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L-Arabinose Metabolism in *Azospirillum brasiliense*†

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An oxidative pathway by which L-arabinose is converted to α-ketoglutarate in crude extracts of *Azospirillum brasiliense* is demonstrated. Specific activities of enzymes involved in the pathway were determined, and several pathway intermediates were identified.

Azospirillum brasiliense grows and fixes dinitrogen optimally when organic acids such as malate, succinate, lactate, and pyruvate are used as carbon and energy sources (7, 19, 24). Under nitrogen-fixing conditions, it grows poorly or not at all in glucose and sucrose (19, 24). Due to its generally poor growth in common sugars and to the fact that cell-free extracts have failed to show enhanced O_2 uptake with several sugars and sugar phosphates as substrates (20), it has been proposed that *A. brasiliense* lacks or has poorly developed glycolytic and pentose phosphate pathways (17, 20). The pentose arabinose, however, has been reported to support the growth of *A. brasiliense* (24). Child and Kurz (3) found that in certain cases nitrogenase activity in *A. brasiliense* could be enhanced by supplementing an organic acid-containing medium with arabinose. Okon et al. (21) have shown chemotactic attraction to arabinose by *A. brasiliense*. We have found that L-arabinose is an excellent sole carbon and energy source for growth and nitrogen fixation, yielding generation times and acetylene reduction rates comparable to those found with malate (N. J. Novick, Ph.D. thesis, University of Florida, Gainesville, 1980).

There are two well-documented pathways for L-arabinose metabolism which do not involve phosphorylated intermediates. In the first pathway, L-arabinose is oxidized to L-arabino-γ-lactone by an NAD(P)-dependent dehydrogenase. The lactone is cleaved by a lactonase to L-arabonic acid, followed by two successive dehydration reactions forming L-2-keto-3-deoxyarabonate (L-KDA) and α-ketoglutaric semialdehyde, respectively. The last step involves the NAD-dependent dehydrogenation of the semialdehyde to α-ketoglutaric acid. The second pathway has the same initial three steps, but L-KDA is cleaved through an aldolase reaction to glycolaldehyde and pyruvate. The first pathway has been demonstrated in *Pseudomonas saccharophila* (27), *Pseudomonas fragi* (4, 26) and the fast-growing rhizobia *R. meliloti*, *R. trifolii*, *R. phaseoli*, and *R. leguminosarum* (9, 10). The second pathway has been demonstrated in *Pseudomonas* sp. strain MSU-1 (5) and in the slow-growing rhizobia *R. japonicum* (9, 22) and *Rhizobium* sp. strain 32H1 (9). In this report, evidence is presented indicating that the first pathway (L-arabinose → α-ketoglutaric acid) is present in *A. brasiliense* (Fig. 1).

A. brasiliense (ATCC 29145) was obtained from the American Type Culture Collection. The growth medium employed was that of Nelson and Knowles (16), with twice the concentration of potassium phosphate buffer (65 mM, pH 7.1). L-Arabinose (37 mM) was filter sterilized and added to the autoclaved and cooled medium. Both NH_4Cl and N_2-grown cells yielded similar specific activities for enzymes involved in L-arabinose metabolism. Because of this and the ease of growing large batches of cells with a fixed nitrogen source, 0.1% NH_4Cl was used as a nitrogen source for enzyme studies.

Cell-free extracts were prepared in the following manner: cells near the middle or end of exponential growth were collected by centrifugation, washed twice in 0.1 M sodium–potassium phosphate buffer (pH 7.5), resuspended to ca. 0.25 g/ml in the same buffer, passed twice through an Aminco French pressure cell at 20,000 lb/in^2, and then centrifuged at 10,000 × *g* for 30 min. The crude extract could be stored for several months at −80°C without significant loss of enzymatic activity.

L-Arabinose dehydrogenase activity was determined by following the reduction of NAD(P) at 340 nm. The product of the dehydrogenation reaction was identified as L-arabino-γ-lactone by paper chromatography with the following solvents: (i) pyridine–1-butanol–water (6:4:3); (ii) 1-propanol–formic acid–water (6:3:1); and (iii) 1-butanol–1-propanol–water (10:7:5). Compounds were detected with alkaline silver nitrate reagent (18). The reaction product was chroma-

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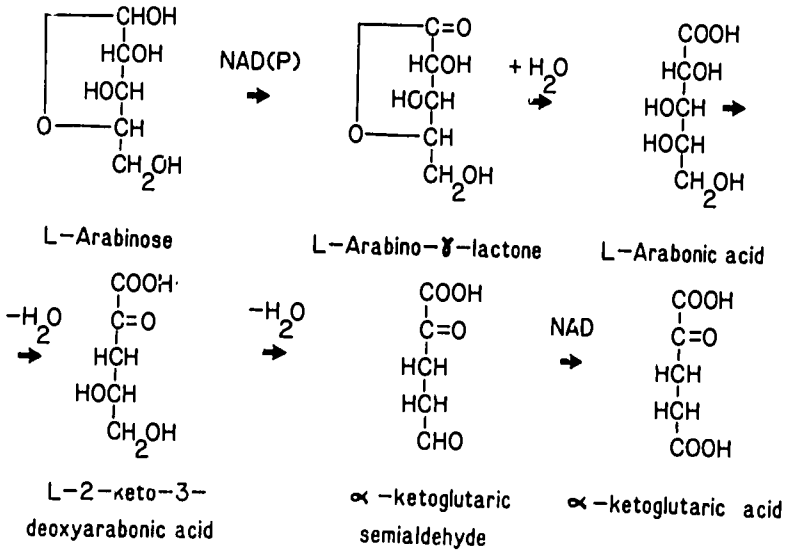

FIG. 1. Pathway of L-arabinose metabolism in *A. brasiliense*.

tographed against L-arabino-γ-lactone, which was produced by boiling potassium arabonate in 0.2 N HCl for 5 min. Potassium arabonate was prepared by the hypoiodite-in-methanol oxidation of L-arabinose (15).

Lactonase activity was measured by the method of Dahms and Anderson (6) with alkaline hydroxylamine (11) to quantitate the disappearance of L-arabino-γ-lactone.

The L-arabonate dehydratase assay was that of Pedrosa and Zancan (22), with the keto acid accumulation quantified using semicarbazide reagent (14). The reaction product was identified as L-KDA by paper chromatography in the solvents described above. Samples were chromatographed against DL-KDA prepared by the method of Stoolmiller and Abeles (23). In addition, the 2,4-dinitrophenylhydrazone of the reaction product comigrated with derivatized DL-KDA on Silica Gel G plates in 1-butanol saturated with 3% NH_4OH. The hydrazones of the acids were prepared by the method of Cavallini et al. (2).

The specific activities of the first three enzymes involved in L-arabinose metabolism are reported in Table 1. KDA aldolase was assayed in the reverse direction (22), with KDA formation being followed by the thiobarbituric acid method (28). Aldolase activity was not detected in any extract tested.

When the route of KDA metabolism was being determined, it was found that potassium arabonate or DL-KDA rapidly reduced NAD in crude extracts. DL-KDA reduced NAD at about three times the rate of potassium arabonate (62 versus 21 nmol/min per mg of protein). This rapid reduction of NAD in the presence of arabonate or KDA has also been demonstrated in *P. saccharophila* (25, 27), a bacterium capable

TABLE 1. Activities of L-arabinose pathway enzymes

Enzyme	Cofactor	Activity[a] (nmol/min per mg of protein)
L-Arabinose dehydrogenase[b]	NAD	70
	NADP	30
Arabonolactonase[c]		13
L-Arabonate dehydratase[d]		17
L-KDA aldolase[e]		ND[f]

[a] Protein was determined by the method of Lowry et al. (13). Values are the average of three crude extract preparations. All assays were carried out at 25°C.

[b] The reaction mixture contained 125 μmol of glycine-NaOH buffer (pH 9.0), 0.5 μmol of NAD or NADP, 0.5 μmol of L-arabinose, and enzyme in a total volume of 1 ml. The reduction of NAD(P) was followed at 340 nm.

[c] The reaction mixture (6) contained 10 μmol of L-arabino-γ-lactone, 40 μmol of Tris-hydrochloride buffer (pH 7.5), and enzyme in a total volume of 0.15 ml. The disappearance of lactone was quantitated with hydroxylamine-NaOH reagent (11).

[d] The reaction mixture (22) contained 20 μmol of HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.2), 25 μmol of potassium arabonate, 5 μmol of magnesium chloride, and enzyme in a total volume of 0.40 ml. Keto acid accumulation was quantitated with semicarbazide reagent (14).

[e] The reaction mixture (22) contained 10 μmol of HEPES buffer (pH 7.8), 5 μmol of sodium pyruvate, 5 μmol of glycolaldehyde, 5 μmol of magnesium chloride, and enzyme in a total volume of 0.35 ml. The production of KDA was determined by the thiobarbituric acid method (28).

[f] ND, Not detected.

TABLE 2. Accumulation of α-ketoglutarate in crude extracts of L-arabinose-grown cells

Reaction mix[a] addition	α-Ketoglutarate accumulation (μmol)
Sodium arsenite	7.6
None	4.4
Control[b]	0.5

[a] The potassium arabonate oxidation mixture contained 60 μmol of potassium phosphate buffer (pH 7.2), 10 μmol of NAD, 10 μmol of potassium L-arabonate, 1 μmol of sodium arsenite (or water), and enzyme in a total volume of 1 ml. Reactions were run at 25°C. After 6 h of incubation, α-ketoglutarate was enzymatically quantified in a reaction mixture containing 80 μmol of potassium phosphate buffer (pH 7.2), 0.32 μmol of excess NADH, 40 μmol of NH_4Cl, 3.3 U of glutamate dehydrogenase (Sigma), and 0.05 ml of potassium arabonate oxidation mixture in a total volume of 1 ml. The amount of NADH oxidized was determined against controls which did not contain glutamate dehydrogenase or ammonium chloride or either to correct for NADH oxidase activity.

[b] Controls were also run using the arabonate oxidation mixture to which potassium arabonate had not been added.

of catalyzing a pathway leading to α-ketoglutarate. This fact plus the lack of KDA aldolase activity indicated a pathway leading to α-ketoglutarate. We confirmed this by examining the accumulation of α-ketoglutarate in crude extracts. Potassium arabonate and NAD (both 10 μmol) were added to crude extracts, and the reaction mixture was incubated for up to 6 h. The accumulation of α-ketoglutarate was quantified enzymatically by the oxidation of NADH upon the addition of glutamate dehydrogenase. In the presence of sodium arsenite, an inhibitor of α-ketoglutarate dehydrogenase (12, 26), 76% of the added potassium arabonate accumulated as α-ketoglutarate (Table 2). In the absence of sodium arsenite, 44% of the arabonate accumulated as α-ketoglutarate. In addition, the 2,4-dinitrophenylhydrazone of the reaction product comigrated with derivatized α-ketoglutarate (Sigma Chemical Co.) by the method previously described. Neither pyruvate, assayed with lactic dehydrogenase, nor glycolaldehyde, detected with diphenylamine reagent (8), accumulated in crude extracts.

α-Ketoglutarate semialdehyde was not identified in any extract tested. The semialdehyde, though, has been demonstrated as the intermediate between L-KDA and α-ketoglutarate in *Pseudomonas* (4, 23) and *Rhizobium* (9, 10) species and is a probable intermediate in the *A. brasiliense* pathway.

A totally oxidative pathway by which L-arabinose is metabolized in crude extracts of *A. brasiliense* has been demonstrated (Fig. 1). L-Arabinose dehydrogenase activity was not detected when *A. brasiliense* was grown on malate or succinate. The ability to metabolize L-arabinose may offer some distinct advantages to this soil bacterium. Plant cell walls are rich in compounds with the L-arabino-configuration (i.e., L-arabinose, D-fucose, and D-galactose) (1). It is possible that root cell death and the sloughing off of cells provide root-associated bacteria which are able to metabolize these sugars (e.g., *A. brasiliense*) with a plentiful substrate.

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