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A Cryptic Melibiose Transporter Gene Possessing a Frameshift from *Citrobacter freundii*[1]

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Wild-type *Citrobacter freundii* cannot grow on melibiose as a sole source of carbon. The melibiose transporter gene *melB* was cloned from a *C. freundii* mutant M4 that could utilize melibiose as a sole carbon source. Although the cloned *melB* gene is closely similar to the *melB* genes of other bacteria, it is cryptic because of a frameshift mutation. Site-directed mutagenesis was used to construct a functional *melB* gene by deleting one nucleotide, resulting in the production of an active melibiose transporter. The active MelB transporter could utilize Na^+ and H^+ as coupling cations to melibiose transport. The amino acid sequence of the *C. freundii* MelB was found to be most similar to those of *Salmonella typhimurium* and *Escherichia coli* MelB. These facts are consistent with the phylogenetic relationship of bacteria and the cation coupling properties of the melibiose transporters.

Key words: *Citrobacter freundii*, cotransport, frameshift mutation, lactose, melibiose.

The melibiose transporter is a well-characterized secondary transport system found in members of the *Enterobacteriaceae* family such as *Escherichia coli* (*1*), *Salmonella typhimurium* (*2*), *Klebsiella pneumoniae* (*3*), and *Enterobacter aerogenes* (*4*). The genes encoding these melibiose transporters have been cloned and sequenced. In addition, these transporters have been expressed and characterized. The deduced amino acid sequences show close similarity. One of the most important characteristics of the melibiose transporter is the versatility of its cation coupling. For instance, the melibiose transporter MelB of *E. coli* utilizes Na^+, H^+ and Li^+ as coupling cations depending on the substrate transported (*5*). Analyses of the structure–function relationships among related proteins are quite important for understanding not only the cation coupling and substrate recognition of MelBs, but also evolutionary aspects of these transporters and bacteria.

Citrobacter freundii is a member of the *Enterobacteriaceae* family. While cells of wild-type *C. freundii* are able to utilize lactose, they are unable to utilize melibiose (*6*). Spontaneous mutants that can grow on melibiose as a sole carbon source were isolated and shown to possess melibiose transport activity coupled with H^+ (*7*). Here we report the cloning and sequencing of the melibiose transporter gene (*melB*) from a *C. freundii* mutant.

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MATERIALS AND METHODS

Bacterial Strains—Wild-type *C. freundii* ATCC 8090 and mutants M4 and M7 (*7*) were used in this study. *E. coli* ER1647 (*8*) was used as a host for λ phage infection. *E. coli* BM25.8 (Novagen) (*9*) was used for automatic subcloning of a λ clone as described below. *E. coli* DW2 (*ΔlacZY, ΔmelB*) (*10*) was used as a host for the *melB* complementation test. *E. coli* RA11r (*ΔlacZY, melA, recA*) (*11*) and NO1 (*Δlac, ΔmelAB*) (*4*) were used as host strains for assays of α-galactosidase and melibiose transport activities, respectively. *E. coli* TG1 (*supE, hsdΔ5, thi, Δ(lac-proAB)*/F'[*traD36, proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15]) was used for all other genetic manipulations.

Cloning of the melB Gene—Chromosomal DNA was isolated from *C. freundii* mutant M4 cells by the method of Berns and Thomas (*12*). The DNA was partially digested with *Sau*3AI and separated by agarose gel electrophoresis. The DNA fragments between 10 and 23 kb were recovered, and a genomic DNA library of *C. freundii* was constructed using a λ phage replacement-type vector, λBlueSTAR, and a PhageMaker *in vitro* packaging system (Novagen). A candidate clone possessing the *melB* gene was selected by plaque hybridization using a *Bam*HI–*Bam*HI fragment (1.1 kb) of the *melB* gene of *E. coli* as a probe, since this fragment has been shown to hybridize to the *C. freundii* chromosome (*7*). The probe DNA was labeled with alkaline phosphatase, and a positive signal was detected by the AlkPhos Direct system (Amersham Pharmacia Biotech) used according to the manufacturer's protocol.

A positive plaque was chosen and the phage was used to infect *E. coli* BM25.8 to subclone the *C. freundii* genomic DNA fragment by Cre recombinase-mediated excision of a plasmid from the λBlueSTAR vector. *E. coli* TG1 cells were

retransformed with this plasmid to prevent further recombination. The subcloned plasmid was designated pCFM1 (Fig. 1).

Sequence of the Melibiose Operon—The nucleotide sequence of the melibiose operon was determined from both the sense and antisense strands via the dideoxy chain termination method (*13*) using a Thermo Sequenase dye terminator cycle sequencing kit (Amersham Pharmacia Biotech) and a DNA sequencer (ABI 373A).

Direct sequencing of the chromosomal DNAs was carried out as follows (*14*). First a 704-bp DNA fragment containing the mutation site was amplified by PCR using *C. freundii* chromosomal DNA as a template. Then it was separated on a polyacrylamide gel and recovered by electroelution from the gel. Next the sequence around the mutation site was determined from both strands by the dideoxy chain termination method described above using primers inside the amplified fragment.

α-Galactosidase Activity—*E. coli* RA11r cells were transformed with pCFM1, and the transformant cells were grown in M9 minimal salt medium (*15*) supplemented with 0.2% casamino acid and 40 mM glycerol in the presence or absence of 10 mM melibiose as an inducer at 30°C under aerobic conditions. α-Galactosidase activity was measured at 30°C as described previously (*16*).

Site-Directed Mutagenesis and Melibiose Transport Activity—Site-directed mutagenesis by PCR was used to construct the full-length *melB* gene in order to express the full-length MelB transporter. The nucleotide sequence corresponding to amino acid residue number 86 in MelB from *C. freundii* was changed from AGCG to GCG or ACG to make alanine (*E. coli* MelB type) or threonine (other type), respectively (see Fig. 3). At the same time, an *Nde*I site was introduced at the initiation codon and a *Bgl*II site was added near the termination codon of the *melB* gene by PCR using a CFmelB(N) primer (5′-TCCATATGAGCATCTCTC-TGAC-3′) and a CFmelB(C) primer (5′-TCAGATCTAGGC-CTTCACATCGC-3′), respectively. The wild-type *melB* gene was also amplified using the CFmelB(N) and CFmelB(C) primers. The mutated and amplified PCR fragments as well as the wild-type fragment were subcloned into the *Hinc*II site of pBluescript II SK- (*17*) and their sequences were confirmed. Then the mutant *melB* gene fragments were cut out with *Nde*I and *Bgl*II and inserted into the *Nde*I and the compatible *Bam*HI sites under the *lpp-lac* promoter of the expression vector pINIII(lpp^{p5})NdeI (*18*). The recombinant plasmids did not produce active MelBs (data not shown), possibly because of the overproduction of inactive membrane transporters. The *melB* upstream regions, including the ribosome binding sequence of pINIII-(lpp^{p5})NdeI, were cut out from the recombinant plasmids using *Xba*I and *Bam*HI, blunted with Klenow DNA polymerase, and ligated into the *Eco*RV site of pBR322 under the *tet* promoter. The *melB* downstream regions were cut out from the pBluescript II SK- recombinant plasmids using *Sac*II and *Bgl*II and ligated into the *Sac*II site of the *melB* gene and the *Bam*HI site of the *tet* gene in the pBR322 recombinant plasmids. Finally the recombinant pBR322 plasmids carrying the wild-type and mutated (Ala-86 and Thr-86) *melB*s were obtained and designated pCFMB1, pCFMB86A, and pCFMB86T, respectively. *E. coli* NO1 cells were transformed with these plasmids and the transformants were grown in M63 medium (*19*) supplemented with 1% Tryptone (Difco). The cells were not induced during growth since *melB* genes were supposed to be expressed from the *tet* promoter of pBR322. [^{3}H]Melibiose (Rotem) transport activities were measured as described previously (*20*).

Na^+ Uptake Elicited by Melibiose Influx—NO1/pCFMB1, NO1/pCFMB86A, and NO1/pCFMB86T cells were grown in Tanaka minimum medium (*21*) supplemented with 1% Tryptone and 50 μg/ml of ampicillin under aerobic conditions at 37°C. Cells were harvested at the late exponential phase of growth, washed three times with 0.1 M Mops-tetramethylammonium hydroxide (TMAH) buffer (pH 7.0), and suspended in the same buffer. The assay mixture contained 0.1 M Mops-TMAH buffer (pH 7.0), 25 μM NaCl, and cells (1 mg protein). The mixture was incubated at 25°C under anaerobic conditions (under N_2 gas), and anaerobic solution of melibiose was added to the mixture to give a final concentration of 10 mM. Changes in the Na^+ concentration of the assay mixture were monitored using an Na^+-selective electrode (Radiometer, Copenhagen).

H^+ Uptake Elicited by Melibiose Influx—NO1/pCFMB1, NO1/pCFMB86A, and NO1/pCFMB86T cells were grown in LB broth (*22*) supplemented with 50 μg/ml of ampicillin under aerobic conditions at 37°C. Cells were harvested at the late exponential phase of growth, washed three times with 120 mM choline chloride containing 2 mM $MgSO_4$, and resuspended in the same solution. The assay mixture contained 120 mM choline chloride, 2 mM $MgSO_4$, and cells (1 mg protein), and was incubated at 25°C under anaerobic

Fig. 2. **Nucleotide sequence of the *C. freundii melB* gene and its flanking regions, and the deduced amino acid sequence of MelB.** The translated amino acid sequence of MelB is shown under the nucleotide sequence starting from nucleotide number 199. Asterisks indicate termination codons. The amino acid sequences that overlap (corresponding to nucleotide numbers 452 to 486) indicate the frameshift (a one nucleotide insertion) region described in the text. The amino acid sequence of the carboxyl-terminal portion of the putative α-galactosidase is also shown in the upstream region of the *melB* gene. The sequence data reported here have been submitted to the DDBJ/GenBank/EMBL nucleotide sequence databases with the accession number AB037372. The sequence data for the *melR* and *melA* genes (data not shown) have also been submitted with the accession number AB053204.

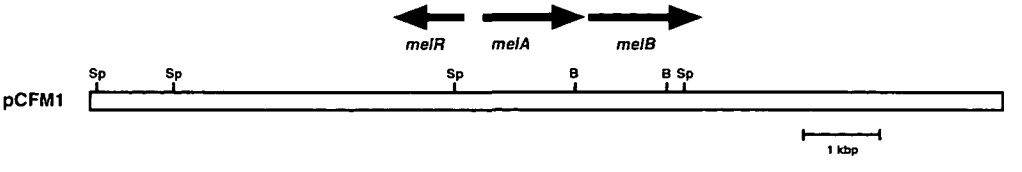

Fig. 1. **A restriction map of cloned *C. freundii* DNA containing the melibiose operon.** The boxed region shown at the top is the DNA fragment cloned from *C. freundii* in pCFM1. The location and direction of each gene are indicated by arrows. The restriction sites shown in the figure are: B, *Bam*HI; Sp, *Sph*I.

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        10         20         30         40         50         60         70         80         90
GGATCCGCATACCGCCGCGGTACTGGGGATTGAAGAGATTTATGCGCTGGTGGACGACCTGATCGCGTCTCACGGCGACTGGCTGCCGGC
 D  P  H  T  A  A  V  L  G  I  E  E  I  Y  A  L  V  D  D  L  I  A  S  H  G  D  W  L  P  A

       100        110        120        130        140        150        160        170        180
CTGGCTGCACCGTTAATACACAATCACGCCGGGCAGAGCATCCGGCGTTTAACCTCTGACGTTTCCTCTTACAGAAGGCGGGCGCTGGTA
 W  L  H  R  *

       190        200        210        220        230        240        250        260        270
ACAGCGACCCGATACCCTATGAGCATCTCTCTGACAACAAAACTCAGTTACGGATTTGGAGCGTTCGGTAAGGATTTCGCTATCGGTATT
                  M  S  I  S  L  T  T  K  L  S  Y  G  F  G  A  F  G  K  D  F  A  I  G  I

       280        290        300        310        320        330        340        350        360
GTGTACATGTATCTGATGTATTACTACACCGACGTGGTGGGATTATCGGTGGGCGTCGTCGGAACCTTGTTCCTGGTGGCAAAAATATGG
V  Y  M  Y  L  M  Y  Y  Y  T  D  V  V  G  L  S  V  G  V  V  G  T  L  F  L  V  A  K  I  W

       370        380        390        400        410        420        430        440        450
AACGCGTTTAACGATCCGGTGATGGGGTGGATAGTGAATGCCACCCGATCGCGCTGGGGAAAATTTAAACCCTGGATTTTACTCGGTACG
N  A  F  N  D  P  V  M  G  W  I  V  N  A  T  R  S  R  W  G  K  F  K  P  W  I  L  L  G  T

       460        470        480        490        500        510        520        530        540
CTGAGCGAACTCATTGGTGCTGTTTTTGCTTTTTAGCGCACATCTTTTTGAGGGTACTGCGCAGGTCGTATTTGTTTGCGTGACGTATAT
L  S  E  L  I  G  A  V  F  A  F  *
 *  A  N  S  L  V  L  F  L  L  F  S  A  H  L  F  E  G  T  A  Q  V  V  F  V  C  V  T  Y  I

       550        560        570        580        590        600        610        620        630
TTTATGGGGAATGACCTACACCATTATGGATATTCCATTCTGGTCACTGGTGCCAACCATTACGCTGGATAAACGCGAGCGTGAACAACT
 L  W  G  M  T  Y  T  I  M  D  I  P  F  W  S  L  V  P  T  I  T  L  D  K  R  E  R  E  Q  L

       640        650        660        670        680        690        700        710        720
GGTGCCGTATCCCCGTTTCTTCGCCAGCCTTGCCGGGTTTGTCACCGCGGGCGTGACGCTACCCTTTGTAAACTATGTTGGCGGCGCGGA
 V  P  Y  P  R  F  F  A  S  L  A  G  F  V  T  A  G  V  T  L  P  F  V  N  Y  V  G  G  A  D

       730        740        750        760        770        780        790        800        810
TCGCGGGTTCGGTTTTCAGATGTTTACGCTGGTGCTGATCGCATTTTTTATTGTTTCTACCCTCGTAACCTTGCGCAATGTGCATGAAGT
 R  G  F  G  F  Q  M  F  T  L  V  L  I  A  F  F  I  V  S  T  L  V  T  L  R  N  V  H  E  V

       820        830        840        850        860        870        880        890        900
GTATTCCTCTGACAGCGAAGCTACCGCAGACAGCACCCGGCTGACGCTGAAATCAATCCTTGCGCTTATCTACAAAAACGATCAGCTTTC
 Y  S  S  D  S  E  A  T  A  D  S  T  R  L  T  L  K  S  I  L  A  L  I  Y  K  N  D  Q  L  S

       910        920        930        940        950        960        970        980        990
CTGCCTGCTGGGGATGGCGCTGGCTTACAACGTGGCGTCCAATATCATTACAGGTTTTGCGATTTACTACTTCACCTACGTGGTGGGTGA
 C  L  L  G  M  A  L  A  Y  N  V  A  S  N  I  I  T  G  F  A  I  Y  Y  F  T  Y  V  V  G  D

      1000       1010       1020       1030       1040       1050       1060       1070       1080
TGCTGACCTTTTCCCATACTACCTCTCCTATGCCGGAGCAGCTAACTTGCTGACATTGATTCTCTTTCCCCGTCTGTCAAAGGCGCTTTC
 A  D  L  F  P  Y  Y  L  S  Y  A  G  A  A  N  L  L  T  L  I  L  F  P  R  L  S  K  A  L  S

      1090       1100       1110       1120       1130       1140       1150       1160       1170
TCGCCGGGTACTGTGGGCTGGCGCTTCGGTTATGCCAGTTCTCAGCGCAGGCGTATTGCTGATTATGGCTCTGGGGGCATTCCACAATAC
 R  R  V  L  W  A  G  A  S  V  M  P  V  L  S  A  G  V  L  L  I  M  A  L  G  A  F  H  N  T

      1180       1190       1200       1210       1220       1230       1240       1250       1260
TTTCCTGATTGTTACCGCAGGGATCCTTCTGAACATTGgTACGGCGCTGTTCTGGGTACTGCAGGTGATTATGGTGGCGGACACCGTAGA
 F  L  I  V  T  A  G  I  L  L  N  I  G  T  A  L  F  W  V  L  Q  V  I  M  V  A  D  T  V  D

      1270       1280       1290       1300       1310       1320       1330       1340       1350
TTACGGTGAATACAAGTTTAACGTCCGCTGCGAGAGTATTGCCTATGCCGTGCAGACAATGGTGGTCAAAGGTGGCTCGGCGTTTTCCGC
 Y  G  E  Y  K  F  N  V  R  C  E  S  I  A  Y  A  V  Q  T  M  V  V  K  G  G  S  A  F  S  A

      1360       1370       1380       1390       1400       1410       1420       1430       1440
TTTCTTTATTGCGGTGGTGCTGGGATTGATTGGTTATGTTCCGAATGCGACGCAATCTGCTGAGACCATCCTCGGCATGCAGTTCATCAT
 F  F  I  A  V  V  L  G  L  I  G  Y  V  P  N  A  T  Q  S  A  E  T  I  L  G  M  Q  F  I  M

      1450       1460       1470       1480       1490       1500       1510       1520       1530
GATCGCGCTGCCCACGCTTTTTTTCGTTGTGACACTGGTGCTCTATTTCCGTTACTACCGGTTGAATGGCGATATGCTGCGTAAGATTCA
 I  A  L  P  T  L  F  F  V  V  T  L  V  L  Y  F  R  Y  Y  R  L  N  G  D  M  L  R  K  I  Q

      1540       1550       1560       1570       1580       1590       1600       1610       1620
GATCCACCTGCTGGATAAGTACCGAAAAGTGCCGTCGCCCGTCAAACAGTCGAATACTCCGGAGATTCCGGTGGTAGTGGCCAGCGATGT
 I  H  L  L  D  K  Y  R  K  V  P  S  P  V  K  Q  S  N  T  P  E  I  P  V  V  V  A  S  D  V

      1630       1640       1650       1660       1670       1680       1690       1700       1710
GAAGGCCTAGCTATGGAGTGGCATTCCTGGATTGGCTATCTGGCGGCAACGCTGACGACGCTCTCTTTTTTGCCGCAGGCGATTAAGGTT
 K  A  *

      1720       1730       1740       1750       1760       1770       1780       1790       1800
ATTACCACCCGCAACACCCAGGGTATCTCCGGTTTAATGTATGTGATGTTTGTGTGCGGGCTGGTGTTGTGGCTGGCTTATGGGTTATTG

      1810       1820       1830       1840       1850
ATTGAAGATACCGCTGTCAGCATGGCGAACTTTCTGACACTGTTGTTCGCC
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conditions (under N_2 gas). An anaerobic solution of melibiose was added to the mixture to give a final concentration of 10 mM.

β-Galactosidase Activity—Cells of each *C. freundii* strain were grown in Tanaka minimal salt medium (*21*) supplemented with 1% Tryptone in the presence or absence of 10 mM melibiose at 37°C under aerobic conditions. The β-Galactosidase activity of each strain was measured as described previously (*6, 23*).

Protein Assay—Protein content was determined by the method of Lowry *et al.* (*24*) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Cloning of the melB Gene—Although wild-type *C. freun-*

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C. f.   MSISLTTKLSYGFGAFGKDFAIGIVYMYLMYYYTDVVGLSVGVVGTLFLVAKIWNAFNDP 60
S. t.   MSISLTTKLSYGFGAFGKDFAIGIVYMYLMYYYTDVVGLSVGLVGTLFLVARIWDAINDP 60
E. c.   MSISMTTKLSYGFGAFGKDFAIGIVYMYLMYYYTDVVGLSVGLVGTLFLVARIWDAINDP 60
K. p.   MSISMTTKLSYGFGAFGKDFAIGIVYMYLMYYYTDIVGLSVGVVGTLFLVARILDAIADP 60
E. a.   MSISMTTKLSYGFGAFGKDFAIGIVYMYLMYYYTDIVGLSVGVVGTLFLVARILDAIADP 60
        **** ******************************** ****** ******** *  *  **

C. f.   VMGWIVNATRSRWGKFKPWILLGTLXNSLVLFLLFSAHLFEGTAQVVFVCVTYILWGMTY 120
S. t.   IMGWIVNATRSRWGKFKPWILIGTLTNSLVLFLLFSAHLFEGTAQVVFVCVTYILWGMTY 120
E. c.   IMGWIVNATRSRWGKFKPWILIGTLANSVILFLLFSAHLFEGTTQIVFVCVTYILWGMTY 120
K. p.   IMGWIVNCTRSRWGKFKPWILIGTITNSVVLYMLFSAHHFSGGALLAWVWLTYLLWGFTY 120
E. a.   IMGWIVNCTRSRWGKFKPWILIGTITNSVVLYMLFSAHHFSGGALLAWVWLTYLLWGFTY 120
         ****** ************* **  **  *  ***** * *      *  ** *** **

C. f.   TIMDIPFWSLVPTITLDKREREQLVPYPRFFASLAGFVTAGVTLPFVNYVGGADRGFGFQ 180
S. t.   TIMDIPFWSLVPTITLDKREREQLVPFPRFFASLAGFVTAGITLPFVSYVGGADRGFGFQ 180
E. c.   TIMDIPFWSLVPTITLDKREREQLVPYPRFFASLAGFVTAGVTLPFVNYVGGGDRGFGFQ 180
K. p.   TIMDVPFWSLVPTITLDKREREQLVPYPRFFASLAGFVTAGVTLPFVNAVGGADRGFGFQ 180
E. a.   TIMDVPFWSLVPTITLDKREREQLVPYPRFFASLAGFVTAGVTLPFVSAVGGADRGFGFQ 180
        **** ********************** ************** *****  *** *******

C. f.   MFTLVLIAFFIVSTLVTLRNVHEVYSSDSEATADSTRLTLKSILALIYKNDQLSCLLGMA 240
S. t.   MFTLVLIAFFIASTIVTLRNVHEVYSSDNGVTAGRPHLTLKTIVGLIYKNDQLSCLLGMA 240
E. c.   MFTLVLIAFFIVSTIITLRNVHEVFSSDNQPSAEGSHLTLKAIVALIYKNDQLSCLLGMA 240
K. p.   MFTLVLIAFFVVSTLVTLRNVHEVYSSDSGVSEDSSHLSLRQMVALIYKNDQLACLLGMA 240
E. a.   MFTLVLIAFFVISTLVTLRNVHEVYSSDSGVSEDSSHLSLGQMVALIYKNDQLACLLGMA 240
        ********** **  ******** ***         * *     ******** ******

C. f.   LAYNVASNIITGFAIYYFTYVVGDADLFPYYLSYAGAANLLTLILFPRLSKALSRRVLWA 360
S. t.   LAYNIASNIINGFAIYYFTYVIGDADLFPYYLSYAGAANLLTLIVFPRLVKMLSRRILWA 360
E. c.   LAYNVASNIITGFAIYYFSYVIGDADLFPYYLSYAGAANLVTLVFFPRLVKSLSRRILWA 360
K. p.   LAYNTAANIIAGFAIYYFTYVIGSAEMFPYYMSYAGAANLLTLILFPRLVKGLSRRILWA 360
E. a    LAYNTAANIIAGFAIYYFTYVIGSAEMFPYYMSYAGAANLLTLILFPRLVKGLSRRILWA 360
        **** * *** ******* ** * *  **** ******** **  **** * **** ***

C. f.   GASVMPVLSAGVLLIMALGAFHNTFLIVTAGILLNIGTALFWVLQVIMVADTVDYGEYKF 360
S. t.   GASVMPVLSCAGLFAMALADIHNAALIVAAGIFLNIGTALFWVLQVIMVADTVDYGEFKL 360
E. c.   GASILPVLSCGVLLLMALMSYHNVVLIVIAGILLNVGTALFWVLQVIMVADIVDYGEYKL 360
K. p.   GASIMPVLGCGVLLLMALGGVYNIALISLAGVLLNIGTALFWVLQVIMVADTVDYGEYTM 360
E. a.   GASIMPVLGCGVLLLMALSGVYNIALISLAGVLLNIGTALFWVLQVIMVADTVDYGEYTM 360
        ***  ***    *  ***    *  **  **  ** ********************* *****

C. f.   NVRCESIAYAVQTMVVKGGSAFSAFFIAVVLGLIGYVPNATQSAETILGMQFIMIALPTL 420
S. t.   NIRCESIAYSVQTMVVKGGSAFAAFFIALVLGLIGYTPNVAQSAQTLQGMQFIMIVLPVL 420
E. c.   HVRCESIAYSVQTMVVKGGSAFAAFFIAVVLGMIGYVPNVEQSTQALLGMQFIMIALPTL 420
K. p.   NIRCESIAYSVQTLVVKAGSAFAAWFIAIVLGIIGYVPNVVQSSHTLLGMQAIMIALPTL 420
E. a    NIRCESIAYSVQTLVVKAGSAFAAWFIAIVLGIIGYVPNTAQSPHTLLGMQAIMIALPTL 420
          ******* *** *** **** * *** *** *** **  **     *** *** ** *

C. f.   FFVVTLVLYFRYYRLNGDMLRKIQIHLLDKYRKVPSPVKQSNTPEIPVVVASDVKA     476
S. t.   FFMMTLVLYFRYYRLNGDMLRKIQIHLLDKYRKTPPFVEQPDSPAISVVATSDVKA     476
E. c.   FFMVTLILYFRFYRLNGDTLRRIQIHLLDKYRKVPP---EPVHADIPVGAVSDVKA     473
K. p.   FFALTLFLYFRYYKLNGDMLRRIQIHLLDKYRRVPE---NDVEPERPIVVPNQV       471
E. a.   FFALTLFLYFRYYKLNGDMLRRIQIHLLDKYRRVPE---NVVEPERPIVVPNQV       471
        **  ** **** * **** ** **********  *                 *
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Fig. 3. **Alignment of the amino acid sequences of five MelBs.** The deduced amino acid sequences of MelBs from *C. freundii (C. f.), S. typhimurium (S. t.), E. coli (E. c.), K. pneumoniae (K. p.),* and *E. aerogenes (E. a.)* are aligned. Asterisks indicate identical amino acid residues among the five melibiose transporters. The amino acid residue corresponding to the frameshift point of *C. freundii* MelB is indicated as "X" and circled.

dii can not grow on melibiose as a sole source of carbon, the mutants M4 and M7, which can utilize melibiose, have been isolated (*7*). Intensive efforts have been made to clone the *melB* gene from *C. freundii* mutants by complementation of a melibiose-negative mutant, which is the method used to clone other *melB* genes. However the attempts failed and led to the cloning of the lactose transporter gene *lacY* (*25*). Here, we cloned the *melB* gene by the plaque hybridization method described in "MATERIALS AND METHODS." The cloned DNA fragment was about 12 kb in length (Fig. 1) and shown to hybridize with the *E. coli melB* gene by the Southern hybridization method (data not shown). To confirm that pCFM1 carries the *melB* gene, *E. coli* DW2 (*ΔlacZY, ΔmelB*) (*10*) was transformed with pCFM1 and plated on a MacConkey melibiose agar plate. The DW2/pCFM1 cells formed white colonies, meaning that the transformant cells did not ferment melibiose. This result indicates that the *melB* gene cloned in pCFM1 was partial or cryptic.

Sequences of the melB Gene and the MelB Transporter—The sequencing data suggest that the cloned DNA fragment in pCFM1 contains the *melA* gene, which encodes α-galactosidase, and the *melR* gene, which encodes the transcriptional positive regulator for the melibiose operon (data not shown), and that the organization of this operon in *C. freundii* is the same as the melibiose operon of *E. coli* (Fig. 1) (*11, 26*). Although pCFM1 contains the whole *melB* gene of *C. freundii*, there is a one-nucleotide insertion, resulting in a frameshift mutation compared with functional *melB* of *E. coli* (Fig. 2) (*1*). According to the amino acid sequence alignment data of *C. freundii* MelB with other MelBs, the frameshift mutation must have taken place at the position corresponding to amino acid residue 86 (X in Fig. 3). This could account for the fact that pCFM1 did not complement the *melB* deletion in *E. coli* DW2 as described above. To rule out the possibility that the frameshift mutation occurred during cloning, we determined the chromosomal sequence around the mutation site of mutant M4 as well as mutant M7 and the wild-type *C. freundii* by direct DNA sequencing of double-stranded PCR products as described in "MATERIALS AND METHODS." The results showed that the M4, M7, and wild type chromosome contain the same frameshift mutation (data not shown).

The amino acid sequence of *C. freundii* MelB was compared with those of MelBs from *S. typhimurium* (*2*), *E. coli* (*1*), *K. pneumoniae* (*3*), and *E. aerogenes* (*4*), as shown in Fig. 3. If the frameshift mutation at amino acid residue 86 is ignored, the *C. freundii* MelB shows sequence identities of 86, 84, 79, and 78% with MelBs from *S. typhimurium, E. coli, K. pneumoniae*, and *E. aerogenes*, respectively.

α-Galactosidase Activity—In order to examine whether only the *melB* gene is inactive or the expression of the melibiose operon is cryptic, α-galactosidase activity was measured as described previously (*16*). When RA11r/pCFM1 cells were grown at 37°C, they had almost no α-galactosidase activity (data not shown). When the same cells were grown at 30°C, however, they showed constitutive α-galactosidase activity (Table I). This type of temperature-sensitive expression of the melibiose operon has been well characterized in *E. coli* (*27*). The melibiose operon from *C. freundii* seems to be regulated in the same manner. In contrast, RA11r/pSTY81-30 cells carrying the positive regulatory gene (*melR*) and *melA* from *E. coli* (*11*) had inducible α-galactosidase activity. This result reveals that the cloned *melA* gene of *C. freundii* in pCFM1 produces active α-galactosidase and that the expression of the melibiose operon is not cryptic. Since the *C. freundii* mutant M4 shows inducible α-galactosidase activity (*7*) and pCFM1 possesses the *melR* positive regulatory gene (see Fig. 1), the constitutive expression of the cloned *melA* indicates that another repressor might be required for full repression in *C. freundii* cells.

Site-Directed Mutagenesis and Melibiose Transport Activity—To investigate whether the cryptic *melB* gene has the potential to produce an active melibiose transporter, we introduced frameshift mutations (one nucleotide deletion) to construct full-length MelB transporters as described in "MATERIALS AND METHODS." NO1/pCFMB1 cells, which express the wild-type *melB* gene, showed no melibiose transport activity (Fig. 4). On the other hand, NO1/pCFMB86A cells, which have alanine (*E. coli* type) at amino acid residue 86, and NO1/pCFMB86T cells, which have threonine (other bacterial type) at the same position, showed melibiose transport activities. These results indicate that only the frameshift found in the *melB* gene of *C. freundii* causes the inactivation of MelB, and that the *C. freundii melB* has the potential to produce an active melibiose transporter when the frameshift is corrected.

Cation Coupling in Mutagenized Melibiose Transporters—Cation coupling to melibiose transport in NO1/pCFMB86A and NO1/pCFMB86T cells was examined. We used ion-selective electrodes (Na^+-electrode and H^+-elec-

TABLE I. **α-Galactosidase activity in *E. coli* transformants.** Cells were grown in the absence or presence of 10 mM melibiose as described in "MATERIALS AND METHODS."

Strain	α-Galactosidase activity (units/mg of cell protein)[a]	
	Without melibiose	With melibiose
RA11r/pUC19	0	0
RA11r/pCFM1	17	21
RA11r/pSTY81-30	6.0	48

[a]One unit of α-galactosidase activity is defined as that hydrolyzing 1 nmol of *p*-nitrophenyl-α-D-galactopyranoside per min.

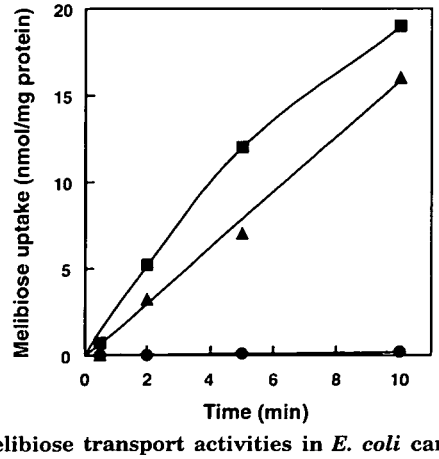

Fig. 4. **Melibiose transport activities in *E. coli* carrying recombinant *melB* genes from *C. freundii*.** Uptake of [^{3}H]melibiose (0.1 mM) was measured in NO1/pCFMB1 (●), NO1/pCFMB86A (■), and NO1/pCFMB86T (▲). 10 mM NaCl was added to the assay mixture.

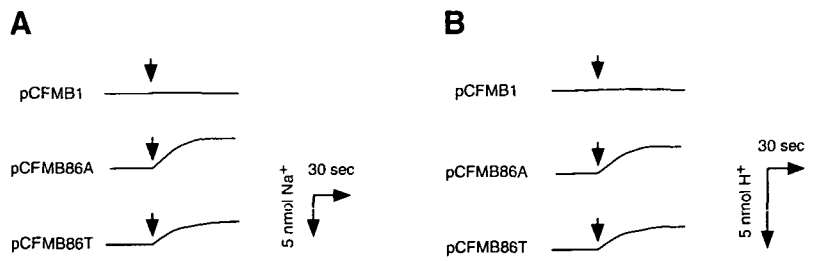

Fig. 5. **Uptake of cations elicited by melibiose influx *via* the melibiose transporter in *E. coli* NO1/pCFMB1, NO1/pCFMB86A, and NO1/pCFMB86T.** A, Uptake of Na^+. Cells (1 mg protein) were suspended in buffer comprising 0.1 M Tricine-TMAH (pH 8.0) and 25 μM NaCl. Na^+ uptake was measured using an Na^+-selective electrode under anaerobic conditions. B, Uptake of H^+. Cells (1 mg protein) were suspended in a solution containing 120 mM choline chloride and 2 mM MgSO4. H^+ uptake was measured using an H^+ electrode under anaerobic conditions. At the time points indicated by the arrows, an anaerobic solution of melibiose was added (final concentration 10 mM). Upward deflections of the chart correspond to the uptake of cations by the cells.

TABLE II. **β-Galactosidase activity in wild type and mutant *C. freundii* cells.** Cells were grown in the absence or presence of 10 mM melibiose as described in "MATERIALS AND METHODS."

Strain	β-Galactosidase activity (units/mg of cell protein)[a]	
	Without melibiose	With melibiose
Wild type	0	0
M4	155	349
M7	0	248

[a]One unit of β-galactosidase activity is defined as that hydrolyzing 1 nmol of *p*-nitrophenyl-β-D-galactopyranoside per min.

trode) to investigate whether Na^+ and/or H^+ uptake can be observed when melibiose is added to a cell suspension. Na^+ uptake elicited by the addition of melibiose was observed in both NO1/pCFMB86A and NO1/pCFMB86T cells (Fig. 5A), but not in NO1/pCFMB1 cells. This result is consistent with the results of the melibiose transport assay described above. Both NO1/pCFMB86A and NO1/pCFMB86T cells also showed H^+ uptake elicited by the addition of melibiose (Fig. 5B). Thus we conclude that the coupling cations for melibiose transport in the full-length melibiose transporter constructed by site-directed mutagenesis are Na^+ and H^+. These results are different from those observed in *C. freundii* mutants M4 and M7 (*7*), in which only H^+ uptake is coupled to melibiose transport. It is likely that melibiose is transported through the lactose transporter in the M4 and M7 mutants, as discussed below in conjunction with the results of β-galactosidase activities.

One of the most important amino acid residues for Na^+ uptake coupled to melibiose is the asparagine-58 residue. In *K. pneumoniae*, only H^+ is a coupling cation for melibiose transport, and the melibiose transporter in this bacterium has an alanine at amino acid residue number 58. However, when this alanine is replaced by asparagine, the mutagenized transporter is able to couple Na^+ to melibiose transport (*28*). Since the melibiose transporter of *C. freundii* has an asparagine residue position 58 (see Fig. 3), the Na^+ coupled melibiose transport we observed is consistent with the previous findings.

β-Galactosidase Activities in C. freundii Mutants—*C. freundii* has been shown to have inducible β-galactosidase and lactose transport activities (*6*). We next investigated whether melibiose can induce the lactose operon in *C. freundii*. As shown in Table II and as described by Okazaki *et al.* (*6*), the β-galactosidase activity of wild-type *C. freundii* is not induced by melibiose. In contrast, the β-galactosidase activities of the melibiose-utilizing mutants M4 and M7 are induced by melibiose, suggesting that the repressor for the lactose operon and/or some other unknown transcriptional regulator(s) are able to recognize melibiose as an inducer in those mutants.

It is likely that the melibiose transport activities in *C. freundii* mutants M4 and M7 are due to the lactose transport system. In fact, *E. coli* carrying the cloned lactose transporter gene (*lacY*) from *C. freundii* accumulate melibiose (*25*). In wild-type *C. freundii*, the lactose operon is not induced by melibiose (see Table II). On the other hand, β-galactosidase activities are induced by melibiose in mutant M7 and semi-constitutively in mutant M4. Therefore the lactose transporter gene (*lacY*) and the α-galactosidase gene (*melA*) are induced by melibiose in the M4 and M7 mutants and melibiose is transported through LacY. Thus, the M4 and M7 mutants are able to grow on melibiose as a sole source of carbon.

Evolutionary Aspects of the MelB Transporter and Bacteria—The MelB transporter of *C. freundii* shows the highest amino acid sequence homology with that of *S. typhimurium* and the second highest with that of *E. coli*, as described above (Fig. 3). Judging from the amino acid sequence homologies among MelB transporters of the five bacteria examined, *C. freundii*, *S. typhimurium*, and *E. coli* seem to be closely related microorganisms forming one group, while *K. pneumoniae* and *E. aerogenes* form another group. In fact, according to the phylogenetic relationship, these two groups diverged in the past, and *Citrobacter* is placed between *Salmonella* and *E. coli* (*29*). These facts are consistent with the cation coupling properties of the melibiose transporters. The MelB transporters of the former group including *C. freundii* show Na^+ coupling to melibiose transport (Fig. 5). On the other hand, the latter group does not show Na^+-coupled melibiose transport (*3, 4*).

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