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CYS$_{154}$ IS IMPORTANT FOR *LAC* PERMEASE ACTIVITY IN *ESCHERICHIA COLI*

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SUMMARY. The *lac Y* gene of *Escherichia coli* which encodes the *lac* permease has been modified by oligonucleotide-directed, site-specific mutagenesis such that *cys*$_{154}$ is replaced with either *gly* or *ser*. Permease with *gly* in place of *cys*$_{154}$ exhibits essentially no transport activity, while substitution of *cys*$_{154}$ with *ser* also causes marked, though less complete loss of activity. The findings suggest that *cys*$_{154}$ plays an important role in lactose:H$^+$ symport. © 1985 Academic Press, Inc.

INTRODUCTION. The *lac* permease of *Escherichia coli* is an integral membrane protein encoded by the *lac Y* gene that catalyzes symport of β-galactosides with H$^+$ (cf. refs. 1-3 for reviews). The *lac Y* gene has been cloned and sequenced, and the permease has been purified to a single polypeptide species in a completely functional state, thus demonstrating that the product of the *lac Y* gene is solely responsible for β-galactoside transport.

A secondary structure model for the *lac* permease based on circular dichroic measurements and an analysis of sequential hydropathic character suggests that the polypeptide is organized into 12 hydrophobic, α-helical segments that traverse the bilayer in a zig-zag fashion connected by hydrophilic, charged segments. Preliminary support for certain general aspects of the model has been provided by proteolysis experiments and binding studies with monoclonal and site-directed polyclonal antibodies in right-side-out (RSO) and inside-out membrane vesicles.

Abbreviations: RSO, right-side-out; ss, single-stranded; SAM, S-adenosyl-L-methionine; EMB, eosin methylene blue; Mab, monoclonal antibody; NEM, N-ethylmaleimide.

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Lately, we have begun to utilize oligonucleotide-directed, site-specific mutagenesis (4) to study the structure and function of the *lac* permease (cf. ref.5). The technique provides a means of altering given amino acid residues in a protein with a high degree of specificity, and the approach was used initially (6,7) to evaluate the role of cys_{148} in the permease. More recently, each of the four *his* residues in the molecule was replaced with *arg* (8), and the results provide a strong indication that the *his* residues at positions 205 and 322 of the permease play an important role in lactose:H^+ symport.

Although cys_{148} is essential for substrate protection against sulfhydryl inactivation, it is not obligatory for lactose:H^+ symport; furthermore, it appears that another *cys* residue(s) may be obligatory for permease activity (6,7). The experiments described here are consistent with the interpretation that cys_{154} is this residue.

MATERIALS AND METHODS. The following materials were purchased from commercial sources: T4 DNA ligase, bacterial alkaline phosphatase, *Eco*RI, *Hind*III, *Hinc*II and dideoxynucleotide sequencing reagents were from BRL; *dam* methylase was from New England Biolabs; S-adenosyl-L-methionine (SAM) was from Boehringer/Mannheim; [γ-^{32}P]ATP, [α-^{32}P]dATP, [1-^{14}C]lactose and T4 polynucleotide kinase were from Amersham; DNA polymerase I (Klenow fragment) was from BRL, Boehringer/Mannheim and New England Biolabs; nitrocellulose (BA85) and Elutip-d columns were from S & S; Sea Plaque and Sea Kem agarose were from FMC. All other chemicals were reagent grade obtained from commercial sources (5).

Bacterial Strains. The following strains of *E. coli* K12 were used: JM101 (Δ*lacpro, supE, thi/F', traD36, proAB, lacI^{q}ZΔM15*) (9); T206 [*lacI$^+$O$^+$Z$^-$Y$^-$(A$^+$), rpsL, met$^-$, thr$^-$, recA, hsdM, hsdR/F', lac I^{q}O$^+$Z^{118} (Y$^+$A$^+$)*] harboring plasmid pGM21 [*lac Δ(I)O$^+$P$^+$Δ(Z)Y$^+$Δ(A), tet^r*] (10); T184 [T206 cured of plasmid pGM21] (10); HB101 [*hsdS20 (r^-_B, m^-_B), recA13, ara-14, proA2, lacY1, galK2, rpsL20(Sm^r), xyl-5, mtl-1, supE44, λ$^-$/F$^-$*] (11); CS71 [*gltC, metB, lacY1/Hfr*] (12).

Site-directed mutagenesis. Oligonucleotide-directed, site-specific mutagenesis (4) was performed essentially as described with the following modifications (5). The single-stranded (ss) template used for mutagenesis was M13mp19 DNA containing the antisense strand of the 2.3 kbp *Eco*RI restriction fragment which contained the *lac Y* gene from the plasmid pGM21. Cys_{154} was replaced with *gly* or *ser* residues by using separate mutagenic primers (5'-GGCACCCAGCGCCCAGCC-3'or 5'-GGCACTCAGCGCCCAGCC-3, respectively), each of which contained a single mismatch (*). Closed-circular heteroduplex DNA synthesized *in vitro* was methylated by using *dam* methylase and SAM, nicked with *Hind*III in the presence of ethidium bromide and heat denatured prior to transfection.

Phage harboring the mutation were identified initially by colony-blot hybridization using the appropriate [^{32}P]-labeled mutagenic primer (13).

Phage from positive colonies were plaque purified, and the mutation was verified by dideoxynucleotide sequencing (14,15) using a synthetic oligonucleotide sequencing primer. *Lac* permease activity of the mutants was tested qualitatively by infecting the cryptic strain *E. coli* CS71 (Z^+Y^-) with phage harboring mutated *lac Y* and plating the cells on lactose/eosin methylene blue (EMB) indicator plates (8).

Cloning of the mutated 2.3 kbp fragment into the original vector pACYC184 and subsequent transformation of *E. coli* T184 with recombinant plasmids are described elsewhere (5,6). Cloned T184 harboring recombinant plasmids with the mutated *lac Y* were grown overnight at 37°C in LB medium containing 100 µg/ml streptomycin and 12.5 µg/ml tetracycline, and plasmids were prepared by alkaline lysis (16). The orientation of the mutant *lac Y* gene in the recombinant plasmids was determined by *Hinc*II restriction enzyme analysis as described (5). Plasmids in which the orientation of the mutated 2.3 kbp fragment is identical to that in pGM21 were selected (10).

Lactose transport. Cells were grown in LB medium and induced with *i*-propyl 1-thio-ß-D-galactopyranoside (0.2 mM) at an A_{420} of 0.4-0.5. After 1.5 h, the cells were harvested, washed in 100 mM potassium phosphate (pH 7.5)/10 mM $MgSO_4$ and resuspended to an A_{420} of 12. Assays were carried out at 25°C, and transport was initiated by addition of 0.38 mM [1-^{14}C]lactose (59 mCi/mmol) to 50 µl aliquots of the cell suspensions. At indicated times, reactions were terminated and the samples processed as described (6).

Preparation of membrane vesicles. RSO membrane vesicles were prepared as described (17,18) and resuspended to a final concentration of about 3 mg of protein/ml in 100 mM potassium phosphate (pH 6.6). Aliquots were frozen and stored in liquid nitrogen for subsequent use.

Binding of monoclonal antibodies (Mabs). In order to estimate the amount of permease present in the membrane, immunoblot analyses were performed with Mab 4A10R and [^{125}I]-labeled protein A (19).

Protein determinations. Protein was measured as described by Lowry *et al.* (20) using bovine serum albumin as standard.

RESULTS. The *lac Y* gene was ligated into the *Eco*RI site of the replicative form (RF) of M13mp19 DNA, and ss phage DNA was isolated and used as template for site-directed mutagenesis. Two 18-base synthetic oligodeoxynucleotides, each with a single base-pair mismatch designed to convert cys_{154} in the *lac* permease to either *gly* or *ser*, were used to prime *in vitro* synthesis. Phage harboring the mutations were identified initially by colony-blot hybridization using [^{32}P]-labeled mutagenic primers. A typical autoradiograph obtained from a colony-blot screen of suspected S154 mutants demonstrates that the frequency of mutant recovery is about 36% using the conditions described (Fig. 1). Phage from positive colonies were plaque purified, and the mutations were verified by dideoxynucleotide sequencing (14,15) using a synthetic primer complementary to a region approximately 60 base pairs

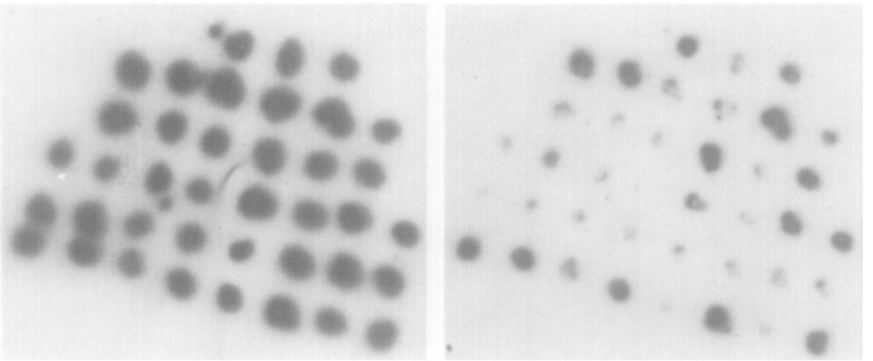

Fig. 1. Colony-blot hybridization of putative S154 mutants. Plaques from *E. coli* JM101 tranfected with synthetic heteroduplex DNA were grown on LB plates for 16 h at 37°C. Nitrocellulose filter replicas were hybridized with [^{32}P]-labeled S154 mutagenic primer, washed sequentially at 23°C and 67°C as indicated and autoradiographed for 30 min as described (13).

downstream from the mutation (Fig. 2). Both mutants contain single-base replacements at position 460 of *lac Y* (A → C and A → T, respectively) which change the anti-codon for cys_{154} in the *lac* permease (ACA) to *gly* or *ser* anti-codons (ACC or ACT, respectively).

Mutated double-stranded *lac Y* DNA was restricted from M13mp19 RF DNA and ligated into the *Eco*RI site of the plasmid vector pACYC184 from which *lac Y* was obtained initially. The resulting plasmids, pG154 and pS154, were used to transform *E. coli* T184, yielding the mutant strains G154 and S154, respectively, which should be identical genotypically to *E. coli* T206 with the sole exception of single nucleotide substitutions in *lac Y*.

Transformation of the cryptic strain *E. coli* HB101 (Z^+Y^-) with pG154 results in white colonies on EMB indicator plates, even at high lactose concentrations (25 mM), suggesting that the permease encoded by pG154 is completely unable to catalyze lactose transport. The suggestion is confirmed by direct measurements of [1-^{14}C]lactose transport which demonstrate that both the initial rate and steady-state level of lactose accumulation in G154 are almost negligible (Fig. 3). That is, lactose transport in this strain is comparable to that observed in *E. coli* T184, a strain devoid of *lac* permease.

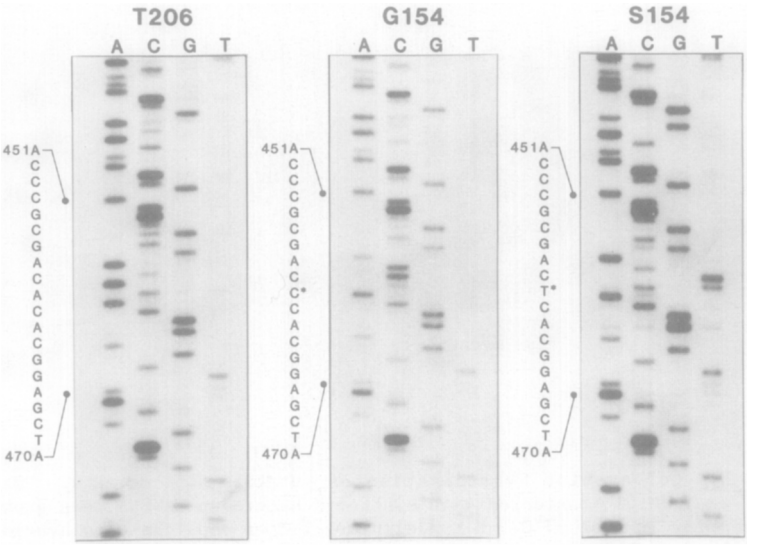

Fig. 2. Dideoxynucleotide sequencing of recombinant M13mp19 ss DNA containing wild type, G154 or S154 *lac Y*. Sequencing was performed as described (14,15) using a synthetic oligodeoxynucleotide (5'-TGCACCGCCAGAGCCCAG-3') that is complementary to base pairs 514 to 531 of the *lac Y* antisense strand. The autoradiograph shows the sequence in the region of the mutations with (*) denoting the base change.

In contrast, transformation of HB101 with pS154 results in light red colonies on EMB indicator plates, indicating that some permease activity is retained. Furthermore, although the initial rate of [1-^{14}C]lactose transport in S154 is only about one-tenth of that observed in T206, it is signifi-

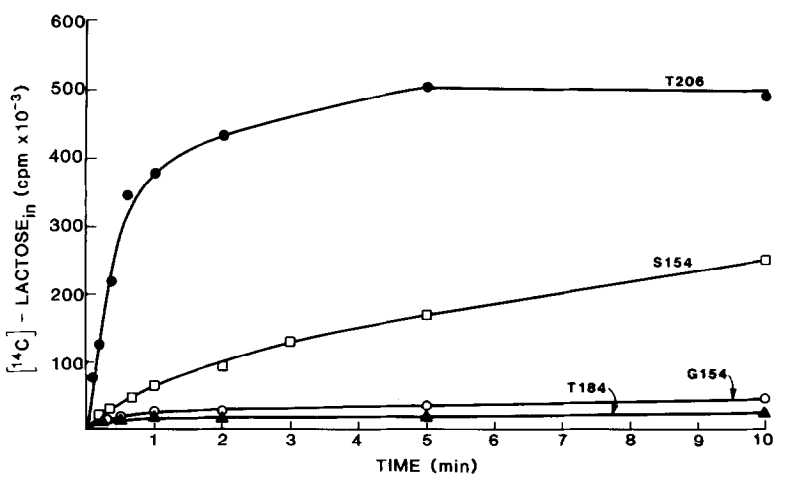

Fig. 3. Lactose transport in *E. coli* T206 (●), G154 (○), S154 (□) and T184 (▲). Transport was measured in 100 mM potassium phosphate (pH 7.5)/10 mM $MgSO_4$ with 0.38 mM [1-^{14}C]lactose (59 mCi/mmol).

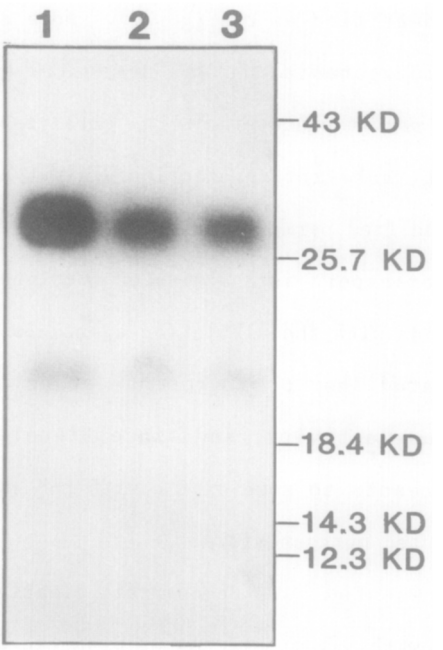

Fig. 4. Quantitation of *lac* permease in membranes from *E. coli* T206, S154 and G154. RSO membrane vesicles (3.8 μg of protein) from T206 (lane 1), S154 (lane 2) and G154 (lane 3) were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis, and the protein bands were electroblotted on to nitrocellulose. The material was then incubated sequentially with Mab 4A10R and [^{125}I]-labeled protein A and radioautographed for 20 h as described (19). Arrows correspond to prestained molecular weight standards (Bethesda Research Laboratories): ovalbumin (43 kDa), α-chymotrypsinogen (25.7 kDa), β-lactoglobulin (18.4 kDa), lysozyme (14.3 kDa) and cytochrome *c* (12.3 kDa).

cantly higher than the initial rate of transport in T184 and G154, and the steady-state level of accumulation is at least one-third of that observed in T206 (Fig. 3).

The possibility that the permease molecules encoded by pG154 and pS154 are not incorporated into the membrane secondary to a defect in insertion or enhanced proteolysis is ruled out by immunoblot analyses with Mab 4A10R and [^{125}I]-labeled protein A (Fig. 4). As shown, the intensities of the 33-kDa bands corresponding to *lac* permease in G154 and S154 membranes are slightly less, but comparable to that observed in T206 membranes.

DISCUSSION. Based on substrate protection against N-ethylmaleimide (NEM) inactivation, it was postulated (21) that there is an essential sulfhydryl group in the *lac* permease located at or near the active site, and the

residue was later identified as cys_{148} (22). More recently, however, by using site-directed mutagenesis, it was demonstrated (6,7,23) that *lac* permease with *gly* or *ser* in place of cys_{148} catalyzes lactose:H^+ symport, but does not exhibit substrate protection against alkylation. Furthermore, although the modified permease molecules are relatively resistant to NEM, active lactose transport is inactivated completely over sufficiently long incubation periods with the alkylating agent. Based on these observations, it was postulated that a *cys* residue other than cys_{148} might be obligatory for permease activity, and since Bieseler *et al.* (24) demonstrated that cys_{154} reacts to some extent with [^{14}C]NEM, this residue was subjected to site-directed mutagenesis.

The experiments presented here demonstrate clearly that replacement of cys_{154} in the permease with *gly* or *ser* causes dramatic diminution in lactose transport activity, thereby implying that cys_{154} plays a critical role in the symport mechanism. However, the weight of the conclusion should be tempered by the following considerations: (i) *Gly* residues are "helix breaking", while *ser* residues, like *cys*, are "helix indifferent" (25). Therefore, the observation that transport activity is virtually nil in G154 may reflect a secondary structural alteration *in addition to* replacement of *cys*, since substitution of cys_{154} with *ser* also causes marked, though less complete loss of activity. (ii) In addition to cys_{148} and cys_{154}, there are six other *cys* residues in the permease that are yet to be mutagenized, and substitutions at one or more of these residues may also compromise permease activity. In any event, *lac* permease with *ser* in place of cys_{154} retains some ability to catalyze lactose:H^+ symport. Possibly, therefore, the hydroxyl function in *ser* is able to mimic the thiol function in *cys* to some extent.

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