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Dichloromethane as the Sole Carbon Source for an Acetogenic Mixed Culture and Isolation of a Fermentative, Dichloromethane-Degrading Bacterium

SUSANNA A. BRAUS-STROMEYER, RENÉ HERMANN, ALASDAIR M. COOK, AND THOMAS LEISINGER*

Institute of Microbiology, Swiss Federal Institute of Technology, ETH-Zentrum, CH-8092 Zürich, Switzerland

Dichloromethane (DCM) is utilized by the strictly anaerobic, acetogenic mixed culture DM as a sole source of carbon and energy for growth. Growth with DCM was linear, and cell suspensions of the culture degraded DCM with a specific activity of 0.47 mkat/kg of protein. A mass balance of 2 mol of chloride and 0.42 mol of acetate per mol of DCM was observed. The dehalogenation reaction showed similar specific activities under both anaerobic and aerobic conditions. Radioactivity from [^{14}C]DCM in cell suspensions was recovered largely as $^{14}CO_2$ (58%), [^{14}C]acetate (23%), and [^{14}C]formate (11%), which subsequently disappeared. This suggested that formate is a major intermediate in the pathway from DCM to acetate. Efforts to isolate from culture DM a pure culture capable of anaerobic growth with DCM were unsuccessful, although overall acetogenesis and the partial reactions are thermodynamically favorable. We then isolated bacterial strains DMA, a strictly anaerobic, gram-positive, endospore-forming rod, and DMB, a strictly anaerobic, gram-negative, endospore-forming homoacetogen, from culture DM. Both strain DMB and *Methanospirillum hungatei* utilized formate as a source of carbon and energy. Coculture of strain DMA with either *M. hungatei* or strain DMB in solid medium with DCM as the sole added source of carbon and energy was observed. These data support a tentative scheme for the acetogenic fermentation of DCM involving interspecies formate transfer from strain DMA to the acetogenic bacterium DMB or to the methanogen *M. hungatei*.

Several aerobic bacteria capable of growth with dichloromethane (DCM) as the sole source of carbon and energy have been isolated over the past decade (6, 15, 16, 22, 27). Some of these organisms were identified as representatives of the genera *Methylobacterium*, *Methylophilus*, and *Hyphomicrobium* (16), while others are unidentified facultatively methylotrophic bacteria. DCM dehalogenase, the key enzyme in aerobic DCM degradation, has been purified from some of these organisms. Characterization of this enzyme, of its structural gene, and of the regulatory gene governing its expression (16) has led to a considerable understanding of aerobic DCM metabolism.

In contrast, little is known about the utilization of DCM or other halomethanes as sole carbon and energy sources by strictly anaerobic bacteria. Chloromethane serves as a growth substrate for a strictly anaerobic homoacetogenic bacterium (30), and two anaerobic mixed cultures capable of growth with DCM have recently been described (10, 26). The latter mixed cultures were enriched under methanogenic conditions, and they produce both methane and acetate from DCM. Inhibition of methanogenesis with 2-bromoethanesulfonate did not prevent degradation of DCM by these mixed cultures. These results suggest that nonmethanogenic bacteria convert DCM to products which can be used by methanogenic bacteria.

Insight into the anaerobic utilization of DCM thus requires microbiological studies to define the component(s) of the mixed cultures responsible for dehalogenation of DCM. A pure culture or a defined mixed culture capable of growth on DCM would then allow progress towards elucidating the biochemistry of DCM dehalogenation in an anaerobic system. Anoxic systems for DCM degradation are of interest not only for their presumably novel dehalogenation mechanism(s) (31) but also because of their potential for the cost-effective treatment of contaminated groundwater.

The mixed culture which converts DCM to carbon dioxide, methane, and acetate (26) was simplified to yield the acetogenic mixed culture DM (4). We now report properties of the culture DM and isolation from it of a fermentative DCM-dehalogenating bacterium and of an acetogenic bacterium. These organisms apparently form a syntrophic culture involving interspecies formate transfer in the homoacetogenic fermentation of DCM.

MATERIALS AND METHODS

Materials and apparatus. [^{14}C]DCM (281 GBq/mol), [U-^{14}C]acetic acid (2.1 TBq/mol), and [2-^{14}C]acetic acid (2.0 TBq/mol) were purchased from Sigma Chemical Co. (St. Louis, Mo.). The sources of other chemicals are given elsewhere (8, 26).

A gas chromatograph (GC) equipped with a flame ionization detector (FID) and a thermal conductivity detector (4), an ion chromatograph (CDM-2; Dionex, Sunnyvale, Calif.), a high-pressure liquid chromatograph (HPLC) (8), a liquid scintillation counter (8), and a spectrophotometer (8) were used routinely. The transmission electron microscope (H-600; Hitachi, Tokyo, Japan) was used at a 100-kV accelerating voltage. The anaerobic glove box (3) was also standard equipment.

Organisms, growth conditions, and microbiological tests. The anaerobic, DCM-utilizing culture was enriched (26) and developed to a state in which it consisted of about three

* Corresponding author.

major components (4). We entitled this culture DM. The growth medium used was modified from that described previously (1, 23, 26, 32), in that yeast extract was eliminated, the phosphate concentration was reduced to 10 mM, and the pressure of the gas phase was set at 1.5×10^5 Pa.

Methanospirillum hungatei (DSM 3595) was kindly supplied by B. Schink (Universität Konstanz, Konstanz, Germany). It was cultivated with H_2 plus CO_2 (80:20 [vol/vol]) in minimal salts medium (see above) supplemented with 2 mM acetate, which is required by *M. hungatei* for fatty acid biosynthesis (34). All cultures were incubated at 30°C in the dark and shaken once a day.

Spore formation was tested indirectly by measuring growth after heat treatment (80°C, 20 min) of the inoculum. Endospore formation was observed directly by the modified staining method of Schaeffer-Fulton (7).

DCM degradation in cell suspensions. A 5-liter batch of culture DM was harvested (5,000 × *g*, 15 min, 4°C) in the late growth phase after it had consumed about 7 mM DCM. The cells were suspended aerobically (50 mM potassium phosphate buffer, 2 mM $NaHCO_3$, 0.4 mM $MgSO_4$, pH 7.8), and centrifuged (10,000 × *g*, 30 min, 4°C). The pellet was then suspended in assay buffer containing reductant (50 mM potassium phosphate buffer, 2 mM $NaHCO_3$, 0.4 mM $MgSO_4$, 3.3 mM Na_2S, 4 μM resazurine, pH 7.8). The dehalogenation of DCM in 2-ml reaction mixtures was assayed under an atmosphere of N_2 plus CO_2 (80:20 [vol/vol]) in 15-ml bottles with butyl rubber stoppers. DCM (to give 3 mM in the assay) was added to 1 ml of assay buffer and equilibrated for 1 h prior to the addition of the cell suspension. The final protein concentration was 1.1 to 1.5 mg/ml, and bottles were incubated on a shaker in the dark at 30°C. Levels of DCM and the products formed were determined at intervals. All bottles were set up in duplicate. The experiment was done twice.

The degradation of [^{14}C]DCM by culture DM was monitored in cell suspensions under the conditions described in the previous paragraph, but with the addition of 33 kBq of [^{14}C]DCM. The transformation of DCM was followed by GC-FID. After 24 h NaOH (to pH 10) was added to the cell suspension to stop the reaction and to trap $^{14}CO_2$. Samples for metabolic products were taken after shaking for 4 h. Cell suspensions for replicate experiments were taken from independently grown cultures, and each determination was done in duplicate.

Isolation of a DCM-dehalogenating organism. Bacteria from culture DM were isolated by serial dilution (eight tubes) in agar shakes as described by Widdel and Bak (33). We used our minimal salts medium containing growth substrate (5 mM) and solidified with 1% (wt/vol) agar; the medium for cocultures was supplemented with 2 mM acetate and 5% (vol/vol) of a culture of *M. hungatei* in the late exponential phase. Cultures were incubated under an atmosphere of N_2 plus CO_2 (80:20 [vol/vol]) in the dark at 30°C. Distinct single colonies were visible after 6 weeks in DCM-minimal medium, and they could be picked into drawn Pasteur pipettes after 8 weeks. Each picked colony was suspended in 1 ml of sterile minimal salts medium and found to consist of two organisms, one of which was a rod-shaped bacterium which we termed strain DMA and the other of which was *M. hungatei*. Strain DMA was purified from possible contaminants from culture DM by two further sets of serial dilutions in coculture with *M. hungatei*. Strain DMA was found to utilize glucose as a sole source of carbon and energy for growth, so strain DMA was obtained in pure culture by serial dilution in agar shakes containing 5 mM glucose minimal medium; colonies with 1- to 2-mm diameters were obtained in 5 days. The organism was stored anaerobically in 50% glycerol at −80°C and has been deposited with the Deutsche Sammlung für Mikroorganismen, Braunschweig, Germany.

A second organism, strain DMB, from culture DM was isolated by serial dilution in solid medium. The carbon source was ethylene glycol (plus 0.01% yeast extract), and colonies could be picked within 2 weeks. Two further sets of serial dilutions were done to purify the organism, which was then grown in liquid medium. The organism was stored anaerobically in 50% glycerol at −80°C and has been deposited with the Deutsche Sammlung für Mikroorganismen, Braunschweig, Germany.

DCM degradation by agar cultures. DCM degradation by defined mixed cultures was studied in minimal medium solidified with agar. Minimal salts medium containing 0.8% agar (25-ml portions) was added anaerobically to 100-ml serum bottles which were then sealed with butyl rubber stoppers and aluminum crimps. The gas phase was set to N_2 plus CO_2 (80:20 [vol/vol]) at 1.5×10^5 Pa, and the bottles were autoclaved. Each of the following inocula (1 ml) was added singly or in combination to the liquid (40°C) agar medium: strain DMA grown with 10 mM glucose, strain DMB grown with 10 mM formate, and *M. hungatei* grown with H_2 plus CO_2 and supplemented with 2 mM acetate. The inocula were mixed with the medium, which was then solidified by cooling in ice-water. DCM (63 μmol) was added to each bottle, and DCM concentrations in the headspace were determined after an equilibration period of 24 h. DCM in the headspace of the cultures was analyzed at intervals. After 8 weeks, the bottles were heated in a boiling water bath to melt the agar, from which samples were taken to determine the concentration of chloride ion.

Analytical methods. DCM, chloromethane, methanethiol, dimethylsulfide, H_2, and methane were sampled (0.3 ml) from the headspace of the culture bottles. DCM, chloromethane, and methane were determined by GC-FID after separation on Poropak P (4). Methanethiol and dimethylsulfide were determined routinely by GC-FID after separation on Chromosyl 330 (4) and identified by GC coupled to mass spectrometry (4). Hydrogen was determined by GC-thermal conductivity detection after separation on a Poropak Q column (1.8 m by 2 mm [inside diameter], 80/100 mesh) at 50°C. Acetate, formate, chloride, and bromide ions were quantified by ion chromatography with suppressor (IONPAC AS10 analytical column; Dionex). Acetic acid and formic acid were occasionally separated by HPLC on a reversed-phase column (8, 12). $^{14}CO_2$ was quantified as described by Fuchs et al. (11) and Stromeyer et al. (25). Unidentified water-soluble radioactive products from the degradation of [^{14}C]DCM were defined as the radioactivity which remained in aqueous solution after acidification (to pH 2.5 with 1 M H_2SO_4) and extraction with 1 volume of hexane. The acidification step caused precipitation of material in which radioactivity was also measured. Protein in whole cells was measured in a Lowry-type reaction (14).

[^{14}C]acetate in the growth medium was protonated by acidification of the samples to pH 2.5 with 3 M perchloric acid, separated by distillation (2, 8) or by HPLC (10), and subjected to a modified Schmidt degradation (11, 24). [U-^{14}C]acetate and [2-^{14}C]acetate were degraded in parallel reactions as controls. Radioactivity from the [2-^{14}C]acetate standard was recovered largely (92%) as $^{14}CH_3NH_2$ (from the methyl carbon) with traces (2%) of $^{14}CO_2$ (from the carboxyl carbon); radioactivity from the [U-^{14}C]acetate

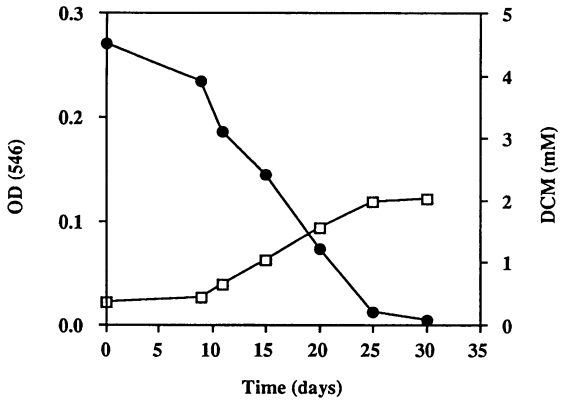

FIG. 1. Growth of the anaerobic mixed culture DM on DCM minimal medium. ●, DCM; □, optical density at 546 nm [OD (546)]. The chloride released amounted to 93% of the theoretical yield, and the final protein concentration was 4.7 mg/liter.

standard was equally distributed between $^{14}CH_3NH_2$ (44%) and $^{14}CO_2$ (47%).

Electron microscopy. Bacteria were cryoimmobilized for the preparation of thin sections by high-pressure freezing (18, 19, 28) in cellulose capillary tubes (200 μm in diameter, 2 mm in length [13]). The subsequent freeze-substitution in acetone containing 2% osmium tetroxide, embedding in Epon-Araldite, and staining of the sections were carried out according to the methods of Studer et al. (28). Whole cells and flagella were visualized by rotary shadowing with platinum-carbon at an angle of 30°, after adsorption onto carbon-coated copper grids and subsequent air drying.

RESULTS

Enrichment of the acetogenic mixed culture DM. The original DCM-utilizing anaerobic mixed culture (26) was subjected to serial transfer through minimal medium with 5 mM DCM as the sole source of carbon and energy (4), and we obtained the homoacetogenic mixed culture DM. Culture DM was examined by phase-contrast microscopy. It consisted of three morphologically distinguishable types of bacterial cells, none of which fluoresced on irradiation with UV light. Methanogenic bacteria had thus been eliminated from the culture.

Culture DM exhibited a maximum dehalogenation rate of 0.63 mkat/kg of protein, considerably lower than the rate of 2.6 mkat/kg of protein (26) observed with the primary enrichment culture. Culture DM grew apparently linearly in 5 mM DCM-minimal medium to an optical density of about 0.1 (Fig. 1). A lag phase of between 5 and 15 days was consistently observed when fresh medium was inoculated (10% [vol/vol]) with a growing culture; an inoculum that had been stored for 1 week at 4°C typically had a lag phase that lasted for about 2 months. Attempts to obtain exponential growth of culture DM with DCM by modifications of the growth medium were unsuccessful. We tested variations in the composition and the concentration of the trace elements and the vitamins as well as the addition of yeast extract (100 mg/ml).

DCM degradation by cell suspensions of culture DM. Washed cells of culture DM were used to study the kinetics of product formation from DCM. As shown in Fig. 2A, suspensions of resting cells formed 1.25 mM acetate and 5.9 mM chloride as the major products from 3 mM DCM. This

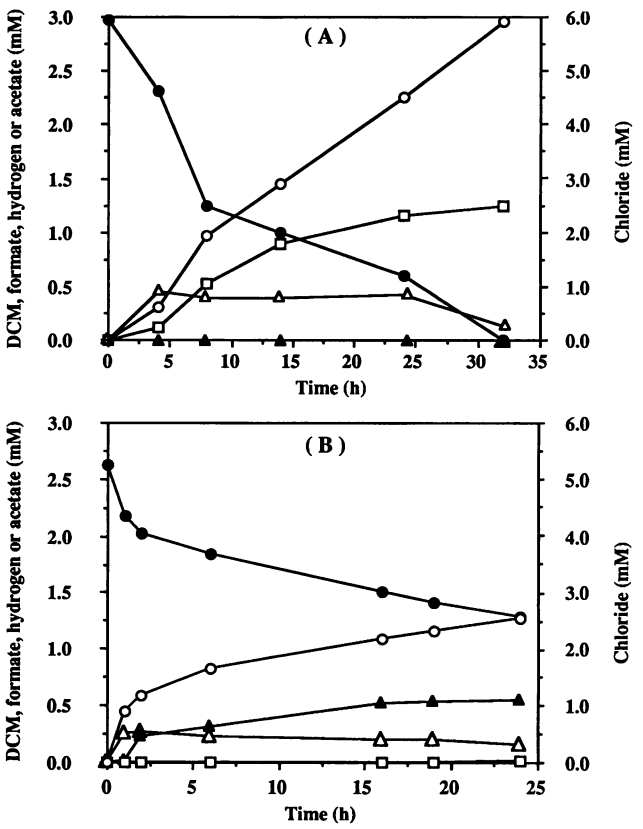

FIG. 2. Dehalogenation of DCM by cell suspensions of the mixed culture DM. (A) Standard conditions; (B) in the presence of 1 mM cyanide. ●, DCM; ○, chloride; △, formate; ▲, hydrogen; □, acetate. The results shown are from one representative culture of four replicates.

partial mass balance suggested the following fermentation balance: $2CH_2Cl_2 + 2H_2O \rightarrow CH_3COO^- + 4Cl^- + 5H^+$. During the conversion of DCM to acetate we observed a transient accumulation of formate which disappeared towards the end of the reaction. Hydrogen was not detected.

Cyanide, an inhibitor of CO-dehydrogenase (36), did not markedly affect the initial dehalogenation rate of DCM by culture DM, but it led to an 80% decrease in the rate after the

TABLE 1. Effects of gas phase and inhibitors on the dehalogenation of DCM in cell suspensions of culture DM

Exptl condition	Sp act[a] (mkat/kg of protein)
No cells	<0.01
Standard reaction mixture	0.47
Autoclaved cell suspension	<0.01
Gas phase	
Air[b]	0.38
H_2 plus N_2 (80:20 [vol/vol])	0.14
H_2 plus CO_2 (80:20 [vol/vol])	0.32
Addition	
CO (10% [vol/vol]) in gas phase	0.43
KCN (1 mM)	0.35
Chloroacetonitrile (5 mM)	0.32

[a] Dehalogenation rate over the first 2 h.
[b] The assay bottles were flushed with sterile air for 10 min.

TABLE 2. Products formed from 3.1 mM [^{14}C]DCM by cell suspensions of culture DM

Product[a]	Radioactivity (% of total ± SD[b])	Specific radioactivity (GBq/mol of C)
Carbon dioxide	58 ± 6.8	ND[c]
Formate	11 ± 2.4	3.8
Acetate	23 ± 1.8	2.8 [2-^{14}C] 0.4 [1-^{14}C]
Biomass	5 ± 0.8	0.008
Undefined in water	4 ± 0.0	ND
Total recovery	101 ± 3.6	

[a] The educt, DCM, had 100% radioactivity; its specific radioactivity was 4.8 GBq/mol of C.
[b] The experiment was done in quadruplicate.
[c] ND, not determined.

reaction had proceeded for about 2 h (Fig. 2B). This inhibitor prevented the formation of acetate and led to the accumulation of hydrogen which represented about 25% of the reducing equivalents theoretically available if the DCM consumed were anaerobically oxidized to CO_2. The data in Fig. 2 suggest that DCM is oxidized by culture DM to formate and CO_2 which then give rise to acetate via the acetyl coenzyme A pathway.

Trace amounts of chloromethane (<0.02 mM) and of methanethiol (<0.05 mM) were identified and quantified in cell suspensions of culture DM after the exhaustion of DCM. We examined whether chloromethane was an intermediate in the dehalogenative pathway leading from DCM by supplying this compound at the end of growth with DCM. Chloromethane is not utilized as a carbon and energy source but is transformed to methanethiol and dimethylsulfide (4). We presume the formation of chloromethane from DCM to be a side reaction in culture DM.

The initial rate of DCM degradation in cell suspensions was not markedly altered when the standard gas phase was replaced with air or H_2 plus CO_2 (Table 1). In contrast, if the reaction was seriously depleted of CO_2 (N_2 plus H_2; Table 1), degradation was significantly retarded, though the rate recovered over 1 h. The addition of CO, as a carbon and energy source for acetogens, failed to improve the rate of dechlorination. Chloroacetonitrile, a nonreversible inhibitor of glutathione-dependent DCM dehalogenases (17), had little effect on the initial rate of DCM degradation.

Products formed from [^{14}C]DCM. The degradation of [^{14}C]DCM was examined to test the hypothesis that formate is a major intermediate in the pathway from DCM to acetate. There was complete recovery of radioactivity (Table 2), mostly in the defined products CO_2 (58%), acetate (23%), and formate (11%). The specific activity of formate (3.8 GBq/mol) was lower than that of DCM (4.8 GBq/mol). The radioactivity in acetate was unevenly distributed, 88% being in the methyl group (2.8 GBq/mol) at about half the specific activity observed in the educt (Table 2). These data support the transformation of DCM to formate, which gives rise to the methyl group of acetate without prior oxidation to CO_2.

Isolation of pure cultures from culture DM. Culture DM contained at least three different bacteria. Attempts to obtain a pure culture growing with DCM as the sole source of carbon and energy by terminal dilution in selective liquid medium or by serial dilution in solid medium were not successful. We therefore considered the possibility of syntrophy, and we chose the formate-utilizing *M. hungatei* as the partner for the dechlorinative organism from culture

TABLE 3. Characteristics of organisms DMA and DMB[a]

Organism	Morphology	Colonies in agar medium	Cell wall	pH optimum for growth	Growth factor requirement(s)	Growth with DCM	Nutrients for growth	
							Utilized	Not utilized
DMA	Straight rod; 3.5–7.5 by 1.0–1.3 μm; ≥1 subpolar flagellum	Uneven margins; whitish; diam, 1–2 mm after 6–8 wk with DCM	Gram positive	6.5–7.5	Unidentified vitamin(s)	Growth in agar medium in coculture with *M. hungatei*	Glucose, sucrose	Acetate, formate, H_2 + CO_2, yeast extract
DMB	Curved rod; 2.0 by 0.6 μm; usually in pairs; 1 polar flagellum	Entire margins; beige; diam, 2–3 mm after 1 wk with ethylene glycol	Gram negative	6.8–7.5	Yeast extract and unidentified vitamin(s)	No growth	Formate, H_2 + CO_2, methanol, vanillic acid, ethylene glycol	Glucose, sucrose, yeast extract

[a] Both organisms are motile, strictly anaerobic, endospore-forming bacteria that grow at 30°C, and both form lens-shaped, circular colonies in agar medium.

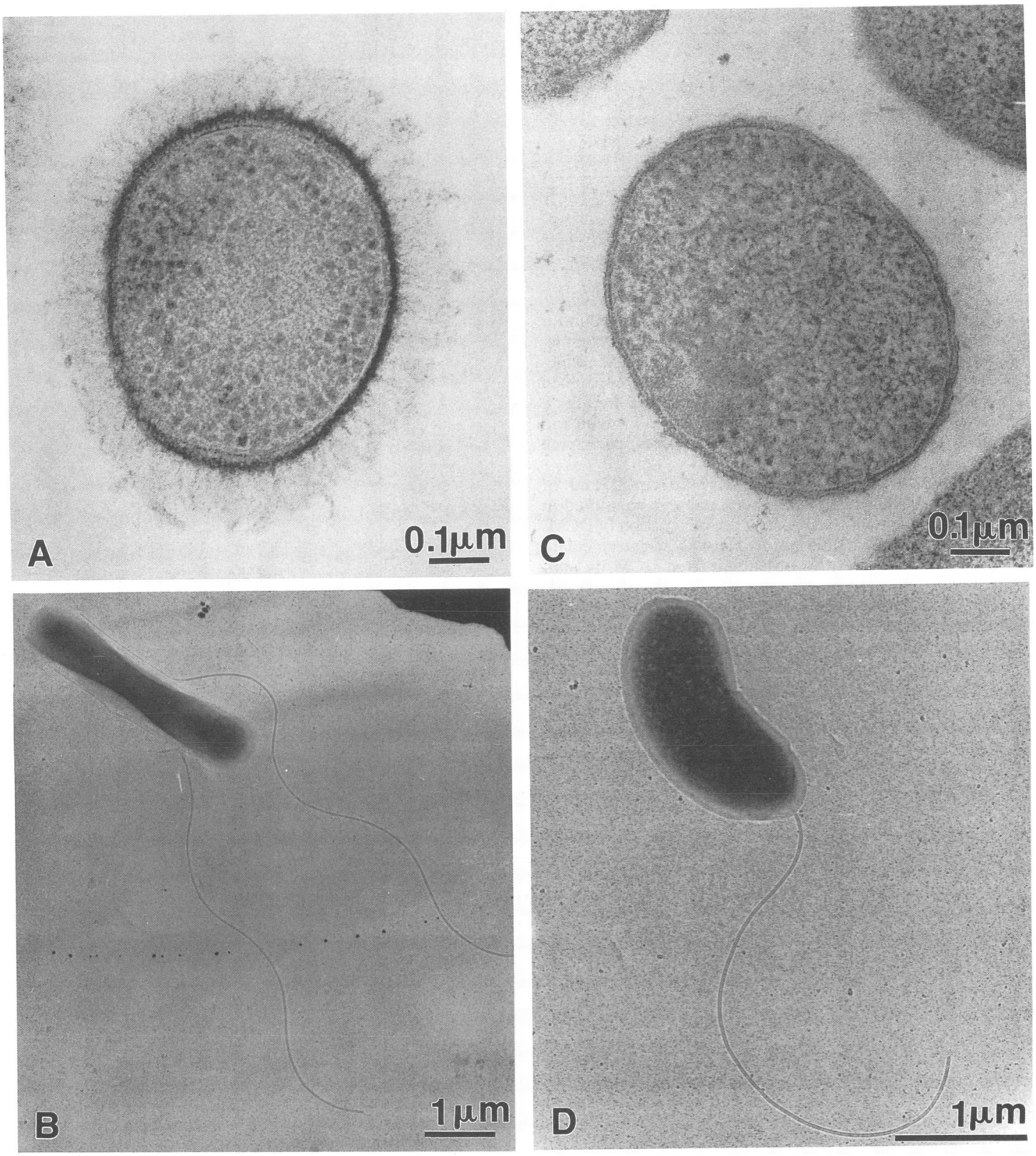

FIG. 3. Electron micrographs of strains DMA and DMB. (A) Thin section of a vegetative cell of strain DMA showing a gram-positive cell wall; (B) strain DMA with flagella; (C) thin section of strain DMB showing the inner and outer membranes, typical for a gram-negative cell wall; (D) strain DMB with polar flagellum.

TABLE 4. Degradation of 2.5 mM DCM by pure cultures and defined cocultures of anaerobic bacteria in solid medium

Organism(s)	Addition(s) to minimal medium containing 63 μmol of DCM/culture	DCM consumed in 65 days (μmol/culture ± SD[a])	Cl^- released in 65 days (μmol/culture ± SD[a])
None	None	3.8 ± 0.4	7.8 ± 0.4
M. hungatei	80 μmol of acetate	3.3 ± 0.4	5.8 ± 0.4
DMA	0.01% Yeast extract and 80 μmol of acetate	1.3 ± 0.3	1.5 ± 0.2
DMB	0.01% Yeast extract	3.2 ± 0.4	5.2 ± 0.4
DMA + DMB	0.01% Yeast extract	14.4 ± 1.1	25.1 ± 2.1
DMA + *M. hungatei*	80 μmol of acetate	23.8 ± 2.2	43.2 ± 2.9

[a] Means for three individual cultures are shown.

DM. This approach allowed us to isolate strain DMA (see Materials and Methods). Strain DMA was found to be a strictly anaerobic, rod-shaped, spore-forming bacterium whose properties are listed in Table 3. Analyses of strain DMA by electron microscopy indicated a gram-positive cell wall (Fig. 3A) and ≥1 subpolar flagellum (Fig. 3B).

Isolation of strain DMA via coculture with *M. hungatei* suggested that utilization of DCM in the acetogenic culture DM involved strain DMA and an acetogenic syntrophic partner. We enriched from culture DM the putative acetogenic syntrophic partner of strain DMA by selecting for growth with ethylene glycol, a substrate typically utilized by acetogens (21). This led to the isolation of strain DMB, a gram-negative, motile, vibroid, spore-forming bacterium (Fig. 3C and D) whose properties are summarized in Table 3.

Strain DMB was able to grow with H_2 plus CO_2, formate, methanol, vanillic acid, and ethylene glycol, but it did not grow with glucose, sucrose, or DCM. It thus shares three properties—(i) a gram-negative cell wall, (ii) the ability to form endospores, and (iii) substrate range—with representatives of the genus *Sporomusa* (5).

Confirmation of strain DMA as a DCM-degrading organism. There was no growth in liquid DCM-salts medium of mixtures of strain DMA plus *M. hungatei* or of strain DMA plus strain DMB. We thus had no direct proof that strain DMA catalyzed dechlorination of DCM. We knew, however, that all three organisms could be grown in solid medium, so each was tested individually and in combination for the ability to degrade DCM and to liberate chloride during growth in DCM-salts medium solidified with 0.8% (wt/vol) agar. None of the pure cultures alone degraded DCM (Table 4). In contrast, strain DMA grew in coculture with either *M. hungatei* or strain DMB. These cocultures degraded significant amounts of DCM during the test period and liberated about 2 mol of chloride per mol of DCM degraded. This indicates that strain DMA is indeed the organism responsible for DCM degradation.

DISCUSSION

The strictly anaerobic mixed culture DM utilizes DCM as the sole source of carbon and energy for growth and produces acetate as the major organic catabolic product. Experiments with suspensions of resting cells suggest that formate is a key intermediate in the production of acetate, which is formed via the acetyl coenzyme A pathway (Fig. 2; Table 2). A simple pathway based primarily on known reactions can be deduced for the formation of acetate from DCM (Fig. 4).

The dehalogenation reaction(s) in this tentative scheme leads from DCM to an intermediate at the oxidation state of formaldehyde. The reaction mechanism is unknown, despite its superficial resemblance to known systems (16). Oxygenation of DCM can be eliminated as a mechanism in these strict anaerobes, and a reduction to chloromethane, though present at a low level, leads to, e.g., methanethiol (4) and not to acetate. There remains a formal hydrolysis, and, presumably, a free or bound intermediate at the oxidation state of formaldehyde. This formal hydrolysis is independent of the glutathione (GSH)-dependent DCM dehalogenases of aerobic DCM-utilizing bacteria: culture DM, like extracts of all anaerobes tested to date (9, 35), does not contain GSH (3); dehalogenation by cell extracts of culture DM is not stimulated by GSH (3); and chloroacetonitrile, a specific inhibitor of GSH-dependent DCM dehalogenases (17), does not significantly inhibit dehalogenation by cell suspensions of culture DM (Table 1).

The overall transformation of DCM to acetate (Table 5, equation 1) exhibits a $\Delta G^{\circ\prime}$ value of −492.7 kJ/mol of acetate and is thus thermodynamically favorable. A major portion of this free energy, however, is associated with the dehalogenation step (−344 kJ/mol; Table 5, equation 2). In view of the presumably hydrolytic nature of the dehalogenation and the

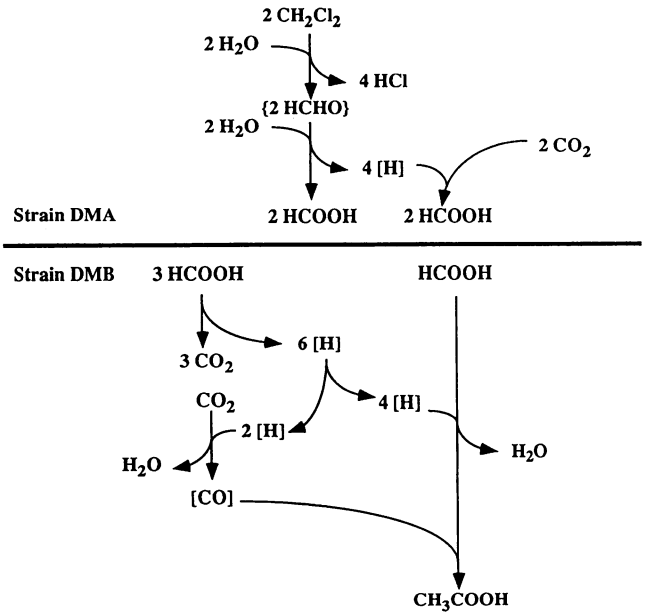

FIG. 4. Tentative scheme for the conversion of DCM to acetate by interspecies formate transfer in the mixed culture DM. {HCHO}, hypothetical intermediate at the oxidation state of formaldehyde; [CO], enzyme-bound carbonyl.

TABLE 5. Gibbs free energy changes of the reactions involved in the degradation of DCM under acetogenic conditions[a]

Reaction type, equation no., and organism	Reaction[b]	$\Delta G^{\circ\prime}$ (kJ/reaction)	$\Delta G'$ (kJ/reaction)
Mixed (syntrophic) DCM conversion, **1**	**$2^*CH_2Cl_2 + 2H_2O \rightarrow {}^*CH_3COO^- + 4Cl^- + 5H^+$**	**−492.7**	**−528.4**
Interspecies H_2 and formate transfer			
2	$2^*CH_2Cl_2 + 2H_2O \rightarrow 2H^*CHO + 4Cl^- + 4H^+$	−344.0	−409.9
3	$2H^*CHO + 2H_2O \rightarrow 2H^*COO^- + 2H_2 + 2H^+$	−46.8	−89.0
4 DMA	**$2^*CH_2Cl_2 + 4H_2O \rightarrow 2H^*COO^- + 2H_2 + 4Cl^- + 6H^+$**	**−390.8**	**−498.9**
5	$H^*COO^- + H_2O \rightarrow H^*CO_3^- + H_2$	+1.3	−5.7
6	$H^*COO^- + HCO_3^- + 3H_2 + H^+ \rightarrow {}^*CH_3COO^- + 3H_2O$	−103.0	−23.9
7 DMB	**$2H^*COO^- + HCO_3^- + 2H_2 + H^+ \rightarrow {}^*CH_3COO^- + H^*CO_3^- + 2H_2O$**	**−101.7**	**−29.5**
Interspecies formate transfer			
2	$2^*CH_2Cl_2 + 2H_2O \rightarrow 2H^*CHO + 4Cl^- + 4H^+$	−344.0	−409.9
8	$2H^*CHO + 2HCO_3^- \rightarrow 2H^*COO^- + 2HCOO^- + 2H^+$	−49.4	−77.7
9 DMA	**$2^*CH_2Cl_2 + 2HCO_3^- + 2H_2O \rightarrow 2H^*COO^- + 2HCOO^- + 4Cl^- + 6H^+$**	**−393.4**	**−487.6**
10	$3H(^*)COO^- + 3H_2O \rightarrow 3H(^*)CO_3^- + 3H_2$	+3.9	−16.9
6	$H(^*)COO^- + HCO_3^- + 3H_2 + H^+ \rightarrow (^*)CH_3COO^- + 3H_2O$	−103.0	−23.9
11 DMB	**$4H(^*)COO^- + HCO_3^- + H^+ \rightarrow (^*)CH_3COO^- + 3H(^*)CO_3^-$**	**−99.3**	**−40.8**

[a] Gibbs free energies were calculated on the basis of data from Thauer et al. (29) and Reid et al. (20). $\Delta G'$ values were calculated from $\Delta G^{\circ\prime}$ assuming the following typical conditions during growth on DCM: H_2, 10^{-4} atm (10^{-2} kPa); Cl^-, 10^{-2} M; H^+, 10^{-7} M; HCO_3^-, 6×10^{-2} M; DCM, 3×10^{-3} M; HCHO, 5×10^{-5} M; $HCOO^-$, 10^{-4} M; and CH_3COO^-, 5×10^{-4} M. Boldface type indicates the sum of partial reactions (e.g., equation 4 = equation 2 + equation 3).

[b] *C symbolizes ^{14}C with a specific radioactivity of 1.0, and (*)C indicates ^{14}C with a relative specific radioactivity of 0.5.

growth yield of culture DM of about 5 g of protein per mol of DCM, it appears unlikely that this energy is conserved. To evaluate the thermodynamic feasibility of the overall process, it is thus necessary to consider the partial reactions leading from the hypothetical intermediate formaldehyde to acetate. Table 5 lists two possible pathways for the reaction in syntrophic associations between the DCM-fermenting strain DMA and the acetogenic strain DMB. One pathway is based on interspecies transfer of formate and hydrogen, and the other is based on interspecies transfer of formate alone. In the former case strain DMA would produce formate plus H_2 from formaldehyde, whereas in the latter case it would ferment 2 mol each of formaldehyde and bicarbonate to 4 mol of formate. As shown in Table 5, hydrogen-formate transfer leads to conservation of the specific radioactivity of DCM in the methyl-carbon of acetate while formate transfer results in a 50% reduction of the specific radioactivity in this position. The experimental data in Table 2 show this 50% reduction, which indicates that interspecies formate transfer predominates in culture DM.

Equation 8 (Table 5) indicates that the formation of formate in strain DMA is thermodynamically favorable, as is acetogenesis from formate (equation 11). Consequently, strain DMA should be able to utilize DCM for growth in pure culture. Our experiments showed the requirement for a coculture for growth with DCM (Table 4), so factors other than thermodynamic limitations seem to be responsible for the obligatory association of strain DMA with a syntrophic partner during growth with DCM. The dependence of strain DMA on a syntrophic partner may be caused by its requirement for growth factors or for a carbon source secreted by the partner. The carbon source utilized by strain DMA during growth on DCM is not known. The organism does not grow on formate, the product of DCM oxidation, nor does it utilize acetate, the product released by the acetogenic organism DMB. Strain DMA thus presumably depends on an unidentified product provided by strain DMB as a carbon source, and this nutritional limitation may be the cause of the slow growth with DCM and of the difficulties in transferring reconstituted cultures comprising strain DMA and a syntrophic partner from solid to liquid medium with DCM. Indeed, the mixed culture DM, a three-component system, may involve the third organism in the food web based on the degradation of DCM. Strain DMA may then be able to utilize DCM in pure culture if provided with a suitable auxiliary carbon source. Strain DMA does not dehalogenate DCM during growth in glucose- or sucrose-salts medium (3). Possibly, strain DMA grows with DCM and a more oxidized compound which serves both as a carbon source and as a terminal electron acceptor.

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