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

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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^R 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 lac Y 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 of 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 [^{14}C]maltose transport, the maltose/TDG^R 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^R 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-methylthiogalactopyranoside. The locations of the amino acid substitutions are discussed with regard to their possible role in sugar recognition.

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 lac Y 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, we report the isolation and characterization of another class of lactose permease mutants which recognize maltose but are resistant to inhibition by thiodigalactoside.

MATERIALS AND METHODS

Reagents—Lactose (*O*-β-D-galactopyranosyl-(1,4)-α-D-glucopyranose), maltose (*O*-α-D-glucopyranosyl-(1,4)-α-D-glucopyranose), TDG[1] (*S*-β-D-galactopyranosyl-(1,1)-β-D-galactopyranose), and TMG (methyl-β-D-thiogalactopyranoside) were purchased from Sigma. [^{14}C]Maltose and [^{14}C]TMG were from Du Pont-New England Nuclear and [^{14}C]lactose was from Amersham Corp. All other chemicals were analytical grade.

Bacterial Strains and Methods—The bacterial strain HS4006/F′I^{Q}Z$^+$Y$^-$ (14) has the genotype Δ(Lac-Pro)ΔMal B 101/F′Lac I^Q Lac Z$^+$ Lac Y$^-$. The Mal B 101 deletion renders the normal maltose transport system inactive. Plasmid DNA, isolated by the NaOH-sodium dodecyl sulfate method (18), was introduced into this strain by the $CaCl_2$ transformation procedure of Mandel and Higa (19).

Stock cultures of plasmid-carrying strains were grown in YT media (13) supplemented with ampicillin (0.1 mg/ml) and tetracycline (0.01 mg/ml). For transport assays, cells were grown to midlog phase at 37 °C in YT media containing ampicillin (0.05 mg/ml), tetracycline (0.005 mg/ml), and 0.25 mM isopropylthiogalactoside.

Transport Assays—Midlog cells were washed and resuspended in phosphate buffer, pH 7.0, containing 60 mM K_2HPO_4 and 40 mM KH_2PO_4. Cells were then equilibrated at 30 or 37 °C and radioactive

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[1] The abbreviations used are: TDG, thiodigalactoside (*S*-β-D-galactopyranosyl-(1,1)-β-D-galactopyranose); TMG, methyl-β-D-galactopyranoside.

sugar (final concentration = 0.1 mM) was added. At appropriate time intervals, 0.2-ml aliquots containing approximately 10^9 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 uphill TMG accumulation, 10 mM $HgCl_2$ was included in the wash buffer to prevent efflux during washing. As a control, the lac Y$^-$ strain, HS4006/F′I^{Q}Z$^+$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′I^{Q}Z$^+$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-glucoside) but a diminished recognition for cellobiose (a β-glucoside). 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/cellobiose^R 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/cellobiose^R mutants.

In order to isolate and identify maltose/TDG^R 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/TDG^R, 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. 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/cellobiose^R 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/cellobiose^R mutants (pTG and pBA) exhibit a significant sensitivity to TDG. Both of these mutants contain Ser-306 changed to threonine.

TABLE I

Phenotype of bacterial strains on maltose MacConkey plates

The bacterial strain HS4006/F′I^{Q}Z$^+$Y$^-$ (14) was transformed with the plasmids listed in the table.

Plasmid	Amino acid substitutions[a]	Color on maltose MacConkey plates[b]	
		No TDG	+0.01% TDG
pTE18	Wild type	White	White
pB15	Val-177	Red	White
pTB	Phe-236	Red	Red
pTT	His-236	Red	Red
pTG	Thr-306	Red	Pink
pTL	Pro-389	Red	Red
pBL	Val-177 Phe-236	Red	Red
pBN	Val-177 Asn-236	Red	Red
pBA	Val-177 Thr-306	Red	Pink
pBB	Val-177 Leu-306	Red	Red
pBI	Val-177 Asn-319	Red	Red[c]
pBX	Val-177 Tyr-322	Red	Red
pBF	Val-177 Asn-322	Red	Red
pBZ	Val-177 Gln-322	Red	Red

[a] The plasmid, pTE18 (25), is a high-copy plasmid carrying the wild-type lac Y gene. The plasmid, pB15 (which was derived from pTE18), encodes a mutant lactose permease which recognizes maltose (14). The remaining plasmids encode mutant lactose permeases which recognize maltose but are resistant to inhibition by cellobiose (16). The plasmids pTB, pTT, pTG, and pTL were derived from pTE18, whereas pBL, pBN, pBA, pBB, pBI, pBX, pBF, and pBZ were derived from pB15.

[b] The plates contained MacConkey media (13) supplemented with 1% maltose, 0.01 mM isopropylthiogalactoside, ampicillin (0.1 mg/ml), tetracycline (0.01 mg/ml), with or without TDG (0.01%).

[c] The pBI mutant strain formed extremely small pinpoint-sized colonies on these plates.

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/TDG^R 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/cellobiose^R 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

TABLE II

DNA sequences of the maltose/TDG^R 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
From pTE18 parent		
pT-A-1, pT-A-2, pT-B-1, pT-C-1, pT-D-2, pT-D-3, and pT-E-1	236 (TAC to CAC)	Tyr-236 to His
pT-D-1, pT-F-3, pT-H-2, and pT-H-3	236 (TAC to TTC)	Tyr-236 to Phe
pT-F-2	236 (TAC to AAC)	Tyr-236 to Asn
From pB15 parent		
pB-4-B, pB-6-A, pB-7-B, pB-7-C, pB-8-A, pB-8-B, pB-8-C, and pB-8-D	236 (TAC to CAC)	Tyr-236 to His
pB-303-A	303 (ATT to TTT)	Ile-303 to Phe
pB-5-A	322 (CAT to AAT)	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 [^{14}C]maltose uptake by thiodigalactoside

Plasmid	Maltose uptake[a]	% maltose uptake in the presence of 1.0 mM TDG
	nmol/min·mg protein	
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	68.8
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	0.36	8.1
pBA-1-A	0.62	70.5

[a] 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 [^{14}C]

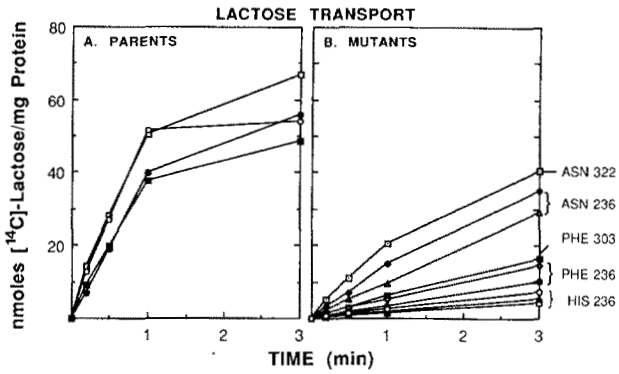

FIG. 1. **Downhill lactose transport by parental and mutant strains.** The uptake of lactose was measured at 30 °C as described under "Materials and Methods." The parental strains pTE18 (□), pB15 (○), pTG (■), and pBA (●) are shown in *A*. The mutant strains pT-A-1 (▲), pT-F-2 (△), pT-H-2 (◇), pB-4-B (□), pB-5-A (⊡), pB-303A (■), pTG-3-B (●); pTG-4-A (○), and pBA-1-A (◆) are shown in *B*.

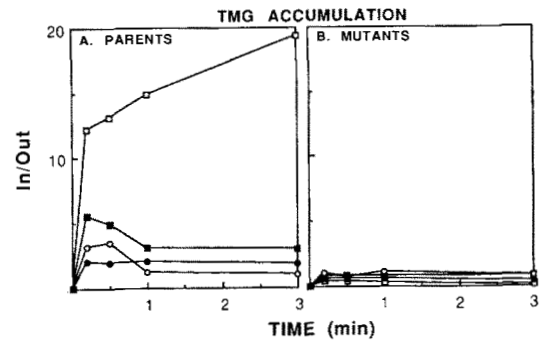

FIG. 2. **Uphill TMG accumulation by parental and mutant strains.** TMG accumulation was measured at 30 °C as described under "Materials and Methods." The parental strains pTE18 (□), pB15 (■), pTG (○), and pBA (●) are shown in *A*. The mutant strains pT-1A-1 (▲), pT-F-2 (△), pT-H-2 (◇), pB-4-B (□), pB-5-A (⊡), pB-303A (■), pTG-3-B (●), pTG-4-A (○), and pBA-1-A (◆) are shown in *B*.

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 strain 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. 2*A*, 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

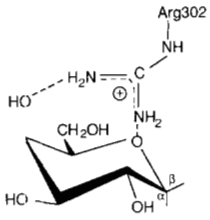

FIG. 3. **Hypothetical interaction between Arg-302 and galactosides.** See text for details.

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^R 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^R 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^R 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^R mutants (16). Position 236 mutants exhibit an enhanced recognition of maltose and a diminished recognition of β-galactosides (lactose, TDG, and TMG), β-glucosides (cellobiose), and α-galactosides (melibiose). With regard to the four possibilities listed above, it would appear that this mutant does not fall into category 3 or 4. Although position 236 mutants possess an inability to recognize β-galactosides and β-glucosides, they are defective in the recognition of α-galactosides as well. In addition, the aglycone region appears of unlikely importance, since these mutants are defective in the transport of galactosides with very small aglycones (*i.e.* TMG). Tyr-236 could be (directly or indirectly) involved with hydrogen bonding at the OH-4 position so that a substitution of this residue may diminish the bond. However, this idea does not explain why maltose recognition is enhanced, nor does it explain cellobiose resistance. Finally, it is worthwhile to consider the possibility that Tyr-236 makes important van der Waals contacts with the sugar. For sugar binding proteins which have been crystallized, it has been shown that aromatic amino acid residues can partially stack with the nonpolar sugar ring structure (22). Such an interaction may confer additional specificity and stability for sugar binding. In the case of the lactose permease, therefore, a substitution at Tyr-236 could diminish galactoside specificity conferred by the presence of the tyrosine residue while better accommodating an α-glucoside. Such a hypothesis would account for the observation that a Tyr-236 substitution in the wild-type parent (which has a poor recognition for maltose) simultaneously enhances maltose recognition while diminishing the recognition of other sugars.

The position 322 mutant obtained in this study involves a change of a histidine residue to an asparagine. Either of these residues could be involved with hydrogen bonding at the OH-4 position or at the linkage site. It would appear less likely that histidine 322 is important for hydrogen bonding at the axial OH-4 position, since the Asn-322 mutant showed relatively good recognition for lactose. It may be more likely that position 322 is involved with linkage recognition, since this particular mutant exhibited a marked specificity with regard to linkage recognition. The Asn-322 mutant had poor recognition of galactosides containing β-thio linkages (*i.e.* TDG and TMG) but had moderate affinity for lactose which contains a *O*-β linkage. In addition, previous studies have shown that Asn-322, Gln-322, and Tyr-322 mutants have low affinities for glucosides with a β linkage (16). The Asn-322 and Gln-322 mutants (which have chemically similar side chains) have moderate recognition for lactose, whereas the Tyr-322 mutant exhibits poor lactose recognition. Overall, these observations suggest that amino acid substitutions at position 322 result in specific alterations in linkage recognition.

Perhaps the most intriguing mutant in this study is the Ile-303 to Phe. Isoleucine is a nonaromatic nonpolar amino acid. This position would not be directly involved with hydrogen bonding at the linkage site or at the OH-4 position on the hexose ring. It remains a possibility that this location makes important van der Waals contacts with the sugar and that a phenylalanine substitution diminishes galactoside binding. However, rather than proposing a direct involvement with sugar recognition, the Ile-303 to Phe substitution may cause a minor change in the secondary structure of the protein and thereby alter the positions of neighboring amino acids. Isoleucine is a weaker α-helix former compared with Phe (23). Therefore, such a substitution might be expected to cause a minor perturbation in α-helical structure. In this light, it is worth noting that Ile-303 is immediately adjacent to Arg-302. In the case of the arabinose binding protein which has been crystallized, Arg-151 forms hydrogen bonds with both the OH-4 group (which is axial) and the ring oxygen (22). In addition, it has been proposed that Arg-197 in the lactose

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.

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