JOURNAL OF BACTERIOLOGY, Mar. 1984, p. 953–955
0021-9193/84/030953-03$02.00/0

Vol. 157, No. 3

Genetic Evidence for Glucitol-Specific Enzyme III, an Essential Phosphocarrier Protein of the *Salmonella typhimurium* Glucitol Phosphotransferase System

MARK V. SARNO, L. GREGORY TENN, ANJANA DESAI, A. MICHAEL CHIN, FRANK C. GRENIER, AND MILTON H. SAIER, JR.*

Department of Biology, C-016, University of California at San Diego, La Jolla, California 92093

Received 28 July 1983/Accepted 28 November 1983

Positive selection procedures were developed for the isolation of mutants defective in components of the glucitol-specific catabolic enzyme system in *Salmonella typhimurium*. *gutA* (enzyme II^{gut}-negative), *gutB* (enzyme III^{gut}-negative), and *gutC* (constitutive for the glucitol operon) mutants were isolated and characterized biochemically and genetically. The gene order was shown to be *gutCAB*.

Hexitols such as mannitol, glucitol, and galactitol are transported into enteric bacteria and phosphorylated by the phosphoenolpyruvate:sugar phosphotransferase system. This enzyme system consists of two general energy-coupling proteins, enzyme I and HPr, as well as one or two sugar-specific proteins, the enzymes II and III (2). Extensive work with the mannitol enzyme II (enzyme II^{mtl}) has shown that this protein is the only mannitol-specific protein required for the phosphorylation of this sugar (4–7). Earlier genetic evidence led to the suggestion that each of the three hexitol operons encoding the respective enzymes responsible for the catabolism of each sugar contains only a single hexitol-specific transport protein (10, 11). Recent biochemical analyses, however, have led to the conclusion that glucitol phosphorylation in vitro requires both an integral membrane glucitol enzyme II (enzyme II^{gut}) and a peripheral membrane glucitol enzyme III (enzyme III^{gut}) (F. C. Grenier, I. Hayward, M. J. Novotny, J. E. Leonard, and M. H. Saier, Jr., submitted for publication). There is, as yet, no direct in vivo evidence for the involvement of enzyme III^{gut} in glucitol utilization and transport. In this study we therefore isolated mutants lacking either enzyme II^{gut} or enzyme III^{gut}, showed that these mutants lack the abilities to utilize, transport, and phosphorylate glucitol, and provided preliminary evidence that both proteins are encoded within a single operon with the gene order *gutCABD*.

Representative strains of *Salmonella typhimurium* described in this report are listed in Table 1. The parental strain, SB1744 (*mtlA61*) (15), unable to utilize mannitol, was spread on a minimal agar plate containing 0.05% mannitol as the sole source of carbon. Spontaneous mutants arose which could utilize mannitol slowly (10). Among these mutants were some which synthesized the glucitol catabolic enzymes constitutively. One such mutant, LJ529 (*mtlA61 gutC151*) was characterized and shown to synthesize enzyme II^{gut}, enzyme III^{gut}, and glucitol-6-phosphate dehydrogenase constitutively (Grenier et al., submitted). This strain was used for the isolation of *gutA* and *gutB* mutants by positive selection.

Employing strains SB1744 and LJ529, it was found that growth of the latter strain in glycerol minimal medium was selectively inhibited by two glucitol analogs: D-arabinitol (14) and 2-deoxy-D-glucitol. Mutants resistant to D-arabinitol were isolated after plating strain LJ529 on minimal glycerol (0.2%) plus arabinitol (0.05%) plates. Mutation was induced with a crystal of nitrosoguanidine. Clones capable of growth on this medium were isolated and shown to be negative for glucitol fermentation but positive for glucose, fructose, and xylose fermentation. All such mutants showed reduced rates of [^{14}C]glucitol uptake, and in vitro phosphorylation assays conducted with purified protein constituents of the phosphotransferase system revealed that 5 to 10% of these mutants were defective for enzyme III^{gut} function, whereas the remaining mutants showed low enzyme II^{gut} activity. None of the mutants isolated lacked both activities.

Two representative strains, LJ530 and LJ531, were totally incapable of glucitol utilization, took up [^{14}C]glucitol at less than 2% of the rate exhibited by the parental strain, and showed low enzyme III^{gut} and enzyme II^{gut} activities, respectively, in in vitro phosphorylation assays (Table 1).

Careful examination of the fermentation properties of the strains revealed that although both LJ530 and LJ531 were negative for glucitol fermentation (Table 1) an interesting difference occurred with respect to mannitol fermentation. Although strains SB1744 and LJ531 could not ferment mannitol, strain LJ530 showed a positive response. This response was less than that exhibited by strain LJ529, but greatly in excess of that shown by either SB1744 or LJ531. This observation suggests that a cooperative interaction between the mutant enzyme II^{mtl} and the wild-type enzyme II^{gut} allows mannitol transport and phosphorylation and that this interaction does not depend on a functional enzyme III^{gut}. Although the molecular basis for this interaction is not understood, it provides a simple model for distinguishing *gutB* mutants from *gutA* mutants.

Transductional analyses of the strains with phage P22 (3) were carried out as follows. Strain SB1744 served as the donor, whereas either LJ530 or LJ531 served as the recipient. Selection was on minimal glucitol (0.2%) plates. Control plates revealed that rates of reversion were insignificant. Eighteen transductants of each cross were cloned and characterized. All fermented glucitol as did strain SB1744. All but two of the transductants grew on glycerol plus D-arabinitol minimal plates but could not ferment mannitol efficiently. Two transductants from the SB1744 × LJ530 cross showed weak mannitol fermentation and did not grow on glycerol plus arabinitol medium. These two strains were shown to synthesize the glucitol enzymes constitutively as

* Corresponding author.

TABLE 1. Characteristics of representative *gutA* and *gutB* mutants of *S. typhimurium*

Strain	Genotype[a]	Glucitol fermentation[b]	Generation time (h)[c]	Uptake of [^{14}C]glucitol after growth in medium containing:[d]		Phosphorylation of [^{14}C]glucitol with limiting amounts of:[e]	
				Glycerol	Glycerol + glucitol	Enzyme III^{gut}	Enzyme II^{gut}
SB1744	*mtlA61*	+	1.2	0.04	0.5		
LJ529	*mtlA61 gutC151*	+	1.1	6.2	5.2	25	10
LJ530	*mtlA61 gutC151 gutB152*	−	>20	0.03	0.03	1	12
LJ531	*mtlA61 gutC151 gutA153*	−	>20	0.02	0.05	28	0.7

[a] The *gut* designation is preferred to the *srl* designation used in reference 1 as discussed (2, 10, 12).

[b] Fermentation responses (15) were recorded after 24 h at 37°C on EMB agar plates containing 1% glucitol. Fermentation responses for other sugars did not differ for the four strains listed.

[c] Growth was conducted at 37°C employing medium 63 supplemented with 0.5% glucitol as described previously (15). All strains grew at the wild-type rate in medium containing 0.5% fructose.

[d] Cells were grown for 4 h in medium 63 supplemented with 0.5% glycerol with or without 0.5% glucitol, harvested during exponential growth, washed three times with medium 63, and suspended to a cell density between 0.04 and 0.16 mg (dry weight) per ml. Uptake of [^{14}C]glucitol (10 μM; 5 μCi/μmol) at 37°C was linear with time and cell density for all strains (15). Values represent micromoles of [^{14}C]glucitol taken up per minute per milligram of dry weight.

[e] Cells were grown in Luria broth (13), harvested during early stationary phase, washed three times with medium 63, and suspended in medium 63 containing 1 mM dithiothreitol. The cells were ruptured by passage through a French pressure cell (10,000 lb/in^2), and cell debris was removed by centrifugation. Extracts were assayed for enzyme II^{gut} and enzyme III^{gut} activities. Saturating amounts of partially purified enzyme I, HPr, and enzyme II^{gut} were employed for enzyme III^{gut} assays, and the assay mixture included 0.5% Lubrol. Enzyme II^{gut} was assayed by using saturating amounts of a high-speed supernatant from strain LJ409 (*mtlA309*) (11) after growth in glucitol-containing medium as a source of enzyme I, HPr, and enzyme III^{gut}. Phosphoenolpyruvate-dependent phosphorylation assays were conducted as described previously (5) with [^{14}C]glucitol at 10 μM. Values represent micromoles of sugar phosphorylated per minute per milligram of protein.

did strain LJ529, whereas representatives of the remaining transductants showed inducible enzyme synthesis as is characteristic of strain SB1744. These results led to the suggestion that the gene order of the three *gut* genes analyzed here is *gutCAB*.

Previous studies have shown that the gene order of the three known genes of the *mtl*, *gut*, and *gat* operons, encoding the mannitol, glucitol, and galactitol catabolic enzyme systems, respectively, is CAD (10). No evidence for an additional gene has been obtained. Subsequent work with purified enzymes of the mannitol phosphotransferase system (4, 5) and with the cloned *mtl* operon (6, 8) confirmed that this operon encodes only two proteins, the membrane-associated enzyme II^{mtl} and the soluble mannitol-1-phosphate dehydrogenase. Nevertheless, the present work showed that the glucitol operon contains an additional structural gene, the *gutB* gene, which encodes enzyme III^{gut}. Based on the transductional analyses, the presumed gene order is CABD.

Enzyme II^{mtl} has a molecular weight by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of 60,000, whereas those for enzyme II^{gut} and enzyme III^{gut} are 45,000 and 16,000, respectively, by the same criterion (4, 5; F. C. Grenier et al., submitted; F. Grenier, unpublished data). Based on this observation it is reasonable to suggest that enzyme II^{mtl} performs the essential functions of the enzyme II^{gut}-enzyme III^{gut} pair and that these two operons differ with respect to a chain termination codon within the first structural gene. Relevant to this suggestion is the fact that the C-terminal half of enzyme II^{mtl} exhibits the hydropathic properties of a soluble protein, whereas the N-terminal half is embedded within the membrane (7). These observations suggest that the mannitol- and glucitol-specific phosphotransferase proteins may have arisen evolutionarily from a common precursor and that studies of the glucitol enzyme II-enzyme III pair will be relevant to the mechanism of enzyme II^{mtl} action. It is not known whether the number of hexitol-specific phosphotransferase proteins (one or two) is determined translationally or posttranslationally, but we favor the former possibility. Further biochemical and genetic studies, including sequence analysis of the *gut* operon, will be aimed at testing these hypotheses and establishing the transport mechanism catalyzed by the enzymes II of the phosphotransferase system.

LITERATURE CITED

1. **Bachmann, B. J.** 1983. Linkage map of *Escherichia coli* K-12, edition 7. Microbiol. Rev. **47**:180–230.
2. **Dills, S. S., A. Apperson, M. R. Schmidt, and M. H. Saier, Jr.** 1980. Carbohydrate transport in bacteria. Microbiol. Rev. **44**:385–418.
3. **Ely, B., R. M. Weppelman, H. C. Massey, Jr., and P. E. Hartman.** 1974. Some improved methods in P22 transduction. Genetics **76**:625–631.
4. **Jacobson, G. R., C. A. Lee, J. E. Leonard, and M. H. Saier, Jr.** 1983. The mannitol-specific enzyme II of the bacterial phosphotransferase system. I. Properties of the purified permease. J. Biol. Chem. **258**:10748–10756.
5. **Jacobson, G. R., C. A. Lee, and M. H. Saier, Jr.** 1979. Purification of the mannitol-specific enzyme II of the *Escherichia coli* phosphoenolpyruvate:sugar phosphotransferase system. J. Biol. Chem. **254**:249–252.
6. **Lee, C. A., G. R. Jacobson, and M. H. Saier, Jr.** 1981. Plasmid-directed synthesis of enzymes required for D-mannitol transport and utilization in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **78**:7336–7340.
7. **Lee, C. A., and M. H. Saier, Jr.** 1983. Mannitol-specific enzyme II of the bacterial phosphotransferase system. III. The nucleotide sequence of the permease gene. J. Biol. Chem. **258**:10761–10767.
8. **Lee, C. A., and M. H. Saier, Jr.** 1983. Use of cloned *mtl* genes of *Escherichia coli* to introduce *mtl* deletion mutations into the chromosome. J. Bacteriol. **153**:685–692.
9. **Lengler, J.** 1975. Nature and properties of hexitol transport systems in *Escherichia coli*. J. Bacteriol. **124**:39–47.
10. **Lengler, J.** 1975. Mutations affecting transport of the hexitols D-mannitol, D-glucitol, and D-galactitol in *Escherichia coli* K-12: isolation and mapping. J. Bacteriol. **124**:26–38.
11. **Leonard, J. E., and M. H. Saier, Jr.** 1981. Genetic dissection of

catalytic activities of the *Salmonella typhimurium* mannitol enzyme II. J. Bacteriol. **145**:1106–1109.
12. **Lin, E. C. C.** 1970. The genetics of bacterial transport systems. Annu. Rev. Genet. **4**:225–262.
13. **Miller, J. H.** 1977. Experiments in molecular genetics, appendix I, p. 431. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Reiner, A. M.** 1977. Xylitol and D-arabitol toxicities due to derepressed fructose, galactitol, and sorbitol phosphotransferases of *Escherichia coli*. J. Bacteriol. **132**;166–173.
15. **Saier, M. H., Jr., R. D. Simoni, and S. Roseman.** 1976. Sugar transport: properties of mutant bacteria defective in proteins of the phosphoenolpyruvate:sugar phosphotransferase system. J. Biol. Chem. **251**:6584–6597.