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Deletion Mutants of the *Escherichia coli* K-12 Mannitol Permease: Dissection of Transport-Phosphorylation, Phospho-Exchange, and Mannitol-Binding Activities

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We have constructed a series of deletion mutations of the cloned *Escherichia coli* K-12 *mtlA* gene, which encodes the mannitol-specific enzyme II of the phosphoenolpyruvate (PEP)-dependent carbohydrate phosphotransferase system. This membrane-bound permease consists of 637 amino acid residues and is responsible for the concomitant transport and phosphorylation of D-mannitol in *E. coli*. Deletions into the 3′ end of *mtlA* were constructed by exonuclease III digestion. Restriction mapping of the resultant plasmids identified several classes of deletions that lacked approximately 5% to more than 75% of the gene. Immunoblotting experiments revealed that many of these plasmids expressed proteins within the size range predicted by the restriction analyses, and all of these proteins were membrane localized, which demonstrated that none of the C-terminal half of the permease is required for membrane insertion. Functional analyses of the deletion proteins, expressed in an *E. coli* strain deleted for the chromosomal copy of *mtlA*, showed that all but one of the strains containing confirmed deletions were inactive in transport and PEP-dependent phosphorylation of mannitol, but deletions removing up to at least 117 amino acid residues from the C terminus of the permease were still active in catalyzing phospho exchange between mannitol 1-phosphate and mannitol. A deletion protein that lacked 240 residues from the C terminus of the permease was inactive in phospho exchange but still bound mannitol with high affinity. These experiments localize sites important for transport and PEP-dependent phosphorylation to the extreme C terminus of the mannitol permease, sites important for phospho exchange to between residues 377 and 519, and sites necessary for mannitol binding to the N-terminal 60% of the molecule. The results are discussed with respect to the fact that the mannitol permease consists of structurally independent N- and C-terminal domains.

The D-mannitol-specific enzyme II (EII^{Mtl} or mannitol permease) of the *Escherichia coli* K-12 phosphoenolpyruvate (PEP)-dependent carbohydrate phosphotransferase system (PTS) is an integral membrane protein that both transports and phosphorylates its substrates. This process requires the participation of the general phosphocarrier proteins of the PTS, enzyme I (EI) and a small, heat-stable protein (HPr) (reviewed in references 28 and 34), as follows:

$$\text{HPr} + \text{PEP} \overset{\text{EI}}{\leftrightarrow} \text{phospho-HPr} + \text{pyruvate} \quad (1)$$

$$\text{Phospho-HPr} + \text{mannitol}_{(\text{out})} \xrightarrow{\text{EII}^{\text{Mtl}}} \text{HPr} + \text{mannitol 1-phosphate}_{(\text{in})} \quad (2)$$

A partial reaction of step 2 has also been shown to be catalyzed by the mannitol permease alone (11, 21, 36):

$$\text{Mannitol 1-phosphate} + [^{14}\text{C}]\text{mannitol} \xleftrightarrow{\text{EII}^{\text{Mtl}}} [^{14}\text{C}]\text{mannitol 1-phosphate} + \text{mannitol}$$

This phospho-exchange reaction (transphosphorylation) has been shown to occur vectorially in whole cells and membrane vesicles (35, 36).

The mannitol permease has been purified (11) and extensively characterized (7, 10, 30, 32), and its gene, *mtlA*, has been cloned and sequenced (17). These studies have revealed that the protein is made up of a single kind of polypeptide chain consisting of 637 amino acid residues (11, 17) that is highly specific for D-mannitol (12, 19) and may function as a dimer in the membrane (24, 30, 33, 41). The topography of the mannitol permease with respect to the membrane has also been studied, and it is clear that this protein consists of a hydrophobic, membrane-bound N-terminal domain (residues 1 to 334) and a hydrophilic C-terminal domain (residues 335 to 637) that is exposed on the cytoplasmic surface of the inner membrane (9, 17, 42). In addition to carrying out the transport and phosphorylation functions described above, the mannitol permease functions as the primary chemotactic receptor for D-mannitol in *E. coli* (20).

The domain structure of the mannitol permease and its multiple functions suggest that structure-function relationships might be conveniently studied by analyzing the structural and functional properties of mutant permease molecules in which portions of the amino acid sequence have been deleted. In this report, we describe the construction of a number of such mutants and the characterization of the resultant truncated protein products. The results define at least three different regions in the permease molecule that are important for various functions: the extreme C terminus (transport and PEP-dependent phosphorylation), residues 337 to 519 (phospho exchange), and amino acids proximal to residue 337 (mannitol binding).

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TABLE 1. *E. coli* strains used

Strain	Genotype	Reference or source
L146-1	F⁻ *lacY1 galT6 xyl-7 thi-1 hisG1 argG6 metB1 rpsL104 mtlA2 gutA50 gatA50 mal*⁺	18
L181	F⁻ *thi-1 hisG1 argG6 metB1 tonA2 supE44 rpsL104 lacY1 galT6 gatR49 gatA50 gutP,O49 gutA50*	20
LGS3	L181 Mtl⁻ Δ(*mtlA'p*) *mtlD*⁺ (no *mtlD* expression)	This study
LGS31	LGS3, but constitutive for *mtlD* expression	This study
LGS322	LGS31 Δ(*gutR'MDBAp-recA*)	This study
JC10279	F⁻ *leuB6 hisG1 argG6 metB1 tonA2 supE44 rfD1? lacY1 gal-6 galP63? malT1* (λ⁻) *xyl-7 mtlA2 gatC300 gatA50 gutR301*::Tn*10 gutD50 rpsL104*	4

MATERIALS AND METHODS

Chemicals and enzymes. [^{14}C]mannitol (45 mCi/mmol), [^{3}H]mannitol (19.1 Ci/mmol), [^{3}H]glycerol (40 Ci/mmol), and ^{125}I-labeled *Staphylococcus aureus* protein A (9.4 mCi/mg) were purchased from Dupont, NEN Research Products (Boston, Mass.). The Klenow fragment of DNA polymerase I was obtained from Pharmacia, Inc. (Piscataway, N.J.); exonuclease III was obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). S1 nuclease and T4 DNA ligase were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Restriction enzymes were products of either Boehringer or New England BioLabs, Inc. (Beverly, Mass.). Other chemicals used were reagent grade.

Bacterial strains and cell growth. The bacterial strains used in this study are listed in Table 1. Cells were grown in liquid culture on Luria broth (LB; 1% tryptone, 0.5% yeast extract, 0.5% NaCl), on agar plates containing LB, or on MacConkey agar base plates (sugarfree; Difco Laboratories, Detroit, Mich.), each containing 1% carbohydrate unless otherwise indicated. Chloramphenicol, when present, was added to a final concentration of 30 mg/liter. Tetrazolium plates contained (per liter) 25.5 g of antibiotic medium 2 (Difco) and 50 mg of 2,3,5-triphenyl tetrazolium chloride. For functional assays and for immunoblots, cells were grown in liquid culture only to mid-exponential phase (A_{550}, 0.6 to 0.8) to minimize endogenous proteolysis of truncated permease molecules (see Results).

Plasmids and cloning techniques. The plasmids used in this study are listed in Table 2. Plasmid DNA was purified from *E. coli* lysates by equilibrium density centrifugation in cesium chloride-ethidium bromide gradients (23). Transformation was carried out by the procedure of Kahn et al. (13). Restriction endonucleases and other enzymes were used as recommended by the supplier unless otherwise indicated.

TABLE 2. Plasmids used

Plasmid	Genotype	Reference or source
pLC15-48	ColE1^{imm} *mtlCAD*⁺	J. Carbon; 15
pACYC184	Cm Tc	3
pGJ9	Cm *mtlAp* on pACYC184	This study
pCD7.5	Tc *mtlCAD*⁺ on pBR322	16
pPG1	pCD7.5 Δ(*mtlA'p*) *'A mtlD*⁺	This study

DNA fragments were analyzed by electrophoresis in 0.7% agarose gels, using bacteriophage λ DNA restriction fragments as molecular weight markers.

Plasmid pGJ9 (Table 2), containing the *mtlA* gene inserted into the tetracycline resistance gene of plasmid pACYC184 (3), was constructed as follows. A 2.1-kilobase base pair (kbp) fragment containing the *mtlA* gene and its promoter was prepared by digestion of plasmid pLC15-48 (15) with *Sal*I and *Stu*I (17) and purified by electrophoresis in a low-melting-point agarose gel. Plasmid pACYC184 was digested with *Sal*I and *Eco*RV to remove most of the tetracycline resistance gene, and the fragment containing *mtlA* was ligated into the vector. The resulting plasmid (pGJ9; ca. 5.65 kbp) contained *mtlA* in an orientation opposite that of the *tet* promoter (Fig. 1A). The structure of pGJ9 was confirmed by restriction mapping.

Construction of deletions of *mtlA*. Deletions into the 3' end of *mtlA* were constructed by cleavage of pGJ9 at its unique *Cla*I site, located approximately 120 bp distal to the TAA stop codon (Fig. 1A). This procedure was followed by digestion with exonuclease III (8), which specifically catalyzes the stepwise release of 5'-nucleotides from the 3'-hydroxyl end of duplex DNA (31). Samples were removed from the reaction mixture at various times, heated to 70°C for 10 min to stop the reaction, ethanol precipitated, and treated with S1 nuclease in the supplier-recommended buffer to remove the single-stranded 5' overhanging ends. Subsequent treatment with DNA polymerase I (Klenow fragment) and T4 DNA ligase resulted in plasmids deleted for various lengths of pGJ9. These plasmids were then transformed into *E. coli* L146-1 (*mtlA2 rec*⁺) and further characterized by restriction mapping. Five classes of deletions within *mtlA* were identified by the presence or absence of restriction sites in the gene (Fig. 1B and Results). A deletion in *mtlA* with a precisely defined end was also constructed by taking advantage of the unique *Sna*BI site of pGJ9 within *mtlA* (Fig. 1A). Digestion at this site cuts within the codon for Arg-378 in the permease sequence (17), and digestion of pGJ9 with both *Sna*BI and *Cla*I followed by purification of the larger fragment and treatment with DNA polymerase I (Klenow fragment) and DNA ligase resulted in plasmid pGJ9-ΔSnaBI, containing the first 377 codons and lacking the last 260 codons of *mtlA* (see Results).

Since many of the deletion plasmids recombined with significant frequency with the defective chromosomal *mtlA* gene of strain L146-1, into which these plasmids were first transformed (see Results), it was necessary to construct a host strain in which the chromosomal *mtlA* copy was deleted. It was also advantageous for functional assays of deletion proteins to introduce into the same strain a deletion in the genes encoding the glucitol-specific EII-EIII pair (*gutAB*), since this complex does recognize mannitol, albeit with low affinity (19). Such a strain was constructed as follows.

Plasmid pCD7.5, which contains the entire mannitol operon (16), was digested with *Bst*XI, followed by treatment with DNA polymerase I (Klenow fragment) and DNA ligase as described by Maniatis et al. (23). The resulting plasmid, pPG1, contained a 3.9-kbp deletion extending from the *Bst*XI site approximately 2 kbp in front of the start codon of *mtlA* to a *Bst*XI site within *mtlA* located 38 bp before its stop codon (16, 17). Therefore, pPG1 contains the entire *mtlD* gene (encoding mannitol 1-phosphate dehydrogenase) and the intracistronic region between *mtlA* and *mtlD*, while the promoter of the mannitol operon and most of *mtlA* are deleted. Plasmid pPG1 was transformed into *E. coli* L181

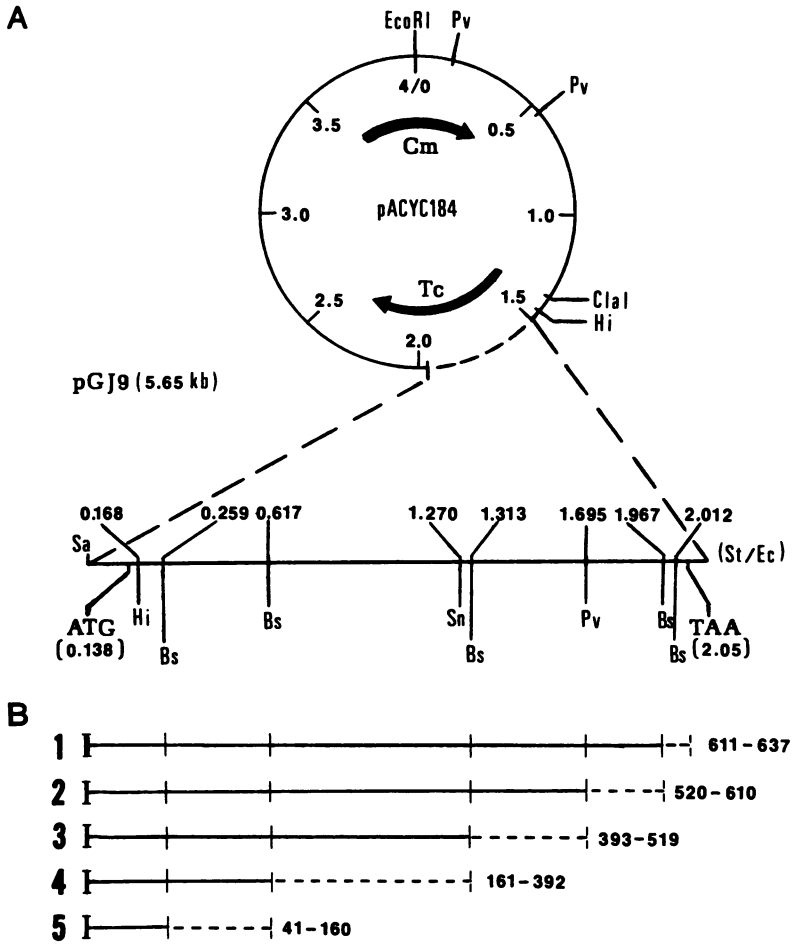

FIG. 1. Construction of pGJ9 and of deletions into the 3′ end of *mtlA*. (A) A 2.1-kbp *Sal*I-*Stu*I fragment containing *mtlA* (17) was isolated from plasmid pLC15-48 (15) and inserted into the tetracycline resistance region of plasmid pACYC184 (3) after removal of a *Sal*I-*Eco*RV fragment from the latter (– – –). The insert contains the endogenous mannitol promoter and a ribosome-binding site (between the *Sal*I site and the ATG start codon) and is oriented in a direction opposite that of the *tet* promoter. Numbers within the pACYC184 portion refer to kilobase pairs from the unique *Eco*RI site before deletion of the region indicated by the dashed line. Numbers on the *mtlA* insert, below, refer to kilobase pairs from the 5′ end of the insert. Abbreviations for restriction sites: Bs, *Bst*XI, Ec, *Eco*RV; Hi, *Hin*dIII; Pv, *Pvu*II; Sa, *Sal*I; Sn, *Sna*BI; St, *Stu*I. (B) Deletions into the *mtlA* region of pGJ9 were constructed after cleavage at the unique *Cla*I site and treatment with exonuclease III for various times. Restriction mapping of over 200 such deletion plasmids established the five groups shown on the basis of the presence or absence of the *Bst*XI and *Pvu*II sites indicated by the vertical lines (compare with panel A). Regions within which the deletion endpoints lie for each group are indicated (– – –) (restriction site on the left is present, and restriction site on the right is absent). The corresponding numbers refer to the codons of *mtlA* between which the deletions in each group lie. Thus, all deletion proteins in group 1 and some in group 2 contain phosphorylation site 1 (His-554), whereas all deletions in groups 1 to 3 and some in group 4 contain proposed phosphorylation site 2 (Cys-384). See Results and Discussion for details.

(Table 1), and the transformants were grown overnight in medium containing tetracycline (10 μg/ml) to enable homologous recombination of the plasmid with the chromosome. The transformants were then grown for 10 to 15 generations in antibiotic-free medium to allow plasmid segregation as described by Kiel et al. (14). The resulting cells were plated onto 100 tetrazolium plates containing 1% mannitol (0.5×10^6 to 1×10^6 cells per plate), and four derivatives with an Mtl^- phenotype were isolated. One of these, LGS3, was used for further experiments.

Transformation of plasmid pGJ9 (*mtlA*$^+$ *mtlD*$^-$) into strain LGS3 resulted in a mannitol-sensitive phenotype because, although LGS3 contains the *mtlD* gene, it lacks the mannitol promoter and thus cannot express mannitol 1-phosphate dehydrogenase and cannot further catabolize mannitol 1-phosphate. It was possible, however, to isolate derivatives of LGS3 that gave an Mtl^+ phenotype when transformed with pGJ9 but were themselves Mtl^-, indicating the presence of a chromosomal mutation leading to expression of the cryptic *mtlD* gene. One of these, LGS31, was selected for construction of a strain that also contained a deletion in the glucitol operon by using a method similar to that of Csonka and Clark (4), as follows.

The glucitol region of *E. coli* JC10279 (*gutR301*::Tn*10* *gutD50*) was transduced into strain LGS31 (which is Gut^-) by selecting for a Tc^r Gut^s phenotype. The resulting transductants were highly sensitive to glucitol because of the loss of glucitol 6-phosphate dehydrogenase (encoded by *gutD*) and constitutive expression of the glucitol-specific EII complex. From one of these transductants, 800 glucitol-resistant derivatives were isolated and tested for glucitol, tetracycline, and UV sensitivity; two were found to be glucitol resistant, tetracycline sensitive, and UV sensitive, which indicated inexact Tn*10* excision including (parts of) the

glucitol operon and the adjacent *recA* region. One of these, LGS322, was used for structural and functional tests of deletion plasmid protein products, since no homologous recombination between these plasmids and the chromosome would be expected (because of the chromosomal deletions in *mtlA* and *recA*), nor could any mannitol permease activity observed be due to any activity of the glucitol-specific EII (because of the glucitol-operon deletion).

Cell fractionation and assays of mannitol permease activities. Cells of *E. coli* LGS322 containing various plasmids were lysed in a French pressure cell, and membrane and cytoplasmic fractions were prepared as described previously (1, 9, 18). Assays of PEP-dependent phosphorylation of D-mannitol and of transphosphorylation were also carried out as previously described (1, 9, 18). Uptake of [^{14}C] mannitol by whole cells was monitored by the procedure of Lengeler (19).

Determinations of binding of radiolabeled mannitol to membrane fractions were carried out as follows. To a suspension of membranes (15 μl) in 30 mM Tris hydrochloride–1 mM dithiothreitol–1 mM phenylmethylsulfonyl fluoride (pH 8.4) was added an equal volume of [^{3}H]mannitol to achieve a final concentration of between 0.1 and 100 μM mannitol (500 to 10 μCi/μmol). Samples were incubated at 30°C for 10 min and centrifuged at 12,800 × *g* for 45 min at 4°C in an Eppendorf microcentrifuge. Both the supernatant and the pellet were counted in scintillation fluid containing Triton X-100. To correct for trapping or nonspecific binding, an identical procedure was followed by using [^{3}H]glycerol, which is not recognized as a substrate by the mannitol permease (12). The amount of mannitol specifically bound was calculated as the difference between the amount of mannitol and the amount of glycerol associated with the membrane pellets; the amount of free mannitol was that present in the supernatant after centrifugation. The data were analyzed by the method of Scatchard (40).

Protein concentrations were estimated by the method of Lowry et al. (22), with bovine serum albumin as the standard.

Protein electrophoresis and immunoblotting. Polyacrylamide gel electrophoresis of proteins in sodium dodecyl sulfate (SDS) was carried out by the procedure of Weber and Osborn (46). Electrophoretic blotting of proteins from these gels onto nitrocellulose was carried out by the method of Towbin et al. (43), with the addition of 1% SDS to the transfer buffer. Visualization of proteins cross-reacting with mannitol permease antibody (15) by using ^{125}I-labeled *S. aureus* protein A was done by the procedure of Stephan and Jacobson (42). Lanes containing molecular weight markers were cut from the original gel before blotting and stained separately with Coomassie brilliant blue.

RESULTS

Mapping of *mtlA* deletion mutations. Deletion mutations of *mtlA* in pGJ9 were constructed by using exonuclease III digestion as described in Materials and Methods. Samples from five time points taken at 30-s intervals from 30 s to 2.5 min were transformed into *E. coli* L146-1, carrying a point mutation in *mtlA*, after treatment of the reaction mixtures with the Klenow fragment of DNA polymerase I and with DNA ligase. The resultant transformants were plated onto MacConkey-mannitol plates containing chloramphenicol. The majority of transformants gave white colonies, although a number of red colonies and one pink colony were also observed. A total of 224 colonies, including some red and the single pink colony, were picked; plasmids were isolated from each and analyzed by restriction endonuclease digestion. Treatment of the deletion plasmids first with either *Pvu*II or *Hin*dIII and then separately with *Bst*XI (Fig. 1) allowed placement of each of the deletions into one of five groups on the basis of the presence or absence of the five *Bst*XI sites and the single *Pvu*II site within *mtlA* (Fig. 1B) (two of the *Bst*XI sites within the *mtlA* insert, at bp 1967 and 2012, could not be distinguished in this analysis). Plasmids from all of the red transformants, the single pink transformant, and several white transformants mapped within deletion group 1 (no deletion or a very short deletion of <83 bp). All other white deletions mapped in groups 2 to 5 (Fig. 1B and Table 3).

TABLE 3. Properties of membranes containing truncated mannitol permease derivatives

LGS322 harboring plasmid[a]:	Deletion group[b]	Mol wt (10^3)[c]		Activity (%)		
		Obs.	Pred.	PEP dependent[d]	Exchange[e]	Transport[f]
pGJ9		65	68	100	100	100
pGJ9-Δ69	1	67	65–68	22	95	65
pGJ9-Δ65	1	64	65–68	0	130	0
pGJ9-Δ90	2	64	55–65	0	130	0
pGJ9-Δ124	2	63	55–65	0	52	0
pGJ9-Δ100	2	62	55–65	0	96	0
pGJ9-Δ63	2	61	55–65	0	100	0
pGJ9-Δ158	2	60	55–65	0	135	0
pGJ9-Δ104	2	60	55–65	0	82	0
pGJ9-Δ145	2	58	55–65	0	210	0
pGJ9-Δ130	2	56	55–65	0	100	0
pGJ9-Δ134	2	53	55–65	0	44	0
pGJ9-Δ117	2	52	55–65	0	480	0
pGJ9-Δ148	2	52	55–65	0	174	0
pGJ9-Δ137	3	51	41–55	0	197	0
pGJ9-ΔSnaBI	4	34	39	0	0	0
pGJ9-Δ21	4	33	19–41	0	0	0
pGJ9-Δ149	5	ND	4–19	0	0	0

[a] All strains listed gave white colonies on MacConkey-mannitol plates except for LGS322(pGJ9) (red) and LGS322(pGJ9-Δ69) (pink).

[b] Determined by restriction mapping (see text and legend to Fig. 1).

[c] Obs., Observed, determined by immunoblotting of membrane preparations (see Materials and Methods); Pred., predicted from restriction map; ND, not determined.

[d] Percent PEP-dependent phosphorylation of mannitol (0.1 mM) relative to that of membranes from LGS322(pGJ9) (wild-type). Assays were conducted with membranes from the indicated strains and saturating amounts of EI and HPr (see Materials and Methods); 100% = 11 nmol of mannitol 1-phosphate formed per min per mg of membrane protein.

[e] Percent phospho exchange activity relative to that of membranes from LGS322(pGJ9) (see Materials and Methods); 100% = 6.3 pmol of mannitol 1-phosphate formed per min per mg of protein.

[f] Percent mannitol transport in whole cells relative to that of LGS322(pGJ9) (see Materials and Methods); 100% = 10.3 nmol of mannitol (25 μM) taken up per min per mg of protein.

A deletion in *mtlA* with a defined end was also constructed by digestion of pGJ9 with *Cla*I and *Sna*BI (see Materials and Methods), which should yield a plasmid containing only the first 1,270 bp of the *mtlA* insert (Fig. 1A). Strain L146-1 harboring this plasmid gave white colonies on MacConkey-mannitol plates, and restriction mapping of this deletion plasmid (pGJ9-ΔSnaBI) showed that it fell into deletion group 4, as expected (Fig. 1).

Structural analyses of proteins encoded by the deletion plasmids. L146-1 strains harboring a number of the deletion plasmids and that initially gave white colonies on MacConkey-mannitol plates developed red papillae (spotted colonies) after prolonged incubation at 37°C. Since the strain alone did not show this phenotype, it was likely that these deletion plasmids could recombine with the defective *mtlA*

gene of the host chromosome to give a normal copy of the *mtlA* gene (also see below). Therefore, an *E. coli* strain (LGS322) that was *recA* and contained chromosomal deletions in both *mtlA* and *gutA* (see Materials and Methods) was constructed. The latter gene encodes the glucitol-specific EII, which also shows a low affinity for mannitol in transport and phosphorylation (19). All of the deletion plasmids were transformed into strain LGS322, and all that initially gave spotted colonies in strain L146-1 gave only white colonies in strain LGS322. All further structural and functional analyses of the truncated permease molecules encoded by the deletion plasmids were therefore carried out with this strain.

Membranes from strain LGS322 harboring a number of deletion plasmids were tested for the presence of truncated mannitol permease molecules by immunoblotting after SDS extraction and polyacrylamide gel electrophoresis. An immunoblot of an electrophoretogram of several of these membrane preparations (Fig. 2) showed that many gave truncated proteins cross-reacting with the mannitol permease that were approximately the size predicted by restriction mapping of the corresponding plasmid (see below). Several membrane preparations also gave additional bands at 50 kilodaltons (kDa), 34 kDa, or both that were not detected in membranes of cells harboring the undeleted pGJ9 (Fig. 2), which suggested that these deletion proteins were more sensitive to endogenous proteolysis than was the native protein. Membranes from an additional seven strains harboring deletions in group 3, which would be expected to give truncated proteins of 41 to 55 kDa in size, showed only a band at 34 kDa in immunoblots (not shown), which suggested that these proteins were very sensitive to endogenous proteolysis. This 34-kDa band undoubtedly corresponds to the N-terminal, protease-insensitive domain of the permease (42). A small amount of this band is often detected, even in immunoblots of membranes containing the native protein (42). No proteins cross-reacting with antipermease antibodies were detected in the cytoplasmic fractions from any of the strains tested (not shown).

Functional analyses of strains harboring deletion plasmids. Whole cells and membrane preparations from strains harboring deletion plasmids representative of all five deletion groups were tested for transport and phosphorylation activities, respectively, of the mannitol permease. Table 3 summarizes the results of these experiments as well as the sizes of the truncated proteins detected by the immunoblotting experiments described above. As shown, all strains tested that gave white colonies on MacConkey-mannitol plates had no detectable mannitol transport activity in whole cells, and membrane preparations from all of these were inactive in PEP-dependent phosphorylation as well. These included two deletions which gave proteins on immunoblots that were only ca. 1,000 Da smaller than the native protein (pGJ9-Δ65 and -Δ90) as well as others that were only slightly smaller than this. In contrast, phospho-exchange activity (transphosphorylation) was detected in nearly normal or elevated levels in most of the deletions in group 2 and in one deletion in group 3 (Table 3). Phospho-exchange activities that were higher than wild-type levels (two- to fivefold) occurred predominantly in the deletions giving the smallest proteins (51 to 52 kDa) that still had detectable phospho-exchange activities. However, both deletion proteins in group 4 that were tested, pGJ9-ΔSnaBI (34 kDa) and pGJ9-Δ21 (33 kDa), were inactive in phospho exchange. These results show that the extreme C terminus of the mannitol permease is necessary for PEP-dependent phosphorylation of mannitol but not for the phospho-exchange activity.

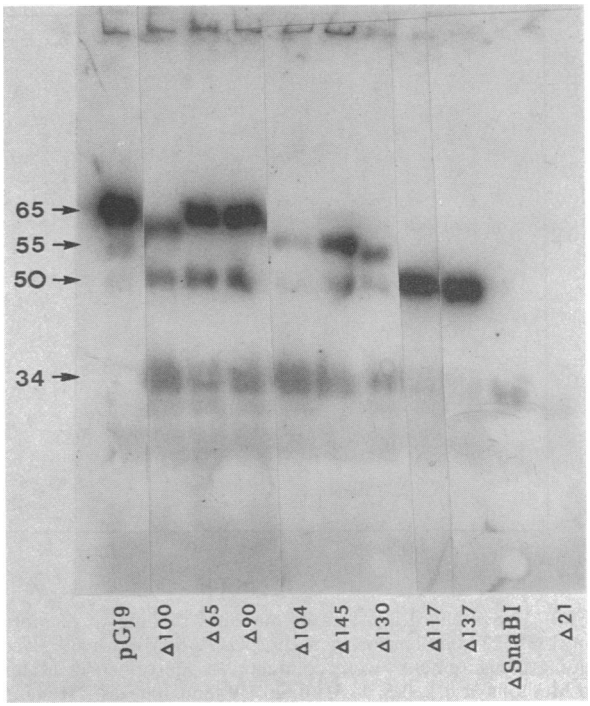

FIG. 2. Truncated mannitol permease molecules in membranes of strains harboring *mtlA* deletion plasmids. Membranes were isolated from LGS322 cells harboring intact pGJ9 or various deletion plasmids and were subjected to SDS-polyacrylamide gel electrophoresis (50 μg of membrane protein per lane). The gel was then electroblotted onto a nitrocellulose sheet, which was probed with antipermease antibody and ^{125}I-labeled *S. aureus* protein A and autoradiographed (see Materials and Methods). Molecular sizes on the left (in kilodaltons) were deduced from standard proteins run in parallel. All of the deletions shown gave bands within the size range predicted on the basis of restriction mapping of the corresponding plasmid, although many also gave bands at lower molecular sizes (50 kDa, 34 kDa, or both), indicating sensitivity of the deletion proteins to endogenous proteolysis. Staining of the deletion proteins from pGJ9-ΔSnaBI (34 kDa) and pGJ9-Δ21 (33 kDa) is weak because most of the epitopes recognized by the antibody are within the C-terminal half of the molecule (42). Expression of these two plasmids in a minicell-producing strain of *E. coli* labeled with [^{35}S]methionine, however, has shown that the corresponding deletion proteins are synthesized in amounts comparable to the amount of intact permease synthesized in the same strain harboring pGJ9 (C. Briggs and G. Jacobson, unpublished observations).

Interestingly, the one deletion (pGJ9-Δ69) that gave a pink phenotype on MacConkey-mannitol plates contained a deletion protein that exhibited an apparent molecular weight on SDS-polyacrylamide gel electrophoresis that was actually slightly larger than that of the wild-type permease (Table 3). This plasmid most likely represents a very short deletion into *mtlA* which, however, encodes a protein larger than 65 kDa by virtue of run-on translation into vector sequences until a stop codon is reached. Strain LGS322(pGJ9-Δ69) transported mannitol at a somewhat lower rate than did the wild type and had a much lower PEP-dependent phosphorylation activity in vitro (Table 3). Nonetheless, the properties of this mutant suggest that very short deletions may still give rise to a partially active protein.

Localization of the mannitol-binding site. The mannitol permease binds mannitol with high affinity, even in its unphosphorylated form (26). To attempt to localize this

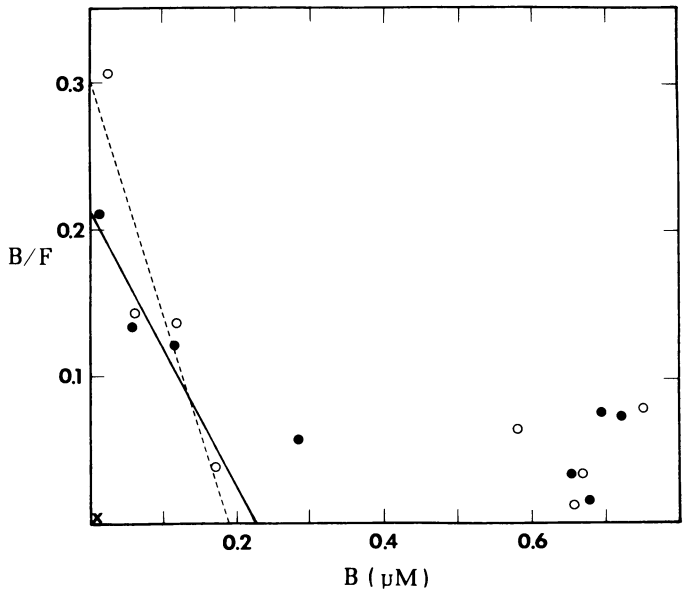

FIG. 3. Scatchard analysis of mannitol binding to membranes from LGS322 cells harboring pGJ9, pGJ9-ΔSnaB1, or no plasmid. Binding measurements were conducted as described in Materials and Methods at 0.1, 0.5, 1, 5, 10, 20, 50, and 100 μM [^{3}H]mannitol and at final membrane protein concentrations of 5.2 mg/ml [LGS322(pGJ9) membranes], 6.1 mg/ml [LGS322(pGJ9-ΔSnaBI) membranes], and 4.6 mg/ml (LGS322 membranes). Estimations of the K_D and stoichiometry for high-affinity mannitol binding (see text) were made from least-squares analyses (lines), using data from the measurements at 0.1, 0.5, and 1 μM mannitol: ● and —, LGS322(pGJ9) membranes; ○ and – – –, LGS322(pGJ9-ΔSnaBI) membranes. No specific binding was detected at any mannitol concentration to membranes from strain LGS322 alone (×). Each point is the average of at least three determinations at a single ligand concentration. B and F, Concentrations of bound and free mannitol, respectively.

binding site, we conducted mannitol-binding assays by using membranes derived from several of the deletion strains giving the smallest detectable truncated protein products (see Materials and Methods). Figure 3 shows a Scatchard plot of the data obtained by using membranes from strain LGS322 carrying undeleted pGJ9, pGJ9-ΔSnaBI, or no plasmid. As shown, membranes from the strain harboring pGJ9-SnaBI bound mannitol with an affinity (K_D, 0.60 μM; high-affinity component) and stoichiometry (32 pmol/mg of membrane protein) that were similar to those for membranes from cells harboring undeleted pGJ9 (K_D, 1.0 μM; 43 pmol/mg of membrane protein) as calculated from the data obtained between 0.1 and 1 μM mannitol. No detectable binding was observed in membranes of strain LGS322 alone at any mannitol concentration tested (Fig. 3). Strain LGS322 (pGJ9-Δ21) gave results similar to those for membranes from cells harboring pGJ9-ΔSnaBI (not shown). These dissociation constants are somewhat greater than that recently determined for mannitol binding to a single site per dimer of the purified permease (0.1 μM; 26) and may reflect a difference in affinity for mannitol between the membrane-bound and purified permeases. Although binding to a lower-affinity site (or sites) is also apparent from the data in Fig. 3 (as also shown in reference 26), the scatter of the data at higher mannitol concentrations for membranes from the strains harboring either pGJ9 or pGJ9-ΔSnaBI prevented an accurate estimation of either the affinity or the stoichiometry of this binding. Nonetheless, it is apparent that all of the amino acid residues constituting at least the high-affinity mannitol-binding site must be present in the truncated protein.

DISCUSSION

We have described the construction and analysis of a series of deletions in the cloned *mtlA* gene encoding the mannitol-specific EII (mannitol permease) of the *E. coli* PTS. The deletions extended into the 3′-terminal end of the gene, giving rise to permease molecules lacking less than 5% to over 75% of the protein. Functional analyses in a strain deleted for the chromosomal copy of *mtlA* defined at least three regions necessary for the various functions of the permease. These coincide remarkably well with three functional domains deduced from sequence homologies between EII^{Mtl} and EIIs specific for sucrose (Scr), β-glucosides (Bgl), D-glucose (Glc), and *N*-acetylglucosamine (Nag) (5). They are also consistent with structure-function relationships proposed before for EII^{Mtl}, using specific antibodies and protease treatment (9, 42). The three domains (Fig. 4) are as follows.

(i) There is a hydrophilic domain of 162 amino acids (residues 475 to 637) at the C terminus, the equivalent of an EIII in those systems in which the EII and EIII are separate proteins (e.g., the glucose PTS). This domain also shows homology with the separate, soluble EIII^{Mtl} of two gram-positive bacteria, *S. aureus* and *Staphylococcus carnosus* (29), and also with part of FPr from *Salmonella typhimurium*, a soluble protein of the fructose PTS that has both an EIII^{Fru} activity and an HPr-like activity (R. H. Geerse, F. Izzo, and P. W. Postma, Mol. Gen. Genet. in press). The

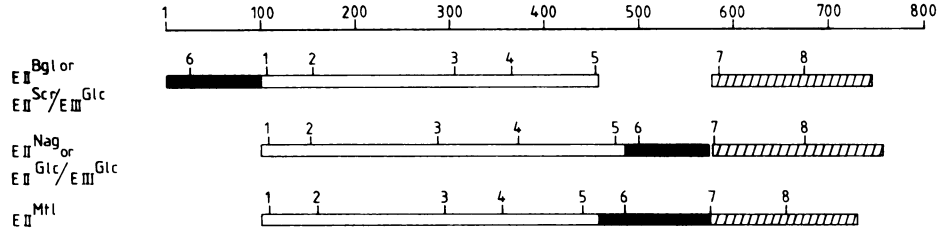

FIG. 4. Similarities of EIIs and EII-EIII pairs and their possible functional domains. Amino acid sequences of EIIs were schematically aligned according to similarities in their primary structures. Symbols: ▭, integral membrane hydrophobic regions of the EIIs; ▨, EIII^{Glc} and the EIII-like regions in EII^{Bgl}, EII^{Nag}, and EII^{Mtl}; ▬, postulated domain with the second phosphorylation site. EII^{Bgl} or EII^{Scr}-EIII^{Glc} and EII^{Nag} or EII^{Glc}-EIII^{Glc} are shown together because they represent the fused or split versions of otherwise similar molecules. The top line indicates length in 100-amino-acid increments. Numbers above the boxes indicate highly conserved areas (numbers and sequences correspond to EII^{Mtl}; see also references 2, 5, 27, and 38): 1, amphipathic helix; 2, -LPLLIG-7N-GGNNGG-; 3, His-195; 4, -GITE-; 5, series of charged amino acids; 6, Cys-384; 7, proline-alanine-rich hinge, or charged amino acids in EII^{Mtl}; 8, His-554.

HPr-like domain of this protein is located at the C terminus, whereas the EIII domain homologous to the *E. coli* EII^{Mtl} is N terminal (Geerse et al., in press).

As shown for EII^{Mtl}, fusion of an EIII domain in some other large EIIs such as EII^{Bgl} and EII^{Nag} (Fig. 4) has been demonstrated conclusively by a series of complementation tests. When overexpressed, both of these EIIs are able to complement the lack of a soluble $EIII^{Glc}$ and to phosphorylate EII^{Glc} and EII^{Scr} in mutants lacking $EIII^{Glc}$ (44). Moreover, truncated EII^{Nag} molecules lacking the C-terminal, EIII-like domain can be phosphorylated by means of the soluble $EIII^{Glc}$ (45). Such fusions of an EIII domain to an EII domain had been postulated previously to explain the differences in the molecular weights of the large and small EII complexes (37) and is also supported by amino acid sequence homologies among these proteins (2, 5, 27, 38, 39). These regions of homology include His-554 of EII^{Mtl} in its EIII domain (Fig. 4), which is phosphorylated by HPr (25).

Our data also suggest that the EIII-like domain of EII^{Mtl} may constitute a separate structural domain. Although many plasmids from group 2 (Table 3) produced proteins with a size consistent with the restriction mapping data, most also gave smaller bands on immunoblots at 50 and 34 kDa. In addition, the protein expressed from pGJ9-Δ117 (the smallest from group 2) and the protein from pGJ9-Δ137 (the largest from group 3) both gave only one band on immunoblots at 52 and 51 kDa, respectively. These deletion proteins, and presumably the 50-kDa breakdown products of the larger proteins, end in a region with an enrichment (9 of 14) of charged amino acids (residues 475 to 488), immediately followed by a region of high beta-turn probability (residues 487 to 492) (G. R. Jacobson and M. M. Stephan, FEMS Microbiol. Rev., in press). We postulate that this region may be a hinge connecting the EIII-like domain to the rest of the protein. The presence of such a hinge could explain why the larger deletion proteins are partially degraded to 50-kDa fragments and are relatively stable.

(ii) There is a hydrophobic domain (residues 1 to 334), which begins with an amphipathic helix (residues 1 to 19) and again ends with a region enriched (8 of 12) in charged amino acids (residues 335 to 346). It contains two areas of homology with the four EIIs mentioned above (residues 59 to 78 and 246 to 262) as well as a highly conserved histidine (His-195) situated in a characteristic hydrophilic loop. This half of the protein is the intramembrane domain and contains the mannitol-binding site (see above) and probably also at least a transient channel through which mannitol enters the cell (Jacobson and Stephan, in press). Since even the smallest deletion proteins detected were still found in the membrane and bound mannitol, we conclude that little, if any, of the C-terminal, hydrophilic half of the protein (residues 335 to 637) is necessary for proper membrane insertion.

(iii) A third, hydrophilic domain is located between the former two domains (residues 347 to 475). This region was first recognized as a domain showing homology to several of the other EIIs. It is located at the N-terminal end of EII^{Bgl} and EII^{Scr}, at the C-terminal end of EII^{Glc}, and between the hydrophobic and EIII-like domains of EII^{Nag} (as in EII^{Mtl}; Fig. 4) (5, 6). It contains a characteristic conserved sequence that flanks a cysteine residue (Cys-384 in EII^{Mtl}). In EII^{Mtl}, this cysteine has been proposed to be a second site of covalent phosphorylation and may be phosphorylated by phospho transfer from His-554 in the EIII-like domain mentioned previously (25, 26).

Functional studies of the deletion proteins revealed that these three domains perform discrete but interrelated functions in EII^{Mtl}. Thus, even relatively short deletions of less than ca. 30 amino acids from the C terminus (e.g., pGJ9-Δ65) were sufficient to inactivate both the transport and PEP-dependent phosphorylation activities of the permease. However, deletions removing as many as ca. 117 amino acids from the C terminus (e.g., pGJ9-Δ137) were still active in the mannitol 1-phosphate:[^{14}C]mannitol phospho-exchange activity of the permease (transphosphorylation). Indeed, several of these deletions were more active than the wild-type protein in phospho exchange. It has been shown that permease dimers are necessary for this activity (21, 41), and it is possible that these deletion proteins form more stable dimers, which could explain their high activities in phospho exchange. All of our deletion proteins that retain phospho-exchange activity still have Cys-384; at least one, the product of pGJ9-Δ137, lacks His-554, as may some others from group 2 (e.g., pGJ9-Δ117 and pGJ9-Δ148). This result shows that only proposed phosphorylation site 2 (Cys-384) is necessary for permease-catalyzed phospho transfer between mannitol and mannitol 1-phosphate and is consistent with the proposed role of this site in direct phospho transfer to mannitol in the PEP-dependent reaction (25, 26; M. M. Stephan, S. S. Khandekar, and G. R. Jacobson, submitted for publication). The phospho-transfer reactions of the mannitol permease can therefore be presumed to occur as depicted below, with phospho exchange (step 3) requiring only proposed site 2 (Cys-384) and not His-554:

$$\text{Phospho-HPr} + EII^{Mtl} \longleftrightarrow \text{HPr} + EII^{Mtl}\text{ (His-554)-P} \quad (1)$$

$$EII^{Mtl}\text{ (His-554)}-\text{P} \longleftrightarrow EII^{Mtl}\text{ (Cys-384)}-\text{P} \quad (2)$$

$$EII^{Mtl}\text{ (Cys-384)}-\text{P} + \text{mannitol} \longleftrightarrow EII^{Mtl} + \text{mannitol 1-phosphate} \quad (3)$$

Except for the very small deletion pGJ9-Δ69, and as judged by their sizes on polyacrylamide gels, some of the deletion proteins in group 2 and all in groups 3 to 5 lack His-554. However, one group 1 deletion protein (from pGJ9-Δ65) as well as others from group 2 should still contain His-554. Since none of the deletion plasmids tested thus far produced a protein that was functional in PEP-dependent vectorial phosphorylation or transport, it is likely that residues C terminal to His-554 are important for phospho transfer from phospho-HPr to the mannitol permease. Indeed, it has recently been hypothesized that the last two residues (Arg-636 and Lys-637), also conserved in other EIIIs and EIII-like domains (5), may be involved in phospho-HPr recognition (38). Sequencing of some of the deletion plasmids or construction of mutants lacking these residues will be necessary to test this possibility.

Finally, because all but one of the group 3 deletions tested gave only bands at 34 kDa in immunoblots instead of the predicted 41 to 55 kDa, it seems likely that amino acid sequences within this region are important for proper folding of the cytoplasmic, C-terminal half of the permease (42). Improper folding of these truncated molecules may lead to the endogenous proteolytic breakdown of the C-terminal part and to detection of only the 34-kDa intramembrane domain. Further analysis of deletion products from groups 4 and 5, most of which have not yet been extensively characterized, may provide information on parts of the N-terminal domain and its conserved structures that are important for mannitol recognition and transport as well as for chemotaxis toward this hexitol (20). Experiments to further define the

roles of the various domains in these processes are currently in progress in our laboratories.

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