122 selected for by growth on erythromycin. Chromosomal DNAs were isolated from several transformants and screened by Southern hybridization (Southern, 1975) to determine that only one copy of pYA3122 had integrated into the chromosome at the correct site (data not shown). One such S. mutans isolate was designated as SMS207 and was used for further study.

Chromosomal DNA was isolated from strain SMS207 and was digested with various restriction endonucleases which would only cut outside of the origin of replication and the chloramphenicol resistance marker of the integrated plasmid. The DNA in these restriction digests was phenol/chloroform extracted and ligated to itself at low DNA concentrations which favour intramolecular ligation. The ligated DNA was used to transform E. coli strain CC118 and the transformation mixture grown in TSB with selection for chloramphenicol resistance. This procedure, done with the restriction endonuclease AvaI, allowed the isolation of a plasmid that contained a 5.5 kb chromosomal DNA fragment originating from the HindIII site in pYA3122 and was designated pYA3123. The chromosomal insert contained in pYA3123 is depicted in Fig. 1.

**DNA sequence analysis.** The complete nucleotide sequence of the 4063 bp AvaI–PstI fragment contained in pYA3123 (this study, Fig. 1) was determined on both DNA strands. Several sequencing strategies were used. A series of random Tn5seq1 transposon insertions were used as the sequencing primer site as described by Nag et al. (1988). Also, a nested deletion series was generated by the procedure of Henikoff (1984) from either end of the cloned fragment. In addition, the nucleotide sequence of various regions was determined using synthetic oligonucleotides from Midland Certified Reagents as sequencing primers. In all cases, Sequenase-modified T7 DNA polymerase (Tabor & Richardson, 1987) from USB was used in the double-stranded sequencing technique of Chen & Seeburg (1985). Nucleotide sequence analysis was done using the following computer programs: Genepro (Riverside Scientific), Seqence (Access Biosystems), GCG (Devereux et al., 1984) and BLAST (Altschul et al., 1997).

**Transcription–translation assays.** These assays were done on selected plasmid constructs using a transcription–translation reaction kit from Promega. Aliquots of the labelled proteins were analysed using SDS-denaturing polyacrylamide gel electrophoresis (Laemmli, 1970) on a 10% gel. Following electrophoresis, the gel was treated with Enhance (Amersham) prior to drying. The dried gel was subjected to autoradiography and/or fluorography.

**Characterization of growth of mannitol mutants.** S. mutans strains were grown overnight in chemically defined medium (CDM) (van de Rijn & Kessler, 1980) containing 0.5% glucose and 0.5% mannitol. Three millilitres of an overnight culture was added to 100 ml pre-warmed medium containing the appropriate carbohydrate in a 250 ml flask plugged with a stopper surrounding an inverted 16×100 mm glass tube. At various time points, the flask was inverted to fill the test tube, which was inserted into a Spectronic 20 colorimeter. The optical density was read at 600 nm.

**RESULTS AND DISCUSSION**

**Isolation of the S. mutans mtlA gene**

We have previously described the isolation and characterization of the S. mutans mtlF gene (the EIIA domain) and mtlD gene (the mannitol-1-phosphate dehydrogenase) (Honeyman & Curtiss, 1992). These loci are located on the 2.3 kb PstI fragment in pYA3121 (Fig. 1). The nucleotide sequence analysis of this restriction fragment revealed the presence of an ORF S to these genes. This, and the fact that no apparent promoter structure was present on the PstI fragment, indicated that the presumed operon structure of the mannitol genes may extend in that direction. These data also indicate that the overall operon structure may be similar to that found in the mannitol operons of Enterococcus faecalis (Fischer et al., 1991) and Staphylococcus carnosus (Fischer et al., 1989). Fischer et al. (1991) stated that the mannitol-specific PTS Enzyme II gene, mtlA, is located approximately 2000 bp 5’ to the mtlF and mtlD genes in Ent. faecalis. Based upon the similarity between the S. mutans and Ent. faecalis operons (Honeyman & Curtiss, 1992), we hypothesized that the S. mutans mtlA gene was located on the chromosome adjacent to the PstI fragment contained in pYA3121. This hypothesis was confirmed by the isolation of pYA3123 as described in Methods.

**Nucleotide sequence of the S. mutans mannitol genes**

The nucleotide sequence of the entire AvaI–PstI fragment contained in pYA3123 was determined (GenBank accession no. AF210133). Nucleotide sequence analysis of this region revealed the presence of two ORFs. The deduced protein from the first ORF exhibits extensive homology to the mannitol-specific EIIs of various organisms. This confirmed our hypothesis about the overall operon structure of the mannitol genes in S. mutans. This operon structure is depicted in Fig. 1.

The nucleotide sequence reported here extends from the MaeIII site shown in Fig. 1 to the PstI site contained in pYA3123. We have previously reported the nucleotide sequence of the 2.3 kb PstI fragment contained in pYA3121 (Honeyman & Curtiss, 1992). Because the second ORF of the operon reported here crosses the previously described PstI site, we have included the nucleotide sequence data obtained previously which is necessary to extend the ORF to a termination codon so that a complete analysis of the deduced protein can be done.

The first ORF spans 1770 nucleotides, from base-pair 39 to base-pair 1808. The deduced protein from this ORF would consist of 589 amino acids with a molecular mass of 62.0 kDa. This ORF is immediately preceded by the nucleotide sequence AAGGAGG (bases 23–29) which resembles the ribosome-binding site of Bacillus subtilis (Moran et al., 1982) and the putative ribosome-binding sites described in S. mutans (Honeyman & Curtiss, 1992; Rosey & Stewart, 1992). These sequences
are used for reference because the 3’ end of the *S. mutans* 16s rRNA has not been described at this time.

The second ORF starts 18 bp after the end of the first ORF and continues for 1967 bp until 1 bp 5’ to the start of the *mtlF* gene. This ORF is not preceded by an apparent ribosome-binding site. Other *S. mutans* genes have sequences which are complementary to the 3’ end of the 16S rRNA molecule of *B. subtilis* and which may serve as ribosome-binding sites (Honeyman & Curtiss, 1992, 1993; Rosey & Stewart, 1992). The first sequences 3’ to the first ORF which resemble potential ribosome-binding sites are located at nucleotides 2029–2033 (AAGAG) and 2150–2156 (AGGAGG). These potential ribosome-binding sites are located 221 bp and 349 bp, respectively, from the end of the first ORF. The ORF following these potential ribosome-binding sites does not contain a methionine initiation codon for another 83 or 280 nucleotides, respectively. Generally, the spacing between the ribosome-binding site and the start site of translation is 8–10 bases (Moran et al., 1982). Therefore, based upon the DNA sequence analysis, it is not possible to determine the potential start site of translation of the second ORF. The possibility exists that a codon other than methionine is used to initiate translation of this protein. It is also possible that this ORF is not preceded by a strong ribosome-binding site as generally found with Gram-positive genes (Vellanoweth & Rabinowitz, 1992).

Analysis of the nucleotide sequence from the start of the first ORF to the end of the *mtlD* gene contained in pYA3121 for the presence of other transcriptional control elements such as other promoter elements or transcriptional terminators does not reveal any other potential regulatory elements. Thus, it appears that the ORFs reported here are part of an operon structure which includes the *mtlF* and *mtlD* genes as previously described.

**Homology analysis of the *S. mutans* proteins**

To determine the potential functions of the deduced proteins from these ORFs, the deduced proteins were compared to all GenBank entries using the BLAST program (Altschul et al., 1997). The deduced protein from the first ORF exhibited significant similarity only with the *mtlA* gene products from various other organisms (Table 1a). For example, the deduced protein from *S. mutans* has 55% identity and 73% similarity with the mannitol EIICB gene product from *Bacillus stearothermophilus*. Based upon the similarity of the deduced protein from *S. mutans* with the proteins from other *mtlA* loci, we conclude that this locus is the *S. mutans* *mtlA* gene.

The similarity displayed between the *S. mutans* MtlA protein and the various MtlA proteins from other species that encode only EIICB domains was restricted to the amino-terminal region of the *S. mutans* MtlA protein. This similarity generally covered approximately 470 amino acids of the *S. mutans* protein. This number of amino acids is the general size of molecules encoding EIICB domains. This similarity would localize the EIICB domains to the amino-terminal end of the *S. mutans* MtlA protein. We have previously reported that the *mtlF* protein from *S. mutans* is highly homologous (40.7% identity, 76.6% conserved amino acid substitution) to the 143 carboxyl-terminal amino acids of the *E. coli* MtlA protein (the EIIA domain) and to other EIIA domains. Thus, the gene for the EIICB domains is located approximately 2250 bp 5’ to the EIIA domain gene in *S. mutans* and the genes are separated by the second ORF reported here. This separation of the EIICB and EIIA domains is consistent with the results found in the mannitol operons from other Gram-positive bacteria which utilize an EIICB-EIIA pair (Fischer et al., 1989, 1991; Henstra et al., 1999). This also supports the hypothesis that various functional domains of the PTS proteins have been switched and that gene fusions and/or gene separations have occurred during the evolutionary process (Saier et al., 1988).

The *S. mutans* MtlA protein is approximately 120 amino acid residues longer than the mannitol EIICB proteins from *Clostridium acetobutylicum* (GenBank accession no. U53868), *B. stearothermophilus* (Henstra et al., 1996) and other organisms. These additional amino acids are located at the carboxyl-terminal end of the *S. mutans* MtlA protein, outside the previously described EIICB domains. This raises questions about the origin and function of this region of the MtlA molecule. When the amino acid sequence of this extended region of the *S. mutans* MtlA protein is compared to the GenBank database, similarity is found with the carboxyl-terminal end of various mannitol EIICB molecules (Table 1b). Thus, *S. mutans* has two regions within the *mtlA* gene product that have similarity with various EIIB domains (Fig. 2). At some point during evolution of the mannitol PTS in *S. mutans*, the EIIB domain must have undergone duplication. It is not
Table 1. Protein identities and similarities

<table>
<thead>
<tr>
<th>Species</th>
<th>Function</th>
<th>Identity (%)</th>
<th>Similarity (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) MtlA proteins with similarity to the deduced MtlA protein from <em>S. mutans</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. stearothermophilus</em></td>
<td>PTS EIICB</td>
<td>55</td>
<td>73</td>
<td>Henstra <em>et al.</em> (1996)</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>PTS EIICBA</td>
<td>51</td>
<td>68</td>
<td>Lee &amp; Saier (1983)</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>PTS EIICBA</td>
<td>52</td>
<td>70</td>
<td>GenBank AF166095</td>
</tr>
<tr>
<td><em>C. acetobutylicum</em></td>
<td>PTS EIICB</td>
<td>53</td>
<td>70</td>
<td>GenBank US53868</td>
</tr>
<tr>
<td><em>S. carnosus</em></td>
<td>PTS EIICB</td>
<td>47</td>
<td>65</td>
<td>Fischer &amp; Hengstenberg (1992)</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>PTS EIICBA</td>
<td>47</td>
<td>64</td>
<td>Yamane <em>et al.</em> (1996)</td>
</tr>
<tr>
<td>(b) Proteins with similarity to the carboxyl-terminal end of the MtlA protein from <em>S. mutans</em></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><em>Ent. faecalis</em></td>
<td>Unidentified</td>
<td>29</td>
<td>46</td>
<td>Fischer <em>et al.</em> (1991)</td>
</tr>
<tr>
<td><em>S. carnosus</em></td>
<td>EIICB</td>
<td>27</td>
<td>52</td>
<td>Fischer &amp; Hengstenberg (1992)</td>
</tr>
<tr>
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<td>22</td>
<td>48</td>
<td>GenBank US53868</td>
</tr>
<tr>
<td><em>B. stearothermophilus</em></td>
<td>EIICB</td>
<td>23</td>
<td>50</td>
<td>Henstra <em>et al.</em> (1996)</td>
</tr>
<tr>
<td>(c) Protein similarity to the deduced MtlR protein from <em>S. mutans</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td><em>B. subtilis</em></td>
<td>Putative regulator</td>
<td>20</td>
<td>44</td>
<td>Kunst <em>et al.</em> (1997)</td>
</tr>
<tr>
<td><em>B. stearothermophilus</em></td>
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<td>44</td>
<td>Henstra <em>et al.</em> (1999)</td>
</tr>
<tr>
<td><em>C. acetobutylicum</em></td>
<td>MtlR</td>
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<td>42</td>
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<td><em>Bacillus halodurans</em></td>
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<td>46</td>
<td>Takami <em>et al.</em> (1999)</td>
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<tr>
<td><em>B. subtilis</em></td>
<td>Putative regulator</td>
<td>21</td>
<td>41</td>
<td>Kunst <em>et al.</em> (1997)</td>
</tr>
</tbody>
</table>

*Fig. 2.* Diagram showing the duplication of the EIIB domain in the *S. mutans* MtlA protein. The *S. carnosus* EIICB protein is shown as an example of the various EIICBs that display similarity with the MtlA protein. The entire *S. carnosus* protein displays similarity with MtlA, which has an extended 120 amino acid region (top two lines). This extended region also displays similarity to the *S. carnosus* protein, but with lower similarity values (bottom two lines).

known, at this time, which of the duplicated domains of the *S. mutans* MtlA protein is the functionally active part of the PTS machinery. However, the similarity displayed by the terminal 119 amino acids with various EIICB domains is less than that displayed by the preceding 119 amino acids of the *S. mutans* MtlA protein. When the preceding amino acids (residues 332–470) are compared to the GenBank database, similarities with the carboxyl-terminus of several EIICB proteins are found. These similarity indexes are generally in the range of 44% identity with 65% similarity (data not shown). These values are greater than those displayed by the terminal 119 amino acids (Table 1b). These data would tend to indicate, based upon similarity alone, that the terminal 119 amino acids of the MtlA protein may not be the functionally active region of the EIICB domain. However, the possibility exists that both duplicated regions of the EIIB domain of this molecule are functional.

The deduced protein from the second ORF exhibits limited homology to several putative transcriptional regulators (Table 1c) and some of these comparisons are shown in Fig. 3. These proteins include the MtlR proteins from *Clostridium acetobutylicum* (GenBank

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*S. carnosus*  
*S. mutans*  
*S. carnosus*  

*Fig. 3.* Partial similarity alignment of the *S. mutans* mtlR gene product with the MtlR proteins from *B. stearothermophilus* and *C. acetobutylicum* and a putative protein from *B. subtilis*, showing the alignment with the 51 amino-terminal amino acids of the *S. mutans* MtlA protein. Residues identical in all the proteins are shown as white letters in a black box. Conservative residue substitutions are contained in the boxed regions. Note the conservation of residues in the second half of the predicted helix–turn–helix region which spans residues 21–43 of the *S. mutans* MtlR protein.
accession no. U53868) and B. steatorophilus (Henstra et al., 1999). These proteins have 20 and 22% identity and 42 and 44% similarity, respectively, with the S. mutans gene product. The other molecule which displays similarity to the deduced protein from the second ORF is a putative protein from B. subtilis whose function has not been determined. The MtlR protein in B. steatorophilus has been shown to be a regulator of the mannitol operon in that organism (Henstra et al., 1999). Because of its function and because of the fact that the mannitol operon from B. steatorophilus has the same overall structure and gene order as that of the S. mutans operon, we feel that an appropriate designation of this gene locus is the mtlR gene. However, the exact function of this gene product must be determined.

All of the proteins that exhibit similarity with the S. mutans MtlR protein have been postulated to be transcriptional regulators based upon the prediction of helix–turn–helix regions within their secondary structure. Within the deduced S. mutans MtlR protein, there is a 22 amino acid sequence (residues 20–41) near the amino-terminal end which has a 100% probability to form a helix–turn–helix structure based upon the prediction method of Dodd & Egan (1990). Several of the residues in this region are conserved in the homology alignments (Fig. 3). Thus, a potential function of the S. mutans mtlR gene product is of a regulatory nature which involves this region as a potential DNA-binding motif. It is interesting to note that the sequence encoding the potential regulatory region is located 5' to the potential ribosome-binding sites discussed previously for the mtlR gene. The fact that the helix–turn–helix region is conserved in several proteins displaying similarity to MtlA provides evidence that the translation of the second ORF initiates from the first methionine located after the end of the mtlA gene. There is no apparent ribosome-binding site located adjacent to this amino acid residue, which may explain the low expression level for the mtlR gene product seen in the in vitro transcription–translation reactions (data not shown). In vitro transcription–translation reactions and coupled transcription–translation expression systems were unable to detect the expression of the S. mutans MtlR protein in E. coli.

It is interesting to note that the S. mutans MtlR protein does not have similarity to the OrfX protein from Ent. faecalis. This is interesting in light of the overall operon structure and protein homology in other regions between the mannitol operons of S. mutans and Ent. faecalis (Fischer et al., 1991; Honeyman & Curtiss, 1992). Fischer et al. (1991) have described an orfX gene within the mannitol operon of Ent. faecalis similar to the second ORF described here. We have previously reported a high degree of homology (55–4 and 61–1% identity, 87–2 and 87.5% conserved amino acid substitutions) between the MtlF and MtlD proteins of these organisms (Honeyman & Curtiss, 1992). Although the sequence data for the orfX gene from Ent. faecalis is limited (Fischer et al., 1991), its deduced protein (104 amino acid residues) does not display any homology to the deduced MtlR protein of S. mutans, which is at the same location within the operon structure. These data seem incongruent with the existing homology data and the data on the overall structure of the mtl operons of these organisms.

Insertional inactivation of mtlR

To determine if mtlF and mtlD are part of a single transcriptional unit which includes mtlR, the mtlR locus was insertionally inactivated in vitro. The interposon Ω-Kan (Perez-Casal et al., 1991), which is flanked by transcriptional and translational terminators, was cloned into the BgII site of pYA3124 (Fig. 1) to generate pYA3125. This plasmid was linearized by restriction endonuclease digestion and used to transform UA130 (Murchison et al., 1986), with selection for kanamycin resistance. Kanamycin-resistant colonies would contain the interposon integrated into the chromosome interrupting the mtlR gene by means of a homologous double crossover. Kanamycin-resistant colonies were obtained and one transformant, ALH154, was used for characterization of the mtlR::Ω-Kan mutation. This in vitro-generated mutation should have a polar effect on the loci 3' to the insertion.

To determine the effect of the interposon insertion into mtlR, S. mutans was grown in CDM (van de Rijn & Kessler, 1980) supplemented with 0–2% glucose, 0–2% mannitol, or 0–2% glucose plus 0–2% mannitol (Fig. 4a). Wild-type cells grown with
glucose plus mannitol exhibited diauxic growth, reaching higher final OD_600 values than cells grown with either glucose or mannitol alone. In contrast, cells of the mtlR insertion mutant strain showed similar growth curves in either glucose or glucose plus mannitol, and there was no growth with mannitol as the sole carbon source (Fig. 4b). Therefore, mannitol utilization is blocked in the mtlR::Ω-Kan mutant, ALH154.

The above data indicate either that mtlF and mtlD are part of a single transcriptional unit which begins 5' to the mtlR gene reported here or that mtlR encodes a product which is necessary for utilization of mannitol. However, the second possibility seems remote because the only genes in any other mannitol PTS system which are necessary for mannitol utilization are mtlA, mtlF and mtlD (Fischer & Hengstenberg, 1992; Fischer et al., 1991; Henstra et al., 1996, 1999; Lee & Saier, 1983; Reich et al., 1988). These genes have not been interrupted in this case, and the observed effects are therefore probably due to the polar insertion of the interposon.

To determine if the effect was due to the polar insertion of Ω-Kan into mtlR, a mutation with a non-polar insertion element was also generated in vitro. The kanamycin-resistance gene from the Ω-Kan cassette was amplified by PCR. The primers for this PCR reaction were generated in such a manner as to produce a promoter-less kanamycin cassette which does not have a transcriptional terminator following the antibiotic resistance gene. This cassette was positioned at the same site in the S. mutans chromosome and in a manner as previously described for the Ω-Kan cassette. Strains containing this non-polar insertion into the mtlR gene (exemplified by ALH124) grow normally on mannitol media and have the ability to ferment mannitol (data not shown). Therefore, the MtlR gene product is not necessary for growth on mannitol or for expression of the operon. Thus, based upon DNA sequence analysis and insertional mutagenesis data, mtlA and mtlR appear to be co-transcribed with mtlF and mtlD and polar insertions into mtlR block mannitol utilization.

In this report, we have described the mtlA and mtlR genes of S. mutans. The mtlA gene encodes the EIICB domains of the PTS permease for mannitol transport. The MtlA protein is approximately 120 amino acids longer than several other EIICB molecules. The extended region of the MtlA protein also displays homology to the carboxyl-terminal end of the EIIB domain. Thus, a part of this domain was duplicated during evolution of the mannitol PTS in S. mutans. It is not currently known which region of the MtlA protein acts as the functional region of the EIIB domain. The mtlR locus, based upon deduced protein similarity, appears to encode a regulatory protein which has a helix-turn-helix region located at its amino-terminal end. Based upon DNA sequence analysis and insertional mutagenesis data, mtlA and mtlR appear to be part of a multiple cistronic message which includes the mtlF and mtlD genes previously described. Insertion of a polar interposon into mtlR blocks mannitol utilization, while insertion of a non-polar cassette does not. This indicates that the MtlR protein is not necessary for mannitol utilization or expression of the operon. Investigations into the exact function of the mtlR gene product are currently under way. Studies on the regulation of the mannitol PTS operon, in relation to the effects of catabolite repression, are also in progress.

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