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Replacement of a Phosphoenolpyruvate-dependent Phosphotransferase by a Nicotinamide Adenine Dinucleotide-linked Dehydrogenase for the Utilization of Mannitol[1]

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ABSTRACT

Mannitol is dissimilated by *Aerobacter aerogenes* via an inducible pathway initiated by a phosphotransferase system dependent upon phosphoenolpyruvate as the phosphoryl donor. A mutational block in this pathway can be suppressed either at the phenotypic level by induction of D-arabitol dehydrogenase, an enzyme fortuitously capable of converting mannitol to fructose, or genotypically by a constitutive mutation in the D-arabitol system.

It has gradually become apparent that the catalytic potential of a cell is invariably more versatile than what is normally exploited. This is due to the fact that many enzymes capable of acting on a number of structurally similar compounds are highly restricted in their actual function by precise cellular control mechanisms, permitting synthesis of the enzymes only under special conditions. Nonetheless, possession of a sizable hidden catalytic repertoire at times must have been crucial to a species in its adaptive responses to novel biochemical situations. For example, it has been shown that a single mutation enabled *Aerobacter aerogenes* to grow on xylitol as a result of a genetic derepression of ribitol dehydrogenase, an enzyme for which xylitol is a fortuitous substrate, but not an inducer (10, 16).

This paper describes the way in which mannitol is utilized by a wild-type population of *A. aerogenes*, and how a mutant which had lost the original catabolic enzymes for this hexitol could have its ability to grow on the compound restored by derepression of an enzyme normally acting on a pentitol. The net outcome of these two mutations was the emergence of a new line of cells with a different pattern for mannitol dissimilation, which provides another example of how metabolic innovation and divergence can arise in a microbial species.

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MATERIALS AND METHODS

Bacteria. A. aerogenes strain 1033-5P14 was provided by Boris Magasanik. This strain was employed as our starting cell line, because its double nutritional requirement for arginine and guanine permitted ready distinction of mutant progeny from contaminating organisms. For simplicity, this double auxotroph will be called strain 2002 in the present work.

Strain 2006, a mutant unable to ferment mannitol, was isolated from strain 2002. Approximately 2×10^9 cells of the parental strain were suspended in inorganic medium, and were irradiated in an open petri dish with ultraviolet light at a dosage giving 0.1% survival. The survivors were allowed to grow out in the dark in rich broth (Antibiotic Medium 3; Difco). This mutagenic procedure was repeated twice. The cells were then grown for about 10 generations in simple glucose medium supplemented with arginine and guanine to eliminate unwanted auxotrophs. The cultures were plated on E M B Agar (Difco) with mannitol as the major carbon source, and strain 2006 was isolated and purified from a pale colony.

Strain 2007, a mutant constitutive for D-arabitol utilization, was isolated from strain 2006 according to a previously published procedure (12).

Chemicals. Mannitol-1-phosphate, prepared according to the method described by Seegmiller and Horecker (22), was a gift of R. D. Hotchkiss. D-

Mannitol-*1*-C^{14} (21 c per mole) was obtained from Nuclear-Chicago Corp., Des Plaines, Ill.; D-mannitol, from Eastman Kodak Co., Rochester, N.Y.; D-arabitol, from Pfanstiehl Chemical Co., Waukegan, Ill.; nicotinamide adenine dinucleotide (NAD), from Pabst Laboratories, Inc., Milwaukee, Wis.; sodium phosphoenolpyruvate (PEP), from Calbiochem; 3-(4,5 dimethyl thiazolyl 1-2)-2,5 diphenyl tetrazolium bromide (MTT) and acid-hydrolyzed casein, from Nutritional Biochemicals Corp., Cleveland, Ohio; phenazine methosulfate, from Eastern Chemical Corp., Pequannock, N.J.; and Antibiotic Medium 3 (for rich broth), from Difco.

Growth of cells. The inorganic components of the basal medium consisted of 0.034 M NaH_2PO_4, 0.064 M K_2HPO_4, 0.02 M $(NH_4)_2SO_4$, 3×10^{-4} M $MgSO_4$, 10^{-6} M $FeSO_4$, 10^{-6} M $ZnCl_2$, and 10^{-5} M $CaCl_2$. The *p*H of the solution was adjusted to 7.0 with HCl. Arginine and guanine were each added at 4×10^{-4} M to all growth media except rich broth. Unless otherwise specified, single sources of carbon and energy were added to a final concentration of 0.2%. Casein hydrolysate was added to a final concentration of 1%.

Studies on growth rates were carried out at 37 C in 15 ml of medium. The cultures were incubated in 300-ml Erlenmeyer flasks, each fitted with a side arm, and swirled on a rotary shaker at about 240 cycles per min. Growth was monitored by reading the turbidity in the side arm in a Klett colorimeter with a 420-mμ filter (1 Klett unit = 10^6 cells per milliliter). For assay or preparation of enzymes, cells were grown in 1 liter of medium in 2-liter Erlenmeyer flasks incubated under the same conditions.

Preparation of cell-free extracts. Cells from 1 liter of fully grown culture (usually about 10^9 cells per milliliter) were harvested by centrifugation at 0 C. The collected cells were washed once with ice-cold 1% NaCl, suspended in 10 ml of 0.1 M tris(hydroxymethyl)aminomethane (Tris) buffer at *p*H 7.6, and disrupted by ultrasonic disintegration for 3 min in a model 60 W MSE apparatus (Measuring and Scientific Equipment Ltd., London, England) while being chilled in a −10 C bath. The resulting suspension was centrifuged at 35,000 × *g* for 20 min at 0 C, and the supernatant fraction was used for enzyme assays.

Enzyme I and heat-stable protein (HPr). Purified enzyme I and HPr, the two components in the PEP-dependent phosphotransferase system (8), were prepared from *Escherichia coli* and were made available to us by Saul Roseman. A crude extract containing these two components was prepared from a mannitol phosphotransferase-negative mutant of *A. aerogenes* strain 2006 grown to stationary phase on 2% Antibiotic Medium 3 supplemented with 1.5% glucose. [In this paper, "mannitol phosphotransferase" will be used to refer to phosphoprotein:mannitol phosphotransferase (enzyme II of the phosphoenolpyruvate: mannitol phosphotransferase system [8]).] The supernatant fraction of a sonic-treated preparation centrifuged for 20 min at 35,000 × *g* was diluted to contain approximately 20 mg of protein per ml, and was stored at −60 C.

Enzyme assays. Unless otherwise specified, mannitol phosphotransferase activity was measured by the amount of C^{14}-mannitol phosphate formed during a 10-min incubation period at 25 C in the following mixture: 40 μliters of 5×10^{-2} M PEP, 40 μliters of 5×10^{-4} M $MgCl_2$, 40 μliters of 5×10^{-4} M labeled mannitol (diluted with carrier to 2.1 c per mole), 0.1 ml of crude enzyme I and HPr (see previous section), cell-free extract to be assayed, and 0.1 M Tris (*p*H 7.6) to a final volume of 0.4 ml. The reaction was stopped by adding 0.6 ml of 0.2 M unlabeled mannitol, followed by chilling in an ice bath. C^{14}-mannitol phosphate was separated from the free hexitol by a procedure originally devised for the separation of galactose phosphate from free glactose (K. Ebisuzaki, *personal communication*). The carrier-quenched reaction mixture was transferred quantitatively onto a Dowex-1 formate column (0.6 by 1.3 cm). The column was washed with 3 ml of water and was eluted with 2 ml of 0.2 M formic acid in 0.5 M ammonium formate. The eluate was collected in a planchet, and was dried and sublimed under a heating lamp. The radioactivity was measured in an end-window gas-flow counter. The cell-free extract to be assayed was omitted from the blank. Under the assay conditions, the reaction proceeded linearly for at least 15 min. The 0.1 ml of enzyme I and HPr employed as reagents in the assay did not contribute any appreciable mannitol phosphotransferase activity. The stability of the labeled product, prepared by boiling a complete assay mixture at the end of the 10-min incubation period, was tested by re-exposure to the crude enzyme I and HPr under the standard assay condition. The recovery was found to be greater than 85%. The enzyme activity is expressed as micromoles of product formed per minute per milligram of protein, and no correction was made for the slight loss of the product during the incubation period.

Mannitol-1-phosphate dehydrogenase was assayed at 25 C by following the rate of reduction of a tetrazolium dye monitored at 558 mμ. The assay mixture contained: 0.2 ml of 0.01 M mannitol-1-phosphate, 0.1 ml of 0.02 M NAD, 0.1 ml of MTT (1 mg/ml), 0.1 ml of phenazine methosulfate (1 mg/ml), 0.1 ml of 0.1 M KCN, 0.2 ml of 0.5 M Tris (*p*H 7.5), enzyme extract, and water to a final volume of 1 ml. Substrate was omitted from the blank. The coupling of the enzymatically formed reduced NAD ($NADH_2$) to the dyes permitted the assay to be carried out at a lower *p*H. Under the conditions of the assay, the reduction of MTT was proportional to added $NADH_2$, and the reaction was virtually instantaneous without enzymatic catalysis. The enzyme activity is expressed as micromoles of $NADH_2$ generated per minute per milligram of protein.

Arabitol dehydrogenase was assayed at 25 C by following the reduction of NAD essentially according to a previously described procedure (11). The assay mixture contained 0.3 ml of 0.5 M substrate (either D-arabitol or D-mannitol), 0.1 ml of 0.01 M NAD, 2.0 ml of 0.2 M Tris (*p*H 9.0), enzyme extract, and water to a final volume of 3 ml. Substrate was omitted from the blank. The enzyme activity is expressed as micromoles of $NADH_2$ formed per minute per milligram of protein.

Protein concentrations were measured with the biuret reagent (4).

RESULTS

Since it had been shown that *A. aerogenes* grown on mannitol contained mannitol phosphate dehydrogenase activity (13, 24), but no mannitol dehydrogenase activity (11), we decided to search for a mannitol phosphorylating reaction as the first step of dissimilation of this hexitol. Using adenosine triphosphate (ATP) as the phosphoryl group donor, we were able to detect a weak enzymatic activity, and upon further exploration we found that ATP or other ribonucleoside triphosphates were much less effective than PEP. This unusual dependence led us to test the possibility that the mannitol phosphorylating activity under investigation was analogous to enzyme II in the PEP phosphotransferase system described by Kundig et al. (8):

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

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

(or mannitol phosphotransferase)

where HPr stands for a small, heat-stable protein containing histidine, and P-HPr for the phosphorylated protein. As shown in Table 1, purified enzyme I and HPr from *E. coli* did mediate the transfer of the phosphoryl group from PEP to mannitol in conjunction with the mannitol phosphotransferase present in the extract of *A. aerogenes*. The heat-labile enzyme I and the heat-stable HPr were also found in *A. aerogenes*, and could replace the two *E. coli* components used in this assay system. (Using the stimulation of mannitol phosphotransferase activity in turn as approximate assays for enzyme I or HPr, we observed that the activities of these two components in crude extracts were comparable to that of the induced mannitol phosphotransferase. A more detailed description of these two components will be published separately.) Under conditions adopted for the assay, the amount of mannitol phosphate formed was proportional to enzyme II (Fig. 1A), which has a K_m for mannitol of 4×10^{-5} M (Fig. 1B).

Table 2 shows that the mannitol phosphotransferase was inducible by mannitol. Cells grown on this compound also contained much higher levels of a NAD-linked mannitol-1-phosphate dehydrogenase. The specific catabolic role of these two enzymes is indicated not only by their induction by mannitol, but also by the correlation of the inability to form these enzymes with failure to grow on this compound (Table 2, strain 2006).

TABLE 1. *Dependence of mannitol phosphorylation on a phosphotransferase system*

Reaction mixture[a]	Crude extract[b]	Enzyme I[c]	HPr[d]	Activity
	μliters	*μliters*	*μliters*	*counts/min*
1	0	100	40	52
2	2	0	40	195
3	2	100	0	423
4	2	100	40	5,250

[a] All reaction mixtures contained C^{14}-mannitol, PEP, $MgCl_2$, and Tris buffer. For further details, see Materials and Methods. The incubation period was 20 min.

[b] From cells of strain 2002 grown on mannitol. The cell-free extract contained 30 mg of protein per ml.

[c] Purified from *Escherichia coli*, 0.5 mg of protein per ml.

[d] Purified from *E. coli*, 1 mg of protein per ml.

A. aerogenes 2002 has been shown to contain the genetic information for the synthesis of another enzyme which acts on mannitol, i.e., the NAD-linked D-arabitol dehydrogenase. This enzyme was found to catalyze in vitro the dehydrogenation of mannitol reversibly to fructose. However, under in vivo conditions this potential was not utilized, both because mannitol could not induce the enzyme and because the dissimilation of mannitol through the normal pathway exerted strong catabolite repression on the formation of the D-arabitol enzyme (11).

The question nevertheless remained as to whether D-arabitol dehydrogenase could be made to serve as a catabolic enzyme for mannitol under special circumstances. For example, in cells genetically lacking the original mannitol pathway, would the presence of the enzyme restore their ability to grow on the hexitol by converting it to the metabolizable sugar, fructose?

A preliminary experiment was carried out to test this possibility. Cells of strain 2006 which lacked mannitol phosphotransferase and mannitol-1-phosphate dehydrogenase were grown on D-arabitol to induce the D-arabitol dehydrogenase. When these cells were collected, washed, and transferred to a medium containing mannitol as the carbon source, linear growth commenced. No growth was observed with control cells which had been pregrown on glucose (Fig. 2). Logarithmic growth could not be expected of the cells pregrown on D-arabitol, because further formation of the dehydrogenase could not take place after transfer to the mannitol medium.

If the interpretation of the above observation was correct, then the presence of D-arabitol in a mannitol medium should have a catalytic effect

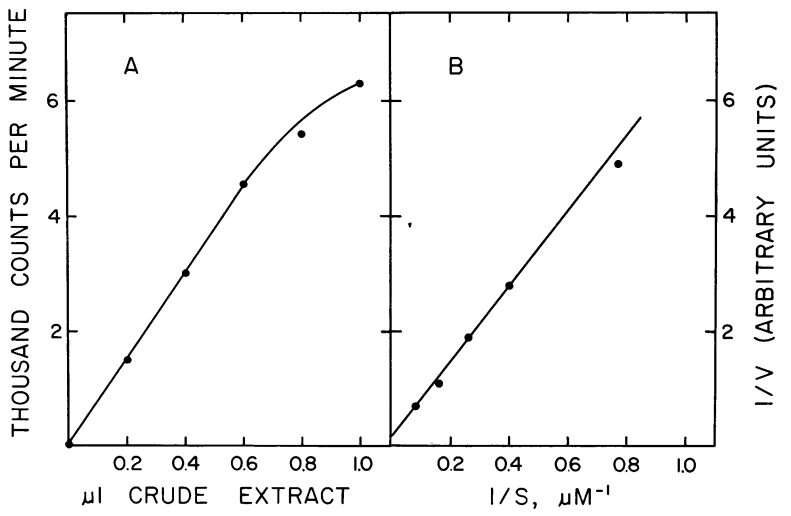

FIG. 1. *(A) Activity versus concentration of mannitol phosphotransferase. (B) Lineweaver-Burk plot of the rate of phosphorylation as a function of mannitol concentration.*

TABLE 2. *Mannitol phosphotransferase and mannitol-1-phosphate dehydrogenase in wild-type and mannitol-negative cells*

Strain	Carbon source for growth	Mannitol phosphotransferase[a]	Mannitol-1-phosphate dehydrogenase[a]
2002	Casein hydrolysate	0.001	0.13
	Mannitol	0.030	3.9
	Casein hydrolysate + mannitol	0.027	6.0
2006	Casein hydrolysate + mannitol	0.0001	0.18

[a] Expressed as micromoles per minute per milligram of protein.

in restoring growth of the mutant on mannitol. As shown in Fig. 3, the presence of a low concentration of D-arabitol, insufficient to permit appreciable growth by itself, restored essentially normal growth on the hexitol, even with cells pregrown on glucose. The provision of 1 molecule of D-arabitol promoted the utilization of over 100 molecules of mannitol. This efficient effect of the pentitol was probably due to the fact that the presence of excess mannitol competitively protected the inducer from consumption by the D-arabitol dehydrogenase. The K_m values of the enzyme for mannitol and D-arabitol have been shown to be 6.1×10^{-3} M and 3.5×10^{-3} M, respectively (11). The growth phenomenon observed was phenotypic in nature; it did not represent the multiplication of cells genetically reverted in the original pathway for mannitol,

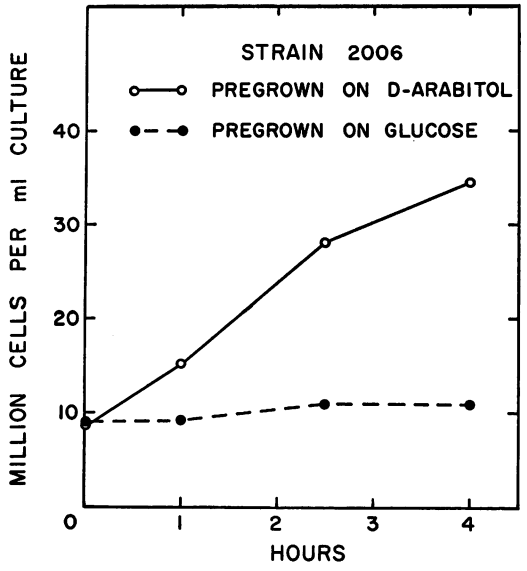

FIG. 2. *Limited phenotypic restoration of the mannitol-utilizing capacity of strain 2006 by pregrowth on D-arabitol.*

since fewer than 0.5% of the cells formed colonies that fermented mannitol in the absence of D-arabitol.

Results from the phenotypic experiments predicted that a mutation of the D-arabitol system to constitutivity should enable mannitol phosphotransferase-negative cells to grow on the hexitol without the intervention of an inducer. This expectation was likewise fulfilled. Figure 4 shows that cells of strain 2007, a D-arabitol constitutive

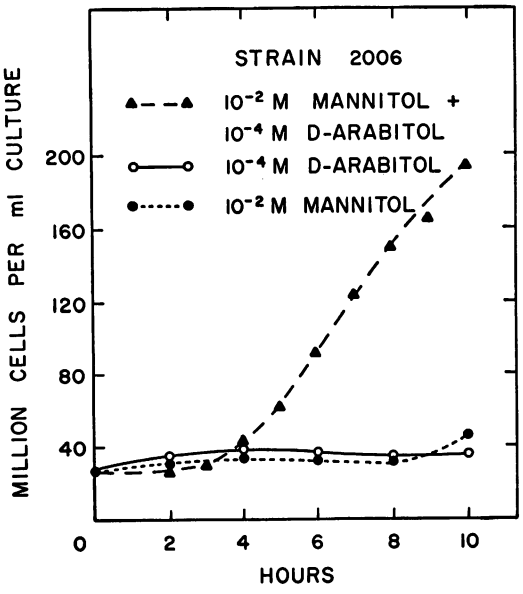

FIG. 3. *Promotion of growth of strain 2006 on mannitol by a catalytic amount of* D-*arabitol.*

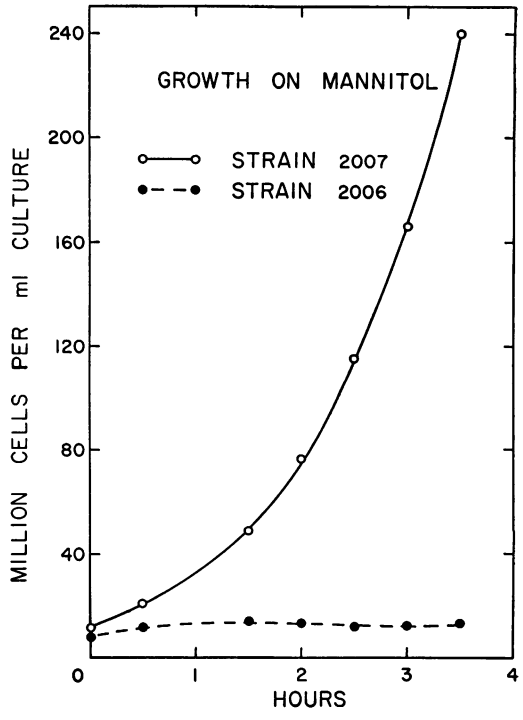

FIG. 4. *Genetic restoration of the ability of the mutant blocked in the mannitol phosphotransferase pathway (strain 2006) to grow on the hexitol by a constitutive mutation in the* D-*arabitol system (strain 2007). The cells were pregrown on fructose.*

derivative of strain 2006, could grow exponentially on mannitol without aid. Such cells, however, could be readily distinguished by their enzyme profile from wild-type cells grown on mannitol. Table 3 shows that the cells of strain 2002 contained high mannitol phosphotransferase activity with negligible D-arabitol dehydrogenase activity. The reverse was true for cells of the suppressed mutant, strain 2007. To test whether the constitutive D-arabitol dehydrogenase was in fact responsible for the restoration of growth on mannitol, three independent mannitol-negative, but fructose-positive, mutants were isolated from strain 2007. All three had also lost the ability to grow on D-arabitol and were unable to produce D-arabitol dehydrogenase when grown on casein hydrolysate in the presence of the pentitol. Cells of one of these mutants, pleiotropically negative for D-arabitol and mannitol, were plated on agar containing D-arabitol as the sole carbon source. Three independent revertants were collected and characterized. All produced D-arabitol dehydrogenase constitutively and had recovered concomitantly the ability to grow on mannitol. Hence, we concluded that the constitutive NAD-linked enzyme in strain 2007 served physiologically as a dehydrogenase for mannitol and D-arabitol.

Although a mutation to constitutivity in the D-arabitol system clearly could suppress physiologically the genetic block in the mannitol phosphotransferase pathway, it remained to be seen whether such suppressions occur at significant frequencies among spontaneous mannitol-positive "reversions." Cells from each of 10 clones of strain 2006 were therefore plated on agar with mannitol as the carbon source. A revertant colony was isolated from each and was examined for its enzymatic pattern. Nine of these were found to produce D-arabitol dehydrogenase constitutively while remaining mannitol phosphotransferase-negative, as in the case of strain 2007. Only one revertant resembled the wild-type strain 2002, in that both the D-arabitol dehydrogenase and the mannitol phosphotransferase were positive and inducible by their respective substrates.

TABLE 3. *Enzymatic composition of strains 2002 and 2007 grown on mannitol*

Strain	D-Arabitol dehydrogenase[a]	Mannitol phosphotransferase[a]
2002	0.007	0.018
2007	0.72	0.0001

[a] Expressed as micromoles per minute per milligram of protein.

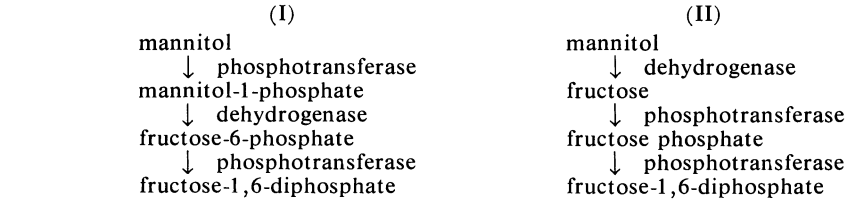

FIG. 5. *Two pathways by which bacterial species dissimilate mannitol. Scheme I: metabolism initiated by phosphorylation; scheme II: metabolism initiated by dehydrogenation. Fructose phosphate in scheme II is explained as follows. Bacteroides symbiosus* and *Aerobacter aerogenes strain PRL-R3 are known to have a phosphotransferase which converts fructose-1-phosphate, rather than fructose-6-phosphate, to fructose-1,6-diphosphate (5, 19). Presumably, fructose is phosphorylated to fructose-1-phosphate. Other organisms may metabolize fructose via fructose-6-phosphate.*

We have not considered in this report the mechanism of entrance of mannitol into cells of strain 2002 and 2007. A recent study by Kundig et al. (9) implicated the HPr system in the active transport of glycosides into cells of *E. coli*. Whether the HPr system plays a similar role for the transport of mannitol in *A. aerogenes* is under investigation.

DISCUSSION

Bacterial species can be divided into two groups with respect to their mode of dissimilation of mannitol: those which initiate its metabolism by phosphorylation (scheme I, Fig. 5) and those which initiate the pathway by dehydrogenation (Scheme II, Fig. 5). In both cases, fructose-1,6-diphosphate is the common intermediate.

On the basis of the ability to form an NAD-linked mannitol-1-phosphate dehydrogenase, the following organisms have been postulated to employ scheme I: *A. aerogenes* (13, 24), *Bacillus subtilis* (6), *Diplococcus pneumoniae* (15), *E. coli* (24, 25), *Lactobacillus plantarum* (2, 3, 24), *Salmonella gallinarum* (26), and *Staphylococcus aureus* (17). The failure to demonstrate mannitol phosphorylating activity in the extracts of most of these organisms might be due to the requirement for the unusual phosphoryl donor system. Although an ATP-dependent mannitol phosphotransferase activity has been reported for *E. coli* (7), its requirement for a heat-stable factor suggests that the transfer of the phosphoryl group from ATP to mannitol is indirect.

Among the organisms presumed to employ pathways depicted by scheme II on the basis of their possessing a mannitol dehydrogenase are *Acetobacter suboxydans* (1, 18), *Azotobacter agilis* (14), *Cellvibrio polyoltrophicus* (20), and *Pseudomonas fluorescens* (21, 23).

The demonstration that two genetic events, a loss mutation followed by a constitutive mutation, could shift the mode of dissimilation of mannitol from type I to type II, and that the suppression occurred more frequently than reversion to the original pathway, raises the question of whether mutational sequences of this nature might have played a role in evolutionary divergence. For example, a genetic modification of the phosphotransferase might have permitted the utilization of a novel compound at the expense of the catalytic activity on mannitol (for further discussion of the problem of developing new metabolic pathways, see references 10 and 16). A secondary mutation causing the derepression of the D-arabitol system would have allowed the new cell line to retain this ability to exploit the novel compound without having to forego mannitol as a carbon source. Although the contribution of such suppressor mutations to bacterial variations cannot be evaluated at present, the reported example should serve to emphasize the danger of employing metabolic characteristics as taxonomic criteria.

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