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Acetogenesis from Dichloromethane by a Two-Component Mixed Culture Comprising a Novel Bacterium

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A strictly anaerobic two-component culture able to grow exponentially with a doubling time of 20 h on a medium containing dichloromethane as the carbon and energy source was characterized. On a medium without sulfate, we observed (per mol of dichloromethane) a mass balance of 2 mol of chloride, 0.26 mol of acetate, 0.05 mol of formate, and 0.25 mol of carbon in biomass. One component of the culture, strain DMB, was identified by a 16S ribosomal DNA analysis as a *Desulfovibrio* sp. The other component, the gram-positive organism strain DMC, could not be isolated. It was possible, however, to associate strain DMC on a medium containing dichloromethane in a coculture with *Acetobacterium woodii* or *Methanospirillum hungatei*. Coculture of strain DMC with the *Archaeon M. hungatei* allowed us to specifically amplify by PCR the 16S rRNA gene of strain DMC. A phylogenetic analysis of the 16S ribosomal DNA sequence revealed that this organism groups within the radiation of the *Clostridium-Bacillus* subphylum and exhibits the highest levels of sequence similarity (89%) with *Desulfotomaculum orientis* and *Desulfitobacterium dehalogenans*. Since the novel organism strain DMC was able to grow acetogenically with dichloromethane when it was associated with one of three metabolically different partners and since, in contrast to strain DMB, strain DMC contained carbon monoxide dehydrogenase activity, this bacterium is responsible for both the dehalogenation of dichloromethane and the acetogenesis observed in the original two-component culture. The obligatory dependence of strain DMC on a partner during growth with dichloromethane is thought to stem from the need for a growth factor produced by the associated organism.

Anaerobic microbial processes offer attractive possibilities for the detoxification of organohalogen compounds (for reviews see references 10 and 19), including the chlorinated methanes, ethanes, and ethenes, which are important representatives of this class of compounds. There are two principal possibilities for metabolic utilization of the chlorinated ethenes by anaerobic bacteria. Chlorinated ethenes may serve as physiological electron acceptors for bacteria that couple reductive dehalogenation of these compounds to growth (14). Alternatively, it is conceivable that chlorinated C_1 and C_2 hydrocarbons are utilized as carbon and energy sources by anaerobic bacteria, a process that has been observed with a homoacetogenic bacterium that utilizes chloromethane (18) and with two anaerobic mixed cultures that are capable of growth with dichloromethane (11, 25).

We have previously described the properties of anaerobic mixed culture DM, which converts 5 mM dichloromethane to 2.1 mM acetate, 10 mM chloride, and biomass within 30 days. The two major organisms present in this culture were isolated and shown to be a gram-positive, endospore-forming rod (strain DMA) and a strictly anaerobic, gram-negative, curved rod (strain DMB). In pure culture neither organism was capable of growth with dichloromethane, but when strain DMA was associated in solid growth medium with either strain DMB or *Methanospirillum hungatei*, it dehalogenated dichloromethane (3). However, dichloromethane dehalogenation in this reconstituted coculture was extremely slow, as was growth of mixed culture DM with dichloromethane. Therefore, studies were performed with a fast-growing anaerobic mixed culture, mixed culture DC. In contrast to culture DM, this culture grows exponentially and converts 5 mM dichloromethane to acetate within 7 days. Culture DC arose spontaneously after subcultivation of mixed culture DM with dichloromethane as the carbon source. The occurrence of fast growth was associated with the appearance of a major new organism, strain DMC, and with the loss of strain DMA from the system. In this paper we describe the characteristics of dichloromethane-utilizing anaerobic mixed culture DC and the properties of its components.

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MATERIALS AND METHODS

Materials. The chemicals which we used were reagent grade or better and were purchased from Fluka, Buchs, Switzerland. Yeast extract was obtained from Oxoid, Basingstoke, Hampshire, England, and agar was obtained from Difco Laboratories, Detroit, Mich.

Organisms. Fast-growing, dichloromethane-utilizing, anaerobic mixed culture DC was obtained spontaneously after subcultivation of mixed culture DM under the growth conditions described by Braus-Stromeyer et al. (3). Strain DMB has been isolated previously (3) and has been deposited in the DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany, as strain DSM 9266. *Acetobacterium woodii* DSM 1030 was obtained from the DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen, and *Methanospirillum hungatei* DSM 3595 was kindly supplied by B. Schink, Universität Konstanz, Konstanz, Germany.

Media and growth conditions. Defined minimal salts medium was prepared anaerobically with bidistilled water and contained 10.9 mM KH_2PO_4, 9.4 mM NH_4Cl, 35.7 mM $NaHCO_3$, 0.9 mM Na_2S, and 5 μM resazurin. The pH of this medium was adjusted to 7.3, and after the medium was autoclaved under an N_2-CO_2 (80:20, vol/vol) atmosphere (2×10^5 Pa), the following sterile anaerobic stock solutions were added aseptically: (i) 1 M $MgSO_4$ (1 ml/liter), (ii) trace element solution SL10 (28) (1 ml/liter) supplemented with 4.1 g of EDTA per liter and 3 mg of Na_2SeO_3 per liter, and (iii) a 20-fold-concentrated vitamin solution (1) (1 ml/liter) supplemented with 50 mg of vitamin B_{12} per liter. In experiments involving quantification of chloride release, the medium contained

NH_4Br instead of NH_4Cl, and for experiments requiring sulfate-free conditions $MgSO_4$ was replaced by $MgBr_2$.

Mixed culture DC was maintained in infusion bottles (total volume, 583 ml) that were sealed with rubber stoppers and contained 500 ml of minimal salts medium and an N_2-CO_2 (80:20, vol/vol) gas phase (2×10^5 Pa). Dichloromethane (5 mM), yeast extract (final concentration, 50 mg/liter), and inoculum (2%, vol/vol) were added with a syringe. The cultures were incubated at 30°C on a shaker and were subcultured when the dichloromethane had been completely utilized. Terminal dilution experiments and tests to determine alternative growth substrates were performed in 35-ml (or 58.7-ml) serum bottles that were sealed with rubber stoppers and contained 10 ml (or 20 ml) of minimal salts medium in which the concentration of $NaHCO_3$ was increased to 60 mM and to which 4 μg of $Na_2WO_4 \cdot 2H_2O$ per liter and 55.5 mg of $CaCl_2$ per liter were added. Potential growth substrates were added to a concentration of 5 mM in the presence of 1 to 5 mM sulfate and 50 mg of yeast extract per liter.

A. woodii was cultivated with H_2-CO_2 (80:20, vol/vol) in minimal salts medium (see above) supplemented with 1 g of yeast extract per liter. H_2-CO_2 (80:20, vol/vol) and minimal salts medium supplemented with 2 mM acetate were used for cultivating *M. hungatei.*

Cocultures of strain DMC with *A. woodii* were grown under an N_2-CO_2 (80:20, vol/vol) atmosphere in minimal salts medium containing 5 mM dichloromethane and 100 mg of yeast extract per liter, and cocultures of strain DMC with *M. hungatei* were grown in minimal salts medium containing 5 mM dichloromethane, 2 mM acetate, 50 mg of yeast extract per liter, and 55.5 mg of $CaCl_2$ per liter. For subcultivation we used a coculture (2%, vol/vol) and an early-stationary-phase culture of either *A. woodii* or *M. hungatei* (2%, vol/vol) as inocula. Pure cultures and cocultures were incubated with shaking at 30°C in the dark.

Agar shake dilution series. Agar shake dilution series were performed as described by Widdel and Bak (27). Minimal salts medium containing 35.7 mM $NaHCO_3$, 10 g of agar per liter, 50 mg of yeast extract per liter, and 5 mM dichloromethane was used. The agar shake cultures were incubated under an N_2-CO_2 (80:20, vol/vol) atmosphere (2×10^5 Pa) at 30°C in the dark.

Analytical methods. Amounts of dichloromethane and methane were determined by performing a gas chromatographic analysis of the headspace gas. A 300-μl portion of the headspace gas was analyzed with a model PE8700 gas chromatograph (Perkin Elmer, Beaconsfield, Buckinghamshire, England) equipped with a flame ionization detector and a Teflon-coated stainless steel column (1.8 m by 2 mm [inside diameter]) packed with Poropak P (80/100 mesh; Supelco Inc., Bellefonte, Pa.). The operating conditions were as follows: injector temperature, 200°C; detector temperature, 250°C; oven temperature, 150°C; nitrogen carrier gas flow rate, 30 ml/min.

Amounts of acetate, formate, and chloride were determined by ion chromatography with a suppressor-based system (Dionex Corp., Sunnyvale, Calif.) and an Ionpac AS10 analytical column (Dionex). A 50-μl portion of a filtered sample was injected by using a model LKB 2157 autosampler (Pharmacia, Uppsala, Sweden). The operating conditions were as follows: 30 mM NaOH for 3.4 min, 60 mM NaOH for 5.7 min, and 180 mM NaOH for 6.5 min; flow rate, 1.0 ml/min.

Protein contents were determined by a method derived from the methods of Kennedy and Fewson (16) and Bradford (2); 1 ml of culture liquid was mixed with 200 μl of 3 M trichloroacetic acid and frozen for at least 24 h at −20°C. After thawing and centrifugation, the resulting pellet was resuspended in 100 μl of 1.32 M NaOH and incubated for 20 min at 80°C and then for 24 h at 30°C. The sample was then diluted with 100 μl of distilled water and centrifuged. The assay mixture consisted of 650 μl of water, 100 μl of sample, 50 μl of 3 M H_3PO_4, and 200 μl of dye reagent (Bio-Rad, Munich, Germany). The optical density at 595 nm was determined, and the protein concentration was calculated from a standard curve established with bovine gamma globulin. Sulfide contents were determined by the methylene blue method (21).

The amount of total carbon in culture DC biomass was calculated from the protein content by using the conversion factor 0.105 ± 0.014 mol of carbon per g of protein. This factor was obtained by determining both the total organic carbon contents (with a model TOC-500 total organic carbon analyzer [Shimadzu, Kyoto, Japan]) and the protein contents in phosphoric acid precipitates of nine separate cultures grown to different final optical densities. Alternatively, the growth of the mixed culture was measured by determining the optical density at 650 nm. An optical density at 650 nm of 0.17 corresponded to 20 μg of protein per ml.

Carbon monoxide dehydrogenase activity was measured as described by Diekert and Thauer (7) by using benzyl viologen as an electron acceptor; 1 kat of activity was defined as the activity that oxidized 1 mol of carbon monoxide to carbon dioxide per s.

16S rDNA sequence and analysis. Genomic DNA extraction, PCR-mediated amplification of the 16S ribosomal DNA (rDNA), and purification of PCR products were carried out as described previously (22, 23). Purified PCR products were sequenced by using a *Taq* Dye-Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Weiterstadt, Germany) as directed in the manufacturer's protocol. Sequence reaction mixtures were electrophoresed by using an Applied Biosystems model 373 DNA sequencer. The 16S rDNA sequences were aligned manually with the sequences of representatives of the main lines of descent.

Pairwise evolutionary distances were computed by using the correction of Jukes and Cantor (15). The least-squares distance method of De Soete (6) was used to construct a phylogenetic dendrogram from distance matrices. The starting materials used for the 16S rDNA sequence analysis were a pure culture of strain DMB and a coculture of strain DMC and *M. hungatei*. In the latter case it was possible by using PCR primers specific for members of the *Bacteria* to separately amplify the 16S rDNA gene of strain DMC and the 16S rDNA gene of the *Archaeon* in the mixture.

Electron microscopy. For transmission electron microscopy bacteria were cryoimmobilized to prepare thin sections by high-pressure freezing (20) in cellulose capillary tubes (12). Samples were then freeze-substituted in acetone containing 2% osmium tetroxide and finally embedded in Epon-Araldite (12).

For scanning electron microscopy bacteria were adsorbed onto glow-discharged carbon platelets, frozen by rapid plunging into liquid propane, and freeze-substituted as described above. After samples were washed in acetone, they were critical point dried and planar-magnetron sputtered with 6 nm platinum in a model MED 010 apparatus (Baltec, Balzers, Fürstentum Liechtenstein).

Nucleotide sequence accession numbers. The 16S rDNA nucleotide sequences of strains DMB and DMC have been deposited in the EMBL data library under accession numbers X86689 and X86690, respectively.

RESULTS

Composition and growth properties of mixed culture DC. Like previously described culture DM (3), culture DC grew with dichloromethane as the sole carbon and energy source and produced acetate as the major catabolite. This culture was subcultured at weekly intervals on dichloromethane-mineral salts medium amended with yeast extract and was stable for 18 months. Culture DC was examined by phase-contrast microscopy and scanning electron microscopy and consisted of two morphologically distinct types of bacterial cells. One of these was a curved rod resembling strain DMB, the organism previously isolated from culture DM (3). The other organism present in culture DC was a short, plump, rod-shaped organism which we designated strain DMC. An approximate ratio of one cell resembling strain DMB to two cells of strain DMC was observed by microscopy, and the relative amounts of the two organisms appeared to remain constant during growth.

Figure 1 shows the growth, substrate utilization, and product formation of culture DC under standard culture conditions (that is, with 1 mM sulfate in the medium); 4.86 mM dichloromethane was converted to 9.00 mM chloride, 0.61 mM acetate, and 2.06 mM carbon in biomass. Only trace amounts of formate (<0.02 mM) were observed. These values are net values corrected for the values measured in control cultures grown with standard mineral salts medium amended with yeast extract but not containing dichloromethane. The culture grew with a doubling time of 20 h and achieved a maximum degradation rate for dichloromethane of 1.8 mkat/kg of protein, which is about three times higher than the rate measured in culture DM. While the balance of substrate and products proved that complete dehalogenation of dichloromethane occurred, the data were unsatisfactory with respect to carbon recovery. As shown in Table 1, the level of carbon recovery was only 67.4%, and this indicated that a product that was not accounted for was formed from dichloromethane during growth.

Since our standard medium contained 1 mM sulfate, oxidation to carbon dioxide (with sulfate as a terminal electron acceptor) of part of the carbon of dichloromethane was a possible explanation for the deficiency in products formed from dichloromethane by culture DC. Carbon dioxide could not be quantified in our system since it formed part of the carbonate buffer used. The culture produced 0.22 mM sulfide during growth with 5 mM dichloromethane. To test the effect of sulfate on product formation, we performed a growth experiment with dichloromethane-mineral salts medium that was amended with yeast extract but did not contain sulfate. Figure 2 shows that under these conditions the net level of acetate formation (1.3 mM) was significantly higher than the level of

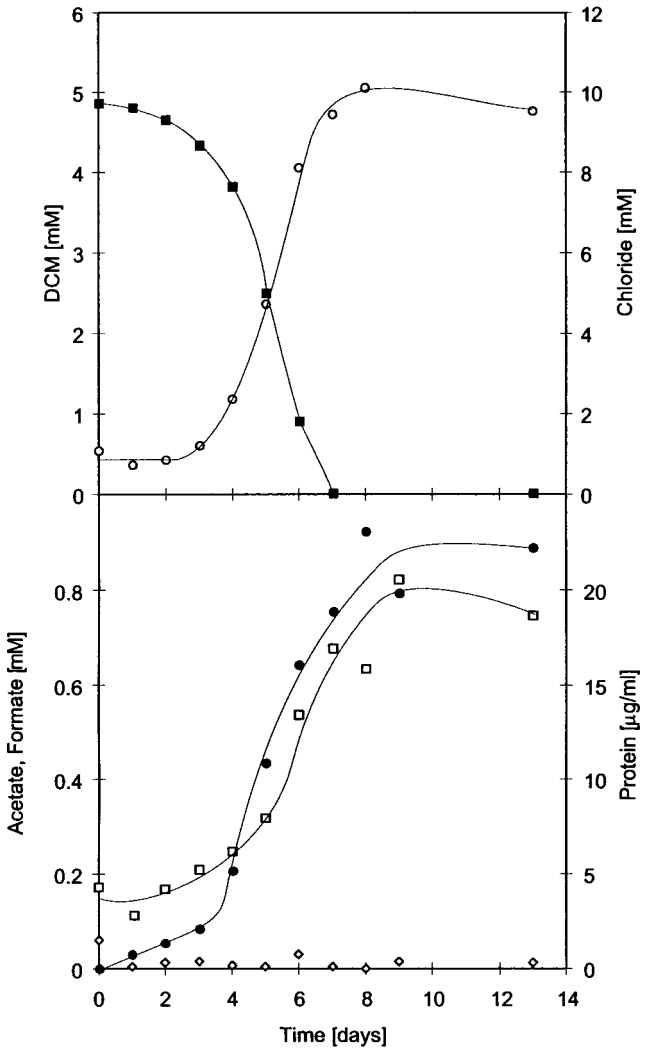

FIG. 1. Dehalogenation of dichloromethane (DCM), growth, and product formation by anaerobic mixed culture DC in standard medium (1 mM sulfate). Symbols: ■, dichloromethane; ○, chloride; ●, protein; □, acetate; ◇, formate.

acetate formation under standard conditions, while less protein and marked amounts of formate were produced. On the sulfate-free medium, culture DC exhibited a doubling time of 40 h and the level of carbon recovery was 82.2% (Table 1). The difference in product formation in the presence and absence of sulfate indicated that one of the two organisms in culture DC was a sulfate-reducing bacterium, and this conclusion was supported when we observed an increase in the concentration of sulfide when sulfate was added to the medium (data not shown). Thus, additional experiments were directed at determining which of the two components of culture DC was the sulfate-reducing organism and which component was responsible for the dehalogenation of dichloromethane.

Strain DMB is a *Desulfovibrio* sp. When mixed culture DC was inoculated into a medium containing lactate and sulfate, growth of a motile organism with the same morphology as strain DMB was observed and sulfide was produced. There was, however, no growth on a medium containing lactate but no sulfate. Similarly, inoculation of a medium containing acetate, sulfate, and H_2-CO_2 resulted in growth of the organism resembling strain DMB, whereas no growth was observed

TABLE 1. Levels of carbon recovery after fermentation of dichloromethane by mixed culture DC in the presence and absence of sulfate

Sulfate concn (mM)	Concn of[a]:					% Recovery[b]
	Substrates		Products			
	Dichloromethane consumed	Yeast extract added	Acetate	Formate	Biomass	
1	0.000	2.580	0.252	0.008	0.186	
1	4.860	2.580	1.466	0.009	2.246	67.4
0	0.000	2.580	0.410	0.010	0.176	
0	5.054	2.580	3.012	0.288	1.449	82.2

[a] Expressed as millimolar concentrations of carbon.
[b] Sum of the products formed in the culture grown with dichloromethane minus sum of the products in the culture grown without dichloromethane related to the amount of dichloromethane supplied.

when acetate was omitted. We concluded from these observations (Table 2) that strain DMB and one of the organisms in mixed culture DC are identical.

Table 2 summarizes the results of growth experiments performed with a pure culture of strain DMB. These results demonstrate that this organism is not able to grow autotrophically with C_1 compounds, but needs acetate for growth with C_1 compounds as energy sources. This growth behavior is reminiscent of the behavior of *Desulfovibrio vulgaris*, a heterotrophic sulfate-reducing bacterium that is not able to use C_1 compounds as carbon sources (cited from reference 4).

The conclusion that strain DMB is related to *Desulfovibrio vulgaris* was supported when the 16S rDNA sequence of strain DMB was determined and analyzed. This sequence exhibited 99.8% similarity to the 16S rDNA sequence of *Desulfovibrio vulgaris*. On the basis of its morphological, physiological, and phylogenetic relatedness to *Desulfovibrio vulgaris*, we concluded that strain DMB is a representative of the genus *Desulfovibrio*. This conclusion is in contrast to the previous assumption (3) that strain DMB is a homoacetogenic bacterium. We believe that in culture DC *Desulfovibrio* sp. strain DMB uses formate (or possibly hydrogen or carbon monoxide) and acetate produced by strain DMC from dichloromethane for growth.

Morphological and physiological properties of strain DMC. Strain DMC was found to be a strictly anaerobic, nonmotile, spherical rod-shaped organism whose average cell length was 1.8 μm and cell diameter was 1.1 μm. The cells occurred predominantly in pairs. Analyses of strain DMC by electron microscopy indicated that it had a gram-positive cell wall (Fig. 3).

Attempts to isolate strain DMC in pure culture, either with dichloromethane or with another compound as the substrate, have failed. Dilution series cultures in liquid medium with dichloromethane as the substrate consistently yielded the original mixed culture DC, containing both strain DMC and strain DMB. We found that it was also impossible to subculture single colonies of strain DMC obtained in agar shake dilution series cultures in liquid medium or on solid medium. However, we did isolate strain DMC in coculture with *A. woodii* or *M. hungatei*. To do this, material from mixed culture DC was diluted in agar shake tubes containing 5 mM dichloromethane in minimal salts medium amended with yeast extract. After 3 weeks of incubation two types of colonies were observed. One of these types was colonies that were slightly brownish and lens shaped and contained an organism morphologically identical

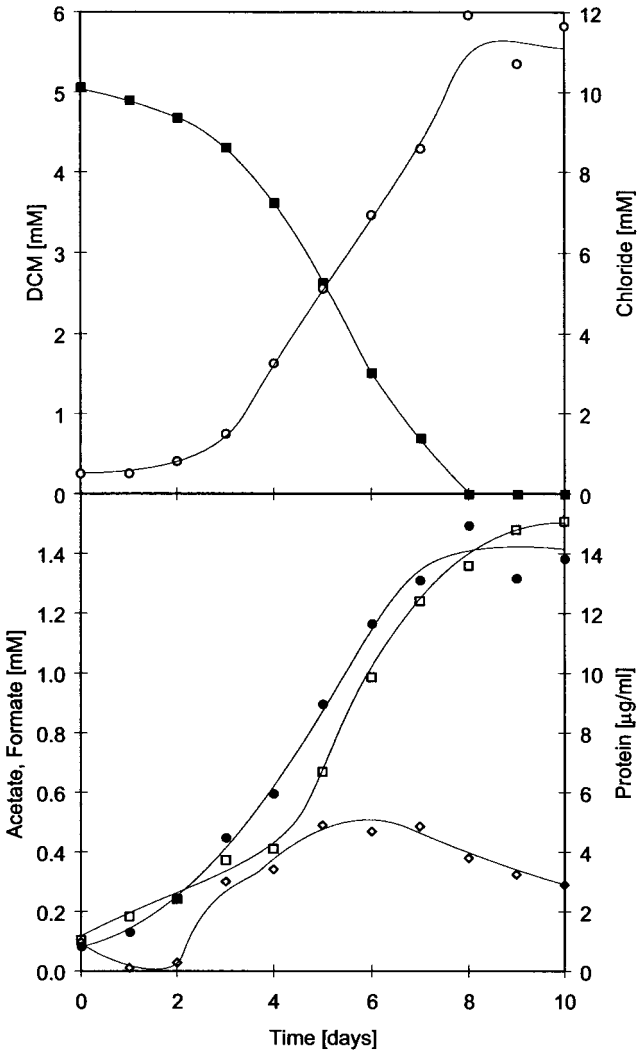

FIG. 2. Dehalogenation of dichloromethane (DCM), growth, and product formation by anaerobic mixed culture DC in sulfate-free medium. Symbols: ■, dichloromethane; ○, chloride; ●, protein; □, acetate; ◇, formate.

to strain DMC. Material from these colonies was used to prepare two agar shake dilution series, one consisting of cultures in dichloromethane medium containing an early-stationary-phase *A. woodii* culture (2%, vol/vol) and the other consisting of cultures in medium containing an early-stationary-phase *M. hungatei* culture (2%, vol/vol). After 5 weeks, colonies similar to the colonies observed in the first dilution series were observed, and these colonies were subcultured in liquid dichloromethane medium supplemented with the appropriate partner organism (see Materials and Methods).

The liquid cocultures of strain DMC with either *A. woodii* or *M. hungatei* were stable for at least three transfers when we used a 2% (vol/vol) inoculum and a culture of the partner organism (2%, vol/vol) for subcultivation. These cultures degraded 5 mM dichloromethane within 3 weeks. After 3 weeks, the coculture with *A. woodii* had produced 1.78 mM acetate, and the coculture with *M. hungatei* had formed 0.95 mM acetate and 1.03 mM formate. Both cocultures were found to be free of strain DMB since they produced no growth when they were inoculated into a medium containing 5 mM lactate and 5 mM sulfate. The coculture with *M. hungatei* produced insignificant amounts of methane, and we concluded that the methanogen was metabolically inactive in this system.

TABLE 2. Heterotrophic growth of and sulfate reduction by strain DMB

Incubation conditions[a]	Growth[b]
H_2-CO_2 + SO_4^{2-}	0.016
H_2-CO_2 + SO_4^{2-} + acetate	0.065
CO + SO_4^{2-}	0.012
CO + SO_4^{2-} + acetate	0.050
Formate + SO_4^{2-}	0.011
Formate + SO_4^{2-} + acetate	0.019
Acetate + SO_4^{2-}	0.008
Lactate	0.009
Lactate + SO_4^{2-}	0.050

[a] The final concentrations of the medium components were 5 mM sulfate, 5 mM lactate, 2 mM acetate, 2.5 mM formate, 2.0×10^5 Pa of H_2-CO_2 in the gas phase, and 30×10^2 Pa of CO in the gas phase. Cultures were grown in 58.7-ml serum flasks containing 20 ml of medium.

[b] Optical density at 650 nm after 5 days of growth.

Mixed culture DC and the coculture with *M. hungatei* were used to search for growth substrates for strain DMC other than dichloromethane. No growth of the organism was observed on media containing one of the following compounds or mixtures of compounds: acetate, acetoin, butyrate, crotonate, ethylene glycol, ethanol, ferulate, formate, formate plus acetate, fructose, fumarate, glucose, H_2-CO_2, H_2-CO_2 plus acetate, 4-hydroxybenzoate, lactate, methanol, nutrient broth, oxalate, pyruvate, ribose, saccharose, succinate, syringate, syringate plus H_2-CO_2, and 3,4,5-hydroxycinnamate.

Formation of acetate from dichloromethane was observed whenever strain DMC was present in a coculture with one of three different partners, suggesting that this organism was re-

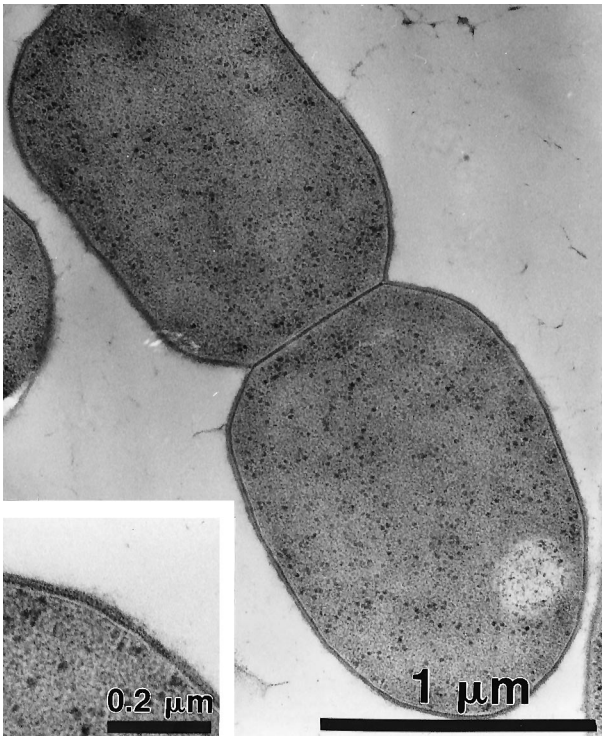

FIG. 3. Electron micrograph of a thin section of strain DMC showing the gram-positive cell wall.

sponsible for both dichloromethane degradation and acetate formation. This hypothesis was supported by determining the specific activity of CO dehydrogenase, the key enzyme of the acetyl-coenzyme A pathway (8), in culture DC and in *Desulfovibrio* sp. strain DMB grown on H_2-CO_2 plus acetate. The specific activity of this enzyme was 14.2 mkat/kg of protein in an extract obtained from exponentially growing cell material from culture DC, and the specific activity was below the limit of detection (0.6 mkat/kg of protein) in an extract obtained from exponentially growing strain DMB cells.

Phylogenetic characterization of strain DMC. Coculture of strain DMC with the *Archaeon M. hungatei* allowed us to amplify by PCR the 16S rRNA gene of strain DMC without interference by bacterial 16S rRNA genes. A phylogenetic analysis of the strain DMC 16S rDNA sequence extending from nucleotide position 200 to nucleotide position 1500 showed that this organism groups within the radiation of the *Clostridium-Bacillus* subphylum of the gram-positive bacteria. Figure 4 shows the phylogenetic position of strain DMC in relation to representatives of the genus *Clostridium* and related taxa. The highest levels of 16S rDNA sequence similarity were found with *Desulfotomaculum orientis* and *Desulfitobacterium dehalogenans*, both of which exhibited 89% similarity (Table 3).

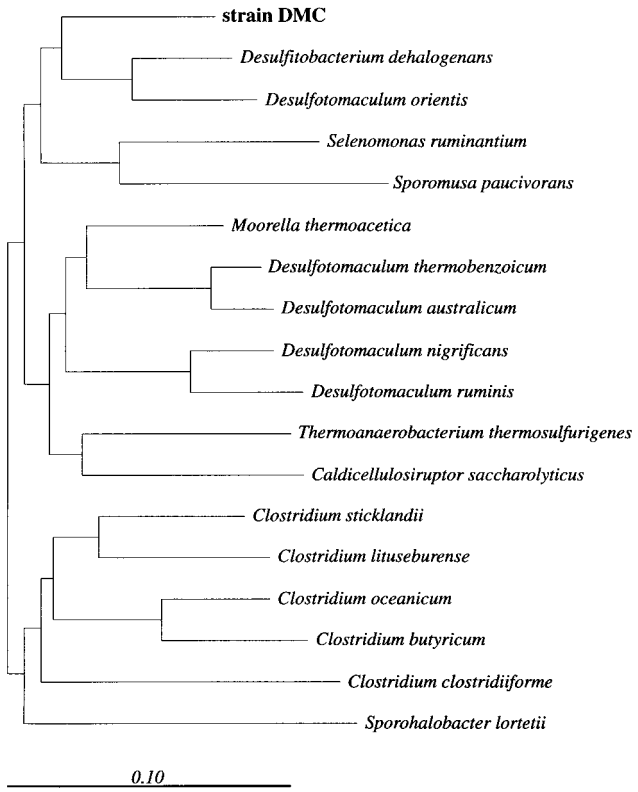

FIG. 4. Phylogenetic dendrogram based on 16S rDNA sequence data indicating the position of strain DMC within the radiation of the clostridia and related taxa. Bar = 10 nucleotide substitutions per 100 nucleotides.

DISCUSSION

Workers have made a range of observations on the metabolic utilization of chlorinated aliphatic C_1 and C_2 hydrocarbons by anaerobic bacteria in enrichment cultures, but data obtained with pure cultures are rare. This is true for the respiratory dehalogenating bacteria that use chlorinated ethenes as physiological electron acceptors (5, 9, 29); so far one of these organisms is available in quasipure culture (13) and another is available in pure culture (24). It is also true for bacteria that anaerobically dehalogenate dichloromethane to yield a carbon and energy source and whose existence was deduced from the growth properties of mixed cultures (3, 11, 25). In this study we analyzed a two-component mixed culture (culture DC) that produces acetate from dichloromethane. A principal question was whether acetogenesis from dichloromethane was catalyzed by a single organism or whether it resulted from interspecies formate or hydrogen transfer between the components of the mixed culture.

The partial mass balance for culture DC during growth with dichloromethane in sulfate-free medium (Table 1) is compatible with the following fermentation balance:

$$2CH_2Cl_2 + 2H_2O \rightarrow CH_3COO^- + 4Cl^- + 5H^+$$

Metabolism of dichloromethane according to this equation has been observed in the mixed culture that preceded culture DC. This metabolism was interpreted to result from the dehalogenation (by an unknown mechanism) of dichloromethane to an intermediate at the oxidation state of formaldehyde, the subsequent oxidation of this intermediate to formate, and the conversion of formate to acetate via the reactions of the acetyl coenzyme A pathway (3). The hypothesis that this pathway occurs in culture DC is supported by the fact that formate accumulates during growth with dichloromethane. The overall conversion of dichloromethane to acetate and the individual steps of the proposed pathway are thermodynamically favorable (3), so that a single organism should be capable of acetogenesis from dichloromethane.

Our data indicate that this is the case and that the novel bacterium strain DMC is responsible for both dehalogenation of dichloromethane and conversion of the dehalogenation product to acetate. We concluded this from the following observations: (i) when strain DMC was associated with one of three metabolically different partners, it was able to grow with dichloromethane; (ii) the coculture of strain DMC with the nonacetogenic archaeon *M. hungatei* formed acetate from dichloromethane; (iii) the coculture of strain DMC with *Desulfovibrio* sp. strain DMB, which is not able to use C_1 compounds as carbon sources, utilized dichloromethane as a carbon source; and (iv) CO dehydrogenase was present in culture DC but not in *Desulfovibrio* sp. strain DMB grown with hydrogen as an electron donor. It must be postulated that strain DMB expresses a dissimilatory CO dehydrogenase when it uses carbon monoxide as an electron donor (Table 2), and this activity cannot be distinguished from the activity of CO dehydrogenase-acetyl coenzyme A synthase by the assay which we used. However, it appears unlikely that the dissimilatory CO dehydrogenase of strain DMB would be expressed during growth with substrates other than carbon monoxide.

The obligatory dependence of strain DMC on a partner during growth with dichloromethane is thought to stem from the need for a growth factor produced by the associated organism. This hypothesis is supported by the observation that *M. hungatei* was metabolically inactive in the coculture with strain DMC and needed to be supplied anew upon subcultivation.

The ability to specifically amplify the 16S rDNA of strain DMC from a coculture with *M. hungatei* allowed us to determine the phylogenetic position of this strain. Some of the characteristics of strain DMC, including anaerobic growth and a gram-positive cell wall, are consistent with a phylogenetic position within the radiation of the clostridia and related taxa.

TABLE 3. 16S rDNA similarity values between strain DMC and related taxa within the *Clostridium-Bacillus* subphylum

Strain or species	% Similarity to strain or species:																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. Strain DMC																			
2. *Desulfitobacterium dehalogenans*	89.0																		
3. *Desulfotomaculum orientis*	89.0	92.4																	
4. *Sporomusa paucivorans*	83.5	83.5	83.7																
5. *Selenomonas ruminantium*	86.0	85.2	85.3	86.0															
6. *Desulfotomaculum ruminis*	84.0	86.3	84.7	80.2	84.8														
7. *Desulfotomaculum nigrificans*	84.8	86.7	84.7	81.4	83.7	93.2													
8. *Desulfotomaculum australicum*	86.0	86.2	85.7	80.4	84.0	85.8	87.1												
9. *Desulfotomaculum thermobenzoicum*	85.7	86.3	85.8	81.4	83.5	86.0	87.5	96.0											
10. *Caldicellulosiruptor saccharolyticus*	86.3	83.0	82.7	79.4	85.0	82.5	83.4	85.0	85.3										
11. *Moorella thermoacetica*	89.6	85.7	85.5	82.5	83.5	83.9	86.5	89.3	90.1	86.5									
12. *Thermoanaerobacter thermohydrosulfuricus*	89.1	85.2	85.3	81.2	83.2	84.2	84.7	88.0	88.5	87.3	91.3								
13. *Thermoanaerobacterium thermosulfurigenes*	84.8	83.0	81.9	78.9	84.0	82.9	83.5	85.3	87.0	86.3	88.8	88.3							
14. *Clostridium butyricum*	83.5	83.4	82.4	79.9	81.2	80.6	82.9	81.1	81.5	80.9	83.7	81.9	83.4						
15. *Clostridium oceanicum*	86.8	84.5	84.3	81.5	83.2	82.0	82.7	82.4	81.9	82.7	85.7	83.5	83.7	91.8					
16. *Clostridium sticklandii*	86.3	86.7	85.7	82.9	83.4	82.2	83.5	83.9	84.5	83.9	85.5	84.4	83.0	84.8	86.3				
17. *Clostridium lituseburense*	86.2	85.3	84.0	79.2	82.0	82.5	82.9	83.0	82.7	82.4	84.7	83.2	82.9	87.3	86.0	89.8			
18. *Clostridium clostridiiforme*	82.9	85.0	81.7	78.3	83.0	80.4	81.5	79.7	80.7	81.1	81.9	79.9	81.9	82.7	83.5	84.0	84.0		
19. *Sporohalobacter lortetii*	83.2	79.9	80.1	80.1	80.6	78.1	79.1	81.5	81.4	81.9	84.5	81.5	82.5	80.9	82.7	83.2	82.5	79.7	

The distinct phylogenetic position and the lack of a high level of sequence similarity with any previously described taxon indicate that strain DMC represents a new genus. Since strain DMC exists only in a coculture, it is impossible to determine a full range of physiological and phenotypic characteristics for comparison with characteristics of other related organisms. Determination of the G+C content of the DNA is also not possible at the present time.

Strain DMC is most closely related to *Desulfotomaculum orientis* and *Desulfitobacterium dehalogenans*. The former organism is a sulfate reducer that possesses the acetyl coenzyme A pathway (17). It appears that this pathway is also operative in strain DMC since this strain utilizes a C_1 substrate for growth and since CO dehydrogenase was detected in the coculture containing the organism. Strain DMC, however, was not able to reduce sulfate. The presence of sulfate did not support growth with a series of potential substrates, and sulfate provided to cocultures of strain DMC with *M. hungatei* or *A. woodii* was not reduced to sulfide.

The ability to dehalogenate chlorinated organic compounds is a feature that is common to *Desulfitobacterium dehalogenans* and strain DMC. However, the former organism reductively dehalogenates certain chloroaromatic compounds (26), whereas strain DMC dehalogenates dichloromethane presumably by a substitutive mechanism. *Desulfitobacterium dehalogenans* needs electron acceptors such as sulfite, thiosulfate, sulfur (but not sulfate), fumarate, nitrate, or *ortho*-chlorinated phenols for growth with electron donors other than pyruvate. Attempts to grow strain DMC by using a limited range of electron donors and sulfite or thiosulfate as an electron acceptor have been unsuccessful, but it will certainly be worthwhile to test other pairs of electron donors and acceptors to find conditions for growth of this organism on substrates other than dichloromethane.

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