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Preparation of Cell-Free Extracts and the Enzymes Involved in Fatty Acid Metabolism in *Syntrophomonas wolfei*

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***Syntrophomonas wolfei* is an anaerobic fatty acid degrader that can only be grown in coculture with H_2-using bacteria such as *Methanospirillum hungatei*. Cells of *S. wolfei* were selectively lysed by lysozyme treatment, and unlysed cells of *M. hungatei* were removed by centrifugation. The cell extract of *S. wolfei* obtained with this method had low levels of contamination by methanogenic cofactors. However, lysozyme treatment was not efficient in releasing *S. wolfei* protein; only about 15% of the L-3-hydroxyacyl-coenzyme A (CoA) dehydrogenase activity was found in the lysozyme supernatant. Cell extracts of *S. wolfei* obtained with this method had high specific activities of acyl-CoA dehydrogenase, enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase. These activities were not detected in cell extracts of *M. hungatei* grown alone, confirming that these activities were present in *S. wolfei*. The acyl-CoA dehydrogenase activity was high when a C_4 but not a C_8 or C_{16} acyl-CoA derivative served as the substrate. *S. wolfei* cell extracts had high CoA transferase specific activities and no detectable acyl-CoA synthetase activity, indicating that fatty acid activation occurred by transfer of CoA from acetyl-CoA. Phosphotransacetylase and acetate kinase activities were detected in cell extracts of *S. wolfei*, indicating that *S. wolfei* is able to perform substrate-level phosphorylation.**

The complete anaerobic degradation of organic matter to CH_4 and CO_2 involves the concerted action of four major metabolic groups of bacteria (9, 27, 45). First, fermentative bacteria hydrolyze the primary substrate polymers such as polysaccharides and proteins and ferment the products mainly to volatile fatty acids, CO_2, and CH_4. The H_2-producing acetogenic bacteria degrade propionate and longer-chain fatty acids and some aromatic acids to acetate, H_2, and sometimes CO_2. The methanogenic bacteria use H_2 to reduce CO_2 to CH_4, and some species cleave acetate to CO_2 and CH_4. A fourth group of bacteria, H_2-using acetogens, produces acetate and some butyrate from H_2/CO_2, methanol, CO, and methoxy moieties of some aromatic compounds (1, 2, 38).

Propionate and longer-chain acids are important intermediates in the complete degradation of organic matter to CO_2 and CH_4 (21, 23, 25), and the degradation of these compounds is often the rate-limiting step in methane fermentation (26). Only recently have the bacteria responsible for the degradation of these compounds been isolated in coculture with H_2-using bacteria (7, 29, 32). *Syntrophomonas wolfei* degrades C_4 to C_8 straight-chain fatty acids to acetate and H_2 or to acetate, propionate, and H_2 (25, 26); isoheptanoate is degraded to isovalerate, acetate, and H_2. *Syntrophobacter wolinii* degrades propionate to acetate, H_2, and presumably CO_2 (7). *Clostridium bryantii*, in coculture with an H_2-using bacterium, degrades C_4 through C_{11} straight-chain fatty acids to acetate and H_2 or to acetate, propionate, and H_2 (3, 9). Long-chain fatty acids such as stearate are degraded mainly to acetate and presumably H_2 by a bacterium morphologically similar to *S. wolfei* (W. H. Lorowitz and M. P. Bryant, Abstr. Annu. Meet Am. Soc. Microbiol. 1985, I21, p. 150). Thermophilic, H_2-producing, acetogenic bacteria have been isolated in coculture with H_2-using methanogens that degrade acetate (46) or butyrate (18). The anerobic degradation of fatty acids with H_2 production is energetically unfavorable unless the H_2 concentration is maintained at a very low level by the H_2-using bacterium (9, 27). Because of this, the anaerobic fatty acid-degrading syntrophic bacteria can only be grown in coculture with H_2-using bacteria.

S. wolfei does not use any other common bacterial energy source or combination of electron donor and acceptor that would enable it to grow in pure culture (28, 29). Growth of *S. wolfei* in coculture with an H_2-using sulfate reducer or methanogen is slow, with the most rapid generation time being 56 and 84 h, respectively (28). Due to the slow growth rates, the low cell yields, and the inability of the syntrophic bacteria to grow in pure culture, the biochemical characterization of these organisms has been hampered.

In this paper, we describe methods to mass culture *S. wolfei* in association with *Methanospirillum hungatei* and to obtain cell extracts of *S. wolfei* essentially free from contamination by cellular components of the methanogen. These methods were used to study the enzymes involved in the activation and beta-oxidation of fatty acids and substrate-level phosphorylation reactions in *S. wolfei*.

MATERIALS AND METHODS

Organisms and growth conditions. *S. wolfei* (Göttingen strain) in coculture with *M. hungatei* JF1 was grown in the butyrate basal medium of McInerney et al. (29). Methods for the preparation and use of anaerobically prepared media were essentially those of Bryant (10) as modified by Balch and Wolfe (3). Ten-liter cocultures in 20-liter carboys were autoclaved for 45 min at 121°C and immediately sparged with an 80% N_2–20% CO_2 gas mixture for 2 to 3 h before the addition of sterile, anaerobically prepared sodium bicarbonate buffer, cysteine-sulfide reducing solution, and butyrate. The final concentration of butyrate was 40 mM. The medium for a 10-liter culture was allowed to equilibrate and reduce for at least 24 h before inoculation with the methanogenic

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coculture. Cultures of 300 ml were grown in 2-liter Schott (Bellco Glass, Inc., Vineland, N.J.) bottles for inoculation of 10-liter cultures. Ten-liter and 300-ml cocultures had stoppers in which a stoppered top of a Balch tube was inserted to facilitate anaerobic additions, samplings, and transfers (3). The purity of each coculture was checked by microscopic analysis and by inoculation of thioglycolate broth (Difco Laboratories, Inc., Detroit, Mich.), which does not support the growth of either species. *M. hungatei* JF1 was grown in the butyrate basal medium of McInerney et al. (29) with the addition of 0.2% sodium formate and an 80% H_2–20% CO_2 gas phase. Cultures were grown in a 3-liter fermentor that was agitated and sparged with the 80% H_2–20% CO_2 gas mixture (11). *Escherichia coli* ATCC 11303 was grown in an aerated 4-liter culture containing a mineral salts medium with 4 mM palmitic acid suspended in Triton X-100 (13).

Cell suspensions. Cells of *S. wolfei-M. hungatei* were harvested by centrifugation (3,840 × *g*, 30 min, 4°C), and the pellet was suspended in basal medium (29) to give a concentration of 5.5 mg (dry weight) per ml. A 10-ml volume of the cell suspension was transferred to an anaerobic, sealed serum tube, and 1 mM sodium butyrate was added. Samples were withdrawn every hour, and the concentration of butyrate and acetate was determined by gas chromatography (38). The K_s and V_{max} for butyrate use were estimated by nonlinear regression analysis of the butyrate depletion curve (36). The concentration of the cells was determined by drying a known volume at 75°C until a constant weight was obtained. The dry weight was corrected for the weight of the medium components.

Preparation of cell extracts. Anaerobically collected cells of the *S. wolfei-M. hungatei* coculture or *M. hungatei* grown in pure culture were suspended in 0.1 M Tris hydrochloride buffer (pH 8.0) and washed three times by centrifugation (12,100 × *g*, 10 min, 4°C), and the cell pellet was suspended in the same buffer. After the final wash the pellet was suspended in 0.1 mM Tris hydrochloride buffer (pH 8.0) containing 0.4 mg of the sodium salt of EDTA per ml and 50 μg of lysozyme per ml (44). A 10-ml volume of this buffer was used per g (wet weight) of cells. The suspension was incubated for the indicated times before the reaction was stopped by the addition of a concentrated Tris hydrochloride buffer (pH 8.0) that brought the concentration of Tris hydrochloride to 10 mM. The number of *S. wolfei* cells was determined before and after lysozyme treatment by using a Petroff-Hausser counting chamber as described previously (28). The suspension was centrifuged (12,000 × *g*, 10 min, 4°C), and the supernatant was collected. The pellet remaining after lysozyme treatment was suspended in 10 mM Tris hydrochloride buffer (pH 8.0). Cell suspensions of the *S. wolfei-M. hungatei* coculture, *M. hungatei* grown in pure culture, and unbroken cells of the lysozyme pellet were broken by two passages through a French pressure cell (American Instruments Co., Silver Spring, Md.) at 16,000 lb/in^2. Suspensions were loaded into and collected from the French pressure cell under a stream of O_2-free N_2 gas. Cells of *E. coli* were disrupted in air by two passages through a French pressure cell at 8,000 lb/in^2 after washing and resuspension of the cells in 0.1 M phosphate buffer. All manipulations of the cells of the coculture and methanogen were performed in an anaerobic chamber (Coy Manufacturing Co., Ann Arbor, Mich.) using anaerobic solutions and sealable centrifuge tubes (Du Pont Institute, Bridgeport, Conn.) (3). The cell extracts were placed in crimp-seal vials on ice and used immediately for the determination of enzymatic activities.

Methanogenic cofactors. Contamination by cellular components of *M. hungatei* in cell extracts of *S. wolfei* prepared by lysozyme treatment was monitored each time by spectrofluorometric determination of the methanogenic cofactor factor$_{420}$ (F_{420}; 12) using an Aminco spectrofluorimeter (American Instruments Co.). F_{420} was quantitated using a highly purified fraction supplied by D. Nagle as a standard. The extinction coefficient used for F_{420} was 51.5 ml/mg per cm at pH 8.0 (14). In some experiments, the amounts of coenzyme M and F_{342} were determined by bioassay for coenzyme M (3) and spectrofluorimetrically for F_{342} (16).

Enzyme assays. Unless otherwise indicated, assays were performed spectrophotometrically in air at room temperature. Activity was corrected for endogenous activity in the crude extracts. The activity of each enzyme under these conditions was proportional to the amount of protein added and was linear with respect to time. Controls using boiled extracts or lacking the substrate were performed for each assay. One unit of enzymatic activity is defined as the amount of enzyme catalyzing the conversion of 1 μmol of reactant to product per min. Specific activity is reported as units per milligram of protein. Protein was determined colorimetrically by the Lowry method (24) using bovine serum albumin as the standard. Protein concentrations were corrected for the amount of lysozyme added to the cell suspension. Protein concentrations for extracts containing deoxycholate were measured using standards containing 1% deoxycholate.

Acyl-coenzyme A (CoA) synthetase (acid:CoA ligase [AMP forming], EC 6.2.1.3) activity was assayed by the hydroxamate assay of Kornberg and Pricer (22) as modified by Overath et al. (35). The reaction mixture contained 1.3 mM butyrate, 13.3 mM NaF, 0.3 mM Triton X-100, 83 mM Tris hydrochloride (pH 8.5), 330 mM hydroxylamine hydrochloride, 3.3 mM ATP, 225 μM CoA, 6.7 mM $MgCl_2$, and 13.3 mM 2-mercaptoethanol in a volume of 1.5 ml. The reaction mixture was incubated for 1 h at 30°C after the addition of the cell extract. The reaction was stopped with 0.1 ml of 70% $HClO_4$ after the addition of 1.25 mg of bovine serum albumin. The mixture was centrifuged and washed by centrifugation, and the cell pellet was suspended in 0.5 ml of 3.5% $HClO_4$. Th amount of hydroxamic acid formed was measured by the addition of an Fe reagent (20), using a molar extinction coefficient of 1.1×10^3 M^{-1} cm^{-1} at 520 nm. A sample lacking ATP was used as the blank.

CoA transferase (EC 2.8.3.–) activity was determined by the arsenolysis method of Barker et al. (4). The reaction mixture contained 20 mM sodium acetate, 44 mM sodium perarsenate, 66 μM butyryl-CoA, 3 U of phosphotransacetylase, and cell extract in a total volume of 1 ml. The reaction was started by the addition of butyryl-CoA. The difference in molar extinction coefficients between butyryl-CoA and its hydrolysis products is 4.5×10^5 M^{-1} cm^{-1} at 232 nm.

Acyl-CoA dehydrogenase (EC 1.3.99.3) activity was assayed with dichlorophenolindophenol as an electron acceptor and phenazine methosulfate as an intermediate electron carrier by modifying the procedure of Thorpe (42). The molar absorption coefficient used for dichlorophenolindophenol at 600 nm was 21,000. The 1-ml assay mixture contained 35 mM potassium phosphate, 0.3 mM EDTA, 30 μM dichlorophenolindophenol, 1.4 mM phenazine methosulfate, and the cell extract. After equilibration in subdued light, the reaction was initiated by the addition of 150 μM butyryl-CoA. Assays were also performed using 150

μM octanoyl-CoA and palmitoyl-CoA as substrates to examine the effect of chain length of the acyl-CoA substrate on activity.

The activity of enoyl-CoA hydratase (EC 4.2.1.17) was determined by a modification of the method described by Fong and Schulz (15). Hydration of the substrate was determined indirectly using a coupled assay containing L-(−)-3-hydroxyacyl-CoA dehydrogenase. The reduction of NAD that accompanied the NAD-dependent oxidation of L-(−)-3-hydroxyacyl-CoA produced by enoyl-CoA hydratase activity was followed. The addition of three times the amount of L-(−)-3-hydroxyacyl-CoA dehydrogenase did not affect activity. The reaction mixture contained 0.1 M Tris hydrochloride (pH 9.0), 0.1 M KCl, 0.1 mg of bovine serum albumin per ml, 200 μM NAD, 2 U of L-(+)-3-hydroxyacyl-CoA dehydrogenase, and cell extract. The reaction was started by the addition of crotonyl-CoA to give a final concentration of 0.2 mM.

The activity of L-(+)-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) was measured by monitoring the decrease in absorption of NADH at 340 nm which accompanied the conversion of an *S*-acetoacetyl-CoA to the corresponding 3-hydroxybutyryl-CoA. The 1-ml reaction mixture used was a modification of that of Bradshaw and Noyes (8), containing 12.5 mM sodium pyrophosphate buffer (pH 7.3), 0.25 mM NADH, and the cell extract. The addition of 1 mM *S*-acetoacetyl-CoA initiated the reaction.

The activity of 3-ketoacyl-CoA thiolase (EC 2.3.2.9) was determined by following the CoA-dependent acetoacetyl-CoA cleavage. The decrease in absorbance at 303 nm was measured spectrophotometrically as described by Middleton (30). The reaction mixture contained 100 mM Tris hydrochloride (pH 8.1), 25 mM $MgCl_2$, 50 mM KCl, 10 μM *S*-acetoacetyl-CoA, and cell extract. The reaction was initiated by the addition of 0.01 mM CoA. The apparent molar extinction coefficient was 16,900 under these standard assay conditions.

Phosphotransacetylase (acetyl-CoA:orthophosphate acetyltransferase, EC 2.3.1.8) was assayed by measuring the formation of acetyl-CoA from acetyl-phosphate. The procedure used was that of Bergemeyer et al. (5) as modified by Hartmanis and Gatenbeck (17). The 1.0-ml reaction mixture contained 100 mM Tris hydrochloride (pH 8.0), 100 mM KCl, 0.6 mM CoA, and 10 mM lithium acetyl-phosphate. The appearance of the thioester bond was measured spectrophotometrically at 233 nm using an extinction coefficient of $4.44 \times 10^3\ M^{-1}\ cm^{-1}$. Controls lacking acetyl-phosphate or CoA had no activity.

Acetate kinase (ATP:acetate phosphotransferase, EC 2.7.2.7) was assayed by the hydroxamate method of Rose (37). The reaction was assayed in the reverse direction using acetate as a substrate. The reaction mixture contained 770 mM sodium acetate, 50 mM Tris hydrochloride (pH 7.4), 1 mM $MgCl_2$, 10 mM ATP, 10% hydroxylamine hydrochloride, and cell extract in a total volume of 1 ml. The reaction was stopped after 2 min by the addition of 1 ml of 10% trichloroacetic acid. The absorbance was measured at 540 nm against a blank which contained all reagents except ATP. The molar extinction coefficient under these conditions was $828\ M^{-1}\ cm^{-1}$.

Chemicals. CoA derivatives, L-(+)-3-hydroxyacyl-CoA dehydrogenase, dichlorophenolindophenol, phenazine methosulfate, hydroxylamine hydrochloride, CoA (lithium salt), acetyl-phosphate (lithium salt), and phosphotransacetylase were purchased from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

Growth studies. Cocultures containing *S. wolfei* grow very slowly and have low cell densities (28, 29). However, large-volume cocultures of *S. wolfei* and *M. hungatei* were consistently obtained when anaerobic procedures were carefully followed during the addition of medium components and the inoculation of the culture. The absorbance of 300-ml cocultures of *S. wolfei* and *M. hungatei* doubled in about 7 days, and these cultures were easily maintained by transferring exponential-phase cultures. Visible gas production in 10-liter cocultures occurred after 1 week of incubation, and an increase in absorbance was noticeable within 2 to 3 weeks after inoculation. About 0.1 to 0.25 g (wet wt) of cells per liter was obtained after about 4 to 6 weeks of incubation. The K_s and V_{max} for butyrate use by cell suspensions of *S. wolfei* with *M. hungatei* were 0.47 mM and 0.35 μmol/ml per mg (dry weight) of cells, respectively.

Preparation of cell extracts. Cell-free extracts of *S. wolfei* were prepared by lysing cells of *S. wolfei* with lysozyme and removing the unlysed cells of *M. hungatei* by centrifugation. No cells were observed microscopically in the cell extract of *S. wolfei*. Also, no viable cells of either *S. wolfei* or *M. hungatei* were detected (less than 40 cells per ml) when the appropriate procedures to grow each strain in roll-tube medium (29) were used. Direct cell counts showed that the number of *S. wolfei* cells decreased from 1.8×10^8 to 0.5×10^8 cells per ml after 15 min of incubation with lysozyme, while the numbers of *M. hungatei* cells increased from 4.3×10^8 to 9.0×10^8 cells per ml. These data show that little lysis of *M. hungatei* cells occurred.

About 3 to 12 ml of *S. wolfei* cell extract (supernatant obtained after incubation with lysozyme and centrifugation) with protein concentrations of 0.1 to 0.6 mg/ml was obtained when 0.3 to 0.9 g (wet weight) of cells was used. Cell extracts of *S. wolfei* contained low levels of the three methanogenic cofactors coenzyme M, F_{342}, and F_{420} (Table 1). The amount of contamination by cellular components of *M. hungatei* as measured by F_{420} was low, ranging from 0.7 to 6.0%. Lysozyme treatment released about 10 to 17% of the total amount of protein and about 15% of the L-3-hydroxyacyl-CoA dehydrogenase activity (Table 1). The amount of activity recovered in the lysozyme supernatant differed depending on the beta-oxidation activity measured, but the amount of activity present in the lysozyme supernatant was 5- to 22-fold higher than the amount of F_{420}, indicating that cells of *S. wolfei* were selectively lysed. These data also suggest that lysozyme treatment was not very efficient in lysing *S. wolfei* cells.

Longer incubation times with lysozyme (up to 40 min) and the addition of 1% (wt/vol) sodium deoxycholate to the breakage buffer slightly increased the total amount of F_{420} (20 to 30 ng) and of protein (9 to 10.8 mg) in the lysozyme supernatant. The specific activity of acyl-CoA dehydrogenase in the lysozyme supernatant decreased when deoxycholate or longer incubation times were used (data not shown). A sevenfold higher concentration of lysozyme did not increase the specific activity of acyl-CoA dehydrogenase in the lysozyme supernatant. Placing the lysozyme-treated cell suspension in a ultrasonic water bath or in a tissue homogenizer did not increase the efficiency of breakage of *S. wolfei* cells. Similar results were obtained when cell pellets stored at −4°C for less than 1 month were used. When cell pellets which were stored at −4°C for 2 months were used, the amount of contamination as measured by F_{420} was about 43%. The above data show that lysozyme treatment is

TABLE 1. Release of methanogenic cofactors, beta-oxidation enzymes, and protein by lysozyme treatment of the *S. wolfei-M hungatei* coculture

Fraction[a]	Coenzyme M			F_{420}			F_{342}			L-3-hydroxyacyl-CoA dehydrogenase		Protein	
	nmol	nmol/mg of protein	% Total[b]	ng	ng/mg of protein	% Total[b]	FIU[c]	FIU/mg of protein	% Total[b]	U	% Total[b]	mg	% Total[b]
Supernatant	9	8.5	2.6	10.5 ± 8.8	9.2 ± 7.5	3.2	3.9	0.8	5	9.3	15.4	1.1 ± 0.2	10.7
Pellet	336	36.5	97.4	285 ± 60	31.7 ± 6.8	96.8	74.8	8.4	95	50.7	84.6	9.0 ± 0.6	89.3

[a] Cell suspensions of *S. wolfei-M. hungatei* coculture were anaerobically treated with lysozyme for 10 to 20 min as described in Materials and Methods. The suspension was then centrifuged, and the supernatant was removed and analyzed. The pelleted cells were suspended in buffer, broken in a French pressure cell, and centrifuged as described above to remove unbroken cells. The supernatant obtained after this treatment is called the pellet fraction.
[b] Amount in the supernatant divided by the amount in both fractions times 100.
[c] FIU, Fluorescence intensity units.

effective in selectively lysing *S. wolfei* cells and that cell extracts of *S. wolfei* can be obtained that contain low levels of contamination by cellular components of the methanogen.

Beta-oxidation enzymes. Cell extracts of the *S. wolfei-M. hungatei* coculture prepared by lysozyme treatment contained high specific activities of acyl-CoA dehydrogenase, enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase (Table 2). Aerobically prepared cell extracts also had high specific activities of these enzymes. The cell pellet obtained after lysozyme treatment was assayed for the above enzyme activities after passage through a French pressure cell. This fraction had high specific activities of each of the above enzymes. This agrees with the above data, indicating that lysozyme treatment did not release all of the *S. wolfei* protein. The specific activities of acyl-CoA dehydrogenase and enoyl-CoA hydratase were higher in the lysozyme supernatant, while L-3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase were higher in the lysozyme pellet. The specific activities of the four beta-oxidation enzymes in the lysozyme supernatant or pellet fraction were much higher than the respective enzyme activity in *E. coli* cell extracts assayed under identical conditions. The specific activities found for the four beta-oxidation enzymes in *E. coli* were in agreement with published data from other laboratories (8, 34). Cell extracts of *M. hungatei* grown in the butyrate basal medium with sodium formate and a H_2/CO_2 gas phase did not have detectable amounts of any of the four beta-oxidation enzyme activities. Addition of *M. hungatei* cell extracts to reaction mixtures containing appropriate amounts of the lysozyme supernatant did not inhibit the activity of any of the four beta-oxidation enzymes (data not shown). This plus the fact that the cell extracts of the *S. wolfei-M. hungatei* coculture obtained by French pressure cell treatment contained high activities of these four enzymes showed that the *M. hungatei* did not contain an inhibitor of these enzymes. These data strongly indicate that *S. wolfei* and not *M. hungatei* contains the beta-oxidation activity.

The acyl-CoA dehydrogenase was assayed using acyl-CoA substrates with different chain lengths. The cell extract of *S. wolfei* obtained by lysozyme treatment had a much higher specific activity with butyryl-CoA as the substrate (2.34 μmol/min per mg of protein) than when octanoyl-CoA was the substrate (0.27 μmol/min per mg of protein). No activity was observed when palmitoyl-CoA served as the substrate.

The specific activities of enoyl-CoA hydratase and 3-ketoacyl-CoA thiolase were higher and that of L-3-hydroxyacyl-CoA dehydrogenase was lower when the ionic strength of the assay mixture was increased by the addition of 100 mM NaCl or sodium acetate (data not shown). The addition of 5 mM dithiothreitol to the assay mixture increased the specific activity of acyl-CoA dehydrogenase by 22%, indicating that reducing conditions increased activity.

Fatty acid activation enzymes. The lysozyme supernatant contained high levels of CoA transferase, but acyl-CoA synthetase activity was not detected (Table 3) although the latter activity was detected in the lysozyme pellet of the

TABLE 2. Specific activities of beta-oxidation enzymes in cell extracts of *E. coli*, *M. hungatei*, and *S. wolfei-M. hungatei*

Strain	Sp act[a] (μmol/min per mg of protein)			
	Acyl-CoA dehydrogenase	Enoyl-CoA hydratase	L-3-hydroxyacyl-CoA dehydrogenase	3-ketoacyl-CoA thiolase
S. wolfei				
Lysozyme supernatant[b]	1.850	164.0	3.50	1.20
Lysozyme pellet[c]	1.030	60.1	6.70	6.73[d]
S. wolfei-M. hungatei	0.83	29.4	10.80	2.20
M. hungatei[c]	<0.001	<0.1	<0.01	<0.01
E. coli[c]	0.011	3.1	0.70	0.02

[a] Activities were corrected for low levels obtained in the absence of substrate and when boiled extracts were used. Activities were linear with respect to protein concentration.
[b] Cell extracts were obtained by treatment of *S. wolfei-M. hungatei* cell suspension with lysozyme and centrifuged to remove unbroken cells (see Table 1).
[c] Cell extracts were obtained by breakage in a French pressure cell.
[d] Determined using a different cell suspension.

TABLE 3. Specific activities of enzymes invovled in fatty acid activation in cell extracts of *E. coli*, *M. hungatei*, and *S. wolfei-M. hungatei* coculture

Strain	Acyl-CoA synthetase		CoA transferase	
	Sp act (nmol/min per mg of protein)	% Total	Sp act (μmol/min per mg of protein)	% Total
S. wolfei-M. hungatei				
Lysozyme supernatant[a]	<0.01	0	16.6	94.8
Lysozyme pellet[b]	3.57	100	1.2	5.2
M. hungatei[b]	1.56		<0.1	
E. coli[b]	26.1		ND[c]	

[a] Cell extracts were prepared anaerobically by 20 min of lysozyme treatment using cell pellets frozen for 2 months. The supernatant obtained after lysozyme had 43% of the total F_{420}.
[b] Cell extracts obtained by anaerobic breakage in a French pressure cell.
[c] ND, Not determined.

coculture and in cell extracts of *M. hungatei* and of *E. coli*. In this experiment and the one shown in Table 4, the lysozyme supernatant used had very high levels of F_{420}, about 43% of the total F_{420} present in both fractions. In both experiments, extracts were prepared from cells stored at −4°C for more than 2 months. However, 94.8% of the total CoA transferase activity was found in the lysozyme supernatant, and all of the acyl-CoA synthetase activity was found in the lysozyme pellet. This plus the fact that cell extracts of *M. hungatei* grown alone contained acyl-CoA synthetase activity but undetectable levels of CoA transferase activity indicates that *S. wolfei* contains CoA transferase activity but not acyl-CoA synthetase activity.

Enzymes involved in substrate-level phosphorylation. Both the lysozyme supernatant and pellet of the *S. wolfei-M. hungatei* coculture contained phosphotransacetylase and acetate kinase activities (Table 4). However, most of the activity was present in the lysozyme supernatant, suggesting that these enzymes are present in *S. wolfei*. *M. hungatei* did not contain phosphotransacetylase activity but did contain acetate kinase activity. The acetate kinase activity present in the lysozyme supernatant and in *M. hungatei* was inhibited to the same extent by the addition of ADP or AMP plus pyrophosphate. Thus, it was not possible to determine how much of the observed activity was due to acetate thiokinase.

DISCUSSION

S. wolfei is a gram-negative bacterium and possesses a eubacterial cell wall as previously indicated by the presence of muramic acid, inhibition of growth by penicillin, and increased sensitivity to lysis after treatment with lysozyme (28). *M. hungatei* is a member of the archaebacteria and is not affected by lysozyme. Lysozyme treatment was effective in selectively lysing *S. wolfei* cells (Table 1). The supernatant obtained after lysozyme treatment and centrifugation contained low levels of the methanogenic cofactors coenzyme M, F_{420}, and F_{342}, when freshly harvested cells or cells frozen for less than 3 weeks were used. Since these cofactors are low-molecular-weight compounds, this indicates that little lysis or leakage of cellular material from *M. hungatei* occurred. The lysozyme supernatant had high specific activities for the beta-oxidation enzymes, showing that active cell extracts are obtained. The low levels of methanogenic cofactors present in the lysozyme supernatant, the distribution of the fatty acid metabolism enzymes in the lysozyme supernatant and pellet, the lack of detectable activity in *M. hungatei*, and the lack of inhibition of beta-oxidation activity by *M. hungatei* extracts strongly support the contention that the beta-oxidation enzyme activities are present in *S. wolfei* and not *M. hungatei*. The lysozyme breakage procedure should be effective in studying the metabolism of other syntrophic eubacteria so long as a methanogen is used for the H_2-consuming partner.

TABLE 4. Specific activities of acetate kinase and phosphotranacetylase in cell extracts of *M. hungatei* and the *S. wolfei-M. hungatei* coculture

Strain	Phosphotransacetylase		Acetate kinase	
	Sp act (μmol/min per mg of protein)	% Total	Sp act (μmol/min per mg of protein)	% Total
S. wolfei-M. hungatei				
Lysozyme supernatant[a]	9.75	85	1.09	78
Lysozyme pellet[b]	2.15	15	0.40	22
M. hungatei[b]	<0.01		0.57	

[a] Cell extract obtained by 20 min of lysozyme treatment using cell pellets frozen for 2 months. The supernatant obtained after lysozyme treatment had 43% of the total F_{420}.

[b] Cell extracts obtained by anaerobic breakage in a French pressure cell.

The high specific activities found in the pellet fractions for all four beta-oxidation enzymes (Table 2) indicate that much of the total activity is not released by this method of lysozyme treatment. Disruption of the coculture in a French pressure cell was more effective for recovering activity of the L-3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase than was the lysozyme treatment (Table 2). Further work is needed to determine whether room-temperature incubation during lysozyme treatment results in the loss of activity of these two enzymes. The ability of mechanically disrupted cell extracts to exhibit such high specific activities even after being diluted with contaminating methanogen protein suggests that the lysozyme treatment released only a small amount of the total activity of these enzymes. Increasing incubation time with lysozyme, adding deoxycholate, or increasing lysozyme concentration did not greatly increase the amount of protein released or the specific activity of acyl-CoA dehydrogenase in the lysozyme supernatant.

The activities found in cell extracts of *S. wolfei* are consistent with a pathway for fatty acid degradation (Fig. 1). Activation of the fatty acid in *S. wolfei* seems to occur by the transfer of the CoA group from acetyl CoA to the fatty acid by a CoA transferase reaction (4), rather than by an acyl-CoA synthetase reaction in which the acyl-CoA derivative is synthesized from the free fatty acid and CoA by coupling the reaction to the hydrolysis of ATP to AMP and pyrophosphate, potentially using two high-energy bonds. Cell extracts of *S. wolfei* contained high levels of CoA transferase, but acyl-CoA synthetase was not detected (Table 3). The degradation of butyryl-CoA to two acetate molecules would yield only two high-energy bonds. Therefore, one would expect that *S. wolfei* would use the CoA transferase rather than the acyl-CoA synthetase reaction. The acyl-CoA synthetase activity found in the lysozyme pellet and in *M. hungatei* could be due to the presence of acetate thiokinase as this activity has been found in methanogens (33). CoA transferase activity was not detected in cell extracts of *M. hungatei*.

The specific activities of the four beta-oxidation enzymes in *S. wolfei* are much higher than the respective activities in *E. coli* (6, 34, 43) (Table 2), but were similar to the specific activities found in *Neurospora crassa* (19). The levels are high enough to account for the observed rate of butyrate degradation in whole-cell suspensions. The chain length specificity of acyl-CoA dehydrogenase activity corresponds to the substrate specificity observed for *S. wolfei* grown on different fatty acids. *S. wolfei* cocultures grow best on butyrate, poorly on octanoate, and not at all on palmitate or stearate (28, 29).

The specific activity of enoyl-CoA hydratase was much higher than that of the other beta-oxidation enzymes in cell extracts of *S. wolfei* (Table 2). The possibility that the observed activity resulted from hydratase activities involved in poly-beta-hydroxybutyrate synthesis is ruled out by the assay method used. The activity was determined by a coupled enzyme assay using an added L-(+)-3-hydroxyacyl-CoA dehydrogenase that uses only the L-(+)-stereoisomer as substrate. The known crotonase involved in poly-beta-hydroxybutyrate synthesis produces D-(−)-beta-hydroxybutyrate, the monomeric unit of the product (31). It is possible that another enzyme exists that converts crotonyl-CoA to

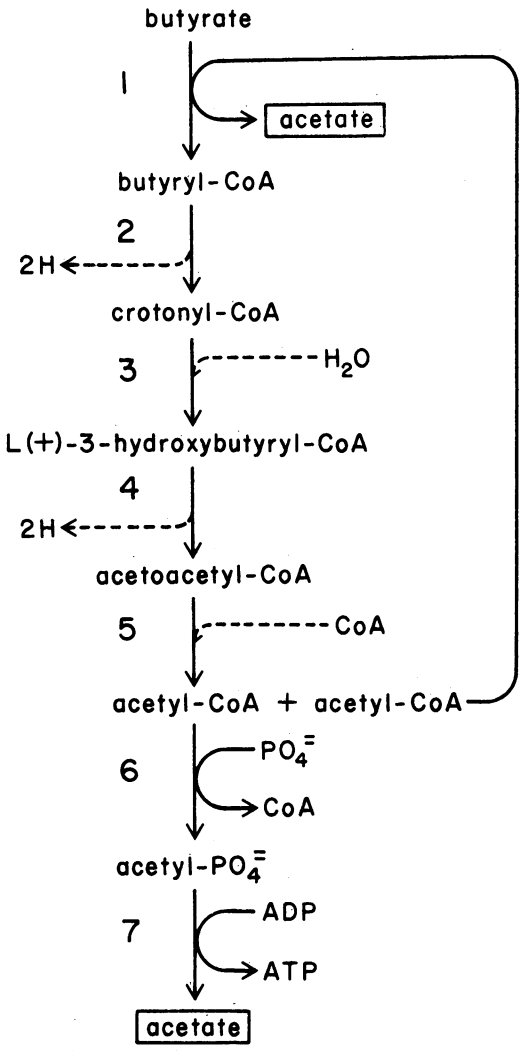

FIG. 1. Proposed pathway for butyrate degradation in *S. wolfei*. Enzymes: 1, CoA transferase; 2, acyl-CoA dehydrogenase; 3, enoyl-CoA hydratase; 4, L-(+)-3-hydroxybutyryl-CoA dehydrogenase; 5, 3-ketoacyl-CoA thiolase; 6, phosphotransacetylase; 7, acetate kinase.

D-(−)-beta-hydroxybutyryl-CoA, which could serve as the substrate for poly-beta-hydroxybutyrate synthesis. The high specific activity of the enoyl-CoA hydratase relative to that of the other beta-oxidation enzymes is also observed in other organisms that have the beta-oxidation pathway (19, 34). The high specific activity of the enoyl-CoA hydratase might shift the equilibrium of the first dehydrogenation step. The standard free energy change at pH 7 for butyryl-CoA to crotonyl-CoA is +75.0 kJ/mol (40). The high activity of the hydratase might function to ensure removal of crotonyl-CoA faster than the acyl-CoA dehydrogenase can catalyze its formation. This would make the change in free energy more negative for the in vivo acyl-CoA dehydrogenation step by removal of end products.

The formation of acetyl-CoA during beta-oxidation suggests that energy can be conserved by substrate-level phosphorylation reactions involving acetyl-phosphate. The lysozyme supernatant contained most of the phosphotransacetylase and acetate kinase activities (Table 4). *M. hungatei* cell extracts did not contain phosphotransacetylase activity, nor did they inhibit this enzyme activity in the lysozyme supernatant. This strongly indicates that *S. wolfei* contains the phosphotransacetylase activity even though the extracts used for the determination of activity had high levels of F_{420}. Since the lysozyme supernatant had most of the acetate kinase activity and the specific activity of acetate kinase in the lysozyme supernatant was much higher than that found in the lysozyme pellet or *M. hungatei* extracts, this indicates that *S. wolfei* also contains acetate kinase activity.

The proposed butyrate degradation pathway (Fig. 1) predicts that a net synthesis of 1 mol of ATP per mol of butyrate degraded occurs by substrate-level phosphorylation. However, the $\Delta G'$ for butyrate degradation to acetate and H_2 is very low, about −17 kJ per mol when calculated according to Thauer et al. (40) using the observed concentrations of reactants and products in *S. wolfei* cocultures (D. F. Dwyer, D. R. Shelton, and J. M. Tiedje, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, I27, p. 126; P. S. Beaty and M. J. McInerney, unpublished data). This is much less than that believed to be required to drive the synthesis of 1 mol of ATP in vivo (−44 kJ/mol) (40), which suggests that ATP synthesis occurs via a process(es) other than substrate-level phosphorylation. However, electron transport linked to H_2 production in *S. wolfei* is probably energy requiring rather than energy yielding since H_2 (E_0' of −414 mV) (40) is produced from electrons of much higher potential. The E_0' values of butyryl-CoA/crotonyl-CoA and 3-hydroxybutyryl-CoA/acetoacetyl-CoA couples are −10 and −280 mV, respectively (40). Thauer and Morris (41) have proposed that about two-thirds of the ATP potentially generated by substrate-level phosphorylation is used to drive the unfavorable redox reaction involving the dehydrogenation of butyryl-CoA to crotonyl-CoA with protons as the electron acceptor through the generation of a proton motive force by ATP hydrolysis. This results in a net synthesis of about one-third ATP per mol of butyrate, which is consistent with the amount of ATP expected from thermodynamic considerations. Our work demonstrates that *S. wolfei* has many of the enzyme activities in the proposed bioenergetic scheme of Thauer and Morris (41). Work is currently in progress on the mechanism for the generation of a proton motive force and the nature of the electron transport system for H_2 production in *S. wolfei*.

We have recently been able to separate cells of *S. wolfei* from those of *M. hungatei* by centrifugation using a Percoll gradient or by growing *S. wolfei* alone on crotonate (P. S. Beaty, N. Q. Wofford, and M. J. McInerney, unpublished data). Preliminary work using cell extracts obtained with the above material supports the conclusion that fatty acid degradation occurs as outlined in Fig. 1.

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