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The DeLey-Doudoroff Pathway of Galactose Metabolism in *Azotobacter vinelandii*

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Azotobacter vinelandii **cell extracts reduced NAD^+ and oxidized D-galactose to galactonate that subsequently was converted to 2-keto-3-deoxy-galactonate. Further metabolism of 2-keto-3-deoxy-galactonate required the presence of ATP and resulted in the formation of pyruvate and glyceraldehyde 3-P. Radiorespirometry indicated a preferential release of CO_2 at the first carbon position of the D-galactose molecule. This suggested that *Azotobacter vinelandii* metabolizes D-galactose via the DeLey-Doudoroff pathway. The first enzyme of this pathway, D-galactose dehydrogenase, was partially characterized. It has a molecular weight of about 74,000 Da and an isoelectric point of 6.15. The pH optimum of the galactose dehydrogenase was about 9. The apparent K_ms for NAD^+ and D-galactose were 0.125 and 0.56 mM, respectively. Besides D-galactose, the active fraction of this galactose dehydrogenase also oxidized L-arabinose effectively. The electron acceptor for D-galactose or L-arabinose oxidation, NAD^+, could not be replaced by $NADP^+$. These substrate specificities were different from those reported in *Pseudomonas saccharophila*, *Pseudomonas fluorescens*, and *Rhizobium meliloti*.**

Carbohydrate metabolism has been studied extensively among the enteric bacteria. However, much less is known about the carbohydrate metabolism of those microbes living in soil. Unlike the intestinal environment, where nutrients are always available, soil is often poor in nutrients. It is likely that soil microbes may use a different strategy to utilize their resources. *Azotobacter vinelandii* is a free-living soil bacterium. This organism can grow on a wide variety of carbohydrates to support nitrogen fixation and growth (2).

The metabolism of D-glucose in *A. vinelandii* has been documented by Mortenson and Wilson (8, 9) and subsequently confirmed by Still and Wang (11). These authors showed that *A. vinelandii* hydrolyzed D-glucose via the Entner-Doudoroff (ED) pathway: D-glucose → D-glucose 6-P → 2-keto-3-deoxy-6-P-gluconate → pyruvate and D-glyceraldehyde 3-P. The carboxyl group of the pyruvate originates from the first carbon of the D-glucose molecule. Oxidation of pyruvate to acetyl coenzyme A releases the first carbon as CO_2. This preferential release of CO_2 from the first carbon of D-glucose during early stages of catabolism is a major characteristic of the ED pathway. In contrast, the more commonly used Embden-Meyerhof-Parnas pathway releases the third carbon of D-glucose preferentially.

A. vinelandii metabolizes melibiose exocellularly to D-glucose and D-galactose (16). Cells growing on melibiose transport both sugars simultaneously (19). It seems logical that galactose, once inside the cell, would be converted to D-glucose or an intermediate of glucose metabolism. Most organisms convert galactose to glucose 6-P by the Leloir pathway (7): D-galactose→ D-galactose 1-P → D-glucose 1-P → D-glucose 6-P. A few species of bacteria have been shown to possess enzymes similar to those of the ED pathway to metabolize D-galactose (5). This enzyme pathway, called the DeLey-Doudoroff (DD) pathway, metabolizes D-galactose as follows: D-galactose → D-galactonate → 2-keto-3-deoxy-galactonate → 2-keto-3-deoxy-6-P-galactonate → pyruvate and D-glyceraldehyde 3-P. The aim of the present study was to determine the metabolic pathway of galactose in *A. vinelandii*.

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MATERIALS AND METHODS

Chemicals. D-galactose, D-glucose, pyruvic acid, glyceraldehyde 3-P (G-3-P), 2-deoxy-D-galactose, 6-deoxy-α-galactopyranose, D-fructose, D-mannose, D-ribose, L-arabinose, 6-phosphogluconic acid, glucose 6-P, D-galactonate-γ-lactone, 2,6-dichlorophenol-indophenol (DCIP), lactate dehydrogenase, G-3-P dehydrogenase, pyruvate kinase, NAD^+, $NADH^+$, coenzyme A, and 1-^{14}C-D-galactose were obtained from Sigma Chemical Co. (St. Louis, Mo.). All other chemicals were reagent grade from Fisher Scientific Co.

Organism and growth conditions. *A. vinelandii* OP (ATCC 13705) was grown in modified Burk's buffer (17) containing 1% D-galactose under conditions described previously (15). The mid-exponential-phase cells were lysed by a chilled French pressure cell at 11,000 lb/in^2. The cytoplasmic fraction was obtained by differential centrifugation as described previously (15).

Enzyme assays. The cytoplasmic fraction was used to determine the metabolic pathway of D-galactose. Galactose dehydrogenase activity was determined by monitoring the reduction of NAD^+ at 340 nm. The reaction mixture contained 1 mM D-galactose and 0.5 mM NAD^+ in a total of 1 ml of 50 mM Tris-HCl buffer (pH 9). Reaction was initiated by the addition of the cytoplasmic fraction. Galactonate dehydratase was assayed by the method of Dahms et al. (4). The formation of 2-keto-3-deoxy-galactose (KD-gal), the product of galactonate dehydratase, was trapped by semicarbazide reagent and quantitated spectrophotometrically at 250 nm. A suitable substrate was not available commercially for the detection of 2-keto-3-deoxy-6-P-gluconate aldolase (KDP-glu aldolase). Therefore, KDP-glu aldolase was assayed as 6-P-gluconate dehydratase–KDP-glu aldolase with gluconate 6-P as the substrate. The products of aldolase, pyruvate and G-3-P, were quantitated as described previously (1, 5). Each tube of a set of 10 reaction mixtures contained 1 mM tested substrate, 1 mM ATP, 1 mM NAD^+ or $NADP^+$, 1.5 mM hydrazine, and 3.3 mM $MgCl_2$ in a final volume of 6 ml of 50 mM Tris-HCl buffer (pH 7.8). The

reaction mixture was incubated at 33°C and initiated by the addition of 0.3 to 0.6 ml of the cytoplasmic fraction. At intervals (ranging from 0 to 90 min), the reaction in the tube was stopped by placing the tube in a boiling water bath for 60 s. The protein precipitated was removed by centrifugation. Hydrazine was extracted three times by a total of 1 ml of benzaldehyde. Excess benzaldehyde was removed by 3 ml of ether. The recovered pyruvate and G-3-P were quantitated by enzymatic methods with lactate dehydrogenase and α-glycerophosphate dehydrogenase, respectively. The recovery rates of pyruvate and G-3-P by the procedure described above with authentic compounds were about 87 to 93%. Hexokinase and galactokinase were detected according to the methods of Porter and Chassy (10) and Wilson and Schell (14), respectively. Galactose-1-P-uridyl-transferase (UDP-transferase) was detected following the procedures described in assay kit 195 from Sigma.

Radiorespirometric assay. Carbon dioxide released by *A. vinelandii* growing on D-galactose was determined gravimetrically. Exponential-phase cells growing on D-galactose (150 ml) were washed twice in Burk's buffer. Cells were suspended in 10 ml of Burk's buffer. Seventy microliters of the radioactive 1-^{14}C-galactose (specific activity, 59 μCi/μmol) was transferred and dried in a 250-ml flask. The isotope was suspended in 40 ml of Burk's buffer containing 1.11 mmol of galactose. The experiment was initiated with the addition of 10 ml of washed cells. The flask was immediately sealed with a rubber stopper with two outlets. The sample was aerated rapidly by a magnetic stirrer. Atmospheric moisture and CO_2 entering the flask from one outlet were removed through a phosphorus pentoxide trap and an ascarite trap, respectively. The air outlet of the flask was connected to a U-tube containing phosphorus pentoxide and then to a preweighed train of U-tubes to trap CO_2 and moisture. The trapping train consisted of two U-tubes containing 10 ml of CO_2-trapping solution composed of ethanolamine and ethyl cellosolve (1:6). A tube of phosphorus pentoxide was used to recount the moisture evaporated from the CO_2 traps. Two guard tubes were used to prevent atmospheric CO_2 and water from entering the U-tubes to be weighed. A vacuum pump was connected to the end of the guard tubes to generate a negative pressure sufficient to allow a slow stream of air to bubble through the U-tubes. After 1 h of incubation, the amount of CO_2 trapped was weighed, and the $^{14}CO_2$ in the CO_2-trapping solution was determined by counting the sample in a scintillation counter (Beckman Model LS7000). CO_2 recovery by the procedure described above was better than 96%.

Kinetic assay of galactose dehydrogenase. The enzyme kinetics and substrate specificity of the D-galactose dehydrogenase were analyzed with partially purified preparations. The cytoplasmic fraction of galactose-grown cells was concentrated and dialyzed by Diaflo ultrafiltration XM50 membrane (Amicon Corp., Lexington, Mass.) under a nitrogen atmosphere. The concentrated sample was separated by fast protein liquid chromatography (FPLC) (Pharmacia LKB Biotechnology); the sample was adsorbed in a Mono Q anion-exchange column and was eluted by 20 ml of linear NaCl gradient (0 to 0.5 M) in 10 mM Tricine buffer (pH 8.2). The active fraction was detected by the decolorization of 2,6-dichlorophenol-indophenol in the presence of galactose. Once separated from the column, the active fraction was very unstable (half-life of the enzyme at 4°C was about 2 h). The active fraction was immediately desalted with a Sephadex G-20 column. Once desalted, the enzyme was assayed immediately.

The isoelectric point of the galactose dehydrogenase was determined with a PBE 94 chromatofocusing column (Pharmacia LKB Biotechnology) at a pH range of 5 to 8. The molecular weight of the galactose dehydrogenase was determined by high-pressure liquid chromatography (HPLC) (ISCO model 2350) with a GF-250 column.

Protein determinations were made by the method of Lowry et al. (6) with bovine serum albumin as the standard.

TABLE 1. Detection of various enzymes in the cytoplasmic fractions of galactose- and glucose-grown cells

Enzyme	Specific activity (μmol oxidized/min/mg of protein)[a]	
	Galactose-grown cells	Glucose-grown cells
Galactokinase	0.00	0.00
UDP-transferase	0.00	0.00
Galactose dehydrogenase	60.00	0.00
KDP-glu aldolase	0.00	431.00
Hexokinase	0.01	88.00

[a] Data are the means of two different cultures.

RESULTS AND DISCUSSION

Analysis of the presence of the key enzymes of the Leloir pathway (galactokinase and UDP-transferase), the DD pathway (galactose dehydrogenase), and the ED pathway (hexokinase and 2-keto-3-deoxy-6-P-glu aldolase) found in the cytoplasmic fraction of galactose-grown cells is summarized in Table 1. Similar fractions from glucose-grown cells were also used for comparison. *A. vinelandii* growing on D-galactose lacked hexokinase and galactokinase to phosphorylate D-galactose. Instead, galactose dehydrogenase was detected in galactose-grown cells.

Detection of an active galactose dehydrogenase in galactose-grown cells strongly suggested the presence of the DD pathway. If such a pathway was indeed functional, galactonate would be metabolized to KD-gal, which subsequently is converted to equal molar concentrations of pyruvate and G-3-P. To elucidate this, the cytoplasmic fraction was coincubated with D-galactose (or galactonate) in the presence of appropriate substrates. At the end of the incubation, KD-gal, pyruvate, and G-3-P were extracted from the reaction mixture and quantified.

The rates of KD-gal, pyruvate, and G-3-P formed by the cytoplasmic fraction of galactose-grown cells with various substrates are shown in Table 2. No KD-gal was detected when only D-galactose was added to the cytoplasmic fraction. The formation of KD-gal from D-galactose required the presence of NAD^+. KD-gal was detected when galactonate was added to the cytoplasmic fraction without the presence of NAD^+.

Pyruvate and G-3-P were detected when ATP was added to samples containing KD-gal. The ratio of pyruvate formed to G-3-P formed was close to 1. Although the lack of a suitable substrate (2-keto-3-deoxy-6-P-galactonate [KDP-gal]) prevented the direct assay of aldolase activity, our results suggested the following sequence of D-galactose oxidation: D-galactose → galactonate → KD-gal → KDP-gal → pyruvate and G-3-P.

The in vitro demonstration of the DD pathway was confirmed by in vivo analysis of CO_2 formation. Like KDP-glucose, hydrolysis of KDP-gal should result in early release of the first carbon of the D-galactose molecule as CO_2. To the best of our knowledge, however, no radiorespirometry data have been reported on the DD pathway. Difficulty in obtaining isotopes labeled at specific positions may be the cause. To

TABLE 2. Formation of 2-keto-3-deoxy-galactonate, pyruvate, and G-3-P by the cytoplasmic fractions of galactose-grown cells with various substrates[a]

Substrate(s)	Concn (μmol/min/mg of protein) of[b]:		
	KD-gal	Pyruvate	G-3-P
Galactose	0.00	0.00	0.00
Galactose + NAD^+	47.3 (±8)	0.00	0.00
Galactose + ATP	0.00	0.00	0.00
Galactose + ATP + NAD^+	4 (±2)	33.30 (±0.5)	32.17 (±0.7)
Galactonate	51 (±2)	0.00	0.00
Galactonate + NAD^+	52 (±2)	0.00	0.00
Galactonate + ATP	3 (±1)	34.80 (±0.4)	32.96 (±0.6)

[a] The reaction mixtures contained 1 mM substrate, 3.3 mM $MgCl_2$, and 1.5 mM hydrazine in a total of 6 ml of Tris-HCl buffer (50 mM; pH 7.8). NAD and ATP, when needed, were at 1 mM concentrations. The reaction mixtures were incubated at 33°C.
[b] Data are means (± standard deviations) from four independent experiments.

overcome this problem, we calculated the percentage of carbon (as CO_2) released from the total carbon (as galactose) added to the medium. We then calculated the percentage of radioactive carbon released from the total radioactive carbon (as 1-^{14}C-galactose) included in the medium.

About 23% of the total galactose carbon was trapped after 1 h of incubation, yet the radioactivity count found in the same trapping solution was about 39% of that of the original counts added to the Burk's medium. Since the first carbon of the galactose molecule was labeled with ^{14}C, our results suggested that the DD pathway was functional in *A. vinelandii* in vivo.

The ratio of isotopic CO_2 to total CO_2 released was highest during the initial growth period. This ratio decreased after prolonged incubation because carbons from other positions were eventually oxidized to CO_2. Shorter incubation (less than 1 h) should produce an even higher ratio. However, the small amount of CO_2 produced made the gravimetric measurement less accurate.

A literature search of the last 30 years revealed only limited information on the DD pathway. The first enzyme of the DD pathway in *Gluconobacter liquefaciens* involves a membrane-bound galactose dehydrogenase (12). This membrane-bound dehydrogenase oxidizes galactose to galactonate without NAD^+ and $NADP^+$. All other galactose dehydrogenases reported (1, 3, 13) are cytoplasmic and require NAD^+.

The cytoplasmic DD pathway and its first enzyme from *Pseudomonas saccharophila* (13) and *Pseudomonas fluorescens* (3) and partially from *Rhizobium meliloti* (1) have been characterized. Attempts to purify the galactose dehydrogenase from *A. vinelandii* by the procedures described in the literature were unsuccessful. This, together with the kinetics results, suggested that the composition of the *A. vinelandii* enzyme differed from that in other organisms.

Chromatofocusing showed that the galactose dehydrogenase eluted at an isoelectric point of 6.15. The apparent molecular weight of the enzyme was estimated to be 74,000 Da by HPLC gel filtration chromatography. The enzyme could be partially purified by FPLC with a Mono Q column. The active fraction (about four- to fivefold purification) from FPLC exhibited multiple peptide bands in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). Further purification of the enzyme from the active FPLC fraction was unsuccessful as the enzyme rapidly degraded and enzyme recovery was low. Thus, the partially purified enzyme was used immediately for kinetics and substrate specificity studies.

The optimum pH for galactose dehydrogenase activity was about 9.0. Kinetic analysis of galactose dehydrogenase showed typical Michaelis-Menten kinetics with regard to both NAD^+ and D-galactose. The K_ms for D-galactose and NAD^+ were 0.56 and 0.125 mM, respectively.

The oxidation of D-galactose was highly specific for NAD^+ (specific activity, 260 μmol/min/mg of protein), which could not be replaced by $NADP^+$ (specific activity, 0.3 μmol/min/mg of protein). Besides D-galactose, this fraction could oxidize 6-deoxy-D-α-galactopyranose (1 mM) partially (specific activity, 80 μmol/min/mg of protein). L-arabinose (1 mM) was a better substrate than D-galactose (specific activity, 390 μmol/min/mg of protein). The enzyme could not oxidize D-glucose, D-fructose, D-mannose, D-ribose, or 1-deoxy-D-galactose.

We previously showed that *A. vinelandii* cannot grow on L-arabinose (18). Interestingly, we found that cells growing on galactose could oxidize L-arabinose at a rate even faster than that of galactose. Although the enzyme was not purified to homogeneity, evidence suggested that the arabinose dehydrogenase activity was an integral part of the galactose dehydrogenase because (i) active fractions of galactose dehydrogenase

TABLE 3. Characteristics and substrate specificities of D-galactose dehydrogenases from *A. vinelandii*, *P. fluorescens*, *P. saccharophila*, and *R. meliloti*

Organism	K_m (mM)		Specific activity (μmol/min/mg of protein)[a]			Physical characteristics			Reference
	NAD^+	Galactose	NAD^+		$NADP^+$, D-galactose	Mol wt	pI	Optimal pH	
			D-Galactose	L-Arabinose					
A. vinelandii	0.125	0.5	260 (100)	390 (150)	0.5 (0.2)	73,000	6.15	9.0	This study
P. fluorescens	0.24	0.7	849 (100)	849 (100)	272 (32)	67,000	4.28	9.1–9.5	3
P. saccharophila	0.14	1.0	202 (100)	<6 (3)	0.4 (0.2)	103,000	5.13	8–9	13
R. meliloti	NA[b]	NA	36 (100)	76 (211)	30 (86)	NA	5.1	NA	1

[a] Specific activity is reported as a reduction in NAD^+ or $NADP^+$. Parenthetical numbers are percentages of reduction.
[b] NA, not available.

isolated with different methods (including ammonium sulfate precipitation, chromatofocusing, gel filtration, and FPLC) all exhibited arabinose dehydrogenase activity, (ii) no arabinose dehydrogenase was detected in cells growing on glucose or acetate, and (iii) the rates of NAD^+ reduction with a mixture of arabinose plus galactose and with arabinose alone were the same.

The galactose dehydrogenases among the bacteria so far examined were heterogeneous (Table 3). The molecular weights of these enzymes ranged from 103,000 to 67,000. The isoelectric points ranged from 4.28 to 6.15. The cofactor binding moiety of the enzyme could recognize either NAD^+ or both NAD^+ and $NADP^+$. Although the steric configurations of the C-2 and C-6 positions of galactose and the C-1 and C-5 positions of arabinose were similar (1), the substrate binding moiety of the enzyme in *P. saccharophila* was highly specific for galactose. The enzyme in *P. fluorescens* oxidized arabinose as effectively as galactose while those in *A. vinelandii* and *R. meliloti* oxidized arabinose faster than galactose.

Galactose is an important component of the outer cell wall of most gram-negative bacteria. It is possible that the DD pathway can serve in galactose genesis for these microbes. The heterogeneity of the key enzyme of the DD pathway found in these bacteria may reflect the physiological differences among these organisms.

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