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Genetic Dissection of Catalytic Activities of the *Salmonella typhimurium* Mannitol Enzyme II

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Approximately 60 mutants of *Salmonella typhimurium* were isolated which exhibited altered levels of the activities of the mannitol enzyme II. The mutants were grouped into six distinct categories based on their mannitol fermentation, transport, chemotaxis, and phosphorylation activities.

The bacterial phosphoenolpyruvate (PEP): sugar phosphotransferase system functions in the coupled transport and phosphorylation of a number of simple carbohydrates (6, 12, 14). Concomitant with translocation of the substrate across the membrane, the sugar is phosphorylated in a sequential process in which the phosphoryl moiety of PEP is transferred first to enzyme I, second to HPr, third to a sugar-specific protein (enzyme II or III), and finally to the substrate. The first two proteins in this cascade, enzyme I and HPr, are cytoplasmic and function in the phosphorylation of all of the substrates accumulated by the phosphotransferase system (3, 6, 7, 12). The enzymes II are integral membrane proteins which typically recognize only one, or at most a few, specific sugars (5, 12-14).

In addition to the vectorial, PEP-dependent phosphorylation process, the enzymes II catalyze exchange group transfer reactions in which the phosphoryl moiety of a specific sugar phosphate is transferred to the corresponding free sugar. This process occurs in the complete absence of any of the other phosphotransferase system components (15, 17, 18) and in a vectorial manner (16, 17). The enzymes II have also been shown to be required for cellular chemotactic responses (2, 10). Thus, these enzymes represent a class of multifunctional, integral membrane proteins which participate in sugar recognition, transport, and phosphorylation.

Considerable effort has been directed toward the characterization of the enzymes II (6-9, 12-17). We have focused our recent efforts on the mannitol enzyme II. This enzyme has been purified to apparent homogeneity, and the gene coding for its synthesis has been cloned (5). To further our understanding of the catalytic properties of the protein we have attempted genetic dissection of its activities. In this communication we describe the different classes of mutants obtained.

Nonradioactive sugars were purchased from Sigma Chemical Co. or Calbiochem-Behring. ^{14}C-labeled sugars were obtained from New England Nuclear Corp. The mutants were isolated from strain SB2956 (*ppc-201*) as previously described (4, 19). Briefly, cloned overnight cultures of strain SB2956 were spread on minimal salts agar plates containing citrate and mannitol, each at a final concentration of 0.2%. A crystal of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was then placed on each plate, and the plates were incubated at 37°C for 48 h. One small and one large colony arising within the zone of clearing were picked from each plate for purification and further characterization. Fermentation studies were performed by using eosin-methylene blue indicator plates without lactose (BBL Microbiology Systems) containing the carbon source at a final concentration of 0.5% (mannitol, glucose, and fructose) or 1.0% (glucitol); carbon sources were filter sterilized and added to the media after autoclaving. Bacterial strains were grown aerobically at 37°C in medium LB (11) containing 0.2% D-mannitol; the cells were harvested and washed, and transport assays were conducted as described by Willis and Furlong (20). Soft-agar chemotaxis assays were performed essentially as described by Adler (1). All of the mutants described were motile and exhibited normal chemotactic behavior in a gradient of aspartate. D-Mannitol 1-phosphate (barium salt) was converted to the sodium salt by passage over a Bio-Rad AG 50-X8 ion exchange column (sodium form). Mannitol transphosphorylation assays were conducted as follows. The reaction mixtures contained: 66 mM sodium acetate buffer, pH 5.0; 0.66 mM dithiothreitol; 20 mM EDTA (sodium salt, pH 7.0); 0.66 mM mannitol 1-phosphate (sodium salt); 0.5 μM [^{14}C]mannitol (45 μCi/μmol); and 0.15 to 0.20 mg of membrane protein in a final volume of 100 μl. The PEP-dependent phosphorylation of mannitol was conducted essentially as described previously (17). Cell-free membrane fractions were prepared by suspending the washed cells in 10 ml of 50 mM potassium phosphate buffer (pH 7.0)

containing 1 mM dithiothreitol and passing them through a French pressure cell at 12,000 lb/in^2. Intact cells were removed by low-speed centrifugation, and the membranes were collected by centrifugation at 100,000 × *g* for 90 min at 4°C. The membrane pellets were each suspended in 2 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol and stored at −70°C until used.

After isolation and purification, the mutants were screened for their ability to catalyze the various enzyme II-mediated functions listed in Table 1 and were subsequently divided into six general classes. Of these, classes 1 and 6 were the trivial classes, representing mutants that exhibited less than 10% of the parental levels of all five mannitol-specific activities assayed (class 1) and isolates that possessed intermediate levels of all of these activities (class 6). Together, these two classes represented slightly more than 50% of the mutants obtained. The mutants which comprised class 2 exhibited intermediate mannitol 1-phosphate-dependent mannitol phosphorylation activities but essentially none of the other activities tested. For example, strain LJ413 (Table 1) could neither ferment mannitol nor respond chemotactically to it. This strain possessed less than 0.5% of the parental mannitol transport activity and about 2% of the wild-type PEP-dependent phosphorylation activity. However, it exhibited greater than 50% of the parental ability to catalyze mannitol transphosphorylation. This phenotype is typical of the other mutants in class 2 (cf. Table 2).

The mutants belonging to class 3 showed reduced levels of all mannitol-specific activities except for chemotaxis. The chemotactic responses of these mutants to mannitol were equal to or greater than that of the parental strain. The mutants of class 4 were similar to those of class 3 in that they exhibited reduced mannitol fermentation, transport, and phosphorylation activities, but positive chemotactic responses of these isolates to mannitol could not be detected. It should be noted (Table 2) that mutants in classes 3 and 4 exhibited considerable variation with respect to the activities of the other enzyme II-mediated functions assayed.

The isolates which comprised class 5 exhibited parental levels or higher of all of the mannitol enzyme II activities tested with the exception of PEP-dependent phosphorylation (Tables 1 and 2). Mutant LJ432, for example, exhibited a positive fermentation response to mannitol and showed elevated levels of mannitol transport and transphosphorylation. By contrast, the PEP-dependent phosphorylation activity was approximately one-third that observed with the wild-type strain. The observation that LJ432 grew poorly on minimal salts agar containing both citrate and mannitol suggested that there may be a critical level of mannitol transport

TABLE 1. *Characteristics of a representative bacterial strain from each class of mannitol enzyme II mutants*

Class	Strain	Fermentation of mannitol[a]	Growth on citrate plus mannitol[b]	Transport of[c]:		Chemotaxis toward mannitol[d]	Mannitol phosphorylation[e]	
				Mannitol	Sorbitol		Mannitol 1-phosphate dependent	PEP dependent
1	LJ409	−	+	0.1	0.3	−	0.1	0
2	LJ413	−	+	0.03	0.3	−	2.7	0.5
3	LJ419	±	+	5.1	0.1	++	3.2	2.1
4	LJ424	−	+	2.8	0.05	−	1.7	0.8
5	LJ432	+	±	21.7	0.1	+	9.4	7.2
6	LJ446	±	+	5.2	0.1	±	3.2	2.8
Wild type	SB2956	+	−	14.4	0.1	+	5.2	21.5

[a] Determined on eosin-methylene blue agar plates containing mannitol at a final concentration of 0.5%.

[b] Determined with minimal salts agar plates containing 0.2% potassium citrate and 0.2% mannitol; none of the mutants grew when mannitol was replaced by glucose at the same final concentration.

[c] Cultures were grown in medium LB containing 0.2% D-mannitol. Transport experiments were conducted at a final substrate concentration of 10 μM; rates are in nanomoles per minute per milligram of protein.

[d] Determined by the soft agar assay (1). All of the mutants showed responses at least equivalent to that of the parental strain when L-aspartate was used as the attractant.

[e] Rates of mannitol 1-phosphate-dependent and phosphoenolpyruvate-dependent phosphorylation are in picomoles per minute per milligram of protein and nanomoles per minute per milligram of protein, respectively.

above which synthesis of the citrate permease is repressed (4, 19).

In this communication we have described our initial attempts to apply genetic techniques to the functional dissection of an enzyme II. Although enzyme II mutants have been described previously (8, 9, 13, 17, 19), this appears to be the first quantitative description of distinct classes of enzyme II mutants exhibiting partial loss of function (Table 3). Whereas the mutants in classes 1 and 6 showed severely reduced and intermediate levels, respectively, of the various mannitol enzyme II-specific activities tested, the members of class 2 lacked all such activities, with the exception of transphosphorylation. The mutants comprising class 3 showed specific enhancement of chemotactic function, whereas those comprising class 4 lacked this response. Finally, the isolates of class 5 showed reduced levels of PEP-dependent mannitol phosphorylation activity but parental levels or higher of all other activities catalyzed by this enzyme.

Any alterations in the mannitol-specific activities reported here can be attributed specifically to genetic defects in the *mtlA* gene, which codes for the mannitol enzyme II. First, a mutation affecting the activity of the soluble phosphotransferase system components would be expected to be pleiotropic (19). Second, enzyme I and HPr, used for assaying the PEP-dependent phosphorylation of mannitol, were purified from strain LJ144, which contains no lesions in the genes coding for these two proteins (3, 7, 17). Third, the mannitol 1-phosphate-dependent

TABLE 2. *Range of activities exhibited by the isolates which comprise the six mutant classes*

Class	No. of mutants	Strain no.	Fermentation of mannitol[a]	Growth on citrate plus mannitol[b]	Transport of[c]:		Chemotaxis toward mannitol[d]	Mannitol phosphorylation[e]	
					Mannitol	Sorbitol		Mannitol 1-phosphate dependent	PEP dependent
1	9	LJ401-409	−	+	0.02–0.3	0.1–0.3	−	0–0.6	0–0.6
2	6	LJ410-415	−	+	0.01–0.1	0.3	−	0.9–4	0–0.6
3	5	LJ416-420	±	+	0.8–11	0.05–0.2	+ to ++	3–4	2–7
4	6	LJ421-426	− to ±	+	1.4–6.0	0.05–0.7	−	1.0–4	0.8–7
5	7	LJ427-433	+ to ++	± to +	10–31	0.1	+ to ++	4–12	2.2–21
6	17	LJ434-452	±	± to +	0.8–6	0.03–0.4	±	0.8–7	1.1–6
Wild type		SB2956	+	−	14–18	0.1	+	5	19–29

[a] Determined on eosin-methylene blue agar plates containing mannitol at a final concentration of 0.5%; all of the isolates showed wild-type fermentation responses when mannitol was replaced by glucitol, glucose, or fructose.

[b] Determined by growth on minimal salts agar plates containing 0.2% potassium citrate and 0.2% mannitol.

[c] Cultures were grown in medium LB containing 0.2% D-mannitol. Transport experiments were conducted at a final substrate concentration of 10 μM; rates are in nanomoles per minute per milligram of protein.

[d] Determined by the soft agar assay (1).

[e] Rates of mannitol 1-phosphate-dependent and PEP-dependent phosphorylation are in picomoles per minute per milligram of protein and nanomoles per minute per milligram of protein, respectively.

TABLE 3. *Qualitative summary of the phenotypes of the six classes of mannitol enzyme II mutants*[a]

Class	Fermentation of mannitol	Transport of mannitol	Chemotaxis toward mannitol	Mannitol phosphorylation	
				Mannitol 1-phosphate dependent	PEP dependent
1	−	−	−	−	−
2	−	−	−	±	−
3	±	±	++/+	±	±
4	±/−	±	−	±	±
5	+	++/+	++/+	++/+	±
6	±	±	±	±	±
SB2956	+	+	+	+	+

[a] See footnotes of Tables 1 and 2 for assay details.

phosphorylation of mannitol has been shown to depend exclusively on the mannitol enzyme II (5, 15–18). Thus, variations in the activities of the phosphorylation reactions must reflect alterations in the enzyme II. A similar conclusion for mannitol-specific chemotaxis and transport seems justified (2, 5, 8–10, 12, 14).

Although the *mtlA* mutants discussed above can clearly be grouped into distinct classes, it seems likely that collectively they represent a continuum of the various phenotypes presented. Our results underscore the multifunctional nature of the mannitol enzyme II and suggest that distinct amino acid residues in the protein are responsible for the different catalytic activities studied. Tight coordination in the loss of mannitol chemotaxis, transport, and phosphorylation was not observed. It should be noted that further studies of additional *mtlA* mutants may reveal mutant classes not reported here. More detailed analysis of *mtlA* mutants will be the subject of future publications.

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