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CHARACTERIZATION AND SEQUENCING OF AN UNCOUPLED LACTOSE CARRIER MUTANT OF *Escherichia coli*

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SUMMARY. A lactose carrier mutant of *Escherichia coli* (ML308-22) showed a severe defect in thiomethylgalactoside accumulation but a faster than normal entry of o-nitrophenyl-galactoside. Sequencing of the mutant *lacY* gene revealed a point mutation resulting in the substitution of glycine-159 by a cysteine residue. The mutant showed an increased sensitivity to sulfhydryl reagents, a property that is consistent with the view that the Cysteine-159 is in or near the sugar recognition site and the energy coupling region of the carrier.

The lactose carrier of Escherichia coli is an example of a substrate cation cotransport system. This carrier shows obligatory coupling between sugar transport and proton transport. Thus, the inward movement of H^+ down its electrochemical gradient drives the accumulation of lactose and other sugar substrates against large concentration gradients(see reviews 1,2). A number of mutants of this carrier have been isolated which show a defect in the coupling between cation entry and sugar accumulation (1-3). One of these "uncoupled" transport mutants (ML308-22) was isolated using thio-*o*-nitrophenyl-β-galactoside (TONPG) (3,4). ML308-22 is capable of recognizing lactose and can grow on high concentrations of this sugar. However, it is highly defective in sugar accumulation because it fails to couple proton movement to sugar entry (5,6).

This communication describes the cloning and sequencing of the *lac Y* gene as well as further physiological studies.

MATERIALS & METHODS

Reagents--Lactose, N-ethylmaleimide (NEM), p-chloromercuribenzoate (pCMB), and p-chloromercuriphenylsulfonic acid (pCMPS) were

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purchased from Sigma. *o*-Nitrophenyl-β-D-galactopyranoside (β-ONPG) was purchased from Schwarz-Mann. [^{14}C]Lactose was obtained from Amersham Corp. [^{14}C]Methyl-β-D-thiogalactopyranoside ([^{14}C]TMG) from Du Pont-New England Nuclear. Other chemicals were reagent grade.

Bacterial Strains--The original *lac Y* mutation, was isolated from *E. Coli* ML308 by the general method of Müller-Hill, Crapo, and Gilbert (4). The parental cell (ML-308) was grown in a minimal salts medium with glycerol as carbon source plus TONPG. This non-metablolizable sugar is accumulated by the carrier by proton cotransport but because of its hydrophobic nature the sugar leaks out of the cell. This futile cycle of accumulation and leak uses protonmotive force, reduces cellular ATP and stops the growth. Cells which grow normally under these conditions are those that are transport negative or fail to couple proton uptake to sugar entry.

Transport Assays ([^{14}C]TMG and [^{14}C]lactose)--Cells were equilibrated to 25°C and incubated in 0.9 ml medium 63 with the indicated concentrations of sulfhydryl reagents for 10 min. The transport assay was started by the addition of 0.1 ml of 1mM ([^{14}C]lactose) or 1mM [^{14}C] thiomethylgalactoside (TMG). Data points were obtained by filtering 0.2 ml aliquots through 0.65μ membrane filters. Cells were then washed with 3ml of medium 63, and radioactivity was measured by liquid scintillation counting. Counts obtained with a *lac Y*-negative mutant (ML35) served as a negative control and were subtracted from the values for the experimental samples.

(β-ONPG)--Cells were equilibrated to 25°C and incubated in 1.9 ml medium 63 with the indicated concentrations of sulfhydryl reagents for 10 min. Addition of 0.1 ml of 20 mM β-ONPG started the assay. Samples were incubated for 5 minutes, and the reac-

Table 1. Primers used for PCR

Name[a]	Sequence	Location[b]
Y174A	TGAGTGCACAGCCAGAGC	518 TO 535
YA	CTCTTATTCTTTCGGTCATTGGC	1326 to 1349
Y102C	CCACTGTTACAATACAAC	289 to 306
YC	AAGTCATCTGAATTCCATTACCAGTTTGGTCTGGTGTC	-60 to -80

[a]The final letter (A) indicates anticoding strand; (C) indicates coding strand. The numbers (102 and 174) are the amino acid corresponding to the 3′ end of the primer. The other two primers are outside the *lac Y* gene. YC is in the terminal region of the *lac Z* gene and YA is in the first portion of the *lac A* gene.

[b]The location is given by the number of bases following the first base of the *lac Y* gene.

tion was quenched by the addition of 4 ml of 0.6M Na_2CO_3. Samples were then centrifuged and the o-nitrophenol determined with a Klett-Summerson colorimeter (No. 42 filter).

Sequencing of lacY *Gene*--Cells were grown overnight in 2 ml LB medium. Chromosomal DNA was isolated (7). DNA amplification was carried out with Taq DNA polymerase as follows: 1 min at 94°C; 29 cycles of 1 min at 92°C, 1 min at 37°C and 1 min at 72°C ; 5 min at 75°C. Primers (see Table 1) were added to give a final concentration of 1 μM and chromosomal DNA was at a concentration of 2 ng/μl in a final volume of 50 μl. First, primers YC and Y174A gave an N-terminal fragment of approximately 632bp . The second fragment was the C-terminal region of approximately 1060bp was prepared with primers Y102C and YA. These two fragments were cloned into the plasmid pCR (from INVITROGEN). Each fragment was sequenced with universal primers and sequencing primers used in the laboratory for sequencing the *lac Y* gene of *E. coli*.

RESULTS

A lactose carrier mutant ML308-22, previously isolated in this laboratory (3),showed an uncoupling of sugar transport from proton uptake. Therefore, it was of interest to clone and sequence the *lac Y* gene of this mutant in order to correlate structural alterations in the protein with physiological changes of the transport. The *lac Y* genes for the parental cell (ML308) and the mutant (ML308-22) were cloned by the PCR technique. Two overlapping fragments of the *lac Y* gene were amplified and cloned into a multicopy plasmid for sequencing as described in the METHODS. The *lac Y* gene of the ML308 was 100% identical to that found in the *E. coli* K12 strain. The mutant was found to have a single point mutation at base 580 (G-->T) which resulted in the substitution of cysteine for Glycine-159.

Several physiological and biochemical studies were carried out in order to further characterize this mutant. The capacity to accumulate sugars against a concentration gradient was tested with the non-metabolizable lactose analog TMG. During a 10 minute incubation, the wild-type ML308 accumulated TMG 90-fold, whereas the mutant ML308-22 accumulated 19-fold (Fig. 1).

In spite of the decreased capacity for uphill sugar accumulation due to the defect in coupling, the mutant was able to carry out thermodynamically "downhill" transport of metabolizable sugars. ML308-22 transported lactose approximately 80% as fast as ML308. It is extremely interesting to note that for the metabolizable analog β-ONPG, at all temperatures between 10°C and

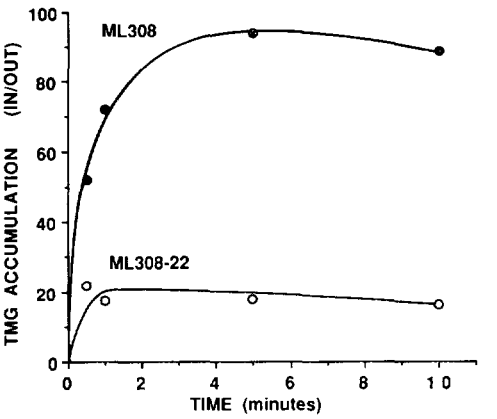

Fig. 1. Accumulation of TMG by ML308-22. ^{14}C-TMG was added to a cell suspension ($3x10^9$ cells/ ml) to give a final sugar concentration of 0.1 mM (0.1 μc/ml). Samples of 0.2 ml were filtered at the times indicated, washed with 3 ml buffer and counted.

37°C, the entry rate (Vmax) into the mutant was about 400% of the parental cell (Table 2). In contrast, the affinity of the mutant carrier for β-ONPG was less than that of the parental cell.

Because the mutation resulted in a cysteine substitution for glycine it was possible that sulfhydryl blocking reagents might have a particularly strong inhibitory effect on transport. Table 3 shows that pCMPS has a much stronger inhibitory effect on lactose transport of the mutant (ML308-22) than the parent (ML308). For example, 0.4 μM pCMPS inhibited transport 67% in ML308-22 and 5% in ML308. This extreme sensitivity of the mutant to sulfhydryl reagents in lactose transport was also observed for β-ONPG entry. pCMPS, pCMB and NEM all inhibited β-ONPG transport in the mutant much more strongly than in the parental strain (Table 4).

Table 2. Effect of temperature on ONPG transport into mutant and parental cells. Data represent mean values of 3 experiments.

Temperature (°C)	Km (mM)		Vmax nmol/min/10^9 cells	
	ML308	ML308-22	ML308	ML308-22
37	2.4	4.0	150	711
35	1.0	3.7	119	547
25	0.37	1.6	37	267
10	0.16	0.77	5	28

Table 3. Inhibition of lactose uptake by pCMPS. Data represent the mean values of 3 experiments.

Conc of pCMPS	Inhibition of transport	
	ML308	ML308-22
0.4 μM	5%	67%
0.8 μM	12%	82%
8.0 μM	64%	94%

DISCUSSION

The inability of ML308-22 to accumulate TMG to normal levels is consistent with the severe defect in proton uptake reported for this mutant (5). Thus, there appears to be a failure of the normal coupling between proton uptake and sugar accumulation. Unlike accumulation, sugar recognition and sugar entry "downhill" are only slightly altered. The entry rate of lactose, the natural galactoside substrate, into ML308-22 was approximately 80 % of the rate of the parent. It is quite remarkable that the entry rate of β-ONPG for the mutant is 4 times faster than that for the parental strain.

Fig. 2 shows a hypothetical model, modified from a proposal by Brooker (2), for the lactose carrier which shows a hydrophilic channel structure composed of seven transmembrane α-helices. This model also shows residues whose modification result in altered sugar recognition, energy coupling, or both.

Table 4.Inhibition of ONPG entry by sulfhydryl reagents. Data represent the mean values of 3 experiments.

Inhibitor	Concentration (μM)	Inhibition of transport	
		308	308-22
pCMPS	0.4	15%	58%
	0.8	17%	83%
	8.0	41%	97%
	16.0	72%	99%
pCMB	2.5	32%	95%
	5.0	43%	98%
NEM	30	12%	53%
	60	43%	59%

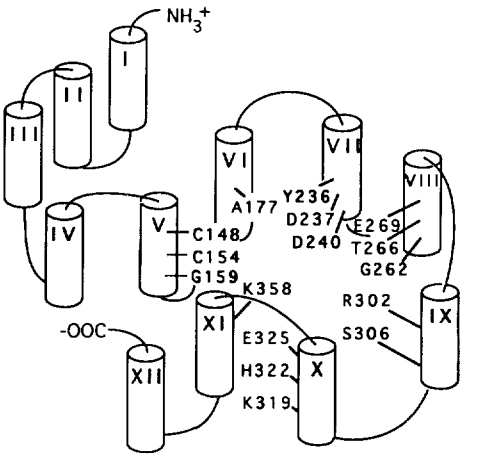

Fig. 2. Hypothetical model of the lactose carrier showing the position of Gly-259. Modified from the model of Brooker (2). The external surface of the cell is at the base of the diagram.

The sensitivity of the mutant ML308-22 to sulfhydryl reagents (8) is consistent with the view that Cysteine-159 is in or near the sugar recognition site and the region responsible for cation coupling. The fact that pCMPS, a hydrophilic reagent, is strongly inhibitory in ML308-22 is consistent with the location of Cysteine-159 in a region close to the external face of the hydrophilic channel. If the diameter of this channel were relatively wide at this location it would permit interaction of pCMPS with Cysteine-159.

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