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Metabolism of Various Carbon Sources by *Azospirillum brasilense*

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Azospirillum brasilense Sp7 and two mutants were examined for 19 carbon metabolism enzymes. The results indicate that this nitrogen fixer uses the Entner-Doudoroff pathway for gluconate dissimilation, lacks a catabolic but has an anabolic Embden-Meyerhof-Parnas hexosephosphate pathway, has amphibolic triosephosphate enzymes, lacks a hexose monophosphate shunt, and has lactate dehydrogenase, malate dehydrogenase, and glycerokinase. The mutants are severely deficient in phosphoglycerate and pyruvate kinase and also have somewhat reduced levels of other carbon enzymes.

An important relationship exists in *Azospirillum* spp. between nitrogen fixation and certain organic acids (e.g., malate) and sugars supplied in nature by host grasses. These compounds occur in C-4 grass root exudates (19, 22), exert a chemotactic effect on *Azospirillum brasilense* (18) and stimulate nitrogenase activity (16). Carbon metabolism and regulation in this nitrogen fixer have received little attention (15, 17).

A. brasilense Sp7 (ATCC 29145), the wild type, was obtained from the American Type Culture Collection, Rockville, Md. Mutants CW-1 (ATCC 35212) and CW-2 (ATCC 35213) were isolated on nutrient agar plates after 45 min of exposure of Sp7 to diethyl sulfate at 1.5 and 3.0% for CW-1 and CW-2, respectively. The mutants were sought so as to have one more tool (besides Sp7) for studying the regulation of carbon pathways in *A. brasilense*.

Dobereiner-Day-NH_4Cl (DDN) medium (pH 6.8) used throughout contained inorganic salts (4), 2.3 g of NH_4Cl per liter, 5 g of a single carbon source (filter-sterilized sodium, calcium, or potassium salt for acids) per liter, and where plates were required, 15 g of agar per liter. In a typical enzyme run, 4 to 6 flasks (2-liter, wide-mouthed, 250 ml of medium per flask) inoculated 1:15 (vol/vol) with logarithmic-phase cells were incubated at 30 ± 1°C on a reciprocal shaker (64 strokes per min, 10-cm displacement). Cultures were grown to an absorbance of 0.6 to 1.3 at 520 nm (3×10^8 to 8×10^8 CFU/ml).

After reversion checks, cells were harvested by centrifugation at 10,000 × *g* for 15 min at 4°C, washed twice with 0.85% NaCl (250 ml per flask), and suspended in 1 to 3 ml of 0.02M Tris-hydrochloride buffer, pH 7.5. Washed cells were ruptured by four 30-s sonic bursts (4 A) at 0 to 4°C with a Branson model S-110 sonifier (small probe). Centrifugation at 39,900 × *g* for 30 min at 0 to 4°C removed particulates, and the supernatant was a crude cell extract.

Enzymes 1 to 21, the trivial names and EC numbers of which are given in the legend to Fig. 1, were assayed by the following referenced procedures: 1, 2, 5, 6, 13, and 15 (9, 21); coupled 3 and 4 (12, 13); 7 and 8 (*Sigma Technical Bulletin*); 9 (3); 10 (7); 11 (23); 12 (*Worthington Enzyme Manual*, 1972); 14 and 16 (5); and 20 and 21 (*Sigma Technical Bulletin* no. 340-UV).

The carbon metabolism of *A. brasilense* was initially explored by checking the growth response of the wild type, Sp7, and two mutants, CW-1 and CW-2, to various single-carbon sources. The results (Table 1) are in general agreement with the findings of others (1, 17, 20) and indicate that *A. brasilense* can use either certain sugars or organic acids for carbon and energy. The two mutants grew poorly on organic acids, indicating deficiency in gluconeogenesis; this was supported by the longer generation times of the mutants compared with Sp7 on gluconate (Table 1).

To further analyze carbon metabolism in Sp7, enzymes of gluconate, glycerol, lactate, and malate metabolism (Fig. 1) were measured in crude extracts from cells grown singly on these substrates. The mutants were tested for the same enzymes in extracts from cells grown on gluconate.

Some of the enzymes were present in markedly higher amounts in Sp7 under certain growth conditions (Table 2). This was the case for glyceraldehyde-phosphate dehydrogenase ($NADP^+$) (no. 8), enolase (no. 11), glyceroki-

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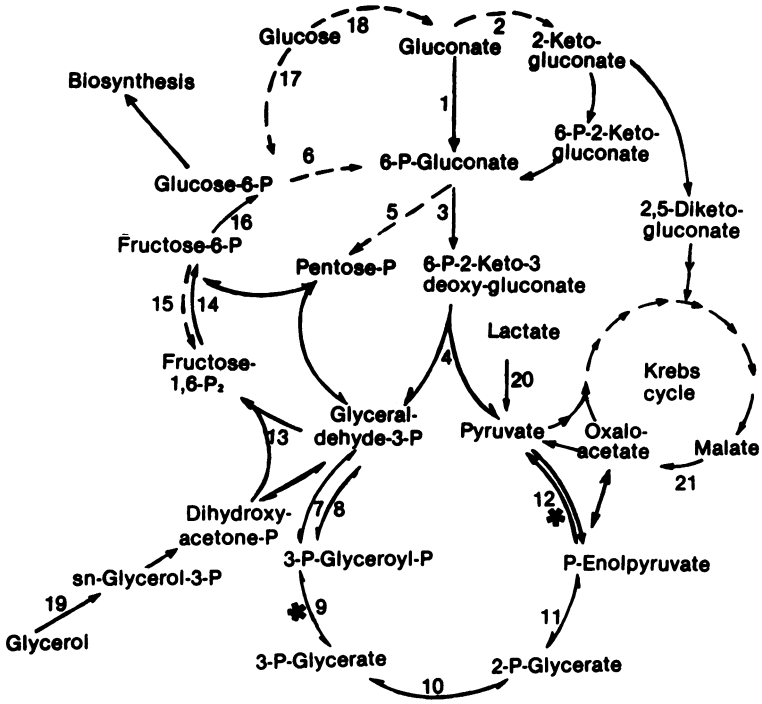

FIG. 1. Carbon metabolism of *A. brasilense*. Enzymes are numbered as follows: 1, gluconokinase (EC 2.7.1.12), 2, gluconate 2-dehydrogenase (EC 1.1.99.3), 3, phosphogluconate dehydratase (EC 4.2.1.12), 4, phospho-2-keto-3-deoxygluconate aldolase (EC 4.1.2.14), 5, phosphogluconate dehydrogenase (EC 1.1.1.44), 6, glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 7, glyceraldehyde-phosphate dehydrogenase (NAD^+) (EC 1.2.1.12), 8, glyceraldehyde-phosphate dehydrogenase ($NADP^+$) (EC 1.2.1.13), 9, phosphoglycerate kinase (EC 2.7.2.3), 10, phosphoglycerate mutase (EC 2.7.5.3), 11, enolase (EC 4.2.1.11), 12, pyruvate kinase (EC 2.7.1.40), 13, fructose-bisphosphate aldolase (EC 4.1.2.13), 14, fructose bisphosphatase (EC 3.1.3.11), 15, 6-phosphofructokinase (EC 2.7.1.11), 16, glucose-phosphate isomerase (EC 5.3.1.9), 17, glucokinase (EC 2.7.1.2), 18, glucose dehydrogenase (EC 1.1.1.47), 19, glycerokinase (EC 2.7.1.30), 20, lactate dehydrogenase (EC 1.1.1.27), 21, malate dehydrogenase (EC 1.1.1.37). Dashed arrows correspond to reactions that are missing in this bacterium or are detectable at only very low levels in extracts. Unnumbered solid arrows correspond to reactions not tested in the present study. The asterisk at phosphoglycerate kinase (no. 9) and pyruvate kinase (no. 12) indicates the most probable site of mutation in CW-1 and CW-2.

TABLE 1. Growth characteristics of *A. brasilense* Sp7 and mutants CW-1 and CW-2

Carbon source[b]	Growth on single-carbon sources[a]		
	Sp7	CW-1	CW-2
D-Gluconate	+++ (5.2)[c]	+++ (7.3)	+++ (7.4)
D-Arabinose	++	+	+
D-Fructose	+++	+++	+
Ethanol	++	++	++
Glycerol	++ (11.2)	++	++
Fumarate, 2-ketoglutarate, pyruvate, or succinate	++/+++	+/++	+/++
DL-Malate	+++ (3.0)	+	+
DL-Lactate	+++ (2.9)	+	+
2-Ketogluconate	+++	++	++

[a] The carbon sources were singly present in DDN agar plates at 5 g/liter. Acids were present as sodium, calcium, or potassium salts. After streak inoculation from DDN-gluconate or BHI cultures, the plates were incubated at 32°C for 3 to 4 days before being scored for surface growth. Symbols for growth on agar: +++, excellent growth; ++, fair growth; and +, little growth.

[b] The following permitted little or no growth of Sp7 or mutants: acetate, D-galactose, D-glucose, glycerate, D-mannitol, D-mannose, D-ribose, and D-xylose.

[c] Numbers in parentheses are the generation times (hours) at 30 ± 1°C in DDN liquid medium under aerobic conditions with the carbon source at 5 g/liter.

nase (no. 19), lactate dehydrogenase (no. 20), and malate dehydrogenase (no. 21).

Our enzyme results suggest that the Entner-Doudoroff pathway is a major route of gluconate dissimilation in *A. brasilense*. Levels of the three Entner-Doudoroff enzymes, 1, 3, and 4 (Fig. 1), in gluconate-grown cells (Table 2) were

TABLE 2. Carbon pathway enzymes in *A. brasilense* Sp7 and mutants CW-1 and CW-2

Enzyme (no.)[b]	Sp act on given growth substrate (mU/mg of protein)[a]					
	Sp7				CW-1	CW-2
	Glycerol	Lactate	Malate	Gluconate	Gluconate	Gluconate
Gluconokinase (1)	0[c]	0.2	0	19.1	20.4	23.0
Gluconate 2-dehydrogenase (2)	0.8	1.0	2.1	1.2	0.7	0.5
Phosphogluconate dehydratase-6-phospho-2-keto-3-deoxygluconate aldolase (3 and 4)[d]	69.9	121	81.9	92.7	41.0	56.0
Phosphogluconate dehydrogenase (5)	0	0	0	0	0	0
Glucose-6-phosphate dehydrogenase (6)[e]	0.5	1.6	0.3	0.2	0.3	0.2
Glyceraldehyde-phosphate dehydrogenase (NAD^+) (7)	2,223	1,573	1,262	2,514	1,197	1,146
Glyceraldehyde-phosphate dehydrogenase ($NADP^+$) (8)	3.7	23.5	2.9	24.3	2.7	3.0
Phosphoglycerate kinase (9)	107	61.4	66.7	172	12.9	19.9
Phosphoglycerate mutase (10)	16.6	36.3	17.4	26.5	16.0	15.2
Enolase (11)	38.8	383	445	379	403	285
Pyruvate kinase (12)[f]	52.8	72.0	136.9	136	14.9	34.0
Fructose-bisphosphate aldolase (13)	15.5	22.0	13.5	36.0	12.8	14.9
Fructose bisphosphatase (14)	28.0	29.0	20.0	41.0	33.0	35.0
6-Phosphofructokinase (15)	0	0	0	0	0	0
Glucose-phosphate isomerase (16)	69.0	134	77.5	61.0	52.5	73.3
Glycerokinase (19)	17.5	1.2	2.1	1.1	1.2	0.6
Lactate dehydrogenase (20)	1.3	21.6	20.6	2.6	9.0	11.1
Malate dehydrogenase (21)	7,233	10,961	5,167	21,712	6,020	6,331

[a] Each substrate was tested singly in a medium with the basal salts of Dobereiner and Day (4) and 2.3 g of NH_4Cl per liter. Cultures were incubated aerobically at 30 ± 1°C. Enzyme activities in crude cell extracts were measured at room temperature, as described in the text. One enzyme unit is the amount of enzyme which catalyzes the formation of 1 μmol of product per min under the conditions of the assay. Specific activities represent averages of activities from at least two extracts from separate cell batches. All assays for each extract were in triplicate. The protein concentration in the extracts was determined by the Bio-Rad procedure.

[b] Glucokinase (no. 17) and glucose dehydrogenase (no. 18) were not assayed. The following millimolar extinction coefficients were used to calculate enzyme activities: NAD(P) and NAD(P)H at 340 nm, 6.22 (9); NADH at 366 nm, 3.4 (2); phosphoenolpyruvate at 230 nm, 3.0 (9), and 1.75 at 240 nm, (8); and the formazan product of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide reduction at 550 nm, 8.1 (9).

[c] Undetectable activity; <1.5 mU for gluconokinase and phosphogluconate dehydrogenase (9); <0.8 mU for 6-phosphofructokinase (9). Yeast phosphogluconate dehydrogenase (type V; Sigma Chemical Co.) and rabbit 6-phosphofructokinase (type III; Sigma) were run as positive controls to verify the assay procedures.

[d] Combined activity of two enzymes was measured.

[e] Yeast glucose-6-phosphate dehydrogenase (type XV; Sigma) was run as a positive control to verify the assay.

[f] It was necessary in the assay to use 7.1 mM phosphoenolpyruvate instead of the prescribed 0.71 mM to obtain anticipated activity.

comparable to induced levels found in *Pseudomonas* spp. (9) and *Rhizobium* spp. (11), where this pathway has been shown to be important in gluconate catabolism. The absence of phosphogluconate dehydrogenase (no. 5) in *A. brasilense* (Table 2) indicates the absence of the hexose monophosphate shunt (11) (Fig. 1). This observation and the presence of only very low levels of gluconate 2-dehydrogenase (no. 2) (Table 2) emphasizes the importance of the Entner-Doudoroff pathway. *A. brasilense* Sp7 appears to have a 2-ketogluconate pathway because it can grow on 2-ketogluconate as the sole carbon source (Table 1).

Glucokinase (no. 17) and glucose dehydrogenase (no. 18) (Fig. 1) are assumed to be absent in *A. brasilense* or unimportant catabolically because the organism cannot use glucose as a sole carbon source (20). A glucokinase (no. 17), even if it were present, would be of little assistance for glucose dissimilation in *A. brasilense* because the necessary follow-up enzymes, 6-phosphofructokinase (no. 15) and glucose-6-phosphate dehydrogenase (no. 6) are missing (Table 2). The absence of 6-phosphofructokinase (no. 15) indicates the absence of a catabolic Embden-Meyerhof-Parnas hexosephosphate route (Fig. 1) (9, 21) in this organism. On the other hand, the presence of fructose-bisphosphate aldolase (no. 13), fructose bisphosphatase (no. 14) and glucose-phosphate isomerase (no. 16) in all of the extracts (Table 2) testifies to an operation-

al anabolic hexosephosphate Embden-Meyerhof-Parnas route (6) (Fig. 1).

All of the Embden-Meyerhof-Parnas triosephosphate enzymes (no. 7 to 12) were present in substantial amounts in gluconate-grown cells of *A. brasilense* (Table 2), indicating that the triose part of glycolysis is functional. This triose pathway is most likely used anabolically when malate or lactate is available and indeed the triose enzymes were present when these substrates were the sole source of carbon for Sp7 (Table 2).

Among the substrates tested in *A. brasilense*, the shortest generation times were with malate and lactate (Table 1), suggesting an active Krebs cycle. This cycle has in fact been shown to be present through respirometry experiments (W. H.-T. Loh, personal communication). Furthermore, we have demonstrated that the necessary entry enzymes, malate (no. 21) and lactate dehydrogenase (no. 20), are present and inducible (Table 2). Another entry enzyme, glycerokinase (no. 19), is induced by glycerol in *A. brasilense* (Table 2) but growth here is very slow (Table 1).

If multiple mutations are discounted as the cause for the assorted enzyme defects in CW-1 and CW-2 (Table 2), and this seems likely (10, 14), then either phosphoglycerate (no. 9), or pyruvate kinase (no. 12) can be considered the logical target of the mutation in the mutants because these two enzymes are the most severely affected (Table 2). It would be premature at this point to suggest that repression or some other secondary response arising from a single mutation caused the other enzyme deficiencies in the mutants (Table 2). Multiple gene lesions have not yet experimentally been ruled out.

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