

## Proline Transport in *Salmonella typhimurium*: *putP* Permease Mutants with Altered Substrate Specificity

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Received 23 May 1986/Accepted 21 July 1986

The *putP* gene encodes a proline permease required for *Salmonella typhimurium* LT2 to grow on proline as the sole source of nitrogen. The wild-type strain is sensitive to two toxic proline analogs (azetidine-2-carboxylic acid and 3,4-dehydroproline) also transported by the *putP* permease. Most mutations in *putP* prevent transport of all three substrates. Such mutants are unable to grow on proline and are resistant to both of the analogs. To define domains of the *putP* gene that specify the substrate binding site, we used localized mutagenesis to isolate rare mutants with altered substrate specificity. The position of the mutations in the *putP* gene was determined by deletion mapping. Most of the mutations are located in three small (approximately 100-base-pair) deletion intervals of the *putP* gene. The sensitivity of the mutants to the proline analogs was quantitated by radial streaking to determine the affinity of the mutant permeases for the substrates. Some of the mutants showed apparent changes in the kinetics of the substrates transported. These results indicate that the substrate specificity mutations are probably due to amino acid substitutions at or near the active site of proline permease.

Very few ion-driven transport proteins have been studied in detail, and the precise mechanism of substrate binding and translocation is not yet known for any transport system. About 40% of the substrates transported by *Escherichia coli* and *Salmonella typhimurium* enter the cell by ion-driven transport systems (30). The energy required for concentration of these substrates inside the cell is obtained either directly (e.g., proton symport) or indirectly (e.g., sodium symport) from the chemiosmotic gradient. Specificity of a transport protein must be determined by a unique binding site for the substrate. In addition, ion-driven transport systems must have a binding site for the specific counterion. When both the substrate and counterion bind to the transport protein it undergoes a conformational change and releases them into the cytoplasm (J. Cronan, R. Gennis, and S. Maloy, in F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger, ed., *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology, in press).

Proline transport by *S. typhimurium* is a good model system for studying the molecular mechanism of sodium symport. The *putP* gene encodes proline permease, an integral membrane protein with an apparent molecular weight of 25,000 (34; R. Menzel, personal communication). The *putP* permease catalyzes active transport of proline by sodium-proline symport (6, 7). The  $K_m$  for proline uptake by this system is 2  $\mu$ M (33). Two betaine transport systems can also transport proline (4, 5), a low-affinity permease ( $K_m$ , 300  $\mu$ M) encoded by the *proP* gene (16) and a permease encoded by the *proU* gene which is induced by high osmolarity (8). However, the *putP* permease is the primary proline transport protein, since *putP* mutants will not grow on proline as the sole nitrogen or carbon source (14, 17, 22).

In addition to the *putP* gene the *put* operon contains the *putA* gene, which encodes a bifunctional degradative enzyme required for the use of proline as a sole nitrogen or carbon source (14, 17, 22). The *putP* and *putA* genes map at 22 min on the *S. typhimurium* chromosome (24). Mutations in the *putP* gene can be selected by using two proline analogs

(23), L-azetidine-2-carboxylic acid (AZT) and 3,4-dehydroproline (DHP) (Fig. 1). Both analogs are transported into the cell by the *putP* permease and are toxic when incorporated into proteins in place of proline (28). Typically, mutations in *putP* confer resistance to both AZT and DHP. By using these phenotypes many *putP* mutants have been isolated and characterized in *S. typhimurium* (14, 17, 22). An extensive fine structure map of the *putP* gene has been constructed, allowing new mutations to be placed precisely within the gene.

The ease of selecting and characterizing *putP* mutants allowed us to isolate unique mutants of proline permease with altered substrate specificity. Genetic and biochemical characterization of *putP* mutants with altered specificity of the proline and sodium binding sites should provide insight into the molecular mechanism of sodium-driven transport systems.

### MATERIALS AND METHODS

**Strains and media.** All strains used in this study were derived from *S. typhimurium* LT2 and are listed in Table 1. Rich media contained 0.8% Difco nutrient broth with 0.5% NaCl. Minimal media were E medium lacking citrate (NCE) (22) and a medium lacking both carbon and nitrogen sources (NCN) (1). NCE and NCN media were supplemented with 0.6% sodium succinate as a carbon source. When tetracycline-resistant cells were selected, tetracycline hydrochloride was added to rich media at a final concentration of 20  $\mu$ g/ml and to minimal medium at a final concentration of 10  $\mu$ g/ml.

**Genetic screens for *put* mutants.** NCN medium with succinate and 0.2% L-proline (PSN medium) was used to check the ability of cells to utilize proline as the sole nitrogen source.

Sensitivity to toxic proline analogs was tested by replica plating onto NCE-succinate plates that contained 27  $\mu$ g of AZT per ml or 12  $\mu$ g of DHP per ml. Analog sensitivity was quantitated by measuring the zone of inhibition after radial streaking (23) on NCE-succinate plates that contained 20  $\mu$ l of a 20-mg/ml solution of AZT or 20  $\mu$ l of a 5-mg/ml solution of DHP added to a sterile filter disk.

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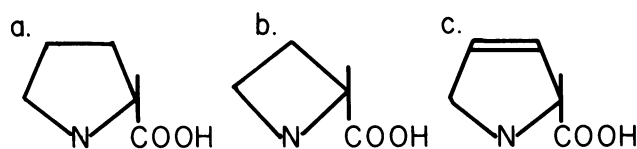


FIG. 1. Structures of three *putP* permease substrates: a, proline; b, AZT; c, DHP.

Glycyl-proline and leucyl-proline are both transported into *S. typhimurium* by a dipeptide transport system (19). After these dipeptides are cleaved, the proline residue can be utilized as a nitrogen source if proline oxidase (encoded by *putA*) activity is present. Leucine cannot be utilized as a sole nitrogen source (10), and glycine is utilized very slowly. Hence, *putP putA*<sup>+</sup> mutants can grow on the dipeptides, but *putP*<sup>+</sup> *putA* or *putP putA* mutants cannot. Previously leucyl-proline was used to differentiate *putP* from *putA* mutants of *S. typhimurium*. However, results with glycyl-proline are faster and easier to score than those with leucyl-proline. Therefore the ability to use glycyl-proline as a sole nitrogen source on NCN-succinate plates was tested to confirm that mutants were *putA*<sup>+</sup>. Approximately 5 mg of the dipeptide was placed directly on NCN-succinate plates.

**Genetic techniques.** All transductions were done with a derivative of bacteriophage P22. P22 HT105/1 *int-201* contains mutations that increase the frequency of generalized transduction and prevent the formation of stable lysogens (25). Phage lysates were prepared as described by Davis et

al. (9). For transductions, phage and cells were mixed directly on selective agar at a multiplicity of infection of approximately 1 PFU per cell. Transductants were screened for mutant *putP* phenotypes by replica plating onto PSN, AZT, and DHP plates. The addition of 10 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid] to the replica plates greatly increased the number of phage-sensitive mutants recovered from the screening process by preventing the readsorption of phage to the cells. Green plates were used for the isolation of phage-free transductants (2, 9). Transductants were tested for sensitivity to phage P22 infection by cross-streaking against the P22 clear plaque mutant H-5 (9).

**Localized mutagenesis.** A bacteriophage P22 lysate was grown on TT1797 (*zcc-7::Tn10*) and centrifuged at 27,000 × g for 2 h. The phage pellet was gently suspended in 1 ml of LBSE (1% tryptone [Difco Laboratories, Detroit, Mich.], 0.5% yeast extract, 1 M NaCl, 1 mM EDTA). The concentrated phage lysate was mutagenized with hydroxylamine in vitro (9, 12). When the titer reached 1.0 to 0.1% of the initial titer, the mixture was centrifuged, and the pellet was gently suspended in LBSE. Approximately 1% of the final phage titer was clear plaque mutants. LT2 was transduced to Tet<sup>r</sup> with the mutagenized phage, and transductants were screened for *putP* mutants.

**Chemicals.** L-Proline, AZT, DHP, EGTA, succinate, glycyl-L-proline, leucyl-L-proline, and tetracycline hydrochloride were obtained from Sigma Chemical Co., St. Louis, Mo. Media and agar were obtained from Difco.

## RESULTS

**Isolation and screening of mutants.** The major proline permease, encoded by the *putP* gene, mediates active transport of proline and two toxic proline analogs, AZT and DHP. Cells containing the wild-type permease are phenotypically Put<sup>+</sup> AZT<sup>s</sup> DHP<sup>s</sup> (Table 2). Typically, *putP* mutations abolish permease function, leaving the cell Put<sup>-</sup> AZT<sup>r</sup> DHP<sup>r</sup> (Table 2); these mutations map throughout the *putP* gene. We reasoned that it should be possible to isolate rare classes of *putP* mutants that exclude the transport of only one or two of the substrates. These rare mutants may be due to mis-sense mutations that change the substrate binding site such that its ability to recognize one of the substrates is diminished.

To obtain rare substrate specificity mutants, we performed localized mutagenesis of the *putP* gene. A P22 transducing lysate grown on a strain (TT1797) with a *Tn10* insertion 80% linked to the *putP* gene (*zcc-7::Tn10*) was mutagenized with hydroxylamine in vitro. LT2 was transduced to Tet<sup>r</sup> with this mutagenized phage lysate, and transductants were replica plated from selection plates directly onto PSN, AZT, and DHP plates. The replica plates were screened for colonies showing phenotypes expected for *putP* mutations that alter substrate binding specificity (i.e., the ability to transport one or two but not all three of the substrates).

To confirm the chromosomal location of the mutations, P22 was grown on each mutant, and the lysate was used to transduce LT2 to Tet<sup>r</sup>. Since the *put* genes are about 80% linked to the *Tn10* insertion, this fraction of the Tet<sup>r</sup> transductants should also inherit the *put* region of the donor strain. If the mutant phenotype is due to a mutation in or near *putP*, about 80% of the Tet<sup>r</sup> transductants will inherit the mutant phenotype. However, if the mutation is unlinked to *putP*, none of the Tet<sup>r</sup> transductants will inherit the mutant phenotype. All of the substrate specificity mutants

TABLE 1. Bacterial strains used

Strain <sup>a</sup>	Genotype	Source <sup>b</sup>
LT2	Wild type	J. Roth
TR4937	<i>Δput-550</i>	Ratzkin and Roth
TR4944	<i>Δput-557</i>	Ratzkin and Roth
TR4946	<i>Δput-559</i>	Ratzkin and Roth
TR5023	<i>Δput-715</i>	Ratzkin and Roth
TR3400	<i>Δput-515</i>	Ratzkin and Roth
TR4921	<i>Δput-534</i>	Ratzkin and Roth
TR4950	<i>Δput-563</i>	Ratzkin and Roth
TR4959	<i>Δput-572</i>	Ratzkin and Roth
TR4981	<i>Δput-594</i>	Ratzkin and Roth
MS325	<i>Δput-516</i>	S. Maloy
TT2264	<i>Δput-679 zcc-7::Tn10</i>	J. Roth
TT1797	<i>zcc-7::Tn10</i>	Menzel and Roth
MS893	<i>putP1082::Tn10</i>	This study
MS120	<i>putP1161</i>	This study
MS124	<i>putP1162</i>	This study
MS144	<i>putP1163</i>	This study
MS146	<i>putP1164</i>	This study
MS148	<i>putP1165</i>	This study
MS1542	<i>putP1149 zcc-7::Tn10</i>	This study
MS1543	<i>putP1150 zcc-7::Tn10</i>	This study
MS1546	<i>putP1151 zcc-7::Tn10</i>	This study
MS1547	<i>putP1152 zcc-7::Tn10</i>	This study
MS1548	<i>putP1153 zcc-7::Tn10</i>	This study
MS1552	<i>putP1154 zcc-7::Tn10</i>	This study
MS1553	<i>putP1155 zcc-7::Tn10</i>	This study
MS1554	<i>putP1156 zcc-7::Tn10</i>	This study
MS1555	<i>putP1157 zcc-7::Tn10</i>	This study
MS1558	<i>putP1158 zcc-7::Tn10</i>	This study
MS1562	<i>putP1159 zcc-7::Tn10</i>	This study
MS1566	<i>putP1160 zcc-7::Tn10</i>	This study

<sup>a</sup> All strains were derived from *S. typhimurium* LT2.

<sup>b</sup> The strains constructed by Ratzkin and Roth (23) and Menzel and Roth (18) were obtained from J. Roth, Department of Biology, University of Utah, Salt Lake City.

TABLE 2. Phenotypes of *PutP* mutants isolated in this study

Strain	Allele	Phenotype <sup>a</sup>			Zone of inhibition (mm) <sup>b</sup>	
		Put	AZT	DHP	AZT	DHP
LT2	Wild type	+	S	S	20	20
MS893	<i>putP1082::Tn10</i>	—	R	R	0	0
MS124	<i>putP1162</i>	—	R	R	0	0
MS144	<i>putP1163</i>	—	R	R	0	0
MS148	<i>putP1165</i>	—	R	R	0	0
MS1554	<i>putP1156</i>	—	R	R	0	0
MS1555	<i>putP1157</i>	—	R	R	0	0
MS1558	<i>putP1158</i>	—	R	R	0	0
MS120	<i>putP1161</i>	—	R	S	0	20
MS146	<i>putP1164</i>	—	R	S	0	19
MS1542	<i>putP1149</i>	—	R	S	0	18
MS1543	<i>putP1150</i>	—	R	S	0	17
MS1546	<i>putP1151</i>	—	R	S	0	17
MS1547	<i>putP1152</i>	—	R	S	3	20
MS2548	<i>putP1153</i>	—	S	S	25	25
MS1552	<i>putP1154</i>	± <sup>c</sup>	R	R	0	4
MS1553	<i>putP1155</i>	±	R	S	0	16
MS1562	<i>putP1159</i>	+	S	R	15	19 <sup>d</sup>
MS1566	<i>putP1160</i>	+	R	R	0	0

<sup>a</sup> Mutants were screened for growth (+, growth; —, no growth) on medium with proline as the sole nitrogen source (Put) and for their sensitivity (S) or resistance (R) to the toxic proline analogs AZT and DHP.

<sup>b</sup> Mutant sensitivity to the analogs was quantitated by measuring zones of inhibition (24).

<sup>c</sup> ± indicates leaky growth on PSN plates.

<sup>d</sup> This mutant (selected as DHP<sup>r</sup>) had a 19-mm zone of leaky growth near the DHP disk, indicating weak transport of DHP.

obtained in this study were closely linked to the *zcc-7::Tn10* insertion.

Since *putA* mutants are also unable to grow on PSN and some *putA* mutants are resistant to proline analogs (S. R. Maloy, in Neidhardt et al., ed., *Escherichia coli* and *Salmonella typhimurium: Cellular and Molecular Biology*, in press), each of the mutant strains was screened to determine

whether the mutation was in the *putP* or *putA* gene. Transport of the dipeptide glycyl-proline does not require the *putP* gene, but its utilization as a nitrogen source does require the *putA* gene product. Therefore, the *putA* phenotype of our mutants was tested by radial streaking away from glycyl-proline on NCN-succinate plates. All of the mutants characterized in this study were *putA*<sup>+</sup>.

In one experiment, out of 2,000 Tet<sup>r</sup> transductants only 12 appeared to have mutations in the *putP* gene that altered substrate binding specificity of the permease. In contrast, null *putP* mutants occurred at a frequency of 1 per 20 Tet<sup>r</sup> transductants. A total of 24 independent mutants with the altered substrate specificity phenotypes were isolated; 11 of these specificity mutants were thoroughly characterized.

**Deletion mapping.** To map the substrate specificity mutations within the *putP* gene, we carried out transductional crosses with donor phage P22 lysates grown on each of the mutants. The recipients for these crosses were strains carrying deletions of various segments of the *putP* gene (Fig. 2). The available deletions subdivide the *putP* gene into approximately 100-base-pair intervals (S. Maloy, unpublished results). The deletion mutants are phenotypically Put<sup>−</sup>. Overnight cultures of the deletion mutants were mixed with P22 lysates of the Put<sup>−</sup> substrate specificity mutants directly on PSN plates and incubated for 48 to 72 h at 37°C. If the mutation being mapped lies outside the region deleted from the chromosome, then recombination between the deletion endpoint and the donor mutation can yield wild-type progeny. However, if the mutation lies within the deletion, recombination events that replace the sequence deleted from *putP* must bring in the Put<sup>−</sup> donor mutation, resulting in no Put<sup>+</sup> recombinants. Out of 13 Put<sup>−</sup> mutations mapped, 11 (including some null mutations) were located in three deletion intervals of the *putP* gene. Three different analog sensitivity phenotypes were represented among these Put<sup>−</sup> mutants: AZT<sup>r</sup> DHP<sup>r</sup>, AZT<sup>r</sup> DHP<sup>s</sup>, and AZT<sup>s</sup> DHP<sup>s</sup>. The mutations responsible for the different phenotypes did not segregate into separate deletion intervals (Fig. 2).

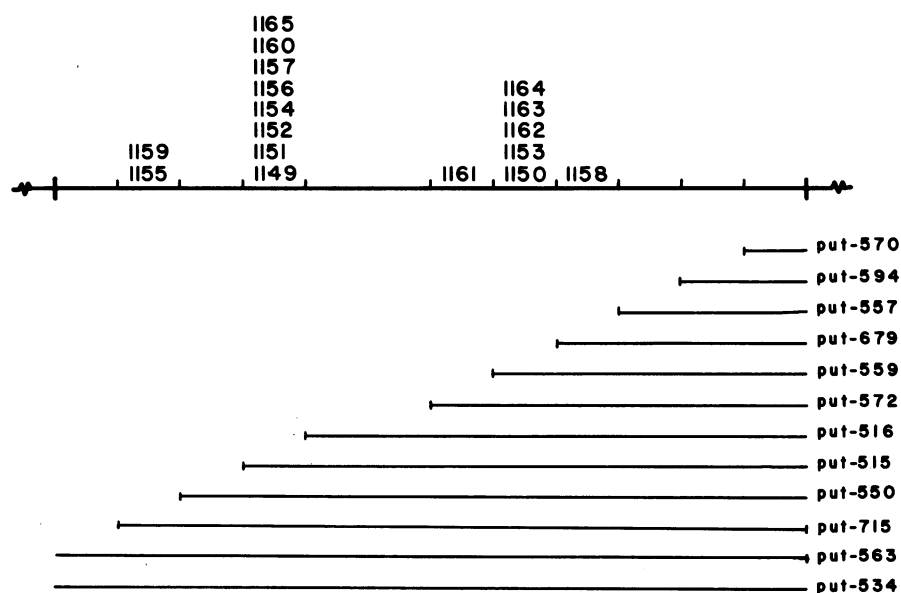


FIG. 2. Deletion map of the substrate specificity mutations in *putP*. The map positions and allele numbers of the substrate specificity mutations are indicated at the top, and the deletion mutations are shown below. The *putP* promoter is on the right side of the diagram. Allele 1153 is Put<sup>−</sup> AZT<sup>s</sup> DHP<sup>s</sup>; 1154 is Put<sup>+/−</sup> AZT<sup>r</sup> DHP<sup>r</sup>; 1155 is Put<sup>+/−</sup> AZT<sup>r</sup> DHP<sup>r</sup>; 1159 is Put<sup>+</sup> AZT<sup>s</sup> DHP<sup>r</sup>; 1160 is Put<sup>+</sup> AZT<sup>s</sup> DHP<sup>r</sup>; 1161, 1164, 1149, 1150, 1151, and 1152 are Put<sup>−</sup> AZT<sup>r</sup> DHP<sup>s</sup>; 1162, 1163, 1165, 1156, 1157, and 1158 are Put<sup>−</sup> AZT<sup>r</sup> DHP<sup>r</sup>.

Selection for Put<sup>+</sup> transductants on PSN plates could not be used to directly map the substrate specificity mutants that were phenotypically Put<sup>+</sup>, since all recombination events replacing the deletion would give a Put<sup>+</sup> phenotype. For these mutations, the frequency of transfer of the mutant phenotype to the Put<sup>+</sup> transductants was used to define the deletion interval carrying the mutation. Phage lysates grown on mutants that retained the ability to transport proline, but could not transport one or both of the analogs, were used to transduce deletion strains to Put<sup>+</sup>. To map the mutations the transductants were screened on PSN, AZT, and DHP plates for the frequency of transfer of the mutant phenotype to the transductants. When a mutation was crossed with a deletion that removes the corresponding sequence from the chromosome, 100% of the Put<sup>+</sup> transductants coinherited the mutant phenotype. However, when a mutation was crossed with a deletion that removes chromosomal sequences adjacent to but not including the corresponding chromosomal sequences, only 90 to 96% of the transductants inherited the mutant phenotype. In crosses with other deletions, less than 90% of the transductants inherited the mutant phenotype. Based on these results, the Put<sup>+/−</sup> AZT<sup>r</sup> DHP<sup>r</sup> and the Put<sup>+</sup> AZT<sup>r</sup> DHP<sup>r</sup> mutations, *putP1154* and *putP1160*, respectively, mapped in the same deletion interval that contained many of the Put<sup>−</sup> mutations (Fig. 2). In contrast, *putP1155* (Put<sup>+/−</sup> AZT<sup>r</sup> DHP<sup>s</sup>) and *putP1159* (Put<sup>+</sup> AZT<sup>s</sup> DHP<sup>r</sup>) mapped in the distal end of the *putP* gene (corresponding to the carboxy terminus of proline permease).

**Levels of analog sensitivity.** Each of the altered substrate specificity mutants was analyzed by radial streaking against AZT and DHP to quantitate the relative sensitivity to the analogs. The zone of inhibition for each of the analogs was also measured in the wild type and in null *putP* mutants. LT2 showed zones of inhibition of 20 mm for both analogs (Table 2). Null *putP* mutants grew to the edge of the analog-saturated disks, showing no signs of inhibition at the analog concentrations used (Table 2). Many of the *putP* substrate specificity mutants (e.g., strains MS1547 and MS1552) showed intermediate sensitivity to one or both of the analogs (Table 2). This simple in vivo test measures the apparent kinetics of transport of these substrates by proline permease. The *putP1152* mutant showed a zone of inhibition of 3 mm for AZT, although it was selected as AZT<sup>r</sup> (Table 2). Therefore, this mutant can still transport AZT with a reduced affinity. The *putP1154* mutant was selected as AZT<sup>r</sup> DHP<sup>r</sup> but retained leaky growth on proline (Table 2). This mutant showed a zone of inhibition of 4 mm for DHP, which suggests that the permease has a decreased affinity for both proline and DHP. Several other mutants with intermediate sensitivity to the analogs are shown in Table 2. These results suggest that some of the mutants with altered binding specificity may have altered  $K_m$ s for the substrates tested, supporting the hypothesis that these mutations affect the substrate binding site of proline permease.

## DISCUSSION

In this study we isolated and characterized strains with mutations in the *putP* permease that alter its substrate binding specificity. The wild-type *putP* permease transports three substrates: proline, DHP, and AZT. Although these substrates share common structural features, the three-dimensional structure of the substrates is different. Therefore, the substrate specificity mutations must alter the conformation of the substrate binding site without destroying the residues critical for substrate binding. Most of the substrate

specificity mutations mapped in three deletion intervals of the *putP* gene. We believe that these regions may specify domains of the active site for substrate binding and translocation. Some of the mutations might be substitutions in domains that have no direct interaction with the substrate but cause conformational changes in the protein that in turn alter the binding site. However, it seems likely that this class of mutation would cause a major change in the binding site, preventing the uptake of all substrates, unlike the mutants we have collected which can transport at least one of the three substrates. Seven different substrate specificity phenotypes were distinguished; each phenotype is presumably due to a different amino acid substitution. Some of the mutations that confer similar phenotypes map in different deletion intervals of the *putP* gene. These data indicate that multiple regions of the protein are involved in binding the substrates. We are presently determining the DNA sequence of the *putP* gene from the wild type and the altered specificity mutants to determine the amino acid substitutions caused by the mutations. The amino acid substitutions in the mutants may indicate how residues at the active site of the permease recognize the substrates. Determining the amino acids involved in substrate binding is necessary to determine the molecular mechanism of substrate translocation by proline permease.

It is difficult to localize the active site of a permease biochemically. Although analysis of substrate specificity mutations is a direct genetic approach for localizing the amino acids at the active site, very few permeases have been extensively analyzed genetically. The major reason for this is the difficulty of directly selecting rare missense mutations in the permease gene. Localized mutagenesis allowed us to rapidly screen for rare mutants with substrate specificity mutations in the *putP* gene. Previous genetic studies have helped define the active site of lactose permease in *E. coli*. The wild-type *lacY* permease is an H<sup>+</sup>-galactoside symporter that transports maltose poorly (13, 21). Several groups have isolated mutants that transport maltose at much higher levels than the wild type (3, 11, 18, 27). Some of these mutants retained the ability to transport galactosides at wild-type levels, and others had a decreased ability to transport galactosides. One of three amino acid residues of lactose permease was substituted in each of the mutants: Ala 177, Tyr 236, or Thr 266 (3, 15). These amino acids are believed to be involved in substrate binding and translocation by lactose permease.

Additionally, ion-driven transport systems must have a unique binding site for the specific counterion. It is also possible to isolate permease mutants with an altered cation specificity. Two *lacY* mutants that have lost the ability to accumulate galactosides were previously thought to have carriers with an altered ability to couple H<sup>+</sup> and galactoside transport (31, 32). However, it appears that these mutations actually cause a decreased rate of active transport that is unrelated to cation binding (35). Bona fide cation binding mutations have been isolated in the *melB* gene (20, 26), which encodes melibiose permease. Melibiose permease can use either an H<sup>+</sup> or an Na<sup>+</sup> electrochemical gradient to accumulate melibiose (29). One *melB* mutant lost the ability to use H<sup>+</sup> as a cotransport ion but can still use Na<sup>+</sup> (20, 26). This mutant can also use Li<sup>+</sup> as a counterion at Li<sup>+</sup> concentrations that normally inhibit melibiose uptake (20). The mutation caused the substitution of Ser for Pro at position 122 of the melibiose permease (27). Preliminary results suggest that cation specificity mutants can also be obtained in the *putP* system (S. Maloy, unpublished results).

Comparison of the amino acid sequence of the substrate and cation binding sites of proline permease ( $\text{Na}^+$  symport), melibiose permease ( $\text{H}^+$ - $\text{Na}^+$  symport), and lactose permease ( $\text{H}^+$  symport) may suggest a molecular mechanism of substrate translocation by ion-driven transport systems.

#### ACKNOWLEDGMENTS

We thank Linda DeVeaux, Tom Elliott, and Jordan Konisky for critically reviewing this manuscript.

This work was supported by Public Health Service grant GM34715 from the National Institutes of Health to S.R.M.

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