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Oxidation of acetate through reactions of the citric acid cycle by *Geobacter sulfurreducens* in pure culture and in syntrophic coculture

Abstract *Geobacter sulfurreducens* strain PCA oxidized acetate to CO_2 via citric acid cycle reactions during growth with acetate plus fumarate in pure culture, and with acetate plus nitrate in coculture with *Wolinella succinogenes*. Acetate was activated by succinyl-CoA:acetate CoA-transferase and also via acetate kinase plus phosphotransacetylase. Citrate was formed by citrate synthase. Soluble isocitrate and malate dehydrogenases reduced $NADP^+$ and NAD^+, respectively. Oxidation of 2-oxoglutarate was measured as benzyl viologen reduction and was strictly CoA-dependent; a low activity was also observed with $NADP^+$. Succinate dehydrogenase and fumarate reductase both were membrane-bound. Succinate oxidation was coupled to $NADP^+$ reduction whereas fumarate reduction was coupled to NADPH and NADH oxidation. Coupling of succinate oxidation to $NADP^+$ or cytochrome(s) reduction required an ATP-dependent reversed electron transport. Net ATP synthesis proceeded exclusively through electron transport phosphorylation. During fumarate reduction, both NADPH and NADH delivered reducing equivalents into the electron transport chain, which contained a menaquinone. Overall, acetate oxidation with fumarate proceeded through an open loop of citric acid cycle reactions, excluding succinate dehydrogenase, with fumarate reductase as the key reaction for electron delivery, whereas acetate oxidation in the syntrophic coculture required the complete citric acid cycle.

Keywords Anaerobic acetate degradation · Reversed electron transport · Fumarate reduction · Syntrophy · Iron-reducing bacteria · *Wolinella succinogenes*

Abbreviations *CTAB* Cetyltrimethylammonium bromide · *DCCD* N,N-dicyclohexylcarbodiimide · *HOQNO* 2-n-Heptyl-4-hydroxyquinoline-N-oxide

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Introduction

Geobacter sulfurreducens strain PCA is a mesophilic freshwater bacterium that can grow by acetate oxidation coupled to reduction of Fe(III), elemental sulfur, or fumarate as electron acceptor (Caccavo et al. 1994). It can also grow on acetate, releasing electrons via interspecies electron transfer to *Wolinella succinogenes* or *Desulfovibrio desulfuricans*, with nitrate as terminal electron acceptor (Cord-Ruwisch et al. 1998). Previously described syntrophic acetate-degrading cocultures are either thermophilic or mesophilic and couple to methanogenic (Zinder and Koch 1984; Schnürer et al. 1994; Hattori et al. 2000) or sulfidogenic (Galouchko and Rozanova 1996) partners. All these cultures grow very slowly, with doubling times of several days.

Two main pathways for acetate oxidation by anaerobic bacteria are known: the CO dehydrogenase/acetyl-CoA pathway and the citric acid cycle, with either CO dehydrogenase/acetyl-CoA synthase or 2-oxoglutarate:electron acceptor oxidoreductase as the key enzymes, respectively (Thauer 1988; Ragsdale 1991). The CO dehydrogenase pathway is more likely to operate in syntrophic acetate-oxidizing cocultures if electrons are transferred between the partners in the form of hydrogen or formate (Thauer 1988; Schink 1997) because the average redox potential of the oxidation steps in this pathway (–339 mV) is closer to that of the proton/hydrogen couple (–414 mV) than to that of the oxidation steps in the citric acid cycle (–247 mV; all redox potential values given for pH 7.0; Thauer 1988), meaning that the former pathway operates closer to the thermodynamic equilibrium than the latter one does. Indeed, the acetate-degrading partners in the thermophilic and mesophilic methanogenic cocultures are homoacetogenic bacteria capable of acetate oxidation or acetate synthesis via the CO dehydrogenase pathway (Lee and Zinder 1988a, b; Schnürer et al. 1996, 1997). The direction of acetate metabolism in these bacteria strongly depends on the hydrogen partial pressure. In the case of *G. sulfurreducens*, some preliminary data indicated that

the mechanism of syntrophic acetate oxidation could be different. Bacteria that are closely related to *G. sulfurreducens* on the basis of 16S rRNA sequence analysis (*Desulfuromonas acetoxidans*, *G. metallireducens* and *G. sulfurreducens* strain AM-1) oxidize acetate through the citric acid cycle (Gebhardt et al. 1985; Champine and Goodwin 1991; Mikoulinskaia et al. 1999; Champine et al. 2000). Moreover, it has been suggested that electrons could be delivered to *W. succinogenes* through a carrier system with a redox potential higher than that of the proton/hydrogen couple (Cord-Ruwisch et al. 1998; Seeliger et al. 1998). In particular, if a cytochrome with a standard redox potential of –167 mV is involved in interspecies electron transfer, this could make acetate oxidation through the citric acid cycle reactions feasible.

In this article, we report that *G. sulfurreducens* strain PCA degrades acetate through citric acid cycle reactions during growth on acetate plus fumarate and also in coculture with *W. succinogenes* on acetate plus nitrate.

Materials and methods

Bacteria and growth conditions

Geobacter sulfurreducens strain PCA (DSM 12127; ATCC 51573) was obtained from R. Cord-Ruwisch (Perth, Australia), and *Wolinella succinogenes* DSM 1740 was provided by A. Kröger (Frankfurt, Germany).

Bacteria were cultivated in a bicarbonate/CO_2-buffered, cysteine-reduced medium for freshwater bacteria (Cord-Ruwisch et al. 1998; Seeliger et al. 1998). For enzyme activity measurements and determination of growth parameters, *G. sulfurreducens* was routinely cultivated in this medium with 20 mM acetate plus 40 mM fumarate. A coculture of *G. sulfurreducens* and *W. succinogenes* was grown with 10 mM each of acetate and nitrate (Cord-Ruwisch et al. 1998). *W. succinogenes* grew in the same medium with 30 mM formate plus 10 mM nitrate. Growth substrates were added from anoxic, sterile stock solutions by syringes flushed with N_2/CO_2 (80:20, v/v).

Preparation of cell extracts

Geobacter sulfurreducens was grown in 1 l medium (in 1.2-l infusion bottles) under an N_2/CO_2 (80:20, v/v) gas phase. Cells were harvested in the late exponential growth phase by centrifugation for 20 min at 10,000×g under anoxic conditions. The cell pellet was suspended in 50 ml of anoxic 50 mM potassium phosphate buffer, pH 7.3, containing 2.5 mM $MgCl_2$ and 2.5 mM dithiothreitol, and centrifuged again as described above. Harvested cells were resuspended in 2.5 ml of the same buffer and passed two to three times through an anoxic French press cell at 137 MPa. Cell debris and intact cells were removed from the homogenate by centrifugation (20 min at 30,000×g). The membrane fraction was separated from the soluble fraction by ultracentrifugation for 1 h at 125,000×g. Fractions were transferred into small glass vials under an N_2 atmosphere and stored on ice. Enzyme activities were measured within 1–8 h after preparation.

All manipulations of cells were performed in an anoxic chamber (Coy Laboratory Products, Ann Arbor, Mich.) under N_2/H_2 atmosphere (95/5, v/v).

Enzyme assays

Enzyme activities were measured photometrically at 30 °C under an N_2 atmosphere (unless otherwise indicated) in glass cuvettes (1 ml assay mixture) sealed with butyl rubber stoppers. Anoxic conditions were achieved by flushing cuvettes with N_2.

Acetate kinase and phosphotransacetylase were assayed according to Bergmeyer (1983). Citric acid cycle enzymes (citrate synthase, ATP-citrate lyase, aconitase, isocitrate dehydrogenase, 2-oxoglutarate:electron acceptor oxidoreductase, succinyl-CoA synthetase, succinyl-CoA:acetate CoA-transferase, succinate dehydrogenase, fumarate reductase, fumarase) were determined according to described procedures (Brandis-Heep et al. 1983; Stams et al. 1984; Möller et al. 1987; Beh et al. 1993). Malate dehydrogenase activity was assayed in the direction of oxaloacetate reduction following the decrease of NADH or NADPH absorption at 365 nm. In addition, malate oxidation was studied by following NAD^+ or $NADP^+$ reduction in the presence or absence of 0.2 mM acetyl-CoA. The assay mixture contained 50 mM potassium phosphate buffer, pH 7.0, 5 mM malate, and 0.4 mM NAD^+ or $NADP^+$. ATP-driven succinate oxidation coupled to NAD^+ or $NADP^+$ reduction, the effect of *N*,*N*-dicyclohexylcarbodiimide (DCCD) on the reaction, and fumarate reduction with NADH or NADPH were determined as described (Paulsen et al. 1986).

Formate dehydrogenase, CO dehydrogenase, and hydrogenase were assayed as benzyl viologen (2 mM) reduction in the presence of the respective substrates (Zeikus et al. 1977). Carbon monoxide and hydrogen were supplied in the gas phase and the buffer used was saturated with these gases in advance. Formate dehydrogenase was tested with 10 mM formate under 100% N_2 gas phase.

Reduction of cytochrome(s) of *G. sulfurreducens* present in cell-free extract by intermediates of the citric acid cycle (isocitrate, 2-oxoglutarate, succinate, malate), NADH, or NADPH was determined by measuring absorption changes at 553 nm after addition of the respective electron donors (ε=31.4 mM^{-1} cm^{-1}). Cell-free extract was prepared in anoxic 50 mM potassium phosphate buffer, pH 7.0, without a reducing agent. The assay volume was 1.0 ml and contained 850 μl buffer and 100 μl extract. Before starting the reaction, by addition of 50 μl of electron donor from anoxic stock solutions, cytochrome(s) were oxidized by shaking the cuvettes after supplying 10–30 μl air to the gas phase.

Analytical procedures

The content of organic acids in the growth medium was measured by HPLC with a Bio-Rad Aminex HPX-87H column (300×7.8 mm). Samples were eluted with 5 mM H_2SO_4 in water (flow rate 0.6 ml min^{-1}). Compounds were detected by an ERC-7512 refraction index detector (Erma).

Growth of *G. sulfurreducens* was followed by measuring the optical density at 660 nm in a 1-ml cuvette with a 1-cm light path. Cell dry mass was calculated via an experimentally determined conversion factor (1 OD at 660 nm of culture grown on acetate plus fumarate mixture corresponded to 340 mg cellular protein l^{-1}). Acetate consumption for cell mass synthesis was calculated according to the equation:

$$17\, CH_3COO^- + 11\, H_2O \rightarrow 8\langle C_4H_7O_3\rangle + 2\, HCO_3^- + 15\, OH^-$$
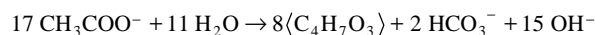
Extraction of menaquinone followed a described procedure (Shestopalov et al. 1997). Menaquinones were detected by their UV absorption spectra at 200–350 nm with an Uvikon-930 spectrophotometer (Kontron Instruments). Vitamin K_1 was used as a reference.

Protein content in cell extracts and cell suspensions was measured with bicinchoninic acid according to a standard and an enhanced protocol, respectively, (BCA protein assay kit; Pierce). Bovine serum albumin fraction V (Pierce) was used for calibration.

Chemicals and gases

All chemicals were of analytical or reagent grade quality and were obtained from Biomol (Ilvesheim, Germany), Boehringer (Mannheim, Germany), Fluka (Neu-Ulm, Germany), Merck (Darmstadt,

Germany), Serva (Heidelberg, Germany), and Sigma (Deisenhofen, Germany). Gases were purchased from Messer-Griesheim (Darmstadt, Germany), and Sauerstoffwerke Friedrichshafen (Friedrichshafen, Germany).

Results

Acetate oxidation in pure culture

Geobacter sulfurreducens strain PCA grew in batch culture on acetate plus fumarate with an average doubling time of 7.7 h (μ=2.2 day^{-1}) (Fig. 1). Up to 5 mM malate accumulated transiently in the medium during growth. The bacteria reduced 4.05 mol fumarate per 1 mol acetate dissimilated (Table 1). The ratio of fumarate metabolized over succinate produced was very close to 1 (0.98–0.99), indicating only an insignificant contribution of these compounds to cell matter formation. Around 46% of metabolized acetate was assimilated. The biomass produced was proportional to the amount of acetate dissimilated, with a yield of 42 g cell dry mass per mol acetate. During growth, the rate of dissimilatory acetate oxidation was around 70 nmol min^{-1} (mg protein)$^{-1}$. When the bacterium was cultivated in medium with excess fumarate (10 mM acetate plus 50 mM fumarate), growth stopped after depletion of acetate.

High specific activities of acetate kinase and phosphotransacetylase (Table 2) demonstrated that acetate could be activated through acetyl phosphate, coupled to hydrolysis of 1 ATP per acetate. Extracts also had substantial succinyl-CoA:acetate CoA-transferase activity. Acetyl-CoA synthetase was not found.

Cell extracts of *G. sulfurreducens* contained all enzymes required for acetate oxidation through citric acid cycle reactions (Table 2). Enzymes dealing with fumarate reduction or succinate oxidation were the only ones found in the membrane fraction. Fumarate reduction by reduced benzyl viologen was stimulated 13-fold in cells permeabilized with cetyltrimethylammonium bromide (CTAB) in comparison to non-permeabilized cells (data not shown).

Citrate synthesis from oxaloacetate and acetyl-CoA in cell-free extracts did not depend on the presence of ADP plus inorganic phosphate. Isocitrate dehydrogenase was strictly NADP$^+$-dependent. Oxidation of 2-oxoglutarate, measured as benzyl viologen reduction, was strictly CoA-dependent (Tables 2,3); a low activity was also observed with NADP$^+$ but not with NAD$^+$. Succinyl-CoA synthetase was absent and succinate was released only in a transferase reaction with acetate as CoA-acceptor.

Fig. 1 Growth of *Geobacter sulfurreducens* on acetate plus fumarate. Time course of changes in concentrations of acetate (▲), fumarate (●), succinate (◆) and malate (■). Growth is shown as the change in optical density of the culture at 660 nm (▫). Acetate concentration changes shown represent the total acetate consumption including dissimilation (54%) and assimilation (46%)
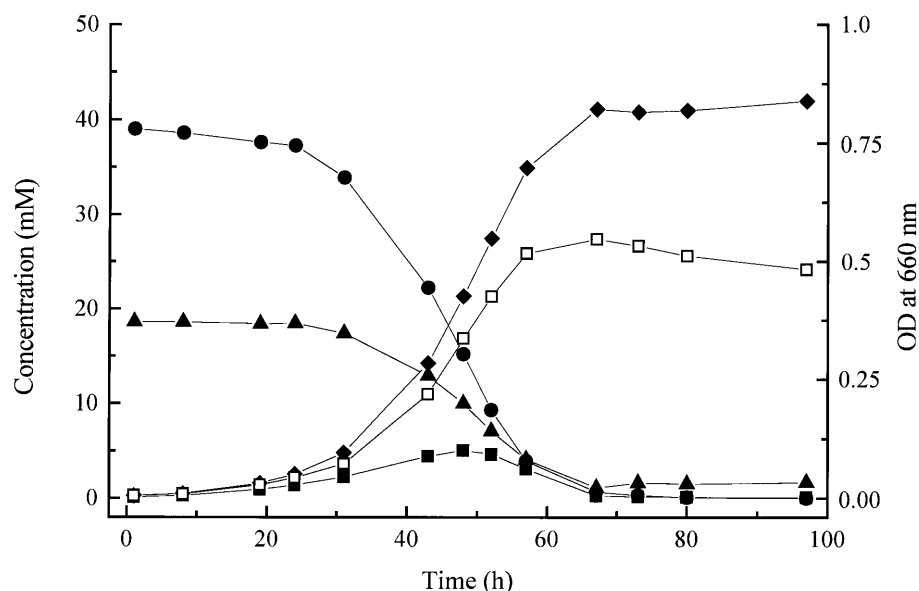
Table 1 Quantification of acetate consumption, fumarate reduction, and growth of *Geobacter sulfurreducens*

Substrate consumed (mM)		Acetate dissimilated (mM)[b]	Succinate produced (mM)	Cell dry mass formed (mg l^{-1})	Electron recovery (%)[c]
Acetate	Fumarate[a]				
10.00	21.65	5.32	21.78	227	102
15.00	32.99	8.10	33.72	334	104

[a]Calculated as change of fumarate plus malate concentrations
[b]The difference between acetate consumed and acetate used for cell synthesis was calculated from the assimilation equation given in the Methods section

[c]Amount of electrons found in produced succinate compared with amount of electrons derived from dissimilated acetate

Table 2 Enzymes involved in acetate activation and oxidation in cell-free extracts of *G. sulfurreducens* grown on acetate plus fumarate. The acetate dissimilation rate was 70 nmol min^{-1} (mg protein)$^{-1}$. *nd* Not determined

Enzymes	Specific activity [nmol min^{-1} (mg protein)$^{-1}$]	Activity in soluble fraction (% of total activity)[a]
Acetate activation		
Acetate kinase	280	105
Phosphotransacetylase	4470	117
Citric acid cycle		
Citrate synthase	860	95
Aconitase	240	102
Isocitrate dehydrogenase (NADP$^+$-dependent)	700	99
2-Oxoglutarate:acceptor oxidoreductase		
benzyl viologen reduction	420	105
NADP$^+$reduction	5	nd
Succinyl CoA:acetate CoA transferase	900	84
Succinate dehydrogenase		
ferricyanide reduction	40	4
NADP$^+$ reduction (plus ATP)	100	nd
Fumarate reductase		
NADPH-dependent	540	0.1
NADH-dependent	80	0.1
Fumarase	1750	120
Malate dehydrogenase		
NADP$^+$-dependent	1090	105
NAD$^+$-dependent	3680	93

[a]Activity recovered in the supernatant after ultracentrifugation (1 h at 125,000×g) expressed as percentage of total activity in cell-free extract

Table 3 Key enzymes of acetate oxidation in cell-free extracts of *Geobacter sulfurreducens, Wolinella succinogenes,* and of the coculture

Enzymes	Specific activity [nmol min^{-1} (mg protein)$^{-1}$]		
	G. sulfurreducens[a]	*W. succinogenes*[b]	Coculture[c]
2-Oxoglutarate:benzyl viologen oxidoreductase	420	30	160
CO dehydrogenase	60	20	20
Hydrogenase	450	2	0
Formate dehydrogenase	7	3480	1320
NADH dehydrogenase	8	850	130
NADPH dehydrogenase	540	1350	1800

[a]*G. sulfurreducens* was grown on acetate plus fumarate
[b]*W. succinogenes* was grown on formate plus nitrate
[c]Coculture of *G. sulfurreducens* and *W. succinogenes* was grown on acetate plus nitrate

Membranes of *G. sulfurreducens* reduced fumarate with NADPH at a rate higher than with NADH [190 vs 70 nmol min^{-1} (mg protein)$^{-1}$, respectively]. The reaction was sensitive to 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HOQNO), a menaquinol analogue (Rothery and Weiner 1998). The presence of 0.5 mM HOQNO in the assay caused a 98.8% decrease of the fumarate reduction rate. In the reverse direction, the reaction was strictly NADP$^+$-dependent. Membranes reduced NADP$^+$ with succinate only in the presence of ATP. The dependence of succinate oxidation on ATP hydrolysis was proven in experiments with DCCD: the rate of ATP-dependent succinate oxidation was decreased by 90% after incubation of membranes with 125 μmol DCCD (mg protein)$^{-1}$ for 20 min at 30 °C. This reagent inhibits ATP synthases due to a reaction with specific amino acid carboxyl residues (Kluge and Dimroth 1993).

Cell-free extracts always possessed high fumarase activity [3.7 μmol min^{-1} (mg protein)$^{-1}$]. The malate produced was dehydrogenated to oxaloacetate by malate dehydrogenase. Since the standard redox potential of the oxaloacetate/malate couple (−166 mV) is higher than that of possible electron carriers (NAD(P)$^+$/NAD(P)H), this reaction is often measured in the thermodynamically favorable direction of oxaloacetate reduction to malate. Under such conditions, malate dehydrogenase activity was found with both NADH and NADPH. However, the activity with NADH was about three times as high as with NADPH (Table 2). Malate oxidation could only be coupled to NADP$^+$ reduction at a considerably lower rate [34

nmol min^{-1} (mg protein)$^{-1}$] (Table 2). Addition of acetyl-CoA caused a complete loss of this activity, which is characteristic for some NADP$^+$-dependent dehydrogenases decarboxylating malate to pyruvate (malic enzymes; Voegele et al. 1999). In contrast to the NADP$^+$-dependent enzyme, the presence of an NAD$^+$-reducing malate dehydrogenase that operates in the oxidative direction [220 nmol min^{-1} (mg protein)$^{-1}$] could only be revealed after addition of acetyl-CoA to scavenge oxaloacetate in the exergonic reaction of citrate formation.

While a low activity of CO oxidation was always detected (Table 3), the even lower activity of formate dehydrogenase (another typical enzyme of the CO/acetyl-CoA pathway) allowed us to conclude that this pathway does not operate in *G. sulfurreducens*. Growth and cell suspension experiments confirmed that this bacterium could not perform a homoacetogenic metabolism: it did not grow on formate (40 mM), on H_2/CO_2 (80/20 vol%), or H_2/CO_2 plus formate. Having active hydrogenase but not formate dehydrogenase (Table 3), it could use hydrogen but not formate for fumarate reduction (data not shown). *G. sulfurreducens* did not grow on CO (1–10 vol% in anoxic gas phase) and could not use this substrate as electron donor for fumarate reduction.

The UV spectrum of lipid extract of *G. sulfurreducens* at 230–350 nm strongly resembled that of vitamin K_1 (data not shown), indicating that it possesses a menaquinone.

Oxidation of acetate by *G. sulfurreducens* in coculture with *W. succinogenes*

Cocultures of *G. sulfurreducens* and *W. succinogenes* grew on acetate as electron donor and nitrate as electron acceptor, with an average doubling time of 5.1 h (μ=3.3 day^{-1}). Small amounts of succinate (30–50 µM) accumulated in the medium during the stationary phase under nitrate-limiting conditions.

To check how acetate was degraded by cocultures of *G. sulfurreducens* and *W. succinogenes*, several enzyme activities in cell-free extracts of the coculture were compared with those of pure cultures of the partner bacteria (Table 3). Coculture extracts had significant 2-oxoglutarate:benzyl viologen oxidoreductase activity. Extracts of *W. succinogenes* exhibited only low activity of this enzyme. Both strains grown in pure cultures and in coculture showed very low CO dehydrogenase activity in cell-free extracts.

Surprisingly, the hydrogenase activity found in cells of *G. sulfurreducens* grown on acetate plus fumarate was not detectable in the coculture (Table 3). On the contrary, the coculture possessed very high formate dehydrogenase activity which, however, could have originated from cells of *W. succinogenes*. All checked extracts had higher activities of NADPH oxidation than of NADH oxidation (measured as potassium ferricyanide reduction).

Since it was proposed that *c*-type cytochrome(s) could play a significant role as an electron carrier system in the

Table 4 Reduction of cytochrome(s) in cell extracts of *G. sulfurreducens* by citric acid cycle intermediates and reduced pyridine nucleotides (concentration in assays 5 and 0.5 mM, respectively). Protein content in 1 ml assay was 2.6 mg

Substrate	Specific activity [µmol min^{-1} (mg protein)$^{-1}$]
Isocitrate	3.13
2-Oxoglutarate	2.72
Succinate	0.17
Succinate+ATP[a]	0.36
Malate	3.16
NADPH	2.49
NADH	0.18

[a]0.1 mM ATP in assay mixture

coculture, we checked the ability of some intermediates of the citric acid cycle, NADPH, and NADH, to serve as electron donors for cytochrome(s) reduction in cell-free extracts of *G. sulfurreducens*. Before assaying, cytochrome(s) were oxidized by addition of a small amount of air. Succinate and NADH showed the lowest rates of cytochrome(s) reduction while the highest ones were caused by isocitrate, 2-oxoglutarate, malate, and NADPH (Table 4). Addition of ATP to the assay mixture stimulated cytochrome reduction by succinate. In contrast, fumarate (concentration in assay 0.25 mM) oxidized dithionite-reduced cytochrome(s) at a rate of 1.7 µmol min^{-1} (mg protein)$^{-1}$.

Discussion

Geobacter sulfurreducens grows by acetate oxidation coupled to reduction of fumarate to succinate, according to the following equation ($\Delta G^{\circ\prime}$ value calculated after Thauer et al. 1977):

$$CH_3COO^- + 4\,C_4H_2O_4^{2-} + 2\,H_2O + H^+ \rightarrow 2\,CO_2 + 4\,C_4H_4O_4^{2-}$$
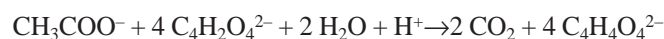
$$\Delta G^{\circ\prime} = -249 \text{ kJ per mol acetate}$$

Acetate metabolism proceeds via reactions of the citric acid cycle – all detected enzyme activities were sufficiently high to allow the observed rate of acetate oxidation.

In general, the citric acid cycle reactions found in *G. sulfurreducens* resemble those described for *D. acetoxidans* (Gebhardt et al. 1985). The most remarkable features of this cycle are the formation of citrate via citrate synthase rather than ATP-citrate lyase, and the presence of succinyl-CoA:acetate CoA-transferase instead of succinyl-CoA synthetase. However, the two pathways differ in ATP-dependent succinate oxidation: the reaction in *D. acetoxidans* is coupled to NAD$^+$ reduction (Paulsen et al. 1986), whereas in *G. sulfurreducens* it is coupled to NADP$^+$ reduction. Also, the enzyme acting in the backwards direction, fumarate reductase, is membrane-bound and reacts with both NADPH and NADH. Increased fumarate reductase activity in CTAB-permeabilized cell sus-
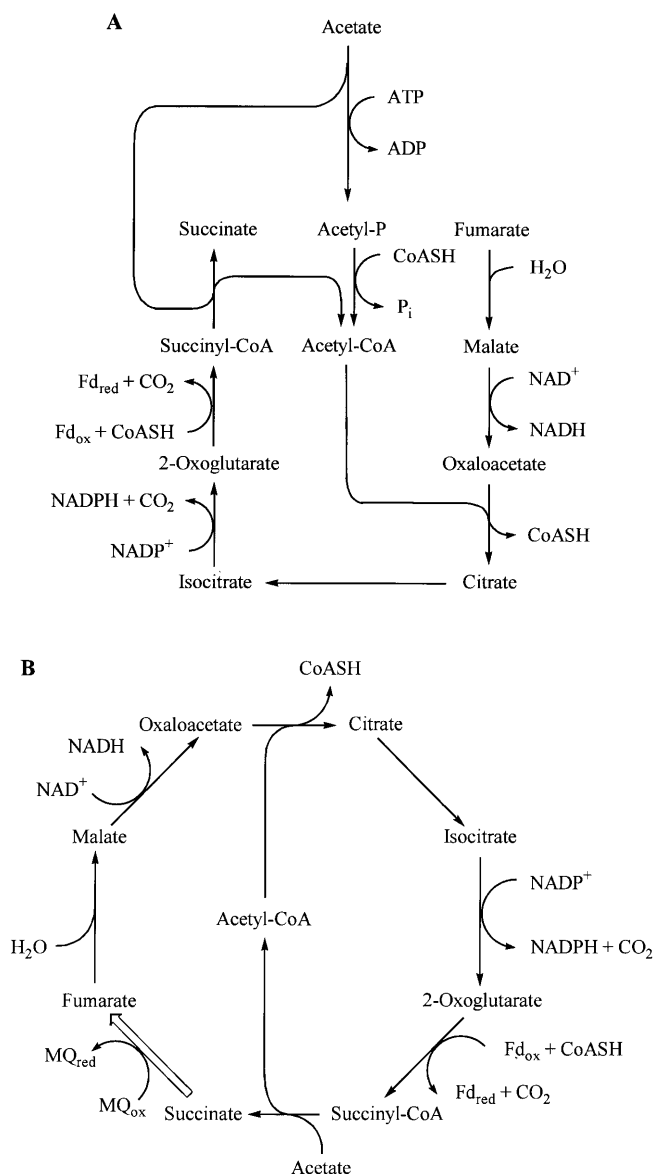
Fig. 2 Pathway of acetate oxidation to CO_2 in *G. sulfurreducens* **A** during growth in pure culture on acetate plus fumarate and **B** during growth on acetate in coculture with *Wolinella succinogenes*. The *broad arrow* refers to membrane-bound succinate dehydrogenase. Ferredoxin is assumed to be the physiological electron acceptor in 2-oxoglutarate oxidation

pensions of *G. sulfurreducens* indicates that this enzyme is localized at the inner face of the cytoplasmic membrane, as is found in *W. succinogenes* and *Escherichia coli* (Kröger et al. 1992; Unden and Bongaerts 1997). In the iron-reducing bacterium *Shewanella putrefaciens*, fumarate reductase has been described as a soluble, periplasmic flavocytochrome *c* enzyme (Pealing et al. 1992).

CoA-dependent 2-oxoglutarate oxidation was measured as benzyl viologen reduction, indicating that in vivo this enzyme couples to ferredoxin reduction. Ferredoxin often serves as physiological electron acceptor in this step in sulfate- and sulfur-reducing bacteria (Brandis-Heep et al. 1983; Gebhardt et al. 1985).

Malate oxidation coupled to NAD^+ reduction is an endergonic process at standard concentrations (standard free energy of the reaction at pH 7 is around +30 kJ per mol malate). To overcome this limitation, *G. sulfurreducens* accumulates malate and keeps the concentration of the reaction product, oxaloacetate, low by efficient removal in the citrate synthase reaction. Malate accumulation is indicated by transient malate release into the medium.

The importance of maintaining a certain intracellular acetyl-CoA level for growth and survival has been proven (Chohnan et al. 1997; Chang et al. 1999). Acetate-degrading anaerobic bacteria often use only one pathway of acetyl-CoA formation (Brandis-Heep et al. 1983; Schmitz et al. 1990), whereas *G. sulfurreducens* possesses two, thus securing a sufficient flux of acetyl-CoA for assimilatory and dissimilatory metabolism. In addition, minor amounts of acetyl-CoA could be formed also from malate via pyruvate in anaplerotic reactions, including decarboxylating $NADP^+$-dependent malate dehydrogenase and CoA-dependent pyruvate:electron acceptor oxidoreductase; the activity of the latter enzyme was low [20 nmol min^{-1} (mg protein)$^{-1}$]. However, the decarboxylating $NADP^+$-dependent malate dehydrogenase is active only at low acetyl-CoA concentrations due to its observed inhibition by acetyl-CoA, and, therefore, cannot sustain growth on fumarate or DL-malate alone in the absence of acetate.

Our results indicate that in this pathway there is no net ATP synthesis through substrate-level phosphorylation, and all ATP has to be produced by electron transport phosphorylation coupled to fumarate reduction. Oxidation of the acetyl residue releases three electron pairs that are transferred to fumarate: two at the redox potential of the $NAD(P)^+/NAD(P)H$ couple, and, presumably, one at that of ferredoxin. The latter one may be further used for $NADP^+$ reduction by ferredoxin:$NADP^+$ oxidoreductase. The presence of such an enzyme in *G. sulfurreducens* is postulated from the observed $NADP^+$ reduction during 2-oxoglutarate oxidation (Table 2). During growth on acetate plus fumarate, succinate is not oxidized to fumarate but excreted into the medium. Oxaloacetate, a substrate for the citrate synthase reaction with acetyl-CoA, is formed from exogenous fumarate via fumarase and malate dehydrogenase. Thus, the pathway does not operate in a cyclic mode but as an open loop (Gottschalk and Andreesen 1979; Fig. 2A).

The electron transport system of *G. sulfurreducens* consists of multiple cytochromes *c* (Gaspard et al. 1998; Seeliger et al. 1998; Lloyd et al. 1999) plus menaquinone. Menaquinone was detected in the present study, and its involvement in electron transport to fumarate is further supported by the observed inhibition of NADPH oxidation with fumarate by HOQNO, which blocks menaquinol-binding sites of fumarate reductase (Rothery and Weiner 1998). Therefore, we assume that during growth on acetate plus fumarate a menaquinone mediates the transfer of reducing equivalents from pyridine nucleotides further on in the electron transport chain. Redox properties of the chain components have not been evaluated yet. So far

there is no evidence for the presence of a *b*-type cytochrome in *G. sulfurreducens*.

Oxidation of NAD(P)H and reduced ferredoxin with ubiquinone via NADH dehydrogenase in aerobic bacteria is coupled to transmembrane translocation of four protons per electron pair (Richardson 2000), which would lead to 12 protons translocated per acetyl residue oxidized, and subsequent synthesis of 3–4 ATP. The energy span of this oxidation with menaquinone as acceptor is smaller. In fumarate reduction via menaquinone by *E. coli* (Unden and Bongaerts 1997), energy is conserved by a NADH-oxidizing proton-translocating dehydrogenase, but the stoichiometry of proton translocation in this reaction step is unknown. Known menaquinol:fumarate oxidoreductases do not translocate protons (Geisler et al. 1994; Iverson et al. 1999). Energy conservation in *G. sulfurreducens* during growth on acetate plus fumarate could be increased by consumption of eight protons on the cytoplasmic side of the membrane if this enzyme faces the cytoplasmic space, analogous to *W. succinogenes* (Geisler et al. 1994). An indication of this orientation has been obtained after partial membrane solubilization with CTAB.

If we assume that a Gibbs free energy change of about –70 kJ is required to synthesize 1 mol ATP (Schink 1997), acetate oxidation to CO_2 coupled to fumarate reduction ($\Delta G^{\circ\prime}$=–249 kJ per mol acetate) can yield ca. 3.6 mol ATP. An ATP yield of 10.0 g dry mass per mol ATP was calculated for growth on acetate in an inorganic salts medium (Stouthamer 1979). In our case, the ATP yield could be slightly higher because cysteine could contribute to a small extent to carbon assimilation. Thus, fumarate-dependent acetate oxidation allows the synthesis of 3–4 ATP via electron transport phosphorylation, but the proton translocation stoichiometry cannot yet be quantitatively assessed.

A comparison of enzyme activities (Table 3) proved that a complete citric acid cycle operates in *G. sulfurreducens* when acetate is oxidized in coculture with *W. succinogenes* (Fig. 2B). The 2-oxoglutarate:acceptor oxidoreductase detected in *W. succinogenes* presumably only operates in anabolic reactions (Bronder et al. 1982). Lack of sufficient hydrogenase or formate dehydrogenase activity indicated that neither hydrogen nor formate served as interspecies electron carrier to the partner bacterium.

Three of the electron pairs liberated in the citric acid cycle can reduce cytochrome(s) *c* directly. Only electrons released in the succinate dehydrogenase reaction would need an ATP-dependent reversed electron transport (Table 4) to reduce cytochrome(s) *c*; perhaps menaquinone acts as electron carrier in this reaction chain. If a small cytochrome *c* of a standard redox potential at –167 mV acts as major extracellular electron carrier (Seeliger et al. 1998), the standard free energy of acetate oxidation coupled to cytochrome *c* reduction is –95 kJ per mol acetate [calculated on the basis of the difference of the standard redox potentials of couples CO_2/acetate (-291 mV) and oxidized/reduced cytochrome *c*]. This value allows synthesis of 1.4 ATP, resulting in 14 g cell dry mass produced per mol acetate dissimilated (calculated after Stouthamer

1979). The latter value matches the estimated cell yield of *G. sulfurreducens* in coculture with *W. succinogenes* (14 g cell dry mass; Cord-Ruwisch et al. 1998). Thus, it appears reasonable that either the described cytochrome(s) or a carrier of similar redox potential transfers electrons between the two partners in this syntrophic association.

Our results indicate that both NADPH and NADH can transfer reducing equivalents to the electron transport chain during fumarate reduction by *G. sulfurreducens*. Similar results were obtained for *G. metallireducens* grown on benzoate plus Fe(III)-citrate (Champine et al. 2000). Other authors reported that Fe(III) reduction in *G. sulfurreducens* depended strictly on NADH (Gaspard et al. 1998; Magnuson et al. 2000) and that the small cytochrome *c* is not an efficient carrier of electrons to Fe(III) oxide (Lloyd et al. 1999). Further studies are required to elucidate the metabolic changes in the energy-conserving system of this bacterium during growth on acetate with Fe(III) as the electron acceptor.

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