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Alteration in Cation Specificity of the Melibiose Transport Carrier of *Escherichia coli* Due to Replacement of Proline 122 with Serine

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The structural genes (*melB*) for the melibiose carrier of five mutants of *Escherichia coli* showing altered cation specificity for melibiose transport were cloned. The mutations were mapped in a 248-base-pair DNA fragment by a recombinational assay by using the mutants transformed with hybrid plasmids carrying various portions of the wild-type *melB* gene. The nucleotide sequences of the corresponding DNA fragments derived from mutated *melB* genes were determined, and the amino acid sequences of the carrier were deduced. Proline 122 was replaced with serine in the melibiose carrier of all five mutants (which were isolated independently). We conclude that this amino acid replacement caused the alteration in cation specificity (loss of coupling to H^+) of the melibiose carrier.

The melibiose carrier of *Escherichia coli* mediates cotransport of galactosides (mainly α-galactosides) with monovalent cations. One of the interesting characteristics of the melibiose carrier is its versatility in cation coupling. Although most cotransport carriers of *E. coli* utilize H^+ as a coupling cation (17), the melibiose carrier utilizes Na^+, H^+, or Li^+ for cotransport, depending on the substrate transported (23, 24). For example, melibiose transport is coupled with either Na^+ or H^+. The lithium ion, which is a potent coupling cation for methyl-β-D-thiogalactopyranoside (TMG) transport, is a very poor coupling cation for melibiose transport and is inhibitory for melibiose transport (22). Therefore Li^+ inhibits *lacY*$^-$ cell growth when melibiose is the sole source of carbon (22). We have isolated mutants which grow on melibiose in the presence of Li^+ (14). These mutants showed altered cation specificity for melibiose transport (14). The melibiose carrier of such mutants lost the ability to utilize H^+ as a coupling cation for melibiose transport. Instead, the carrier acquired the ability to utilize Li^+ for melibiose transport. Normal Na^+-melibiose cotransport was observed in the mutants. Judging from the reversion frequencies, the mutants harbored single mutations (19). The mutations were mapped in the *melB* gene (19), the structural gene for the melibiose carrier.

To understand the molecular mechanism by which cation-substrate cotransport is operated, it is important to know the structure of the carrier. We employed techniques for DNA sequencing to elucidate the primary structure of the carrier. We determined the nucleotide sequence of the wild-type *melB* gene, and the amino acid sequence of the melibiose carrier was deduced (25). Thus, it became possible to determine the substituted nucleotide of the mutated *melB* gene and to infer the amino acid substitution of the carrier of the mutant strains. Here we report cloning of mutated *melB*, intracistronic mapping of the mutation, and replacement of nucleotides and amino acids.

MATERIALS AND METHODS

Bacteria. The *E. coli* strains utilized in these studies are listed in Table 1. All strains are derivatives of strain K-12 and lack the lactose transport system. All *melB* mutants were derived from strain W3133-2. Mutants with altered cation coupling were selected as described previously (14). They were isolated independently but showed similar cation coupling to melibiose transport. That is, they lost the ability to transport melibiose coupled to H^+ and gained the ability to transport melibiose coupled to Li^+ (14).

Media. Cells were grown at 37°C in the following media depending on the experiments. A minimal salts medium (20) (Na^+ salts were replaced with K^+ salts) supplemented with 10 mM melibiose and 1% tryptone (Difco Laboratories) for transport assays, L broth (8) for plasmid propagation, MT medium (19), or the minimal slats medium containing 10 mM melibiose for the recombination assay and growth test. The MT medium was originally developed to minimize the contamination of Na^+ (19). When the MT medium was solidified with agar, the agar had been washed twice with the MT medium, in advance, to reduce the contaminating Na^+.

Plasmids. Plasmids pBR322 (2), pSTY81, and pSTY81-40 (6) were used as starting materials. Plasmids pSTY212, pSTY213, pSTY214, pSTY222, and pSTY231 were constructed as follows. The *Bam*HI fragment obtained from the wild-type *melB* gene (from a plasmid) was digested with *Rsa*I, *Alu*I, or *Hae*III, and the resulting DNA fragments were separated by polyacrylamide gel electrophoresis. Each DNA fragment was ligated to the *Sca*I site (pSTY212, pSTY213, and pSTY214) or the *Bam*HI and *Pvu*II sites (pSTY222 and pSTY231) of pBR322. Cells of strain RE16r were transformed with each plasmid, and appropriate drug-resistant transformants were isolated. Then their drug (ampicillin or tetracycline) sensitivity was checked. Plasmids were prepared from the cells, and insertion of each DNA fragment was confirmed by measuring changes in the sizes of the DNA fragments.

Recombination assay. Competent cells were prepared from each mutant and transformed (13) with each plasmid. Transformed cells were selected on L agar containing either ampicillin or tetracycline. Antibiotic-resistant colonies were then streaked on agar plates containing the MT medium and 10 mM melibiose and were checked to see whether recombinants showing wild-type properties (cells that can grow on melibiose in the absence of Li^+ or Na^+) appeared.

Preparation of DNA. Chromosomal DNA and plasmid DNA were prepared by published procedures (1, 11). DNA

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TABLE 1. Strains utilized

Strain	Relevant properties	Reference
W3133-2	*lacZ-lacY*	(10)
W3133-2S	*melB317*(Cc)[a] *lacZ-lacY*	(14)
W3133-2T	*melB318*(Cc) *lacZ-lacY*	(14)
W3133-2U	*melB319*(Cc) *lacZ-lacY*	This paper
W3133-2V	*melB320*(Cc) *lacZ-lacY*	This paper
W3133-2W	*melB321*(Cc) *lacZ-lacY*	This paper
RE16r	*melB lacZ-lacY recA*	(6)

[a] *melB*(Cc), Altered cation coupling in the melibiose carrier.

was digested with appropriate restriction endonucleases, and DNA fragments were separated by polyacrylamide gel electrophoresis and eluted (12).

Nucleotide sequence. The nucleotide sequence was determined by the method of Maxam and Gilbert (12).

Li^+ transport. Entry of Li^+ into cells induced by melibiose or TMG was measured by a Li^+-selective electrode as described previously (23).

Enzymes and chemicals. Restriction endonucleases, bacterial alkaline phosphatase, and T4 polynucleotide kinase were obtained from Takara Co. [γ-^{32}P]ATP was prepared from $^{32}P_i$ (New England Nuclear Corp.) and ADP (Sigma Chemical Co.) by a published method (18).

RESULTS

Cloning of mutated gene. We found that the mutation of strain W3133-2S was present in the *Bam*HI fragment of the *melB* gene (19), which covers ca. 80% of the *melB* gene (25). Plasmid pSTY88 was constructed as a cloning vector for the *Bam*HI fragment of *melB*. First, a small *Sau*3A fragment was inserted into the *Bam*HI site of pBR322 to inactivate the *Bam*HI site, and pSTY203 was obtained (Fig. 1). Then, the *Eco*RI fragment obtained from pSTY81, which carries the whole melibiose operon, was ligated to the *Eco*RI site of pSTY203, and pSTY85 was obtained. This plasmid possesses *melA* and *melB*. Digestion of this plasmid with *Bam*HI and ligation produced pSTY88, which harbors a deletion of the *melB* gene (Fig. 1). If the *Bam*HI fragment of the *melB* gene is inserted in the *Bam*HI site of the pSTY88 with a proper orientation, a complete *melB* gene should appear. The *Bam*HI fragment of mutant chromosomal DNA was prepared as follows. Chromosomal DNA was prepared from mutant strain W3133-2S. The DNA was digested with *Bam*HI and separated on polyacrylamide gel. A region corresponding to ca. 1,100 base pairs was recovered, and DNA was extracted. This DNA fraction should contain the *Bam*HI fragment derived from the *melB* gene. Then, the mixture of the *Bam*HI fragments was ligated to the *Bam*HI site of the cloning vector pSTY88. Although the lactose carrier, product of the *lacY* gene, transports melibiose, there is no possibility that the DNA fragments prepared from W3133-2S contain the *lacY* gene, because W3133-2S is a *lacZ-lacY*-deleted strain. It seems that no other system which can transport melibiose is present in *E. coli*, since all *melB lacY* cells failed to grow on melibiose (unpublished data).

Competent cells of RE16r (*melB* Δ*lacZ-lacY recA*) were transformed with those plasmids and spread on agar plates containing a minimal salts medium plus melibiose. We obtained several colonies on the plates and checked their properties. The original mutant strain W3133-2S can not grow on melibiose as the sole source of carbon in the absence of Na^+ or Li^+ (14). Since the minimal salts medium used contained 0.1 to 0.2 mM of Na^+ (data not shown), strain W3133-2S grew on melibiose in this medium. W3133-2S cells did not grow on melibiose in the MT medium (19), which contains less than 2 μM Na^+ (data not shown). RE16r cells harboring the new plasmid did not grow in the MT medium containing melibiose, but they grew in it in the presence of 10 mM Na^+. This growth property is same as that of W3133-2S. RE16r cells not harboring the plasmid did not grow on melibiose regardless of the presence of Na^+ (or Li^+).

One of the most striking differences in properties of the melibiose carrier between wild-type cells and mutant cells is Li^+-TMG cotransport. Although TMG induced a large influx of Li^+ in wild-type cells, no Li^+ influx was induced by TMG in mutant W3133-2S (Fig. 2) (23). Addition of melibiose to the cell suspension after the addition of TMG caused no further Li^+ influx in wild-type cells; rather, some Li^+ efflux was elicited. In the mutant cells, however, addition of melibiose in the presence of TMG elicited significant Li^+ influx. It seems that TMG is no longer a substrate of the melibiose carrier of strain W3133-2S (23). Properties of the melibiose transport system of strain RE16r harboring the

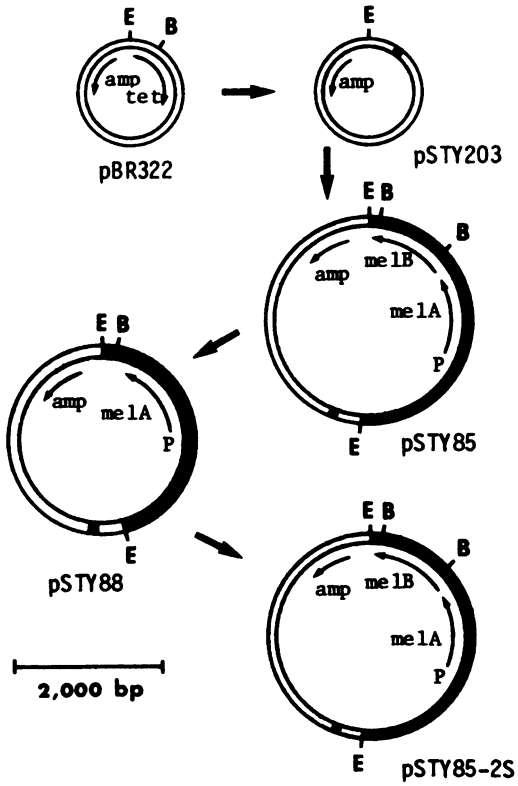

FIG. 1. Construction of plasmids. A small *Sau*3A fragment (130 base pairs) was inserted into the *Bam*HI site of pBR322 to inactivate the *Bam*HI site. The *Eco*RI fragment derived from pSTY81 (6), which carries the melibiose operon, was inserted into the *Eco*RI site of the resulting plasmid, pSTY203. Thus, pSTY85 was obtained. The *Bam*HI fragment of the *melB* gene of pSTY85 was removed by *Bam*HI digestion and ligation. The resulting plasmid pSTY88 is a cloning vector for the *Bam*HI fragment of the *melB* gene. The *Bam*HI fragments of ca. 1,100 base pairs obtained from chromosomal DNA of strain W3133-2S were inserted into the *Bam*HI site of pSTY88. Thus, pSTY85-2S, which carries the mutated region of the *melB* gene of W3133-2S, was obtained. Abbreviations: B, *Bam*HI; E, *Eco*RI; bp, base pairs.

newly constructed plasmid were similar to those of mutant W3133-2S (Fig. 2). A larger Li^+ influx was induced by melibiose in the plasmid-carrying strain, suggesting a gene dosage effect. No Li^+ uptake was induced by the sugars in RE16r not harboring the plasmid. Thus, we have succeeded in cloning the mutated DNA of strain W3133-2S. We designated this plasmid as pSTY85-2S.

The mutated gene (*melB*) for cation coupling of other mutants was cloned by the same methods as described above. Each plasmid carrying each mutated DNA was designated as pSTY85-2T, pSTY85-2U, pSTY85-2V, and pSTY85-2W, which were derived from W3133-2T, W3133-2U, W3133-2V, and W3133-2W, respectively.

Intracistronic mapping. We tried intracistronic mapping of the mutation in the *melB* gene of strain W3133-2S. The *Bam*HI fragment derived from the wild-type *melB* was divided into several smaller fragments; then, each fragment was ligated to pBR322, and plasmids pSTY212, pSTY213, pSTY214, pSTY22, and pSTY231 were constructed (Fig. 3). These plasmids were tested to see whether recombinants that can grow on melibiose (in the absence of Li^+ or Na^+) appeared when strain W3133-2S was transformed with the plasmids. Among the plasmids, only pSTY212 and pSTY231 produced such recombinants; pSTY213, pSTY214, and pSTY222 did not. It was concluded that the mutation was in the region covered by pSTY212, since the whole DNA region of pSTY212 was included in pSTY231. The pSTY212 carries a 248-base-pair DNA fragment derived from the *melB* gene (25). Therefore, the mutation of W3133-2S was mapped in this DNA region. The same results were obtained with W3133-2T, W3133-2U, W3133-2V, and W3133-2W (data not shown).

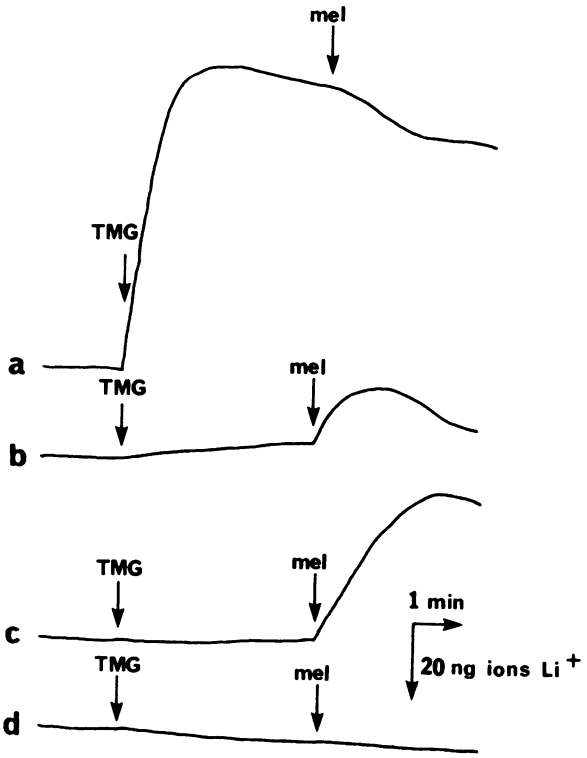

FIG. 2. Uptake of Li^+ induced by sugars via the melibiose carrier. Cells used were W3133-2 (a), W3133-2S (b), Re16(pSTY85-2S) (c), and RE16 (d). Cells were washed three times with 0.1 M 3-(*N*-morpholino)propanesulfonic acid–Tris (pH 6.7). Cells (ca. 5 mg of protein per ml) were incubated in 3 ml of the same buffer containing 100 μM LiCl at 25°C. An anaerobic solution (30 μl) of 1 M TMG or 1 M melibiose (mel) was added at the points indicated. The abscissa shows time, and the ordinate shows the Li^+ concentration. An upward deflection of the record indicates an uptake of Li^+.

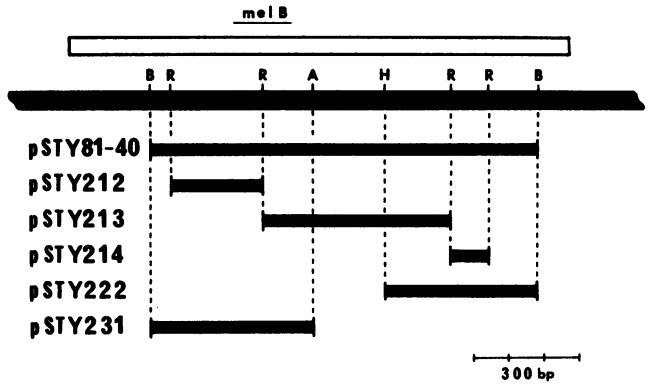

FIG. 3. Plasmids carrying various portions of the *melB* gene. The length of *melB* is indicated at the top. For intracistronic mapping, we constructed plasmids carrying various portions of the *melB* gene. The *Bam*HI fragment of pSTY81-40, which was derived from wild-type *melB*, was digested with *Rsa*I, *Alu*I, or *Hae*III, and each fragment was isolated and ligated to either the *Sca*I site or the *Bam*HI and *Pvu*II sites of pBR322. Abbreviations: A, *Alu*I; B, *Bam*HI; H, *Hae*III; R, *Rsa*I; bp, base pairs.

Nucleotide sequence and amino acid sequence. The nucleotide sequence of the 248-base-pair DNA fragment prepared from pSTY85-2S which carries mutated *melB* derived from strain W3133-2S was determined. We found just one nucleotide substitution in this DNA region. The sequence of the other 247 nucleotides was exactly the same as that of the corresponding region of wild-type *melB* (25). This indicates that the cloned gene was actually *melB*. The *melB* gene consists of 1,407 nucleotides (25). The substituted nucleotide was at position 364 starting from the first letter of the initiation ATG. Cytosine of the antisense strand was replaced with thymine in the mutated gene at this position (Fig. 4). Sequencing of this DNA region was performed twice, and the nucleotide sequence of the corresponding region of the sense strand was determined (Fig. 4). Thus, we conclude that the cytosine · guanine pair of the wild-type gene was replaced with a thymine · adenine pair in the mutant gene at position 364.

This nucleotide substitution results in conversion of CCC to TCC in the genetic code and causes the amino acid replacement of proline at position 122 (starting from NH_2-terminal methionine) with serine (Fig. 5).

We also determined the nucleotide sequence of the 248-base-pair DNA fragments prepared from pSTY85-2T, pSTY85-2U, pSTY85-2V, and pSTY85-2W. To our surprise, exactly the same results were obtained. Namely, a C→T transition at position 364 was found in mutated *melB* of all of the mutants (data not shown). Thus, the replaced amino acid residue in the melibiose carrier of all of the mutants which showed altered cation coupling was from proline to serine at position 122.

DISCUSSION

We determined the nucleotide substitution in the *melB* gene of mutants W3133-2S, W3133-2T, W3133-2U, W3133-2V, and W3133-2W to be thymine for cytosine (wild type) at

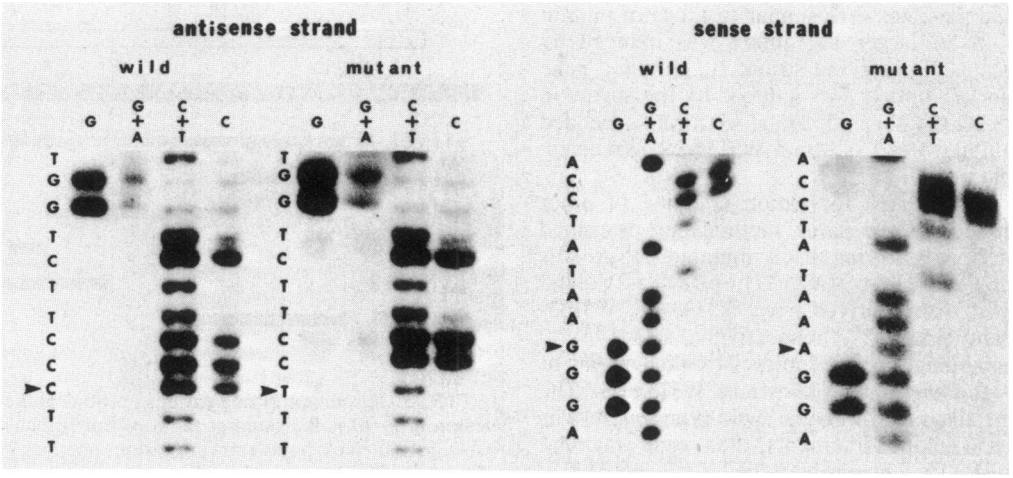

FIG. 4. Nucleotide sequence of the mutated region. The nucleotide sequence of the 248-base-pair DNA derived from mutated *melB* was determined. Sequence ladders of both the antisense and sense strands of the mutated region are shown. Arrows indicate the position of the substitution.

position 364. Since we mutagenized cells with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine when isolating those mutants (14), this transition is consistent with published results that this mutagen was highly specific for the cytosine · guanine to thymine · adenine transition (5).

The results demonstrated that proline 122 of the melibiose carrier of wild-type *E. coli* was replaced with serine in those mutants. This amino acid replacement caused the following changes in the melibiose carrier. (i) The carrier lost the ability to couple transport of any substrate tested to H^+ (14). (ii) The carrier acquired the ability to couple transport of melibiose to Li^+ (14, 23). (iii) TMG and methyl-β-galactopyranoside were no longer substrates of the carrier (23). On the other hand, Na^+-coupled transport of melibiose was unaffected (14). We speculate that the amino acid replacement caused a structural change of the carrier and caused alterations in cation and substrate specificities. It is likely that the mutant carrier protein can not bind H^+ at the proper site, or the carrier cannot function even if H^+ binding occurs. It seems unlikely that proline 122 is directly involved in H^+ binding. Rather, because proline does not possess a dissociable group, the involvement of proline 122 in H^+ transport might be an indirect one. Although a combination of Li^+ and melibiose gave very poor cotransport in the wild type, the conformational change in the mutant carrier made the carrier favorable for Li^+-melibiose cotransport. It is not clear whether serine 122 of the mutant carrier is directly involved in Li^+ binding. It should be pointed out, however, that Li^+ can generally interact directly with a serine residue, because Li^+ is a hard Lewis acid and the hydroxyl group of serine residue is a hard Lewis base in terms of the "hard and soft acids and bases principle" (15, 16). It should be noted that Lewis acids are atoms, ions, or molecules which have at least one atom with a vacant orbital in which a pair of electrons can be accommodated, and bases are similarly units in which there is at least one pair of valence electrons which are not already being shared in a covalent bond (16).

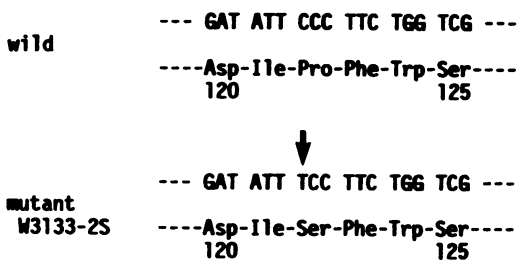

FIG. 5. Amino acid replacement in the melibiose carrier. The nucleotide and amino acid sequences of the mutated region are shown. An arrow indicates the position of the substituted nucleotide. The cytosine-to-thymine transition causes replacement of the proline residue at position 122 of the wild-type carrier with the serine residue in the mutant carrier.

We determined substituted nucleotide of mutated *melB* of five similar mutants and obtained identical results. Those mutants were isolated independently as $Li^+(Na^+)$-dependent mutants (14). Therefore, it seems likely that only the replacement of proline with serine at position 122 of the melibiose carrier can endow the carrier to couple to Li^+ but not H^+.

The replaced amino acid is located in a weakly hydrophobic region (25). The replacement of proline with serine did not significantly effect the hydropathy profile (data not shown), as calculated by a published procedure (7). This is expected because the hydropathy indices of the two amino acids were similar. Furthermore, no significant change was induced in the predicted secondary structure of the carrier by the proline-to-serine transition (data not shown) when calculated by a published procedure (4). Although it is true that both proline and serine are unlikely to occur in α helices, the ability of proline and serine to affect a protein turn might be expected to be rather different. For example, one might expect proline to effect a turn between two neighboring helices so that these two helices would come to lie close to each other (3, 9). With serine substitution this turn might be disrupted, and these two helices might not lie in favorable proximity for cation recognition.

We have isolated many mutants which showed altered cation specificities and substrate specificities and altered activities of the melibiose transport system (manuscript in preparation). We are now trying to characterize the melibiose transport system of such mutants and to determine the replaced amino acid residues. An effect of amino acid replacement in the lactose carrier has been reported by Trumble et al. (21). They modified the lactose carrier by oligonucleotide-directed site-specific mutagenesis. In that case cysteine 148 was converted to glycine. Such alteration

in the carrier caused lower transport activity and less sensitivity to inactivation by *N*-ethylmaleimide. We believe that these strategies are useful to evaluate the role of amino acid residues in transport carrier.

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