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Genetic Analyses of the Mannitol Permease of *Escherichia coli*: Isolation and Characterization of a Transport-Deficient Mutant Which Retains Phosphorylation Activity

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Three positive selection procedures were developed for the isolation of plasmid-encoded mutants which were defective in the mannitol enzyme II (II^{Mtl}) of the phosphotransferase system (*mtlA* mutants). The mutants were characterized with respect to the following properties: (i) fermentation, (ii) transport, (iii) phosphoenolpyruvate(PEP)-dependent phosphorylation, and (iv) mannitol-1-phosphate-dependent transphosphorylation of mannitol. Cell lysis in response to indole acrylic acid, which causes the lethal overexpression of the plasmid-encoded *mtlA* gene, was also scored. No correlation was noted between residual II^{Mtl} activity in the mutants and sensitivity to the toxic effect of indole acrylic acid. Plasmid-encoded mutants were isolated with (i) total or partial loss of all activities assayed, (ii) nearly normal rates of transphosphorylation but reduced rates of PEP-dependent phosphorylation, (iii) nearly normal rates of PEP-dependent phosphorylation but reduced rates of transphosphorylation, and (iv) total loss of transport activity but substantial retention of both phosphorylation activities in vitro. A mutant of this fourth class was extensively characterized. The mutant II^{Mtl} was shown to be more thermolabile than the wild-type enzyme, it exhibited altered kinetic behavior, and it was shown to arise by a single nucleotide substitution (G-895 → A) in the *mtlA* gene, causing a single amino acyl substitution (Gly-253 → Glu) in the permease. The results show that a single amino acyl substitution can abolish transport function without abolishing phosphorylation activity. This work serves to identify a site which is crucial to the transport function of the enzyme.

The bacterial phosphoenolpyruvate (PEP)-dependent sugar phosphotransferase system consists of a pair of energy-coupling proteins (enzyme I and HPr or enzyme I^{Fru} and FPr), as well as the sugar-specific permease-kinasereceptors, the enzymes II or enzyme II-III pairs (15, 16, 18). Although sequence data and purification procedures for several phosphotransferase system proteins are now available (for a review and references, see M. H. Saier, Jr., M. Yamada, B. Erni, K. Suda, J. Lengeler, R. Ebner, P. Argos, B. Rak, K. Schnetz, C. A. Lee, G. C. Stewart, F. Breidt, Jr., E. B. Waygood, K. G. Peri, and R. F. Doolittle, FASEB J., in press). The first enzyme II to be purified (4) and sequenced (8, 9) was the mannitol enzyme II (II^{Mtl}). Indeed, this enzyme is the only phosphotransferase system permease which has been functionally reconstituted in an artificial membrane (12, 19), dissected genetically (11), or studied topologically (3, 4).

Although much is known regarding hexitol catabolism in enteric bacteria, major questions remain regarding the mechanism of hexitol permease function and regarding transcriptional regulation of the respective operons. In this communication, we report the results of studies aimed at the genetic dissection of II^{Mtl} functions. Three distinct positive selection procedures for the isolation of plasmid-encoded II^{Mtl} mutants are described, and the properties of some of the most interesting mutants are reported. One of these mutants was found to have lost its transport activity in vivo, but it retained much of its phosphorylation activity in vitro. Since analogous mutants have never been described previously for any one of the enzymes II of the phosphotransferase system and because such mutants may provide important clues to the transport mechanism, this mutant was extensively characterized. The mutant enzyme was shown to exhibit altered kinetic properties, showing higher apparent affinities for its two substrates, mannitol and phospho-HPr, than the wild-type enzyme. It also exhibited altered thermal stability when in the membrane (but not when solubilized in detergent micelles), altered sensitivities to detergents, and altered pH optima. The defect was shown to be due to a single base substitution, changing amino acyl residue 253 from glycine to glutamic acid. This glycyl residue is three residues from histidyl residue 256, which is postulated to be phosphorylation site 2, the histidyl residue which transfers its phosphoryl group to sugar during the group translocation step.

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MATERIALS AND METHODS

Strains and plasmids. The *Escherichia coli* and *Salmonella typhimurium* strains and plasmids which were used in this study are listed together with their genotypes, selection procedures, and sources in Table 1.

Growth media. LB medium was used as the complex medium, and a modified medium 63, containing mannitol, glucose, or citrate at 0.2% or glycerol at 1% as the carbon source, was used as the minimal medium (5, 21). Amino acids, when required, were present at 20 μg/ml. Eosin-methylene blue (without lactose) indicator plates were used to test for fermentation (21). Tetracycline (10 μg/ml) was included in the plates to ensure retention of plasmid pCAS2.0. Sensitivity to growth inhibition by indole acrylic acid (IAA) was measured on solid minimal medium containing the following ingredients in medium 63: 0.2% glucose; 1.5% Bacto-Agar (Difco Laboratories, Detroit, Mich.); and 0, 5, 10, 20, or 40 μg of IAA per ml.

Enzyme and transport assays. PEP-dependent sugar phosphorylation was accomplished with soluble enzymes and membrane fragments as previously described (5) unless

TABLE 1. Bacterial strains and plasmids used in this study

Strain	Genotype	Isogenic parent	Selection procedure or source
S. typhimurium			
LJ113	*his met trp ilv res mod*$^+$		P. E. Hartman
LJ409	*ppc-201 mtlA309*	SB2956	(11)
LJ538	LJ409(pCAS2.0)		This study
LJ534	LJ409(pCAS2.0)*mtlA354*	LJ538	This study; procedure 1
LJ535	LJ409(pCAS2.0) *mtlA355*	LJ538	This study; procedure 1
LJ536	LJ409(pCAS2.0) *mtlA356*	LJ538	This study; procedure 1
LJ537	LJ409(pCAS2.0) *mtlA357*	LJ538	This study; procedure 1
E. coli			
C600	*F$^-$ thr-1 leu-6 thi-1 supE44 lacY tonA21*		(10)
LJ1008	C600 Δ*mtl-29*	C600	(10)
LJ1009	C600 Δ*mtl-47*	C600	(10)
LJ526	LJ1008(pCAS2.0)	LJ1008	This study
LJ528	LJ1009(pCAS2.0)	LJ1009	This study
LJ523	LJ1008(pCAS2.0) *mtlA359*	LJ526	This study; procedure 2
LJ524	LJ1009(pCAS2.0) *mtlA360*	LJ528	This study; procedure 2
LJ525	LJ1009(pCAS2.0) *mtlA361*	LJ528	This study; procedure 2
L163sr	F$^-$ *thi metB rpsL galT malA mtlA51 gat-50 xyl* Δ*gutrecA*	L163	(10)
LJ510	L163sr(pCAS2.0)	L163sr	(10)
LJ514	L163sr(pCAS2.0) *mtlA363*	LJ510	This study; procedure 3
LJ518	L163sr(pCAS2.0) *mtlA367*	LJ510	This study; procedure 3

detergent-solubilized II^{Mtl} was explicitly used. Except for the kinetic analyses, where the concentrations of both substrates were varied (see below), the 100-μl total volume used contained 12.5 mM $MgCl_2$, 25 mM KF, 2.5 mM dithiothreitol, 5 mM PEP, 50 mM K_2PO_4 (pH 7.5), and 10 μM [^{14}C]mannitol (5 μCi/μmol). The energy-coupling proteins (enzyme I and HPr) were present in excess. Mannitol-1-phosphate-dependent sugar phosphorylation was determined as previously described (17). The final concentrations were the same as those described above, except that 10 mM mannitol-1-phosphate was substituted for PEP and the energy-coupling proteins were omitted. Protein was estimated by the Folin phenol method with bovine serum albumin as the standard (21).

Transport of mannitol into whole cells was measured at room temperature. [^{14}C]mannitol (5 μCi/μmol) was added to a final concentration of 20 μM. At various times, 0.5-ml portions were removed, and the cells were filtered on membrane filters (Millipore Corp., Bedford, Mass.) with a pore diameter of 0.45 μm and washed three times with medium 63. Nonspecific binding was negligible. The filters were dried and transferred to scintillation vials for counting.

Materials. Nonradioactive sugars were from Sigma Chemical Co. (St. Louis, Mo.) or Calbiochem-Behring (La Jolla, Calif.). ^{14}C-labeled sugars were from New England Nuclear Corp. (Boston, Mass.), and [^{35}S]methionine was from Amersham Corp. (Arlington Heights, Ill.). All other chemicals used were obtained commercially and were of the highest purity available.

DNA manipulations. Plasmid isolation, transformation, polyacrylamide gel electrophoresis, restriction endonuclease cleavage, and ligation were performed as described previously (13). In vitro mutagenesis with hydroxylamine was performed as follows. A total of 100 μl of DNA in 20 mM Tris hydrochloride and 1 mM EDTA (pH 8.0) was incubated overnight at 37°C with 200 μl of 0.6 M NH_2OH in 40 mM K_2HPO_4 and 1 mM EDTA. The solution was then dialyzed three times for more than 3 h against 20 mM Tris hydrochloride (pH 8.0) containing 20 mM NaCl and 1 mM EDTA to remove mutagen. This mutagenized preparation was used to transform the appropriate host cell.

Construction of hybrid II^{Mtl} proteins and sequencing strategy. For an illustration of the construction of hybrid II^{Mtl} proteins by gene fusion, see Fig. 3. To sequence the region of *mtlA354* containing the mutation, the mutant gene was cut with two different restriction enzymes, *Sau*3A and *Alu*I. DNA fragments corresponding to 445, 441, and 361 bases from the *Sau*3A digest were isolated and then cloned into the *Bam*HI site of mp18. Furthermore, DNA fragments of 414 and 385 bases from the *Alu*I digest were isolated and cloned into the *Sma*I site of mp18. The resultant fragments were sequenced as described previously (21, 22).

Three positive selection procedures for the isolation of plasmid-encoded *mtlA* mutants. (i) Procedure 1. pCAS2.0 (10) was isolated from *E. coli* C600 and used to transform *S. typhimurium* LJ113. This strain is restriction negative but DNA modification positive. The plasmid was again isolated and used to transform LJ409 (*ppc-201 mtlA309*; Table 1), selecting for tetracycline resistance on citrate minimal medium. LJ538 was thereby isolated.

For the isolation of *mtlA* mutants, pCAS2.0, which was isolated from *S. typhimurium* LJ113, was mutagenized with hydroxylamine in vitro, and the mutagenized plasmid was used to transform LJ409. Selection was on minimal citrate (0.2%)-plus-mannitol (0.2%) plates containing tetracycline (10 μg/ml). Strain LJ538 could not grow on these plates (11). Mutants capable of growth on this medium were isolated, cloned, and streaked onto eosin-methylene blue mannitol, glucose, fructose, mannose, and xylose plates. Mutants showing selective loss of mannitol fermentation were kept for further characterization.

(ii) Procedure 2. *E. coli* LJ1008 and LJ1009 were transformed with pCAS2.0 which had been mutagenized in vitro with hydroxylamine, as outlined above. The chromosomal *mtlA* gene and part of the *mtlD* gene were deleted from these two strains. They consequently lack the two protein products of these genes. The strains still possess low mannitol-1-phosphate dehydrogenase activity caused by the presence of two poorly expressed high-molecular-weight enzymes which are not encoded by the *mtlD* gene (14). However, the accumulation of mannitol-1-phosphate in the presence of a functional II^{Mtl} is, nevertheless, toxic. Cells were grown in

TABLE 2. Properties of plasmid-encoded *mtlA* mutants that were isolated by three different procedures[a]

Procedure and strain	Transport (%)	Transphosphorylation (%)	PEP phosphorylation (%)	IAA sensitivity (concn at which growth is inhibited) (μg/ml)
S. typhimurium; procedure 1				
LJ538 (parent)	100	100	100	<5
LJ534	3	43	42	20 to 40
LJ535	79	67	59	5 to 10
LJ536	20	98	22	10 to 20
LJ537	8	25	3	<5
E. coli; procedure 2				
LJ526 (parent)	100	100	100	<5
LJ523	2	24	2	<5
LJ528 (parent)	100	100	100	<5
LJ524	19	39	88	5 to 10
LJ525	85	103	37	20 to 40
E. coli; procedure 3				
LJ510 (parent)	100	100	100	<5
LJ514	10	10	9	20
LJ518	2	5	5	20

[a] Activities measured were those occurring after growth under uninduced conditions (in the absence of IAA) and represented activity due to transcription from another promoter, probably the *mtl* promoter (10). This fact, together with the relative effects of the mutations on the different II^{Mtl}-catalyzed reactions, demonstrated that the mutations were in the *mtlA* structural gene and not in the *trpOP* region of the plasmid.

liquid or on solid medium consisting of 1% glycerol in medium 63 which was supplemented with 0.2% mannitol, 10 μg of tetracycline per ml, and auxotrophic requirements. Mutants which were resistant to the toxic effects of mannitol were isolated and characterized with respect to their growth and fermentation properties.

(iii) Procedure 3. Plasmid pCAS2.0 expresses sufficient basal II^{Mtl} activity to allow substantial rates of mannitol fermentation, but expression of the *mtlA* gene is under the control of the *trpOP* region, and consequently, IAA, an antirepressor of the tryptophan operon, causes overproduction of II^{Mtl}, which in turn inhibits septation, causes "snake" formation, and eventually results in cell lysis (10). The toxic effect of IAA was used to develop a third *mtlA* mutant selection procedure. *E. coli* L163sr was plated onto minimal glucose medium containing auxotrophic requirements, 10 μg of tetracycline per ml and 20 μg of IAA per ml, following transformation with in vitro-mutagenized pCAS2.0. IAA-resistant mutants were clonally isolated and characterized with respect to their fermentation characteristics. Most of the mutants obtained by all three procedures were specifically defective in mannitol utilization.

Kinetic analysis of II^{Mtl} phosphorylation reactions. PEP-dependent [^{14}C]mannitol phosphorylation reactions catalyzed by II^{Mtl} were carried out in 0.1-ml volumes containing 50 mM potassium phosphate (pH 7.5), 10 mM $MgSO_4$, 10 mM KF, and 10 mM PEP. The enzyme I concentration was 1 μM, whereas the concentrations of HPr and [^{14}C]mannitol varied. Enzyme I was present in excess. Incubations were for 20 min at 37°C and were stopped by the addition of ice-cold water (3 ml). The [^{14}C]mannitol phosphate was separated from free sugar by using Dowex (AG1-X2) columns and quantitated as described elsewhere (7). In all cases, the II^{Mtl} concentration was within the linear range.

Effects of detergents on II^{Mtl} activity in vitro. Deoxycholate, Lubrol-PX, or Triton X-100 was added to a final concentration of 0, 0.1, 0.2, 0.5, or 1.0% in a 0.2-ml reaction volume at 0°C before assaying for either PEP- or mannitol-1-phosphate-dependent phosphorylation, as described above. The duration of the preincubation at 0°C was 15 min.

Sensitivity of II^{Mtl} to protease digestion. Rates of parental and mutant II^{Mtl} inactivation by trypsin and chymotrypsin were followed as a function of time and protease concentration, employing the conditions described previously (3, 4). The PEP-dependent [^{14}C]mannitol phosphorylation assay was used to follow inactivation.

Sensitivity of II^{Mtl} to perseitol inhibition. Perseitol (D-mannoheptitol) inhibition of parental and mutant II^{Mtl} was studied under standard assay conditions, employing the PEP-dependent phosphorylation reactions. The perseitol concentration range which was studied was 0.1 to 10 mM (6).

Preparation of anti-enzyme II^{Mtl} antibody. Antibody against purified enzyme II^{Mtl} was raised in a New Zealand White rabbit as described previously (8). The immunoglobulin G fraction of the serum was enriched by precipitation with $(NH_4)_2SO_4$ at 40% saturation and suspended in 0.1 M potassium phosphate buffer (pH 7.0) to an A_{280} of 20.

RESULTS

Properties of select plasmid-encoded *mtlA* mutants. The properties of representative plasmid-encoded *mtlA* mutants which were isolated by the three selection procedures described in Materials and Methods are summarized in Table 2. In addition to mutants which showed complete loss of all II^{Mtl} functions which were assayed (i.e., LJ518) and mutants which showed partial loss of all II^{Mtl} functions (LJ514 and LJ535), several mutants exhibited the selective loss of one or two of the three functions assayed (Table 2). For example, strain LJ534 was essentially lacking in transport activity in vivo but exhibited about 40% of both in vitro phosphorylation activities. Mutants LJ536 and LJ537 exhibited 98 and 25% of wild-type transphosphorylation activity, respectively, but only about 20 and 5% of transport and PEP-dependent phosphorylation activities, respectively. Strain LJ523 resembled strain LJ537 in this respect. In contrast, strain LJ525 showed selective loss of PEP-dependent phosphorylation activity, with retention of full transphosphorylation and partial transport activities, whereas LJ524 retained 88% of its PEP-dependent activity, but showed only

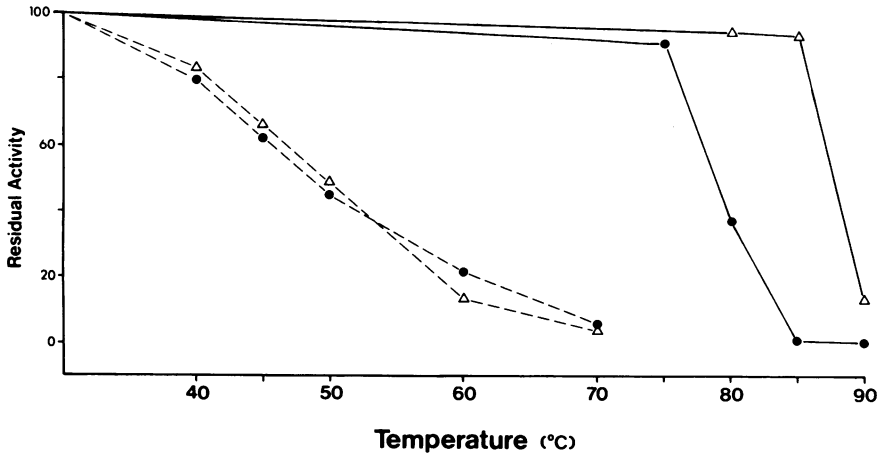

FIG. 1. Heat Inactivation of enzyme II^{Mtl}. *S. typhimurium* membranes (——) and micelles (– – – –) [deoxycholate-extracted membranes (5)] were incubated for 3 min at the temperatures indicated before being chilled to 0°C and assayed for PEP-dependent mannitol phosphorylation at 37°C. In both cases, parental II^{Mtl} from strain LJ538 (△) was compared with mutant II^{Mtl} from strain LJ534 (●).

39% of wild-type transphosphorylation activity and 19% of wild-type transport activity (Table 2).

Determination of the sensitivities of these mutants to the toxic effects of IAA revealed a gradient of sensitivities to this antirepressor of *trp* operon expression, but sensitivity to IAA did not correlate with the activity of any of the three II^{Mtl} functions which were assayed. Thus, LJ514 and LJ518 were deficient in all II^{Mtl} activities and showed resistance to IAA up to 20 μg/ml (as required, since they were isolated on this basis), but LJ525, showing high basal activities, was more resistant to IAA than LJ514 or LJ518. Strain LJ535, also showing high basal activity, was sensitive to 10 μg of IAA per ml. Strains LJ537 and LJ523, with negligible PEP-dependent phosphorylation and transport activities, but 25% residual transphosphorylation activity, were still sensitive to 5 μg of IAA per ml, but LJ536, with full transphosphorylation activity and 20% residual transport and PEP-dependent phosphorylation activities, was much more resistant to IAA. LJ534, with selective loss of transport activity, and LJ525, with selective loss of PEP-dependent activity, were resistant to 20 μg of IAA per ml (Table 2). All studies reported below characterize the *mtlA354* mutation in strain LJ534.

Thermal stability of mutant II^{Mtl} (*mtlA354*). The mutant II^{Mtl} protein caused by the *mtlA354* mutation in LJ534 was characterized by using several approaches. As shown in Fig. 1, the parental enzyme in intact membranes was much more stable to heat inactivation than was the mutant enzyme. In contrast, the detergent-solubilized enzyme was much less stable than the membrane-bound enzyme, and the parental and mutant enzymes showed similar thermal inactivation curves. The results show that the difference in thermal stability of the mutant and parental enzymes was dependent on the integrity of the membrane; i.e., it was only apparent when the enzyme was stabilized by interactions with the lipid bilayer of the membrane.

Kinetic analysis of the mutant II^{Mtl}. Lineweaver-Burk plots of the data which were generated when reaction rate was studied as a function of the concentrations of both substrates (phospho-HPr and mannitol) revealed ping pong kinetics for both the mutant and parental enzymes (data not shown). However, the kinetic constants differed markedly. Surprisingly, the mutation caused the absolute K_m values of both substrates to decrease, i.e., caused the apparent affinity of the enzyme for its substrates to increase. K_m values for mannitol and phospho-HPr were 10.0 ± 6.5 μM and 2.7 ± 1.2 μM, respectively, for the parent and 2.6 ± 0.3 μM and 0.16 ± .08 μM, respectively, for the mutant. Each value represents an average of four determinations. Thus, the affinity for phospho-HPr increased nearly 20-fold, whereas that for mannitol increased fourfold. V_{max} values varied, but these values were much lower for the mutant than for the parental enzyme. To what extent this last observation was attributable to the lability of the mutant II^{Mtl} was not determined.

Effect of the *mtlA354* mutation on the pH optima of II^{Mtl}-catalyzed phosphorylation reactions. Activity-pH curves for the parental (4, 5) and mutant enzymes for both the PEP- and mannitol-1-phosphate-dependent phosphorylation reactions were determined. The mutation caused the optima in both pH curves to shift toward lower pH values by about 1 pH unit (data not shown). This fact may reflect the altered charge or acid-base properties of the mutant enzyme, relative to the parental enzyme.

Immunoblot analysis of mutant II^{Mtl}. To estimate the amounts of II^{Mtl} in the mutant and parental membranes, these proteins were solubilized, and II^{Mtl} was visualized after sodium dodecyl sulfate gel electrophoresis, as described in the legend to Fig. 2 by using the Western blot (immunoblot) technique. The results shown in Fig. 2 establish that the mutant protein was present in an amount similar to that of the parental strain and that it migrated in the gel, as did the parental enzyme. Surprisingly, the detergent-solubilized and purified parental II^{Mtl} migrated substantially more rapidly than did either of the membrane-bound enzymes (see the arrow in Fig. 2). This result suggests that incomplete denaturation of the enzyme or lipid associations influence the migration rate under the conditions of electrophoresis.

Effects of detergents on II^{Mtl} activities. In view of the possibility that lipid interactions were important to the II^{Mtl} structure and function, the effects of a variety of detergents on enzyme activity under various conditions were studied. When the PEP-dependent phosphorylation reaction was studied, the mutant enzyme was uniformly sensitive to inhibition (or inactivation) by all detergents which were studied (deoxycholate, Lubrol-PX, and Triton X-100; 0.1 to 1%) both at pH 6.2 and 8.5. At pH 8.5, the parental enzyme

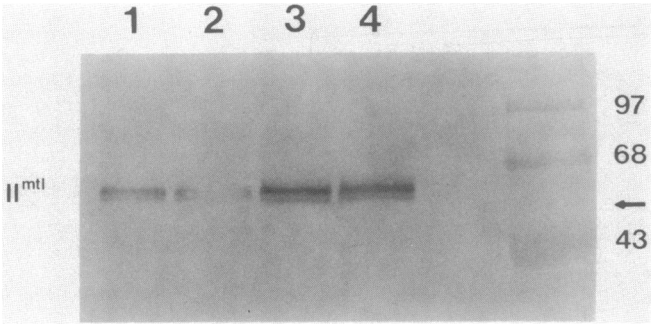

FIG. 2. Immunoblot of enzyme II^{Mtl}. Mutant and parental protein samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Upon completion of the electrophoresis, the proteins were electrophoretically transferred onto nitrocellulose paper, as described in the Bio-Rad information booklet (Bio-Rad Laboratories, Richmond, Calif.). Enzyme II^{Mtl} was detected by incubation with anti-enzyme II^{Mtl} antibody. Lanes: 1 and 3, mutant extracts with protein concentrations at 125 and 250 μg, respectively; 2 and 4, parental extracts at 125 and 250 μg, respectively. Protein standards with the molecular weights indicated in kilodaltons are shown at the right. Double bands for II^{Mtl} (approximate molecular weight of 60 kilodaltons) have been reported previously (4, 5), but the basis for their presence is not known. The arrow on the right indicates the position to which purified, lyophilized parental II^{Mtl} migrated.

was also inhibited, but at pH 6.2, it was subject to activation by all three detergents. The effects of the various detergents on the transphosphorylation reaction were more complex. At low detergent concentrations, the mutant enzyme was activated, whereas at higher concentrations, it was inhibited. Similar behavior was usually, but not always, observed for the parental enzyme. The greater sensitivity of the mutant enzyme to inhibition or inactivation by detergents when the PEP-dependent reaction was studied correlated with its greater thermal lability.

Localization and identification of the *mtlA354* mutation. To determine which region of the *mtlA* gene contained the *mtlA354* mutation, hybrid *mtlA* genes were constructed (Fig. 3). Cloned *mtlA* genes from both the mutant and parent were split with a restriction enzyme, and the mutant N-terminal part was ligated with the parental C-terminal part (i.e., hybrid 1 in Fig. 3). Alternatively, the parental N-terminal moiety was ligated to the mutant C-terminal moiety (i.e., hybrid 2 in Fig. 3). After subsequent insertion into a plasmid and transformation into a strain from which most of the *mtl* operon was deleted, it could be determined whether the N- or C-terminal half of the mutant gene contained the mutation. For example, if the mutation was in the N-terminal half of the gene, then after transformation into an *mtl* deletion strain, hybrid 2 would ferment mannitol, whereas hybrid 1 would not. This was observed to be the case when hybrids were made by using the restriction enzymes *Pvu*II and *Tth*III but not *Hae*II (Table 3). Consequently, the mutation was located to the left of the *Pvu*II (1,697 bases) and *Tth*III (1,413 bases) sites but to the right of the *Hae*II (851 bases) site. Hence, the mutation occurred in the region between base 851 and base 1413.

The sequencing strategy which revealed the site and nature of the *mtlA354* mutation was discussed in Materials and Methods. A single nucleotide substitution (G-895 → A), caused a single amino acyl substitution (Gly-253 → Glu). All of the altered biochemical and kinetic properties thus displayed by the mutant II^{Mtl} are attributable to this amino acyl substitution.

Characteristics of II^{Mtl} which were unaffected by the *mtlA354* mutation. Rates of II^{Mtl} inactivation by both trypsin and chymotrypsin were quantitated by using membrane fragments derived from French-pressed cells of strains LJ538 (parent) and LJ534 (mutant) as described in Materials

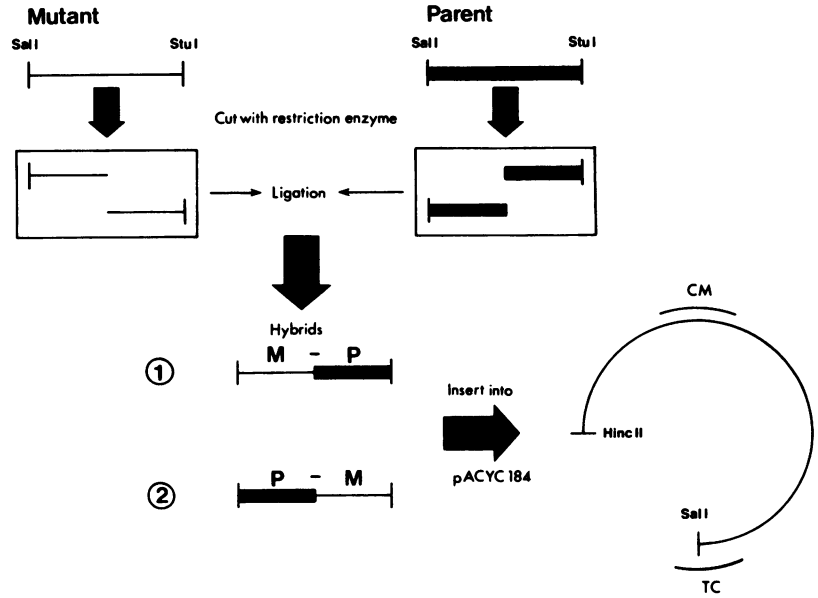

FIG. 3. Construction of hybrid II^{Mtl} proteins. DNA corresponding to the *mtlA* gene in the mutant and parent was isolated and then cut with a restriction enzyme (in each case, the *mtlA* gene was cut with *Pvu*II, *Tth*III, and *Hae*II). Ligations between the mutant N-terminal half and the parental C-terminal half (hybrid 1) or between the parental N-terminal half and the mutant C-terminal half (hybrid 2) were then performed. The resulting hybrids were inserted into pACYC184, and the plasmids were used to transform strains in which the *mtl* operon was defective or from which it was deleted (LJ1006, LJ1008, and HB101).

TABLE 3. Localization of the *mtlA354* mutation by hybrid reconstruction[a]

Restriction enzyme	Cleavage site (no. of bases)[b]	N terminus[c]	C terminus[c]	Fermentation response
*Pvu*II	1,697	M	P	−
		P	M	+
*Tth*III	1,413	M	P	−
		P	M	+
*Hae*II	851	M	P	+
		P	M	−

[a] Hybrid plasmids, constructed according to the protocol outlined in Fig. 3, were transformed into LJ1006, LJ1008, and HB101. Fermentation was tested on eosin-methylene blue indicator plates plus mannitol (2.0%). Chloramphenicol (30 μg/ml) was included in the plates to ensure retention of the hybrid plasmids.

[b] The *mtlA* gene is approximately 2,000 base pairs long. The position which is indicated is the number of bases between the first nucleotide of the structural gene, corresponding to the N terminus of the protein, and the cleavage site.

[c] Abbreviations: M, mutant; P, parental.

and Methods (3, 4). Rates of inactivation for the two preparations were the same, ±5%, with both enzymes at all concentrations of the proteases tested.

Sensitivity to inhibition by the nonphosphorylateable II^{Mtl} competitive inhibitor, perseitol (6), was also unaffected by the *mtlA354* mutation. This last finding showed that while the apparent affinity of the enzyme for its natural substrate, mannitol, was drastically altered, the relative affinities of the enzyme for mannitol and perseitol were unaltered.

DISCUSSION

In this paper, we report the development of three positive selection procedures for the isolation of plasmid-encoded mutants which are defective in the mannitol permease of *E. coli*. A number of these mutants were isolated and characterized with respect to mannitol fermentation, transport, PEP-dependent phosphorylation, and mannitol-1-phosphate-dependent transphosphorylation. Sensitivity to the toxic effects of IAA, which induces synthesis of the plasmid-encoded II^{Mtl}, was also quantitated. The mutant enzymes showed altered relative activities, with any one of the activities catalyzed by the enzymes being preferentially depressed. None of the activities examined was consistently depressed in parallel with sensitivity of the bacterial strain carrying the mutant plasmid to IAA. These results are in agreement with our postulate (10, 21) that overproduction of an integral membrane protein can tie up the insertion machinery, thereby preventing proper or sufficient insertion of other essential membrane proteins, such as those responsible for septation.

We have extensively characterized an *mtlA* mutant which appeared to selectively lose its transport function in vivo while retaining a much larger percentage of its phosphorylation activity in vitro. Analogous mutants had not been described previously, and it was hoped that the characterization of this paralyzed mutant would lead to a better understanding of the transport process. The defect was shown to be due to a single base substitution giving rise to a single amino acyl substitution in the permease, Gly-253 → Glu. An examination of the amino acyl sequence and hydropathy plot of the permease revealed that the mutation occurred in the hydrophobic N-terminal half of the protein at the C-terminal end of a region containing four short hydrophobic stretches (9). These short hydrophobic stretches could pass through the membrane either as four strands of β-structure or as two α-helicies with hydrophilic centers. Alternatively, they may exist on the membrane surface, merely dipping into the phospholipid bilayer. Regardless of the secondary structural element which characterizes this region, we suggest that it functions in sugar recognition and the transmembrane translocation process as an essential part of the active site. This suggestion is supported by the fact that mutation of Gly-253 to Glu not only destroyed transport function, it also drastically altered the apparent binding affinities (absolute K_m values) of the enzyme for its substrates, phospho-HPr and sugar in vitro. Surprisingly, the affinity of the enzyme for these two substrates increased, rather than decreased, in the mutant. The mutation also altered the pH optima for sugar phosphorylation and rendered the membrane-bound enzyme more heat labile. In addition, we have recently found that the *mtlA354* mutation drastically altered the catalytic response of the enzyme to pressure. While the transphosphorylation activity of the wild-type enzyme was markedly stimulated by high pressure, this activity of the mutant enzyme was strongly inhibited (E. DeLong, R. Manyan, and M. H. Saier, Jr., unpublished results). The fact that sensitivity of the mutant enzyme to protease treatment was the same as that of the parental enzyme suggests that the overall conformation of the enzyme was not drastically altered by the *mtlA354* mutation. However, the possibility that the mutation prevents proper insertion of the protein into the membrane can not be excluded.

Gly-253 is adjacent to another glycyl residue (Gly-254), and a histidyl residue is at position 256. The sequence in this region (residues 253 to 256) is Gly-Gly-Ile-His (9). Interestingly, the second phosphorylation site in the β-glucoside enzyme II is probably His-306 (1, 2, 20) on the basis of site-specific mutagenesis studies (K. Schnetz, G. A. Daniels, S. Sutrina, M. H. Saier, Jr., and B. Rak, unpublished results; see Saier et al., in press). When His-306 was changed to a lysyl residue, all transport and phosphorylation activities of the enzyme were lost. The sequence preceding and including this residue in the β-glucoside enzyme II is Phe-Gly-Leu-His, whereas that for the homologous position in the sucrose enzyme II is Thr-Gly-Ile-His, which is similar to the sequence including residues 253 to 256 in II^{Mtl}. These considerations lead to the suggestion that His-256 may be the active site histidyl residue, the dephosphorylation of which is coupled to sugar transport and phosphorylation. Further investigations, particularly by site-specific mutagenesis, should provide conclusive evidence for or against this postulate. These studies are in progress.

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