Altered Cation Coupling to Melibiose Transport in Mutants of
Escherichia coli*

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The α-galactoside transport system of Escherichia coli utilizes either H+ or Na+ as a coupling cation for melibiose transport. Mutants were isolated which showed altered cation recognition for melibiose transport. The transport carrier of the mutants has lost the ability to accept H+ but can utilize either Na+ or Li+ for co-transport with melibiose. Such mutants might be predicted if the melibiose carrier were viewed as a descendant of an evolutionary transition between the H+-substrate co-transport systems of primitive cells and the Na+-substrate systems of more complex organisms.

A second mutation was found in these mutants which involves the Na+(Li+)-H+ exchange carrier. The maximal rate of Li+-H+ exchange in one of the mutants was 11 times higher than the parent and the Km for Li+ by this carrier was one-sixth that of the parent.

Cation-substrate co-transport systems are widely distributed in nature as they are found in bacteria, yeast, molds, plants, and animals (see Ref. 1 for a review). In the primitive anaerobic bacteria, as well as other prokaryotes, proton pumps generate an electrochemical potential difference for H+ across the plasma membrane and H+-substrate co-transport is common. In animal cells, on the other hand, the ATP-driven Na+-K+ pump establishes a large Na+ gradient and the co-transport systems use exclusively Na+.

During evolution there must have been a transition between the "proton economy" of the primitive cell and the "Na+ economy" of the animal cell. It is reasonable to suppose that, during this transition period, a co-transport system existed that recognized both H+ and Na+ and that it later lost the capacity to utilize H+.

From an evolutionary point of view, it is of interest to consider the unusual properties of the Escherichia coli melibiose membrane carrier which shows cation-sugar co-transport with either H+ or Na+ (2). If this carrier represents a descendant of a transport protein that appeared during the period of transition between H+ and Na+ membrane economies, one might predict that it would be possible to isolate by a simple mutation a carrier that recognizes only one of the two cations.

This paper describes two mutants of E. coli in which the melibiose carrier (that normally utilizes either H+ or Na+) has lost the ability to recognize H+ and developed an absolute requirement for Na+ (or Li+). The properties of these two Na+(Li+)-requiring mutants are described.

EXPERIMENTAL PROCEDURES

Strains and Growth of Cells—The primary strain utilized in these studies was W3133-2, lac ZY deleted and possessing a temperature-resistant melibiose carrier (3). A plasmid from the Clarke-Carbon collection (4) carrying the mel A (α-galactosidase) and mel B (melibiose transport) genes was inserted into two transport mutants. The mating experiments were carried out as follows: W3133-2 (or W3133-2T) was grown in Tris medium (see below) containing melibiose and 10 mM LiCl. Cells were diluted 100-fold and 0.1 ml spread on agar plates containing Tris medium plus 10 mM melibiose and 100 μg/ml of streptomycin. Onto this plate were streaked cells of JA200/pLC 25-33 previously grown on a nutrient agar plate. The donor fails to grow due to its sensitivity to streptomycin and amino acid requirements while mutants fail to grow in the absence of added Na+ or Li+. The mating on the plate gave rise to colonies which were purified and tested for the presence of the plasmid.

For the following investigation, cells were grown in a medium (Tris medium) consisting of 100 mM Tris-Cl, pH 7.3, 1 mM (NH4)2SO4, 1 mM MgHPO4, plus 10 mM melibiose with or without 10 mM LiCl and supplemented in some experiments with 1% Bacto-tryptone (Difco).

Cells were grown at 37 °C and harvested at late exponential phase of growth.

Isolation of Mutants with Altered Cation Specificity—E. coli W3133-2 (3) were treated with a mutagen, N-methyl-N'-nitrosoguanidine (5). Treated cells were divided into 10 portions in order to isolate at least 10 independent mutants. Cells in 10 tubes were grown in nutrient medium, then inoculated into the Tris medium described above plus 10 mM melibiose and 10 mM LiCl. Potassium was not added to the medium to minimize the contamination of Na+. The final concentration of both K+ and Na+ in the medium due to impurities in the other salts were 2 to 3 μM. The growth rate and extent of growth was about half that observed in other minimal media. Since Li+ inhibited H+-melibiose co-transport (2, 6), a mutant which can grow on melibiose as sole source of carbon in the presence of Li+ should possess some alteration in the transport carrier. After 2 days of shaking at 37 °C in the Li+-containing medium, the culture medium became turbid. Cells were spread on 10 agar plates which contained various salts as described above (Tris medium plus 10 mM melibiose and 10 mM LiCl) and 1.5% agar. Plates were incubated at 37 °C for 2 days, and the largest colonies on each plate were picked.

Thus, we obtained 10 mutants which can grow on melibiose in the presence of Li+. Six of the mutants were found to require Li+ or Na+ for growth when melibiose was added as a sole carbon source. The four other mutants grew either in the absence or in the presence of Li+. Two of such Li+ (Na+)-dependent mutants (W3133-2S, W3133-2T) were studied in detail.

Preparation of Everted Membrane Vesicles—The cells were grown in the Tris medium supplemented with 1% Bacto-tryptone. The cells were washed once with 10 mM MOPS-KOH, pH 7.2, containing 100 mM KCl, 5 mM MgSO4, and 2 mM 2-mercaptoethanol, and resuspended in the same buffer containing 2 μg/ml of DNase to 5 volumes/g of wet cells. The cells were disrupted by a passage through a French press cell (Ohitake, Co., Tokyo) using a pressure of 20,000 p.s.i. The
resulting suspension was centrifuged at 11,000 × g for 10 min to remove unbroken cells and large cell debris. The supernatant solution was centrifuged at 105,000 × g for 1 h. The pellet was resuspended to give about 40 mg of protein/ml in the same buffer, and an equal volume of glycerol was added. As reported previously (7), glycerol is very useful to stabilize the vesicles. All steps were performed at approximately 4 °C. The vesicles were frozen in dry ice-acetone, and stored at −80 °C until use.

**Measurements of H+ Movement and Na+ Movement**—The pH change or pNa change of the extracellular medium resulting from transport of melibiose were measured as described previously (2) with slight modification. For pH measurement, cells were washed twice with 120 mM choline Cl, and suspended in the same solution. For pNa measurement, experiments were carried out at various concentrations of NaCl ranging from 25 μM to 10 mM. Anaerobic solutions of sugars were added to give a final concentration of 10 mM. pNa was measured with a Na+-selective electrode (Radiometer, Copenhagen). Initial velocity of the Na+ entry was measured.

**Fluorescence Assays**—Fluorescence of 9-aminoacridine was measured with a Hitachi fluorometer using an excitation wavelength of 365 nm and measuring emission at 454 nm as described (8). The vesicles (about 1 mg of protein) were added to a mixture (2.0 ml) containing 20 mM N-tris(hydroxymethyl)methyl glycine KOH, pH 8.5, 100 mM KCl, 2 mM MgSO4, and 5 μM 9-aminoacridine. Relative fluorescence was recorded.

**RESULTS**

**The Melibiose Carrier**—An attempt was made to isolate a series of mutants with abnormal cation requirements for the melibiose transport system. Previous studies indicated that lithium-resistant mutants showed alterations in the cation recognition site of the membrane carrier (6). In this study, mutagenized cells were grown in liquid media containing melibiose as the sole carbon source in the presence of 10 mM Li+. The growth of parental strain was strongly inhibited but after 2 days mutant cells appeared in the culture. These survivors were plated on agar plates containing 10 mM Li+ with melibiose as the sole carbon source. The largest clone on each plate was picked for further study. Ten independent mutants were tested for growth on melibiose in the presence and absence of Li+. Six of the mutants showed a Li+ requirement for growth, four showed no effect of Li+. Fig. 1 compares the effect of Li+ on the parental cell (W3133-2) and one of the mutant cells (W3133-2S). Lithium at a concentration of 10 mM completely inhibited the growth of the parental cell. The mutant on the other hand failed to grow unless Li+ was added to the growth medium. Sodium could replace Li+ in the stimulation of growth of this mutant (data not shown). Five other mutants showed similar growth properties. Sodium at a concentration of 10 mM caused a 40% inhibition of growth of the parental cell. Neither Na+ or Li+ had an effect on the growth of the mutant cells when glucose, galactose, or amino acids were the sole source of carbon. The effect of Li+ concentration on growth of the parent and the mutants was next investigated. The concentration of Li+ which caused 50% inhibition of growth of the parental cell was approximately 0.02 mM (Fig. 2). The stimulation of growth in the mutants (W3133-2S and W3133-2T) required a very much higher concentration of Li+ (approximately 0.4 mM for half-maximal effect). The concentration of Na+ which gave half-maximal effect on the growth of the mutants was approximately 0.6 to 0.7 mM. Thus, Li+ was slightly more effective than Na+ in the stimulation of the growth of the mutants on melibiose.

**Measurement of H+ and Na+ Movements on the Melibiose Carrier**—Proton movement in response to addition of melibiose was measured in washed cells suspended in an unbuffered solution. The pH of the external medium was continuously monitored on a chart recorder. In these experiments, the uptake of protons with melibiose on the carrier of the wild type cell resulted in an alkalinization of the medium (an upward deflection on the chart, Fig. 3A). This alkalinization process was followed by an acidification of the medium (downward deflection on the chart) due to the metabolism of glucose and galactose resulting from hydrolysis of the disaccharide. In contrast, the two mutants showed no initial alkalinization of the medium on melibiose addition but showed only continuous acidification due to metabolism of the hexoses. These experiments were carried out in the presence of 120 mM choline chloride to avoid the presence of Na+ or Li+. The experiment was repeated in the presence of 10 mM LiCl (Fig. 3B).

**Fig. 2. Effect of Li+ concentration on the growth rate of parent and mutant cells.** Cells were grown aerobically at 37 °C in minimal medium supplemented with 10 mM melibiose and the indicated concentrations of LiCl. Growth rate was expressed as doubling per hour. A, parent (W3133-2); B, mutants, W3133-2S (A) and W3133-2T (B).

**Fig. 3. Proton fluxes induced by the addition of melibiose to cell suspensions.** Washed cells (7 mg of protein) of three strains of E. coli were incubated in 3 ml of 120 mM choline Cl in the absence (A) or in the presence (B) of 10 mM LiCl under anaerobic conditions. At the time point indicated by arrows, 30 μl of an anaerobic solution of 0.5 M melibiose was added to the cell suspension, and pH changes of the medium were monitored. An upward deflection represents a rise in the pH of the medium (indicating an entry of H+ into cells). Strains used were: W3133-2 (a), W3133-2S (b), W3133-2T (c).
completely prevented the alkalinization of the medium in the parental cell (W3133-2), owing to its known inhibition of the membrane carrier (2, 6). Lithium had little effect on the acidification reaction arising from metabolism. In the case of the two mutants, the addition of melibiose in the presence of Li⁺ resulted in a rapid acidification of the medium during the first 15 s compared with the Li⁺-free experiment in A. This was followed by a metabolic acidification reaction. It was assumed that in the presence of Li⁺ this cation entered the cell with melibiose on the carrier resulting in a membrane potential (inside positive) which provided a driving force for the exit of protons. A similar melibiose-induced proton exit was previously observed in the presence of sodium ions with wild type cells (2).

Another substrate of the melibiose carrier, TMG, was tested for its effect on proton movement in the parent and two mutant cells. Previous experiments have shown that TMG is transported by the melibiose carrier with Na⁺ or Li⁺ but not protons (2, 3, 9). The addition of TMG to the two mutants (in the absence of Na⁺ or Li⁺) results in no proton movement (Fig. 4A). In the presence of 10 mM Li⁺ or Na⁺ the addition of TMG to the parental cell caused a marked acidification of the medium as a result of the exit of protons from the cell (Fig. 4D). This effect is believed to be due to a positive internal membrane potential due to the entry of Li⁺ (or Na⁺) with TMG. It should be noted that TMG is not metabolized by these cells and thus does not result in the prolonged acidification seen with melibiose. The two mutants showed much less acidification on the addition of TMG in the presence of Li⁺. This is consistent with the view that TMG transport in these two mutants cannot couple to Li⁺ effectively. Na⁺-TMG co-transport appears to be normal. Addition of 10 mM Na⁺ stimulates TMG accumulation by W3133-2s 6-fold.

Cation specificity of methyl-α-galactoside transport in the two mutants was also tested. No proton movement was observed when a-methylgalactoside was added in the absence of Na⁺ or Li⁺. On the other hand, proton efflux (acidification of the medium) was observed when the sugar was added in the presence of either Na⁺ or Li⁺. When sugar was added to cells in the presence of Na⁺, this cation entered the cell as indicated by the fall in external Na⁺ concentration (measured with a Na⁺-electrode).

It has been previously shown that in the presence of Na⁺ melibiose enters the cells on the carrier with Na⁺ (2, 9). The uptake of Na⁺ by the parent and mutant cells was measured with a sodium electrode. The addition of melibiose resulted in a prompt fall in the concentration of Na⁺ in the external medium. The kinetic parameters for Na⁺ stimulation of melibiose transport in the parent and mutant cells were similar. The parent showed a $K_m$ for Na⁺ of 0.3 mM and a $V_{max}$ of 17 ng ions/min/mg of protein, while the mutant (W3133-2s) gave a $K_m$ of 0.4 mM and a $V_{max}$ of 19 ng ions/min/mg of protein.

Additional evidence was sought to demonstrate that the mutation in the lithium-resistant mutant W3133-2S was indeed in the melibiose transport system. A plasmid carrying the melA melB genes (Clarke-Carbon collection) (4) was inserted into the mutant. In Fig. 6A the mutant shows a complete loss of proton entry on melibiose addition (H⁺-melibiose co-transport), while the same cell carrying the plasmid (pLC 25-33) showed a restoration of proton co-transport. Proton exit was observed when melibiose was added to W3133-2S or the plasmid-containing cell in the presence of Li⁺. As indicated above, this acidification results from Li⁺ entry with melibiose. Strain W3133-2S/pLC 25-33 possesses two melibiose genes, one from the mutant and the other from the wild type. Thus, cells of W3133-2S/pLC 25-33 showed properties both of the mutant and of the wild type with respect to its pH response to melibiose in the presence of Li⁺. From these results, we conclude that the change in cation coupling in the melibiose transport system in the mutants is due to a mutation in the melibiose operon.

$\text{Na}^+/(\text{Li}^+)/\text{H}^+$ Antipporter Activity in Mutant Cells—The possibility of a second mutation in the Li⁺(Na⁺)-dependent mutants was considered. During growth on melibiose, these mutants take up large quantities of Li⁺ which would be expected to inhibit growth of the cell unless this ion were continuously extruded from the cell (10). Thus, it was of
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FIG. 7. Activity of the Na+(Li+)/H+ antiporter in membrane vesicles of wild type and the mutants. Membrane vesicles were prepared by the French press method from W3133-2 (a), W3133-2S (b), and W3133-2T (c). Quenching of 9-aminoacridine fluorescence, used as a probe of ΔpH was measured as described under "Experimental Procedures." Each experiment was initiated by the addition of K+ lactate (6 mM final concentration) which resulted in a fall in fluorescence over a 2- to 4-min period. LiCl and NaCl were added to the assay mixture, at the points indicated by arrows to give a final concentration of 10 mM. The chart speed of the recorder was increased at the point indicated by dotted lines to facilitate the measurement of the initial rate of change of fluorescence. A downward deflection indicates a decrease in fluorescence intensity, indicating the establishment of ΔpH, acidic interior. An upward deflection represents a dissipation of the ΔpH.

The effect of Li+ concentration on proton movement was next investigated. Fig. 8 and Table I show that the mutant W3133-2S has a higher affinity for lithium and also increases in the maximum velocity of the reaction. The affinity of the antiporter for Na+ in the mutant (W3133-2S) was higher and the maximum velocity was also higher than the parent (W3133-2) (Table I).

A second method for the assay of Li+/H+ antiporter was investigated. In this technique, the extrusion of protons resulting from the addition of Li+ to cell suspension was monitored with a pH electrode (11). Fig. 9 shows that when Li+ was added to the mutant W3133-2S, there was a more rapid acidification of the external medium (0.77 ng ions H+/s X mg of protein) than in the comparable experiment with the parental cell (0.33 ng ion H+/s X mg of protein). These data are in general agreement with the findings obtained with the 9-aminoacridine.

It was important to demonstrate that the abnormality of the Li+(Na+)/H+ antiporter activity was completely unrelated to the melibiose operon. To establish this fact, a mutant containing a normal melibiose operon from the plasmid was compared with the mutant strain W3133-2S. The insertion of a normal melibiose operon into the mutant did not modify the abnormality in the antiporter activity (Fig. 8 and Table I). Thus, the mutant possesses two separate mutations, one in the melibiose transport system and a second in the Na+(Li+)/H+ antiporter.

TABLE I

Kinetic parameters of the Na+(Li+)/H+ antiporter in wild type and the mutant cells

| Cation | Strain            | K_m  | V_max |
|--------|-------------------|------|-------|
| Li+    | W3133-2           | 5.5  | 1.6   |
|        | W3133-2S          | 0.9  | 18    |
|        | W3133-2S/pLC 25-33| 0.9  | 18    |
| Na+    | W3133-2           | 16   | 0.8   |
|        | W3133-2S          | 4.5  | 2.8   |
|        | W3133-2S/pLC 25-33| n.d. | n.d.  |

*% fluorescence increase/s X mg protein.

*Not determined.

The measurement of the Na+(Li+)/H+ antiporter activity was carried out with the 9-aminoacridine fluorescence technique. The internal pH of the everted membrane vesicles was monitored by changes in fluorescence of 9-aminoacridine. The addition of lactate activated the respiratory chain and acidification of the vesicle lumen which resulted in accumulation of 9-aminoacridine within the vesicle and quenching of its fluorescence (Fig. 7). When a new steady state was reached LiCl or NaCl were added to give a final concentration of 10 mM. The entry of Li+ (or Na+) on the carrier resulted in a loss of H+ which decreased the ΔpH and resulted in an increase in fluorescence of 9-aminoacridine. The rate and extent of the increase in fluorescence is a good measure of the activity of the Na+(Li+)/H+ antiporter (8, 12). The response to the addition of Li+ (and Na+) was greater than normal in the mutant W3133-2T and was very much more active than normal in the mutant W3133-2S. Lithium caused larger change of fluorescence than Na+.

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when melibiose was added, indirect evidence for uptake of Na⁺ or Li⁺. Thus, these two mutants utilize Na⁺ or Li⁺.

The second mutation in these two mutants involves the presence of melibiose presumably by sugar-Na⁺ co-transport. From this evolutionary standpoint, it is interesting to find a class of mutants that no longer accepts H⁺ but utilize Na⁺ (or Li⁺). Preliminary attempts to isolate the opposite extreme, a mutant utilizing H⁺ but not Na⁺, have been reported (17). In this mutant, Na⁺ has far less effect on the carrier than in the parental cell. Thus, it appears that the mutants predicted based on the evolutionary hypothesis can be isolated.

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