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Impairment of Melibiose Utilization in *Streptococcus mutans* Serotype c *gtfA* Mutants

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The *Streptococcus mutans* serotype c *gtfA* gene encodes a 55-kilodalton sucrose-hydrolyzing enzyme. Analysis of *S. mutans gtfA* mutants revealed that the mutant strains were specifically impaired in the ability to use melibiose as a sole carbon source. *S. mutans gtfA* mutant strains synthesized less α-galactosidase activity inducible by raffinose than wild-type strains. Melibiose (an inducer in wild-type strains) failed to induce significant levels of α-galactosidase in the mutant strains. We hypothesize that melibiose use by *S. mutans* requires the interaction of the GtfA enzyme, or another gene product under the control of the *gtfA* promoter, with other gene product(s) involved in melibiose transport or hydrolysis.

Sucrose metabolism plays a critical role in caries formation by *Streptococcus mutans* (15). Recently, the genes for several of these enzymes have been cloned and thoroughly analyzed (1, 10, 14, 21), yet there is no full description of the importance, function, and interactions of these enzymes (5).

We have previously reported the cloning, characterization, and properties of a unique *S. mutans* sucrose-hydrolyzing activity, the GtfA enzyme (21). It was hypothesized that the GtfA enzyme synthesizes a glucan primer for the synthesis of larger glucan products, perhaps for the synthesis of water-insoluble glucans (19–21). Further analysis of the product formed from sucrose by purified enzyme encoded by the ggg gene indicates that the GtfA enzyme is most likely a sucrose phosphorylase, which catalyzes the transfer of a glucosyl moiety from sucrose to P_i to generate α-D-glucose-1-phosphate and free fructose (22). Since both glucan and glucose-1-phosphate have similar mobilities in paper and thin-layer chromatography (21, 22), and they are also methanol insoluble, this new finding (22) is consistent with previous reports (19–21). However, the identity of the reaction products of the GtfA enzyme with sucrose as the substrate has not been fully solved. We have previously reported that the product made from sucrose had an apparent molecular mass of 1,500 kilodaltons (kDa) by gel filtration (21). Russell et al. (22) suggested that charge interaction between glucose-1-phosphate and the column matrix led us to an erroneous estimate of size. However, we used charged standards to calibrate the column, and most importantly, we analyzed the product synthesized early (30 min) in the reaction kinetics (21). Furthermore, the product synthesized late (18 h) in the reaction kinetics (Russell et al. analyzed the product of an overnight incubation) had a lower apparent molecular mass (less than 0.75 kDa; unpublished observations). Therefore, it is still possible for the GtfA enzyme to synthesize a phosphorylated glucan product with P_i as the initial acceptor. This phosphorylated glucan could then serve as the primer for the synthesis of other extracellular (19–21) or storage (22) glucan products or be degraded to glucose-1-phosphate (22).

On the other hand, the final elucidation of the function of the GtfA enzyme requires the biochemical analysis of *S. mutans* mutants with specific lesions in the *gtfA* gene. Such mutants have been constructed and partially analyzed (2, 19, 20). In this regard, we have shown that these mutants were fully virulent in the rat model system (2). This result suggests that either the GtfA enzyme is not a major virulence determinant or its function is or can be supplied by another gene. In this manuscript, we describe and analyze the unexpected inability of *S. mutans gtfA* mutants to use melibiose as a carbon source. We hypothesize that the GtfA enzyme, or a gene product regulated by the *gtfA* promoter, interacts with other proteins involved in melibiose transport or hydrolysis.

Bacterial strains are listed in Table 1. Growth of *Escherichia coli* and streptococci, complex media and their components (Difco Laboratories, Detroit, Mich.), and nutritional supplements (Sigma Chemical Co., St. Louis, Mo.) have been described before (21, 24).

Wild-type serotype c *S. mutans* strains are able to ferment and grow on various sugars as sole carbon sources (8). Therefore, we tested the ability of *S. mutans gtfA* mutants to utilize different carbohydrates as carbon sources. Carbohydrate utilization by *S. mutans* was evaluated by fermentation tests in thioglycolate medium plus purple agar base (16), supplemented with 1% fructose, 1% galactose, 1% lactose (4-O-β-D-galactopyranosyl-D-glucoside), 1% maltose (4-O-α-D-glucopyranosyl-D-glucoside), 1% melibiose (6-O-α-D-galactopyranosyl-D-glucoside), 1% raffinose (6-O-α-D-galactopyranosyl-1α-D-glucoside-2β-fructofuranoside; Pfanstiehl Laboratories, Inc., Waukegan, Ill.), or 1% sucrose (β-D-fructofuranosyl-α-D-glucopyranoside; ultrapure; Schwarz/Mann, Cambridge, Mass., or Sigma). Plates were incubated anaerobically for 36 to 48 h at 37°C. Fermentation of the sugar was evidenced by a color change from purple to yellow. We also tested the ability of *S. mutans* strains to grow on defined FMC medium (24) with the same carbohydrates as sole carbon sources. Wild-type strains MT8148, UA101, UA130, and V403 fermented and grew on 1.0% (wt/vol) fructose, galactose, lactose, maltose, mannitol, melibiose, raffinose, sorbitol, and sucrose. On the other hand, *S. mutans gtfA* insertion-duplication mutant strain UAB751 and deletion mutant strain V1362 fermented and grew on all carbon sources except melibiose. Five other independent insertion-duplication mutant strains gave the same results (data not shown). The mutant strains were

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TABLE 1. Bacterial strains

Strain	Phenotype or genotype	Source, reference, or derivation
E. coli K-12		
M1900	F⁻ *lacY96 glnV44*? *rpsL45* or *rpsL110 metB1 melB4*	R. Schmitt[a] (23)
M2701	F⁻ *galK rpsL metB1 melA7*	R. Schmitt (23)
Streptococci[b]		
MT8148	*S. mutans* serotype c, rough, Mel⁺	18
UA101	*S. mutans* serotype c, rough, Mel⁺	P. Caufield[c] (17)
UA130	*S. mutans* serotype c, smooth, Mel⁺	P. Caufield (17)
UAB751	*S. mutans* serotype c, smooth, *gtfA* Mel⁻	Derived from UA130 (2)
V403	*S. mutans* serotype c, smooth, GtfA⁺	F. L. Macrina[d] (12)
V1362	*S. mutans* serotype c, smooth, Δ*gtfA*	Derived from V403 (20)

[a] Institut für Biochemie Genetik und Mikrobiologie, Universität Regensburg, Regensburg, Federal Republic of Germany.
[b] Rough or smooth phenotype refers to the colony morphology on brain-heart agar plates after 48 h of incubation at 37°C under anaerobic conditions. It is indicated for identification purposes and does not correlate with other traits described.
[c] School of Dentistry, University of Alabama at Birmingham.
[d] Department of Microbiology and Immunology, Virginia Commonwealth University, Richmond, Va.

incapable of fermenting melibiose when the sugar was added at higher concentrations (2 to 8% [wt/vol]). The inability of mutant strains to utilize melibiose suggested that the *S. mutans gtfA* gene, or a gene under the control of the *gtfA* promoter, was involved in melibiose metabolism. *S. mutans gtfA* mutants also displayed a reduced ability to grow on FMC-raffinose agar, suggesting a common pathway for melibiose and raffinose catabolism in *S. mutans*.

We performed reversion tests to verify the tight linkage between the presence of the GtfA enzyme and melibiose use by *S. mutans*. Insertion-duplication mutant strains were stable in the absence of antibiotic selective pressure (2). However, melibiose-utilizing (Mel⁺) true revertants were obtained at frequencies between 1.1×10^{-6} and 3.0×10^{-5} by plating *S. mutans gtfA* mutant cells onto FMC agar supplemented with 1% melibiose. Revertants were also obtained by repeated passages on thioglycolate medium plus purple broth (or agar) base with 1% melibiose. All revertants displayed the same patterns of carbohydrate utilization as wild-type strains and gave the wild-type colony size on FMC-raffinose agar. Furthermore, analysis of one revertant by Southern blotting analysis showed the loss of the DNA sequence inserted to inactivate the *gtfA* gene (data not shown). Six independent revertants analyzed by Western blot had a wild-type GtfA protein (data not shown).

Since *S. mutans gtfA* mutants were unable to utilize melibiose, we investigated whether the GtfA enzyme was able to transport and hydrolyze melibiose. We tested these properties of the GtfA enzyme in vivo by the use of appropriate *Escherichia coli gtfA* clones. Fermentation of *E. coli* Mel⁻ strains M1900 and M2700 (Table 1) and their transformants with pYA601 (GtfA⁺ [21]) was tested at 25 and 37°C in MacConkey base agar supplemented with various carbohydrates: 1% (wt/vol) galactose, 1% glucose, 1% lactose, 1% melibiose, 1% raffinose, or 1% sucrose. The wild-type *gtfA* gene failed to complement either a mutation in the α-galactosidase gene (*melA*) or a mutation in the thiomethylgalactoside permease II gene (*melB*). Since the *E. coli melA* mutant has all the machinery necessary to transport melibiose inside the cells, we concluded that the GtfA enzyme cannot hydrolyze melibiose. Furthermore, the GtfA enzyme is partly accessible to the periplasmic space (21) (see below), where melibiose can diffuse freely and react directly with the enzyme. The lack of complementation of the *melB* mutation shows that the GtfA enzyme, by itself, cannot function as a melibiose permease, at least not in *E. coli*. This result should be viewed with caution, since the *S. mutans* gene product might not behave in *E. coli* exactly as it would in *S. mutans*.

E. coli melA GtfA⁺ clones were also unable to ferment raffinose. This result indicated that fermentation of raffinose by *E. coli* GtfA⁺ clones is dependent on the hydrolysis of raffinose (into sucrose and galactose) by α-galactosidase, as we have suggested before (21). Furthermore, strains M1900(pYA601) and M2701(pYA601) grew on sucrose as the sole carbon source. Colonies on sucrose minimal agar were readily visible after 2 to 3 days of incubation at 37°C. No growth was observed when the host strains were incubated for 7 days in the same conditions. This result is consistent with the localization of the GtfA enzyme on the external side of the *E. coli* cytoplasmic membrane in a functional way so as to utilize sucrose as a substrate (21).

We investigated whether melibiose had any effect on sucrose hydrolysis by the GtfA enzyme. If the GtfA enzyme binds melibiose and sucrose through the same site, melibiose should competitively inhibit product (glucose-1-phosphate or a phosphorylated glucan) formation from sucrose. If it binds elsewhere, it should inhibit product formation noncompetitively. For the inhibition experiment, sucrose was used at 1.0 mM, a concentration slightly below the K_m of 1.25 mM (21). Melibiose was added to reaction mixtures at concentrations ranging from 12.5 to 100 mM. The unrelated sugar lactose was also tested at 100 mM. Quantitation of product synthesis for the various reaction mixtures (data not shown) showed that neither melibiose nor lactose had any inhibitory effect on product synthesis by the GtfA enzyme. This result suggests that the GtfA enzyme does not bind melibiose, at least not at a site that would impair sucrose hydrolysis.

Use of melibiose and raffinose by *S. mutans* requires their uptake and subsequent hydrolysis of the product of the transport reaction to monosaccharides. Alternatively, melibiose could be hydrolyzed by an extracellular α-galactosidase into galactose and glucose and metabolized via the homolactic fermentation pathway, which is prevalent in streptococci (6). The initial step in raffinose catabolism may involve various alternative routes: uptake of raffinose followed by intracellular hydrolysis, hydrolysis by an extracellular α-galactosidase into sucrose and galactose, or hydrolysis by extracellular invertase (4) or fructosyltransferase (11) into melibiose and fructose. The latter route may explain the reduced ability of *S. mutans* Mel⁻ mutants to grow on raffinose, as we have observed. So far, it remains unknown whether the transport of raffinose and melibiose also in-

TABLE 2. Measurement of α-galactosidase activity in *S. mutans* wild-type and *gtfA* mutant strains[a]

Strain	Phenotype	Sugar(s) added and concn (wt/vol)	Mean α-galactosidase activity (nmol of *o*-nitrophenol/ml per min) ± SD	
			Intact cells	Permeabilized cells
UA130	GtfA$^+$ Mel$^+$	Galactose (1%)	1.9 ± 0.2	1.0 ± 0.2
		Galactose (1%), melibiose (0.1%)	4.0 ± 0.2	2.2 ± 0.2
		Galactose (1%), raffinose (0.1%)	6.2 ± 0.2	5.0 ± 0.2
		Melibiose (1%)	7.0 ± 0.2	5.8 ± 0.2
		Raffinose (1%)	11.5 ± 0.2	8.9 ± 0.2
UAB751	GtfA$^-$ Mel$^-$ (insertion-duplication mutant derived from UA130)	Galactose (1%)	1.8 ± 0.2	0.6 ± 0.2
		Galactose (1%), melibiose (0.1%)	2.1 ± 0.2	0.7 ± 0.2
		Galactose (1%), raffinose (0.1%)	3.3 ± 0.21	1.6 ± 0.2
		Raffinose (1%)	6.0 ± 0.2	2.6 ± 0.2
V403	GtfA$^+$ Mel$^+$ (wild type)	Galactose (1%)	0.8 ± 0.2	<0.2
		Galactose (1%), melibiose (0.1%)	1.7 ± 0.2	0.2 ± 0.2
		Galactose (1%), raffinose (0.1%)	3.1 ± 0.2	0.6 ± 0.2
		Raffinose (1%)	4.9 ± 0.2	1.2 ± 0.2
V1362	GtfA$^-$ Mel$^-$ (deletion mutant derived from V403)	Galactose (1%)	0.4 ± 0.2	0.2 ± 0.2
		Galactose (1%), melibiose (0.1%)	0.6 ± 0.2	<0.2
		Galactose (1%), raffinose (0.1%)	1.5 ± 0.2	0.3 ± 0.2
		Raffinose (1%)	3.0 ± 0.2	0.7 ± 0.2

[a] *S. mutans* cells were grown at 37°C to the stationary phase as standing cultures in modified FMC medium (containing 0.5% casein hydrolysate instead of the standard amino acid mixture) supplemented with various sugars as indicated. Erythromycin (25 μg/ml) was added for growth of strain UAB751 (2). Cells (from 50-ml cultures) were harvested at 4°C and washed once with 5 ml and resuspended in 2 ml of buffered saline with 0.01% gelatin. Cells were permeabilized as described by LeBlanc et al. (13). All samples were kept at 2°C pending assay. α-Galactosidase activity was measured by the hydrolysis of *o*-nitrophenyl-α-D-galactoside (α-ONPG; Sigma) to the chromogenic product *o*-nitrophenol. All assays were run in duplicate, and the final data were calculated from averages for at least two time points when the hydrolysis of α-ONPG was linear with time and cell concentration. The assay mixture contained 50 mM Tris hydrochloride (pH 7.5), 5.0 mM $MnCl_2$, 5.0 mM dithiothreitol, 5.0 mM α-ONPG, 15 μg of chloramphenicol per ml (to prevent α-galactosidase induction by α-ONPG), and *S. mutans* intact or permeabilized cells at an optical density of approximately 1.0 at 600 nm. Reactions were terminated by adding stop reagent (166 mM sodium carbonate and 8.3 mM EDTA at final concentrations). The amount of *o*-nitrophenol was determined by measuring the A_{420}.

volves the phosphoenolpyruvate:sugar phosphotransferase system, responsible for the translocation of all major sugars catabolized by *S. mutans* (9).

Since α-galactosidase levels might be an important parameter in melibiose and raffinose use by *S. mutans*, we measured α-galactosidase activity in *S. mutans* wild type, *gtfA* insertion-duplication mutants (2), and *gtfA* deletion mutants (20). The results (Table 2) showed that (i) wild-type and mutant strains displayed approximately the same level of basal activity in FMC medium with 1% galactose; (ii) wild-type strains displayed a moderate but significant increase (about four to nine times the basal levels) of α-galactosidase activity when grown in the presence of 1% melibiose or 1% raffinose (raffinose was a slightly better inducer); (iii) addition of 0.1% melibiose or 0.1% raffinose to FMC medium with 1% galactose also raised the α-galactosidase levels in wild-type strains, but only to two to five times the basal levels; (iv) melibiose failed to induce α-galactosidase activity in *S. mutans gtfA* mutants, and raffinose induced only between 30 and 60% of the enzyme levels of their wild-type parents grown under the same conditions; and (v) intact cells displayed higher (up to three times) enzyme activities than permeabilized cells, suggesting that *S. mutans* α-galactosidase activity is (at least in part) accessible from the cell surface.

In summary, the effect of a mutation in the *gtfA* gene seemed to be quite specific on melibiose utilization, since *S. mutans gtfA* mutants were able to grow on various other sugars as sole carbon sources. The tight linkage between the *gtfA* gene and melibiose use was further verified by the isolation of *S. mutans* Mel$^+$ revertants. All revertants had a wild-type GtfA enzyme. The impairment of melibiose use in *S. mutans gtfA* mutants could be a direct consequence of inactivation of the *gtfA* gene or of a polar effect on the expression of another gene. In this regard, Burne et al. identified a gene coding for a 38-kDa protein following the *gtfA* gene (3). DNA sequence analysis of this region seemed to indicate that the 38-kDa protein gene is under transcriptional control by the *gtfA* gene (7). However, analysis of *S. mutans gtfA*$^+$/*gtfA* merodiploid strains (unpublished observations), constructed from insertion-duplication mutants, suggests a direct involvement of the *gtfA* gene. The studies with *E. coli* Mel$^-$ mutants showed that melibiose is not hydrolyzed or transported by the GtfA enzyme. Furthermore, melibiose had no effect on sucrose hydrolysis by the GtfA enzyme. The analysis of α-galactosidase activity in *S. mutans gtfA* mutant and parent strains showed that *S. mutans* may have two α-galactosidase activities, one inducible by melibiose and raffinose and another inducible by raffinose only. Induction of the former α-galactosidase activity by melibiose or raffinose might require the cooperation of the GtfA enzyme with the melibiose transport or hydrolysis machinery. In this regard, the finding of Russell et al. (22) that the GtfA enzyme is a sucrose phosphorylase led us to speculate that the GtfA enzyme might phosphorylate melibiose (without hydrolysis of the glycosidic bond) at the reducing carbon of the glucose moiety.

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