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Biodegradation of Dichloromethane and Its Utilization as a Growth Substrate under Methanogenic Conditions

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Biodegradation of dichloromethane (DCM) to environmentally acceptable products was demonstrated under methanogenic conditions (35°C). When DCM was supplied to enrichment cultures as the sole organic compound at a low enough concentration to avoid inhibition of methanogenesis, the molar ratio of CH_4 formed to DCM consumed (0.473) was very close to the amount predicted by stoichiometric conservation of electrons. DCM degradation was also demonstrated when methanogenesis was partially inhibited (with 0.5 to 1.5 mM 2-bromoethanesulfonate or approximately 2 mM DCM) or completely stopped (with 50 to 55.5 mM 2-bromoethanesulfonate). Addition of a eubacterial inhibitor (vancomycin, 100 mg/liter) greatly reduced the rate of DCM degradation. $^{14}CO_2$ was the principal product of [^{14}C]DCM degradation, followed by $^{14}CH_4$ (when methanogenesis was uninhibited) or $^{14}CH_3COOH$ (when methanogenesis was partially or completely inhibited). Hydrogen accumulated during DCM degradation and then returned to background levels when DCM was consumed. These results suggested that nonmethanogenic organisms mediated DCM degradation, oxidizing a portion to CO_2 and fermenting the remainder to acetate; acetate formation suggested involvement of an acetogen. Methanogens in the enrichment culture then converted the products of DCM degradation to CH_4. Aceticlastic methanogens were more easily inhibited by 2-bromoethanesulfonate and DCM than were CO_2-reducing methanogens. When DCM was the sole organic-carbon and electron donor source supplied, its use as a growth substrate was demonstrated. The highest observed yield was 0.085 g of suspended organic carbon formed per g of DCM carbon consumed. Approximately 85% of the biomass formed was attributable to the growth of nonmethanogens, and 15% was attributable to methanogens.

Dichloromethane (DCM) has been in widespread use for several decades. Since 1970, annual U.S. production has ranged from 212 × 10^6 to 287 × 10^6 kg, with the principal application being paint removal (34). DCM is one of 14 volatile organic compounds regulated under the Safe Drinking Water Act Amendments of 1986; it has been shown to cause lung and liver cancer in mice (25). Because of its relatively low Henry's constant (14), DCM is difficult to remove from contaminated groundwater by air stripping, one of the most commonly applied remediation techniques for volatile organics (22).

Surprisingly little information is available concerning the behavior of DCM under methanogenic conditions. DCM is a potent inhibitor of methanogenesis (28, 32, 35). However, inhibition typically diminishes as cultures acclimate to continuous DCM addition. Stuckey et al. (28) observed the ability of a mixed culture to acclimate even when the digestor concentration of DCM reached 10 mg/liter. Biological degradation of DCM has been demonstrated conclusively by Gossett (13), who used mixed batch cultures which repeatedly consumed 8-mg/liter additions of DCM. The principal biotransformation products of [^{14}C]DCM were $^{14}CO_2$ (approximately 73%) and a nonstrippable residue (NSR; 21%), about one-half of which was soluble. Because the identity of the NSR was not determined, it was impossible to ascertain whether or not this major product was environmentally acceptable. Evidence for biodegradation of DCM has also come from studies with continuous-flow reactors (2, 28). However, these studies did not investigate the products of DCM transformation or the organisms responsible for carrying them out. Formation of DCM from biodegradation of carbon tetrachloride (7) and chloroform (6, 13) has also been shown; both compounds inhibit further degradation of the DCM formed.

In this study, anaerobic enrichment cultures and specific inhibitors were used to investigate the roles of methanogens and nonmethanogens in mediating DCM degradation. When DCM was supplied as the sole organic-carbon and electron donor source, essentially stoichiometric amounts of methane were produced from the DCM consumed. The use of DCM as a growth substrate was also demonstrated. Volatile and nonvolatile products of DCM biotransformation were identified by using [^{14}C]DCM and measurements of hydrogen formation. On the basis of the cumulative evidence, a model for DCM degradation under methanogenic conditions is proposed.

MATERIALS AND METHODS

Chemicals. DCM was obtained in neat liquid form (99 mol% pure; Fisher Scientific); it was added to cultures from saturated-water stock solutions (approximately 240 mM DCM). Chloromethane was purchased dissolved in methanol (200 mg/liter, 1-ml ampoule; Supelco, Inc.). Methane and ethylene were obtained in gaseous form (99+%; Scott Specialty Gases). 2-Bromoethanesulfonic acid (BES; sodium salt, 98%) was purchased from Aldrich Chemical Co. Vancomycin hydrochloride (963 μg/mg) was obtained from Sigma Chemical Co. (St. Louis, Mo.) and dissolved in water (10.4 g/liter). [^{14}C]DCM (Sigma Radiochemical) was diluted in 150 ml of distilled deionized water and stored in a 160-ml serum bottle capped with a grey butyl rubber septum (Wheaton) and an aluminum crimp cap. The [^{14}C]DCM stock

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solution contained 2.80×10^7 dpm/ml (4.62 mM DCM); gas chromatographic (GC) analysis of the stock bottle headspace indicated the presence of an unidentified contaminant, which was not radiolabeled. There was no indication that this contaminant interfered (e.g., as an inhibitor or electron donor) with the DCM degradation studies. $^{14}CH_3COOH$ and $CH_3{}^{14}COOH$ (Sigma Radiochemical) were diluted in distilled deionized water. To ensure purity, aliquots of each were processed by high-pressure liquid chromatography (HPLC; described below), and the fraction corresponding to acetic acid (HAc) was added to 150 ml of 0.013 N H_2SO_4. The final $^{14}CH_3COOH$ stock contained 1,710 dpm/ml; the final $CH_3{}^{14}COOH$ stock contained 1,850 dpm/ml. ScintiVerse-E liquid scintillation cocktail was employed (Fisher Scientific).

Cultures and enrichment procedures. All experiments used 160-ml serum bottles containing 100 ml of liquid. The bottles were sealed with slotted grey butyl rubber septa and aluminum crimp caps. Incubation was conducted at 35°C, with the liquid in contact with the septum (to minimize loss of volatiles) and under quiescent conditions except where noted. Degradation of DCM was initially achieved in "first-generation" cultures that were prepared by anaerobically transferring 100-ml mixed-liquor samples directly from a laboratory reactor to serum bottles. The laboratory reactor was a 15-liter, stirred, semicontinuous, anaerobic digestor operated at 35°C with a residence time of 20 days and fed a complex substrate (Ensure) as previously described (10).

In this study, we report the total mass of each compound present in a serum bottle. Most of the DCM resided in the liquid, and most of the CH_4 resided in the headspace: based on Henry's constants [(moles per cubic meter of gas)/(moles per cubic meter of aqueous solution)] of 0.128 (14) and 33.1 (10) for DCM and CH_4, respectively, at 35°C, 93% of the DCM and 4.8% of the CH_4 were present in the 100 ml of liquid, with the balance in the 60-ml headspace.

A typical initial DCM dose was 8 to 12 μmol per bottle or an aqueous concentration of 6.3 to 9.5 mg of DCM per liter. Whenever DCM dropped near or below its detection limit, more DCM-saturated stock solution was added. After being operated in this mode for at least 80 days, first-generation cultures were used to inoculate second-generation cultures, which were then used to inoculate third-generation cultures, and so on. A 2 to 10% (vol/vol) inoculum was employed with each transfer, the balance consisting of basal medium (plus 50 mg of HAc per liter where noted) that was anaerobically prepared and dispensed as previously described (10). It included bicarbonate as pH buffer and 50 mg of yeast extract per liter.

Each set of experiments with inoculated bottles was accompanied by duplicate water controls (100 ml of distilled deionized water plus DCM) and duplicate inoculated bottles which were autoclaved (121°C, 30 min) and cooled before addition of the DCM.

Eighteen bottles (all third and fourth generation) were used to examine the fate of [^{14}C]DCM. Prior to receipt of [^{14}C]DCM, they were subjected to differing conditions. In B-1 through B-8, methanogenesis was uninhibited and DCM (10 to 12 μmol per bottle) was added repeatedly; 10 mg of HAc was added along with each DCM dose to bottles B-1 and B-2, 1 mg of HAc was added to B-3 and B-4, 0.1 mg of HAc was added to B-5 and B-6, and no HAc was added to B-7 and B-8. In B-9 through B-18, no HAc was added and methanogenesis was inhibited (either partially or completely) by adding BES, high doses of DCM, or both. In B-9 and B-10, DCM additions as high as 240 μmol per bottle were made and no BES was added; in B-11, B-12, and B-13, the DCM dose was 10 to 12 μmol per bottle along with 0.5 to 1.5 mM BES; in B-14, B-15, and B-16, the DCM dose was 10 to 12 μmol per bottle along with 50 to 55.5 mM BES; and in B-17 and B-18, the DCM dose was 53 to 84 μmol per bottle along with 50 to 55.5 mM BES. Since all 18 bottles were used in various other experiments, they had been actively degrading DCM (for 51 to 284 days) prior to receiving [^{14}C]DCM.

In 14 of the bottles (B-1 through B-8 and B-11 through B-16), 100 μl of [^{14}C]DCM stock (2.49×10^6 to 2.94×10^6 dpm) was added once along with the usual dose of unlabeled DCM (10 to 12 μmol per bottle every other day). The same amount of [^{14}C]DCM stock was also added to a water control bottle. Following a 2-day incubation, the inoculated bottles and the water controls were sacrificed to determine the distribution of ^{14}C. Average results for the water control bottles (11 analyzed) indicated that nearly all of the ^{14}C stock consisted of $^{14}CH_2Cl_2$ (97.0%), with the balance being $^{14}CO_2$ (2.7%) and a trace of ^{14}C-labeled NSR, all soluble (0.3%).

The four bottles (B-9, B-10, B-17, and B-18) that received high DCM doses were handled differently; each received five 25-μl additions of [^{14}C]DCM stock (0.66×10^6 to 0.72×10^6 dpm) every other day along with the usual doses of unlabeled DCM. Water controls received the same additions of [^{14}C]DCM. Two days after the fifth DCM addition, all of the bottles were sacrificed for ^{14}C analysis. The amount of DCM-saturated water needed to provide the dose of unlabeled DCM used in these bottles was high enough (e.g., 0.9 ml for 200 μmol of DCM) that it had to be accounted for in order to keep the liquid volume in the serum bottles at 100 ml. An equivalent volume of mixed culture was therefore withdrawn prior to adding the DCM, and a 0.10-ml aliquot was counted in liquid scintillation cocktail. The final accounting for ^{14}C in these bottles was adjusted for the number of disintegrations per minute removed in the five effluent samples by assuming that the composition of the effluent was the same as that of the liquid in the bottle at the time it was sacrificed for complete ^{14}C analysis.

The growth experiment involved four serum bottles. B-9 and B-10 were started with 90 ml of basal medium (containing 50 mg of yeast extract per liter) plus 10 ml of DCM-degrading inoculum; they were incubated in an orbital shaker bath. DCM additions were gradually increased from approximately 10 μmol to as high as 240 μmol per bottle; these high levels afforded the greatest opportunity to observe an accumulation of biomass within a reasonable time. Control bottles (BC-9 and BC-10) were prepared and operated identically but received no DCM.

Volatile and ^{14}C-labeled volatile compounds. Volatile organics (CH_4, chloromethane, and DCM) were routinely determined by GC (Perkin-Elmer model 8500) analysis of a 0.5-ml headspace sample by using a flame ionization detector in conjunction with a stainless-steel column (3.2 mm by 2.44 m) packed with 1% SP-1000 on 60/80 Carbopack-B (Supelco) as previously described (10). Detection limits (in nanomoles per bottle) were 19.4 for chloromethane, 49.2 for DCM, and 3.52 for CH_4. Identification and confirmation of DCM and chloromethane were made by GC-mass spectrometry (MS; Finnigan model 3500 GC-MS coupled with a Teknivent Interactive GC-MS Data System). Identification was subsequently confirmed by injection of authentic material into the same GC system routinely used for headspace monitoring (SP-1000–Carbopack-B column). Identification of methane was accomplished by matching the retention time of authentic material with its peak from headspace samples of enrichment cultures. This was done with four GC columns oper-

ated under different temperature conditions as previously described (10).

GC calibration factors were measured to directly relate the total mass of compound present in a serum bottle to the GC peak area obtained from a 0.5-ml headspace injection. Known masses of DCM and chloromethane were added to replicate serum bottles containing 100 ml of distilled deionized water, allowed to equilibrate at 35°C, and then analyzed (0.5-ml headspace) by GC (10, 14). Standard additions of CH_4 were effected from a neat gaseous stock. Coefficients of variation (100× standard deviation/mean) for the calibration factors were 1.89% for DCM, 1.64% for chloromethane, and 2.28% for CH_4.

The headspace-monitoring method relied on the volatiles being freely available, i.e., unsorbed and uncomplexed. This was a reasonable assumption, judging from a comparison of water controls with inoculated bottles. Soon after preparation of a new generation of enrichments, the GC peak areas obtained for DCM in inoculated bottles were consistently close to those obtained in the water controls.

Volatile ^{14}C-labeled compounds (DCM and CH_4) were analyzed with a GC-combustion technique as previously described (10). A 0.5-ml headspace sample was injected onto the GC, and the well-separated compounds were routed to a catalytic combustion tube, where they were converted to CO_2. Each fraction was then trapped in NaOH and added to liquid scintillation cocktail. [^{14}C]DCM was resolved on the SP-1000–Carbopack-B column (4.6- to 7.1-min fraction), while $^{14}CH_4$ and $^{14}CO_2$ were resolved on a Carbosieve S-II column. Henry's constants for DCM and CH_4 at 35°C (given above) were used to relate the disintegrations per minute measured from a 0.5-ml headspace injection to the total DCM and CH_4 disintegrations per minute in a bottle (10).

$^{14}CO_2$ and ^{14}C-labeled nonvolatile compounds. $^{14}CO_2$ and ^{14}C-labeled nonvolatile compounds were measured as previously described (10) once analysis of the ^{14}C-labeled volatile compounds was completed. NaOH was added to serum bottles to drive virtually all of the CO_2 into the aqueous phase. Aliquots were then removed, acidified (with HAc), and purged with N_2 through an NaOH trap; the ^{14}C recovered in NaOH corresponded to $^{14}CO_2$, while the ^{14}C not purged at acid pH corresponded to NSR. When methanogenesis was inhibited, a significant portion of the [^{14}C]DCM was recovered as NSR, most of which was soluble. Composition of the ^{14}C-labeled soluble NSR was determined by HPLC (Hewlett Packard 1090) (39). Samples (250 μl; preparation described below) were pumped (0.7 ml/min, 0.013 N H_2SO_4) through a 300-mm-long ion-exchange column (Bio-Rad Laboratories HPX-87H) into a refractive-index detector (Perkin-Elmer LC-25). As fractions eluted, they were collected in 15 ml of liquid scintillation cocktail.

By operating the ion-exchange column at 30 and 65°C, we were able to resolve acetate and methanol from several other suspected degradation products, including formate, formaldehyde, propionate, butyrate, isobutyrate, and ethanol (9). Acetate did not coelute with any of the other compounds tested. There was some overlap between methanol, isobutyrate, and propionate at 30°C but virtually none at 65°C. Conversely, there was overlap between methanol and butyrate at 65°C but none at 30°C. The collection intervals were determined by injecting each compound (100 μl at approximately 100 mM), collecting 0.5-min fractions, and then measuring the amount of compound present in each fraction.

Samples were prepared for HPLC analysis by filtering (0.45-μm-pore-size filter) 20.0 ml of culture that had received NaOH, acidifying it with HCl (0.5 ml), purging it with N_2 for 30 min, and then diluting it to 25.0 ml. Filtering prior to acidification minimized the possibility of acid hydrolyzing (and therefore solubilizing) any of the nonfilterable material. Also, using HCl for acidification instead of HAc (as used in the ^{14}C-labeled NSR analysis) made it possible to quantify the total HAc present.

In all of the samples tested, the only HPLC fractions that contained significant amounts of radioactivity were those corresponding to HAc and methanol. Confirmation that these fractions actually did contain HAc and methanol was obtained by GC analysis with a Nukol capillary column (0.53 mm by 15 m, 0.5-μm film; Supelco) and a flame ionization detector. The column temperature was 80°C for 2 min, increased at 20°C to 200°C, and then held for 4.5 min; the carrier gas (helium) flow rate was 5 ml/min. The retention time of the predominant peak in the methanol fraction (1.7 min) matched the retention time of a methanol standard; likewise, the retention time of the predominant peak in the HAc fraction (6.3 min) matched the retention time of an HAc standard.

Additional confirmation of the HAc HPLC fraction was obtained by electron impact GC-MS (Finnigan model 3300). MS results for the HAc HPLC fraction matched those for an HAc standard (9). The low concentration of methanol in its HPLC fraction prohibited similar direct confirmation by GC-MS. Instead, single-ion monitoring of the methanol HPLC fraction was employed. The presence of a compound having an *m/e* value of 31 was confirmed (9). This limited the possible structure to $\cdot OCH_3$, $\cdot OCH_2O$, or CH_3NH_2, with the possible origin of the fragment being a primary aliphatic alcohol, methoxy derivatives, methyl esters, dimethyl acetals and ketals, and CH_2OH branched chains (15). The combined evidence from HPLC, GC, and GC-MS analyses support the conclusion that the compound was methanol.

The efficiency of the HPLC method for recovering the disintegrations per minute in soluble NSR samples was evaluated as the cumulative disintegrations per minute recovered from fractions collected off the HPLC (HAc plus methanol plus other soluble substances) divided by disintegrations per minute from a direct addition of soluble NSR to scintillation cocktail. For samples in which the soluble NSR represented at least 5.0% of the total disintegrations per minute in a bottle, the average degree of recovery from the HPLC analysis was 96.2% (coefficient of variation = 3.01%).

An abbreviated version of the Schmidt degradation using the reaction vials described by Fuchs et al. (11) was used to determine which of the acetate carbons was labeled. In order to minimize the presence of organic compounds other than HAc, samples were prepared by processing them through HPLC and collecting only the HAc fraction. The procedure was carried through the splitting of HAc into CO_2 and CH_3NH_2. CO_2 (from the carboxyl carbon) was absorbed in NaOH, transferred to liquid scintillation cocktail, and counted. CH_3NH_2 (from the methyl carbon) was likewise transferred to liquid scintillation cocktail and counted. From a $^{14}CH_3COO^-$ standard, nearly 93% was recovered as $^{14}CH_3NH_2$, with only a trace of $^{14}CO_2$; from a $CH_3{}^{14}COO^-$ standard, nearly 91% was recovered as $^{14}CO_2$, with only a trace of $^{14}CH_3NH_2$.

^{14}C activity was assayed with a Beckman model 9800 liquid scintillation counter. Corrections for counting efficiency were made according to a quench curve (sample H no. versus efficiency).

Hydrogen. Hydrogen levels were monitored with an RGA2 reduction gas detector (Trace Analytical, Menlo Park, Calif.). Headspace samples were injected onto a 1% SP-1000–

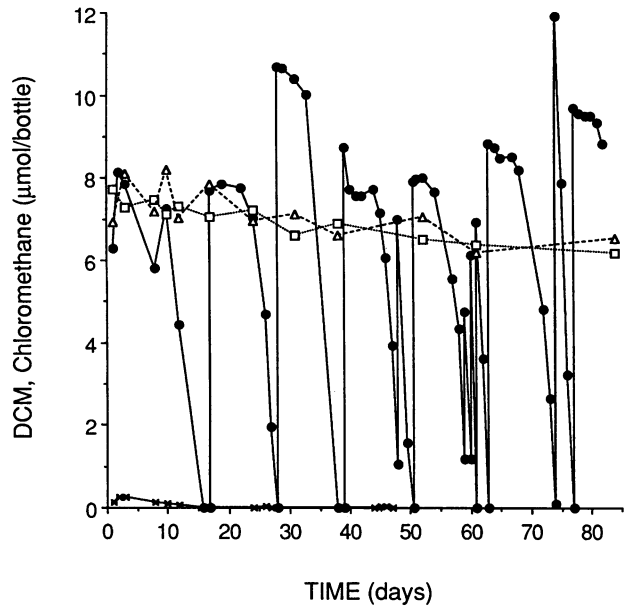

FIG. 1. DCM (●) degradation and chloromethane (×) formation in bottle A-1. DCM levels in duplicate water controls (△) and autoclaved and inoculated controls (□) are shown.

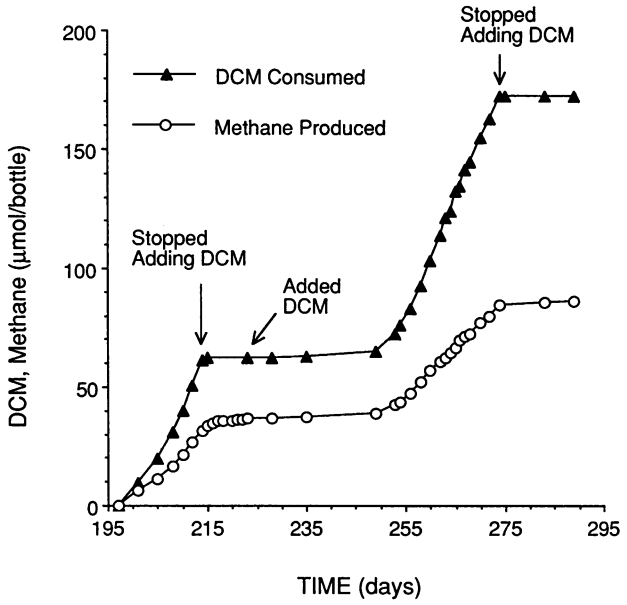

FIG. 2. Cumulative DCM consumed and methane produced in bottle A-2 when DCM was the sole organic compound added.

Carbopack-B column as described above. By using an automated switching valve timed to change at 1.5 min, the first compounds eluting off the Carbopack-B column—H_2, CH_4, and CO_2—were routed to a 60/80 Carbosieve G column (3.2 mm by 3.05 m, stainless steel; Supelco), while any DCM that was present eluted later from the Carbopack column and was routed to the flame ionization detector. On the Carbosieve G column, H_2 was resolved from the other compounds before being sent to the reduction gas detector. The GC was operated isothermally at 100°C for a total of 6 min, with a helium carrier gas flow rate of 30 ml/min. H_2 eluted at approximately 1.25 min. Standards were prepared in serum bottles containing 100 ml of distilled deionized water which were then purged with N_2 (about 60 ml/min for 5 min) and capped with grey butyl septa before H_2 was added.

Biomass. Biomass was determined by measuring suspended organic carbon (SOC), calculated as total organic carbon in unfiltered samples minus total organic carbon in filtered (0.45-μm-pore-size filter) samples. A model 700 TOC Analyzer (OI Corp., College Station, Tex.) was used to measure organic carbon (1-ml samples, duplicate injections), with sodium persulfate serving as the oxidant (100 mg per sample); the effectiveness of the procedure was confirmed by the method of standard additions.

RESULTS

Stoichiometry of DCM degradation and methane formation. Results for one of the first-generation bottles (A-1) are shown in Fig. 1. The initial DCM addition of 8 μmol was consumed after a lag period of about 10 days. Subsequent 8- to 11-μmol DCM additions were also degraded with little or no lag. Chloromethane appeared in only trace amounts over the first 46 days. By day 84, losses of DCM from duplicate water control and autoclaved-inoculated control bottles were only 5.74 and 10.8%, respectively. Thus, the much faster disappearance of DCM in the inoculated bottle was a consequence of biological activity rather than abiotic processes. Similar results for water controls and autoclaved-inoculated controls were obtained in all subsequent experiments. Over the longest period a set of controls was monitored (707 days), only 17.2% of the DCM was lost from water controls and only 25.7% was lost from autoclaved-inoculated controls.

After a long lag period (64 days), a second-generation enrichment culture (A-2) began to degrade repeated 10- to 11-μmol additions of DCM within 3 or 4 days. Prior to each DCM addition, 4 ml of mixed culture was withdrawn and replaced with 4 ml of new basal medium containing 50 mg of HAc per liter. This mode of operation continued until day 193, after which only DCM-saturated water was added. On day 197, monitoring of cumulative DCM degradation and CH_4 formation began. (It was necessary to purge the bottle's headspace of accumulated methane before this monitoring began in order to keep the CH_4 output below the upper detection limit of the flame ionization detector.)

The absence of HAc additions did not deter DCM degradation. Between days 197 and 215, six additions of DCM (10 to 11 μmol per addition) were degraded, totaling 61.3 μmol of DCM consumed. Over the same 17-day interval, 31.4 μmol of methane was produced, i.e., 0.51 mol of methane was produced per mol of DCM consumed. DCM additions were then stopped, while monitoring of methane production continued. Between days 215 and 217, 1.82 μmol of methane was produced; only 0.94 μmol of additional methane was produced over the next 6 days (Fig. 2). Thus, the absence of DCM resulted in a levelling off of methane output. This suggested that the CH_4 produced in the presence of DCM was a consequence of DCM degradation and did not come from some other electron donor (e.g., yeast extract or HAc) possibly left over from previous use of the bottle.

DCM was added again on day 223. An acclimation period was observed before a rapid rate of DCM consumption

TABLE 1. Correlation of methane production and DCM degradation[a]

Bottle no.	n	Total CH_4 produced (μmol)	Total DCM consumed (μmol)	Slope	
				CH_4/DCM[b]	R^2 (%)
A-2	17	85.5	173	0.492	99.1
B-7	12	62.7	131	0.483	99.9
B-8	11	66.4	121	0.551	99.5
B-11	4	22.8	43.5	0.521	99.8
B-12	22	104	241	0.428	99.8
B-13	15	80.1	165	0.499	99.8
B-14	56	284	617	0.453	99.9
B-15	10	55.6	118	0.456	99.7
B-16	12	69.3	140	0.469	99.0
B-19	108	547	1,150	0.477	100.0
B-20	151	763	1,580	0.477	100.0
B-21	46	240	518	0.458	99.9

[a] All bottles received DCM as sole organic substrate, typically 10 to 12 μmol per bottle every other day; n = number of points included in the regression analysis of CH_4 produced versus DCM consumed; each addition of DCM represented one point. The average slope was weighted according to n.
[b] Weighted average, 0.473.

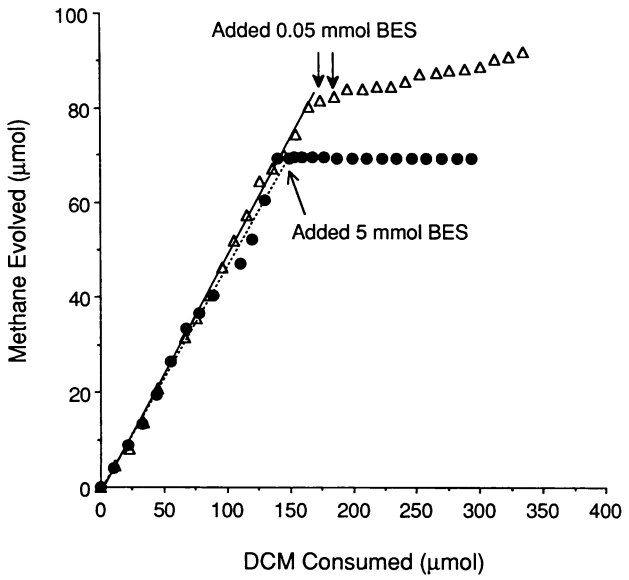

FIG. 3. Cumulative CH_4 formed and DCM consumed in bottles B-13 (△) and B-16 (●).

resumed. When it did, DCM consumption and methane production were once again linked. With 17 cycles of DCM consumption completed, the slope of the best-fit line through the data indicated 0.492 mol of methane produced per mol of DCM consumed (R^2 = 99.1%). DCM additions were stopped on day 275, and methane output correspondingly leveled off a second time (Fig. 2).

Analogous experiments correlating methane formation with DCM degradation were conducted in 11 other bottles to which DCM was added as the sole organic substrate. The duration of the studies varied, but in no instance was it less than 8 days. One bottle received only DCM for more than 300 days (151 additions, totaling 1.575 mmol of DCM), with no indication of a need for any non-DCM organic-carbon or electron donor source. As shown in Table 1, similar results were obtained, with the overall average being 0.473 mol of methane produced per mol of DCM consumed.

Effect of inhibiting methanogenesis on DCM degradation. In several bottles used to arrive at the above ratio, subsequent addition of 0.5 to 1.5 mM BES did not slow the cultures' abilities to continue degrading DCM. This amount of BES caused a partial inhibition of methanogenesis; i.e., methane output was not completely stopped but was considerably below the above ratio. For example, in bottle B-13, 0.499 mol of CH_4 was produced per mol of DCM degraded prior to BES addition. Following two 50-μmol BES additions, DCM degradation continued unabated (about 10 μmol per bottle every other day), but CH_4 output was reduced by 86% compared with output before the BES addition (Fig. 3). In bottles B-11 and B-12, similar results were obtained, with 74 and 84% reduction in methanogenesis following the BES addition, respectively.

In several other bottles described in Table 1, subsequent addition of 50 to 55.5 mM BES completely stopped CH_4 production, but the cultures continued to degrade DCM. For example, in bottle B-16, 0.469 mol of CH_4 was produced per mol of DCM degraded (Fig. 3). Addition of 5 mmol of BES completely stopped CH_4 production. DCM degradation was temporarily slowed, with 8 days instead of 2 days required to degrade 9.1 μmol of DCM. Lower initial DCM doses were subsequently added and then gradually increased until the previous rate (about 10 to 12 μmol of DCM degraded every other day) was restored and maintained for 20 days. Results for B-14 and B-15 were similar.

Concurrent with the addition of 5 mmol of BES was the accumulation of ethylene. Ethylene formation was also noted, though only in trace amounts, in bottles subjected to lower doses of BES. The probable source of ethylene was degradation of BES, as has been observed by Belay and Daniels (1) in pure cultures of methanogens exposed to BES.

Effect of vancomycin on DCM degradation. Four bottles (C-1 through C-4) were prepared identically and continuously shaken in an orbital water bath. Over the first 29 days of operation, the ability of each bottle to repeatedly degrade increasing levels of DCM was established. On day 29, C-1 and C-2 received 10 mg of vancomycin while C-3 and C-4 continued to receive only DCM. As shown in Fig. 4, C-3 was able to repeatedly degrade increasing levels of DCM of up to 120 μmol per bottle on day 41; no effort was made to increase the DCM dose above this, although in other cultures, amounts twice as high were readily degraded. DCM degradation in C-1 continued at a rapid rate for only one spike after the vancomycin was added and then declined considerably. Despite the tailing off in DCM degradation, CH_4 increased in C-1 by 67 μmol between days 29 and 49. Virtually identical results were obtained from bottles C-2 and C-4.

Hydrogen formation during DCM degradation. The possible appearance of H_2 as a product of DCM degradation was evaluated in bottles B-19, B-20, and B-22. While these bottles were continuously shaken in an orbital water bath, the amount of DCM added (as sole organic substrate) was gradually increased from approximately 10 to 200 μmol per bottle every other day and then maintained at that level for at least 18 days prior to the monitoring of H_2. At these high DCM levels, HAc accumulated in all three bottles, though HAc was not fed (data not shown).

Results for B-22 are shown in Fig. 5. Prior to DCM addition, the background level of H_2 was less than 20 nmol per bottle (8×10^{-6} atm [1 atm = 101.29 kPa]). Following

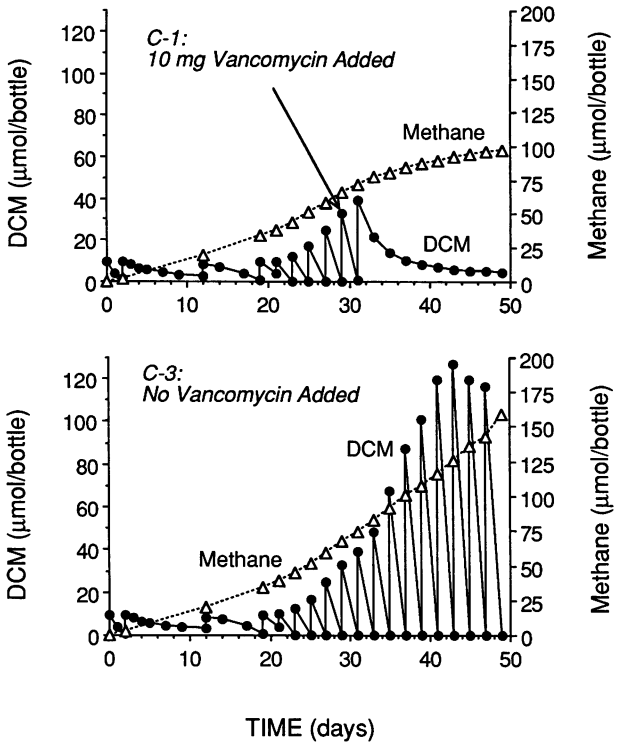

FIG. 4. DCM degradation in bottle C-1 before and after vancomycin was added and in C-3, which received no vancomycin.

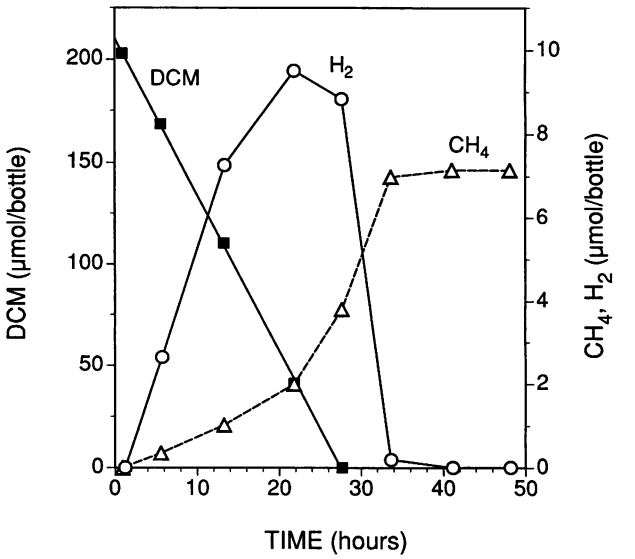

FIG. 5. H_2 and CH_4 levels following addition of a large amount of DCM to bottle B-22.

DCM addition, H_2 accumulated as DCM degraded, reached a peak of 9.5 μmol per bottle, and then returned to background levels. CH_4 increased as H_2 accumulated and then leveled off when the H_2 returned to background levels. Results for B-19 and B-20 were similar; the peak H_2 levels reached were 11.3 and 1.5 μmol per bottle, respectively.

The average rates of DCM disappearance were 6.23, 6.19, and 7.65 μmol h^{-1} $bottle^{-1}$ in B-19, B-20, and B-22, respectively. Biomass levels were measured prior to DCM addition and after DCM was degraded; no significant change occurred, with an average of 4.51, 4.59, and 4.16 mg of SOC per bottle in B-19, B-20, and B-22, respectively. The reason for the higher DCM utilization rate in B-22 is unknown; it had been in operation approximately one-third as long as the other two bottles.

Operation of B-19, B-20, and B-22 at high DCM doses resulted in partial inhibition of methanogenesis, just as low doses of BES (0.5 to 1.5 mM) did in other bottles. For example, when 200 μmol of DCM was added to B-22, it was degraded below the detection limit in 27.5 h (Fig. 5). Twenty hours later, cumulative methane output was only 7 μmol, or 0.035 mol of CH_4 per mol of DCM; this is 93% lower than the ratio observed when cultures received only 10 to 12 μmol of DCM every other day (Table 1).

Biodegradation pathway analysis using [^{14}C]DCM. The fate of [^{14}C]DCM added to bottles B-1 through B-18 is presented in Table 2. These results have been corrected for the small amount of $^{14}CO_2$ present in the [^{14}C]DCM stock solution (averaging 2.7%, as indicated by analysis of the water controls) and represent the percent of total disintegrations per minute recovered in each bottle. On average, 96.5% (coefficient of variation = 4.2%) of the disintegrations per minute added (measured by direct addition of [^{14}C]DCM stock to scintillation cocktail) was recovered (as DCM + CH_4 + CO_2 + HAc + methanol + other soluble NSR + nonsoluble NSR [assumed to be biomass]). Recoveries from water controls also averaged 96.5%.

Four general cases based on the condition of methanogenesis are represented in Table 2. In the first, methanogenesis was uninhibited (B-1 through B-8), with various levels of HAc added along with DCM. In the second, methanogenesis was partially inhibited with high doses of DCM (B-9 and B-10). In the third, methanogenesis was partially inhibited with low doses of BES (B-11, B-12, and B-13). In the fourth, methanogenesis was completely inhibited with high doses of BES (B-14 through B-18). The following major results emerged.

Under all of the conditions examined, the principal product of [^{14}C]DCM degradation was $^{14}CO_2$, which represented at least 61% of the ^{14}C recovered. $^{14}CH_4$ was formed in appreciable amounts (9 to 36%) only when methanogenesis was uninhibited. The higher the amount of HAc added with each dose of DCM, the higher the percentage of $^{14}CH_4$. When methanogenesis was partially inhibited with either high doses of DCM or low doses of BES, little or none of the methane that was produced was radiolabeled. As would be expected, no $^{14}CH_4$ was measured when methanogenesis was completely inhibited with BES.

When methanogenesis was partially inhibited, the most important product after $^{14}CO_2$ was [^{14}C]HAc (20 to 30%). Partial inhibition of methanogenesis in B-9 and B-10 (no BES added) was caused by high doses of DCM, just as it was in B-19, B-20, and B-22 (described above). Between days 140 and 150, when [^{14}C]DCM was added every other day, B-9 degraded 0.986 mmol of DCM and produced 0.140 mmol of CH_4, or 70% less than the ratio reported in Table 1. Over the same interval, B-10 degraded 1.114 mmol of DCM and produced 0.067 mmol of CH_4, amounting to an 87% inhibition of methanogenesis.

When methanogenesis was completely inhibited, the most important product after $^{14}CO_2$ was also [^{14}C]HAc (22 to 41%). Bottles B-14, B-15, and B-16 received low doses of DCM and a single addition of [^{14}C]DCM, while B-17 and

TABLE 2. Distribution of ^{14}C from degradation of [^{14}C]DCM

Inhibition of methanogenesis	HAc added (mg/bottle)	Bottle no.	% of total dpm recovered as:						
			DCM	CH_4	CO_2	Soluble NSR			Biomass
						HAc	MeOH[a]	Other	
None	10	B-1	0.85	32.5	64.1	0.14	0.11	0.31	2.02
		B-2	0.98	36.1	60.5	0.16	0.12	0.24	1.91
		Avg	0.92	34.3	62.3	0.15	0.12	0.28	1.97
None	1	B-3	0.00	26.7	69.1	0.40	1.04	0.44	2.31
		B-4	0.81	28.7	66.9	0.63	0.00	0.49	2.45
		Avg	0.41	27.7	68.0	0.52	0.52	0.47	2.38
None	0.1	B-5	2.85	24.4	67.7	0.69	1.07	0.60	2.65
		B-6	0.48	22.6	70.2	1.46	1.77	0.45	3.02
		Avg	1.67	23.5	69.0	1.08	1.42	0.53	2.84
None	0	B-7	2.97	18.2	73.6	0.74	0.00	0.63	3.83
		B-8	1.03	9.09	81.5	5.83	0.00	0.63	1.89
		Avg	2.00	13.7	77.6	3.29	0.00	0.63	2.86
Partial (high DCM)	0	B-9	0.62	1.07	68.7	27.8	0.17	1.54	0.13
		B-10	0.27	0.79	67.1	29.8	0.14	1.59	0.32
		Avg	0.45	0.93	67.9	28.8	0.16	1.57	0.23
Partial (BES)	0	B-11	0.54	0.076	73.4	19.6	4.78	0.27	1.30
		B-12	0.07	0.00	69.0	26.4	2.13	1.44	1.03
		B-13	0.59	0.029	72.1	23.2	2.41	1.28	0.40
		Avg	0.40	0.035	71.5	23.1	3.11	1.00	0.91
Complete (BES)	0	B-14	1.23	0.00	65.5	22.5	9.46	0.80	0.52
		B-15	0.20	0.00	70.8	22.3	3.57	1.58	1.55
		B-16	0.60	0.00	67.7	22.5	6.30	1.85	1.01
		B-17	0.052	0.015	54.0	40.7	3.17	2.04	0.00
		B-18	0.15	0.008	61.1	36.1	0.42	2.27	0.00
		Avg	0.45	0.005	63.8	28.8	4.58	1.71	0.62

[a] MeOH, methanol.

B-18 received higher doses of DCM and five additions of [^{14}C]DCM.

When BES was added to cultures, the next most important product after $^{14}CO_2$ and [^{14}C]HAc was $^{14}CH_3OH$. On average, the higher the amount of BES, the higher the amount of $^{14}CH_3OH$. The "other soluble substances" category—never of much consequence under any of the conditions examined—tended to be slightly higher when methanogenesis was partially or totally inhibited. A relatively small amount of the ^{14}C label (0.23 to 2.84%) was recovered as nonsoluble NSR, which was assumed to be biomass.

Schmidt degradation of the HAc produced in two bottles was used to determine the distribution of the label on the carbons. In B-11 ([^{14}C]DCM added when methanogenesis was partially inhibited with BES), HAc represented 19.6% of the total disintegrations per minute recovered; in B-14 ([^{14}C]DCM added when methanogenesis was completely inhibited with BES), HAc represented 22.5% of the total disintegrations per minute recovered. In both bottles, replicate analyses indicated that the label was almost entirely (>94%) on the methyl carbon of HAc.

Use of DCM as a growth substrate. Figure 6A presents results for bottles receiving DCM (B-9 and B-10) and the associated controls receiving no DCM. There was no statistically significant change in SOC levels in the controls over time; their overall average was 40.9 μmol of SOC per bottle. However, SOC in B-9 and B-10 rose definitively above that in the control bottles beginning on day 49, just as cumulative

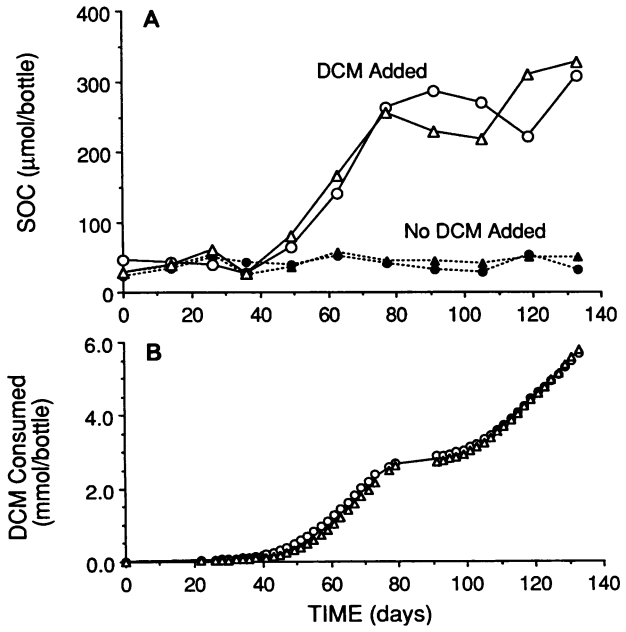

FIG. 6. Growth experiment results. DCM was added to bottles B-9 (○) and B-10 (△); no DCM was added to control bottles BC-9 (●) and BC-10 (▲).

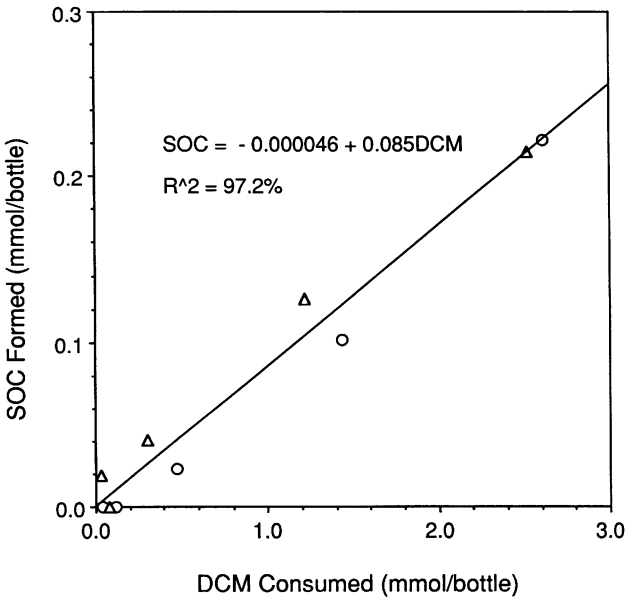

FIG. 7. Correlation between SOC formed and DCM consumed in bottles B-9 (○) and B-10 (△) for days 26 to 77 of the growth experiment. SOC formation was calculated as the measured SOC minus the overall average SOC in the controls.

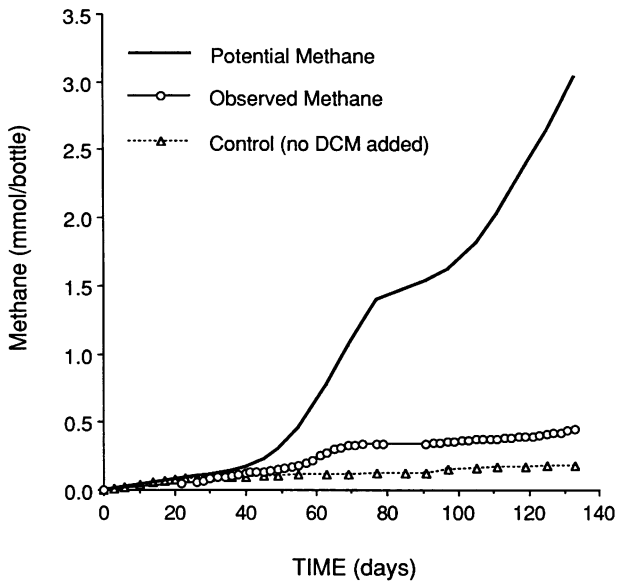

FIG. 8. Average CH_4 output during the growth experiment. DCM was added to bottles B-9 and B-10 (○); no DCM was added to control bottles BC-9 and BC-10 (△). Maximum potential methane output in B-9 and B-10 = [0.5 × (DCM consumed) + CH_4 from controls].

DCM consumption began to rise significantly (Fig. 6B). The accumulation of SOC and increase in cumulative DCM degradation continued through day 77, at which point the rates of DCM degradation and SOC formation slowed markedly. The likely reason was a significant drop in pH, presumably as a consequence of the HCl produced from DCM biotransformation; prior to day 77, B-9 and B-10 were dechlorinating more than 200 μmol of DCM per bottle every other day. On day 91, the pHs were 5.10 in B-9 and 5.44 in B-10 versus 7.37 and 7.56 in the controls. $NaHCO_3$ was added to B-9 and B-10 in order to raise the pH to 7.0 to 7.7 and they, as well as the controls, were reinoculated with 5 ml of a culture actively degrading DCM. This restoration effort was successful. On day 91, the rate of DCM degradation was about 35 μmol per bottle every other day; this was gradually built up to more than 160 μmol by day 111. Starting on day 105, appropriate amounts of 8 M NaOH were added along with DCM in order to maintain the pH in B-9 and B-10 at between 6.5 and 7.0.

SOC formation did not respond immediately to the restoration of rapid DCM degradation. Between days 77 and 119, the SOC in B-9 actually decreased slightly and then rose well above the day 77 level on day 133. In B-10, a similar decrease occurred through day 105, but consecutive increases in SOC over the day 77 level were observed on days 119 and 133 (Fig. 6A).

Before the decrease in pH, SOC formation and DCM degradation in B-9 and B-10 were highly correlated, with the slope of the best-fit line indicating 0.085 g of biomass carbon formed per g of DCM carbon consumed (Fig. 7). This translates to an observed yield of 0.060 g of biomass (dry weight organic matter) formed per g of oxygen demand consumed, assuming a typical composition for bacterial-cell organic matter of $C_5H_7O_2N$ (18) and 32 g of oxygen demand per mol of DCM.

Figure 8 shows that the observed methane output from B-9 and B-10 was well below the amount that could have been produced, assuming 0.5 mol of methane per mol of DCM degraded (plus CH_4 from the controls). By day 133, methane output in B-9 and B-10 was only 16.8 and 12.6%, respectively, of the potential methane output. This partial inhibition of methanogenesis was a consequence of the high levels of DCM added during the growth experiment.

The fraction of observed growth attributable to methanogens and nonmethanogens was estimated on the basis of the amount of methane produced, since methanogens presumably cannot grow without producing methane (19). By day 77, net methane formation in B-9 was 237 μmol, which is equivalent to 15.2 mg of oxygen demand used for energy (1 mmol of CH_4 = 64 mg of oxygen demand), or 1.90 meq (8 mg of oxygen demand = 1 meq [23]). For CO_2-reducing methanogens, the ratio of electron equivalents used for energy to electron equivalents of cells formed is approximately 12.5 (the ratio is even higher for aceticlastic methanogens) (23). Therefore, 0.152 meq of biomass was formed as a consequence of methanogenesis (1.90 divided by 12.5); this amounts to 0.859 mg of biomass, assuming that $C_5H_7O_2N$ describes the composition of bacterial cell organic matter (1 eq = 0.05 $C_5H_7O_2N$ = 5.65 g of biomass [18, 23]). By day 77, net biomass formation in B-9 was 222 μmol of SOC, or 5.02 mg of biomass. Thus, in B-9, only 17.1% (0.859 divided by 5.02) of the net biomass formed can be credited to methanogens. The same calculations applied to B-10 indicate that only 15.1% of the net biomass formed can be credited to methanogens, and the balance is credited to nonmethanogens.

DISCUSSION

The experimentally determined ratio of methane formed to DCM consumed (Table 1) was very close to the ratio expected (0.5 mol of CH_4/mol of DCM) on the basis of stoichiometric conservation of electrons:

$$CH_2Cl_2 + 2H_2O \rightarrow CO_2 + 2Cl^- + 6H^+ + 4e^-$$
$$\tfrac{1}{2}CO_2 + 4H^+ + 4e^- \rightarrow \tfrac{1}{2}CH_4 + H_2O$$
$$CH_2Cl_2 + H_2O \rightarrow \tfrac{1}{2}CH_4 + \tfrac{1}{2}CO_2 + 2HCl$$

This relationship was followed only when the DCM dose was low enough (≤12 μmol per bottle) to avoid inhibition of methanogenesis. A ratio of slightly less than 0.5 is consistent with the finding that DCM serves as a growth substrate.

The accumulated evidence suggests that nonmethanogens were responsible for DCM degradation. First, cultures continued to degrade DCM after methanogenesis was completely inhibited by BES. Second, vancomycin, an antibiotic that prevents cell wall synthesis in eubacteria by interfering with the formation of the UDP-MurNAc-pentapeptide precursors of murein (3), essentially stopped DCM degradation. Third, when DCM was provided to cultures as the sole organic compound, the majority of the growth observed was attributable to nonmethanogens. And fourth, the formation of HAc (a major product of DCM degradation) from C_1 compounds is characteristic of acetogenic bacteria (21). Although Westermann et al. (36) recently demonstrated that methanogens are also capable of synthesizing acetate from C_1 substrates (H_2-CO_2 and methanol), the other evidence tends to rule out the possible role of methanogens in DCM degradation.

A proposed model for DCM degradation under methanogenic conditions is presented in Fig. 9. The principal mode of DCM degradation under all conditions examined was oxidation to CO_2; the organisms mediating this reaction have therefore been termed DCM oxidizers. The other major mode of DCM transformation was fermentation to acetic acid, with the methyl carbon coming directly from DCM and the carboxyl carbon coming from unlabeled CO_2, which was available in the large pool of carbonates present in the basal medium. DCM degradation is thus a disproportionation: a portion of the DCM is oxidized, making reducing equivalents available for reduction of an equal amount of DCM.

Methanogens consumed the products of DCM degradation. CO_2-reducing methanogens used some of the electrons made available from DCM oxidation to form methane. At least a portion of those electrons were available in the form of H_2 (Fig. 5). Recent evidence indicates that the majority of electron transfers in methanogenic systems occur via formate rather than H_2 (4, 33), but this possibility was not explored with the DCM-degrading cultures. Methane formed by CO_2-reducers was unlabeled because the $^{14}CO_2$ formed from oxidation of $^{14}CH_2Cl_2$ was essentially diluted out by the large unlabeled carbonate pool in the basal medium. Aceticlastic methanogens produced methane from the acetic acid formed by fermentation of DCM. This route was the probable source of $^{14}CH_4$; methane from aceticlastic methanogens was derived from the methyl carbon of HAc, which was shown to be the carbon carrying the ^{14}C label from DCM.

Figure 9 also shows where inhibitors exerted their effects. DCM degradation was stopped by vancomycin. We did not test the specificity of vancomycin on the DCM-degrading enrichment cultures. However, Murray and Zinder (24) reported that 100 mg of vancomycin per liter (the dose used in this study) inhibits eubacteria without effecting archaebacteria, specifically methane production or growth of *Methanosarcina barkeri* 227. Our data indicate qualitatively that vancomycin did not totally stop methanogenesis. As shown in Fig. 4, methane production continued after the addition of vancomycin. A possible source of the methane formed was previously accumulated acetate.

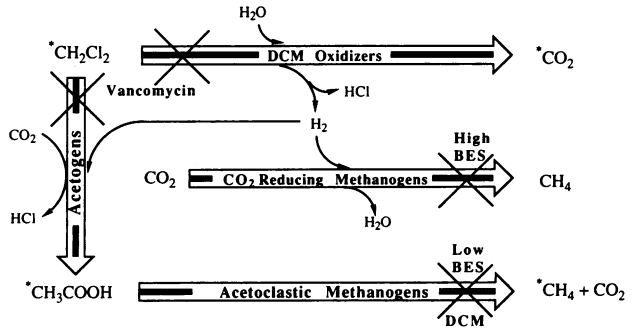

FIG. 9. Proposed model for DCM degradation under methanogenic conditions, including points at which inhibitors (high-dose DCM, low-dose BES, and high-dose BES) exert their effects.

Inhibition of methanogenesis is also indicated in Fig. 9. With low doses of BES (0.5 to 1.5 mM) and high doses of DCM (about 2 mM), methane production was significantly reduced (i.e., partially inhibited) but not stopped (Fig. 3). However, of the methane that was formed, none was labeled (Table 2). This result is consistent with the hypothesis that $^{14}CH_4$ came from $^{14}CH_3COOH$ and not $^{14}CO_2$, since it appears that the aceticlastic methanogens were inhibited to a much greater extent than the CO_2-reducing methanogens by low levels of BES and high doses of DCM. Less-than-stoichiometric formation of methane and accumulation of acetate (but not H_2) were observed in all cultures receiving repeated high doses of DCM, such as those used in the growth experiments (9). Zinder et al. (38) also reported that a low dose of BES (1.0 mM) inhibited aceticlastic methanogenesis whereas a much higher dose (50 mM) was required to completely inhibit CO_2 reduction; the same level was used in this study to completely inhibit methanogenesis.

The recently elucidated acetyl coenzyme A pathway (21) makes it possible to speculate on how DCM might enter the metabolic pathway of acetogenic bacteria. Since DCM is incorporated into the methyl carbon of acetate, DCM probably enters at the level of one of the tetrahydrofolates, such as hydroxymethyltetrahydrofolate. Transformation of DCM to hydroxymethyltetrahydrofolate may occur by a mechanism similar to that described for *Hyphomicrobium* sp. strain DM2 (5), i.e., dechlorination to a bound chloromethyl compound (mediated by a dehalogenase) followed by nonenzymatic hydrolytic formation of a bound hydroxymethyl compound. The reducing power needed ($4e^-$) to complete formation of acetate from hydroxymethyltetrahydrofolate would come from oxidation of another mole of DCM to CO_2. An analogous situation has been described for synthesis of acetate from methanol, the methyl group of which is incorporated directly into the methyl group of acetate. Methanol appears to enter the acetyl coenzyme A pathway at the level of the protein-bound Co-methylcorrinoid (21).

It is less clear how DCM oxidation might occur, be it in the same or a different organism. If an acetogen does carry out oxidation, the pathway may be similar to that for methanol. Some of the methanol consumed by acetogens must be oxidized to CO_2 to provide the reducing equivalents needed for formation of the methyl carbon of acetate (21); the same holds for DCM.

As shown in Table 3, the thermodynamics of DCM degradation are very favorable, in part because of the substantial amount of free energy associated with removal of Cl^-. Half reactions were calculated from ΔG°_f data (all values

TABLE 3. Thermodynamic considerations related to DCM degradation

Reaction	$\Delta G^{\circ a}$	ΔG^{b}
Half-reactions		
1. $\frac{1}{4}CH_2Cl_2 + \frac{1}{2}H_2O = \frac{1}{4}CO_2 + \frac{1}{2}Cl^- + \frac{3}{2}H^+ + e^-$	−6.908	
2. $\frac{1}{2}H_2 = H^+ + e^-$	0.0	
3. $\frac{1}{8}CH_3COO^- + \frac{3}{8}H_2O = \frac{1}{8}CO_2 + \frac{1}{8}HCO_3^- + H^+ + e^-$	+2.981	
4. $\frac{1}{6}CH_3OH + \frac{1}{6}H_2O = \frac{1}{6}CO_2 + H^+ + e^-$	+0.725	
Reactions		
1. $\frac{1}{4}*CH_2Cl_2 + \frac{1}{2}H_2O = \frac{1}{4}*CO_2 + \frac{1}{2}H_2 + \frac{1}{2}H^+ + \frac{1}{2}Cl^-$	−6.91	−14.6
2. $\frac{1}{8}*CH_2Cl_2 + \frac{1}{4}H_2 + \frac{1}{8}HCO_3^- = \frac{1}{8}*CH_3COO^- + \frac{1}{8}H_2O + \frac{1}{4}H^+ + \frac{1}{4}Cl^-$	−6.44	−7.84
3. $\frac{1}{4}*CH_2Cl_2 + \frac{1}{8}H_2O + \frac{1}{8}HCO_3^- = \frac{1}{8}*CH_3COO^- + \frac{1}{8}*CO_2 + \frac{1}{2}H^+ + \frac{1}{2}Cl^-$	−9.89	−15.2
4. $\frac{1}{6}*CH_2Cl_2 + \frac{1}{6}H_2O + \frac{1}{6}H_2 = \frac{1}{6}*CH_3OH + \frac{1}{3}H^+ + \frac{1}{3}Cl^-$	−5.33	−8.67
5. $\frac{1}{6}CO_2 + \frac{1}{2}H_2 = \frac{1}{6}CH_3OH + \frac{1}{6}H_2O$	−0.725	+1.10
6. $\frac{1}{8}CH_3COO^- + \frac{1}{4}H_2O = \frac{1}{8}CH_3OH + \frac{1}{8}HCO_3^- + \frac{1}{8}H_2$	+2.44	+1.34
7. $\frac{1}{8}CO_2 + \frac{1}{8}HCO_3^- + \frac{1}{2}H_2 = \frac{1}{8}CH_3COO^- + \frac{3}{8}H_2O$	−2.98	−0.519

[a] 25°C, 1 atm, with all species in their standard states at unit activity.

[b] $\Delta G = \Delta G^{\circ} + RT \ln \left[\frac{(C)^c(D)^d}{(A)^a(B)^b}\right]$ for the reaction aA + bB → cC + dD; $R = 1.987 \times 10^{-3}$ kcal/mol · K (1 cal = 4.184 J); $T = 298$ K.

except those for DCM are from Stumm and Morgan [31]; values for DCM_g from Reid et al. [26] were converted to values for DCM_{aq} by using a Henry's constant of 2.135 liter · atm/mol at 25°C [14]). ΔG values were calculated from ΔG°, assuming the following typical conditions: $CO_2 = 0.3$ atm, $H_2 = 10^{-4}$ atm, $Cl^- = 37$ mM, $H^+ = 10^{-7}$ M, $HCO_3^- = 59.5$ mM, $CH_3COO^- = 0.5$ mM, $CH_3OH = 31.3$ μM, and DCM = 2.0 mM. The oxidation of DCM to CO_2 plus H_2 (reaction 1) remains favorable even if a hydrogen partial pressure of 1 atm is assumed.

One of the products observed from [^{14}C]DCM degradation but not included in Fig. 9 is $^{14}CH_3OH$, which tended to accumulate significantly (i.e., >1% of total disintegrations per minute) only when BES was used to inhibit methanogenesis (Table 2). The source of the methanol formed is not known. Some of the possibilities, shown in Table 3, include direct production from DCM (reaction 4), synthesis from H_2-CO_2 (reaction 5), and synthesis from acetate (reaction 6). Under the typical conditions experienced by the enrichment cultures, the only thermodynamically favorable possibility is methanol formation from DCM (reaction 4). Since BES promoted $^{14}CH_3OH$ formation, it appears that BES had some effect on the DCM-degrading organism. The rate of DCM degradation in enrichment cultures exposed to 50 to 55.5 mM BES was approximately one-half that of cultures without BES. No reports regarding the effect of BES on acetogens were found in the literature. However, formation of methanol by an acetogen from a single carbon substrate has been demonstrated previously with *Clostridium thermoaceticum* in the presence of carbon monoxide and methylviologen (37).

Biotransformation under methanogenic conditions of several other chlorinated aliphatics in widespread use (e.g., tetrachloroethylene and trichloroethylene) occurs primarily by reductive dechlorination. This process requires the supply of an external electron donor (8, 10). With DCM, the principal transformation pathways were oxidation to CO_2 and fermentation to acetate rather than reductive dechlorination; chloromethane never accumulated beyond trace levels, even in first-generation cultures previously unexposed to DCM (Fig. 1). Furthermore, DCM degradation was sustained for extended periods without the need for an external electron donor.

The results of this research appear to be the first demonstration of growth under methanogenic conditions using a chlorinated aliphatic compound as the sole organic-carbon and energy source. Other studies have shown that growth on a chlorinated organic compound supplied as the sole carbon and energy source is possible under aerobic conditions. For example, use of DCM as a growth substrate has been demonstrated in enrichment cultures (27) and by pure cultures of *Pseudomonas* and *Hyphomicrobium* species (5, 12, 20, 29). Other chlorinated alkanes shown to be capable of supporting growth of aerobic organisms include methyl chloride (17), vinyl chloride (16), and 1,2-dichloroethane (30).

For some very good reasons, most studies examining dehalogenation under anaerobic conditions have not addressed the question of whether the halogenated compound can serve as a growth substrate. Low concentrations (e.g., in the microgram-per-liter range) have usually been employed, often to avoid toxicity. However, very low concentrations are unlikely to support growth (and furthermore, present difficulty in detecting growth, should it occur). An additional problem is presented in those instances when reductive dehalogenation is the predominant halogen removal mechanism, because an auxiliary electron donor is required to sustain the transformation. If the auxiliary electron donor supports growth apart from reductive dechlorination reactions (e.g., via methanogenesis, sulfate reduction, or fermentation), it becomes very difficult to ascertain whether energy is derived from dechlorination of the halogenated compound. With DCM, the lack of a requirement for an auxiliary electron donor allowed relatively easy determination that this compound can serve as a growth substrate.

The principal products of DCM biotransformation under methanogenic conditions, i.e., CO_2 and HAc, are environmentally acceptable. Since the fate of other chlorinated solvents (including tetrachloroethylene, trichloroethylene, carbon tetrachloride, and chloroform) under methanogenic conditions often results in some residual hazardous transformation product(s), DCM may be viewed from an environ-

mental standpoint as a lesser evil. To the extent that chlorinated solvents remain in widespread use, the specific ones employed should be restricted to those that have the least hazardous environmental fate.

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