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Plasmid-directed synthesis of enzymes required for D-mannitol transport and utilization in *Escherichia coli**

(phosphotransferase system/minicell/mannitol-specific enzyme II/mannitol-1-phosphate dehydrogenase)

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ABSTRACT **A transformant *Escherichia coli* colony bank [Clarke, L. & Carbon, J. (1976) *Cell* 9, 91–99] has been screened for hybrid ColE1 plasmids carrying the genes for D-mannitol utilization. Two of the plasmids, pLC11-7 and pLC15-48, were shown to contain the mannitol operon, which includes the structural genes for the mannitol-specific enzyme II of the phosphotransferase system and mannitol-1-phosphate dehydrogenase. One *E. coli* strain harboring plasmid pLC15-48 overproduced mannitol-1-phosphate dehydrogenase activity 4- to 5-fold. However, there was no corresponding increase in mannitol enzyme II activity. Plasmid pLC15-48 was shown to direct the synthesis of two polypeptides in *E. coli* minicells in the presence of cyclic AMP and mannitol. The larger (M_r = 60,000) was membrane bound and was specifically precipitated by antibody directed against purified mannitol-specific enzyme II. The smaller (M_r = 40,000) was soluble and had an electrophoretic mobility indistinguishable from that of the major component in a partially purified mannitol-1-phosphate dehydrogenase preparation. These data are consistent with previous genetic studies of the mannitol locus and confirm an independent conclusion [Jacobson, G. R., Lee, C. A. & Saier, M. H., Jr. (1979) *J. Biol. Chem.* 254, 249–252] that mannitol enzyme II consists of a single type of polypeptide chain that has a M_r of 60,000. The plasmid pLC15-48 DNA was characterized by mapping of restriction endonuclease cleavage sites.**

D-Mannitol and several other hexitols and hexoses are taken up into *Escherichia coli* cells via a phospho*enol*pyruvate (*P-e*Prv)-dependent phosphotransferase system first described by Roseman and coworkers (1). The sugar-specific components of this system (enzymes II) appear to be integral membrane proteins responsible for the transport and concomitant phosphorylation of their sugar substrates (for review, see refs. 2–4). Genetic studies have shown that mannitol utilization in *E. coli* is governed by three closely linked genes: *mtlA*, the structural gene for the mannitol-specific enzyme II of the phosphotransferase system; *mtlD*, the structural gene for mannitol-1-phosphate (Man-1-*P*) dehydrogenase; and *mtlC*, a *cis*-dominant regulatory gene (5, 6). The unit comprising these genes behaves as a single operon with the gene order *C*, *A*, *D* and maps at 80 min on the recalibrated *E. coli* K-12 linkage map (7).

We have screened a transformant *E. coli* colony bank (8) for hybrid ColE1 plasmids containing the mannitol operon. In this study, we report the identification of two such plasmids and the properties of cells harboring them. The proteins produced by the plasmid pLC15-48 and its DNA restriction map are described.

MATERIALS AND METHODS

Bacterial Strains Used. The strains of *E. coli* used and their relevant genotypes and sources are given in Table 1.

Identification of Hybrid ColE1 Plasmids Containing the Mannitol Operon. Replica mating experiments, as described by Clarke and Carbon (8), were carried out between a transformant colony bank of *E. coli* strain JA200 harboring synthetic ColE1 hybrid plasmids and *E. coli* strain AI214 (F^-, mtl^-). Minimal salt agar plates (medium 63) containing 0.1% mannitol as the sole carbon source were spread with a thin layer (0.1 ml) of washed *E. coli* AI214 cells (10^9/ml) derived from a culture growing exponentially on nutrient broth, and the plates were allowed to dry. Replica plating of the Clarke–Carbon colony bank from nutrient agar plates onto these seeded plates resulted in visible growth (37°C, 2 days) from colonies that had received plasmids pLC11-7, 15-48, and 22-40, respectively. Plasmid pLC22-40 was removed from further consideration because it had been shown to contain the sorbitol (D-glucitol) operon (8). Growth of this clone could be explained by overproduction of the sorbitol enzymes, allowing utilization of the small amount of sorbitol in commercial mannitol, by the ability of the sorbitol enzyme II to transport mannitol with low efficiency, or both (9). Liquid mating experiments confirmed the ability of plasmids pLC11-7 and pLC15-48 to convert a number of F^- strains with a mannitol-negative phenotype into cells capable of growth on mannitol (see Table 2). Both pLC11-7 and 15-48 were therefore presumed to carry the genes for mannitol utilization.

Expression of Plasmid pLC15-48 in an *E. coli* Minicell Strain. Plasmid pLC15-48 was transferred to the *E. coli* minicell strain MV1009 by liquid mating. Minicells were purified from stationary phase cultures in L broth by differential centrifugation followed by two discontinuous sucrose gradients and selective killing of contaminating cells with penicillin (1600 units/ml) (10). The purified minicells were suspended in medium 63 supplemented with the 19 amino acids other than the radiolabeled one (2 μg/ml each). Protein labeling was initiated by addition of 0.1 mCi (1 Ci = 3.7×10^{10} becquerels) of [^{35}S]methionine or 0.5 mCi of [^{3}H]leucine per ml of medium. Protein was labeled in the presence or absence of 0.5% mannitol, 1.0 mM cyclic AMP, and the inhibitors of membrane-bound proteases phenethyl alcohol (0.5–10 mg/ml) and procaine (1–100 mg/ml). After 3 hr of incubation at 37°C, the minicells were collected and treated with lysozyme (1 mg/ml) in 30 mM Tris·HCl/5 mM potassium EDTA, pH 8.0, at 37°C for 1 hr. The membrane and cytoplasmic fractions were obtained by osmotic lysis or sonication of the spheroplasts. The membranes were centrifuged and washed at 100,000 × *g* for 90 min. The

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Abbreviations: *P-e*Prv, phospho*enol*pyruvate; *M*an-1-*P*, mannitol-1-phosphate; enzyme II^{mtl}, mannitol-specific enzyme II of the *P-e*Prv-dependent phosphotransferase system.

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Table 1. *E. coli* strains and sources

Strain	Genotype	Source
AI214	*srl tet^r recA r_k^- m_k^+ B_1 pyrE60 argE3 his-4 proA2 leu-6 mtl-1 xyl-5 ara-14 galK2 lac41 strA31*	D. W. Smith*
JA200	*F$^+$ trpE5 recA thr leu lacY* harboring hybrid ColE1 plasmids	D. W. Smith
KL141	*F$^-$ argG6 pyrE41 thi recA56*	D. W. Smith
MV1009	*F$^-$ thr ara leu azi^r tonA^r lacY T6^s minA gal$^-$ minB str^r malA xyl mtl thi sup srl tet^r recA56*‡	M. Volkert†

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‡ *minA* and *minB* refer to defects that allow minicell production (5).

cytoplasmic fraction was retained and lyophilized. [^{35}S]-Methionine-labeled whole cells or cell fractions were boiled in NaDodSO$_4$ sample buffer, subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis as described below, dried, and autoradiographed. Gels of samples labeled with [^{3}H]leucine were treated with EN^{3}HANCE according to the manufacturer's instructions, dried, and fluorographed.

Preparation of Anti-Enzyme II^{mtl} Antibody. Purified mannitol-specific enzyme II (enzyme II^{mtl}; 100 μg; ref. 11) was dialyzed overnight against 2 liters of distilled water to remove excess Lubrol PX. The solution was lyophilized, and the product was redissolved in 0.1 ml of water and homogenized with 0.1 ml of complete Freund's adjuvant to form an emulsion that was then injected directly into the hind leg lymph nodes of a New Zealand rabbit. After 4 weeks, a second subcutaneous injection was made with 100 μg of similarly prepared enzyme II^{mtl}, except that incomplete adjuvant was used. Ten days later, the animal was bled (50 ml), and bleedings were continued every 5 days as long as the antibody titer remained high (2 weeks). The resulting antiserum specifically inhibited *P-e*Prv-dependent and Man-1-*P*-dependent mannitol phosphorylation activities of both crude membranes and the purified protein and formed a single precipitin line in double-diffusion tests against deoxycholate extracts of *E. coli* membranes (12). For some experiments, the IgG fraction was enriched by precipitation with $(NH_4)_2SO_4$ at 40% saturation and resuspended in 100 mM potassium phosphate, pH 7.0, to an A_{280} of 20.

Immunoprecipitation of the Membrane-Bound Protein Synthesized from pLC15-48. Membranes were prepared from MV1009/pLC15-48 minicells as described above. The labeled minicell membranes were suspended in 160 mM Tris·HCl, pH 6.8/20% (vol/vol) glycerol/4% NaDodSO$_4$ and boiled 8 min. Insoluble components were removed by centrifugation at 13,000 × *g* for 5 min. Primary binding of antibody to protein was initiated by adding 20 μl of NaDodSO$_4$-solubilized sample and 2 μl of rabbit serum protein to 200 μl of 1.25% Nonidet P-40/2.5% bovine serum albumin/0.035% sodium azide/25 mM Tris·HCl, pH 7.4/75 mM NaCl/2.5 mM EDTA. The binding was complete after 20 min at 37°C and at least 2 hr at 4°C. Secondary precipitation of antibody by staphylococcal A adsorbant (13), added to the above solution to a final concentration of 2% (vol/vol), was allowed to occur for 30 min at room temperature. The adsorbant was washed twice with 0.5% Nonidet P-40 in TNE buffer (0.05 M Tris·HCl/0.05 M NaCl/5 mM EDTA, pH 7.4) and twice with TNE buffer alone. Antibody and immunoprecipitated proteins were removed from the staphylococcal A adsorbant by a 15-min incubation with NaDodSO$_4$ sample buffer at 60°C. The adsorbant was removed by centrifugation, and the supernatant was subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis.

Assays. *P-e*Prv-dependent phosphorylation of mannitol catalyzed by enzyme II^{mtl} was assayed by a DEAE–filter paper technique (11). Man-1-*P* dehydrogenase activity was monitored by the reduction of NAD$^+$ at 340 nm. Reaction mixtures, at 22°C, contained 0.1 M Tris·HCl, pH 9.1, 1 mM NAD$^+$, and the protein sample to be assayed. After the reaction mixture was placed in the cuvette, any sugar-phosphate independent reduction was allowed to occur (generally, there was none), and the reaction was then started by addition of Man-1-*P* to a final concentration of 10 mM. Enzyme II^{mtl} was routinely measured in cell membranes (100,000 × *g* pellet) and Man-1-*P* dehydrogenase was measured in 100,000 × *g* cell supernatants after rupture of cells by passage through a French pressure cell at 10,000 psi (1 psi = 6.9 kPa). Comparisons of levels of these enzymes in various strains (see Table 2) were made after preparation of extracts from cells that had been harvested during the midexponential growth phase (6–8 × 10^8 cells per ml).

Partial Purification of Man-1-*P* Dehydrogenase. A 100-ml culture of *E. coli* strain JA200/pLC15-48, which overproduces this enzyme 4- to 5-fold was grown to stationary phase in medium 63/0.5% mannitol supplemented with L-tryptophan at 50 μg/ml. Cells were harvested, washed, taken up in 5 ml of TD buffer (0.02 M Tris·HCl/1 mM dithiothreitol, pH 7.5) and broken by passage through a French pressure cell (10,000 psi). After 90 min of centrifugation at 100,000 × *g* (4°C) the supernatant was brought to 55% saturation with $(NH_4)_2SO_4$, and the precipitate was discarded (14). Solid $(NH_4)_2SO_4$ was then added to 65% saturation, and the pellet, containing most of the Man-1-*P* dehydrogenase activity, was then dissolved in 2 ml of TD buffer and dialyzed against 100 ml of the same buffer at 4°C. The sample was further fractionated by loading on, and stepwise elution from, a 1-ml Bio-Rad Affi-Gel Blue column, previously equilibrated with TD buffer. Eluting buffers were (2 ml each) TD buffer/0, 1, 5, or 10 mM NAD$^+$, pH 7.5. Most of the Man-1-*P* dehydrogenase activity eluted with the buffer containing 10 mM NAD$^+$. This eluate was dialyzed against 1 liter of 5 mM $(NH_4)HCO_3$, and the product was lyophilized and analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis. The sample was judged to be at least 20-fold purified relative to the crude cell lysate by specific activity measurements and consisted of a major stained band with an apparent M_r of 40,000 (see Fig. 2). The M_r of native Man-1-*P* dehydrogenase is 40,000 as determined by gel filtration (unpublished observations).

Purification and Restriction Endonuclease Cleavage of Plasmid pLC15-48. Plasmid pLC15-48 DNA was isolated from chloramphenicol-amplified JA200/pLC15-48 cells by using a cleared lysate procedure followed by equilibrium centrifugation in dye/CsCl gradients (15). The purified DNA (0.4 μg) was digested with 1 unit of restriction endonuclease at 37°C for 90 min. The reaction mixtures were (*i*) for *Eco*RI, 100 mM Tris·HCl, pH 7.4/50 mM NaCl/10 mM MgCl$_2$/6 mM 2-mercaptoethanol; (*ii*) for *Bgl* II, 6 mM Tris·HCl, pH 7.4/50 mM NaCl/6 mM MgCl$_2$/6 mM 2-mercaptoethanol; (*iii*) for *Pst* I and *Hin*dIII, either the *Eco*RI or the *Bgl* II mixture; and (*iv*) for *Kpn* I, 6 mM Tris·HCl, pH 7.4/6 mM NaCl/6 mM MgCl$_2$/ 6 mM 2-mercaptoethanol. The digests were subjected to electrophoresis through a 0.6% agarose slab gel with *Hin*dIII-digested λDNA and *Hae* II-digested φX174 DNA as standards.

Analytical Procedures. Protein was estimated according to Lowry *et al.* (16) with bovine serum albumin as the standard. NaDodSO$_4$/polyacrylamide gel electrophoresis was performed according to Laemmli (17) in 0.5-mm-thick slabs.

Materials. Man-1-*P*, phenethyl alcohol, and procaine were from Sigma. [^{14}C]Mannitol, [^{3}H]leucine, and [^{35}S]methionine were products of New England Nuclear. EN^{3}HANCE was also obtained from New England Nuclear. Affi-Gel Blue affinity

resin was from Bio-Rad. Nonidet P-40 was from Particle Data Laboratories (Elmhurst, IL). Restriction enzymes were obtained from Bethesda Research Laboratories (Rockville, MD) or New England BioLabs. All other chemicals used were of the highest purity available.

RESULTS

Phenotypes of Cells Harboring Plasmids pLC11-7 or pLC15-48. Replica mating experiments suggested that plasmids pLC11-7 and pLC15-48 contained part or all of the mannitol operon. This was confirmed by the following observations. *E. coli* strains AI214 and MV1009, both defective in the utilization of mannitol, could grow in this sugar when either pLC11-7 or pLC15-48 was introduced via liquid mating experiments with strain JA200 harboring the appropriate plasmid (Table 2). Unexpectedly, strain JA200, which had not been reported to be mannitol negative (8), also could not grow on mannitol as the sole source of carbon unless it carried either plasmid. Levels of enzymes encoded in the mannitol operon, enzyme II^{mtl} of the phosphotransferase system and Man-1-*P* dehydrogenase (5, 6), were also measured in some of these strains, and the results are included in Table 2. In each strain tested, growth on mannitol minimal medium, conferred by either plasmid, was accompanied by the appearance of nearly normal levels of the mannitol enzymes. (Both AI214 and JA200 had very low levels of both enzyme II^{mtl} and Man-1-*P* dehydrogenase when grown on minimal media containing both glycerol and mannitol.) Interestingly, however, strain JA200/pLC15-48 produced elevated levels of dehydrogenase activity, even when grown in the absence of mannitol. In contrast, enzyme II^{mtl} activity, although present, was low in mannitol-grown cells (Table 2). The explanation for this noncoordinate expression of the two activities is not known. But the data presented in Table 2 with three independent mannitol-negative strains confirm the presence of the mannitol operon on plasmids pLC11-7 and pLC15-48. The inducibility of the mannitol enzymes in all of the plasmid-bearing strains (data not shown) also argues for the presence of regulatory genes, including *mtlC*, controlling induction.

Identification of Proteins Whose Synthesis Is Directed by Plasmid pLC15-48. Minicells containing plasmid pLC15-48 were isolated, radiolabeled, and fractionated. When labeled in the presence of mannitol and cyclic AMP, the minicell membranes contained one dominant radiolabeled polypeptide (M_r = 60,000 on $NaDodSO_4$/polyacrylamide gels); the cytoplasmic fraction also contained a major protein of M_r 40,000 (Fig. 1). These proteins were not produced in significant amounts in minicells incubated without mannitol and cyclic AMP. Cyclic AMP alone partially induced synthesis of the two proteins, but maximal synthetic rates required the simultaneous presence of mannitol (data not shown). These results are consistent with *in vivo* observations that the mannitol operon is under dual control by cyclic AMP and mannitol. The 40,000-dalton soluble protein comigrated on electrophoresis with the major band in a partially purified preparation of Man-1-*P* dehydrogenase (Fig. 2), and the 60,000-dalton membrane-bound protein comigrated with purified mannitol-specific enzyme II (not shown). Furthermore, the membrane-bound protein, when solubilized in $NaDodSO_4$, could be immunoprecipitated by antiserum against

Table 2. Growth characteristics and levels of mannitol enzymes for various *E. coli* strains grown on mannitol minimal medium

Strain	Growth*	Enzyme II^{mtl}	Man-1-*P* DHase
KL141 (mtl^+)	++	8.3	2.7
AI214†	−	<0.01	0.47
AI214/pLC11-7	+	ND	ND
AI214/pLC15-48	++	6.8	2.8
JA200†	−	<0.01	<0.1
JA200/pLC11-7	++	5.5	3.1
JA200/pLC15-48‡	+	2.9	13.8

Enzyme levels are expressed as (μmol/30 min)/mg and (μmol/min)/mg for enzyme II^{mtl} and Man-1-*P* DHase, respectively. DHase, dehydrogenase; ND, not determined.

* Symbols: −, no growth (<1 doubling); +, good growth (but $<10^9$ cells/ml at stationary phase); ++, excellent growth ($>2 \times 10^9$ cells/ml at stationary phase).

† Medium also contained 0.5% glycerol to allow growth.

‡ When grown on glycerol minimal medium in the absence of mannitol, there was no detectable enzyme II^{mtl} activity in this strain, but considerable DHase activity was still present [4.5 (μmol/min)/mg].

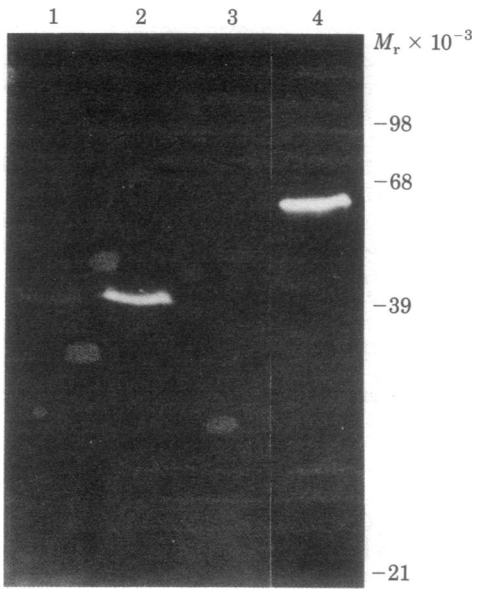

FIG. 1. Proteins encoded by pLC15-48. Minicells isolated from *E. coli* strain MV1009/pLC15-48 were labeled with [^{35}S]methionine in the presence and absence of 0.5% mannitol and 1.0 mM cyclic AMP. The cells were fractionated into cytoplasmic and membrane fractions. The fractions were subjected to $NaDodSO_4$ polyacrylamide gel electrophoresis, fixed, dried, and autoradiographed. Lanes: 1 and 2, cytoplasmic fractions from cells incubated without and with cyclic AMP and mannitol, respectively; 3 and 4, membrane fractions from cells incubated without and with cyclic AMP and mannitol. Identical results were obtained from minicells labeled with [^{3}H]leucine (data not shown).

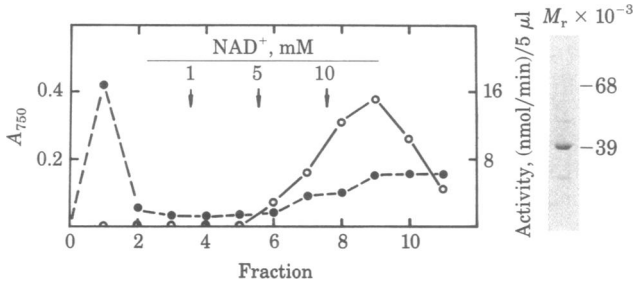

FIG. 2. Partial purification of Man-1-*P* dehydrogenase standard. Partially purified Man-1-*P* dehydrogenase was chromatographed on Bio-Rad Affi-Gel Blue resin. Most of the protein applied to the column passed through unretarded (●). Successive stepwise elutions with NAD^+ at increasing concentrations resulted in the appearance of most of the Man-1-*P* dehydrogenase activity in the eluate containing 10 mM NAD^+ (○). The $NaDodSO_4$ gel profile (*Right*) shows the polypeptide composition of pooled fractions 8–10 with a prominent band at M_r 40,000.

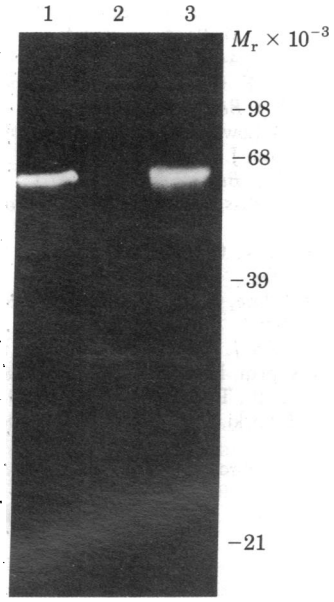

FIG. 3. Immunoprecipitation of membrane-bound protein synthesized from pLC15-48. Membranes from minicells labeled with [^{35}S]methionine in the presence of cyclic AMP and mannitol (see Fig. 1, lane 4) were immunoprecipitated. Lanes: 1, total membranes; 2, immunoprecipitate using preimmune serum; 3, immunoprecipitate using immune serum directed against purified enzyme II^{mtl}.

purified enzyme II^{mtl} (Fig. 3). When phenethyl alcohol (0.5–10 mg/ml) or procaine (1–100 mg/ml) was incubated with the minicells, no accumulation of a larger form of the radiolabeled minicell membrane-bound protein was seen on $NaDodSO_4$ gels of intact minicells (data not shown). Phenethyl alcohol (5 mg/ml) (18) and procaine (25 mg/ml) (19) have been shown to inhibit processing of *E. coli* outer membrane protein precursors to their mature membrane-bound forms.

Endonuclease Cleavage Mapping of Plasmid pLC15-48 DNA. Plasmid pLC15-48 DNA was purified and cleaved with several restriction endonucleases. The plasmid was 15-kilobase pairs, of which ≈8.7 was *E. coli* chromosomal DNA inserted into the plasmid vector ColE1. The inserted DNA contained unique cleavage sites for *Bgl* II, *Eco*RI, *Hin*dIII and *Kpn* I (Fig. 4).

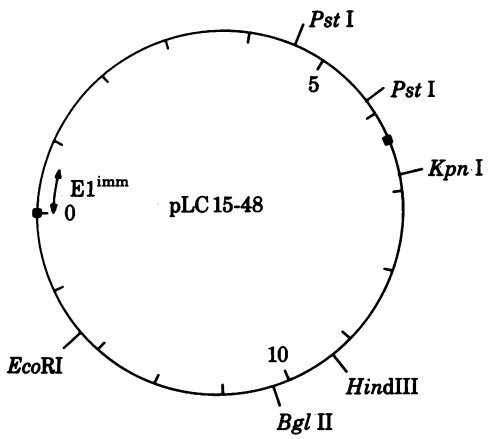

FIG. 4. Restriction enzyme cleavage map of plasmid pLC15-48. Physical map of pLC15-48 (15 kilobases) showing ColE1 vector DNA with colicin immunity gene and poly(dA·dT) "connector" regions (■) used to construct hybrid plasmid (8).

DISCUSSION

We have used the *E. coli* minicell system and a hybrid plasmid containing the mannitol operon to complement previous genetic (5, 6) and biochemical (11) studies on mannitol utilization in this organism. The uptake of mannitol is governed by the product of a single gene, *mtlA*, that directs the synthesis of the membrane-bound enzyme II^{mtl}, a protein consisting of a single kind of polypeptide chain (M_r, 60,000). No other inducible membrane protein was detected in minicells harboring plasmid pLC15-48. This, and other evidence, suggests that the mannitol enzyme II is the sole protein responsible for mannitol transport, phosphorylation, and chemotaxis in *E. coli* (11, 20, 21).

Intracellular Man-1-*P*, the product of the enzyme II^{mtl}-catalyzed reaction, is converted to fructose-6-phosphate by the product of a second gene, *mtlD*, that is soluble and consists of a single-sized subunit of M_r 40,000. No other soluble gene products inducible by cyclic AMP and mannitol were observed in minicells containing plasmid pLC15-48. Parallel experiments with pLC11-7 gave similar results, but background synthesis of other polypeptides in the absence of inducer was much higher.

It is interesting to compare the *E. coli* mannitol phosphotransferase system with those of other bacteria. In *Spirochaeta aurantia*, mannitol is the only sugar transported into intact cells via the phosphotransferase system, although sorbitol and fructose can serve as phosphorylation substrates *in vitro* (22). No evidence for a sugar-specific soluble enzyme III was obtained in this organism. In contrast, an enzyme III specific for mannitol and sorbitol has been identified in *Staphylococcus aureus* (23, 24). This soluble factor is reversibly phosphorylated in the presence of enzyme I, HPr, and *P-e*Prv, and then donates its phosphoryl group to mannitol in the presence of the membrane-bound enzyme II^{mtl} (23). The results presented in this study and elsewhere (11) suggest that mannitol transport and phosphorylation in *E. coli* is independent of a soluble enzyme III. Furthermore, preliminary kinetic analyses of the *P-e*Prv-dependent reaction catalyzed by purified *E. coli* enzyme II^{mtl} in the presence of enzyme I and HPr indicate the formation of a covalent phosphoryl–enzyme II^{mtl} intermediate (unpublished results). We suggest that enzyme II–enzyme III pairs, such as those in *S. aureus*, might have evolved from sugar-specific enzymes II, such as those in *E. coli* and *S. aurantia*, by segmentation of the enzyme II gene into two genes, one coding for a membrane-bound function and the other coding for a soluble enzyme III-like protein. If this is the case, then *E. coli* enzyme II^{mtl} might consist of two domains, a hydrophobic moiety spanning the phospholipid bilayer and a hydrophilic moiety exposed on the inner surface of the cytoplasmic membrane containing the site of phosphorylation by phospho~HPr. In this regard, it is also interesting to note that kinetic analyses of the phosphoryl transfer from the lactose-specific enzyme III in *S. aureus* to lactose, catalyzed by the enzyme II^{lac}, are consistent with a sequential rather than a ping-pong mechanism (25) as would be predicted by this evolutionary model (cf. also ref. 2).

We have used the minicell system to investigate the possibility that enzyme II^{mtl} might be synthesized as a larger precursor molecule, as is the case with a number of other membrane and periplasmic enzymes in *E. coli* (26–28). No evidence has been found for the synthesis of a larger precursor form of the enzyme II^{mtl} using the inhibitors of membrane protein processing, phenethyl alcohol and procaine.

We have observed that, although strain JA200/pLC15-48 has elevated levels of Man-1-*P* dehydrogenase activity, even when grown in the absence of mannitol, a parallel increase in enzyme II^{mtl} activity is not seen. Failure of fully induced cells to express

high levels of the activity is easily explained if there is only a limited number of insertion sites for the protein in the membrane or if an essential and minor phospholipid is limiting. However, because *uninduced* JA200/pLC15-48 cells contain undetectable levels of enzyme II^{mtl} but significant dehydrogenase activity (Table 2), an additional phenomenon must be involved. Because overproduction of dehydrogenase is presumably linked to multiple copies of the plasmid in the cell, it is possible that, under these conditions, a second promoter site becomes selectively activated (by an unknown mechanism), leading to noncoordinate synthesis of the two enzymes. Further experiments will be necessary to test these possibilities.

Finally, the minicell system will be useful to probe the structure and topography of enzyme II^{mtl} in the plasma membrane. Membranes from plasmid-bearing minicells radiolabeled in the presence of cyclic AMP and mannitol contain enzyme II^{mtl} as the predominant labeled species. Chemical cleavage of the polypeptide with membrane-impermeable reagents or enzymatic cleavage with specific proteases could thus be used to identify and orient specific sites of the protein in appropriate vesicle or spheroplast preparations. Chemical crosslinking reagents might also be used with these minicell membranes to attempt to define the subunit structure of enzyme II^{mtl} in the phospholipid bilayer.

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