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Lactose transport mutants of *Escherichia coli* resistant to inhibition by the phosphotransferase system

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The lactose carrier activity of *Escherichia coli* in inhibited by the binding of dephosphorylated glucose enzyme III. Saier et al. ((1978) J. Bacteriol. 133, 1358–1367) isolated *lacY* mutants that escaped this inhibition. This communication reports the cloning and sequencing of one of the Saier mutants and the isolation, cloning and sequencing of another similar mutant. Both mutations resulted in amino acid substitutions on the middle cytoplasmic loop of the carrier (alanine-198 to valine and serine-209 to isoleucine). It is concluded that this cytoplasmic loop may be one of the sites of binding of glucose enzyme III.

Introduction

When cells of *E. coli* are exposed to a mixture of glucose and lactose they preferentially utilize the glucose. Only after all of the glucose is exhausted lactose is used for growth. This phenomenon, called diauxic growth, has been studied for many years [7,10,15]. One of the regulatory mechanisms that prevents the utilization of lactose when glucose is present, involves the direct inhibition of lactose carrier activity by glucose Enzyme III (III^{Glc}), an element of the phosphotransferase system [13–16]. The entry of the glucose involves phosphorylation of the sugar via glucose Enzyme II at the expense of phosphorylated III*Glc* and the dephosphorylated III^{Glc} inhibits the lactose carrier. This inhibition of the carrier prevents entry of the inducer and is therefore called inducer exclusion [7,17]. Dills et al. [3] showed that partially purified III^{Glc} could be shocked into *E. coli* membrane vesicles which possessed high levels of lactose carrier. Intravesicular (but not extravesicular) III^{Glc} inhibited lactose transport [3,9]. Direct binding of III^{Glc} (but not phosphorylated III^{Glu}) to the lactose carrier could be demonstrated in inside-out membrane vesicles [12] or in reconstituted proteoliposomes with purified lactose carrier [11]. In each case binding was stimulated by the presence of added lactose or lactose analog. In the experiments with purified III^{Glc} and purified lactose carrier it was estimated that there were 1 to 1.5 molecules of III^{Glc} bound per molecule of lactose carrier protein [11]. Binding of III^{Glc} did not occur to a mutant lactose carrier with three mutations in the first 24 amino acids at the N-terminal region of the molecule. It was concluded that the N-terminal segment of the carrier had a cytoplasmic location and was probably a binding site for Enzyme III.

Saier et al. [18] isolated *lac*Y mutants which had escaped the inhibitory effect of III^{Glc} on lactose transport. He showed that the transfer of the lactose genes to a lactose-deleted strain by conjugation resulted in the transfer of the mutant phenotype. This communication reports the cloning and sequencing of one of the Saier mutants and the isolation and sequencing of a second similar mutant, both of which show single amino acid substitutions in the middle cytoplasmic loop of the carrier protein.

Materials and Methods

Materials. DNA sequencing reagents were from a kit by Pharmacia. Lactose, Melibiose, ONPG and TMG were from Sigma. Deoxyadenosine 5′-[α-^{35}S]triphosphate and [D-*glucose*-1-^{14}C]lactose were from Amersham

Abbreviations: ONPG, *o*-nitrophenyl β-D-galactopyranoside; TMG, methyl β-D-thiogalactopyranoside; IPTG, isopropyl β-D-thiogalactopyranoside; XG, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; αMG, methyl α-D-glucopyranoside; PTS, phosphotransferase system.

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International. [*Me*-^{14}C]TMG and [^{3}H]β-D-ONPG were from New England Nuclear. [^{3}H]Melibiose was a generous gift of Dr. Gérard Le Blanc (CEA, Villefranche sur Mer, France). All radioactive sugars were purified by descending paper chromatography (Whatman No. 1 paper) using propanol/water (3 : 1, v/v). Bacteriological media were from Difco. Other chemicals were obtained from usual sources and were of the highest quality commercially available.

Strains. The parental strains for the isolation of mutants was 1101 *Hfr thi ptsH315.* A methyl α-D-glucoside-resistant mutant of 1101 (designated 3004) was kindly provided by Dr. Milton Saier of The University of California, San Diego, DW2 (*lacI*$^{+}$Δ*ZY melA*$^{+}$Δ*B rpsL*) was the recipient for mating of *Hfr* strains. DW2/pRAF-S11 is a strain containing a raffinose-positive plasmid coding for a constitutivley expressed invertase [5]. When a plasmid expressing a *lacY*$^{\text{sucrose}+}$ gene is inserted into this strain the cell becomes sucrose-positive and can ferment this sugar.

Isolation of mutants. Overnight-grown cells of strain 1101 (10 μl) were spread on lactose minimal plates containing 1% methyl α-D-glucoside (αMG). Clones that grew after 2–3 days were restreaked on the same plates to purify. Mutants were streaked on several different plates to exclude a variety of mutations unrelated to the lactose carrier. Out of 180 mutants which grew on lactose in the presence of methyl α-D-glucoside 37% grew on mannitol minimal plates and were presumed to be *Hpr* revertants, 53% were white on maltose, αMG fermentation plates and presumed to be *Crr* mutants; 15% were blue on XG plates (*lacI*$^{-}$); 4% failed to show αMG inhibition of glycerol or melibiose fermentation and were presumed to be additional *Crr* mutants.

Mating. The *lac* genes were transferred from the original *Hfr* strain to a lac-deleted strain (DW2) by conjugation. The mated mixture was plated on lactose minimal plates plus streptomycin. The donor was selected against by the antibiotic and the recipient failed to grow since it does not possess lactose transport or β-galactosidase. Several lactose positive conjugants were tested for αMG inhibition of TMG transport. With mutant TW3 all conjugants showed the αMG inhibition of TMG transport. A similar experiment had previously been carried out for mutant 3004 by Saier et al. [18].

Cloning the lacY by homologous recombination. The plasmid used in these studies was derived from pMB050-680 (*lacZ*$^{-}$*Y*$^{+}$ *Amp*^{R}), kindly provided by Dr. Michael Malamy [8]. A *lacY* gene (*Y*^{suc}), which codes for a lactose carrier recognizing sucrose, was inserted into the above plasmid to give pSCK1 (*lacZ*$^{-}$*Y*^{suc} *Amp*^{R}) [5,6]. The mutants 3004 and TW3 were trnasformed by pSCK1 and spread on LB plates plus ampicillin. The transformed cells were mated to DW2 (*lacI*$^{+}$Δ*ZY rpsL*) and spread on LB plates plus XG, ampicillin and streptomycin. The transfer of the ampicillin-resistant gene by conjugation involves the formation of a cointegrant between chromosome and plasmid, a process requiring recombination. Six blue clones were picked, pooled together and plasmid DNA isolated. This mixed plasmid DNA should contain plasmids of the original pSCK1 phenotype (*lac Z*$^{-}$ *Y*$^{\text{sucrose}+}$) as well as examples of the recombinant *lacZ*$^{+}$ *Y*$^{\text{sucrose}-}$ phenotype. Screening for the recombinant was performed by placing the plasmids in the genetic background of DW2/pRAF-S11 by transformation. This cell contains a raffinose plasmid with constitutive expression of invertase. The sucrose-positive phenotype was readily distinguished from recombinants as only the former gave red colonies on MacConkey agar containing 2% sucrose and ampicillin. The non-fermenting (white) colonies were considered recombinants. Further evidence of recombination was obtained by confirming that the sucrose-negative clones were *lacZ*$^{+}$ (blue on XG agar) and retained the raffinose plasmid (fermentation positive on MacConkey agar containing 1% raffinose). We found that all sucrose-negative cells were *lacZ*$^{+}$ and *raf*$^{+}$.

Plasmid DNA purification and sequencing. Double-stranded plasmid DNA was extracted from cells and purified by the method of Qiagen (Studio City, CA) following the directions of the manufacturer. The entire *lacY* gene was sequenced by the method of Sanger et al. [19] using the T7 sequencing kit from Pharmacia (Piscataway, NJ). Appropriately spaced oligonucleotides complementary to the lacY coding strand were used in order to synthesize the second strand which was labeled with [α-^{35}S]dATP (> 600 Ci/mmol).

Transport studies. Cells were grown to mid-logarithmic phase at 37° C in medium 63 [2], plus 1% tryptone (Difco). When methyl α-D-glucoside inhibition was to be tested (Figs. 1 and 2) cells were grown in the above tryptone medium plus added 0.2% lactose (to induce the lactose carrier). Glucose was added to the culture to give a final concentration of 0.2% 45 min prior to harvesting (to produce maximum induction to the pts enzymes). Cells were harvested and washed once with 100 mM potassium phosphate buffer (pH 7.0) and resuspended to a density of $3 \cdot 10^{9}$ cells per ml in the same buffer. In the experiments using αMG as the inhibitor this glucose analog was added to the cells 30 s prior to the addition of [^{14}C]TMG. The transport reaction was initiated by adding 900 μl of cell suspension to 100 μl of 1 mM stock sugar (1 μCi/ml). At the indicated times 100-μl or 200-μl aliquots were rapidly filtered through 0.65 μm pore size nitrocellular membrane filters (Sartorius Filters, Inc., Haywood, CA) and washed with 5 ml of buffer. Reactions were carried out at room temperature (approx. 21° C). Radioactivity within the cells was quantified by liquid scintillation counting using Liquiscint (National Diagnostics, Somerville, NJ).

Results

Isolation of methyl α-D-glucoside-resistant mutant

The *Hfr* strain 1101 was spread on lactose minimal plates containing 1% αMG, following the method of Saier et al. [18]. This *Hpr* leaky strain has a limited capacity to phosphorylate III^{Glc} so that when cells are exposed to αMG, a substrate for the glucose Enzyme II, most of the III^{Glc} is converted to the non-phosphorylated form which inhibits the lactose carrier. This selection procedure results in many types of diverse mutations which must be excluded by several methods (see Materials and Methods). Out of 180 αMG-resistant mutants only one proved to be in the *lacY* gene.

The lactose transport in the mutants was tested for sensitivity to inhibition by αMG. Fig. 1 shows that the transport of the lactose analog thiomethylgalactoside by the parental strain was inhibited by about 60% by added αMG. In contrast, the sugar transport by the two mutants was not inhibited by the glucoside. The degree of inhibition by the αMG in the normal cell varied with the extent of induction of the lactose system. When cells were fully induced with IPTG rather weak inhibition by glucoside was observed while partial induction by lactose resulted in much stronger inhibition. This is consistent with the view that there is only a limited quantity of III^{Glc} and thus with low levels of the lactose carrier strong inhibition of transport by αMG would be expected.

Additional evidence was sought to identify the lactose carrier as the point of mutation. The lactose operon from the parent and the two mutants was transferred from the *Hfr* to a lac-delected F^- strain by conjugation. This *Hfr* transfers the *lac* genes as early markers. Lactose-positive conjugants resulting from this mating were tested for αMG-sensitive lactose transport. Fig. 2

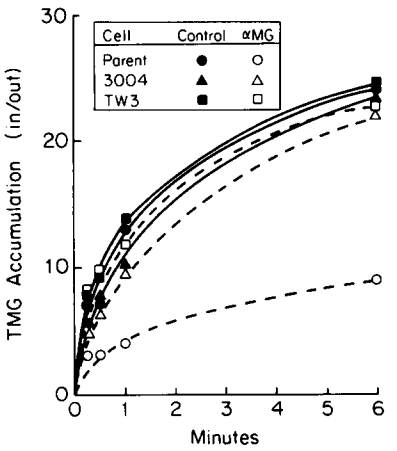

Fig. 1. The effect of αMG on TMG transport by the parent (1101) and the two mutants (3004 and TW3). The growth conditions and transport assays are given in the Materials and Methods. Transport of [^{14}C]TMG (0.1 mM; 0.1 μCi/ml) was carried out with or without 1 mM αMG.

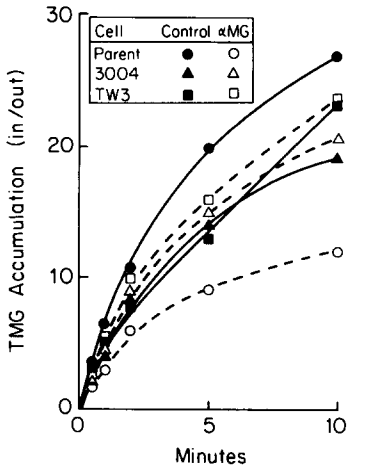

Fig. 2. The effect of αMG on TMG transport by conjugants of DW2 possessing the lac genes from the parent (1101) or the two mutants (3004 and TW3). The three stains 1101, 3004 and TW3 were mated to DW2 and lactose-positive conjugants isolated. These were tested in the same manner as that in Fig. 1.

shows that the conjugant from the parental strain shows inhibition of TMG transport by added αMG. TMG transport by the conjugants derived from the mutant strains was insensitive to the glucoside. Several conjugants of TW3 were isolated and all of them showed inhibition of TMG transport by αMG. Saier et al. [18] had previously demonstrated that all conjugants of 3004 retained the phenotype of the mutant. This result is consistent with the view that the mutation resided in or near the *lacY* gene which was transferred from the *Hfr* to the recipient strain.

In order to determine possible changes in sugar recognition in the mutant strains transport was tested with three additional sugars. The conjugants induced by isopropyl thiogalactoside were exposed to radioactive lactose, melibiose, or *o*-nitrophenyl galactoside. The initial rates of transport (not shown) and the uptake at 5 min (Table I) by the mutants were rather similar to those of the parental strain. It was concluded that although there were differences between the mutants and parent there were no major alterations.

TABLE I

Transport of several sugars by αMG-resistant mutants

The DW2 conjugants containing the lac genes of the parent and two mutants were grown in 1% tryptone plus 0.5 mM IPTG. Cells were washed once and transport assayed as given in Materials and Methods. The concentration of sugar was 0.1 mM.

	Parent 1101	Mutant TW3	Mutant 3004
ONPG (nmol/mg protein per 5 min)	9.6	11.6	9.9
Lactose (nmol/mg protein per 5 min)	112	136	158
Melibiose (in/out ratio in 5 min)	55	63	32

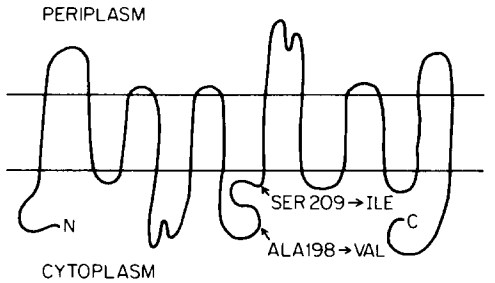

Fig. 3. The location of the two mutants in the carrier protein. The model of lactose carrier is redrawn from that of Foster et al. [4].

Cloning and sequencing

The cloning procedure by homologous recombination is one suggested by Malamy [8] and modified by King and Wilson [5]. This method involves introduction of a plasmid containing a *lac* operon with a defective *lacZ* gene but *lacY*^{suc} (lactose carrier recognizes sucrose) as well as an ampicillin resistance gene. Recombination between chromosomal *lac* genes and the *lac* genes on the plasmid was facilitated by mating this strain to a *Δlac* recipient. A recombination event is required for the transfer of the *Amp*^{R} gene via a cointegrant and a second recombination occurs when the plasmid separates from the cointegrant in the recipient cell. The identification of the cell containing the mutant *lacY* gene on the plasmid was facilitated by picking cells which had lost the original *lacY*^{suc} from the plasmid (see Materials and Methods).

Plasmid DNA was isolated and sequenced by conventional techniques. Mutant 3004 was found to have alanine 198 substituted by valine. Mutant TW3 was found to have serine-209 substituted by isoleucine. Fig. 3 shows the approximate positions of these mutations on a cytoplasmic loop of the lactose carrier assuming the 12 membrane-spanning model proposed by Foster et al. [4]. Carrasco et al. [1] have confirmed the cytoplasmic location of the loop on which the altered amino acids of these two mutants were found. These authors showed that an antibody to a syhthetic peptide corresponding to amino acid 185–198 of the carrier protein binds to the cytoplasmic surface of membrane vesicles.

Discussion

There is good evidence for direct regulation of the lactose carrier activity by III^{Glc}. Non-phosphorylated III^{Glc} has been shown to bind directly to the lactose carrier while phosphorylated III^{Glc} does not bind. This binding of III^{Glc} results in inhibition of lactose transport by the lactose carrier. Nelson et al. [11] showed that III^{Glc} fails to bind to a *lacY* mutant with three different mutations in the N-terminal 24 amino acids (Thr-7 → Ile; Met-11 → Ile and Gly-24 → Arg). They conclude that one of the binding sites for Enz III may be the N-terminal segment of the carrier. The studies presented in this communication suggest that a cytoplasmic loop is also a site of interaction. If there were a single binding site for III^{Glc} then these two regions of the lactose carrier molecule would be adjacent to one another.

The mechanism by which III^{Glc} inhibits the activity of the carrier is not yet understood. It is of interest, however, that the binding of III^{Glc} to the carrier increases the affinity for galactosides 3–5-fold [11]. Thus there must be some complex allosteric change in the structure of the lactose carrier resulting from binding of the inhibitory protein III^{Glc}.

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References

1 Carrasco, N., Herzlinger, D., Dechiara, S., Danho, W. and Kaback, H.R. (1985) Ann. N.Y. Acad. Sci. 456, 305–306.
2 Cohen, G.N. and Rickenberg, H.W. (1956) Ann. Inst. Pasteur 91, 693–720.
3 Dills, S.S., Schmidt, M.R. and Saier, M.H., Jr. (1982) J. Cell Biochem. 18, 239–244.
4 Foster, D.L., Boublik, M. and Kaback, H.R. (1983) J. Biol. Chem. 258, 31–34.
5 King, S.C. and Wilson, T.H. (1989) Biochim. Biophys. Acta 982, 253–264.
6 King, S.C. and Wilson, T.H. (1990) J. Biol. Chem., in press.
7 Magasanik, B. (1970) in The Lactose Operon (Beckwith, J.R. and Zipser, D., eds.), pp. 189–219, The Cold Spring Harbor Laboratory, New York.
8 Malamy, M.H., Rahaim, P.T., Hoffman, C.S., Baghoyan, D., O'Connor, M.B. and Miller, J.F. (1985) J. Mol. Biol. 181, 551–555.
9 Misko, T.P., Mitchell, W.J., Meadow, N.D. and Roseman, S. (1987) J. Biol. Chem. 262, 16261–16266.
10 Monod, J. (1947) Growth 11, 223–289.
11 Nelson, S.O., Wright, J.K. and Postma, P.W. (1983) EMBO J. 2, 715–720.
12 Osumi, T. and Saier, M.H., Jr. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 1457–1461.
13 Postma, Postma, P.W. and Lengeler, J.W. (1985) Microbiol. Rev. 49, 232–269.
14 Postma, P.W., Broekhuizen, C.P. and Geerse, R.H. (1989) FEMS Microbiol. Rev. 63, 69–80.
15 Roseman, S. and Meadow, N.D. (1990) J. Biol. Chem. 265, 2993–2996.
16 Saier, M.H., Jr. (1989) Microbiol. Rev. 53, 109–120.
17 Saier, M.H., Jr. and Roseman, S. (1976) J. Biol. Chem. 251, 6606–6615.
18 Saier, M.H., Jr., Stroud, H., Massman, L.S., Judice, J.J., Newman, M.J. and Feucht, B. (1978) J. Bacteriol. 133, 1358–1367.
19 Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.