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NOTES

Acetate Catabolism in the Dissimilatory Iron-Reducing Isolate GS-15

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Acetate-grown GS-15 whole-cell suspensions were disrupted with detergent and assayed for enzymes associated with acetate catabolism. Carbon monoxide dehydrogenase and formate dehydrogenase were not observed in GS-15. Catabolic levels of acetokinase and phosphotransacetylase were observed. Enzyme activities of the citric acid cycle, i.e., isocitrate dehydrogenase, 2-oxoglutarate synthase, succinate dehydrogenase, fumarase, and malate dehydrogenase, were observed.

GS-15 (17, 19) is a rare example of a dissimilatory iron-reducing bacterium that can grow by coupling the oxidation of acetate and other organic molecules to the reduction of ferric ion to ferrous ion. Derek Lovley of the U.S. Geological Survey has advanced the idea that iron is available to act as the predominant terminal electron acceptor in some sediments and groundwater systems (10, 11, 13–18, 23). Recently the organism has been found to oxidize a number of aromatic compounds (11, 14) including toluene, phenol, and *p*-cresol. Little is known about the physiology of this microorganism, including the basic pathways for the utilization of acetate (26, 27). The microorganism is similar to other eubacteria in that it can use nitrate as the terminal electron acceptor and hence might be expected to use the citric acid cycle to oxidize acetyl coenzyme A (acetyl-CoA), yet it has characteristics (i.e., strict oxygen sensitivity, low yield and growth rate, and limited substrate range) that suggest that it could resemble those sulfate reducers that oxidize acetyl-CoA through the carbon monoxide dehydrogenase pathway (27). Our approach was to assay detergent-solubilized, whole-cell suspensions of this bacterium, along with eubacterial and archaebacterial controls, to determine whether the organism contains enzymes representative of each of the known oxidative pathways for acetate.

The organisms used in this study were GS-15, the iron-reducing isolate; *Methanosarcina barkeri* MS; *Pseudomonas aeruginosa* PAO; and SR-8, a methanogen isolated from the rumen of a cow. GS-15 and *P. aeruginosa* were grown on the freshwater nitrate medium (FWA-NO_3) described previously (17), with acetate as the sole carbon and energy source. Nitrate-reducing bacteria were grown in 70 ml of anaerobic liquid medium in 160-ml serum bottles under 1 atm (1 atm = 101.29 kPa) of 80% N_2–20% CO_2 or on 2% Noble agar plates (Difco Laboratories, Detroit, Mich.) of the same medium in an anaerobic chamber with an atmosphere of 80% N_2–13% CO_2–7% H_2. The medium for iron-grown GS-15 (FWA-FeIII) contained 50 mM ferric citrate and 50 mM acetate (17). SR-8 was grown in bicarbonate-buffered mineral medium (12, 24) with 80% hydrogen as the energy source. *Methanosarcina barkeri* was grown in phosphate-buffered basal medium (8) with 80 mM acetate as the energy source. All cultures were incubated statically at 30°C in the dark. Cells were anaerobically collected from plates into 5 ml of FWA-NO_3 medium in screw-cap centrifuge tubes. Cells from the liquid medium were collected into the same tubes under continuous flushing with O_2-free N_2 in a manner similar to that previously described (4). The cells were pelleted (15 min, 4°C, 20,000 × *g*), washed anaerobically with 20 mM Tris buffer (pH 7.0) that included 0.3 mM dithiothreitol, and repelleted. Pellets were resuspended with 1 to 2 ml of the same buffer in the anaerobic chamber. These suspensions (generally 0.5 to 2.0 mg of protein per ml) were transferred to 12-ml serum bottles, and the bottles were capped with sleeve-type serum stoppers. The suspensions were then made 0.5% (final concentration) in Triton X-100 nonionic detergent. The vials were then evacuated and flushed with O_2-free nitrogen for several cycles. Vials were kept at 4°C until assayed. Most assays were performed within 4 h, and no assays were performed with suspensions older than 10 h. For each activity reported as zero, the activity was measured with freshly prepared suspensions to ensure that the lack of activity was not due to the age of the suspension.

All enzyme assays were performed at 28°C, using 50 to 200 μg of protein. Activities reported are in nanokatals per milligram of cell protein and are corrected for any nonspecific reaction observed. All aerobic assays were performed in 1.0-ml volumes in quartz cuvettes, and anaerobic assays were performed in 3.0-ml volumes in screw-cap glass cuvettes fitted with serum bottle stoppers. In each case, the path length was 1.0 cm. Anaerobic assay cocktails were prepared and dispensed in the anaerobic chamber and flushed for 5 min with either N_2 or substrate gas to remove traces of H_2. All assays were performed on a Lambda 4B UV/Vis spectrophotometer (model no. C688-0001; Perkin-Elmer, Norwalk, Conn.). Colorimetric determination of protein was performed by the standard Bio-Rad assay (Bio-Rad Laboratories, Richmond, Calif.), a modification of the Bradford method (2), using a Bausch and Lomb Spectronic 21 spectrophotometer. Chicken egg white lysozyme was used as a standard.

Acetate activation by GS-15. The majority of organisms that oxidize acetate anaerobically use the combined action of acetate kinase and phosphate acetyltransferase. Assays of acetate activation enzymes were performed aerobically, as these activities are not sensitive to oxygen, even when cell extracts from strict anaerobes are used (1, 7). Acetate kinase

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TABLE 1. Enzyme activities as observed in detergent-solubilized, whole-cell suspensions of GS-15 grown with acetate and either nitrate or iron as the terminal electron acceptor

Enzyme and EC no.	Coenzyme or artificial dye[a]	Sp act (nkat mg of cell protein^{-1}) of electron acceptor in the following medium[b]:	
		NO_3^{1-}	Fe^{3+}
Acetate kinase, 2.7.2.1		1.03	0.38
Phosphate acetyltransferase, 2.1.3.8		0.80	ND
Isocitrate dehydrogenase, 1.1.1.42	NADP	0.78	0.68
2-Oxoglutarate:methyl viologen oxidoreductase	MV	1.12	0.87
2-Oxoglutarate synthase, 1.2.7.3	Fd	0.17	ND
Succinate dehydrogenase, 1.3.99.1	DCIP	0.33	0.17
Fumarate hydratase, 4.2.1.2		5.35	ND
Malate dehydrogenase, 1.1.1.37	NAD	1.69	7.86
Carbon monoxide dehydrogenase	MV	0.00[c]	0.00
Hydrogenase	MV	0.02[d]	ND
Formate dehydrogenase	MV	0.00[e]	ND

[a] MV, Methyl viologen; Fd, ferredoxin from *C. pasteurianum*; DCIP, dichlorophenol-indophenol.
[b] ND, Not determined.
[c] Specific activity measured in *M. barkeri*, 3.55 nkat mg of cell protein^{-1}.
[d] Specific activity measured in *M. barkeri*, 1.90 nkat mg of cell protein^{-1}.
[e] Specific activity measured in SR-8, 1.53 nkat mg of cell protein^{-1}.

(EC 2.7.2.1) was followed by coupling acetate-dependent ADP formation from acetate and ATP to NADH oxidation through pyruvate kinase and lactic acid dehydrogenase in a reaction mixture described by Aceti and Ferry (1). As with all other reactions involving NAD^+, change in absorbance was followed at 340 nm by using an extinction coefficient of 6.22 mM^{-1} cm^{-1}. Triton detergent in the whole-cell suspensions interfered (data not shown) with the observation of acetyl phosphate formation in the hydroxamate assay of Rose (20). Phosphate acetyltransferase (EC 2.3.1.8) activity was followed directly by observing the increase in A_{233} of the reaction mixture (9), which was due to the formation of acetyl-CoA, by using an extinction coefficient of 4.44 mM^{-1} cm^{-1} (28). As an exception, 10 to 80 μg of protein was used in these assays. When grown with acetate, GS-15 activates acetate to acetyl-CoA by these enzymes (Table 1). Also, acetate kinase levels were similar for nitrate- and iron-grown cells. Acetate kinase activities measured in *P. aeruginosa* and *M. barkeri* were 0.30 and 13.47 nkat mg of cell protein^{-1}, respectively. The transferase activities seen were 3.75 and 1.32 nkat mg of cell protein^{-1}, respectively.

Absence of carbon monoxide pathway enzymes in GS-15. GS-15 is an obligate anaerobe, and many oxygen-sensitive anaerobes that catabolize acetate contain the oxygen-sensitive CO dehydrogenase pathway. To test the possibility that GS-15 may contain enzymes of this pathway, each of the representative activities was observed under strictly anaerobic conditions described elsewhere (4, 29, 30). The oxidation of carbon monoxide, hydrogen, or 10 mM formate was coupled to the reduction of the artificial electron acceptor methyl viologen and quantified by the increase in A_{578} with an extinction coefficient of 9.7 mM^{-1} cm^{-1}. Using a freshly prepared suspension, we did not observe carbon monoxide dehydrogenase or formate dehydrogenase activity in GS-15. This was true for cells grown with either nitrate or ferric iron as a terminal electron acceptor. The level of CO dehydrogenase in *M. barkeri* was 3.55 nkat mg of cell protein^{-1}. In another methanogenic isolate grown on H_2 and CO_2, SR-8, the level of formate dehydrogenase was 1.53 nkat mg of cell protein^{-1}. GS-15 does not consume or produce carbon monoxide when grown on acetate with iron.

GS-15 showed only a trace of uptake hydrogenase activity (0.02 nkat mg of cell protein^{-1}). Cultures of GS-15 exhibited biological incorporation of tritiated hydrogen into the aqueous phase that was directly proportional to the amount of protein present (data not shown). Protein-dependent tritiated hydrogen incorporation into the aqueous phase has been shown to be indicative of the presence of either hydrogen-producing or hydrogen-consuming hydrogenase (22).

Acetyl-CoA oxidation via the citric acid cycle in GS-15. Most of the remaining prokaryotes that oxidize acetate anaerobically do so by using the citric acid cycle under nitrate-reducing conditions. We measured these activities in GS-15 and *P. aeruginosa* under anaerobic conditions. GS-15 contained many of the enzymes of the citric acid cycle (Table 1). GS-15 demonstrated four characteristic dehydrogenase activities of the citric acid cycle. Isocitrate dehydrogenase activity was seen to be Mg^{2+} dependent and NADP specific. Isocitrate dehydrogenase was measured as substrate-dependent NADP reduction but not as NAD reduction. Activity was 10% or less when 10 mM $MgCl_2$ was removed from the reaction mix. The apparent K_m for DL-isocitrate was 0.28 mM. The reaction was followed at 365 nm, using an extinction coefficient of 6.17 mM^{-1} cm^{-1} for NADPH. 2-Oxoglutarate dehydrogenase was able to couple with the low-potential electron acceptors methyl viologen and clostridial ferredoxin but not with either NAD^+ or NADP. The assay mixture described by Brandis-Heep et al. (3) was employed, but with 0.3 mM methyl viologen. 2-Oxoglutarate:ferredoxin 2-oxidoreductase (EC 1.2.7.3) was assayed in the same reaction mixture, but with 0.1 mg of ferredoxin from *Clostridium pasteurianum* per ml. NAD^+ and NADP (both at 0.3 mM) were also tried. Succinate dehydrogenase (EC 1.3.99.1) activity was demonstrated with the artificial electron acceptor 2,6-dichlorophenolindophenol in a manner previously described (4). The K_m for succinate was 0.78 mM. The activity was not seen with menadione (0.1 mM) as the electron acceptor. The reduction of the artificial electron acceptor was followed as bleaching at 578 nm and quantified with an extinction coefficient of 20.6 mM^{-1} cm^{-1}. Malate dehydrogenase coupled with NAD^+ but not with NADP or menadione (0.1 mM). The activity of malate dehydrogenase (EC 1.1.1.37) was observed in the reverse direction by following oxaloacetic acid-dependent NADH oxidation. The reaction mixture contained 50 mM Tris (pH 7.5), 0.26 mM NADH, and 10 mM $MgCl_2$, and the reaction was initiated with 1.5 mM oxaloacetic acid (final concentration). Fumarate hydratase activity (EC 4.2.1.2) was followed by observing the disappearance of UV absorbance due to the loss of the double-bond character of fumarate. The assay described by Brandis-Heep et al. (3) is quantified by using an extinction coefficient of 0.57 mM^{-1} cm^{-1} for $\Delta(A_{290}$ to $A_{260})$. As an exception, this assay was carried out aerobically.

The activities reported for both GS-15 and the *Pseudomonas* control are lower than values for activities of citric acid cycle enzymes from other acetate oxidizers (3, 5, 6, 21). The literature values are from cell extract systems, while the values reported here are all for solubilized cell suspensions. The use of solubilized cell suspensions was necessitated by

the extremely low yield and growth rate of GS-15 and by the need to minimize manipulations to ensure anaerobiosis. Most importantly, the levels of activity of solubilized GS-15 were comparable to the levels of activity in *P. aeruginosa*.

GS-15, like some denitrifiers and sulfidogens, is capable of completely oxidizing acetate under anaerobic conditions (25, 27). On the basis of the results of these experiments, GS-15 oxidizes acetyl-CoA (hence acetate) through the citric acid cycle and not via the carbon monoxide pathway. On the whole, enzyme activities of the citric acid cycle are present at levels similar to those in the denitrifiers, *P. aeruginosa*, and others studied in this laboratory. GS-15 is only the third obligate anaerobe demonstrated to use the citric acid cycle for catabolic purposes. The other two are *Desulfobacter postgatei* (3) and *Desulfuromonas acetoxidans* (6). The absence of carbon monoxide dehydrogenase activity supports the suggestion of Thauer et al. (26) that the redox potentials of nitrate, Mn(IV), and Fe(III) would be so high as to inhibit the CO dehydrogenase enzyme. Continuing studies are examining the presence of other electron acceptors and the topology of the enzymes involved in an attempt to understand energy conservation in this novel, dissimilatory iron-reducing organism. Investigations into electron transfer pathways are a necessary prelude to understanding energy conservation by dissimilatory iron-reducing bacteria.

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