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Pathways for Metabolism of Ketoaldonic Acids in an *Erwinia* sp.

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The pathways involved in the metabolism of ketoaldonic acids by *Erwinia* sp. strain ATCC 39140 have been investigated by use of a combination of enzyme assays and isolation of bacterial mutants. The catabolism of 2,5-diketo-D-gluconate (2,5-DKG) to gluconate can proceed by two separate NAD(P)H-dependent pathways. The first pathway involves the direct reduction of 2,5-DKG to 5-keto-D-gluconate, which is then reduced to gluconate. The second pathway involves the consecutive reduction of 2,5-DKG to 2-keto-L-gulonate and L-idonic acid, which is then oxidized to 5-keto-D-gluconate, which is then reduced to gluconate. Gluconate, which can also be produced by the NAD(P)H-dependent reduction of 2-keto-D-gluconate, is phosphorylated to 6-phosphogluconate and further metabolized through the pentose phosphate pathway. No evidence was found for the existence of the Entner-Doudoroff pathway in this strain.

The ketogenic properties of a variety of microorganisms have been known for many years (6, 18). The conversion of D-glucose to the ketogluconates 2-keto-D-gluconate (2-KDG), 5-keto-D-gluconate (5-KDG), and 2,5-diketo-D-gluconate (2,5-DKG) is mediated by membrane-bound dehydrogenases linked to the cytochrome chain (4, 24, 28). We have been interested in the subsequent metabolism of the ketogluconates, some of which can serve as the sole source of carbon and energy for various bacteria (6, 13). These compounds are converted to 6-phosphogluconate (6-PGA), which is further metabolized through the Entner-Doudoroff and/or pentose phosphate pathways. Two different metabolic routes from the ketogluconates to 6-PGA have been described. The first, observed in *Erwinia herbicola* (16, 17) and a *Corynebacterium* sp. (26), begins with the enzyme-catalyzed reduction of the ketogluconate by NAD(P)H to gluconate (GA); this step is followed by phosphorylation to 6-PGA. The second route has been best studied in *Pseudomonas* spp. and begins with the phosphorylation of 2-KDG at the 6 position; this step is followed by reduction to 6-PGA (13, 18, 21, 33). Soluble NAD(P)H-dependent enzymes capable of reducing either 2-KDG or 5-KDG to GA have been isolated from various acetic acid bacteria (1, 3, 10, 12), suggesting that the former route is used in these organisms.

Erwinia sp. strain ATCC 39140 grows well on glucose, GA, ketogluconates such as 2-KDG, 5-KDG, and 2,5-DKG, and the related compounds 2-keto-L-gulonate (2-KLG) and L-idonate (IA). The pathways for the metabolism of these compounds were deduced from enzyme assays and isolation of mutants unable to grow on GA or the ketogluconates. Our results demonstrate that in this microorganism, the ketogluconates are reduced to GA, which is then phosphorylated to 6-PGA, which is further metabolized via the pentose phosphate pathway.

MATERIALS AND METHODS

Chemicals. Calcium 2,5-DKG, sodium 2-KLG, and sodium IA were provided by T. Kevin Murphy, Pfizer. Purity was ascertained by high-pressure liquid chromatography (HPLC) (see below). Sodium GA, calcium 2-KDG, and potassium 5-KDG were purchased from Sigma.

Bacterial strains. *Escherichia coli* ATCC 31446 and *Erwinia* sp. strain ATCC 39140, previously classified as *Acetobacter cerinus*, were obtained from the American Type Culture Collection. *Pseudomonas putida* ATCC 12633 was obtained from the Pfizer culture collection.

Growth of cells. Cultures were routinely grown at 30°C in Luria broth or in mannitol medium consisting of mannitol (25 g/liter), yeast extract (5 g/liter), and peptone (3 g/liter) (11). For mutant isolation and characterization, a semidefined medium, known as modified ML5, was devised and contained the following: 1 g of $(NH_4)_2SO_4$, 2 g of K_2HPO_4, 3 g of KH_2PO_4, 1 g of $MgSO_4 \cdot 7H_2O$, 10 g of sodium 2-(*N*-morpholino)-ethanesulfonic acid, 0.1 g of yeast extract, 0.4 mg of nicotinic acid, 0.5 mg of calcium pantothenate, 10 mg of nitrilotriacetic acid, 10 mg of $FeSO_4$, and 10 mg of $MnSO_4$ per liter and 27 mM carbon source. The initial pH was adjusted to 6.1 with HCl, and the medium was sterilized by filtration. When solid medium was required, 15 g of agar per liter was added. Mutants that did not utilize GA for growth and that also grew poorly on glucose were given a mixture of glucose and fructose. For Entner-Doudoroff assays, *P. putida*, *E. coli*, and the *Erwinia* sp. were grown in modified ML5 with 2-KDG or GA as the carbon source.

Preparation of crude extracts. Cells were harvested by centrifugation, resuspended in 20 mM Tris-HCl (pH 7.5), and broken by sonication (three 30-s pulses for the *Erwinia* sp. and *E. coli* and five 10-s pulses for *P. putida*). Nonlysed cells and debris were removed by centrifugation at 27,000 × *g* for 1 h.

Assays. Ketoaldonate reductase activities were assayed spectrophotometrically at 340 nm with 100 mM substrate (10 mM for 2,5-DKG) and 0.2 mM NADH or NADPH in 0.2 M bis-Tris-HCl (pH 7.0). Oxidative activities were measured with 100 mM substrate and 0.2 mM NAD^+ or $NADP^+$ in 40 mM sodium carbonate (pH 10).

The overall activities of the Entner-Doudoroff pathway enzymes 6-phosphogluconate dehydratase (EC 4.2.1.12) and 2-keto-3-deoxy-6-phosphogluconate aldolase (EC 4.2.1.14) were assayed with 6-PGA as the substrate under conditions previously described (2). In some experiments, an Aminex

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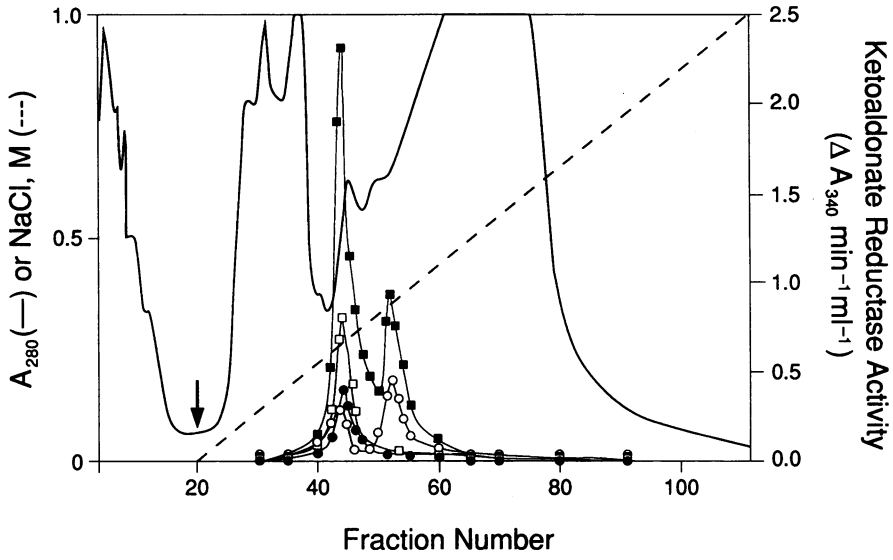

FIG. 1. Separation of ketoaldonate reductase activities in *Erwinia* sp. strain ATCC 39140 by ion-exchange chromatography on DEAE-cellulose. Symbols: ○, 5-KDG activity; ●, 2-KLG activity; ■, 2,5-DKG activity; □, 2-KDG activity. A linear NaCl (0 to 1 M) gradient was started at the arrow. Activities shown were obtained with NADH as a cofactor; similar activities were obtained with NADPH, except for the second peak of 2,5-DKG-dependent activity, which was not present with NADPH. The activity peaks eluted at 0.27 and 0.35 M NaCl.

HPX-87H HPLC column (Bio-Rad) was used to quantitate pyruvate. The column was run isocratically with 0.01 N sulfuric acid and monitored with a UV detector at 210 nm; sodium pyruvate (Sigma) was used as the standard.

6-Phosphogluconate dehydrogenase (GND; EC 1.1.1.44) activity was assayed spectrophotometrically essentially as described previously (32). Gluconate kinase (GNK; EC 2.7.1.12) activity was assayed spectrophotometrically by coupling it to the GND reaction (7). Specific activities are defined as micromoles of product formed per minute per milligram of protein. Protein assays were performed by the method of Bradford with bovine serum albumin as the standard (9).

Partial purification of the ketoaldonate reductases was carried out by DEAE-cellulose chromatography. A crude extract (20 ml) from a culture grown in Luria broth was prepared as described above and loaded onto a column (2.4 by 11 cm^2) of DE 52 resin (Whatman) equilibrated with 20 mM Tris-HCl (pH 7.5). The column was washed with the same buffer until a stable baseline (A_{280}) was obtained, and elution was done with a 600-ml linear gradient (0 to 1 M NaCl) at 1.1 ml/min. Fractions (6.6 ml) were collected and assayed for ketoaldonate reductase activity as described above. Active fractions were further characterized to determine their substrate and cofactor requirements and the products formed.

Determination of the products formed from the different ketoaldonates was carried out as previously described for 2,5-DKG (19) in a total volume of 0.2 ml containing 100 mM bis-Tris-HCl (pH 7.0), 25 mM substrate, 25 mM cofactor, and 0.1 ml of enzyme extract for 12 h at 25°C. Ketoaldonic and aldonic acids were identified by ion-exchange HPLC with Aminex A28 anion-exchange resin; products were confirmed by gas chromatography as previously described (15).

Pyridine nucleotide-linked dehydrogenases present in crude extracts and partially purified fractions were detected by activity staining of nondenaturing polyacrylamide gels (22, 29). Native gel electrophoresis followed by activity staining was used to identify the enzymes described, to verify substrate and cofactor specificities, and to corroborate the absence of activity in putative mutants.

Mutagenesis. Cells were grown in mannitol medium to an A_{550} of 0.4 to 0.5, pelleted, and resuspended in 0.05 M Tris-maleic acid buffer (pH 6.0). *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was added to a final concentration of 150 μg/ml. After incubation for 30 min at 30°C without shaking, cells were washed and plated for viable count determinations. The decrease in viability under these conditions varied between 40 and 60%.

Mutant isolation. Mutagenized cells were plated on modified ML5 medium containing mannitol as the carbon source and then replica plated on the same medium with GA, 2-KDG, 2-KLG, 5-KDG, or 2,5-DKG as the carbon source. Colonies unable to grow on one or more of these carbon sources were analyzed further. In addition, mutagenized cells were plated on a medium with 2-KDG and tetrazolium chloride (8); white (non-2-KDG-utilizing) colonies were picked for further evaluation.

Potential mutants were retested for growth in liquid modified ML5 medium containing GA, 2-KDG, 2-KLG, 5-KDG, 2,5-DKG, or IA as the carbon source. Single colony isolates of putative mutants were assayed for the relevant enzymatic activities as described above.

RESULTS

Enzyme activities. The presence of various cytosolic NAD(P)H-dependent ketoaldonate reductases in *Erwinia* sp. strain ATCC 39140 was initially determined by measuring substrate-dependent activities in the crude cytosolic extract spectrophotometrically. The enzymes were partially purified by chromatography on DEAE anion-exchange resin to further characterize the specific ketoaldonate reductases present (Fig. 1). Column fractions were assayed with either NADH or NADPH and the different ketoaldonate substrates. Product formation was monitored by HPLC and gas chromatography following incubations of substrate and co-

TABLE 1. *Erwinia* sp. strain ATCC 39140 ketoaldonate reductase data

Enzyme	DEAE elution (M NaCl)	Substrate	Product	Cofactor	Mutant
2KR	0.27	2-KLG	IA	NADPH or NADH	None found
			2-KDG	GA	
		2,5-DKG	5-KDG		
5KR(G)	0.27	5-KDG	GA	NADPH or NADH	187-6
5KR(I)	0.35	5-KDG	IA	NADPH or NADH	139-2
2,5-DKGR	0.35	2,5-DKG	2-KLG	NADH	None found

factor with either crude extract or partially purified enzyme (15). In addition, activity staining of native gels was used to identify particular activity bands (22).

The presence of 2-ketoaldonate reductase (2KR) activity was observed by spectrophotometric assays with 2-KLG, 2-KDG, or 2,5-DKG as substrates and partially purified enzyme (Fig. 1). Either NADH or NADPH could be used as a cofactor. As determined by HPLC and gas chromatography, 2KR catalyzed the formation of IA from 2-KLG, GA from 2-KDG, and 5-KDG from 2,5-DKG. Activity gels showed comigrating bands of 2KR activity with 2-KLG, 2-KDG, or 2,5-DKG as a substrate and either NADH or NADPH as a cofactor, indicating that 2KR is relatively nonspecific for both substrate and cofactor. The reactions are reversible, as determined by analysis of reaction products and with activity assays and gels (23). Subsequent work demonstrated that this organism contains two different 2KR enzymes, both able to utilize 2-KDG, 2-KLG, or 2,5-DKG as a substrate and either NADH or NADPH as a cofactor (23).

Two peaks of 5-KDG-dependent activity were observed (Fig. 1). A 5-ketoreductase, named 5KR(G) (for gluconate producing), eluted at 0.27 M NaCl from the DEAE resin and reversibly catalyzed the conversion of 5-KDG to GA with either NADH or NADPH as a cofactor. No 2-KDG was formed from 2,5-DKG, suggesting that 5KR(G) does not use 2,5-DKG as a substrate. A second 5-ketoreductase, named 5KR(I) (for idonate producing), eluted at 0.35 M NaCl from the DEAE resin and reversibly catalyzed the reduction of 5-KDG to IA with either NADH or NADPH as a cofactor.

Two peaks of activity in the DEAE fractions were also observed with 2,5-DKG as a substrate and NADH as a cofactor (Fig. 1). The first peak, eluting at 0.27 M NaCl from the DEAE resin, corresponded to 2KR and produced 5-KDG (see above). The second peak, eluting at 0.35 M NaCl from the DEAE resin, represented a 2,5-DKG reductase (2,5-DKGR), produced 2-KLG, and appeared to be specific for NADH as a cofactor. A summary of the *Erwinia* sp. strain ATCC 39140 ketoaldonate reductases is presented in Table 1.

To characterize GA metabolism in strain ATCC 39140, we performed assays for known GA catabolic enzymes. Activities of both GNK and GND, an enzyme unique to the pentose phosphate pathway, were observed (Table 2). The two enzymes specific for the Entner-Doudoroff pathway, 6-phosphogluconate dehydratase and phosphogluconate aldolase, were assayed together by measuring pyruvate formation from 6-PGA. Only a trace of pyruvate was found following incubation of the assay reagents with *Erwinia* sp. strain ATCC 39140 extracts; similarly prepared extracts of *P. putida* and *E. coli*, organisms known to have the Entner-Doudoroff pathway (33), produced at least 25 and 50 times more pyruvate, respectively.

Mutant isolation and characterization. Isolation of blocked mutants confirmed that the pathways identified by enzymatic analysis operate in vivo. Because *Erwinia* sp. strain ATCC 39140 grows well on a variety of carbon sources in the semidefined medium described in Materials and Methods, it was possible to screen for the desired mutants by picking colonies unable to grow on the different aldonates or ketoaldonates as carbon sources. Four types of mutants differing in their carbon source utilization patterns were obtained; their growth patterns are shown in Table 3. Confirmation of the mutations inferred from the growth phenotypes was obtained by assaying crude extracts for the activities in question. Mutant 187-6 lacked a functional 5KR(G) enzyme, as determined by the absence of 5KR(G) activity on native gels in both the reductive (5-KDG as a substrate) and oxidative (GA as a substrate) directions; normal 2KR activity was observed (Fig. 2). Since 5KR(I) activity could not be easily detected on the activity gels, the cytosolic extract of mutant 139-2 was subjected to DEAE anion-exchange chromatography as illustrated in Fig. 1 and assayed for ketoaldonate reductase activity. The observed activity profile was similar to that of the wild type, except that the 5KR(I) activity peak

TABLE 2. Enzymatic activities in non-GA-utilizing mutants

Strain	Sp act (μmol of product formed/min/mg of protein) of [a]:	
	GNK	GND
Wild type	0.18	0.29
159-34	0.12	0.004
159-45	0.002	0.10

[a] Values are the averages of at least two assays.

TABLE 3. Growth phenotypes of mutants

Mutant	Growth on the following carbon source[a]:						
	Glucose	GA	2-KDG	2,5-DKG	5-KDG	2-KLG	IA
None (wild type)	+	+	+	+	+	+	+
139-2	+	+	+	+	+	−	−
187-6	+	+	±	−	−	−	−
159-45	±	−	−	−	−	−	−
159-34	±	−	−	−	−	−	−

[a] +, good growth; ±, growth slow and extent variable; −, no growth.

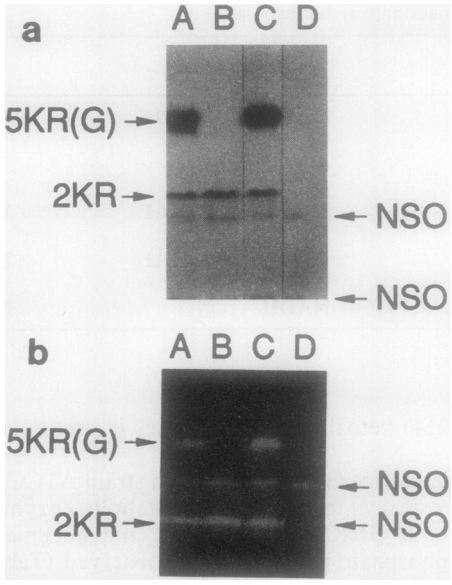

FIG. 2. Activity staining of a native gel (10%) of ketoaldonate reductase activities in *Erwinia* sp. strain ATCC 39140 and mutants. Lanes A, B, C, and D represent crude lysates from strain 139-2, strain 187-6, the wild type, and the wild type with a cofactor only, respectively. NSO, nonspecific oxidoreductase. Reductive staining (a) was carried out with 5-KDG and 2-KLG and with NADPH as a cofactor. Oxidative staining (b) was carried out with GA and with NADP as a cofactor. Conditions for the assays have been previously described (22).

was absent. In addition, only GA was produced from incubations of the crude cytosolic extract with 5-KDG and NADH or NADPH; no IA was observed. Mutant 139-2 had normal activities for 2KR and 5KR(G), as determined by activity gel assays (Fig. 2). Mutants 159-45 and 159-34 were unable to grow on GA and were found to be deficient in GNK and GND activities, respectively (Table 2). From approximately 110,000 colonies screened, 5 mutants lacking 5KR(I) activity, six lacking 5KR(G) activity, one deficient in GNK activity, and two deficient in GND activity were identified. Additional mutants were observed but not fully characterized. No mutants deficient in 2KR activity were isolated.

DISCUSSION

The presence of the enzymatic activities and the growth phenotypes of the mutants described above suggest the pathways shown in Fig. 3 as the degradative routes for ketogluconates in *Erwinia* sp. strain ATCC 39140. The major oxidation product from glucose in this organism is 2,5-DKG, which can be catabolized by sequential reductions to GA via two separate pathways. The first pathway involves the reduction of 2,5-DKG at the 2 position to produce 5-KDG, which is then reduced to GA. This pathway appears to operate in vivo, because 5KR(I)-deficient mutant 139-2 showed normal growth on 2,5-DKG, whereas 5KR(G)-deficient mutant 187-6 failed to grow on this carbon source. The second pathway involves the reduction of 2,5-DKG at the 5 position to produce 2-KLG, which is then reduced to IA, which is then oxidized to 5-KDG, which is then reduced to GA. Evidence that this pathway is functional in vivo comes from the observation that small amounts of 2-KLG and IA are produced from glucose along with 2,5-DKG (23) and from the failure of 5KR(I)-deficient mutant 139-2 to grow on either 2-KLG or IA.

It was initially surprising that no 2KR-deficient mutants were obtained, since this mutation was not expected to be lethal. However, the presence of two different 2KR enzymes explains the failure to find such mutants with NTG; mutations in both genes would be necessary to prevent growth on 2-KDG, 2-KLG, and 2,5-DKG in the primary screening.

The pathways observed in this strain are quite similar to those found in a *Corynebacterium* sp. (26), except that in the *Corynebacterium* sp. 2,5-DKG cannot be reduced at the 2 position to produce 5-KDG but rather is reduced at the 5 position to produce 2-KDG. They also resemble pathways found in *E. herbicola* which, however, lacks the 2,5-DKGR

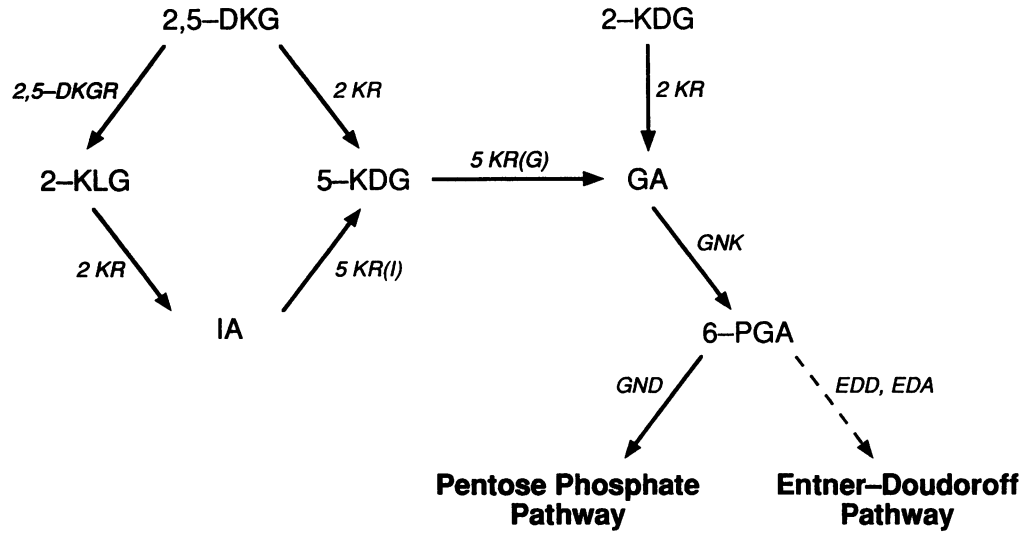

FIG. 3. Proposed model for ketoaldonate metabolism in *Erwinia* sp. strain ATCC 39140. The ketoaldonate reductases catalyze reversible reactions; the proposed in vivo metabolic flow is shown by the solid arrows. The broken arrow represents the Entner-Doudoroff pathway, which is absent in this strain. EDD, 6-phosphogluconate dehydratase; EDA, 2-keto-3-deoxy-6-phosphogluconate aldolase.

and 5KR(I) enzymes and cannot grow on 2-KLG or IA as a carbon source (16).

There are several interesting features of the enzymes described here. They appear to be substrate specific, except for the two 2KR enzymes which, with their broad specificities, resemble the 2KR enzymes found in *E. herbicola* (16, 17, 23). Previously described 2,5-DKGRs are specific for NADPH (19, 27), while the one identified in *Erwinia* sp. strain ATCC 39140 is specific for NADH. Whereas 2,5-DKGR is quite widespread in nature (including another *Erwinia* strain [25]), the production of 2-KLG from glucose via 2,5-DKG has previously only been observed after the introduction of a plasmid carrying a cloned 2,5-DKGR gene into 2,5-DKG-producing strains (5, 14, 16). In contrast to the widespread occurrence of 2,5-DKGR, the only other reported enzyme capable of interconverting 5-KDG and IA occurs in a *Fusarium* sp. which possesses an NADPH-dependent 5KR(I) activity (30).

Acetic acid bacteria have soluble, pyridine nucleotide-dependent ketoaldonate reductases which are similar to those described here and which act on 2-KDG or 5-KDG (24). Since the growth on these compounds is slow, however, it has been suggested that the reductase reactions may be more important in maintaining redox balance than in providing carbon and energy (1). The claim that 2- and 5-ketoaldonate reductase enzymes are found only in acetic acid bacteria (24) would seem to be invalid now that enzymes with very similar activities have been found in both *Corynebacterium* and *Erwinia* spp.

The fact that GNK-deficient mutant 159-45 could not grow on any of the ketogluconates suggests the absence of enzymes capable of directly phosphorylating 2-KDG or 5-KDG. In contrast to these results with *Erwinia* sp. strain ATCC 39140, 2-KDG kinase activity has been found in *Erwinia carotovora* (13). However, the genus *Erwinia*, as currently defined, contains a very diverse group of organisms (31).

The inability of GND-deficient mutant 159-34 to grow on GA and the absence of significant levels of Entner-Doudoroff enzyme activities indicated that the pentose phosphate pathway is the primary, if not the only, route for metabolizing 6-PGA in this organism.

The poor growth of both the GNK- and the GND-deficient mutants on glucose was unexpected, since they should have been able to metabolize glucose via the Embden-Meyerhof-Parnas pathway. The most likely explanation is that most of the glucose was rapidly oxidized to 2,5-DKG by membrane-bound dehydrogenases (28). This explanation is consistent with experiments with *Pseudomonas* spp. (34) and *Gluconobacter* spp. (20) which showed that at high glucose concentrations, glucose was metabolized mainly via the oxidative route, while at low concentrations, it was metabolized mainly via the phosphorylative route.

In summary, we have presented evidence suggesting that *Erwinia* sp. strain ATCC 39140 metabolizes ketogluconates via reductive pathways that lead to GA. GA is then phosphorylated to 6-PGA, which enters central metabolism predominantly through the pentose phosphate pathway.

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