Overlapping substrate specificity for sucrose and maltose of two binding protein-dependent sugar uptake systems in *Streptococcus mutans*

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*Streptococcus mutans*; sucrose; maltose; transport; binding protein-dependent.

**Abstract**

Sugar metabolism by *Streptococcus mutans* is associated with tooth decay. The most abundant sugars in the human diet are sucrose and maltose, a derivative of starch. Previously, we reported a binding protein-dependent transport system (msm) in *S. mutans* that transports sucrose and maltose, but its associated enzymes do not metabolize maltose. By searching the *S. mutans* genomic sequence for a maltose system (mal), we found a gene cluster encoding proteins with homology to those of *msm* and the *Escherichia coli* maltose system. Mutants were constructed by deleting *msm* or *mal*, or both, and tested for sugar utilization. Deletion of the *mal* system diminished the ability of *S. mutans* to ferment maltose, but deletion of only the *mal* transporter genes or *msm* showed reduced utilization of chromogenic maltosides.

Maltose, sucrose, glucose, fructose, mannose, and N-acetyl glucosamine inhibited utilization of chromogenic maltosides by the wild-type strain and mutants. In conclusion, the two binding protein-dependent systems in *S. mutans* appear to transport collaboratively their common substrate sugars, notably sucrose and maltose.

**Introduction**

*Streptococcus mutans* and consumption of dietary sucrose have been associated with dental caries (Tanzer et al., 2001). As a result, sucrose metabolism by *S. mutans*, including extracellular production of glucans and intracellular production of storage polysaccharides, has been extensively studied (Kuramitsu, 1993). *Streptococcus mutans* metabolizes sucrose to lactic acid more rapidly than other oral bacteria (Minah & Loesche, 1977). This property is most likely related to its multiple systems for transporting and metabolizing sucrose (Slee & Tanzer, 1982).

Slee & Tanzer (1982) initially reported that *S. mutans* has at least three systems for sucrose uptake: two phosphoenolpyruvate-dependent phosphotransferase systems (PTS), and one non-PTS, the third transport system (TTS). The two PTS systems have different affinities for sucrose. The high-affinity system has been genetically characterized as the *scr* regulon (Lunsford & Macrina, 1986; Sato et al., 1989). *In vivo* testing of an *scr* mutant in rats showed no loss of virulence (Macrina et al., 1991). Poy & Jacobson (1990) reported that the low-affinity sucrose PTS system is the trehalose-specific PTS system (*tre*), which also recognizes sucrose as a substrate. Although the nature of the TTS is not clear, the *S. mutans*-binding protein-dependent system for multiple sugar metabolism (*msm*) was found to transport sucrose (Tao et al., 1993a). Our preliminary studies showed that deletions of genes encoding these three sugar transport systems (*scr*, *tre*, and *msm*) did not affect the growth of *S. mutans* on sucrose. This suggested that an additional system(s) for efficient utilization of sucrose may exist in *S. mutans*.

At least five enzymes are secreted by *S. mutans* to metabolize sucrose extracellularly (Kuramitsu, 1993). Three glucosyltransferases (GtfB, GtfC, and GtfD) synthesize glucans from sucrose and release free fructose. One fructosyltransferase (Ftf) synthesizes fructan from sucrose and releases free glucose. Fructanase (FruA, exo-β-fructosidase) degrades sucrose because the sugar is structurally both a β-fructoside and α-glucoside (Burne et al., 1987). Collectively, these extracellular enzymes have complicated the study of *S. mutans* sucrose transport.

Besides sucrose, the metabolism of malto- and maltotriosaccharides by *S. mutans* is also important, because they are derived from...
starch, which is abundant in the human diet. Maltosaccharides are also substrates for synthesis of intracellular polysaccharide (Simpson & Russell, 1998), a key virulence factor of S. mutans (Spatafora et al., 1995). Although a system specifically for transport and metabolism of maltosaccharides has been identified in other bacteria, such as Escherichia coli (Dippel & Boos, 2005), a similar system has not yet been reported in S. mutans.

Studies in a number of other microorganisms showed that sucrose can be utilized by systems specific for α-glucosides, such as maltose. These organisms include Staphylococcus xylosus (Egeter & Bruckner, 1995), Sinorhizobium (Si.) meliloti (Jensen et al., 2002), Actinoplanes (Brukhorst & Schneider, 2005), Thermus thermophilus (Silva et al., 2005), Saccharomyces cerevisiae (Stambuk et al., 1999), and Candida albicans (Williamson et al., 1993).

The S. mutans msm system transports and metabolizes raffinose, melibiose, stachyose, isomaltosaccharides, palatinose, panose, and sucrose (Tao et al., 1993a). It also transports but does not metabolize maltosaccharides, glucose, fructose, and galactose. Because an msm deletion mutant can still ferment maltosaccharides, at least one additional system may exist in S. mutans specifically for the transport and metabolism of this group of sugars. The aims of the present study were to identify such a system and to study its interaction with the msm system in transporting the sugars sucrose and maltose.

Materials and methods

Strains, plasmids, and culture media

The bacterial strains and plasmids used in this study are listed in Table 1. Streptococcus mutans was grown at 37 °C in Jordan medium (Jordan et al., 1960), Todd–Hewitt broth (THB), or purple broth (Difco) supplemented with 0.5% glucose, maltose, or other sugars as specified. On agar plates, the cultures were incubated in a candle jar. To select for antibiotic resistance marker-inactivated mutants of S. mutans, antibiotics were added as follows: erythromycin (Em), 15 µg mL⁻¹; tetracycline (Tc), 15 µg mL⁻¹; or kanamycin (Km), 350 µg mL⁻¹.

Mutant construction

For construction of the mal regulon-deletion mutant, DNA fragments of the malP and malK genes, located at the two extremities of the regulon, were amplified by PCR with primers: malF1 (5’-CATCGGATATTTGAGACTG-3’) and malR1 (5’-CCATTATGGACACTAGACC-3’); and malF2 (5’-TAATCGGGATCCGGTCTGATCGCATGCT-3’) and malR2 (5’-AGAATTACGAGCTGGTGACA-3’). malR1 includes an existing XbaI site, while malF2 includes an added BamHI site. The two amplicons digested with XbaI and BamHI, respectively, were ligated overnight at 16 °C with the Km resistance (KmR) cassette derived from pSI170 (a gift from Moses N. Vijayakumar, Oklahoma State University, unpublished) digested with the same enzymes. The ligation mixture was used to transform S. mutans LT11 (Tao et al., 1993b). Transformants were selected on THB agar supplemented with Km after incubation at 37 °C for 24 h and tested for maltose fermentation in purple broth. Gene deletion was verified by PCR. An mal deletion mutant was selected and named Cm111.

For construction of the mal transporter (malEFGK) deletion mutant, DNA fragments of the malE and malK genes were amplified by PCR with two pairs of primers, malF3 (5’-GAATCAGATATTTGAGACTG-3’) and malR3 (5’-GCAGTATCGATCTTAGGCAGTAGC-3’), malF2 and malR2. The two amplicons digested with XbaI and BamHI, respectively, were ligated with the Em resistance (EmR) cassette derived from pAK491 [constructed by inserting the EmR gene of pVA891 (Macrina et al., 1983) into pBluescript] digested with the same enzymes. The ligation mix was amplified by PCR with the primers malF3 and malR2. The amplified DNA was used to transform S. mutans LT11 to

### Table 1. Bacterial strains and plasmids used in the study

<table>
<thead>
<tr>
<th>Strain/Plasmid</th>
<th>Description/Genotype</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>S. mutans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LT11</td>
<td>Wild-type</td>
<td>Tao et al. (1993b)</td>
</tr>
<tr>
<td>SF130</td>
<td>LT11 (Δmsm, Tc)</td>
<td>Tao et al. (1993a)</td>
</tr>
<tr>
<td>Cm111</td>
<td>LT11 (ΔmalEFGK, ΔmalRQP, KmR)</td>
<td>This study</td>
</tr>
<tr>
<td>Cm112</td>
<td>LT11 (ΔmalEFGK, EmR)</td>
<td>This study</td>
</tr>
<tr>
<td>ALH105</td>
<td>Wild-type NG8</td>
<td>A. Honeyman</td>
</tr>
<tr>
<td>ALH698</td>
<td>ALH105 (msmE::ΔKan2, KmR)</td>
<td>This study</td>
</tr>
<tr>
<td>ALH698M</td>
<td>ALH105 (msmE::ΔKan2, ΔmalEFGK, KmR, EmR)</td>
<td>This study</td>
</tr>
<tr>
<td>Plasmid</td>
<td></td>
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</tr>
<tr>
<td>pALH124</td>
<td>pUC7 derivative with a promoterless KmR</td>
<td>A. Honeyman</td>
</tr>
<tr>
<td>pSI170</td>
<td>KmR cassette, AmpR</td>
<td>M. Vijayakumar</td>
</tr>
<tr>
<td>pAK491</td>
<td>EmR cassette, AmpR</td>
<td>This study</td>
</tr>
</tbody>
</table>
EmR. Gene deletion was verified with PCR. One mutant was selected and named as Cm112.

A promoterless KmR marker (ΩKan2) derived from pALH124 was used for insertion into the msmE gene (Cote & Honeyman, 2003). In the resulting mutant, the aga gene encoding the galactosidase was intact, while the gene encoding the sugar-binding protein, msmE, was inactivated. The mutant was named ALH698. An msm-deletion mutant, SF130, was already available from a previous study (Tao et al., 1993a). For the construction of a double mutant with defects in both mal and msm transporters, chromosomal DNA of Cm112 was used to transform ALH698. A transformant with both KmR and EmR phenotypes was selected and named ALH698M.

**Sugar utilization analysis**

To test for maltose fermentation, overnight cultures of wild type and mutants in THB were spun down and washed twice with phosphate-buffered saline (PBS; pH 7.0) and resuspended in purple broth supplemented with various amounts of maltose, ranging from 0.05% to 0.5%. Following incubation at 37 °C, color changes were recorded as a function of time.

To test for utilization (transport and metabolism) of other sugars, two chromogenic sugars (Sigma) were used: 4-nitrophenyl α-d-maltoside (NPG2) and 4-nitrophenyl α-d-maltohexaoside (NPG6). Various nonchromogenic, unconjugated sugars (Sigma) were used as competitors. The reaction mixture included 1 mM chromogenic sugar, 10 mM competing sugar, and 100 mM sodium phosphate buffer (pH 7.0). Overnight S. mutans cultures in THB were diluted 1:50 in 5 mL Jordan broth supplemented with either 0.5% glucose or maltose as an inducing sugar, and grown for 3 h to the mid-exponential phase. The cells were washed three times and resuspended in 100 mM sodium phosphate buffer (pH 7), adjusting the cell concentration to OD600nm = 0.5. One milliliter was centrifuged and resuspended in 0.5 mL chromogenic sugar reaction mix. Following incubation at 37 °C for 3 h, 1 mL 0.6 M Na2CO3 was added to stop the reaction. The cells were removed by centrifugation, and the yellow color of the supernatant was measured with a spectrophotometer (Spectronic® 20 Genesys™) at 420 nm to monitor the release of 4-nitrophenol. A standard curve was obtained using 4-nitrophenol (Sigma) at 500, 400, 300, 200, 100, 50, 25, and 0 μM in 100 mM sodium phosphate buffer (pH 7) with 2 vol of 0.6 M Na2CO3.

**Results and discussion**

A **binding protein-dependent transport system for maltosaccharides in S. mutans**

To identify genes that encode a binding protein-dependent transport system for maltosaccharide in S. mutans, we used the amino acid sequence data (Russell et al., 1991) of the four msm transporter proteins, MsmE, MsmF, MsmG, and MsmK, to search the S. mutans genome (Ajidic et al., 2002) at the University of Oklahoma website (http://www.genome.ou.edu/smutans.html) with the BLAST program. A gene cluster was identified with similarities to the transport module of S. mutans msm and E. coli mal (Blattner et al., 1997; Fig. 1). It includes four genes: malE (putative sugar-binding protein), malf and malG (putative sugar transporter membrane components), and malK (putative ATP-binding protein). We refer to this gene cluster as malEFGK. Divergently oriented from this locus, separated by only 246 bases, is another gene cluster. It includes three genes: malR (putative regulator), malQ (putative amylomaltase), and malP (putative maltodextrin phosphorylase) homologous to E. coli mal genes. We refer to this gene cluster as malRQP. Collectively, we refer to these two gene clusters as the mal locus.

The **mal locus is involved in efficient utilization of maltose**

The mal locus-deletion mutant Cm111 (ΔmalEFGK, ΔmalRQP) did not ferment 0.1% maltose in purple broth, although fermentation of 0.5% maltose was detected after 24 h of incubation. The wild-type strain LT11 rapidly fermented maltose at both concentrations (the purple color changed in 15 min). Therefore, the mal locus may be required for rapid growth of S. mutans in maltose because deletion of this locus caused impairment of maltose utilization by S. mutans. In general, all strains tested, except Cm111 (Δmal) previously grown in Jordan medium with maltose as the sole carbon source, fermented maltose about two-fold more efficiently than cells grown in the same medium with glucose as the sole carbon source (data not shown). Although genes encoding malto-amylase (Simpson & Russell, 1998) and
glycogen phosphorylase (Ajdic et al., 2002) exist in *S. mutans* outside the *mal* locus, these enzymes might not be highly efficient in maltose hydrolysis. Nonetheless, their activities might explain why the *mal*-deletion mutant could still grow in 0.5% maltose-supplemented purple broth at a much reduced rate. To our knowledge, this is the first report of a binding protein-dependent transport system for maltose utilization by *S. mutans*.

**Both msm and mal transporters are involved in transporting maltosaccharides**

When *S. mutans* LT11 (wt) and Cm112 (ΔmalEFGK) were grown in purple broth supplemented with 0.05%, 0.1%, 0.2%, or 0.5% maltose, there was no detectable difference in their abilities to ferment maltose. This suggested that at least one additional system in *S. mutans* also transported maltose efficiently. We previously observed that multiple sugars, including maltose and maltotriose, competitively inhibited [H\(^+-\)]-melibiose uptake by the *S. mutans* msm transporter (Tao et al., 1993a). This suggested that the msm transporter might also recognize maltose. However, the role of msm in maltose transport had not been confirmed by a direct approach. With chromogenic maltosides, we evaluated the roles of *msm* and *mal* transporters in the uptake of maltose by *S. mutans*. As shown in Fig. 2(a), the two wild-type strains (LT11 and ALH105) were not detectably different in their utilization of chromogenic maltoside (NPG2). Their respective *msm*-deletion and *msmE*-insertion mutants, SF130 and ALH698, both had only about a 25% wild-type level of NPG2 utilization. The *malEFGK* deletion mutant Cm112 had about a 45% wild-type level of NPG2 utilization. The *msm* and *mal* double transporter mutant, ALH698M, had only 15% of wild-type levels of NPG2 utilization. These data suggested that both *msm* and *mal* transporters are required for efficient uptake of maltosaccharides, and that the *msm* transporter might transport maltosaccharides more efficiently than the *mal* transporter. Because the double transporter mutant could still utilize maltose, there might be yet another system(s) in *S. mutans* that could transport maltosaccharides, albeit at a much reduced rate.

**The *mal* transporter takes up multiple sugars, including sucrose and maltose**

Owing to the similarity between the transporters encoded by *msm* and *mal*, it is likely that the *mal* system could also transport multiple sugars. We selected two chromogenic maltosaccharides, NPG2 and NPG6, for testing. Inhibition studies with nonchromogenic sugars would suggest competition for transport and/or metabolism. As shown in Table 2, 15 sugars were tested against the utilization of chromogenic maltosides (NPG2 and NPG6) by three *S. mutans* strains: LT11 (wt), SF130 (Δmsm), in which the only remaining major transporter for maltosaccharides is MalEFGK, and ALH698M (ΔmalEFGK, *msmE*).

In the absence of competing sugars, NPG2 metabolism by SF130, in comparison with LT11, released about one half of the chromogen. Interestingly, the metabolism of NPG6 was reduced about 8-fold. Melibiose and raffinose strongly inhibited NPG6 but not NPG2 utilization by LT11. These results suggested that the *msm* transporter may contribute more to transport of higher molecular weight maltosaccharides. When competing sugars were tested,

![Fig. 2. Utilization of NPG2 by *Streptococcus mutans* strains. Data are presented as 4-nitrophenol release in nmol min\(^{-1}\) 10\(^{-9}\) cells. Bar represents SD.](https://academic.oup.com/femsle/article-abstract/266/2/218/565336)
sucrose was the strongest competitor besides maltose against the utilization of the two chromogenic maltosides. This suggested that the _mal_ system may also recognize sucrose as a substrate. Other competing sugars displaying inhibition against utilization of chromogenic maltosides by SF130 included fructose, glucose, mannose, and _N_-acetylglucosamine (NAG). The double transporter mutant ALH698M also showed a low level of NPG2 utilization, which was inhibited by maltose, sucrose, glucose, mannose, trehalose, and NAG. This suggested that systems for utilization of these sugars might transport maltose at low levels.

_Streptococcus mutans_ has two separate binding protein-dependent sugar transport systems for transporting sucrose, maltose, and several other sugars. Likewise, two similar systems have also been reported in the soil bacteria _Streptococcus_ (Jensen et al., 2002) and _Actinoplanes_ (Brunkhorst & Schneider, 2005). Sugar transport systems are critical for bacterial nutrient acquisition in their ecological environment. Photosynthetically derived sucrose is the main source of carbon for legume root nodules (Jensen et al., 2002). Notably, these soil bacteria have two mutually collaborative sugar transport systems. Sucrose and starch are the most common sources of dietary carbohydrates for humans. The presence in _S. mutans_ of two apparently collaborative systems to transport sucrose and maltosaccharides may contribute to its ecological success on the surface of teeth in hosts consuming sucrose and starch. It is of particular interest that the gene organization of the two sugar transport systems in _Streptococcus_ is remarkably similar to those found in _S. mutans_. As no PTS-associated sucrose transport activity has been found in rhizobia (Martinez-de Drets et al., 1974), binding protein-dependent sugar uptake is the major mechanism for _S. meliloti_ to transport sugars. In contrast, _S. mutans_ has two PTS systems for additional sucrose transport (Slee & Tanzer, 1982), which are important for nutrient acquisition. Unlike sucrose in plant roots, which is consistently available to symbiotically colonized soil bacteria, _S. mutans_ is only transiently exposed to the supply of dietary carbohydrates during the meal time of the host. At least four transporters for sucrose and two transporters for maltosaccharides allow _S. mutans_ to take up these important sugars more rapidly when they are only briefly available. The ability to transport sucrose and maltosaccharides by two mutually collaborative binding protein-dependent transport systems may thus contribute to efficient nutrient acquisition and pathogenicity of _S. mutans_. Further studies will be needed to better understand these two sugar transport systems.

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