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Two Closely Related ABC Transporters in *Streptococcus mutans* Are Involved in Disaccharide and/or Oligosaccharide Uptake▽

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Streptococcus mutans **has a large number of transporters apparently involved in the uptake of carbohydrates. At least two of these, the multiple sugar metabolism transporter, MsmEFGK, and the previously uncharacterized MalXFGK, are members of the ATP-binding cassette (ABC) superfamily. Mutation analysis revealed that the MsmEFGK and MalXFGK transporters are principally involved in the uptake of distinct disaccharides and/or oligosaccharides. Furthermore, the data also indicated an unusual protein interaction between the components of these two related transporters. Strains lacking *msmE* (which encodes a solute binding protein) can no longer utilize raffinose or stachyose but grow normally on maltodextrins in the absence of MalT, a previously characterized EII^{mal} phosphotransferase system component. In contrast, a mutant of *malX* (which encodes a solute binding protein) cannot utilize maltodextrins but grows normally on raffinose or stachyose. Radioactive uptake assays confirmed that MalX, but not MsmE, is required for uptake of [U-^{14}C]maltotriose and that MalXFGK is principally involved in the uptake of maltodextrins with as many as 7 glucose units. Surprisingly, inactivation of the corresponding ATPase components did not result in an equivalent abolition of growth: the *malK* mutant can grow on maltotetraose as a sole carbon source, and the *msmK* mutant can utilize raffinose. We propose that the ATPase domains of these ABC transporters can interact with either their own or the alternative transporter complex. Such unexpected interaction of ATPase subunits with distinct membrane components to form complete multiple ABC transporters may be widespread in bacteria.**

Members of the ATP-binding cassette (ABC) transporter superfamily have been identified in all domains of life. An increasing number are known to be involved in bacterial pathogenesis; others are associated with genetic disorders of humans (9, 13). Although a few have been very well characterized, there are no experimental data for the majority of ABC transporters in terms of their function and biological significance. However, because the members of this transporter superfamily are involved in diverse functions, a comprehensive characterization of those present in any given species can provide valuable insight into the lifestyle of an organism. Furthermore, it has been suggested that ABC transporters are promising targets for antimicrobial strategies (9).

The basic structure of ABC transporters consists of four domains: two integral membrane domains and two ATPase subunits. The ATPase subunits of ABC transporters include a characteristic ABC signature motif (33). Bacterial ABC transporters involved in uptake also require an additional solute binding domain, which provides specificity and maintains the direction of transport into the cell (15). In eukaryotes, the domains of ABC transporters are typically combined to form one or two protein subunits, containing both integral membrane and ATPase domains. However, in prokaryotes, the domains are commonly separate proteins. These protein subunits must associate to form an active ABC transporter, consisting of homo- or heterodimers of ATPase and integral membrane domains (13).

The production of organic acids during the fermentation of carbohydrates in oral bacteria, such as *Streptococcus mutans*, is central to dental caries in humans (10). Although sucrose is frequently considered the major cariogenic sugar, other carbohydrates may also contribute to caries (43). Analysis of the genome sequence of *S. mutans* indicates that this bacterium has the capacity to transport and metabolize a wide range of sugars (1). Therefore, a variety of dietary or host-derived carbohydrates may support *S. mutans* growth and contribute to caries. Because starch is a major dietary carbohydrate in modern societies, it is considered a potential contributor to caries, either alone or in combination with sucrose (5, 21, 27). Saliva contains an abundance of α-amylase, an enzyme that degrades starch to yield maltose and maltodextrins [linear α-(1,4)-linked D-glucose polymers] (16). Although it is apparent that *S. mutans* can ferment such starch degradation products, until recently it was not known how maltose and maltodextrins are taken up by this bacterium (40). We have presented data which established that MalT, an EII^{mal} phosphoenolpyruvate-dependent phosphotransferase system (PTS) component, was the principal transporter of maltose in *S. mutans* (40). However, MalT is not the sole transporter of maltodextrins in *S. mutans*: *malT* mutants retain the ability to grow on maltotriose or maltotetraose as a sole carbon source (40).

In some bacteria, maltose and maltodextrins are transported by members of the ABC transporter superfamily (4, 25). Indeed one of the most-studied ABC transporters is the *Escherichia coli* maltose/maltodextrin permease (4). Therefore, we initiated a study to determine whether two ABC transporters in *S. mutans* with significant identity to the *E. coli* maltose

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
UA159	*Streptococcus mutans* ATCC 700610	ATCC
KCL48	UA159 *malK*::*aphA3*	40
KCL57	UA159 *msmK*::*aphA3*	40
KCL63	UA159 *malX*::*aphA3*	This study
KCL81	UA159 *msmK*::*aphA3 malX*::*ery*	This study
KCL82	UA159 *msmK*::*aphA3 malK*::*ery*	40
KCL91	UA159 *msmE*::*aphA3*	This study
KCL92	UA159 *msmE*::*aphA3 malK*::*ery*	This study
KCL93	UA159 *malT*::Sp	40
KCL94	UA159 *malT*::Sp *malK*::*aphA3*	This study
KCL95	UA159 *malT*::Sp *msmK*::*aphA3*	This study
KCL96	UA159 *malT*::Sp *msmK*::*aphA3 malK*::*ery*	This study
KCL97	UA159 *malT*::Sp *malX*::*aphA3*	This study
KCL98	UA159 *malT*::Sp *msmE*::*aphA3*	This study
Plasmids		
pCR2.1-TOPO	Cloning vector for PCR products; Km^r Amp^r	Invitrogen Life Technologies
pCR4-TOPO	Cloning vector for PCR products; Km^r Amp^r	Invitrogen Life Technologies
pGEM-T-easy	Cloning vector for PCR products; Amp^r	Promega
pVA838	*E. coli–Streptococcus* species shuttle vector; Cm^r Em^r	22
pALH124	pALH123 carrying the nonpolar *aphA3* (Km^r) cassette	17
pKCL58	pCR4-TOPO containing *malX* from UA159 amplified using primers P86 and P87	This study
pKCL94	pUC19 containing *malX*, cloned from pKCL58 as a SphI/EcoRI fragment	This study
pKCL98	pUC19 containing *msmK* with *aphA3* from pALH124 cloned into the EcoRV site	40
pKCL101	pUC19 containing *malX* (pKCL94) with *aphA3* (Km^r) cloned into the PstI site	This study
pKCL103	pUC19 containing *malK* (SMU.1571) with *aphA3* from pALH124 cloned into the HpaI site	40
pKCL134	pALH124 containing *ery* (Em^r), amplified from pVA838, replacing *aphA3* between the HindIII sites	40
pKCL144	pUC19 containing *malX* (pKCL94) with *ery* from pKCL134 cloned into the PstI site	This study
pKCL148	pUC19 containing *malK* (SMU.1571) with *ery* from pKCL134 cloned into the HpaI site	40
pKCL149	pGEM-T-easy containing *msmE* from UA159 amplified using primers P82 and P83	This study
pKCL150	pGEM-T-easy containing *msmE* (pKCL149) with *aphA3* from pALH124 cloned into the HpaI site	This study
pKCL163	pCR2.1-TOPO containing *malT*::Sp^r, i.e., spectinomycin resistance gene from pNE1gfp inserted between the HpaI sites in *malT*, deleting 165 bp	40

permease were involved in the transport of maltose and maltodextrin. We particularly wanted to establish whether these two transporters had overlapping substrate specificities. We report that while these two ABC transporters each transport disaccharides and/or oligosaccharides (referred to herein as di/oligosaccharides), they are principally involved in the uptake of distinct subsets of carbohydrates: the previously described multiple sugar metabolism transporter MsmEFGK (29, 37) transports raffinose, melibiose, stachyose, isomaltose, and isomaltotriose, while a previously uncharacterized ABC permease transports maltodextrins (maltotriose, maltotetraose, maltopentaose, and maltoheptaose). We also present data that indicate that the ATPase components of either of these two transporters can compensate for the loss of the other. This means that the two ATPase domains can interact with either transporter complex to energize transport. This is unexpected, since it was previously thought that the constituent domains of ABC transporters were dedicated to only one specific transporter complex (33). More recently however, based on indirect bioinformatic evidence, others have suggested that a single ATPase protein can serve several ABC transporters (32). For example, in *Bacillus subtilis* there are two ATPases of the carbohydrate uptake transporter 1 (CUT1) subfamily (26), but only one of these is located in close genetic proximity to other CUT1 transport components, although both are encoded by monocistronic operons (22). These CUT1 ATPases are believed to interact with as many as eight individual CUT1 transporters (26), but this has yet to be confirmed. Nevertheless, this hypothesis does indicate that the atypical protein interaction described here is not exclusive to *S. mutans*, and so the findings of this study have wider application.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids used in this study are detailed in Table 1.

E. coli strains used for plasmid manipulation and maintenance were grown aerobically at 37°C either on Luria-Bertani (LB) agar or in LB broth. When required, *E. coli* cultures were supplemented with 50 μg/ml spectinomycin, 20 μg/ml kanamycin, 50 μg/ml ampicillin, or 10 μg/ml chloramphenicol. To select for appropriate transformants during cloning, LB agar was also supplemented with 40 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and 100 μg/ml isopropyl-β-D-thiogalactopyranoside (IPTG).

S. mutans strains were routinely grown at 37°C anaerobically (80% N_2, 10% H_2, 10% CO_2) on blood agar, on brain heart infusion agar, or in brain heart infusion broth (Lab M, United Kingdom). When required, *S. mutans* cultures were supplemented with 800 μg/ml spectinomycin, 1 mg/ml kanamycin, or 10 μg/ml erythromycin.

For growth analysis and to prepare cells for uptake assays, *S. mutans* strains were grown in a previously described semidefined growth medium (41). Appropriate carbohydrates were added at the following concentrations: 20 mM glucose, 10 mM raffinose, 10 mM stachyose, 10 mM melibiose, 10 mM maltose, 10 mM maltotriose, or 10 mM maltotetraose. In experiments using strains that could not

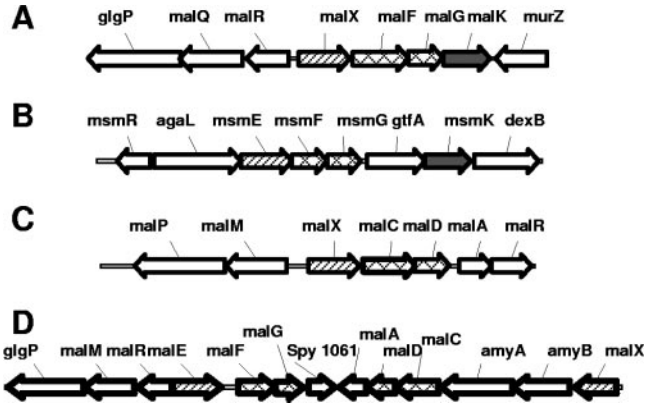

FIG. 1. Genetic maps of the loci encoding related carbohydrate ABC transporters in selected streptococci. The genes within the loci encoding components of the *S. mutans* MalXFGK (A), *S. mutans* MsmEFGK (B), *S. pneumoniae* MalXCD (C), and *S. pyogenes* MalEFG and MalXCD (D) transporters are represented by the arrows, which also indicate the direction of transcription. Shaded arrows, genes encoding the ATPase component of ABC transporters; hatched arrows, genes encoding solute binding proteins; cross-hatched arrows, genes encoding integral membrane proteins; open arrows, other genes, which encode metabolic enzymes or regulatory proteins. Neither of the loci encoding the Mal transporters in *S. pneumoniae* and *S. pyogenes* includes a gene encoding an ATPase component. The regions shown represent SMU.1564 to SMU.1572 (GenBank accession number AE014133) (A), SMU.876 to SMU.883 (accession number AE014133) (B), Spr1916 to Spr1922 (accession number AE008556) (C), and M5005_Spy_1055 to M5005_Spy_1067 (accession number CP000017) (D).

grow on raffinose or maltotriose as a sole carbon source, cultures were also supplemented with 5 mM glucose.

Genetic modification of bacterial strains. Genetic manipulation of bacterial strains and general molecular techniques were carried out essentially as described previously (31). The following oligonucleotides were used in this study: P82 (AAGCTTTACCGTCTAATTAATCCCGAAGT), P83 (CTAATCAACG TCTGCTTTCATTG), P86 (GCATGCGTCACAGCAGTCTCAGCCAT), and P87 (TTATTTAGCAGCTGCTAAATCTTT). Oligonucleotides were designed using the Vector NTI suite, version 10 (Invitrogen, United Kingdom) and were obtained from MWG-Biotech (Germany). *S. mutans* strains were transformed as previously described (23, 41), occasionally with the addition of 1 μg/ml synthetic competence-stimulating peptide (SGSLSTFFRLFNRSFTQALGK; Sigma-Aldrich, United Kingdom) (3, 20).

Insertional mutagenesis of genes of interest was performed essentially as described previously (18, 40, 41); the specific cloning strategies are outlined in Table 1. Briefly, strains containing *malK*::*aphA3*, *malK*::*ery*, *malX*::*aphA3*, *malX*::*ery*, *msmK*::*aphA3*, *msmE*::*aphA3*, or *malT*::Sp were created by transforming *S. mutans* UA159 with the reamplified inserts (only the inactivated gene of interest with the inserted antibiotic resistance gene, without the vector sequence) from pKCL103, pKCL148, pKCL101, pKCL144, pKCL98, pKCL150, and pKCL163, respectively. Double recombination events replaced the wild-type alleles with these linear DNA fragments, thus inactivating the gene of interest. It is known that insertion of the *aphA3* and Spec^r constructs does not have polar effects on downstream genes (18, 40, 41). Furthermore, the genes encoding MalXFGK are in a distinct operon and divergently transcribed from adjacent genes. It is particularly pertinent that the genes encoding the ATPase components, *malK* and *msmK*, are downstream of the genes encoding all the other ABC transporter components (Fig. 1).

Solute uptake by *S. mutans* strains. Uptake of [U-^{14}C]maltose (GE Healthcare, United Kingdom), [U-^{14}C]maltotriose (Biotrend GmbH, Germany), and [galactose-6-^{3}H]raffinose (Biotrend GmbH, Germany) was assayed by the rapid filtration method, essentially as described previously (40, 41). Exponentially growing *S. mutans* cells in semidefined medium were harvested by centrifugation when the optical density at 620 nm was approximately 0.5 and were washed with an equal volume of 50 mM potassium phosphate buffer (pH 7.2) supplemented with 1 mM $MgCl_2$. The cells were resuspended in this buffer to an approximate optical density at 620 nm of 1. Prior to their use in transport assays, the cells were incubated for 1 h at 37°C to deplete any remaining carbohydrate. Uptake assays were performed with 0.8 ml of cells per 1 ml of assay mixture and a final solute concentration of 25 μM containing 0.125 μCi of ^{14}C-labeled solute or 250 μM containing 1μCi ^{3}H-labeled raffinose. Samples (100 μl) were removed at 0, 1, 2, and 3 min, immediately filtered through glass fiber filter paper (GF/F; Whatman, United Kingdom), and rinsed with 10 ml phosphate buffer. Ready Safe liquid scintillation cocktail (Beckman Coulter, United Kingdom) was added to the filters, and the amount of radioactivity retained on the filters was determined in a scintillation counter. We previously determined, using a bicinchoninic acid protein assay (Sigma-Aldrich, United Kingdom), that suspensions of *S. mutans* with an optical density of 1 are equivalent to 0.238 mg of protein ml^{-1}. This figure was used to convert the radioactivity counts to nanomoles of solute transported per milligram of protein. For uptake competition assays, competing solutes were added to final concentrations of 1 mM. Only the initial rate of uptake (0 to 1 min) was used to calculate the K_m and V_{max} from rates obtained using a range of solute concentrations. All transport assays were performed on at least three independent cultures.

RESULTS

The *S. mutans* genome encodes two putative carbohydrate transporters of the CUT1 ABC transporter subfamily. ABC transporters that are known to transport carbohydrates belong to one of three subfamilies: the CUT1, CUT2, or peptide/opine/nickel uptake transporter (PepT) subfamily (30). The genome of *S. mutans* UA159 includes two regions each of which encodes all the components required to constitute a discrete ABC transporter of the CUT1 subfamily (Fig. 1A and B). One of these regions encodes the previously described multiple sugar metabolism transporter MsmEFGK (29). The genes in the second region, SMU.1567 to SMU.1570, have been annotated as a putative maltose transporter (1). Although we now know that it is not the principal maltose transporter (40), we propose that the nomenclature MalXFGK be retained (i.e., SMU.1567 is *malX*, SMU.1568 is *malF*, SMU.1569 is *malG*, and SMU.1570 is *malK*).

Although it is most common for the genes encoding all the components that constitute an ABC transporter complex to be located in close proximity to each other in a genome (8, 30), it is our observation that this is in fact unusual for genes encoding CUT1 transporters in *Streptococcus* species. Most operons encoding CUT1 transporters in streptococci lack a gene encoding an ATPase (see, for example, Fig. 1C and D). However, each of the regions in *S. mutans* encoding CUT1 ABC transporters includes a gene encoding an ATPase (*msmK* or *malK*) in addition to the genes encoding a solute binding protein (SBP) (*msmE* or *malX*) and two membrane proteins (*msmF* and *msmG* or *malF* and *malG*) (Fig. 1A and B).

The ATPase components of ABC transporters are required to energize the transport of substrates, but they do not contribute to solute specificity (33). Therefore, it is typical for these components, rather than the membrane domains or solute binding proteins, to have the greatest identity to the corresponding domains of related ABC transporters. The amino acid sequences of MsmK and MalK are 85% identical, indicating that these proteins have a high degree of conservation: each contains the expected Walker A and ABC transporter signature motifs. In contrast, the solute binding proteins MsmE and MalX share only 28% identity. The MalG and MsmG membrane proteins are 48% identical, whereas the MalF and MsmF membrane proteins are only 29% identical. However, transmembrane prediction

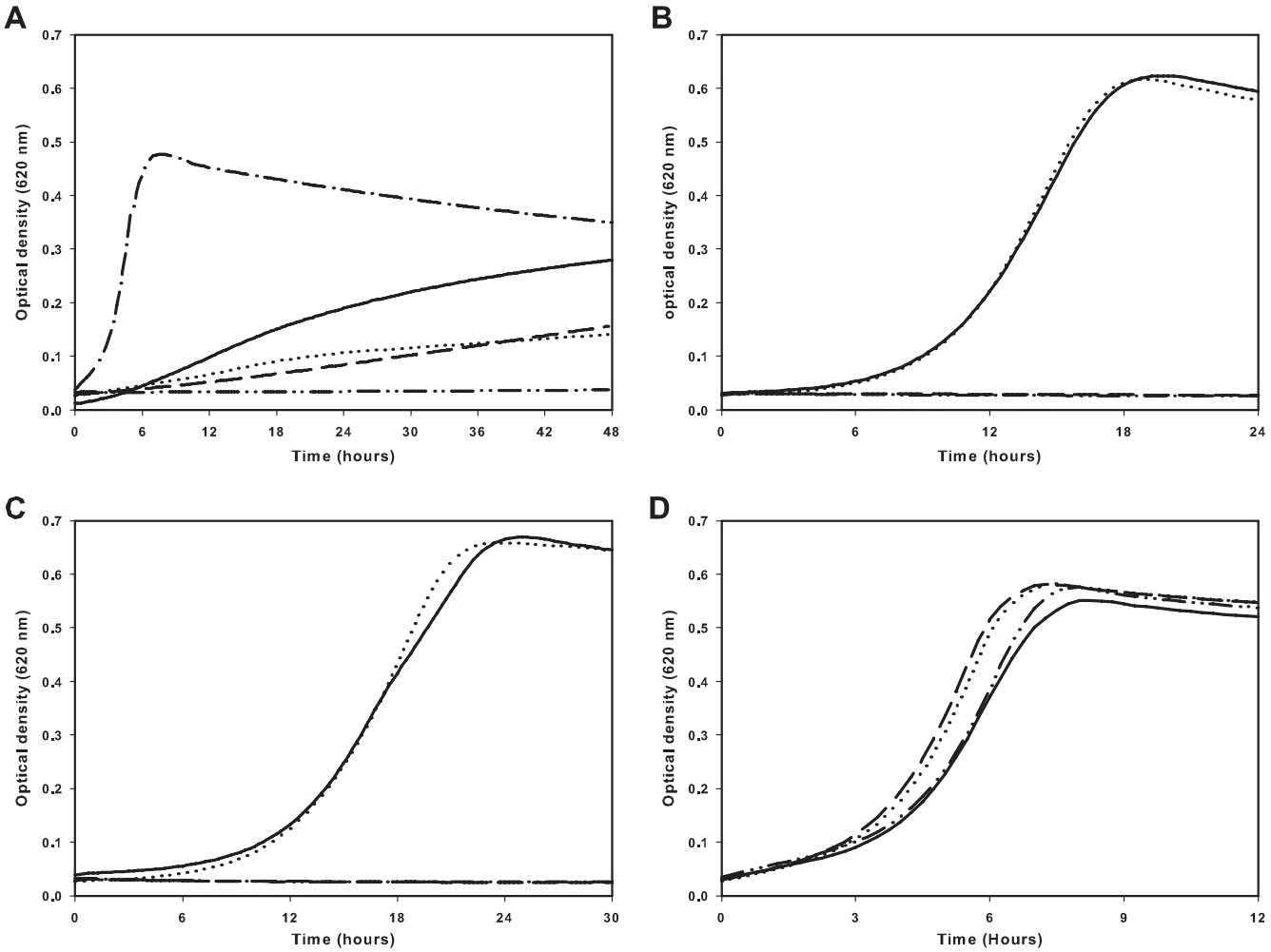

FIG. 2. Growth of the *S. mutans* UA159 *malT* mutant (solid lines), the *malT msmE* mutant (KCL98) (dotted lines), the *malT malX* double mutant (KCL97) (dashed lines), the *malT msmK malK* triple mutant (KCL96) (dash-dot-dot-dash lines), and wild-type *S. mutans* (UA159) (dash-dot-dash lines) (panel A only) in minimal medium containing 10 mM maltose (A), 10 mM maltotriose (B), 10 mM maltotetraose (C), or 20 mM glucose (D) as the sole carbon source. Data are means from at least three independent experiments.

algorithms (TopPred [6]) predict that MalF and MsmF each contain eight transmembrane helices, while MalG and MsmG each have six transmembrane domains. Therefore, although the primary sequence identity is lower than that observed between the ATPase domains, the secondary protein structure is somewhat conserved.

As is the case with the *msmEFGK* operon (29), genes encoding enzymes thought to be involved in carbohydrate metabolism are located adjacent to the region encoding the MalXFGK transporter (Fig. 1A). Divergently transcribed from *malXFGK* are genes encoding a putative glycogen phosphorylase (GlgP) and a putative 4-α-glucanotransferase (MalQ). These putative enzymes are involved in the metabolism of starch, and the annotation of MalXFGK suggests that it is involved in the uptake of maltose and/or maltodextrins (1). However, there is no direct experimental evidence to support this, and it is now known that the principal maltose transporter in *S. mutans* is a PTS (40). Furthermore, it is not known whether the transport specificity of MalXFGK is distinct from that of MsmEFGK or whether they transport similar carbohydrates. Therefore, we initiated a study of the role of MalXFGK in di/oligosaccharide transport in *S. mutans*.

The MalXFGK permease does not support growth on carbohydrates transported by MsmEFGK. It is known that MsmEFGK is required for the uptake of raffinose, melibiose, and isomaltotriose by *S. mutans* and that inactivation of this transporter results in strains that are unable to ferment these substrates (29). In order to compare the function of MalXFGK with that of MsmEFGK, a series of isogenic transport mutants were constructed in *S. mutans* (Table 1). As expected, inactivation of *msmE*, which encodes the solute binding protein of the MsmEFGK transporter, results in a strain (KCL91) that is unable to grow in semidefined medium with raffinose (α-D-galactosyl-α-D-glucosyl-β-D-fructose), stachyose (α-D-galactosyl-α-D-galactosyl-α-D-glucosyl-β-D-fructose), or melibiose (α-D-galactosyl-D-glucose) as a sole carbon source (data not shown). However, inactivation of *malX*, the gene encoding the corresponding solute binding protein in the MalXFGK ABC transporter, did not impair growth on any of these substrates (data not shown). Therefore, the MsmEFGK ABC transporter

is both essential and sufficient to transport raffinose, stachyose, or melibiose for the optimum growth of *S. mutans* on these substrates. This suggests that the MalXFGK ABC transporter cannot transport these substrates sufficiently to support growth and that the specificity of this permease is distinct from that of MsmEFGK.

The MalXFGK permease supports growth on maltodextrins as a sole carbon source. The proximity of genes encoding putative starch metabolism enzymes to *malXFGK* indicates that the ABC transporter encoded by these genes may be involved in the uptake of maltose or maltodextrins. However, MalT, a recently characterized PTS in *S. mutans*, is known to transport maltose and maltotriose (40). Therefore, because the presence of MalT in *S. mutans* could conceal any contribution of MalXFGK to the uptake of these substrates, we constructed a series of *S. mutans* strains containing insertions in *malT*, in addition to either *malX*, *msmE*, or both *malK* and *msmK*, resulting in strains KCL97, KCL98, and KCL96, respectively (Table 1).

Strains of *S. mutans* that lacked MalT could grow only very poorly on maltose as a sole carbon source (Fig. 2A). However, in view of the facts that mutation of either *malX* or *msmE* in addition to *malT* further reduces the growth rate and a *malT malX msmE* triple mutation results in no discernible growth of *S. mutans* on maltose, both the MalXFGK and MsmEFGK ABC transporters can apparently allow some uptake of maltose. Nevertheless, the growth observed is extremely low, so it is apparent that MalT is the principal transporter of maltose in *S. mutans*. In contrast, *S. mutans* without active MalT can grow efficiently on maltotriose or maltotetraose as a sole carbon source (Fig. 2B and C). Inactivation of *malX* in addition to *malT* results in a strain that will not grow on either of these sole carbon sources. Conversely, mutation of *msmE* does not influence growth on maltotriose or maltotetraose (Fig. 2B and C). The investigators who initially characterized MsmEFGK reported that maltotriose could partly inhibit (approximately 50%) the uptake of melibiose, suggesting that maltotriose is also transported by this permease (37). Nevertheless, it is apparent that MsmEFGK alone is not sufficient to allow the growth of *S. mutans* on maltotriose as a sole carbon source. Therefore, in *S. mutans*, MalXFGK, but not MsmEFGK, is involved in the uptake of maltodextrins, such as maltotriose and maltotetraose.

Uptake of maltotriose by the MalXFGK permease. To confirm that MalXFGK is involved in the uptake of maltodextrins, we performed radioactive uptake assays using [U-^{14}C]maltotriose (Fig. 3A). The marked reduction of maltotriose uptake in strains lacking MalT (for example, KCL93) grown in glucose as a sole carbon source indicates that MalT is the dominant maltotriose transporter in *S. mutans* under these conditions. However, when maltotriose is included in the growth medium, a transporter other than MalT is apparent (Fig. 3A). A comparison of the uptake of maltotriose by the mutant strains indicates that the principal induced maltotriose transporter is MalXFGK, since uptake is negligible in the *malT malX* double mutant, KCL97 (Fig. 3A). The data indicate that MsmEFGK may also transport maltotriose, since the rate of uptake in KCL98, the *malT msmE* double mutant, is lower than that observed in the single *malT* mutant, KCL93. Nevertheless, the relevance of any residual uptake of maltotriose by MsmEFGK is doubtful, since the rate of uptake in the *malT malX* mutant (KCL97), which retains MsmEFGK, is insignificant (Fig. 3A) and this strain cannot grow on maltotriose as a sole carbon source (Fig. 2B). Therefore, MalT and MalXFGK are the principal maltotriose transporters in *S. mutans*.

MalXFGK is not involved in significant maltose uptake. Since the growth data indicated that MalXFGK and MsmEFGK are required for the residual growth on maltose as a sole carbon source when *malT* is inactivated (Fig. 2A), we analyzed maltose uptake in our mutant strains. Since the data for maltotriose uptake (Fig. 3A) indicated that MalXFGK was not expressed in cells grown on glucose as a sole carbon source, [U-^{14}C]maltose uptake in the strains was determined after growth on 5 mM glucose and 10 mM maltotriose (Fig. 3B). Each of the strains lacking active *malT* had only a very low rate of maltose uptake. For example, the rate of maltose uptake by KCL93, the *malT* mutant, was 0.41 ± 0.09 nmol of maltose mg of protein^{-1} min^{-1}. Mutation of *msmE* in addition to *malT* did not reduce this rate of maltose uptake further: the rate of maltose uptake by KCL98 was 0.43 ± 0.10 nmol of maltose mg of protein^{-1} min^{-1}. In contrast, the *malT malX* double mutant, KCL97, did show a significant reduction of the rate of maltose uptake (0.06 ± 0.03 nmol of maltose mg of protein^{-1} min^{-1}), indicating that the MalXFGK transporter is indeed responsible for the residual growth observed on maltose as a sole carbon source. Nevertheless, the data further confirm that the contributions of MalXFGK and MsmEFGK to maltose uptake in *S. mutans* are insignificant compared to the principal role of the MalT PTS.

Specificity of carbohydrate uptake by MalXFGK. To obtain an indication of the range of solutes transported by MalXFGK, [U-^{14}C]maltotriose uptake assays were carried out in the presence of 1 mM (4-fold excess) competing solutes. Maltodextrins consisting of 3 to 7 glucose units (maltotriose, maltotetraose, maltopentaose, and maltoheptaose) each inhibited the uptake of [U-^{14}C]maltotriose by $>90\%$ (data not shown). It is most likely that this is competitive inhibition and that these maltodextrins are also transported by MalXFGK. Some of the other sugars tested, particularly stachyose and melibiose, also inhibited maltotriose uptake, but to a much lesser extent ($<70\%$ inhibition) than the maltodextrins. Maltose, isomaltose, and isomaltotriose inhibited maltotriose uptake only by $<50\%$. Therefore, MalXFGK has a much lower affinity for these substrates than for maltotriose, and it is doubtful that these are true substrates of MalXFGK. These data indicate that maltodextrins are the principal sugars transported by MalXFGK.

Maltodextrins do not inhibit the uptake of raffinose by MsmEFGK. Since MsmEFGK and MalXFGK belong to the same subfamily of ABC transporters, it is possible that they transport related substrates. Therefore, to determine whether there is any overlap in their substrate specificity, we analyzed the uptake of [galactose-6-^{3}H]raffinose in a range of *S. mutans* mutants. Preliminary experiments revealed that raffinose uptake by MsmEFGK required induction of this transporter by the inclusion of raffinose in the semidefined growth medium. The rate of uptake observed when wild-type *S. mutans* was previously grown in glucose as a sole carbon source was only 0.99 ± 0.15 nmol of raffinose mg of protein^{-1} min^{-1}. This rate increased to 6.61 ± 1.49 nmol of raffinose mg of protein^{-1} min^{-1} when *S. mutans* UA159 was grown on 5 mM glucose and

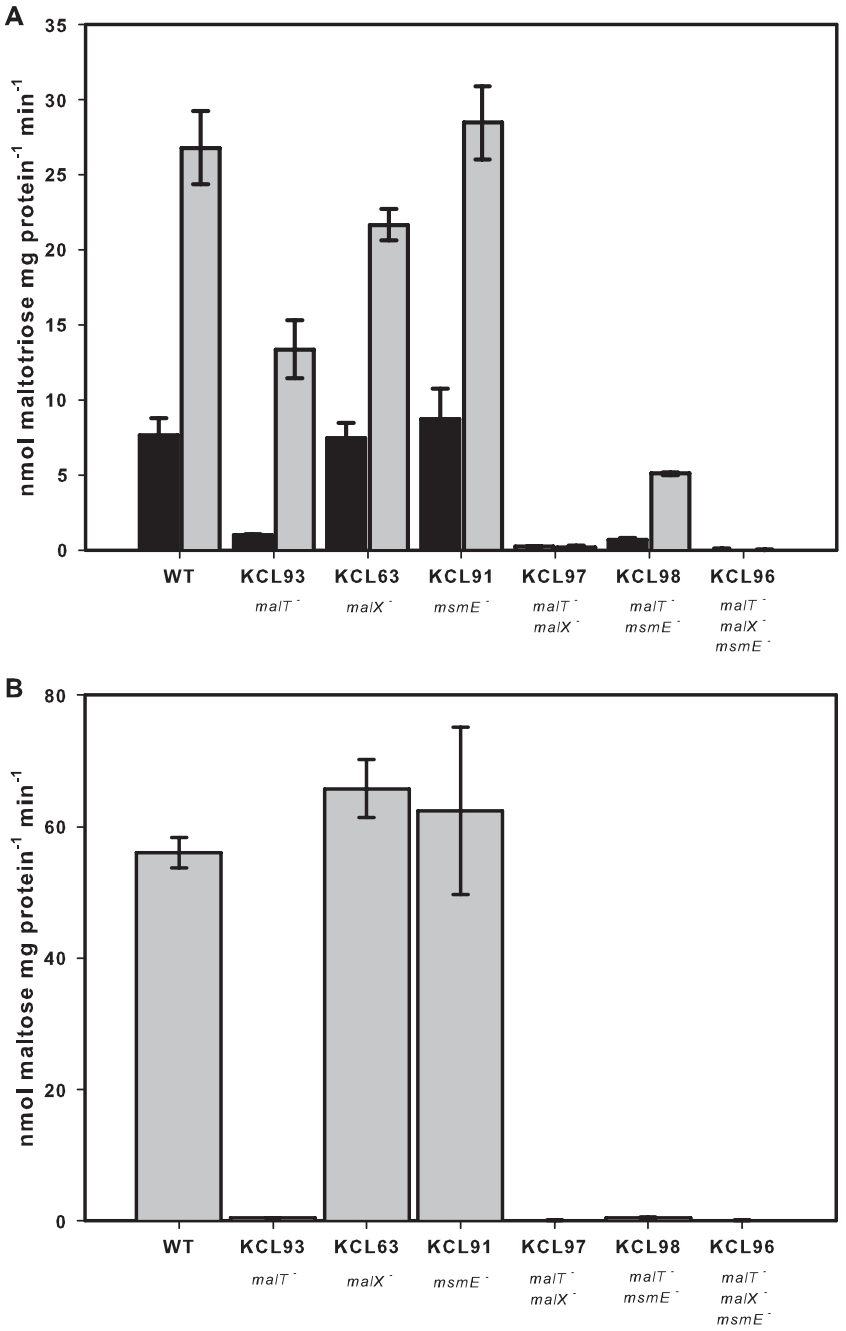

FIG. 3. Uptake of maltotriose and maltose by *S. mutans* strains. The rate of [U-^{14}C]maltotriose (A) or [U-^{14}C]maltose (B) uptake by *S. mutans* strains was determined following the addition of 25 μM substrate containing 0.125 μCi [U-^{14}C]maltotriose or [U-^{14}C]maltose. The uptake from the initial 3 min was used to determine [U-^{14}C]maltotriose uptake rates, but because the uptake of [U-^{14}C]maltose was linear for only 1 min, only the initial minute was used to calculate the corresponding maltose uptake rate. The strains were grown in minimal medium containing either 20 mM glucose (solid bars) (panel A only) or 5 mM glucose plus 10 mM maltotriose (shaded bars). Data are means from at least three independent experiments.

10 mM raffinose. The *msmE* mutant, KCL91, did not exhibit such an increase in raffinose uptake: the rate observed when this strain was grown on glucose and raffinose (0.81 ± 0.45 nmol of raffinose mg of protein^{-1} min^{-1}) was the same as that of glucose-grown cells. Mutation of *malX* alone (KCL63) did not significantly decrease the rate of raffinose uptake (3.37 ± 0.64 nmol of raffinose mg of protein^{-1} min^{-1}). Therefore, using the *malX* mutant KCL63 grown in 5 mM glucose and 10 mM raffinose, [galactose-6-^{3}H]raffinose uptake assays were carried out in the presence of 1 mM (4-fold excess) competing solutes. In accordance with the previously reported specificity of MsmEFGK (37), we observed that isomaltotriose, stachyose, raffinose, isomaltose, and melibiose each inhibited raffinose uptake (between 96 and 42%). In contrast, none of the

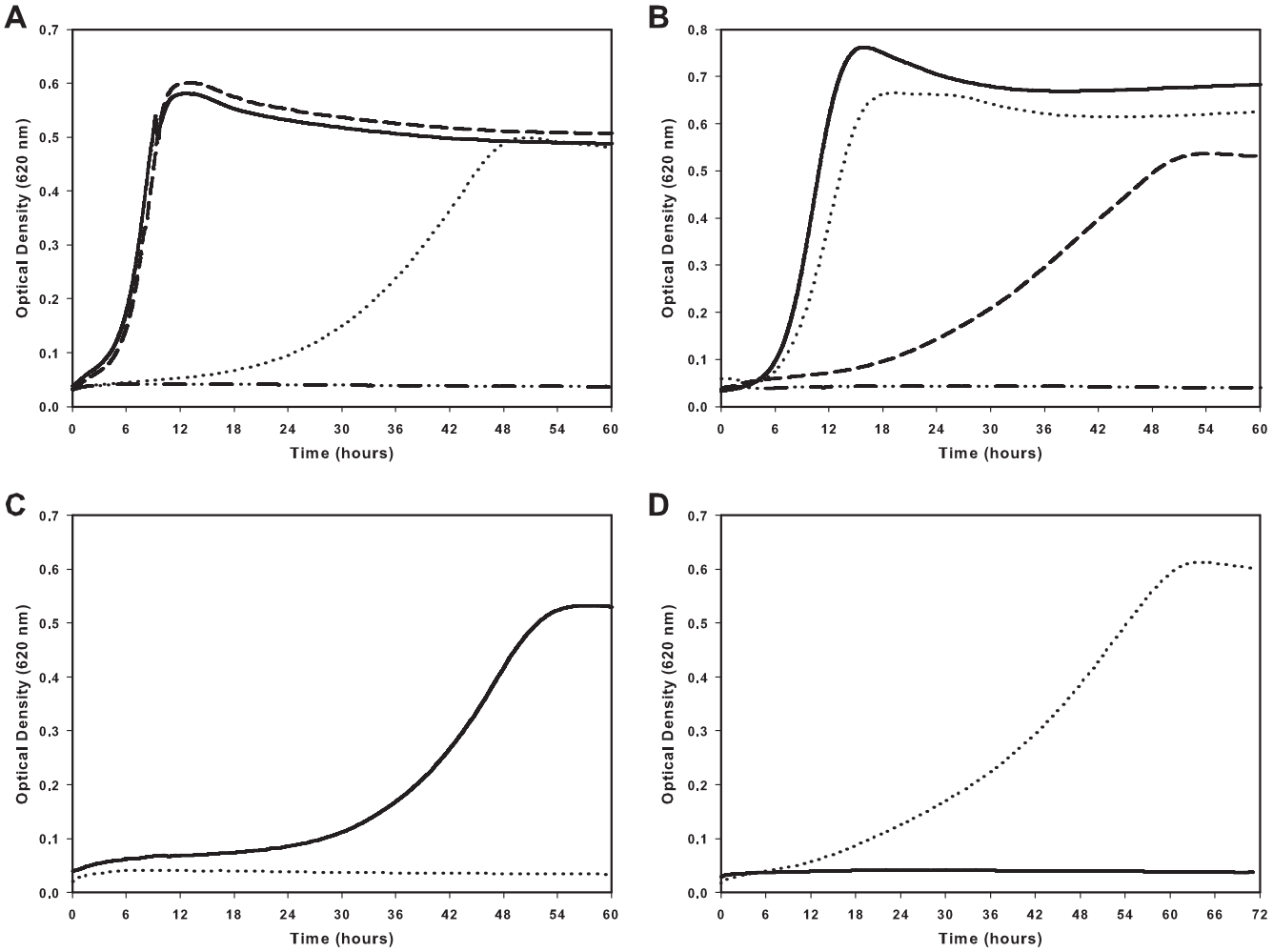

FIG. 4. (A and B) Growth of *S. mutans* UA159 (solid lines), the isogenic *msmK* mutant (KCL57) (dotted lines), the *malK* mutant (KCL48) (dashed lines), and the *msmK malK* double mutant (KCL82) (dashed and dotted lines) in minimal medium containing 10 mM raffinose (A) or 10 mM maltotetraose (B) as the sole carbon source. (C and D) Growth of the *msmK malX* double mutant (KCL81) (solid lines) and the *msmE malK* double mutant (KCL92) (dotted lines) in minimal medium containing 10 mM raffinose (C) or 10 mM maltotetraose (D) as the sole carbon source. Data are means from at least three independent experiments.

maltodextrins tested (maltose, maltotriose, maltotetraose, maltopentaose, and maltoheptaose) inhibited the uptake of raffinose by MsmEFGK. Therefore, the apparent transport specificity of MsmEFGK does not include maltodextrins.

Mutation of the genes encoding the ATPase domains of MalXFGK and MsmEFGK does not prevent carbohydrate uptake by these transporters. We report above that inactivation of *msmE*, which encodes the solute binding protein of MsmEFGK, results in a strain (KCL91) that is unable to utilize raffinose as a single carbon source. In contrast, when the gene that encodes the corresponding ATPase, *msmK*, is inactivated, the resulting strain (KCL57) can still grow on raffinose as a sole carbon source, but at a reduced rate (Fig. 4A). Similarly, mutation of *malX*, which encodes the solute binding protein of MalXFGK, results in a strain (KCL97) that is unable to transport and use maltotetraose as a sole carbon source (Fig. 2C). However, inactivation of the corresponding ATPase, MalK (KCL48), does not abolish growth on maltotetraose (Fig. 4B). Therefore, although the loss of the ATPase decreases the apparent efficiency of MsmEFGK or MalXFGK, the mutated strains can still transport their substrates.

The simplest explanation for this observation would be that the ATPase domains of these transporters are not required for transporter activity. However, this is contrary to the established paradigm of ABC transporter function. Furthermore, when we mutated both of the genes in *S. mutans* encoding ATPase domains of CUT1 transporters, the resulting strain (KCL82) could not grow on either raffinose or maltotetraose as a sole carbon source (Fig. 4A and B). Therefore, in *S. mutans*, the uptake of raffinose and maltotetraose, which are typically transported by MsmEFGK and MalXFGK, respectively, requires the ATPase domains of one of these ABC transporters to be active.

Our current understanding of ABC transporter domain interaction is that it is typical for the component subunits of ABC transporters to interact exclusively in distinct transport complexes. Thus, the MalK ATPase should associate only with the MalXFG solute binding and membrane protein complex to

constitute the MalXFGK ABC transporter. Similarly, MsmK should associate only with MsmEFG to form the active MsmEFGK transporter complex. If this were true, in order to interpret the data presented in Fig. 4, it must be assumed that the MalXFGK transporter complex can transport raffinose and that MsmEFGK can transport maltotetraose. However, the data described above indicate that this is not the case. Furthermore, inactivation of the gene encoding the ATPase of the alternative CUT1 ABC transporter does not affect growth on the principal substrates of MsmEFGK or MalXFGK: inactivation of MalK does not alter growth on raffinose (Fig. 4A), and inactivation of MsmK does not alter growth on maltotetraose (Fig. 4B). Also, if the MalX solute binding protein is inactivated in the same strain as MsmK, growth on raffinose is still observed (Fig. 4C). Therefore, although the MalK ATPase is required for raffinose uptake in the absence of MsmK, the corresponding solute binding protein is not. Similarly, if the MsmE solute binding protein is inactivated in the same strain as MalK, this strain can still grow on maltotetraose (Fig. 4D). Thus, although the MsmK ATPase is required for maltotetraose uptake in the absence of MalK, the MsmE solute binding protein is not.

It is conceivable that the SBPs of the *S. mutans* CUT1 ABC transporters (MalX and MsmE) can interact with either membrane complex (MsmFGK and MalFGK) and alter the solute specificity. However, although there are a number of examples of multiple SBPs interacting with a single ABC transporter and refining the specificity of the transporter complex (12, 19), we know of no precedent for a single SBP interacting with multiple ABC transporters. Moreover, while the solute specificity of ABC transporters is principally determined by the SBP, it is known that the membrane domains also contribute to specificity (7, 24, 36, 38). Also, MalX and MsmE are only 28% identical, and the membrane domains have only 48% and 29% identity. Therefore, it is unlikely that this is sufficient identity to allow interaction of the SBP with the alternative membrane complex and allow transport of atypical substrates through the membrane complex. Indeed, maltotetraose cannot inhibit the uptake of raffinose in the *msmK* mutant (data not shown). It is extremely unlikely that MsmE can bind to MalFG with affinity equal to that of MalX. Therefore, one would expect that, in the presence of maltotetraose, MalX would compete with MsmE for binding to MalFG and inhibit raffinose uptake. Since it does not, it is unlikely that raffinose is being transported by an MsmE-MalFGK complex. Similarly, raffinose cannot inhibit maltotriose uptake in the *malT malK* mutant (data not shown). Therefore, it is unlikely that maltotriose is being transported by a MalX-MsmFGK complex in this mutant.

In contrast to the SBPs, the MalK and MsmK ATPases are very similar (85% identity), especially in the conserved motifs including the Q loop region (centered on Q84 in each ATPase), which is particularly thought to interact with the membrane components. Therefore, we propose that the ATPase domains of the CUT1 ABC transporters in *S. mutans* are interchangeable—that the MalK ATPase can interact with MsmEFG domains to constitute an active MsmEFG-MalK transporter complex and that MsmK can interact with MalXFG to form MalXFG-MsmK. If such transport complexes exist, the affinity for the substrates transported would be unaltered, since it is dependent on the solute binding protein and membrane domains. However, the rate of uptake may be decreased, because the coupling of ATP hydrolysis to the transport process may be suboptimal when each ATPase interacts with an unusual membrane complex. Therefore, we compared the kinetics of maltotriose uptake in strains KCL98 and KCL94, which, respectively, contain MalXFGK and the proposed MalXFG-MsmK transporter complex. The affinities (K_m) for maltotriose uptake in these strains were not significantly different, but the V_{max} was lower for KCL94 (Table 2). This finding supports the conclusion that the ATPase domains of the different CUT1 transporters in *S. mutans* can each compensate for the loss of the other and interact with either transport complex.

TABLE 2. Kinetic constants for maltotriose and raffinose uptake by *S. mutans* strains

Strain	Proposed ABC transporter complex	[U-^{14}C]maltotriose uptake	
		K_m (μM)	V_{max} (nmol mg of protein^{-1} min^{-1})
KCL98	MalXFGK	2.35 ± 0.87	5.10 ± 0.48
KCL94	MalXFG-MsmK	2.67 ± 0.95	1.43 ± 0.13

DISCUSSION

The data presented here are evidence that *S. mutans* has two related ABC transporters that are involved in the transport of di/oligosaccharides. However, as summarized in Fig. 5, these transporters are principally involved in the uptake of distinct substrates. MsmEFGK is principally involved in the uptake of melibiose, raffinose, stachyose, isomaltose, and isomaltotriose (Fig. 5A). Maltodextrins, including maltotriose, maltotetraose, maltopentaose, and maltoheptaose, are transported by MalXFGK (Fig. 5B). Neither MsmEFGK nor MalXFGK is involved in significant uptake of maltose, which is principally transported by MalT, a PTS group translocator (Fig. 5C). Typically, inactivation of any of the genes encoding the different domains of an ABC transporter abolishes the activity of the transporter as a whole, regardless of whether they encode the solute binding protein, the membrane proteins, or the ATPase domain (14, 41). Therefore, it was surprising that we observed a different phenotype when the individual genes encoding the ATPase of MalXFGK or MsmEFGK were mutated rather than the corresponding genes encoding the solute binding proteins. These data indicate that the ATPase domains of either of these CUT1 ABC transporters can interact with either the corresponding transporter complex (MalK with MalXFG; MsmK with MsmEFG), or the alternative CUT1 ABC transporter (MalK with MsmEFG; MsmK with MalXFG) (Fig. 5).

The MsmEFGK permease was one of the earliest characterized ABC transporters in gram-positive bacteria (29). It was established that this permease has a role in the uptake and metabolism of multiple sugars; hence its name (29, 37). Our data are in accord with these early studies and confirm the range of substrates transported by this permease. Although the genes that encode it are not universally present in *S. mutans* strains (28, 39), MsmEFGK is clearly required for the uptake of melibiose, raffinose, and isomaltodextrins. However, the fre-

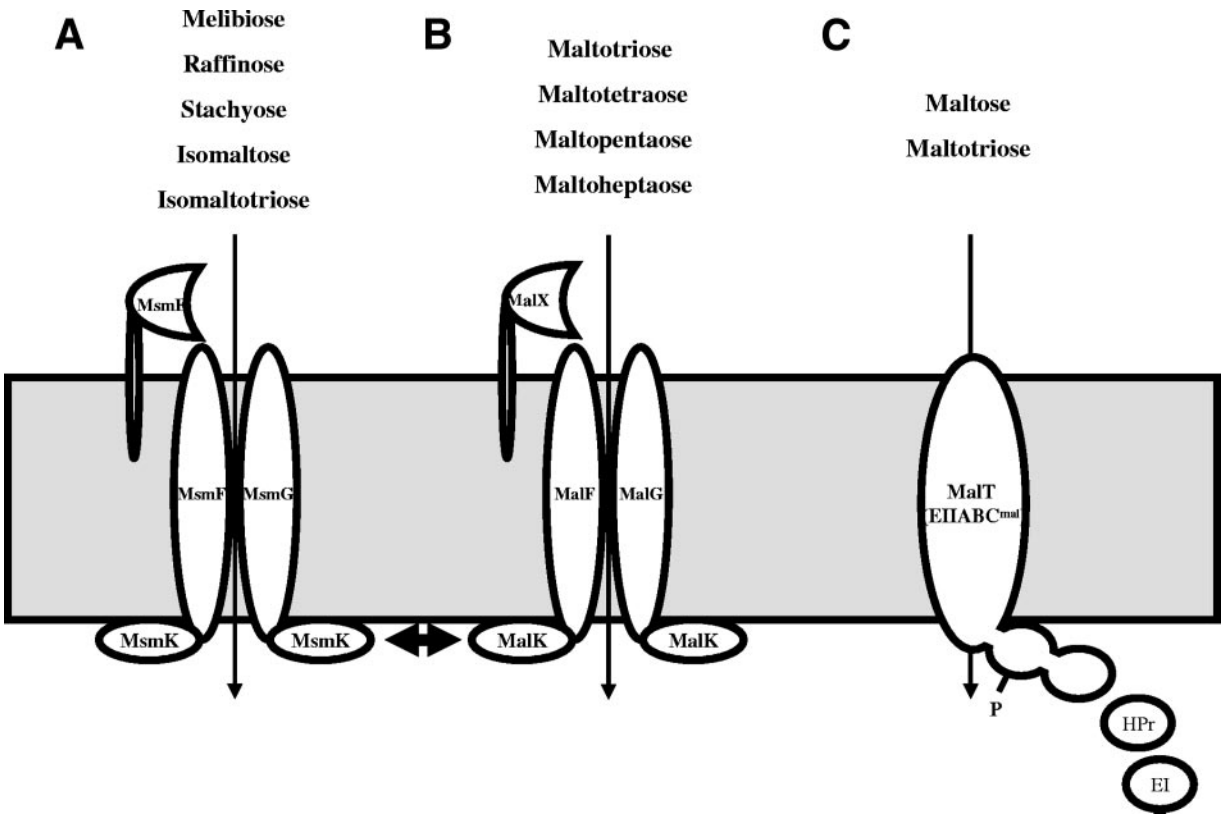

FIG. 5. Summary of carbohydrate uptake by *S. mutans* ABC transporters MsmEFGK (A) and MalXFGK (B) and a PTS transporter, MalT (C). Above each representation of the transport complexes is a list of some key carbohydrates transported by each permease. The bold double-headed arrow indicates that the MsmK and MalK ATPases of the transporters can act with the other components of the alternative ABC transporter in the absence of the normal constituent ATPase.

quent isolation of clinical strains that lack this ABC transporter indicates that the loss of this trait does not affect the ability of *S. mutans* strains to survive and proliferate in vivo (28).

It was apparent from our earlier characterization of MalT that neither MsmEFGK nor MalXFGK is the principal maltose transporter in *S. mutans* (40). The data presented here confirm this. However, it was also apparent that MalT was not the sole transporter of maltodextrins in *S. mutans* (40). Our data establish that the CUT1 ABC transporter MalXFGK is the principal maltodextrin transporter in this bacterium. Maltotriose is taken up by both MalT and MalXFGK, but longer maltodextrins are principally transported only by MalXFGK. Hence, the substrate specificity of MalXFGK is distinct from that of MsmEFGK. This conclusion is apparently contrary to a recent report that these ABC transporters have overlapping specificities (17). It was reported that both MalXFGK and MsmEFGK are required for the efficient uptake of maltosaccharides. Although our growth data suggest that both of these transporters may contribute to a low level of maltose uptake, this is insignificant compared to the role of MalT. Indeed, maltose did not inhibit the uptake of [U-^{14}C]maltotriose by MalXFGK in a way that would suggest that it is transported by this permease. However, the data presented by Kilic et al. (17) are consistent with our conclusions, particularly in light of the recent characterization of MalT (40). In their study, Kilic et al. (17) used chromogenic maltosides (4-nitrophenyl α-D-maltoside and 4-nitrophenyl α-D-maltohexaside), which are physically larger than maltose, having a nitrophenyl ring that could influence transport specificity. Our direct measurement of oligosaccharide uptake by *S. mutans* strains containing *msmEFGK*, *malXFGK*, and *malT* mutations provides a reliable measure of substrate specificity and clarifies the different roles of these transporters.

Although the substrate specificity of MalXFGK is distinct from that of MsmEFGK, it is comparable to the reported specificity of maltodextrin ABC transporters in other streptococci. A *Streptococcus pneumoniae malX* mutant, which lacks the CUT1 ABC transporter solute binding protein MalX, can no longer grow on maltotetraose as a sole carbon source but can grow on maltose (25). Similarly, a solute binding protein from *Streptococcus pyogenes*, MalE, is involved in the transport of linear maltodextrins with as many as 7 glucose units, but it does not bind maltose or cyclic maltodextrins (34). Therefore, the uptake of maltodextrins by ABC transporters of the CUT1 subfamily is common in *Streptococcus* species. Furthermore, such transporters may be important in streptococcal pathogenesis, since there is evidence that such a maltodextrin ABC transporter is required by *S. pyogenes* to colonize the oropharynx (35). However, a comparison of the *S. mutans malXFGK* operon with the regions that encode the putative orthologues indicates apparent differences in transporter organization. Nei-

ther of the operons in *S. pneumoniae* or *S. pyogenes* that encode the components of the putative maltodextrin ABC transporters contains a gene encoding a corresponding ATPase (Fig. 1C and D). In each of these species, a single gene encoding an ATPase of the CUT1 subfamily is located elsewhere on the genome. Furthermore, these species also contain multiple operons encoding solute binding proteins and membrane domains of CUT1 ABC transporters. Therefore, either *S. pneumoniae* and *S. pyogenes* only have one functional ABC transporter of the CUT1 family in addition to a number of redundant, nonfunctional CUT1 operons or the single ATPase must interact with multiple ABC systems. In contrast, although *S. mutans* has two operons encoding ABC transporters of the CUT1 subfamily, each includes a gene encoding an ATPase component (Fig. 1A and B). Thus, each of the CUT1 ABC transporters in *S. mutans* has a dedicated ATPase that can interact with the distinct transporter complex. Nevertheless, our data indicate that each of these *S. mutans* ATPases can compensate for the loss of the other and interact with either of the CUT1 ABC transporter complexes. This unexpected domain interaction in *S. mutans* suggests that the single ATPase in *S. pneumoniae* and *S. pyogenes* can also interact with multiple CUT1 ABC transporter complexes. Because this is contrary to the established paradigm of ABC transporter domain organization, further research—particularly direct protein interaction studies—is required to confirm this.

Some ABC domains of the CUT1 family have been shown to be functionally exchangeable in heterologous transport systems (11, 42). Specifically, complementation experiments with *E. coli* showed that *ugpC*, which encodes an ATPase of a glycerol phosphate permease, can substitute for *E. coli malK*, provided that *ugpC* expression is sufficiently high and the integral membrane components of the Ugp system are absent (11). Similarly, *E. coli malK* can complement the loss of *ugpC*. However, such hybrid transporters are less efficient than the wild-type equivalents (11). CUT1 ATPases from different bacterial species can also be exchanged: an ATPase of a lactose permease, LacK, from *Agrobacterium radiobacter* can functionally substitute for MalK in *Salmonella enterica* serovar Typhimurium (42). Although these previous studies were somewhat artificial in that they required extensive manipulation of the bacterial strains, it is considered likely that the *S. mutans* CUT1 ATPases can naturally interact with multiple ABC transporters.

The data presented indicate that the two CUT1 ABC transporters in *S. mutans* are induced by the presence of transported substrates. The uptake of raffinose by MsmEFGK is increased when raffinose is included in the growth medium. This is consistent with earlier reports that melibiose uptake by this transporter is induced by growth in the presence of melibiose or raffinose (29). Similarly, the rate of maltotriose uptake by MalXFGK is increased by the presence of maltotriose in the growth medium. This corresponds with recent data from a global transcriptional analysis of *S. mutans* sugar transporters, which indicate that *malXFGK* is induced by maltotriose and maltose, while *msmEFGK* is induced by raffinose and stachyose (2). Our data extend the findings of Ajdić et al. (2) by demonstrating that the uptake specificity of MalXFGK, in addition to that of MsmEFGK, corresponds to the carbohydrates that induce transcription of the genes that encode these transporters. The regions of the *S. mutans* genome that encode these ABC transporters include putative transcriptional regulators, *msmR* and *malR* (Fig. 1A and B), which are most probably responsible for controlling the expression of these genes. Indeed, MsmR is reported to be a positive activator of *msmEFGK* (29).

The contribution to dental caries of in vivo uptake and metabolism of starch breakdown products, such as maltose and maltodextrins, by *S. mutans* is still controversial (21). Most emphasis is still placed on the central role of sucrose. However, the significance of maltose and maltodextrin uptake and fermentation cannot be disregarded. This study has clarified the different roles of the two CUT1 ABC transporters and the MalT EII^{mal} PTS transporter in di/oligosaccharide uptake in *S. mutans*. Moreover, it has provided experimental evidence of an unexpected interaction of ATPase components of ABC transporters with distinct ABC transport complexes.

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