Operation of the CO Dehydrogenase/Acetyl Coenzyme A Pathway in both Acetate Oxidation and Acetate Formation by the Syntrophically Acetate-Oxidizing Bacterium *Thermacetogenium phaeum*

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Received 27 July 2004/Accepted 13 February 2005

Thermacetogenium phaeum is a homoacetogenic bacterium that can grow on various substrates, such as pyruvate, methanol, or H₂/CO₂. It can also grow on acetate if cocultured with the hydrogen-consuming methanogenic partner *Methanothermobacter thermautotrophicus*. Enzyme activities of the CO dehydrogenase/acetyl coenzyme A (CoA) pathway (CO dehydrogenase, formate dehydrogenase, formyl tetrahydrofolate synthase, methylene tetrahydrofolate dehydrogenase) were detected in cell extracts of pure cultures and of syntrophic cocultures. Mixed cell suspensions of *T. phaeum* and *M. thermautotrophicus* oxidized acetate rapidly and produced acetate after addition of H₂/CO₂ after a short time lag. CO dehydrogenase activity staining after native polyacrylamide gel electrophoresis exhibited three oxygen-labile bands which were identical in pure culture and coculture. Protein profiles of *T. phaeum* cells after sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated that the strain exhibited basically the same protein patterns in both pure and syntrophic culture. These results indicate that *T. phaeum* operates the CO dehydrogenase/acetyl-CoA pathway reversibly both in acetate oxidation and in reductive acetogenesis by using the same biochemical apparatus, although it has to couple this pathway to ATP synthesis in different ways.

Conversion of acetate to methane and CO₂ can be carried out not only by aceticlastic methanogens but also by syntrophic associations of acetate-oxidizing bacteria and hydrogenotrophic methanogens, especially at enhanced temperatures (>55°C) (19). To date, several syntrophically acetate-oxidizing bacteria cooperating with partner methanogens have been reported (11, 20). All these bacteria oxidize acetate only if cocultured with hydrogen-scavenging methanogens. The reaction is highly endergonic under the standard conditions CH₃COO[−] + H⁺ + 2H₂O → 4H₂ + 2CO₂ (ΔG° = +95 kJ per mol) and requires that the hydrogen partial pressure is maintained low so the reaction can yield a minimum of energy to the acetate-oxidizing partner; at [H₂] = 10^{−4.5} atm, the ΔG' changes to −7.5 kJ per mol, and at this hydrogen partial pressure and enhanced temperature (55°C) the ΔG' is −25 kJ per mol, just sufficient to allow ATP synthesis (19).

The same bacteria can grow in pure culture by homoacetogenic fermentation of other substrates, such as sugars, alcohols, or H₂/CO₂. The ability to use acetate to produce H₂/CO₂ and vice versa by the same organism is an exciting phenomenon, since it implies that the bacterium can change substrate and product reversibly depending on the prevailing conditions. It also illustrates how close to the thermodynamic equilibrium an anaerobic fermentation process can operate.

Measurements of enzyme activities in syntrophically acetate-oxidizing cocultures indicated that the acetate-oxidizing partners used the CO dehydrogenase/acetyl coenzyme A (CoA) pathway both for acetate oxidation and acetate production (12, 21). However, these studies were performed with cocultures composed of syntrophic acetate oxidizers and the partner methanogens because separation of the partner cells was not possible. To date, only three acetate-oxidizing bacteria have been isolated in coculture with methanogens. One was lost later on, and another one was very difficult to grow and could hardly produce sufficient cell mass for biochemical analysis (21).

Recently, a novel thermophilic, syntrophically acetate-oxidizing bacterium, *Thermacetogenium phaeum* strain PB, has been isolated (8). This bacterium can grow homoacetogenically on several substrates and can also grow on acetate in syntrophic cooperation with *Methanothermobacter thermautotrophicus*. Besides hydrogen, also formate appears to act as an electron carrier in this syntrophic association (9). In the present study, we elucidated the pathway of acetate formation and acetate oxidation under both growth conditions with this bacterium which promises to become a model organism for the study of this unusual metabolism.

MATERIALS AND METHODS

Microorganisms and growth conditions. *Thermacetogenium phaeum* strain PB (DSM 12270) in pure culture or in coculture with the hydrogenotrophic methanogen *Methanothermobacter thermautotrophicus* strain TM was taken from our culture collection. Unless otherwise mentioned, cells were cultivated in potassium bicarbonate/CO₂-buffered reduced medium at 55°C as described previously (8). *T. phaeum* was cultured under three different conditions. For syntrophic growth, *T. phaeum* was cocultivated with *M. thermoautotrophicus* strain TM with

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80 mM sodium acetate. For growth in pure culture, *T. phaeum* was cultivated either with 40 mM sodium pyruvate or 40 mM methanol.

Acetate oxidation and acetate formation by cell suspensions. Five-hundred ml of the coculture of *T. phaeum* and *M. thermotrophicus* grown on acetate was harvested under anoxic conditions as described above. The cell pellet was suspended in 12.5 ml of prewarmed (55°C) reduced fresh medium without washing and transferred into 50-ml serum glass vials sealed with butyl rubber stoppers and aluminium crimps. For measuring acetate oxidation, the headspace was flushed with N_2/CO_2 (80:20 [vol/vol]) to purge hydrogen derived from the anoxic chamber. The reaction was initiated by adding sodium acetate to the cell suspensions to 56 mM final concentration. For measuring hydrogen-dependent reductive acetogenesis, an inhibitor specific for methanogens, bromoethanesulfonate, was added to the cell suspension to a final concentration of 50 mM and the reaction was started by flushing the headspace with H_2/CO_2 (80:20 [vol/vol]) at 170 kPa. Both reactions were performed at 55°C in the dark. To determine substrates and products, liquid and gas samples were taken at appropriate intervals. After the reactions were finished, the remaining cells were harvested and extracted by enzymatic lysis.

Preparation of cell extracts. All manipulations were performed inside an anoxic chamber (Don Whitley Scientific Limited, West Yorkshire, England) under $N_2/H_2/CO_2$ atmosphere (80:10:10, vol/vol). All centrifugations were carried out anaerobically. All buffers and reagents were prepared under anoxic conditions. Five-hundred ml of cultures at the late-exponential growth phase was harvested by centrifugation at $8,000 \times g$ and 20°C for 10 min under anoxic conditions. The cell pellet was washed twice with an equal amount of anoxic 10 mM Tris-HCl buffer, pH 7.5, containing 2.5 mM dithiothreitol (DTE). Cells were resuspended in anoxic buffer to a final volume of 5 ml, and aminoethylbenzene sulfonylfluoride (AEBSF) was added as protease inhibitor to the suspension to 1 mM final concentration. For preparation of cell extracts of *T. phaeum* in pure culture or in coculture with partner methanogens, lytic enzymes were used based on previously described methods (22, 27) with slight modifications. Briefly, 1000 units of mutanolysin (Sigma) and 5 mg of lysozyme (Sigma) dissolved in oxygen-free 10 mM Tris-HCl buffer, pH 7.0, were injected into the serum vials to give a final volume of 5.06-ml cell suspension. The suspension was incubated at 37°C for 30 to 45 min with gentle shaking to allow cell lysis. In case of *T. phaeum* cells in coculture, the suspension was centrifuged at $9,600 \times g$ and 4°C for 10 min to remove intact cells of the methanogenic partner. The supernatant was centrifuged again at $30,000 \times g$ and 4°C for 20 min to remove all remaining cell debris, and the resulting supernatant was used as cell extract of *T. phaeum* for enzyme assays. Cell extracts of the partner methanogen were prepared from the residual pellet after lysis of *T. phaeum* cells. The pellet was washed twice with 500 ml of anoxic buffer to remove contaminating extract constituents of strain PB and was resuspended in 5 ml of the same buffer. The methanogen cells were disrupted five times in a cooled, anoxic French pressure cell (Aminco) at 137 MPa. Cell debris and undisrupted cells were removed from the homogenate by centrifugation at $30,000 \times g$ and 4°C for 20 min.

Enzyme assays. Unless mentioned otherwise, enzyme activities in cell extracts were measured spectrophotometrically under N_2 atmosphere at 55°C using quartz cuvettes fitted with rubber stoppers. Each enzyme reaction was initiated by addition of cell extract to the respective reaction mixtures described below. Enzyme activities were measured under strictly anoxic conditions according to the method of Meßmer et al. (14), with slight modifications. For measurements of carbon monoxide dehydrogenase, formate dehydrogenase, hydrogenase, and 2-oxoglutarate dehydrogenase, reduction of benzyl viologen at 578 nm ($\epsilon = 8.65 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) or NAD(P)⁺ at 365 nm ($\epsilon = 3.4 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) was monitored in the presence of the respective substrates (modified, after reference 3). The reaction mixture contained 50 mM Tris-HCl buffer, pH 7.5, 5 mM $MgCl_2$, and 2.5 mM DTE. For carbon monoxide dehydrogenase and hydrogenase assays, the reaction mixture was saturated with CO or H_2 , respectively, flushing the headspace with the respective gas. Before addition of the cell extract, the reaction mixture was reduced slightly with ca. 0.2 mM sodium dithionite to develop a faint blue color. Background reactions in the absence of electron donors were measured as negative controls.

Formyltetrahydrofolate synthetase was measured in the direction of formyltetrahydrofolate formation from ATP, THF, and formate (15). The assay was performed in a 5-ml glass vial under an N_2 headspace sealed with black butyl rubber stoppers. The reaction mixture contained 50 mM Tris-HCl buffer, pH 8.0, with 50 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 5 mM NH_4Cl , 5 mM ATP, 10 mM tetrahydrofolate (THF), and 40 mM sodium formate. Ammonium ions are known to activate formyl tetrahydrofolate synthetase enzymes (16). Before starting the reaction by addition of formate, the mixture containing the cell extract was preincubated for 10 min at 55°C. The product was measured at 350-nm wavelength in samples withdrawn from the mix at 5-min intervals after chemical

conversion of formyltetrahydrofolate to methenyltetrahydrofolate by acidification with $HClO_4$ (15).

Methenyltetrahydrofolate dehydrogenase was assayed in the direction of methenyl tetrahydrofolate formation. The reaction mixture contained 50 mM Tris-HCl, pH 7.5, with 50 mM 2-mercaptoethanol, 2.5 mM formaldehyde, 1 mM THF, and 1 mM NAD⁺ (or NADP⁺) (26). Methenyltetrahydrofolate was chemically synthesized from formaldehyde and THF. The reaction was initiated by adding cell extract. The enzyme activity was measured via absorption of methenyl tetrahydrofolate at 350 nm, as described above (26).

Native PAGE for CO dehydrogenase activity staining. Native polyacrylamide gel electrophoresis (Native PAGE) was performed according to Laemmli (10) on a 4% stacking gel and a 6% separation gel. Gels were prepared under air and introduced inside the anaerobic chamber afterwards. They were soaked in oxygen-free loading buffer (50 mM Tris-HCl, pH 7.5, with 2.5 mM DTE and 2% Triton X-100) overnight. Cell extracts for electrophoresis were prepared as described above, except that they were treated with Triton X-100 (1% [vol/vol] final concentration) for 2 h on ice with gentle stirring to also resolve membrane-associated enzymes. The extracts were loaded onto the gels and separated at 100 V for 12 h. For CO dehydrogenase activity staining, gels were washed with prewarmed (55°C) CO-saturated reaction buffer (50 mM Tris-HCl, pH 7.5, plus 2.5 mM DTE) and soaked in 100 ml of the same CO-saturated buffer inside a polypropylene box. The box was sealed tightly with a semitransparent plastic lid, and the headspace of the box was replaced with pure CO for 10 min to remove the $N_2/H_2/CO_2$ gas mix. After preincubation at 55°C for 10 min, enzymatic reactions in the gels were initiated by addition of benzyl viologen (final concentration 2 mM). The bands of reduced benzyl viologen were fixed by further reaction with triphenyl tetrazoliumchloride (final concentration, 1 mM) (18).

SDS-PAGE of total proteins. SDS-PAGE was carried out according to Laemmli (10) with 4% stacking gels and 8% separation gels that were run at 20 mA for 4 h. Calibration proteins were obtained from Boehringer, Mannheim, Germany. After electrophoresis, the proteins were stained with Coomassie brilliant blue (Bio-Rad CBB staining kit) after Bradford (1) with bovine serum albumin as standard.

Analytical methods. Acetate and formate were determined by high-performance liquid chromatography (HPLC) on an ion-exclusion column as described previously (8). Methane and hydrogen were analyzed by gas chromatography as described previously (8). Protein contents of cell extracts were determined with bicinchoninic acid (BCA protein assay kit; Pierce, Rockford, Ill.) with bovine serum albumin as standard.

RESULTS

Thermacetogenium phaeum strain PB grew in coculture with *Methanothermobacter thermotrophicus* strain TM on 40 mM acetate with a growth rate of 0.012 h^{-1} (corresponding to a doubling time of 56.8 h). The final optical density at 600 nm (OD_{600}) was 0.26, corresponding to a molar growth yield of 2.4 g dry mass per mol acetate.

Shift from acetate oxidation to reductive acetogenesis. Resting cells of *T. phaeum* in coculture with *M. thermotrophicus* degraded acetate rapidly in cell suspensions (Fig. 1). Methane was produced from acetate at a 1:1 ratio. Also a trace amount of H_2 (up to 20 Pa) was detected during acetate oxidation. After acetate was consumed, H_2/CO_2 was added to the culture headspace. The cells started to consume H_2 and to produce acetate after a short lag phase (Fig. 1). Methane production was inhibited due to the presence of bromoethane sulfonate. The biomass ratio of *T. phaeum* to *M. thermotrophicus* cells was approximately 1:1.8 as estimated on the basis of protein determinations in either fraction after selective cell lysis. The specific rates of acetate oxidation and reductive acetate production by *T. phaeum* were 128 and $23 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$, respectively.

We also examined whether cells grown in pure culture on pyruvate or methanol could be converted quickly to syntrophic acetate oxidation by mixing resting cells of *T. phaeum* with *M. thermotrophicus* grown on H_2/CO_2 . However, no acetate

FIG. 1. Acetate oxidation and acetate formation (in the presence of 50 mM BES) by cell suspensions of *T. phaeum* and *M. thermoautotrophicus*. Symbols: (■) acetate; (●) methane; (□) hydrogen. Arrow indicates the point when H_2/CO_2 (170 kPa) was spiked and BES was added. The unit mM with gases means μmol per ml liquid medium.

oxidation was observed even if resting cells of the partner methanogen were used which were selectively obtained from a syntrophically acetate-oxidizing coculture after selective lysis of *T. phaeum* cells (see below). New cocultures recombined from separate pure cultures started acetate formation only after a time lag of about generally three weeks.

Selective enzymatic lysis of *T. phaeum* cells. Simultaneous application of lysozyme and mutanolysin to syntrophically grown cells caused selective cell lysis of *T. phaeum*, whereas the partner methanogens remained intact. *T. phaeum* cells started to lyse soon after addition of the two enzymes, and complete cell lysis was achieved within 40 to 60 min. Analysis of the extract for factor F_{420} by fluorescence spectrometry indicated that the contamination by cell constituents of the methanogen amounted to maximally 1.6% vol/vol. Cell lysis was incomplete if lysozyme or mutanolysin was added separately (Fig. 2). The lytic efficiency of the two enzymes was decreased as well if magnesium ions were present in the sample. This method was successfully applied also with *T. phaeum* in pure culture on pyruvate or methanol.

Enzyme activities of *T. phaeum* in syntrophic and in pure culture. The activities of major enzymes of the acetyl-CoA pathway were determined in cell lysates of *T. phaeum* (Table 1). All enzyme activities were detected both in cells grown syntrophically on acetate and in cells grown in pure culture on pyruvate. High activities of CO dehydrogenase (CODH) were detected in syntrophically grown cells, and slightly lower ones were detected in pyruvate-grown cells. In both cases, the CODH activity was oxygen sensitive. A small amount of benzyl viologen-reducing CODH activity ($0.3 \mu\text{mol min}^{-1} \text{mg protein}^{-1}$) was found in the cell extract of *M. thermoautotrophicus* after separate preparation (data not shown). Formate dehydrogenase and hydrogenase activity both were present at substantially higher activity in syntrophically grown than in pyruvate-grown cells. Oxidation of formate or hydrogen could also be coupled to NAD^+ reduction, however, only at about 1% of

FIG. 2. Enzymatic lysis of *T. phaeum* cells in coculture with *M. thermoautotrophicus*. Protein release from lysing cells after centrifugation was measured as absorption at 280 nm wavelength. Symbols: (■), control (without lytic enzymes); (▲), with mutanolysin; (●), with lysozyme; (□), with lysozyme plus mutanolysin; (○), same as open square except that 2.5 mM $MgCl_2$ was supplied.

the benzyl viologen-dependent activity, and no activity was found with $NADP^+$ as electron acceptor. No activity of 2-oxoglutarate dehydrogenase was found in any extract, neither with BV nor with NAD^+ as electron acceptor.

Formyl tetrahydrofolate synthase activity was low both in extracts of cells grown in pure culture on pyruvate and in those

TABLE 1. Specific enzyme activities in crude extracts of *T. phaeum* grown syntrophically on acetate and axenically on pyruvate^a

Enzyme	Sp act [$\mu\text{mol min}^{-1} \text{mg protein}^{-1}$]	
	Cells grown syntrophically on acetate	Cells grown in pure culture on pyruvate
CO dehydrogenase		
BV reduction	163.3	76.8
Formate dehydrogenase		
BV reduction	47.6	1.3
NAD^+ reduction	0.055	0.03
$NADP^+$ reduction	0.001	0.005
Hydrogenase		
BV reduction	23.4	4.16
NAD^+ reduction	0.126	0.037
$NADP^+$ reduction	0.001	ND
Formyl-THF synthetase		
ATP hydrolysis	0.067	0.010
Methylene-THF dehydrogenase		
NAD^+ reduction	3.46	1.21
$NADP^+$ reduction	0.008	0.004

^a Enzyme activities in the syntrophically grown cells were measured after selective lysis of *T. phaeum* cells. The measured activities within one extract preparation varied within $\pm 15\%$. No activity of 2-oxoglutarate dehydrogenase was found, neither with BV nor with NAD^+ as electron acceptor. ND, not detectable.

FIG. 3. Activity stains of CODH after Native PAGE of *T. phaeum* cell extracts. Proteins in the gel were stained with benzyl viologen and tetrazolium chloride (lanes 1 to 3) or with Coomassie brilliant blue (lanes 4 and 5). Lanes: 1, extract of pyruvate-grown culture; 2, extract of acetate-grown coculture; 3, extract of methanol-grown culture; 4, marker protein (ferritin, 440 kDa); 5, marker protein (β -galactosidase, 116 kDa). Each lane received 5 μ g of protein.

grown syntrophically on acetate, but these activities were still in the range of substrate turnover in exponentially growing cells (210 nmol mg protein⁻¹ min⁻¹). The activities could be stimulated slightly when the concentrations of tetrahydrofolate, formate, ATP, NH₄⁺, and 2-mercaptoethanol were increased (data not shown). Also, GTP and ITP could serve as substrate for the enzyme, but far less efficiently than ATP (data not shown). Methylene tetrahydrofolate dehydrogenase exhibited good activity with NAD⁺ and was hardly detectable with NADP⁺ as electron acceptor.

Activity staining of CODH during acetate oxidation and acetate production. Proteins in cell extracts of *T. phaeum* grown in pure culture or in coculture with the methanogen were separated by Native PAGE, and CODH was visualized by activity staining. In the presence of CO, three major bands appeared within 1 min in both extracts on the gels at the same migration distances (Fig. 3). Thicker bands were observed in cells grown syntrophically on acetate rather than in cells grown in pure culture on pyruvate or methanol. Extended incubation with CO increased the intensity of staining but caused the appearance of nonspecific color smears around the bands.

If gels were exposed to air, the stains disappeared rapidly within 5 min and never reappeared again, even if the gels were incubated again under the reaction conditions. Under N₂ atmosphere, several faint bands appeared as a result of background reactions, but migration distances of these bands differed from those of the bands formed under CO atmosphere (data not shown).

Protein profiles in extracts of *T. phaeum* cells in coculture and in pure culture. SDS-PAGE of crude extracts of *T. phaeum* cells cultivated syntrophically or in pure culture revealed

FIG. 4. SDS-PAGE of extracts of *T. phaeum* cells grown under different conditions. Proteins in the gel were stained with Coomassie brilliant blue. Lanes: 1, marker proteins including α -macroglobulin (340 kDa), β -galactosidase (116 kDa), fructose 6 phosphate kinase (85.2 kDa), glutamate dehydrogenase (55.6 kDa), aldolase (39.2 kDa), trypsin inhibitor (20.1 kDa); 2, extract of *T. phaeum* cells grown on methanol; 3, extract of *T. phaeum* cells grown on pyruvate; 4, extract of *T. phaeum* cells grown on acetate with *M. thermoautotrophicus*; 5, extract of *M. thermoautotrophicus* cells grown on acetate with *T. phaeum*. Each lane received 10 μ g of protein. Cell extract of *M. thermoautotrophicus* (lane 5) was prepared by French pressure cell treatment after *T. phaeum* was separated by selective cell lysis.

basically the same protein profiles (Fig. 4). However, two dense bands observed in syntrophically grown cells (Fig. 4, lane 4) appeared much smaller in cells grown on pyruvate than in those grown on methanol. Molecular masses of these bands were estimated to be 76.6 kDa and 51.2 kDa, respectively. Two further dense bands (estimated to be 40.1 kDa and 30.0 kDa in size) were detected in all three cell preparations. Cell extract of *M. thermoautotrophicus* after SDS-PAGE showed band profiles basically different from those of *T. phaeum*. Comparison of lanes 4 and 5 also documents how efficient the selective cell lysis procedure was that was applied in our study.

DISCUSSION

Anaerobic oxidation of acetate can be catalyzed via two different pathways, the citric acid cycle and the CO dehydrogenase (CODH)/acetyl-CoA pathway (13, 24). The key enzymes of these pathways are 2-oxoglutarate dehydrogenase and CODH/acetyl-CoA synthase, respectively. Whereas the citric acid cycle is used by sulfur reducing and few sulfate-reducing bacteria (2, 7, 17) and by the iron(III) reducer *Geobacter sulfurreducens* (6), the CODH/acetyl-CoA pathway

is employed by most sulfate reducers and by the aceticlastic methanogens (4, 18, 23, 25). Preliminary experiments also indicated that two syntrophically acetate-oxidizing bacteria, strain AOR and *Clostridium ultunense*, metabolize acetate via the CODH/acetyl-CoA pathway (12, 21), but these experiments were performed with mixed extracts of both partners in methanogenic cocultures.

In the present study, we tried to elucidate the acetate degradation pathway of *T. phaeum*, a novel syntrophically acetate-oxidizing bacterium, after growth in pure culture or in syntrophic coculture. To assay selectively for enzymes of the fermenting bacterium, we developed a selective cell extraction method for *T. phaeum*. With a combination of lysozyme and mutanolysin, selective cell lysis of *T. phaeum* was successfully achieved (Fig. 2). Both lysozyme and mutanolysin had to be applied simultaneously, since only incomplete lysis was achieved with either enzyme alone. To achieve complete lysis of *T. phaeum* cells, washing of the cells was essential to remove divalent cations such as magnesium.

Extracts of *T. phaeum* cells grown in coculture exhibited high activities of CODH and of tetrahydrofolate-linked redox enzymes and no activity of 2-oxoglutarate dehydrogenase, indicating that *T. phaeum* possesses the CODH/acetyl-CoA pathway rather than the citric acid cycle for acetate oxidation. Pure-culture cells of *T. phaeum* forming acetate possessed the same enzymes at slightly lower specific activities. These results suggest that this bacterium utilizes the CO dehydrogenase/acetyl-CoA pathway in both directions, i.e., oxidation and synthesis of acetate.

Comparing the enzyme activities in *T. phaeum* with those of previously reported syntrophs, *T. phaeum* exhibited far higher activities, especially of CODH. Formate dehydrogenase and hydrogenase showed high activities with benzyl viologen and far lower but still significant activities with NAD^+ , indicating that a low-potential electron carrier such as a ferredoxin may be the primary acceptor of these enzymes. NAD^+ reduction in our assays may be mediated through a ferredoxin: NAD^+ oxidoreductase as a secondary activity. NADP^+ did not play a major role in electron exchange with these substrates. Also the methylene tetrahydrofolate dehydrogenase activity coupled far more efficiently to NAD^+ than to NADP^+ in cells grown under either condition. Thus, this enzyme differs from the corresponding enzyme activity in *C. ultunense* which showed NAD^+ dependent methylene tetrahydrofolate dehydrogenase activity in the extract of the syntrophically acetate-oxidizing coculture, whereas NADP^+ dependent activity was found in the extract of pure-culture cells (21).

Since cell extracts of *T. phaeum* grown under different conditions exhibited activities of the same enzymes related to the CODH/acetyl-CoA pathway, we supposed that they could operate the pathway reversibly using the same set of enzymes. If an acetate-oxidizing coculture in the absence of acetate was exposed to high amounts of H_2/CO_2 it produced acetate nearly immediately after a short time lag, indicating that *T. phaeum* is likely to use at least a major part of the CODH/acetyl-CoA pathway enzymes both in the oxidative and the reductive direction. One could also assume that the bacterium constitutively expresses the enzymes required for acetate production even during periods of syntrophic acetate oxidation. A shift back from pure culture growth on pyruvate or methanol to

syntrophic cooperation turned out to be much more difficult to achieve and required several weeks until the cooperation was reestablished, thus indicating that the biochemical apparatus used is not identical both ways.

These results indicate that at least part of the enzymes required for syntrophic acetate oxidation may be inducible. Actually, SDS-PAGE analysis showed that two dense bands (corresponding to 76.6 kDa and 51.2 kDa, respectively) are produced only in syntrophically grown cells of *T. phaeum*, although the protein profiles were basically the same among different culture conditions (Fig. 4). This indicates that at least two proteins are inducible or upregulated in coculture. On the contrary, there were several bands that appeared only in pure culture grown on pyruvate or methanol but not in the coculture. Since cells could switch immediately from acetate oxidation to reductive acetogenesis, these proteins found in pure-culture cells might not be related to the central role of the pathway, i.e., formation and oxidation of acetate, but might act as components required for catabolic metabolism (e.g., pyruvate-ferredoxin oxidoreductase or methanol dehydrogenase).

Lee and Zinder (21) demonstrated that a syntrophically acetate-oxidizing bacterium, strain AOR, possessed two different forms of CO dehydrogenases if cocultivated with partner methanogens on acetate. Also, *Desulfobacterium autotrophicum*, an acetate-oxidizing sulfate-reducing bacterium, possessed different forms of the enzyme if cultivated autotrophically on H_2/CO_2 plus sulfate (18). It appears plausible that strain AOR and *D. autotrophicum* use the CODH/acetyl-CoA pathway both in the oxidative and the reductive direction, in the latter case only for autotrophic cell carbon assimilation. In *T. phaeum*, CODH activity staining after PAGE of cell extracts exhibited identical migration band patterns after growth under both conditions, indicating that it might use the same form of CODH both in the oxidative and the reductive CODH/acetyl-CoA pathway.

An open question remains how the bacteria can link the reactions of this pathway in either direction to ATP synthesis. At least one step in either direction has to be linked to ATP synthesis via different membrane-bound ion translocation steps. We have tried to localize the various enzyme activities in cell fractions under both growth conditions, but due to problems with separating cell fractions in very small amounts of cell material the results were not conclusive.

Homoacetogenic bacteria are known to be metabolically very versatile (5). The finding of homoacetogens that can operate the key reactions in their energy metabolism basically in two different directions extends this versatility even further. We do not know whether the acetogenic or the acetotrophic lifestyle is the predominant one of these bacteria in their natural environment. In any case, their capacity to adapt to such basic changes in their lifestyle enables them to cope with dramatic changes in their life conditions.

ACKNOWLEDGMENTS

We are grateful to Seigo Amachi, Toshihiko Suzuki, and Toru Shigematsu for fruitful discussion about biochemistry and ecology of syntrophic anaerobes.

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