Mutants of *Citrobacter freundii* That Transport and Utilize Melibiose

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We have isolated mutants of *Citrobacter freundii* that can grow on melibiose. Inducible α -galactosidase activity and melibiose transport activity were detected in the mutant cells but not in the wild-type cells. We detected a DNA region which hybridized with *melB* (the gene for the melibiose transporter) DNA of *Escherichia coli* in the chromosomal DNA of wild-type *C. freundii*. Protons, but not sodium ions, were found to be the coupling cations for melibiose (and methyl- β -D-thiogalactoside) transport in the mutant cells.

The melibiose transporter of *Escherichia coli* is a secondary transporter which mediates symport of monovalent cations and melibiose or its analogs (16). This transporter is a valuable system for the investigation of structure-function relationships in a cation-coupled symporter. Either Na^+ , H^+ , or Li^+ is utilized as a coupling cation for transport of melibiose or other galactosides (or galactose). The coupling cation utilized varies depending on the substrate transported (16). Na^+ is the most effective coupling cation for melibiose transport, followed by H^+ and Li^+ (Li^+ is a poor coupling cation). With methyl- β -D-thiogalactoside (TMG) as the substrate, both Na^+ and Li^+ , but not H^+ , are utilized (5, 16). We cloned the gene (*melB*) encoding the melibiose transporter and sequenced it (3, 18). Thus, the primary structure of the melibiose transporter (MelB) was deduced. Mutational analysis revealed many amino acid residues that are important for the function of the melibiose transporter, especially for cation recognition (11, 17).

Analyses of functionally and structurally related proteins are valuable for the understanding of structure-function relationships in the proteins. Several microorganisms possess melibiose transporters. The melibiose transporters from *Salmonella typhimurium* (6), *Klebsiella pneumoniae* (2), *Enterobacter aerogenes* (9), and *Enterobacter cloacae* (8), in addition to *E. coli*, have been characterized and sequenced (13). Such analyses are also useful for understanding the evolutionary relationships of the transporters (and microorganisms).

Citrobacter freundii is a member of the *Enterobacteriaceae* and is often found in clinical specimens as an opportunistic or secondary pathogen (12). Although cells of *C. freundii* are able to utilize lactose as a carbon source (10), they are unable to utilize melibiose. Here we report the isolation of *C. freundii* mutants able to grow on melibiose. We also describe the properties of the melibiose transporter in the mutants.

Isolation of mutants. Cells of *C. freundii* ATCC 8090 grown in L medium (4) were densely streaked on agar plates containing a minimal medium (14) supplemented with 10 mM melibiose. Na^+ salts in the minimal medium were replaced with K^+ salts. After incubation at 37°C for 2 days, colonies appeared on the plates. Since these mutant cells utilized melibiose as a

carbon source, they must have expressed a transporter for melibiose and an enzyme for the degradation of melibiose. We isolated the colonies and purified them on agar plates containing minimal medium and melibiose. Thereafter, we measured the growth of two of the mutants, M4 and M7, on melibiose. The mutant cells grew well on melibiose, although the wild-type cells did not (data not shown). Cells of M4 showed better growth than cells of M7. The generation time for M7 was about 1.5 times longer than that for M4.

α -Galactosidase activity in the mutants. Wild-type and mutant cells of *C. freundii* were grown in minimal medium supplemented with 1% tryptone either in the absence or presence of 10 mM melibiose at 37°C under aerobic conditions, and α -galactosidase activity was measured as described previously (15). As shown in Table 1, cells of the wild type and M7 grown in the absence of melibiose had no α -galactosidase activity. Cells of M4 grown in the absence of melibiose, however, showed some α -galactosidase activity. When grown in the presence of melibiose, cells of M4 showed very high α -galactosidase activity, cells of M7 showed moderate activity, and wild-type cells showed no activity. Thus, cells of M4 and M7 possessed inducible α -galactosidase activities, although the activity was partially constitutive in M4 cells (Table 1).

Melibiose transporter in the mutants. For transport experiments, cells were grown in minimal medium supplemented with 1% tryptone and 10 mM melibiose at 37°C under aerobic conditions. Transport of [³H]melibiose (Rotem) and [¹⁴C]TMG (DuPont, NEN, Boston, Mass.) was measured as reported previously (5). Wild-type cells showed no melibiose transport activity, M7 cells showed some activity, and M4 cells showed higher activity than M7 cells (Fig. 1A). When TMG was used as the substrate, M4 cells showed very high activity and M7 cells showed moderate activity (Fig. 1B). M4 cells showed a little TMG transport activity when cells were grown in the absence of melibiose (data not shown).

We tested the effects of several sugars (50-fold excess) on TMG (0.1 mM) transport in M4 cells induced with melibiose. Among the sugars tested, galactosyl- β -D-thiogalactoside (thiodigalactoside) showed the strongest inhibition (82%), followed by melibiose (76%), galactose (48%), and lactose (48%).

Cation coupling in the mutants. Cation coupling to melibiose and TMG transport in the wild-type and mutant cells were investigated. For this experiment, cells of the wild type, M4, and M7 were grown as described above. Since transport of melibiose or TMG in cells of *E. coli* and *S. typhimurium* is stimulated by Na^+ or Li^+ (5, 7, 16), we tested the effects of Na^+

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TABLE 1. α -Galactosidase activity in wild-type and mutant cells of *C. freundii*

Strain	α -Galactosidase activity (units/mg of cell protein) ^a	
	Without melibiose	With melibiose ^b
Wild type	0.0	0.0
M4	4.4	23.5
M7	0.0	9.1

^a One unit is defined as the activity releasing 1 nmol of *p*-nitrophenol from *p*-nitrophenyl- α -D-galactopyranoside per min.

^b Melibiose was added to the culture medium at 10 mM.

or Li^+ on melibiose transport and on TMG transport in the *C. freundii* mutants. However, no significant effect was observed (data not shown). Thus, it seems that neither Na^+ nor Li^+ is a coupling cation for melibiose transport or TMG transport in the *C. freundii* mutants.

We then investigated whether H^+ and/or Na^+ uptake was observed when the transport substrate was added to the cell suspension by using ion-selective electrodes (H^+ electrode and Na^+ electrode), as described previously (16). We observed uptake of H^+ elicited by the addition of melibiose or TMG in M4 cells (Fig. 2), indicating that melibiose or TMG is taken up by cells by a mechanism of symport with H^+ . TMG gave a larger H^+ uptake than did melibiose. Cells of M7 showed some H^+ uptake elicited by the addition of melibiose or TMG. The wild-type cells showed no H^+ uptake. Uptake of Na^+ was not detected when melibiose or TMG was added to the cell suspension of M4, M7, or the wild type (data not shown). Thus, we concluded that the coupling cation for melibiose transport or TMG transport in the *C. freundii* mutants is H^+ .

***melB* homolog in *C. freundii*.** Since both α -galactosidase activity and melibiose transport activity were detected in the mutant cells but not in the wild-type cells, it seemed that wild-type *C. freundii* possesses a cryptic melibiose operon. We tested this possibility by Southern blot analysis with a DNA fragment derived from the *E. coli melB* gene used as a probe. Chromosomal DNA was prepared from cells grown in minimal medium supplemented with 1% tryptone, as described previously (1). Chromosomal DNA prepared from cells of *E. coli*, *S. typhimurium*, *C. freundii*, *Citrobacter amalonaticus*, or *Citrobacter diversus* was digested with *Bam*HI (except *S. typhimurium* DNA) or *Eco*RV (*S. typhimurium* DNA), separated by electrophoresis in a 1% agarose gel, and blotted onto a nitro-

FIG. 1. Melibiose and TMG transport activities in wild-type and mutant cells of *C. freundii*. Cells of the wild type (○), mutant M4 (●), or mutant M7 (▲) were grown in minimal medium supplemented with 1% tryptone and 10 mM melibiose at 37°C under aerobic conditions and assayed for melibiose transport (final concentration, 0.1 mM) (A) or TMG transport (final concentration, 0.1 mM) (B).

FIG. 2. Uptake of H^+ driven by downhill sugar entry into cells of *C. freundii*. Cells of the wild type, mutant M4, or mutant M7 were grown in minimal medium supplemented with 1% tryptone and 10 mM melibiose at 37°C under aerobic conditions. Changes in H^+ concentration in the assay medium (16) were measured with an H^+ electrode under anaerobic conditions at 25°C. At the time points indicated by the arrows, melibiose or TMG was added to the cell suspensions under anaerobic conditions to give a final concentration of 5 mM. Upward deflections of the curves indicate uptake of H^+ into cells.

cellulose membrane. The *melB* probe used was a *Bam*HI-*Bam*HI fragment (1.1 kbp) derived from the *melB* gene of *E. coli* (18). The probes were labeled with [³²P]dCTP by using a Multiprime DNA Labelling Kit (Amersham), as suggested by the manufacturer. The ³²P-labeled *melB* probe hybridized with the DNA blot on the nitrocellulose. As shown in Fig. 3, we detected a band which hybridized with the probe in a DNA digest from *C. freundii*. In a control experiment, we detected a hybridized band in a DNA digest from *E. coli* and *S. typhimurium* (Fig. 3). No hybridized band was detected with a DNA digest from *C. amalonaticus* (ATCC 25405) or *C. diversus* (ATCC 25408). Thus, we conclude that wild-type *C. freundii* possesses a cryptic *melB* homolog in the chromosomal DNA but that the other species of *Citrobacter*, *C. amalonaticus* and *C. diversus*, do not possess such a gene. It should be pointed out that we were unable to obtain mutants from *C. amalonaticus* and *C. diversus* that utilize melibiose (data not shown).

Cloning of the *melB*-like gene from *C. freundii* is now under way.

In all of the mutants tested, we detected both α -galactosi-

FIG. 3. Southern hybridization analysis. Chromosomal DNA prepared from *E. coli*, *S. typhimurium*, *C. freundii*, *C. amalonaticus*, or *C. diversus* was digested with *Bam*HI (except *S. typhimurium* DNA) or *Eco*RV (*S. typhimurium* DNA), separated by electrophoresis in a 1% agarose gel, and blotted onto nitrocellulose. The probe used was a *Bam*HI-*Bam*HI fragment (1.1 kbp) derived from the *melB* gene of *E. coli*. The position of the 1.1-kbp band is indicated.

dase activity and melibiose transport activity. Thus, it seems that a gene for α -galactosidase and a gene for the melibiose transporter are organized into an operon.

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