Isolation and Characterization of Thiodigalactoside-resistant Mutants of the Lactose Permease Which Possess an Enhanced Recognition for Maltose*

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The lactose permease of *Escherichia coli* has provided a model system in which to investigate active transport (see Refs. 1 and 2 for recent reviews). The study of this transport system has been particularly amenable to a variety of molecular genetic techniques. For example, site-directed mutagenesis has been used to investigate the functional importance of many different amino acids within the protein (3). Once generated, the transport properties of site-directed mutants can be easily assayed in whole cells (4, 5), membrane vesicles (6, 7), or reconstituted proteoliposomes (8, 9). This technique has served to rule out the importance of particular amino acids in transport function (i.e. cysteine residues, Refs. 10 and 11) as well as to suggest that certain amino acids (i.e. Arg-302, His-322, and Glu-325) are involved with H⁺ recognition and transport (12).

An alternative genetic approach towards the identification of important amino acid residues within the lactose permease has involved the direct isolation and sequencing of lactose permease mutants with alterations in function. Due to the historical importance of the lactose operon, a variety of indicator plates are available which make it possible to identify lacY mutants (13). In addition, sugar transport mutants can be distinguished by their ability to grow on minimal plates containing a particular sugar as the sole carbon source. This latter approach was used in the successful isolation of lactose permease mutants which recognize maltose (14, 15) and mutants which recognize maltose but are resistant to cellobiose (16). Overall, this type of work has aided in the identification of particular amino acids (i.e. Ala-177, Tyr-236, Thr-266, Ser-306, Lys-319, His-322, and Ala-389) of the lactose permease which may be important for sugar recognition (14, 16, 17).

In the current study, lactose permease mutants were isolated which exhibited an enhanced recognition for maltose (an α-glucoside) but a diminished recognition for thiodigalactoside, TDG (a β-galactoside). Maltose/TDG mutants were obtained from four different parental strains encoding either a wild-type permease (pTE18), a mutant lactose permease which recognizes maltose (pB15) or mutant lactose permeases which recognize maltose but are resistant to inhibition by cellobiose (pTG and pBA). A total of 27 independent mutants were isolated: 12 from pTE18, 10 from pB15, 3 from pTG, and 2 from pBA. DNA sequencing of the 27 mutants revealed that the mutants contain single base pair substitutions within the lacY gene which result in single amino acid substitutions within the lactose permease. All of the mutants obtained from pTE18, pTG, and pBA involved a change in Tyr-236 to histidine, phenylalanine, or asparagine. From pB15, three different types of mutants were obtained: Tyr-236 to histidine, Ile-303 to phenylalanine, or His-322 to asparagine. When assayed for [¹⁴C]maltose transport, the maltose/TDG mutants were seen to transport maltose significantly faster than the wild type. Furthermore, although TDG was shown to inhibit the uptake of maltose in the four parental strains, all of the mutant strains exhibited a dramatic resistance to TDG inhibition. Most of the maltose/TDG mutants were also shown to be very defective in the transport of lactose. However, certain mutants (i.e., Asn-322) exhibited moderate lactose transport activity. Finally, it was observed that all of the mutant strains were unable to facilitate the uphill accumulation of β-methylthiodigalactopyranoside.

The locations of the amino acid substitutions are discussed with regard to their possible role in sugar recognition.

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‡ The abbreviations used are: TDG, thiodigalactoside (S-P-D-galactopyranosyl-(1,1)-D-galactopyranoside); TMG, methyl-D-thiogalactopyranoside.
sugar (final concentration = 0.1 mM) was added. At appropriate time intervals, 0.2-ml aliquots containing approximately 10^8 cells were withdrawn and filtered over a membrane filter (pore size = 0.45 μm). The external medium was then washed away with 5–10 ml of phosphate buffer, pH 7.0, by rapid filtration. For uphll TMG accumulation, 10 mM MgCl₂ was included in the wash buffer to prevent efflux during washing. As a control, the lac Y+ strain, HS4006/pF’PZ’Y’, was also assayed for radioactive sugar uptake in order to obtain an accurate value for nonspecific sugar uptake. The control sample was then subtracted from the experimental samples to determine the amount of lactose permease-mediated uptake. In the case of certain parental strains (i.e. HS4006/F’PZ’Y/pB15) which were sensitive to inhibition by TDG, their amount of sugar uptake observed in the presence of 1 mM TDG was identical to the amount of uptake observed in the lac Y+ plasmidless strain without TDG.

DNA Sequencing—Double-stranded plasmid DNA was isolated and sequenced as described by Kraft et al. (20) using several oligonucleotide primers which anneal at approximately 200-base pair intervals within the lac Y gene.

RESULTS

Isolation of Mutants—An important approach towards the identification of amino acid residues which are involved with sugar recognition has been the isolation of “sugar specificity” mutants. In a previous study (16), lactose permease mutants were isolated and characterized which possessed an enhanced recognition for maltose (an α-galactoside) but a diminished recognition for cellobiose (a β-galactoside). In the current study, our strategy was to isolate mutants which recognize maltose but have a diminished recognition for TDG (a β-galactoside). It seemed reasonable to expect that such a selection would result in two different types of mutants. The first type might involve a generalized defect in the recognition of galactosides. Such mutants would be expected to contain different amino acid substitutions compared to the maltose/cellobioseR mutants identified previously. Alternatively, mutants might be isolated which possess a defect in the recognition of sugars with β linkages. This latter class would be expected to contain some of the amino acid substitutions identified among the maltose/cellobioseR mutants.

In order to isolate and identify maltose/TDGR mutants, MacConkey plates containing 1% maltose and 0.01% TDG were used. Strains encoding a mutant lactose permease which recognizes and transports maltose but is resistant to inhibition by TDG would be expected to form red colonies on these plates due to the ability to metabolize maltose. In contrast, those strains which cannot take up maltose, or those strains which are inhibited by TDG, would be expected to form white colonies. Before proceeding to the direct isolation of maltose/TDGR, we first streaked a variety of strains on maltose MacConkey with or without TDG (Table I). These strains differ only with regard to the plasmids they contain. The pTE18 plasmid encodes a wild-type lactose permease (Val-177) which recognizes maltose but is inhibited by high concentrations of cellobiose (14). The remaining plasmids encode mutant lactose permease molecules which recognize maltose but are resistant to inhibition by cellobiose. As expected, the pTE18 strain is white on maltose MacConkey (with or without TDG) due to an inability to transport maltose. The pB15 plasmid encodes a mutant lactose permease (Val-177) which recognizes maltose but is inhibited by high concentrations of cellobiose (14). The remaining plasmids encode mutant lactose permease molecules which recognize maltose but are resistant to inhibition by cellobiose. As expected, the pTE18 strain is white on maltose MacConkey (with or without TDG) due to an inability to transport maltose. The pB15 strain is red in the absence of TDG but white in the presence of TDG, indicating that TDG is effective at inhibiting maltose uptake in this particular strain. The remaining maltose/cellobioseR strains are generally able to transport maltose even in the presence of TDG. As suggested previously, these mutants already possess a defect in the recognition of β-galactosides. However, two of the maltose/cellobioseR mutants (pTG and pBA) exhibit a significant sensitivity to TDG. Both of these mutants contain Ser-306 changed to threonine.

Thus, the Thr-306 mutation appears to encode a lactose permease which is able to recognize α-glucosides and β-galactosides, but not β-glucosides.

As a starting point for mutant isolation, we chose the four parental strains pTE18, pB15, pBA, and pTG. As discussed above, when streaked on maltose MacConkey plates containing TDG, these parental strains formed white or pink colonies. However, if these plates were allowed to incubate several days at 37 °C, “red flecks” were seen to arise within the primary streak. When picked and restreaked on the same type of plate, these red flecks were seen to form bright red individual colonies. In all, 27 independent mutants (12 from pTE18, 10 from pB15, 3 from pTG, and 2 from pBA) were identified and saved for further study.

DNA Sequencing—As summarized in Table II, the maltose/TDGR mutants were subjected to DNA sequencing. All mutants were found to contain single base pair substitutions within the lac Y gene resulting in single amino acid substitutions within the lactose permease. The overwhelming majority of the mutants obtained in this study involved amino acid substitutions at Tyr-236. Indeed, all of the mutants obtained from pTE18, pTG, and pBA were position 236 mutants. From the pB15 parent, three different types of mutants were obtained (position 236, 303, and 322). Position 236 and 322 mutants were also obtained in the previous maltose/cellobioseR study, suggesting that these mutants possess a defect in the recognition of certain β sugars. However, the Phe-303 substitution represents a novel mutation which
Maltose/TDG<sup>+</sup> Mutants of the lac Permease

**Table II**

DNA sequences of the maltose/TDG<sup>+</sup> mutants

DNA sequencing was performed as described under "Materials and Methods." Each mutation was identified in multiple samples and each type of mutant was sequenced throughout the entire lac Y coding sequence.

| Plasmid          | Codon change | Amino acid substitution |
|------------------|--------------|-------------------------|
| pTE18 parent     |              |                         |
| pT-A-1, pT-A-2,  | 236 (TAC to CAC) | Tyr-236 to His          |
| pT-B-1, pT-C-1,  |              |                         |
| pT-D-2, pT-D-3,  |              |                         |
| pT-E-1           |              |                         |
| pT-D-1, pT-F-3,  | 236 (TAC to TTC) | Tyr-236 to Phe          |
| pT-H-2, and pT-H-3|              |                         |
| pT-F-2           | 236 (TAC to AAC) | Tyr-236 to Asn         |
| From pBA parent  |              |                         |
| pB-303-A         | 303 (ATT to TTT) | Ile-303 to Phe          |
| pB-5-5-A         | 322 (CAT to CAC) | His-322 to Asn      |
| From pTG parent  |              |                         |
| pTG-3-B, pTG-3-C | 236 (TAC to TTC) | Tyr-236 to Phe          |
| pTG-4-A          | 236 (TAC to CAC) | Tyr-236 to His          |
| From pBA parent  |              |                         |
| pBA-1-A, pBA-1-B | 236 (TAC to AAC) | Tyr-236 to Asn         |

**Table III**

Inhibition of <sup>14</sup>C/maltose uptake by thiodigalactoside

| Plasmid | Maltose uptake* | % maltose uptake in the presence of 1.0 mM TDG |
|---------|-----------------|---------------------------------------------|
| pTE18   | 0.08            | <2.0                                         |
| pT-A-1  | 0.17            | 49.6                                         |
| pT-F-2  | 0.12            | 27.5                                         |
| pT-H-2  | 0.33            | 68.4                                         |
| pB15    | 0.38            | <2.0                                         |
| pB-4-B  | 0.29            | 39.4                                         |
| pB-5-A  | 0.72            | 68.3                                         |
| pB-303A | 0.22            | 51.9                                         |
| pTG     | 0.21            | <2.0                                         |
| pTG-3-B | 0.56            | 72.5                                         |
| pTG-4-A | 0.42            | 55.2                                         |
| pBA     | 0.35            | 8.1                                          |
| pBA-1-A | 0.62            | 70.5                                         |

* Maltose uptake was carried out at 37 °C as described under "Materials and Methods."

has not been identified in previous studies.

**Maltose Transport**—To confirm that the phenotype of the mutants was due to enhanced maltose recognition and/or diminished TDG recognition, parental and mutant strains were tested for their ability to transport maltose in the presence or absence of TDG (Table III). As expected all of the strains transport maltose better than the wild-type strain (pTE18) which has a very poor recognition of maltose. In addition, all of the mutant strains exhibit a rather dramatic resistance to inhibition by a high concentration (1.0 mM) of TDG. In contrast, all four parental strains (pTE18, pB15, pBA, and pTG) are inhibited to a great extent. Indeed, with the exception of pBA which shows a small amount of transport activity, the other three parental strains are almost completely inhibited by 1.0 mM TDG.

**Galactoside Transport**—Since the mutants isolated in this study were clearly resistant to TDG (a β-galactoside), it was of interest to investigate their abilities to transport other β-galactosides as well. In the experiment of Fig. 1, parental and mutant strains were tested for their ability to transport <sup>14</sup>C lactose. Since all strains contain β-galactosidase, lactose is rapidly cleaved upon entry into the cell so that transport is always downhill (21). As expected, the four parental strains are able to effectively transport lactose (A). In sharp contrast, the mutants are relatively defective in lactose transport. With regard to position 236, the His-236 mutants are the most defective in lactose transport, followed by the Phe-236 mutants and then the Asn-236 mutants. In addition, the Phe-303 mutant also showed low levels of lactose transport. The Asn-322 mutant, however, appears to show an interesting dichotomy. This particular mutant (pB-5-A) is very insensitive to inhibition by TDG (see Table III) but transports lactose reasonably well (to about 40% of the pB15 parental strain). In this case, the defect in the Asn-322 mutant appears to be more linkage-specific. Whereas the mutant has very poor recognition towards the S-β-1,1 linkage of TDG, it has a fairly good recognition of lactose which contains an O-β-1,4 linkage.

It was also of interest to examine the ability of the mutant strains to actively transport galactosides against a concentration gradient. Therefore, parental and mutant strains were tested for their ability to accumulate the nonmetabolizable sugar, TMG. In Fig. 2A, it is seen that all four parental strains are able to transport TMG against a concentration gradient. The wild-type strain, pTE18, is able to accumulate TMG to the highest level. In contrast, all of the mutant strains are
severely defective in TMG accumulation. This defect in accumulation is seen even in mutant strains (i.e., the Asn-236 and Asn-322 mutants) which show significant levels of downhill lactose transport. Thus, it appears that alterations in sugar specificity seen in the maltose/TDG mutants also results in a defect in active transport.

**DISCUSSION**

The results of the current study present the isolation and characterization of a novel class of lactose permease mutants which recognize maltose but have a diminished recognition for TDG. Although four different parental strains were used, this phenotype could be brought about by amino acid substitutions at one of only three different sites: Tyr-236, Ile-303, and His-322. Only Tyr-236 substitutions were obtained from three of the parental strains (pTE18, pBA, and pTG). The pB15 parent, which already recognizes maltose, produced Tyr-236, Ile-303, and His-322 mutants. As expected, transport assays showed that all of the maltose/TDG mutant strains are able to effectively transport maltose in the presence of TDG. Indeed, a relatively high concentration of TDG (1.0 mM) was shown to dramatically inhibit the four parental strains, whereas the mutant strains showed much less inhibition. All of the mutant strains were defective in the uphill accumulation of galactosides, although certain mutants (i.e., Asn-236 and Asn-322) showed moderate levels of downhill lactose transport.

When considering the nature of the phenotype of the mutants obtained in this study, it is important to compare the structures of maltose and TDG. Maltose is a glucoside with an α-1,4 linkage to an aglycone which is a second D-glucose residue. TDG is a galactoside with a β-1,1 linkage to a second galactose residue. In order to exhibit the maltose/TDG phenotype, the protein must possess the ability to recognize maltose while decreasing its affinity for TDG. To accomplish this, several different types of structural alterations could be hypothesized. 1) The protein could alter the binding site so that van der Waals contacts better accommodate a glucoside. 2) The protein could diminish critical hydrogen bonding with the axial OH-4 group. (Note: the only structural difference between glucose and galactose is the position of the –OH group at C-4. In the case of galactose, the OH-4 group is axial relative to the hexose ring.) 3) The sugar recognition site could be altered so that sugars with β linkages are poorly recognized. 4) The protein could alter its structure so that the aglycone of TDG (i.e., galactose) interacts in an unfavorable manner.

It is interesting to discuss which of the above hypotheses best accounts for the transport properties of the position 236, 303, and 322 mutants. Besides the current study, Tyr-236 mutants have been obtained during the isolation of maltose recognition mutants (14) and during the isolation of maltose/ cellobiose mutants (16). Position 236 mutants exhibit an enhanced recognition of maltose and a diminished recognition of β-galactosides (lactose, TDG, and TMG), β-glucosides (cel-

![Fig. 3. Hypothetical interaction between Arg-302 and galactosides. See text for details.](image-url)
repressor forms hydrogen bonds with the OH-4 and ring oxygen of galactosides (24). Thus, as shown in Fig. 3, one might speculate that such an interaction exists in the lactose permease as well.

The hypothesis that Arg-302 is involved with sugar recognition is consistent with this and other genetic studies. In this study, the Phe-303 mutant was isolated from a parental strain which recognizes both maltose and TDG (pB15). Since the parental strain already possesses maltose recognition, the mutant phenotype can arise from a specific defect in TDG recognition. Thus, if Arg-302 is involved with hydrogen bonding as depicted in Fig. 3, the phenotype could result from a minor change in the position Arg-302 brought about by the Phe-303 substitution. Such a change could disrupt a hydrogen bond between arginine and the axial OH-4 group, thereby lowering the affinity for all galactosides (i.e. TDG, lactose, and TMG). In other studies, Arg-302 has been implicated to be involved with H+ recognition and transport (12). It has been shown that a Leu-302 mutant is able to recognize and transport lactose, although the affinity for lactose is severely reduced compared to the wild-type strain. Such a defect in lactose affinity would be expected if Arg-302 is directly involved with sugar recognition. Overall, these considerations raise the possibility that Arg-302 plays a dual role in both H+ and sugar recognition.

REFERENCES
1. Kaback, H. R. (1986) Annu. Rev. Biophys. Chem. 15, 279-319
2. Wright, J. K., Seckler, R., and Overath, P. (1986) Annu. Rev. Biochem. 55, 225-248
3. Kaback, H. R. (1987) Biochemistry 26, 2071-2076
4. West, I. C. (1970) Biochem. Biophys. Res. Commun. 41, 655-661
5. Flagg, J. L., and Wilson, T. H. (1977) J. Membr. Biol. 31, 233-255
6. Hirata, H., Attendorf, K., and Harold, F. M. (1974) J. Biol. Chem. 249, 2939-2945
7. Ramos, S., Schuldiner, S., and Kaback, H. R. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 1892-1896
8. Newman, M. J., and Wilson, T. H. (1980) J. Biol. Chem. 255, 10583-10586
9. Newman, M. J., Foster, D. L., Wilson, T. H., and Kaback, H. R. (1981) J. Biol. Chem. 256, 11804-11808
10. Brooker, R. J., and Wilson, T. H. (1986) J. Biol. Chem. 261, 11765-11769
11. Menick, D. R., Lee, J. A., Brooker, R. J., Wilson, T. H., and Kaback, H. R. (1987) Biochemistry 26, 1132-1136
12. Menick, D. R., Carrasco, N., Antes, L., Patel, L., and Kaback, H. R. (1987) Biochemistry 26, 6638-6644
13. Miller, J. (1972) in Experiments in Molecular Genetics, p. 433, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
14. Brooker, R. J., and Wilson, T. H. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 3959-3963
15. Brooker, R. J., Fiebig, K., and Wilson, T. H. (1985) J. Biol. Chem. 260, 16181-16186
16. Collins, J. C., Permutt, S. F., and Brooker, R. J. (1989) J. Biol. Chem., in press
17. Markgraf, M., Bocklage, H., and Müller-Hill, B. (1985) Mol. & Gen. Genet. 198, 473-475
18. Birnboim, H. C., and Doly, J. (1979) Nucleic Acids Res. 7, 1513-1523
19. Mandel, M., and Higa, A. (1970) J. Mol. Biol. 63, 154-162
20. Kraft, R., Tardiff, J., Krauter, K. S., and Leinwand, L. A. (1988) BioTechniques 6, 544-547
21. Rickenberg, H. V., Cohen, G., Buttin, G., and Monod, J. (1956) J. Mol. Biol. 7, 89-97
22. Quiocho, F. A. (1986) Annu. Rev. Biochem. 55, 278-315
23. Chou, P. Y., and Fasman, G. D. (1974) Biochemistry 13, 211-222
24. Sams, C. F., Vyas, N. K., Quiocho, F. A., and Matthews, K. S. (1984) Nature 310, 429-430
25. Teather, R. M., Bramhall, H., Riede, I., Wright, J. K., Furst, M., Aichele, G., Wilhelm, U., and Overath, P. (1980) Eur. J. Biochem. 108, 223-231