Identification of a Phosphoenolpyruvate:Fructose 1-Phosphotransferase System in *Azospirillum brasilense*

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An inducible phosphoenolpyruvate:fructose phosphotransferase system has been detected in *Azospirillum brasilense*, which requires a minimum of two components of the crude extracts for activity: (i) a soluble fraction (enzyme I) and (ii) a membrane fraction (enzyme II). The uninduced cells neither show any uptake of fructose nor express activity of either of these two enzyme fractions. C-1 of fructose is the site of phosphorylation. This phosphotransferase system does not accept glucose as a substrate for phosphorylation.

Among the chemoheterotropic spirilla (9), the nitrogen-fixing bacterium *Azospirillum brasilense* has recently come into prominence because of its ability to associate with roots of maize, wheat, and several other tropical grasses and to partially relieve the dependence of these plants for growth on fixed N (18). The bacterium has been found to thrive in the intercellular spaces of roots of the infected plants (17) and appears to depend on circulating photosynthetic products as the C source for growth and N fixation during associative symbiosis (6). With the idea that the ability of the bacterium to transport and utilize sugars may determine the efficiency of this kind of symbiosis in regard to plant productivity, we have studied the initial steps of glucose and fructose metabolism in the bacterium.

In previous investigations with a related species (*Aquaspirillum itersonii*), it was shown that, although the enzymatic complement necessary for glucose catabolism (including glucokinase) was present in the organism, this bacterium could not use glucose as the C source for growth, possibly due to a permeability barrier. In addition, the organism utilized fructose quite efficiently as the C source for growth, but the ATP-dependent, fructose-phosphorylating system could not be detected in the extracts of the bacteria (8). Recent studies with *A. brasilense* (previously known as *Spirillum lipoferum* group I) have shown that the organism can metabolize fructose, but not glucose or sucrose (10). Although previous studies by Hylemon et al. (8) with *Aquaspirillum itersonii* suggested the existence of an inducible fructose uptake system, any biochemical basis for this difference in the metabolism of glucose and fructose in spirilla still remains unknown. In fact, very little is known about carbohydrate transport systems of spirilla, although some elegant studies on the carbohydrate metabolism of a few members of this group have already been carried out (8, 9, 12, 13, 19). We report here that an inducible phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) is present in *A. brasilense* which acts specifically on fructose to mediate its phosphorylation and transport. This fructose-specific PTS has characteristics similar to that of fructose 1-phosphotransferase of *Pseudomonas aeruginosa*, as recently reported by Durham and Phibbs (7). The demonstration of the presence of a highly specific fructose 1-phosphotransferase system in *A. brasilense* shows that the

limited ability of *Azospirillum* spp. and related bacteria to utilize sugars (9, 10) may be due to a possible narrow specificity of PTS-mediated hexose transport.

The strain used for all of our experiments was *A. brasilense* RG, which was selected from a culture of *S. lipoferum* 81 (sent to us by N. R. Krieg) for its ability to form good colonies in an N-free agar plate in 36 h (14); it was also found to be resistant to streptomycin and ampicillin. Utilization and uptake studies of sugars with the bacteria (Fig. 1) showed that the whole cells were unable to utilize fructose if grown previously with succinate as the only C source, but they acquired the capacity for fructose uptake when they were grown previously with fructose. It can also be seen from Fig. 1A that even fructose-grown cells were practically incapable of utilizing glucose. One possible way to interpret these results is that any glucose uptake system is absent, but an inducible fructose uptake system is present in *A. brasilense*.

The PEP:sugar PTS, first reported by Kundig et al. (11), is now known to mediate sugar transport in most facultative anaerobic and many anaerobic bacteria, but the PTS is generally absent in strict aerobes (5). However, there are many exceptions to this pattern of PTS distribution; for example, *Bacillus* spp. (3) and *Arthrobacter pyridinolis* (16) are aerobic but possess the PTS. We therefore looked for glucose- and fructose-phosphorylating systems in the extracts of the aerobic bacterium *A. brasilense*. *A. brasilense* RG was grown on succinate-salt medium (13) containing 0.3% fructose, and after centriguation, the cells were suspended in 4 volumes of 0.01 M KPO_4 (pH 7.6)–1 mM EDTA–1 mM 2-mercaptoethanol and disrupted by sonication. The supernatant fraction, obtained after centrifugation of the sonicated cells at 10,000 × g for 30 min, was dialyzed against the above buffer (crude extracts) and was assayed for enzymatic phosphorylation of glucose and fructose with both ATP and PEP as the phosphoryl donor. The standard incubation mixture per tube for the PTS or kinase assay contained 2.5 μmol of PEP or ATP, 5 μmol of sugar, 40 μmol of KPO_4 (pH 7.6), 2 μmol of $MgCl_2$, and enzyme fraction(s) in a total volume of 0.5 ml. After incubation, sugar phosphate formed in the assay mixture was adsorbed on a small column (1 ml [volume]) of Dowex 1-acetate, free sugar was removed by washing the column with water, and the phosphorylated sugar was finally quantitatively eluted with 3 ml of 1 M NaCl–0.2 M HCl solution. The sugar concentration was measured by the Nelson color reaction method (1). From the results of sugar phosphorylation by the crude
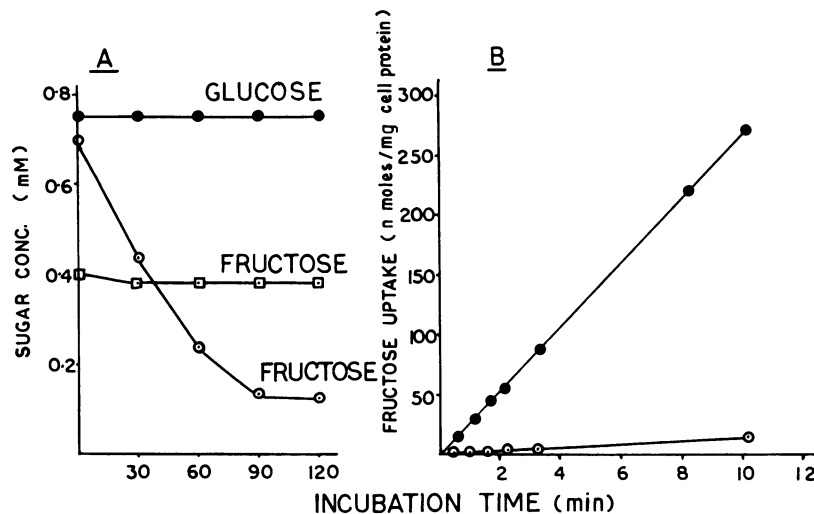
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FIG. 1. Utilization and uptake of sugars by *A. brasilense* RG. (A) Sugar utilization was measured with cells previously grown either in succinate-salt medium (13) (□) or in succinate-salt medium containing 0.3% fructose (○) or glucose (●). After growing to an absorbance at 590 nm of ca. 1.0, the cells were washed, suspended in 0.01 M KPO₄ (pH 7.6) to an absorbance at 590 nm of 2.0 containing either glucose or fructose, and incubated at 30°C in a rotary shaker. Sugar utilization was measured by periodically taking samples and diluting them 10-fold with ice-cold phosphate buffer (0.01 M; pH 7.6), centrifuging off the cells, and measuring the sugar concentration in the supernatant medium as described by Nelson (1). (B) Uptake of [¹⁴C]fructose by the bacteria was measured with cells previously grown in either succinate-salt medium (○) or succinate-salt medium containing 0.3% fructose (●) to an absorbance at 590 nm of ca. 1.0. The cells were washed with 75 mM sodium phosphate buffer (pH 7.0) and suspended in the same buffer to an absorbance at 590 nm of 0.8. The uptake was started by adding 0.1 ml of 1 mM [¹⁴C]fructose (900 cpm/nmol) to 1.9 ml of the cell suspension and incubating with shaking at 30°C. Samples (0.1 ml) were removed and added to 2 ml of 75 mM sodium phosphate buffer (pH 7.0), immediately filtered through a Millipore filter, and washed first with 5 ml of the same phosphate buffer containing 0.01 M cold fructose and finally with 10 ml of the same phosphate buffer. Membrane filters were counted after drying with a liquid scintillation counter.

TABLE 1. Identification of a specific PEP:fructose PTS in *A. brasilense* RG[a]

Expt no.	Enzyme fraction	Substrates in incubation mixture	Amt of sugar phosphate formed/min per incubation mixture (nmol)
1	Crude extracts (dialyzed)	PEP + fructose	5.3
		ATP + fructose	0.8
		ATP + glucose	5.1
		PEP + glucose	0.3
2	Mem (Fru) + Sol (Fru)	PEP + fructose	4.0
		ATP + fructose	0.2
		ATP + glucose	3.6
		PEP + glucose	0.3
	Sol (Fru)	ATP + glucose	3.5
3	Mem (Fru) + Sol (Succ)	PEP + fructose	0.2
	Mem (Succ) + Sol (Fru)	PEP + fructose	0.6
	Mem (Succ) + Sol (Succ)	PEP + fructose	0.0
	Mem (Fruc)	PEP + fructose	0.2
	Sol (Fruc)	PEP + fructose	0.1
4	Mem (*A. brasilense*)+ Sol (*E. coli*)	PEP + fructose	0.0
	Mem (*E. coli*) + Sol (*A. brasilense*)	PEP + fructose	0.0
	Mem (*E. coli*) + Sol (*E. coli*)	PEP + fructose	5.9
	Mem (*A. brasilense*) + Sol (*A. brasilense*)	PEP + fructose	8.9

[a] All incubations were done at 37°C for 1 h. In experiment 1, crude extracts containing 1.1 mg of protein were added to each incubation mixture. In experiments 2 and 3, the following abbreviations were used: Mem and Sol, membrane and soluble fractions, respectively; Fru and Succ (in parentheses), fractions derived from fructose-succinate- and succinate-grown cells, respectively. The amount of protein added with the enzyme fractions per incubation mixture was as follows: Mem (Fru), 0.46 mg; Mem (Succ), 0.45 mg; Sol (Fru), 0.5 mg; and Sol (Succ), 0.53 mg. The enzyme fraction Mem (Fru) + Sol (Fru) catalyzed the synthesis of fructose 1-P from PEP and fructose at a nearly constant rate for up to 1 h under the conditions of assay. In experiment 4, *A. brasilense* and *E. coli* were grown in fructose-containing medium. The amount of protein added per incubation mixture with the enzyme fractions was as follows: Sol (*A. brasilense*), 2 mg; Mem (*A. brasilense*), 1.2 mg; Sol (*E. coli*), 0.34 mg; and Mem (*E. coli*), 0.18 mg. The fructose 1-P values were corrected for a small amount of fructose 1-P formed with each individual fraction.

extracts of *A. brasilense* RG (Table 1, experiment 1), it became apparent that the PTS could mediate fructose but not glucose phosphorylation in this aerobic bacterium. ATP-dependent glucokinase was normal, but PEP-dependent glucose-phosphorylating activity was quite low in the extracts.

Because the uptake experiments with whole cells of *A. brasilense* (Fig. 1) suggested that the PTS for fructose could be inducible, we therefore looked for the possible presence of PTS in both succinate- and succinate-fructose-grown cells. Crude extracts were prepared from these cells and were subsequently fractionated by ultracentrifugation at 100,000 × *g* for 1 h to obtain the membrane fraction (enzyme II) and soluble fraction (enzyme I) as described previously (11). Whereas the glucokinase was found almost entirely in the soluble fraction (Table 1, experiment 2, lines 3 and 5), an efficient, PEP-dependent fructose phosphorylation depended upon the simultaneous presence of both the membrane and the soluble fractions of the extracts (Table 1, experiment 2, line 1 and experiment 3, lines 4 and 5). These results strongly suggest that a fructose-specific PTS is present in *A. brasilense* which may not be active with glucose. We failed to detect any PTS activity in the succinate-grown cells (Table 1, experiment 3). By complementation analysis with the membrane and soluble components of the fructose-induced and uninduced cells, it became evident that both enzyme I and enzyme II of this PTS are inducible. Very low PEP-mediated phosphorylation of glucose, observed with the crude PTS, was possibly due to the presence of contaminating enzymes. We have very recently purified the fructose-specific enzyme I of *A. brasilense* to near homogeneity (M_r of enzyme I, ~80,000), and it became clear during purification that unlike the PTS of *Escherichia coli*, the PTS of *A. brasilense* was devoid of any low-molecular-weight protein component, such as the HPr (unpublished data).

To determine the position of phosphorylation in fructose, we subjected the PTS-catalyzed product from [^{14}C]fructose to thin-layer chromatography which could separate fructose 1-P from fructose 6-P (4). All of the phosphorylated product from [^{14}C]fructose was recovered as [^{14}C]fructose 1-phosphotransferase, indicating that C-1 of fructose was the site of phosphorylation.

We also wanted to determine whether any component of the PTS from *E. coli* could substitute for the similar component of PTS from *A. brasilense* for PEP-dependent fructose phosphorylation. Therefore, a complementation analysis was also performed with the membrane fraction (enzyme II) and soluble fraction (enzyme I plus HPr) derived from fructose-grown *E. coli*. These two fractions of the PTS were obtained from the crude extracts of *E. coli* C600, grown in M-9 medium containing 0.4% fructose and 0.1% yeast extract as described previously (11). The results (Table 1, experiment 4) indicated that neither enzyme I plus HPr (the soluble fraction) nor enzyme II (the membrane fraction) of the PTS from *E. coli* could complement components of PTS from *A. brasilense*, suggesting a wide divergence of these two bacteria during evolution. The absence of HPr-like protein in the PTS was previously observed in the bacteria of *Athiorhodacae* family (2, 15) also, and their PTS, like that of *A. brasilense*, is also very specific for fructose. Very recently, Durham and Phibbs (7) demonstrated the presence of a fructose-specific PTS in *P. aeruginosa* which is also inducible by fructose and contains no HPr-like component, as in members of the family *Athiorhodacae* (2, 15). These findings probably suggest a close evolutionary relationship between these three types of bacteria (*Pseudomonas* and *Azospirilla* species and members of the family *Athiorhodacae*).

LITERATURE CITED

1. **Ashwell, G.** 1957. Arsenomolybdate method of Nelson. Methods Enzymol. **3**:85–86.
2. **Brouwer, M., M. G. L. Elferink, and G. T. Robillard.** 1982. PEP-dependent fructose phosphotransferase system of *Rhodopseudomonas spheroids*: purification and physico-chemical and immunochemical characterization of membrane-associated enzyme I. Biochemistry **21**:82–88.
3. **Delobbe, A., H. Chalumeau, J. M. Claverie, and P. Gay.** 1976. Phosphorylation of intracellular fructose in *Bacillus subtilis* mediated by PEP-1-fructose phosphotransferase. Eur. J. Biochem. **66**:485–491.
4. **Dietrich, C. P., S. M. C. Dietrich, and H. G. Pontis.** 1964. Separation of sugar phosphates and sugar nucleotides by thin layer chromotography. J. Chromatogr. **15**:277–278.
5. **Dills, S. S., A. Apperson, M. R. Schmidt, and M. H. Saier, Jr.** 1980. Carbohydrate transport in bacteria. Microbiol. Rev. **44**:385–418.
6. **Döbereiner, J., and J. M. Day.** 1976. Associative symbiosis in tropical grasses: characterization of microorganisms and dinitrogen fixation sites, p. 518–536. *In* W. W. Newton and C. J. Nyman (ed.), Proceedings of the 1st International Symposium on Nitrogen Fixation. Washington State University Press, Pullman.
7. **Durham, D. L., and P. V. Phibbs, Jr.** 1982. Fractionation and characterization of the phosphoenolpyruvate:fructose 1-phosphotransferase system from *Pseudomonas aeruginosa*. J. Bacteriol. **149**:534–541.
8. **Hylemon, P. B., N. R. Krieg, and P. V. Phibbs, Jr.** 1974. Transport and catabolism of D-fructose by *Spirillum itersonii*. J. Bacteriol. **117**:144–150.
9. **Krieg, N. R.** 1976. Biology of the chemoheterotropic spirilla. Bacteriol. Rev. **40**:55–115.
10. **Krieg, N. R.** 1977. Taxonomic studies of *Spirillum lipoferum*, p. 463–472. *In* A. Hollaender (ed.), Genetic engineering for nitrogen fixation. Plenum Publishing Corp., New York.
11. **Kundig, W., S. Ghosh, and S. Roseman.** 1964. Phosphate bound to histidine in a protein as an intermediate in a novel phosphotransferase system. Proc. Natl. Acad. Sci. U.S.A. **52**:1067–1074.
12. **Novick, N. J., and M. E. Tyler.** 1982. L-Arabinose metabolism in *Azospirillum brasilense*. J. Bacteriol. **149**:364–367.
13. **Okon, Y., S. L. Albrecht, and R. H. Burris.** 1976. Factors affecting growth and nitrogen fixation of *Spirillum lipoferum*. J. Bacteriol. **127**:1248–1254.
14. **Rauth, S., and S. Ghosh.** 1981. Effect of dimethylsulfoxide on derepression of nitrogenase in *Spirillum lipoferum*. FEBS Lett. **126**:77–80.
15. **Saier, M. H., B. U. Feucht, and S. Roseman.** 1971. Phosphoenolpyruvate-dependent fructose phosphorylation in photosynthetic bacteria. J. Biol. Chem. **246**:7819–7821.
16. **Sobel, M. E., and T. A. Krulwich.** 1973. Metabolism of D-fructose by *Arthrobacter pyridinolis*. J. Bacteriol. **113**:907–913.
17. **Vasil, I. K., V. Vasil, and D. H. Hubbell.** 1977. Engineered plant cells of fungal association with bacteria that fix nitrogen, p. 197–209. *In* A. Hollaender (ed.), Genetic engineering for nitrogen fixation. Plenum Publishing Corp., New York.
18. **Von Büllow, J. W. F., and J. Döbereiner.** 1975. Potential for nitrogen fixation in maize genotypes in Brazil. Proc. Natl. Acad. Sci. U.S.A. **72**:2383–2393.
19. **Westby, C. A., D. S. Cutshall, and G. V. Vigil.** 1983. Metabolism of various carbon sources by *Azospirillum brasilense*. J. Bacteriol. **156**:1369–1372.