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A Biochemical Study of the Intermediary Carbon Metabolism of *Shewanella putrefaciens*

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Cell extracts were used to determine the enzymes involved in the intermediary carbon metabolism of several strains of *Shewanella putrefaciens*. Enzymes of the Entner-Doudoroff pathway (6-phosphogluconate dehydratase and 2-keto-3-deoxy-6-phosphogluconate aldolase) were detected, but those of the Embden-Meyerhof-Parnas pathway were not. While several tricarboxylic acid cycle enzymes were present under both aerobic and anaerobic conditions, two key enzymes (2-oxoglutarate dehydrogenase and pyruvate dehydrogenase) were greatly diminished under anaerobic conditions. Extracts of cells grown anaerobically on formate as the sole source of carbon and energy were positive for hydroxypyruvate reductase, the key enzyme of the serine pathway in other methylotrophs, while no hexulose synthase activity was seen.

Shewanella putrefaciens strains have been isolated from many different environments (4, 32, 36–38, 41), many of which are suboxic or anoxic. In a recent report, Brettar and Hoefle characterized these bacteria as redox interface organisms on the basis of their abundance at oxic/anoxic interfaces in the Baltic Sea (4). The prevalence of *S. putrefaciens* in so many different suboxic niches may be the result of their ability to exploit a wide range of compounds as terminal electron acceptors. In addition to growing aerobically, *S. putrefaciens* can use Fe(III), Mn(IV), S^0, $S_2O_3^{2-}$, NO_3^-, NO_2^-, trimethylamine oxide, dimethyl sulfoxide, glycine, and fumarate as terminal oxidants during anaerobic respiration and growth (32–36, 38). With regard to electron donors, *S. putrefaciens* strains can use hydrogen (28) and a limited but diverse group of carbon compounds (36). Carbon sources utilized include glucose, lactate, pyruvate, propionate, ethanol, acetate (aerobically), formate (anaerobically), and a number of carboxylic and amino acids, including serine. Since *S. putrefaciens* is capable of growth on formate as its sole source of energy and carbon, it can be classified as a facultative methylotroph according to the definition of Anthony and others (1, 7, 24, 26). Methylotrophs are characterized by their ability to grow on compounds that contain no carbon-carbon bonds and are distinguished from autotrophs by their ability to fix carbon at the oxidation level of formaldehyde (1, 7, 24, 26). Facultative methylotrophs in general utilize multicarbon compounds that can be converted to acetyl coenzyme A and oxidized by the tricarboxylic acid (TCA) cycle under aerobic conditions (1, 6–8, 13, 26, 30, 39). In contrast to *S. putrefaciens*, most of these organisms are aerobes.

Methylotrophic bacteria utilize one, or more, of three pathways for the assimilation of carbon: (i) the ribulose bisphosphate (RuBP) pathway, used by organisms that fix carbon at the level of carbon dioxide; (ii) the ribulose monophosphate (RuMP) pathway; or (iii) the serine pathway, used by those organisms that fix carbon at the level of formaldehyde. While the oxidation of formate by numerous organisms during anaerobiosis is common (15), the assimilation of formate is usually an aerobic process (1, 18, 26). In those cases in which anaerobic growth on formate is known (2, 13, 18, 40), either the RuBP pathway or the serine pathway has been implicated. A number of "pseudo-methylotrophs" (1, 46), for example, *Paracoccus denitrificans* (2, 40), and several strains of photosynthetic purple non-sulfur bacteria (13, 40), the latter requiring light, are able to grow on methanol or formate anaerobically. Furthermore, they all fix carbon dioxide via the RuBP pathway (1, 6, 11). Conversely, a number of strains of the genus *Hyphomicrobium* are able to grow anaerobically on methanol and formate by assimilating carbon at the level of formaldehyde via the serine pathway.

In this paper we present enzymological data that indicate that *S. putrefaciens* uses the Entner-Doudoroff (EDD) pathway to produce pyruvate, that under aerobic conditions it utilizes the TCA cycle to oxidize carbon, and that under anaerobic conditions it is capable of methylotrophic growth on formate, utilizing the serine pathway for carbon fixation (Table 1). The studies encompass strains MR-4, MR-7, MR-8, and MR-9, all from the Black Sea (37), as well as strain MR-1, from Oneida Lake, N.Y. (32). All strains were grown on either LB broth (35) or a minimal salts medium (M1 [29]) supplemented with a carbon source at 20 mM (or with 100 mM formate). Anaerobic growth was facilitated with nitrate (40 mM) as the electron acceptor. Cells were harvested by centrifugation and lysed by sonication, and the crude preparations were clarified by centrifugation at 30,000 × *g*.

Glucose metabolism of *S. putrefaciens*. *S. putrefaciens* has been previously proposed to dissimilate glucose via the Embden-Meyerhof-Parnas (EMP) pathway (41, 43), seemingly in conflict with the fact that this organism is nonfermentative (29). We thus began our studies with the examination of the pathway used for conversion of glucose to pyruvate. As shown in Table 1, extracts of *S. putrefaciens* exhibited no key enzymes of the EMP pathway (6-phosphofructokinase and 1,6-bisphosphofructoaldolase), while there were key enzymes of the EDD pathway (6-phosphoglucose dehydrogenase and 6-phosphogluconate dehydratase). *S. putrefaciens* is apparently an EDD pathway-utilizing bacterium. In addition, the key enzymes of the pentose phosphate pathway (6-phosphoglucose dehydrogenase and transaldolase) were also shown to be present (Table 1).

TCA cycle activity in *S. putrefaciens*. The activities of selected enzymes of the TCA cycle were examined under aerobic and anaerobic conditions. Extracts of aerobically grown *S.*

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TABLE 1. Enzymatic activities of extracts of *S. putrefaciens* and control strains[k]

Pathway	Enzyme (reference)	Activity (μmol/min/mg of protein), mean ± SD		
		Aerobic		Anaerobic (*S. putrefaciens*)
		S. putrefaciens	Control	
TCA	Isocitrate dehydrogenase (6)	0.033 ± 0.012 (n = 13)	0.040 ± 0.051[a] (n = 1)	0.579 ± 0.051 (n = 2)
	Malate dehydrogenase (6)	0.914 ± 0.153 (n = 4)	0.206[a] (n = 1)	1.259 ± 0.359 (n = 4)
	Pyruvate dehydrogenase (6)	0.139 ± 0.024 (n = 6)	0.058 ± 0.010[a] (n = 6)	0.049 ± 0.026 (n = 6)
	2-Oxoglutarate dehydrogenase (6)	0.196 ± 0.050 (n = 6)	0.039 ± 0.006[a] (n = 4)	0.019 ± 0.012 (n = 6)
	Citrate synthase (22)	0.311 ± 0.053 (n = 3)	0.537 ± 0.283[a] (n = 4)	0.272 ± 0.063 (n = 3), 0.178 ± 0.001[b] (n = 2)
One-carbon metabolism	Hydroxypyruvate reductase (25)	NA[c]	0.477 ± 0.038[d,e] (n = 3)	0.085 ± 0.008[b] (n = 7)
	3-Hexulose-6-phosphate synthase (25)	NA	0.33[h] (n = 1)	0.010 ± 0.004[b] (n = 2)
	Dye-linked formate dehydrogenase (31)	0.079 ± 0.049[f] (n = 8)	ND[i]	0.312 ± 0.219[f] (n = 8)
	NAD-linked formate dehydrogenase (25)	NA	0.501 ± 0.231[d,e] (n = 3)	<0.001[b] (n = 10)
Anaplerotic enzymes	Phosphoenolpyruvate (5)	0.070 ± 0.051 (n = 8)	ND	0.098 ± 0.058 (n = 10)
	Isocitrate lyase (12)	0.009 ± 0.001 (n = 6), 0.009 ± 0.002[g] (n = 3)	ND ND	0.003 ± 0.0001 (n = 11)
Carbohydrate metabolism	6-Phosphoglucose dehydrogenase	0.067 ± 0.021 (n = 21)	0.084 ± 0.024[d,h] (n = 2)	ND
EDD	2-Keto-3-deoxygluconate aldolase (16)	0.095 ± 0.025 (n = 8)	0.117 ± 0.049[a,j] (n = 12)	ND
EMP	1,6-Bis-phosphofructose aldolase (10)	0.015 ± 0.08 (n = 5)	0.306 ± 0.251[a] (n = 1)	ND
	6-Phosphofructose kinase (10)	0.006 ± 0.007 (n = 16)	0.251 ± 0.166[a] (n = 9)	ND
Pentose monophosphate	Transaldolase (20)	0.539 ± 0.26 (n = 7)	0.167 ± 0.005[a,j] (n = 2)	ND

[a] *E. coli.*
[b] Strain(s) grown with 20 to 100 mM formate as the sole source of carbon at 20 to 22°C.
[c] NA, not applicable.
[d] Strain(s) grown with 1.0 or 0.1% methanol as the sole source of carbon at 30°C.
[e] *M. extorquens.*
[f] Strain(s) grown with LB (35) supplemented with 20 mM formate at 20 to 22°C.
[g] Strain(s) grown with 20 mM acetate as the sole source of carbon at 20 to 22°C.
[h] *M. methylotrophus.*
[i] ND, not determined.
[j] Strain(s) grown with LB (35) supplemented with 20 mM gluconate at 20 to 22°C.
[k] See reference 19 for methods.

putrefaciens exhibited all enzymes of the TCA cycle (citrate synthase, pyruvate dehydrogenase, malate dehydrogenase, isocitrate dehydrogenase, and 2-oxoglutarate dehydrogenase [Table 1]), suggesting that the TCA cycle is a principal route of assimilation and catabolism and that acetyl coenzyme A is a pivotal intermediate of intermediary carbon metabolism during aerobic growth.

In marked contrast, extracts of anaerobically grown cells (with nitrate as the terminal electron acceptor) had depressed (2- to 20-fold less) specific activities of 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase, suggesting that the TCA cycle is truncated and no longer plays the important role that it does in aerobic metabolism. These results contradict the supposition of other investigators (41, 43) that the intermediary carbon metabolism of *S. putrefaciens* is akin to that of other nonfermentative anaerobes, such as *Pseudomonas stutzeri*, *Pseudomonas aeruginosa*, *Pseudomonas denitrificans*, and *Paracoccus denitrificans* (41, 42, 44). While *S. putrefaciens* does share a number of metabolic features with nonfermentative pseudomonads (i.e., utilization of the EDD pathway in deference to the EMP pathway for the oxidation of glucose), there is no evidence that it utilizes a complete TCA cycle during anaerobiosis. On the contrary, it appears that the intermediary carbon metabolism of *S. putrefaciens* is more closely related to that of other enteric bacteria, such as *Escherichia coli*, which also display a truncated TCA cycle during anaerobiosis (22).

Formate metabolism in *S. putrefaciens*. *S. putrefaciens* grows anaerobically, but not aerobically, with formate as the sole source of carbon and energy (23). Extracts of formate-grown *S. putrefaciens* exhibited high levels of hydroxypyruvate reductase but no detectable levels of hexulose phosphate synthase, indicating that it is a serine pathway-utilizing methylotroph (Table 1). *S. putrefaciens* is the first member of the gamma-purple family of proteobacteria known to utilize the serine pathway (1, 11, 26, 46); all other serine pathway methylotrophs are in the alpha-purple group (17, 45). This raises the interesting possibility that the serine pathway may be more ubiquitous (possibly present in nonmethylotrophic anaerobes) than was previously thought.

The biochemistry and physiology of formate oxidation to CO_2 have not been elucidated for *S. putrefaciens*, but it has been reported that cytoplasmic membrane vesicles prepared from strain NCMB 1735 (grown anaerobically with trimethylamine oxide as the terminal electron acceptor) use formate as an electron donor (43). In vitro studies with strain MR-1 (31) have shown that formate is the preferred electron donor for the direct reduction of iron. Our own studies with cell extracts from *S. putrefaciens* grown either aerobically or anaerobically have yielded only dye-linked formate dehydrogenase activity (Table 1); no activity for an NAD- or NADP-liked formate dehydrogenase was detected.

On the basis of biochemical data presented here, we propose

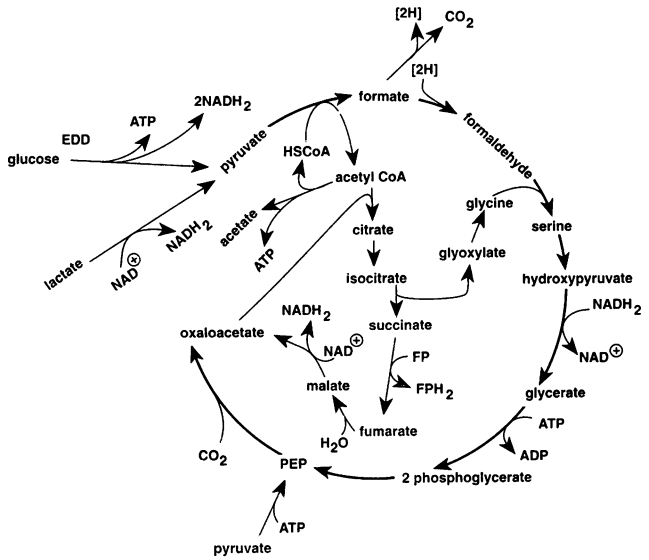

FIG. 1. Proposed route of carbon during anaerobiosis for *S. putrefaciens*.

that, under aerobic conditions, *S. putrefaciens* is metabolically akin to many enteric bacteria, with the EDD pathway feeding pyruvate into the TCA cycle via acetyl coenzyme A. As with many facultative methylotrophs, multicarbon compounds can be converted into acetyl coenzyme A, which can be metabolized to CO_2 aerobically.

In contrast, under anaerobic conditions (Fig. 1), formate, but not acetate, is the central intermediate in carbon assimilation. Substrates that can be converted to formate can be utilized, with carbon being assimilated at the level of formaldehyde. The proposed pathway is similar to that described for other serine pathway methylotrophs (1, 6, 8, 14, 18, 24, 26, 30, 39): an amalgam of the serine pathway and several TCA cycle enzymes. The key intermediate is formate or formaldehyde, but not acetate, which is excreted when glucose, pyruvate, or lactate is the sole source of carbon (23, 27, 28, 41). In fact, Ringo et al. (41) showed that anaerobic growth on lactate leads to the accumulation of acetate, while serine, an intermediate of the serine pathway, was oxidized completely to CO_2. We make this point because some controversy exists in the literature concerning the ability of *S. putrefaciens* to grow anaerobically on acetate, and the view held previously by our laboratory, that acetate can serve as a carbon and energy source for this organism under anaerobic conditions (34, 36), is not compatible with the proposed pathway nor the physiological evidence and is evidently not correct. The validity of our proposed pathway (Fig. 1) can be proven by using a combination of label incorporation and enzymatic studies, which are now a primary focus of work in our laboratory.

Other reports have suggested that *S. putrefaciens* can grow anaerobically with hydrogen as the electron donor, iron as the electron acceptor, and CO_2 as the carbon source (28). We have seen no evidence, either physiological or genetic, for RuBP activity (3), although we cannot eliminate this possibility at this point. Such considerations may be very important in terms of interpreting the hydrogen uptake studies of Klueber and Conrad (21).

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