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D-Mannitol Utilization in *Salmonella typhimurium*

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A biochemical and genetic analysis of D-mannitol metabolism in *Salmonella typhimurium* indicates that D-mannitol is phosphorylated by the phosphoenolpyruvate-dependent phosphotransferase system. D-Mannitol-1-phosphate is converted to D-fructose-6-phosphate by mannitol-1-phosphate dehydrogenase. Two classes of mannitol mutants are described. Both map at about 115 min on the *Salmonella* chromosome. Mutants missing mannitol-1-phosphate dehydrogenase activity are mannitol sensitive; i.e., either growth is inhibited or the cells are lysed in the presence of mannitol. In a strain missing adenyl cyclase activity, the mannitol genes require exogenous cyclic adenosine-3',5'-monophosphate for expression.

Studies of mannitol utilization pathways have led to some key experiments in bacterial genetics and physiology. Transformation of the pneumococcal gene coding for mannitol phosphate dehydrogenase was the first demonstration that a gene coding for a known enzymatic activity could be transformed (20). In the same paper Marmur and Hotchkiss showed that mannitol was oxidized as the polyol phosphate rather than as the free sugar. Wolff and Kaplan (34, 35) showed that it is also the polyol phosphate which is oxidized in *Escherichia coli*, although enzymes from a number of other bacterial species are capable of oxidizing the nonphosphorylated sugar (29). Neither Marmur and Hotchkiss nor Wolff and Kaplan were able to demonstrate a mannitol kinase. The problem of the "missing kinase" led to an interest in pleiotropic carbohydrate negative mutants (28). Shortly after the discovery of the phosphoenolpyruvate (PEP)-dependent phosphotransferase system by Kundig et al. (16), Tanaka and Lin (30) showed that this system served in place of the mannitol kinase. For strains using the phosphorylated route, the mannitol pathway can now be written:

$$\text{HPr} + \text{PEP} \xrightarrow{\text{enzyme I}} \text{HPr-P} + \text{pyruvate}$$

$$\text{HPr-P} + \text{mannitol} \xrightarrow{\text{enzyme II}} \text{mannitol-P} + \text{HPr}$$

$$\text{mannitol phosphate} + \text{NAD}^+ \xrightarrow[\text{dehydrogenase}]{\text{mannitol phosphate}} \text{NADH} + \text{fructose phosphate}$$

where HPr (a histidine-containing protein) and enzyme I are general components of the Roseman phosphotransferase system. Enzyme II is the mannitol-specific phosphotransferase component.

This paper shows that in *Salmonella typhimurium* mannitol is metabolized by the above pathway. Some features of the genetics and regulation of the pathway are also presented.

MATERIALS AND METHODS

Media. The minimal medium E of Vogel and Bonner (32) was used with 0.6 mM adenine, proline, and isoleucine, as required. Carbon sources were added at the concentrations indicated. For measuring growth rates on single carbon sources, modified medium E without citrate was used (4). For solidification, 1.5% agar (Difco) was added. Fermentation indicator plates were made by the addition of 1% sugar to MacConkey Agar Base (Difco). The complete medium used was nutrient broth containing 0.5% NaCl.

Strains. All strains were derived from SL751, which is of the genotype *purC7 purI590 proA46 ilvA405 rha-461 flaA56 strA* (J. Gots, *personal communication*). This strain was obtained from B. Ames. For the isolation of mutants, cultures for mutagenesis were started from less than 100 cells to insure that each mutant would be an independent isolate. Only one mutant was derived from each culture. Nitrosoguanidine (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) was used as the mutagen (1).

For the isolation of mannitol-sensitive mutants (class I), a mutagenized culture of SL751 was plated on nutrient agar. Colonies were printed onto nutrient agar and onto nutrient agar containing 0.1 mg of mannitol per ml. The sensitive mutants grew on nutrient agar, but not on nutrient agar containing mannitol. Mannitol-sensitive mutants occurred at a frequency of about 1/1,000 among the survivors. Several attempts to isolate these mutants by penicillin selection were unsuccessful. The original selection was intended to be a simultaneous selection for actinomycin-sensitive and sucrose-dependent mutants. This was done on plates containing 0.1 mg of mannitol per ml, 25 μg of actinomycin per ml, and 20% sucrose. Some sucrose-dependent mutants were obtained, but all the sensitive mutants were sensitive to mannitol rather than actinomycin. The mannitol was a diluent in the Merck, Sharpe and Dohme actinomycin.

Mannitol-negative mutants (class II) were obtained by plating mutagenized cultures of SL751 on MacConkey mannitol plates. White colonies were picked. Sorbitol-negative mutants were isolated in the same way by using sorbitol MacConkey plates. DB310, 316, and 318 carrying enzyme I mutations were all isolated as sorbitol negatives. DB99 and DB92 were isolated as mannitol negatives.

Hfr strains SA540, SA535, SA464, and SA536 were obtained from K. Sanderson. SA535, 464, and 536 all carry *serA13*. SA540 carries *purE8*. The origins and directions of transfer of these strains are shown in Fig. 1. Strains SB761 and SB762 were kindly provided by Mark Levinthal.

Mapping. Cultures of donors and recipients in mid-log phase were mixed in a ratio of 1:4. Five milliliters of the mixtures were filtered onto membrane filters (0.45-μm pore size; Millipore Corp.). The filters were transferred, cells up, to prewarmed nutrient agar plates and incubated for 2 hr. The filter pads were then removed, and the cells were suspended in minimal medium by agitation in a Vortex mixer. Appropriate portions were plated selecting for Pur$^+$, Ile$^+$, or Pro$^+$ recombinants. The distribution of the carbohydrate mutations relative to each of the auxotrophic markers was determined by printing recombinants onto fermentation indicator plates. This is similar to the method used by Anton (2).

Induction. Unless otherwise indicated in the text, strains were induced by the addition of 10^{-2} M mannitol to nutrient broth cultures 1 hr before harvesting. This allows comparisons to be made between mannitol-sensitive strains and other strains.

Mannitol uptake. Log-phase cultures of each strain were centrifuged, washed with medium E, and suspended in one-fifth of the original volume of medium E. At zero time a portion of this cell suspension was added to medium E containing mannitol-*1*-^{14}C. The final volume was 1 ml and the mannitol concentration 10^{-4} M. Uptake was stopped by filtering the contents of each assay tube onto a membrane filter (type HA; Millipore Corp.). The tube and filter were then washed twice with 2.5 ml of medium E containing 0.1% mannitol. Uptake was measured at room temperature for 3 min. Rates were linear up to 6 or 8 min and were proportional to the amounts of cells used. The rates of uptake varied considerably from day to day. Average values and standard deviations for experiments conducted over several months were: SL751 induced, 62.5 ± 26.3 (8); SL751 noninduced, 20.2 ± 12.1 (8); DB90 induced, 2.83 ± 2.72 (6). The results are expressed in nanomoles per minute per milligram of protein.

Enzyme assays. For enzyme assays, cells were harvested in log phase. Cells were washed once with 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 7.5) and suspended in one-fifth of the original culture volume of Tris buffer. The cells were disrupted by sonic oscillation for three 10-sec intervals with 15 sec of rest between each interval to maintain the temperature close to 0 C. A Branson Sonifier was used. The suspensions were centrifuged at 3,000 × *g* for 10 min to remove whole cells and debris. The supernatant solutions were used as sources of enzyme. For the phosphotransferase assay, the cell suspensions were made 10 times more concentrated and were centrifuged at 12,000 × *g* for 10 min after sonic oscillation.

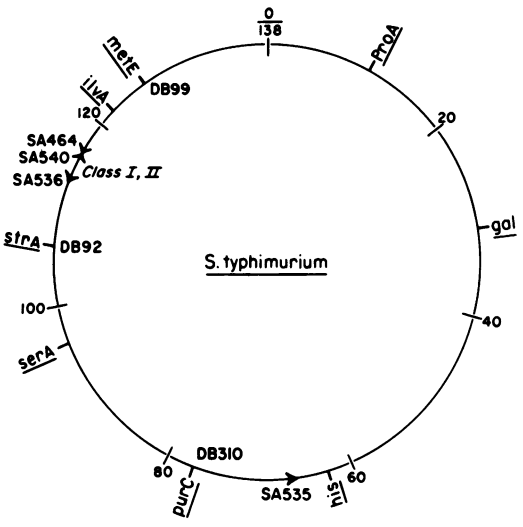

FIG. 1. *Salmonella typhimurium chromosome. Modified from Sanderson (25).*

Mannitol phosphotransferase was measured as described by Tanaka et al. (29). The following changes were made. The ^{14}C-mannitol concentration was 2.5 × 10^{-4} M instead of 5 × 10^{-5} M. The reaction was stopped by the addition of 0.1 ml of nonradioactive 1 M mannitol. Portions (0.05 ml) of each reaction mixture were placed on 2.4-cm diethylaminoethyl filter-paper discs (Reeve-Angel, DE81). The discs were rinsed twice with distilled water, dried, and counted in a scintillation counter (22). Purified enzyme I and HPr fractions were not added; thus, the rates were determined by the rate-limiting step in each extract. With induced extracts of SL751 (parental strain), after a 2-min lag, the rates were linear with time for at least 30 min or until 60% (60 nmoles) of the mannitol was consumed. The rates increased exponentially with increasing protein concentration. Data are presented as nanomoles of mannitol-1-phosphate formed in 30 min. Since protein concentrations for each extract are also given, the reader can readily make comparisons. Extracts prepared from frozen cells had rates within 20% of those prepared from fresh cells.

Mannitol-1-phosphate dehydrogenase was assayed by following nicotinamide adenine dinucleotide (NAD) reduction with mannitol-1-phosphate as substrate or by measuring the rate of reduced nicotinamide adenine dinucleotide (NADH) oxidation with fructose-6-phosphate as substrate. The rates were measured at room temperature (25 C) by following pyridine nucleotide changes at 340 nm. With fructose-6-phosphate as substrate, each cuvette contained 10^{-2} M disodium fructose-6-phosphate, 10^{-4} M NADH, 7 × 10^{-3} M Tris buffer (*p*H 7.5), and enzyme and water to make 1 ml or 0.5 ml (35). With mannitol-1-phosphate as substrate each cuvette contained 2 × 10^{-3} M mannitol-1-phosphate, 1.5 × 10^{-3} M NAD, 7 × 10^{-2} M Tris buffer (*p*H 8.9), and enzyme and water to make 0.5 ml (13). Specific activi-

ties are expressed as international units (IU) per milligram of protein. One IU of dehydrogenase is that amount which converts 1 micromole of substrate to product in 1 min.

Sorbitol-6-phosphate dehydrogenase was measured in the same way as mannitol-1-phosphate dehydrogenase using sorbitol-6-phosphate as the substrate. The final concentration of sorbitol-6-phosphate was 10^{-2} M.

Preparation of mannitol phosphate and sorbitol phosphate. Mannitol-1-phosphate and sorbitol-6-phosphate were prepared by the borohydride reduction of mannose-6-phosphate and glucose-6-phosphate, respectively (35). Reducing equivalents were measured by the Park-Johnson method with glucose as a standard (23). Formaldehyde produced by periodate oxidation was measured by the method of Burton (5). DL-α-Glycerophosphate was used as a standard. Phosphate was determined by the method of Bartlett (3). The polyol phosphates prepared had no reducing equivalents, and the ratios of formaldehyde to phosphate were 1.16 for sorbitol-6-phosphate and 1.37 for mannitol-1-phosphate. The theoretical ratio is 1:1. The higher ratios may be a result of hydrolysis of the phosphate or migration to one of the central hydroxyl groups. The concentrations of these substrates used in the assays are based on the phosphate determinations.

Chemicals. Nitrosoguanidine was obtained from the Aldrich Chemical Co. (Milwaukee, Wis.). Lactose was purchased from Difco Laboratories. All other sugars and polyols were obtained from Mann Research Laboratories (New York), with the exception of D-xylose and D-galactose which were purchased from Pfanstiehl Laboratories (Waukegan, Ill.). Mannitol and sorbitol were each recrystallized as described by Horwitz and Kaplan (13). Experiments in which recrystallized polyols were used are so designated. Mannitol-*1*-^{14}C and ^{14}C-α-methylglucoside were obtained from Amersham/Searle (Des Plaines, Ill.). Fructose-6-phosphate and mannose-6-phosphate were obtained from Sigma Chemical Co. (St. Louis, Mo.). All sugars, sugar phosphates, and polyols used were of the D-configuration unless otherwise specified.

Proteins were determined by the method of Lowry et al. (19), by using bovine albumin as a standard.

RESULTS

In *S. typhimurium* it is likely that mannitol is metabolized by conversion to mannitol-1-phosphate followed by oxidation of the phosphorylated polyol to fructose-6-phosphate. Some evidence that mannitol is metabolized via mannitol-1-phosphate dehydrogenase is presented in Table 1. When the parental strain (SL751) was induced with 10^{-2} M mannitol, there was an 18-fold increase in both a mannitol-1-phosphate-dependent reduction of NAD, and an 18-fold increase in a fructose-6-phosphate-dependent NADH oxidation. In the fructose-6-phosphate assay, no fructose-dependent NADH oxidation was observed with 5×10^{-2} M fructose, nor was there a mannose-6-phosphate-dependent oxidation of NADH in the presence of 10^{-2} M mannose-6-phosphate. A glucose-6-phosphate-dependent NADH oxidation was seen only after a lag and, even at a concentration of 10^{-2} M glucose-6-phosphate, it was not as rapid as the oxidation seen with 2×10^{-4} M fructose-6-phosphate as the substrate. This glucose-6-phosphate-dependent NADH oxidation was probably a result of the conversion of glucose-6-phosphate to fructose-6-phosphate by hexose phosphate isomerase in the extract. In the mannitol-1-phosphate assay, no NAD oxidation was observed when 5×10^{-2} M mannitol or 5×10^{-2} M arabitol was used as a substrate. This substrate specificity, the coordination of induction, and the fact that both the mannitol-1-phosphate-dependent NAD reduction activity and the fructose-6-phosphate-dependent NADH oxidation activity are missing in the same mutants (*unpublished data*) is evidence that in *S. typhimurium* mannitol is oxidized as the phosphorylated polyol by mannitol-1-phosphate dehydrogenase.

Some weaker evidence suggests that sorbitol is converted to sorbitol-6-phosphate and is then oxidized to fructose-6-phosphate by sorbitol-6-phosphate dehydrogenase. The sorbitol-6-phosphate dehydrogenase appears to be distinct from the mannitol-1-phosphate dehydrogenase since it is induced by sorbitol, whereas the mannitol-1-phosphate dehydrogenase is not induced by sorbitol (Table 1). Phosphorylated sugars are required for the reaction in both directions. Sorbitol-6-phosphate could not be replaced by 5×10^{-2} M sorbitol nor could 5×10^{-2} M fructose replace fructose-6-phosphate as the substrate. The rates obtained with sorbitol-6-phosphate as substrate are not reliable since the sorbitol-6-phosphate may contain some mannitol-1-phosphate. Evidence for this is presented in Table 2. Sorbitol-6-phosphate was added to extracts prepared from either mannitol- or sorbitol-induced cultures. When added to the extracts from mannitol-induced cultures, the rate of NADH formation was almost as great as the rate seen when sorbitol-6-phosphate was added to extracts from sorbitol-induced cultures. After an absorbance change of 0.130, the rate of NADH production in the extract from mannitol-induced cells was near zero, whereas in the extract prepared from sorbitol-induced cells, NADH was still being formed at 75% of the initial rate. Contamination of the sorbitol-6-phosphate by mannitol-1-phosphate is a reasonable interpretation, since the sorbitol-6-phosphate was prepared by alkaline borohydride reduction, and under these conditions glucose-6-phosphate epimerizes (35). The mannitol-1-phosphate may be contaminated with sorbitol-6-phosphate, but in this case the contamination is less critical because the oxidation

TABLE 1. *Assay of mannitol-1-phosphate and sorbitol-6-phosphate dehydrogenase activity in extracts from noninduced, mannitol-induced, and sorbitol-induced cultures*[a]

Additions during growth	Fructose-6-phosphate assay	Mannitol-1-phosphate assay	Sorbitol-6-phosphate assay
None	0.06	0.12	0.02
Mannitol, 10^{-2} M	1.11	2.11	0.25
Sorbitol, 10^{-2} M	2.65	0.28	0.27

[a] Recrystallized sugars were used for induction. The data are expressed in international units per milligram of protein.

TABLE 2. *Mannitol-1-phosphate contamination of sorbitol-6-phosphate*[a]

Addition during growth	Initial rate	Rate after absorbancy change of 0.130
Mannitol	0.039	0.002
Sorbitol	0.043	0.033

[a] Assays were done with sorbitol-6-phosphate as the substrate. With the extract from mannitol-induced cells, an absorbancy change of 0.130 required 16 min, from sorbitol-induced cells 3 min. Rates are expressed as absorbancy change per minute. The reaction mixtures contained 11.7 (mannitol) and 12.9 (sorbitol) μg of protein. The volume was 0.5 ml. Recrystallized sugars were used for induction.

of mannitol-1-phosphate is much faster than the rate of sorbitol-6-phosphate oxidation. In the experiment shown in Table 1, the oxidation of sorbitol-6-phosphate by mannitol-induced extracts was probably a result of the oxidation of the mannitol-1-phosphate contaminant, whereas in the sorbitol-induced extract most of the rate probably represents the oxidation of sorbitol-6-phosphate. The oxidation of mannitol-1-phosphate by the sorbitol-induced extract may be due to the oxidation of contaminating sorbitol-6-phosphate. Although it seems likely that sorbitol-6-phosphate is converted to fructose-6-phosphate by a sorbitol-6-phosphate dehydrogenase, other possibilities, such as epimerization to mannitol-1-phosphate and subsequent oxidation by the mannitol-1-phosphate dehydrogenase, cannot be excluded.

Mannitol-1-phosphate and sorbitol-6-phosphate are also converted to fructose-6-phosphate by dehydrogenases induced by their respective polyols, in *E. coli* (34, 35) and in *A. aerogenes* (18). In both of these cases, the mannitol-1-phosphate oxidation activity is much more active than the sorbitol-6-phosphate oxidation activity.

Mannitol-specific mutants. The class I mutants (mannitol-sensitive) are all missing mannitol-1-phosphate dehydrogenase activity (Table 3). The class I mutants also have a decreased ability to take up mannitol. This pleiotropic effect will be discussed in a later section. The class I mutants were all isolated as mannitol-sensitive mutants. These mutants are not only unable to use mannitol as a carbon source, but are inhibited by mannitol concentrations as low as 10^{-6} M. One of the mutants, DB82, lyses rapidly after the addition of mannitol. The addition of mannitol to nutrient broth cultures of other mutants of this class resulted in inhibition or slow lysis. This is shown in Fig. 2. In the absence of mannitol phosphate dehydrogenase, mannitol-1-phosphate probably accumulates, and it is presumably this substance or some derivative which causes the toxicity. This would be similar to inhibition by a number of other sugars under conditions in which

TABLE 3. *Mannitol-1-phosphate dehydrogenase and mannitol uptake in class I and class II mutants*[a]

Strain	Specific activity	
	Dehydrogenase	Mannitol uptake
Parental		
SL751	2.24	74
Class I		
DB76	0	6.23
DB77	0	8.59
DB78	0.01	0.68
DB79	0	0.10
DB80	0	7.70
DB81	0.03	1.29
DB82	0	5.19
DB321	0	0
Class II		
DB89	0.07	1.29
DB90	0.08	3.03
DB91	0.17	2.81
DB93	0.26	5.55
DB95	2.32	6.74
DB96	3.37	11.4
DB97	0.05	14.1
DB303	0.13	3.16
DB305	0.27	3.92
DB306	0.07	0.97
DB320	0.13	4.83
STL$^-$		
DB307	2.87	62.1
DB309	0.87	44.7
DB311	2.63	39.1
DB312	2.24	68.8
DB315	2.71	63.2

[a] Nutrient broth cultures were induced by the addition of 10^{-2} M mannitol 1 hr before harvesting. Mannitol-1-phosphate was used as the dehydrogenase substrate. Dehydrogenase activity is expressed in units per milligram of protein, uptake rates in nanomoles per minute per milligram of protein.

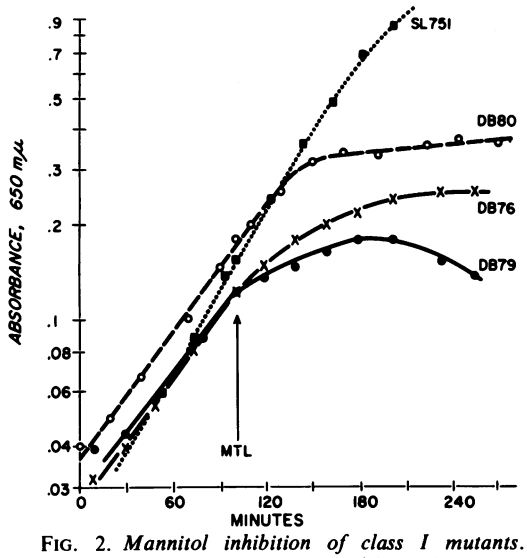

FIG. 2. *Mannitol inhibition of class I mutants. Mannitol at a final concentration of 2 × 10^{-4} M was added to nutrient broth cultures where indicated by the arrow. An absorbancy of 0.100 corresponded to a cell density of approximately 10^8 per ml.*

sugar phosphates accumulate (6, 8–11, 36). Some characteristics of the sensitivity will be published separately.

The class I mutants are able to ferment maltose, glucose galactose, xylose, mannose, and fructose. DB82, the most sensitive mutant, has normal doubling times on all of these sugars. The response of class I mutants to sorbitol is not clear. The mutants are sensitive to sorbitol, but 5 × 10^{-3} to 10^{-2} M sorbitol is about as effective an inhibitor as 10^{-5} M mannitol. The sorbitol sensitivity could be due to the sorbitol itself or be a result of contamination by as little as 0.1% mannitol in the recrystallized sorbitol.

Mutants of class II (mannitol negative) have low levels of mannitol phosphotransferase (Table 4) and of mannitol uptake activity (Table 3). Table 3 shows that class II mutants transport mannitol at about 1 to 10% of the rate of the parental strain. DB95 is leaky and has from 20 to 45% of the parental phosphotransferase activity. Class II mutants have parental growth rates on maltose, galactose, xylose, mannose, glucose, and fructose as carbon sources, but prolonged doubling times with mannitol as the carbon source (Table 5). The class II mutants also have prolonged doubling times when grown on sorbitol as a carbon source, but the sorbitol-negative mutants examined grew with the parental doubling time on mannitol as a carbon source. In the mannitol uptake assay, sorbitol, at concentrations of 10^{-4} or 10^{-3} M, did not compete with mannitol for entry via the mannitol uptake system. Higher concentrations of sorbitol were not tested. Several sorbitol-negative mutants took up mannitol at nearly normal rates (Table 3).

The class II mutants are somewhat "leaky" on mannitol. As shown in Table 6, at 2.8 × 10^{-2} M mannitol, DB90 has a doubling time close to that of the parental strain. Some of this leakiness can

TABLE 4. *Mannitol phosphotransferase activity of class II mutants*[a]

Strain	Amt of product[b]
SL751 (Parental)	78
DB90	1.7
DB91	0.2
DB96	1.8
DB97	10.9

[a] Extracts were prepared from mannitol induced frozen cells. Each assay tube contained 0.2 ml of extract. The extract protein concentrations were (per ml): SL751, 4.03 mg; DB90, 5.00 mg; DB91, 5.56 mg; DB96, 4.29 mg; DB97, 4.94 mg.

[b] Nanomoles of product formed in 30 min.

TABLE 5. *Doubling times of mannitol (MTL)-negative and sorbitol (STL)-negative mutants on glucose, mannitol, and sorbitol*[a]

Strain	Carbon source		
	0.5% glucose	0.1% MTL	0.1% STL
Parental			
SL751	58 ± 5(5)	65 ± 6(6)	75 ± 7(4)
Class II (MTL⁻)			
DB89	62	232	196
DB90	68	>300	138
DB91	73	275	120
DB95	60	>300	154
STL⁻			
DB307	72	61	>300
DB311	51	68	224
DB312	61	65	>300

[a] Standard deviations and the number of experiments averaged are shown for SL751. The sugar concentrations were: glucose, 2.7 × 10^{-2} M; mannitol, 5.6 × 10^{-3} M; and sorbitol, 5.6 × 10^{-3} M. These concentrations correspond to 0.5% for glucose and 0.1% for mannitol and sorbitol.

TABLE 6. *Inhibition of mannitol utilization by α-methylglucoside*[a]

Carbon source	Doubling time	
	Parental (SL751)	Class II (DB90)
0.028 M MTL	61	83
0.028 M MTL + 0.026 M α-Meglu	65	228
0.020 M MTL	65	108
0.02 M MTL + 0.01 M α-Meglu	64	>600
0.01 M MTL	65	125
0.01 M MTL + 0.01 M α-Meglu	66	>600
0.005 M MTL	65	428
0.005 M MTL + 0.01 M α-Meglu	64	>600

[a] Mannitol (MTL) or α-methylglucoside (α-Meglu) were added at the concentrations indicated.

be explained as mannitol uptake by the glucose permease. Mutants missing components of the PEP-dependent phosphotransferase system are less leaky with respect to mannitol uptake than the class II mutants. Thus, the alternate route for mannitol entry may be one requiring the PEP-dependent phosphotransferase system. Nonradioactive glucose and α-methylglucoside inhibited the uptake of ^{14}C-mannitol. In induced cultures, 2×10^{-3} M α-methylglucoside inhibited mannitol uptake by 50%. Further evidence for α-methylglucoside inhibition is shown in Table 6. The parental strain, SL751, had a 65-min doubling time on all concentrations of mannitol over a 10-fold range. α-Methylglucoside, a nonmetabolizable glucose analogue, had no effect on the growth of SL751 on mannitol as the sole carbon source. The growth of DB90, a mutant missing the mannitol-specific transport system, was highly dependent upon the mannitol concentration, and the doubling times were greatly extended by the presence of α-methylglucoside. This might be interpreted to mean that in the absence of the mannitol-specific uptake system, at high concentrations, mannitol can enter the cell via the "glucose permease" or some other permease on which α-methylglucoside competes. The α-methylglucoside would then prevent or slow the entry of mannitol. Since the α-methylglucoside competition is not seen in the parental strain, the mannitol-specific uptake system must function fast enough to support a 65-min doubling time even in the presence of α-methylglucoside.

Pleiotropic carbohydrate-negative mutants. Several different types of pleiotropic carbohydrate-negative strains have been isolated on the basis of either mannitol- or sorbitol-negative phenotypes. The phenotypes of these strains relative to seven other sugars are given in Table 7.

DB310, DB316, and DB318 have the phenotypes of *Salmonella* mutants missing general components of the Roseman PEP-dependent phosphotransferase system (26). The data in Table 8 indicate that the defect in DB310 is in enzyme I. SB761 is a *Salmonella* mutant missing enzyme I, and SB762 is missing HPr activity (17). DB310 complements SB762, but not SB761. DB310 is missing the same component of the phosphotransferase as is missing in SB761, enzyme I. DB90 complemented both SB761 and SB762. DB310, DB316, and DB318 are all missing enzyme I activity.

DB99 is missing adenyl cyclase activity. This strain is unable to use mannitol, sorbitol, maltose, galactose, xylose, or mannose as carbon sources in the absence of cyclic AMP (cyclic adenosine-3′,5′-monophosphate), but in the presence of cyclic AMP DB99 is able to use any of these sugars. Glucose and fructose can be used in the absence of cyclic AMP. Table 9 shows that the induction of mannitol-1-phosphate dehydrogenase in DB99 requires the presence of cyclic AMP. In a phosphotransferase experiment, DB99 grown in the absence of cyclic AMP complemented both SB761 and SB762, indicating that cyclic AMP was required for the induction of a mannitol-specific component, i.e., not enzyme I or HPr. The strain was identified as an adenyl cyclase mutant by Yokota and Gots (37), who have shown that the enzyme activity is missing in extracts of DB99. They have also found that the mutation is linked to *metE* by transduction with P22. DB99 is similar to an adenyl cyclase mutant isolated in *E. coli* by Perlman and Pastan (24). Cyclic AMP is required for the expression of the mannitol genes.

Strain DB92 has the same sugar fermentation phenotype as DB99, but does not respond to cyclic AMP. The mutation appears to be the same type described in *E. coli* by Ullman and Monod (31), Zubay et al. (38), and Emmer et al. (7). The Zubay and Emmer mutants are missing a cyclic AMP-binding protein which is required for β-galactosidase in an in vitro system. The mutation carried by DB92 is 40% linked to *strA* by transduction with P22. The Emmer mutation is closely linked to the corresponding locus in *E. coli* (R. Perlman, *personal communication*).

TABLE 7. *Phenotypes of pleiotropic carbohydrate-negative mutants*[a]

Strain	Sugar							
	MTL	STL	MAL	GAL	XYL	MAN	GLU	FRU
SL751	+	+	+	+	+	+	+	+
DB310	−	−	−	+	+	−	−	−
DB316	−	−	−	+	+	−	−	−
DB318	−	−	−	+	+	−	−	−
DB99	−	−	−	−	−	−	+	+
DB92	−	−	−	−	−	−	+	+

[a] Strains were streaked on MacConkey indicator plates containing 1% of the indicated sugar. Abbreviations: MTL, mannitol; STL, sorbitol; MAL, maltose; GAL, galactose; XYL, xylose; MAN, mannose; GLU, glucose; FRU, fructose.

TABLE 8. *Mannitol phosphotransferase activity*[a]

Strain	Amt of product
SL751	17.8
DB90	0.61
DB310	1.30
SB761	0.07
SB762	0.46
DB310 + SB761	1.12
DB310 + SB762	10.6
DB90 + SB761	10.8
DB90 + SB762	5.6
SB761 + SB762	13.8

[a] Results are expressed as nanomoles of mannitol-1-phosphate formed in each assay tube in 30 min. Extracts were prepared from induced frozen cells. Protein concentrations were (per ml): SL751, 1.87 mg; DB90, 2.58 mg; DB310, 2.59 mg; SB761, 3.60 mg; SB762, 1.74 mg. In addition to the assay components, each tube contained 0.2 ml of the indicated extract, or 0.2 ml of an equal volume mixture of extracts.

TABLE 9. *Induction of mannitol-1-phosphate dehydrogenase in an adenyl cyclase mutant (DB99)*[a]

Additions during growth	Mannitol-1-phosphate dehydrogenase
None	0.05
Mannitol, 10^{-2} M	0.06
Cyclic AMP, 10^{-3} M	0.06
Mannitol, 10^{-2} M + cyclic AMP, 10^{-3} M	1.29

[a] Where indicated, cyclic AMP (adenosine-3',5'-monophosphate) was added to cultures at the time of inoculation, and mannitol 1 hr before harvesting. Results are expressed in units per milligram of protein. Mannitol-1-phosphate was used as the substrate in the assay.

Mapping. The mutations of class I and class II all map in the mannitol locus of the *Salmonella* chromosome (Fig. 1). By conjugation these mutations all show about 65% linkage to *ilvA* with SA536 as the donor and no linkage to *ilvA* with SA464 as the donor. Sanderson and Saeed (*manuscript in preparation*) have found about 10% linkage of both classes to *cysE* and *xyl* with P22. The gene order is *xyl mtl cysE pyrE*. None of the sorbitol-negative mutants isolated maps at the mannitol locus.

The pleiotropic carbohydrate-negative mutation carried by strain DB310 is very closely linked to *purC* (Table 10). This strain is missing enzyme I of the PEP-phosphotransferase system, based on complementation with SB762. The locations of the mutations carried by DB310, SB761, and SB762 were also tested by using SA535 as the chromosome donor in a 30-min mating. The mating was interrupted after 30 min and Pur$^+$ recombinants were selected. The *car* mutations in DB310, SB761, and SB762 were linked 72, 67, and 69% to *purC purI*. None of the recombinants was Ile$^+$ or Pro$^+$. Levinthal and Simoni (18) have located the *Salmonella* enzyme I mutation in SB761 in the 0- to 10-min region of the chromosome. Their data for SB761 are better than the data given here. The reason for this discrepancy is not understood. The *purC* location of enzyme I mutations (carA) is consistent with their location in *E. coli* (33) and with the location of a large number of enzyme I mutations recently isolated in *S. typhimurium*. (Saier and Simoni, *personal communication*).

TABLE 10. *Mapping of enzyme I mutations*[a]

Donor	Recipient	Selected	Per cent of MTL$^+$ recombinants
SA535	SB761	Ade$^+$	84
	SB762	Ade$^+$	92
	DB310	Ade$^+$	96
SA535	SB762	Ile$^+$	31
	DB310	Ile$^+$	77
SA536	SB761	Pro$^+$	0
	SB762	Pro$^+$	0
	DB310	Pro$^+$	2

[a] Strains were mated for 2 hr. Recombinants were selected on minimal medium containing galactose as the carbon source, threonine, and appropriate combinations of adenine, proline, and isoleucine. MTL, mannitol.

DISCUSSION

The class I and class II mutants both have low levels of mannitol-1-phosphate dehydrogenase and mannitol uptake activity (Table 3). This pleiotropy is also seen in *Staphylococcus aureus* (21). Some of our unpublished data indicate that the low uptake rates in the class I mutants are, at least in part, a result of the mannitol toxicity. The low uptake rates appear to be caused by inhibition of the phosphotransferase by mannitol-1-phosphate or some derivative which accumulates in these strains during the induction period (15). The low dehydrogenase levels in the class II mutants may be explained if the class II mutations are regulatory gene mutations. The atypical class II mutant, DB95, has noninduced dehydrogenase levels five to eight times higher than the noninduced dehydrogenase levels in the parental strain. A search for temperature-sensitive class II mutants has not yet been successful. Because of these ambiguities, gene assignments have not been made.

In *S. typhimurium*, mannitol is phosphorylated by the PEP-dependent phosphotransferase system and the mannitol-1-phosphate formed is oxidized to fructose-6-phosphate by mannitol-1-phosphate

dehydrogenase. Evidence for this pathway comes from the fact that mutants selected on the basis of an inability to utilize mannitol are missing a component of the PEP-dependent phosphotransferase system or mannitol-1-phosphate dehydrogenase activity.

The data for sorbitol-6-phosphate dehydrogenase given in Table 1 are peculiar in that the enzyme appears to be induced to a much greater extent in the fructose-6-phosphate assay than in the sorbitol-6-phosphate assay. The sorbitol-6-phosphate assay is not reliable because of probable contamination of the sorbitol-6-phosphate with mannitol-1-phosphate (34). Mannitol-1-phosphate inhibition of sorbitol-6-phosphate dehydrogenase might explain the low sorbitol-6-phosphate-dependent rates. The high level of fructose-6-phosphate-dependent oxidation of NADH induced by sorbitol may be a result of the reduction of fructose-6-phosphate or one of its metabolites by another enzyme. The data of Tables 1 and 2 taken together suggest the presence of a sorbitol-6-phosphate dehydrogenase in *S. typhimurium* but its quantitative significance is unknown.

Several lines of evidence suggest a relationship between mannitol and sorbitol metabolism. One is the extended doubling times of the class II mutants on sorbitol. Another is that the class I mutant, DB82, appears to have no sorbitol-6-phosphate dehydrogenase when measured with either sorbitol-6-phosphate or fructose-6-phosphate as substrates, even when induced with 5×10^{-2} M sorbitol. Finally, 36 mannitol-resistant mannitol-negative revertants of DB82 and other class I mutants have been examined. On MacConkey Agar containing 1% mannitol or sorbitol (5.5×10^{-2} M) half show a mannitol-negative, sorbitol-positive phenotype (like DB90), and the other half a mannitol-negative, sorbitol-negative phenotype. Structural gene and regulatory gene relationships between mannitol and sorbitol metabolism can not be distinguished at this point.

The class II mutants have low levels of mannitol specific phosphotransferase activity (Table 4). The data do not indicate that it is enzyme II which is low, but only that the missing component is not enzyme I or HPr (Table 8). It is possible that these mutants are deficient in a Km factor (12), factor III (27), or some as yet unidentified component.

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