Primary Structure and Characteristics of the Melibiose Carrier of
*Klebsiella pneumoniae*

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The melB gene coding for the melibiose carrier of *Klebsiella pneumoniae* was cloned and sequenced. There were two potential translation initiation sites. It was predicted that the melibiose carrier consists of 471 (or 467) amino acid residues. Seventy-eight percent of the 471 amino acids were identical to the *Escherichia coli* melibiose carrier.

Sugar transport characteristics were studied using an *E. coli* mel-*C* mutant expressing cloned *K. pneumoniae* melB gene. Accumulation of melibiose via the *K. pneumoniae* melibiose carrier was not stimulated by adding NaCl or LiCl which stimulates melibiose accumulation via the *E. coli* melibiose carrier. Lactose was accumulated only in the presence of LiCl. TMG (methyl-1-thio-β-D-galactopyranoside) was accumulated in the absence of added NaCl or LiCl. The accumulation was stimulated by LiCl but not by NaCl. Rapid H⁺ uptake was observed when melibiose or TMG was added to cell suspensions. These results suggest that the preferred cation couplings via *K. pneumoniae* melibiose carrier are H⁺-melibiose, Li⁺-lactose, and H⁺/Li⁺-TMG. This coupling spectrum is quite different from that of the *E. coli* melibiose carrier. It is of special interest that the *K. pneumoniae* melibiose carrier appears to lack the ability to recognize Na⁺ which is a preferred coupling cation of the *E. coli* melibiose carrier for all known sugar substrates. Further investigation of these two carriers may give us insight into the Na⁺ recognition site.

The melibiose carrier of *Escherichia coli* is a cytoplasmic membrane protein which is responsible for cation-galactoside cotransport (see Wilson et al. (1982) and Tsuchiya et al. (1985), for reviews). While H⁺ is the sole coupling cation in many of the bacterial cotransport systems, the melibiose carrier is able to utilize H⁺, Na⁺, or Li⁺ depending on sugar substrate (Tsuchiya and Wilson, 1978; Tsuchiya et al., 1983; Wilson and Wilson, 1987). The melibiose carrier is encoded by the melB gene, and the sequence has been determined (Hanatani et al., 1984; Yazyu et al., 1984). It is predicted that the melibiose carrier is a hydrophobic protein consisting of 469 amino acid residues. Based on hydropathy analysis, binding of anti-carboxyl terminus antibody, and the study of carrier-bacterial alkaline phosphatase fusion proteins, the melibiose carrier is considered to have 12 membrane-spanning domains (Botfield, 1989; Botfield and Wilson, 1989b; Botfield et al., 1992).

Recently, specific involvement of certain amino acid residues in recognition of cation and sugar have been suggested through the analyses of mutant carriers. Amino acid substitutions at positions 122, 142, 232, and 236 have been shown to cause alterations in H⁺ and Li⁺ recognition (Niiya et al., 1982; Tsuchiya et al., 1983; Shiotani et al., 1985; Yazyu et al., 1985; Kawakami et al., 1988).

Botfield and Wilson isolated 71 mutants with impaired methyl-β-D-thiogalactoside (TMG)¹ recognition. When sequenced these mutants had a total of 23 different single amino acid substitutions and one double mutation clustered into four distinct regions near the cytoplasmic surface of the membrane (Botfield and Wilson, 1988; Botfield, 1989). It is of interest that three of the 23 single substitutions were the same substitutions found in the cation recognition mutants described above. Furthermore, all but one of their sugar recognition mutants showed decreased Li⁺ recognition. These observations suggest that recognition sites for H⁺, Li⁺, and sugar are overlapping.

Our knowledge concerning the Na⁺ recognition site is more limited. So far, no Na⁺ recognition mutant has been isolated by random mutagenesis. Through the analysis of a mutant with a substitution at aspartate 55, which was generated by site-specific mutagenesis, it has been suggested that this residue is in or near the Na⁺ binding site and that this cation binding site and sugar binding site overlap (Pourcher et al., 1991).

Although site-specific mutagenesis is a quite useful method to analyze the function of each amino acid residue, the interpretation of the results is often difficult because mutants thus generated are sometimes severely impaired in transport function itself. We therefore undertook a comparative approach to learn more about cation and sugar recognition sites. Our preliminary studies suggested that TMG accumulation in *Klebsiella pneumoniae* was not affected by Na⁺ which is a coupling cation for transport of this sugar in *E. coli.*² It was considered that by comparing the primary structures of the melibiose carriers of *E. coli* and *K. pneumoniae*, the binding sites for Na⁺ and other substrates might be revealed.

In this communication we report the cloning and sequencing of the melB gene of *K. pneumoniae*. Because of two potential translation initiation sites the gene could encode a polypeptide of either 467 or 471 amino acid residues. Seventy-eight percent of the 471 amino acid residues was found to be

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¹ The abbreviations used are: TMG, methyl-1-thio-β-D-galactopyranoside; [35S]dATP, deoxyadenosine-5'-triphosphate; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; MOPS, 3-(N-morpholino)propanesulfonic acid; bp, base pairs; kb, kilobase(s).

² M. Wilson and T. H. Wilson, unpublished observation.
identical to the E. coli melibiose carrier. In spite of this high similarity in primary structure, the K. pneumoniae melibiose carrier fails to use Na+ as a coupling cation.

**MATERIALS AND METHODS**

Reagents—L-a-a-DTAP and [14C]lactose were purchased from Amer sham Corp.; [3H]MG was purchased from Du Pont-New England Nuclear. [3H]Melibiose was a generous gift from Dr. Gérard Lelaine, Commissariat à l'Energie Atomique, France. The nonradioactive sugars were obtained from Sigma. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. The Sequenase kit was purchased from United States Biochemical. All other chemicals were reagent grade.

**Bacterial Strains and Plasmids—** K. pneumoniae 2002 (Tanaka et al., 1967) was grown in LB medium containing 20 μg/ml chloramphenicol or 50 μg/ml ampicillin, harvested at late log phase, washed twice with 150 mM KC1, and resuspended in 120 mM KC1 plus 2 mM 2-mercaptoethanol. Samples were incubated at 37°C for 2 h, and the reaction was terminated by the addition of 1 ml of 0.6 M NaCl. The alkaline pH used to lyse the bacteria allows for the development of p-nitrophenol, a product of hydrolysis of p-nitrophenol-α-D-galactoside by α-galactosidase. Absorbance at 420 nm was taken for each sample after cells were removed by centrifugation.

**α-Galactosidase Activity—** To test α-galactosidase activity of a vector plasmid, cells were grown in LB medium containing 1 mg/ml of 30 μg/ml chloramphenicol until the cell density reached approximately 1 × 10^8 cells/ml. The cells were resuspended in the same buffer at a final concentration of 3 × 10^8 cells/ml. Washed cells were incubated in the same buffer with [14C]lactose (0.1 mM, 0.2 μCi/ml), [3H]melibiose (0.1 mM, 0.2 μCi/ml), or [14C][3H]MG (0.1 mM, 0.2 μCi/ml) at 25°C. NaCl (10 mM) or LiCl (10 mM) was added as indicated. Samples were withdrawn at each time point and filtered through 0.65-μm pore size cellulose nitrate membrane filters (Sartorius). After washing with 5 ml of the same buffer, filters were placed in 4 ml of 1 M NaOH (National Diagnostics) and counted. The volume of intracellular water was taken as 0.4 μ1 per 6 × 10^9 cells to calculate sugar accumulation calculated from 3.7 ± 0.2 μg/mg protein, Shiotz et al., 1985).

**Measurement of H+ Movement—** Proton uptake coupled to sugar transport was measured by the method of West (West, 1970) with slight modifications. OH- wells grown in LB medium containing 20 μg/ml chloramphenicol or 50 μg/ml ampicillin, harvested at late log phase, washed twice with 150 mM KCl, and resuspended in 120 mM KCl plus 30 mM KSCN to a final concentration of 6 × 10^8 cells/ml. Cells (2.5 ml) were placed in a plastic vial, and a combined pH electrode (Radiometer, Copenhagen) was inserted through a hole in the plastic lid. H₂O-saturated Ngas was continuously passed through one of the two vents in the lid to keep cells anaerobic. After stirring for 20 min at room temperature (about 22°C) anaerobic melibiose or TMG solution (0.5 or 1 M) was introduced into the other vent. The pH values were recorded with a Linear Instruments recorder. Calibrations were carried out by adding a known amount of KOH.

**RESULTS**

Cloning of the mel Opolon of K. pneumoniae—Lysozymes of E. coli mel− mutants were used to screen a λ phage library of K. pneumoniae genomic DNA. It was expected that recombination would occur between the λ prophage genome in a mel− lysogenic and infecting recombinant phage DNA carrying the mel operon of K. pneumoniae resulting in mel+ recombinant lysogen. As described under "Materials and Methods" mel+ lysogens arose and recombinant λ phage clones were recovered from these lysogens. One of the clones, X2-12, was further analyzed.

Physical mapping revealed that X2-12 contained an 11-kb insert and that a part of λ genome of the vector was replaced with the wild type λ genome. The insert DNA was cut out by digestion with ScaI which has recognition sites that flank the BamHI cloning sites. When X2-12 DNA was treated with

**Fig. 1. Construction of plasmids and the phenotype of plasmid DNA fragments—** Plasmid DNA fragments carried by each plasmid are shown by bars. Multiple restriction sites are followed by a or b for identification. The size and location of the melA, melB, and melT genes are indicated by arrows. α-Galactosidase activity of plasmid carrying cells is indicated by + or − under α-gal. The ability to ferment melibiose was tested on melibiose MacConkey plate. Results are designated as red (fermentation positive) or white (fermentation negative) under MacConkey. In pSUBS25 the lac promoter of the vector precedes the melB insert.
SacI, 9- and 2-kb fragments appeared in addition to the left and right arms. Both fragments were subcloned into pSU2718. pSUSS90, the one with the 9-kb fragment, conferred mel" phenotype on E. coli (Fig. 1). Subsequently, several plasmids with shorter insert were constructed, and the phenotypes of plasmid carrying cells were tested (Fig. 1). While cells carrying pSUSS90, pSUSS73, or pSUSS60 had α-galactosidase activity, only those with pSUSS90 or pSUSS73 fermented melibiose. Since the fermentation requires transport and hydrolysis of the sugar, this result suggested that the melB gene coding for the melibiose carrier was disrupted by SacI digestion. In fact, the nucleotide sequence of a part of the 1.3-kb SalI(a)-SalI(b) fragment (Fig. 1) was identified as coding for a protein which had considerable similarity to the melibiose carrier of E. coli (from valine at position 353 to threonine at position 425 in Fig. 2). Various portions of the DNA fragment encompassing SalI(a) site were then subcloned into pSU2718, and the entire sequence of the melB gene was determined (Fig. 2).

Interestingly, cells carrying pSUSS63 did not have α-galactosidase activity, although this plasmid should contain the entire melA gene which encodes the α-galactosidase. This result suggests two possibilities. One is that the mel operon of K. pneumoniae has another gene between the promoter and the melA gene and the promoter is located upstream beyond the SalI site. Alternatively the expression of the melAB genes requires an upstream regulatory element, the melR gene, as reported in E. coli (Webster et al., 1987). The melR gene has been identified upstream of the E. coli melAB genes in the opposite direction and deduced to code for a protein with 302 amino acid residues (Webster et al., 1987). If it is the case, the SalI site should disrupt the melR gene. In fact, the sequence from the SalI site in pSUSS63 was identified to have high similarity to the E. coli melR gene (data not shown). By analogy with E. coli, a small part of the K. pneumoniae melR gene which corresponds to the carboxyl-terminal 18 amino acid residues of the E. coli melR gene product seems to be missing in pSUSS63.

**Primary Structure of the Melibiose Carrier**—The melB gene was found to have two potential translation initiation sites resulting in a 1401- or 1413-bp open reading frame (Fig. 2). One of the two initiation sites corresponds to the one for the E. coli melB gene and is preceded by the Shine-Dalgarno sequence A-GAG. The other site is located 12 nucleotides upstream and is preceded by the Shine-Dalgarno sequence GGA. It was deduced that the melibiose carrier of *K. pneumoniae* consisted of either 467 or 471 amino acid residues, and its carboxyl terminus was 2 amino acids shorter than the E. coli melibiose carrier. The calculated molecular weight of the K. pneumoniae melibiose carrier was 51,722 or 52,140. Seven to eight percent of the 471 amino acid residues (or 79% of the 467 amino acid residues) was found to be identical to the E. coli melibiose carrier (Fig. 3). Of the two initiation sites the upstream one is more likely to be the actual site, because the additional 4 amino acid residues are identical to those of *Salmonella typhimurium* (Mizushima et al., 1992). This site was therefore designated as position number 1 in Fig. 2. Alternatively, both of the sites might work resulting in two populations of carrier proteins that are identical except for their different amino termini.

A part of the melA gene was also identified 93 nucleotides upstream from the initiation site of the melB gene (Fig. 2). Thirty-one out of 34 amino acid residues were found to be identical to the carboxyl terminus of the E. coli α-galactosidase (Liljestrom and Liljestrom, 1987).

**Effect of Cations on Sugar Transport**—Sugar accumulation by DW1/pSUSS25 was studied in parallel with DW1/pKKMB to contrast characteristics of the *K. pneumoniae* melibiose carrier with that of *E. coli*. It is known that melibiose transport is coupled not only to H+ but also to Na+ and Li+. When DW1/pKKMB was exposed to radioactive melibiose in the absence of Na+ or Li+ cells accumulated the sugar to a concentration 20 times that observed in the opposite direction (Fig. 4A). In the presence of 10 mM NaCl 220-fold accumulation was observed in 15 min, and with 10 mM LiCl

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**Fig. 3.** Comparison of the melibiose carrier of *K. pneumoniae* and *E. coli*. The deduced amino acid sequence of the *K. pneumoniae* melibiose carrier is compared with the *E. coli* melibiose carrier (Yazay et al., 1985). +, identical amino acids; F, conservative amino acid substitutions. Putative transmembrane regions (Botfield et al., 1992) are underlined.
FIG. 4. Effect of NaCl and LiCl on melibiose accumulation. Washed cells were incubated at 25 °C in the presence or absence of 10 mM NaCl or LiCl. The assay was initiated by addition of 0.1 mM [14C]melibiose (0.2 μCi/ml). A, DW1/pKKMB (the melB gene of E. coli); B, DW1/pSUBS25 (the melB gene of K. pneumoniae).

FIG. 5. Effect of NaCl and LiCl on lactose accumulation. Washed cells were incubated at 25 °C in the presence or absence of 10 mM NaCl or LiCl. The assay was initiated by addition of 0.2 mM [14C]lactose (0.2 μCi/ml). A, DW1/pKKMB; B, DW1/pSUBS25. Dotted line shows the level of equilibration with the medium.

FIG. 6. Effect of NaCl and LiCl on TMG accumulation. Washed cells were incubated at 25 °C in the presence or absence of 10 mM NaCl or LiCl. The assay was initiated by addition of 0.1 mM [14C]TMG (0.2 μCi/ml). A, DW1/pKKMB; B, pSUBS25.

FIG. 7. The uptake of H+ driven by downhill sugar entry into cells. pH measurements were carried out as described under "Materials and Methods." Washed cells of DW1/pKKMB (a, b, c) or DW1/pSUBS25 (d, e) were incubated anaerobically at room temperature (~22 °C) for 20 min. After base-line pH was recorded for 2-3 min, an anaerobic melibiose (a, d) or TMG (b, c, e) solution was added at the point indicated by arrows to give a final concentration of 5 mM (a, b, d, e) or 10 mM (c). The upward change of chart indicates H+ uptake into cells (alkalinization of the medium).

The existence of the melibiose transport system in K. pneumoniae was first predicted by Reeve and Braithwaite (1973). By studying the growth properties of several mutants they suggested that K. pneumoniae possessed multiple galactoside transport systems. One of the systems, the MelP system, was induced by melibiose and responsible for the accumulation of lactose and melibiose. In this study we cloned an agreement with reported results, rapid alkalinization of the external medium, i.e. proton entry into the cells, was observed when 5 mM melibiose was added to DW1/pKKMB cell suspension (Fig. 7a). This observation has been taken as evidence for H+-melibiose cotransport in E. coli (Tsuchiya and Wilson, 1978). Although it has been thought that H+ is not a coupling cation for TMG transport via the E. coli melibiose carrier, slight alkalinization was observed when 5 mM TMG was added to DW1/pKKMB cell suspension (Fig. 7b). This alkalinization was more obvious when 10 mM TMG was added (Fig. 7c). In contrast, rapid H+ uptake occurred by adding either 5 mM melibiose or 5 mM TMG to DW1/pSUBS25 cell suspension (Fig. 7d and e). This result indicates that both melibiose and TMG transport are efficiently coupled to H+ in the K. pneumoniae melibiose carrier.

**DISCUSSION**

The existence of the melibiose transport system in K. pneumoniae was first predicted by Reeve and Braithwaite (1973). By studying the growth properties of several mutants they suggested that K. pneumoniae possessed multiple galactoside transport systems. One of the systems, the MelP system, was induced by melibiose and responsible for the accumulation of lactose and melibiose. In this study we cloned an
pneumoniae which was induced by melibiose and the cells expressing the melB gene in this operon accumulated lactose and melibiose. Therefore it seems that the melB gene corresponds to the MelIP system.

The cloning method we undertook is useful for bacterial genes when multiple copies of a cloned gene are toxic for the growth of host cells or the entire operon is very large. Using a λ replacement vector, fragments up to 23 kb can be cloned without using any radioactive material and genes are carried as a single copy in E. coli cells. Since the recombinant phages constituting the library are lacking genes required to lysogenize, the recombinant lysogens we isolated should carry both the wild type λ genome and a recombinant λ genome. The recombinant λ genome is considered to have been integrated into the prophage genome by recombination. Our result indicates that the integrated recombinant λ genome is excised and packaged in the same way as prophage genome. Once a recombinant lysogen is isolated, the phage clone can be amplified efficiently by infecting a λ" strain with phage-containing culture supernatant of the lysogen. This method can be applied to the introduction of genes into E. coli genomic DNA to avoid the gene dosage effect observed with multicopy plasmids.

The K. pneumoniae mel operon is similar to that of E. coli and consists of a melibiose-inducible promoter, the melA gene coding for the α-galactosidase, and the melB gene coding for the melibiose carrier. Furthermore, the expression of the mel operon requires an upstream element, the melR gene. Thus the genes of the mel operon as well as the regulatory mechanism seem to be similar to that of E. coli.

The deduced primary structure of the K. pneumoniae melibiose carrier was 78% identical to the E. coli melibiose carrier. The K. pneumoniae lacY gene which encodes the lactose carrier has been sequenced, and 60% of the deduced amino acid sequence has been found to be identical to that of the E. coli lactose carrier (McMorow et al., 1988). The similarity between the melibiose carriers is considerably higher than that between lactose carriers of these organisms. This higher similarity may suggest later diversification of the melibiose carrier during evolution.

The differences in amino acids between E. coli and K. pneumoniae carriers were seen throughout the molecule, but the distribution showed an interesting pattern. According to the structural model of the E. coli melibiose carrier (Botfield et al., 1992), 38 substitutions were located in the predicted cytoplasmic region (77% conservation), another 38 substitutions were in the predicted transmembrane region (65% conservation), and 23 substitutions were in the predicted peripheral region (53% conservation). It should be pointed out that one-third of the 38 substitutions in the cytoplasmic region were located at the carboxyl-terminal region.

The K. pneumoniae melibiose carrier has been shown to play no direct role in transport; 14 amino acids may be deleted without loss of function (Botfield and Wilson, 1989a). In this carboxy-terminal region there are only two conserved amino acids in the carriers of the two organisms; 2 amino acids are missing in the K. pneumoniae carrier and 10 amino acids are substituted. This forms a striking contrast to the high similarity between the amino-terminal regions of the carriers of the two organisms. In this comparison, if one disregards the carboxyl-terminal 14 amino acids, 28 substitutions are located in the rest of the cytoplasmic region and the conservation is 81%. These figures may suggest the relative importance of the cytoplasmic region and the transmembrane region in transport function. This idea agrees well with the observation that all of the sugar or cation recognition mutants isolated so far have mutation(s) in either cytoplasmic or transmembrane regions.

In spite of the high similarity of the primary structure, the physiology of cation coupling in sugar transport via the K. pneumoniae melibiose carrier showed striking differences from that of E. coli. Proton uptake experiments revealed that melibiose and TMG transport via the K. pneumoniae melibiose carrier were coupled to H+. Accumulation of TMG and lactose were stimulated by added LiCl. These results indicate that the K. pneumoniae melibiose carrier catalyzes H+-melibiose, Li+-lactose, and H+/Li+-TMG cotransport. It has been shown that the preferred coupling cations in the E. coli melibiose carrier are: H+, Na+, and Li+ for melibiose; Na+ and Li+ for lactose or TMG. We also detected H+-TMG cotransport via the E. coli carrier, although this coupling seemed to be quite inefficient. The failure to detect this weak H+-TMG cotransport in earlier studies (Tsuchiya and Wilson, 1978) was probably due to the fact that the chromosomal expression of the carrier is only about 10% of that of the high copy number plasmid pKKMB. It should be noted that for all known sugar substrates Na+ is a preferred coupling cation in the E. coli melibiose carrier. Although our results are not sufficient to exclude the possibility of Na+-sugar cotransport via the K. pneumoniae melibiose carrier, H+ and Li+ are definitely preferred.

The lack of Na+ recognition by the K. pneumoniae melibiose carrier may make it possible to identify the Na+ recognition site by comparing the amino acid sequences of the melibiose carriers of the two organisms. Pocher et al. (1991) reported that aspartate 55 is in or near the Na+ binding site in the E. coli melibiose carrier. Interestingly, in the K. pneumoniae melibiose carrier the next residue, asparagine 54 in the E. coli carrier, is substituted with alanine. This substitution might influence the ability to recognize Na+. Three other residues which are next to asparagines in the transmembrane regions are also substituted (valine 52 with isoleucine, tryptophan 50 with leucine, and isoleucine 121 with valine (amino acid numbering based on the E. coli carrier) while all the asparagines in the transmembrane regions (positions 31, 53, and 55) are conserved. These substitutions may also have some importance in Na+ recognition. It is thus of particular interest to focus on the amino acid substitutions which affect Na+ recognition. Such a study is now under way.

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