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Toxicity of Chloroform Biotransformation to Methanogenic Bacteria

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Batch serum bottle assays were conducted to determine the effect of exposure to and biotransformation of chloroform (CF) on unacclimated, aceticlastic, methanogenic bacteria. Methanogenesis was negatively impacted both during and following exposure of methanogens to CF: methanogenesis was strongly inhibited in the presence of detectable levels of chloroform and maximum methane production (MMP) rates decreased following CF exposure. Using a general nonlinear model it was found that the loss of methanogenic activity was most strongly correlated with the mass of chloroform transformed (nmol CF; $r^2 = 0.84$), rather than with the time-integrated CF exposure which methanogens received (μM CF-h; $r^2 = 0.60$). Comparison of individual data sets supported this finding. Control studies showed that desorption of CF and exposure of methanogens to DCM or possible nonvolatile CF metabolites did not cause decreased MMP rates. Based on these results, it is concluded that CF biotransformation is toxic to unacclimated, aceticlastic methanogenic bacteria and nonlinear with respect to the mass of CF transformed.

Introduction

The mineralization of organic matter by anaerobic microorganisms is a key process in the carbon cycle, and no compound plays a more critical role than acetate. Acetate is produced under anaerobic conditions by the conversion of fatty acids such as propionate and butyrate by acetogenic bacteria and by the conversion of sugars, amino acids, and fatty acids by fermentative acidogenic bacteria. Aceticlastic methanogens then mineralize acetate, with the major products being methane and carbon dioxide. The mineralization of acetate by aceticlastic methanogens is important because of the following: (1) acetate accounts for two-thirds to three-fourths of the methane produced in man-made anaerobic digesters or natural systems such as river or pond sediments (*1*); (2) the conversion of acetate to methane is the rate-limiting step in the anaerobic degradation of organic matter (*2, 3*); and (3) the removal of acetate from anaerobic ecosystems prevents their acidification and eventual failure.

While methanogenesis in a healthy anaerobic environment occurs at a sufficient rate, the introduction of anthropogenic toxicants into a waste stream can upset this process, both while the toxicant is present and following its removal. Disruption of methanogenesis by chlorinated aliphatic compounds (CACs) has been reported for chloroform (*4*−*7*), 1,1,1-trichloroethane (TCA) (*4*), and many others. Yang and Speece (*5*) studied the effect of chloroform on mixed, methanogenic cultures enriched on acetate. They concluded that the time to recover from exposure to CF increased with the duration of chloroform exposure. Gupta et al. (*7*) reported that aceticlastic methanogenesis did not occur in an acetate enrichment culture after methanogenic cells were exposed to 2.7 μM CF, even following complete CF transformation. Whether this was due to simple exposure to CF or some other cause was not determined. Aside from hindering the normal physiological processes of anaerobic bacteria, CACs may also undergo biochemical transformation by anaerobes. Pure or mixed methanogenic cultures have been reported to transform many CACs including chloroform (*8*−*12*).

While the above-mentioned phenomenon are well-known, there is no documented connection between the transformation of CF by aceticlastic methanogens and toxicity. CF biotransformation is toxic to mammalian cells under anoxic conditions (*13*−*15*), however, and it has been proposed that methanogenic bacteria transform CF by a similar mechanism (*16, 17*). Thus, the objective of our study was to determine if the reported toxicity of CF to methanogens following CF exposure was due merely to exposure to CF, as proposed by Yang and Speece (*5*); due to CF biotransformation; or due to some other factor.

Materials and Methods

Experimental Design. All experiments, unless noted otherwise, were conducted in quadruplicate 38-mL serum bottles using 25 mL of cell suspension. Standard anaerobic techniques were used throughout. Preliminary batch experiments were conducted to examine the role of methanogens in acetate consumption and methane production. Bottles were purged with oxygen-free N_2/CO_2 gas (80%/20%; v/v), sealed with Teflon-coated rubber septa, and capped with aluminum crimp caps. Cell suspension was injected through the septum using a 50-mL glass syringe. One set of bottles received 50 mM bromoethanesulfonate (BES), a specific methanogenic inhibitor (*18*). Bottles were then injected with 10 μL of acetic acid (HAc), producing an acetate concentration of about 7.0 mM (~420 mg L^{-1}), much greater than the saturation coefficient of about 10 mg L^{-1} (*11*). Duplicate bottles were periodically sampled for methane or acetate until acetate was nondetect in the BES-free controls.

A study was conducted to examine the significance of the hydrogenolysis transformation pathway for CF of this culture. Two sets of duplicate bottles were injected with 1 μL of the CF stock and 0 or 10 μL of acetic acid. The average initial aqueous CF concentrations were 1.74 μM CF for both HAc-fed incubations and 1.44 and 1.72 μM CF for the resting cell incubations. Autoclaved controls were used to assess abiotic CF transformation. CF and DCM was monitored until CF was nondetect.

A two-phase experimental protocol using 15 bottle sets was used to examine CF biotransformation toxicity: incubations transformed some amount of CF in phase 1, and maximum methane production (MMP) rates were measured in phase 2. To begin phase 1, incubations received from 0 to 6 μL of CF-saturated stock solution and 0 or 10 μL of acetic acid (Table 1). Bottles were inverted on a shaker table (200 rpm) in the dark and periodically sampled for CF and CH_4. After the transformation of all or some fraction of CF, the liquid within a bottle was sparged for 2 min with oxygen-free N_2/CO_2 gas (80%/20%; v/v) flowing through a needle inserted through the septum. CF-free controls were treated identically. Bottles were then hand-shaken, and headspace analysis was

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TABLE 1. Experimental Results from Two-Phase CF Biotransformation Toxicity Assay[a]

	phase 1					phase 2
set	$[CF]_{aq}$ initial (μM)	$[CF]_{aq}$ final (μM)	HAc[b]	CF transformed (nmol)	CF exposure (μM CF-h)	MMP rate[c] (μmol CH_4 $bottle^{-1}$ h^{-1})
1	0.00	0.00		0	0.0	6.47 ± 0.45
2	0.00	0.00	*	0	0.0	5.86 ± 0.98
3	1.70	0.00	*	45	1.7	3.75 ± 0.06
4	1.70	0.10		43	58.0	4.00 ± 0.13
5	1.79	1.32		12	13.0	5.33 ± 0.43
6	1.79	0.54		33	36.7	4.82 ± 0.49
7	2.87	0.00	*	76	9.2	3.75 ± 0.33
8	4.49	0.20	*	113	40.7	3.00 ± 0.29
9	5.61	5.26		9	1.9	6.46 ± 0.24
10	5.66	3.76		50	177.5	3.85 ± 0.02
11	6.02	0.38	*	149	81.5	2.52 ± 0.24
12	6.02	2.86	*	84	37.6	3.49 ± 0.32
13	7.00	0.19	*	180	114.4	2.77 ± 0.26
14	8.49	3.32	*	137	105.2	2.37 ± 0.29
15	8.49	0.39	*	215	245.7	1.74 ± 0.12

[a] Mean values for each four bottle incubation set, unless otherwise indicated. [b] Incubation sets receiving 10 μL of glacial acetic acid during incubation with CF in the first phase of the assay. [c] Maximum methane production rate, mean ± 1 SD for each four bottle incubation set.

conducted to confirm that CF and DCM were nondetectable. This marked the end of phase 1. Incubations were then amended with 10 μL of acetic acid to start phase 2, with the exception of incubations that received acetic acid and CF in phase 1 because methanogenesis was totally inhibited in the presence of CF (see Results and Discussion). Only one incubation set, IS2 (a CF-free control), received 10 μL of HAc in both phases 1 and 2. This set was used to monitor inhibitory effects due to possible pH changes. Methane was then measured in all bottles until its production ceased. The data from these experiments were analyzed, and it appeared that the phase 2 MMP rates decreased with either CF transformation or CF exposure in phase 1 (see Results and Discussion).

Three control experiments were conducted to examine if some factor other than CF transformation or CF exposure was responsible for depressed phase 2 MMP rates. The first control experiment was examined if phase 2 methane production was inhibited by CF, possibly desorbing from the Teflon-lined rubber stoppers. Duplicate bottles containing 25 mL of cell suspension were injected with 10 μL of acetic acid and given about 5.6 μM CF. Bottles were sampled periodically until CF was nondetect. Cell suspension was then withdrawn from the bottles and replaced with fresh cell suspension. Bottles were amended with 10 μL of acetic acid and periodically monitored for methane.

The second control experiment was examined if decreased phase 2 MMP rates might have resulted from exposure to DCM in phase 1. Duplicate incubations were prepared with an initial DCM concentration of about 10 μM and injected with 10 μL of acetic acid. DCM was measured periodically for 24 h, at which time the bottles were sparged and respiked with 10 μL of acetic acid. Methane was measured periodically until methane production ceased.

The third control experiment was examined if phase 2 methane production might be inhibited by nonvolatile CF metabolites. We inferred the possibility of nonvolatile metabolites from a study which examined the transformation of ^{14}C-labeled CF by a similar mixed, methanogenic, acetate-enrichment culture (*19*). In these experiments, 12.1% of the label was nonstrippable under acetate-starved conditions and 10.1% under acetate-fed conditions. This hypothesis was tested in a "media-swapping" study. One set of eight replicates received 10 μL of acetic acid and an initial CF concentration of about 9.8 μM, while another set of eight replicates received 10 μL of acetic acid only. CF was measured until it was nearly nondetectable. Incubations were then sparged until headspace analysis confirmed all CF and DCM was stripped. Bottles were centrifuged (International Equipment Company, model HN-SII) at 4000 rpm for 10 min to separate cells from supernatant. The supernatant from four of the CF-incubations was then swapped with the supernatant from four of the CF-free incubations. Swapping the supernatants resulted in these final four treatments: (1) CF-exposed cells/nonvolatile metabolite (NVM) medium, (2) CF-exposed cells/uncontaminated medium, (3) CF-unexposed cells/NVM medium, and (4) CF-unexposed cells/uncontaminated medium. Acetic acid (10 μL) was added to each bottle, and methane was measured periodically.

Chemicals and Reagents. CF (high performance liquid chromatography (HPLC) grade) and DCM (certified American Chemical Society (ACS) grade) were purchased from Fisher Scientific (Pittsburgh, PA). Stock aqueous solutions of CF and DCM were prepared by adding about 5 mL of each chemical to 25 mL of autoclaved, distilled deionized, anoxic water in a 43-mL glass serum bottles sealed with Teflon-lined rubber septa and aluminum crimp caps. Other chemicals used included acetic acid (glacial, Mallinckrodt) and methane gas (100%, Scott Specialty Gases).

Stock Culture Reactor. The source of organisms was a magnetically stirred, 9.5-L glass reactor containing a cell suspension volume of 8 L. The reactor was buffered with $NaHCO_3$ at a concentration of 2500 mg L^{-1} as $CaCO_3$ to maintain a pH of about 6.9. Operational details for the reactor have been published previously (*20*). Briefly, for a period of over 3 years, the culture was fed virtually daily a mineral salts medium containing acetate as the sole carbon and energy source. It should be noted that, on the day prior to launching an experiment, the reactor was not fed in order to ensure that the background acetate concentration the following day would be negligible. The volatile suspended solids (VSS) concentration of the reactor averaged 245 ± 20 mg/L (n = 5) at the time the experiments were conducted.

Analytical Methods. CF, DCM, and CH_4 were determined by gas chromatography (GC) using headspace analysis, as described previously (*20*). Detection limits for CF, DCM, and CH_4 were 1.7, 15.3, and 9.3 nmol per bottle, respectively. Acetate concentrations were determined by HPLC, as described previously (*20*). The detection limit for acetate was approximately 0.08 mM (~5 mg L^{-1}) or 2000 nmol per bottle. Biomass was measured as VSS using Method 2540 E in **Standard Methods** *(21)*.

Results and Discussion

Acetate consumption and methane production were severely inhibited by BES, thus establishing the role of aceticlastic methanogens in both processes (Figure 1). Similar results were found with this culture when H_2 gas was used as electron donor (*20*). In addition, the results from the BES-free bottles showed a near-unimolar stoichiometric relationship between acetate consumption and methane production, as expected in a culture dominated by aceticlastic methanogens. In subsequent experiments, methane production was used as a measure of microbial activity rather than acetate consumption because of the greater than 200× higher sensitivity for detection of methane compared to acetate.

Hydrogenolysis was the major CF transformation pathway for the culture; the recovery of DCM from CF varied from 55% to 60% under acetate-starved conditions and from 48% to 58% under acetate-fed conditions (data not shown). CF transformation was negligible in killed cell incubations.

In the two-phase biotransformation toxicity assay, methanogenesis was strongly inhibited until CF reached low levels

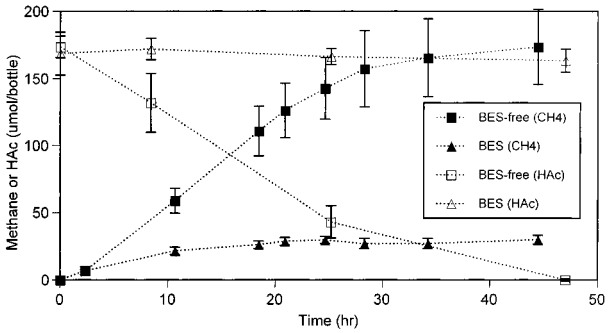

FIGURE 1. Inhibition of aceticlastic methanogenesis by 50 mM BES, a specific methanogenic inhibitor. Thirty-eight-milliliter serum bottles containing 25 mL of cell suspension were amended with 10 μL of acetic acid. Mean values of duplicate bottles are shown. Error bars show $\pm$ 1 SD.

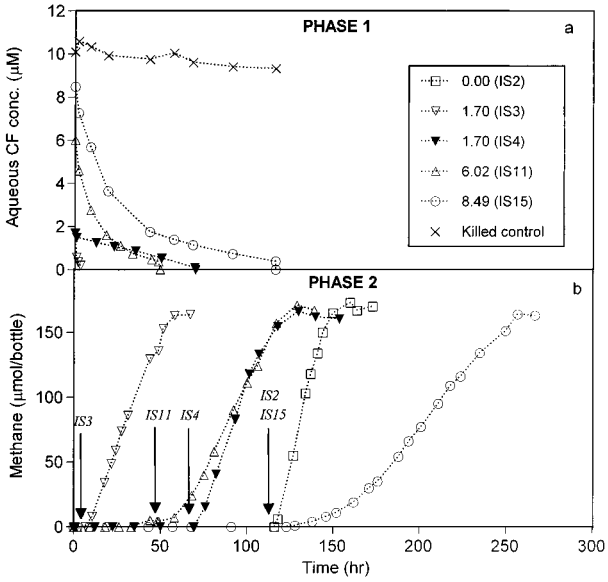

FIGURE 2. CF transformation (a) and methane production (b) in selected incubation sets (IS) from the two-phase, CF biotransformation toxicity experiment. All incubations except those indicated by filled symbols (IS4) received acetic acid in phase 1. Mean values of quadruplicate bottles are shown. Arrows indicate when incubations were sparged with N_2/CO_2 gas. Legend gives initial CF concentration (μm CF) and incubation set number. See Table 1 for the results of all incubation sets.

in the incubations (Figure 2), similar to previous results when H_2 gas was used as electron donor (*20*). Despite this, CF degradation was enhanced by acetate, similar to results when incubations were supplied with H_2 gas (*20*). For example, incubation sets IS3 and IS4 each had initial CF concentrations of 1.70 μM, but IS3 was amended with acetic acid in phase 1 while IS4 was not. Complete CF transformation in IS3 occurred within 7 h, while about 70 h were required for IS4 (Figure 2). These two incubation sets thus had equal initial CF concentrations and transformed the same mass of CF but were subjected to different time-integrated CF exposure histories. The significance of this point is discussed below.

In phase 2, methane production rates gradually increased in all CF-exposed bottles before MMP rates were attained. Furthermore, MMP rates of CF-exposed treatments were depressed compared to MMP rates of CF-free controls (Figure 2; Table 1). A statistical comparison of MMP rates for the two CF-free control sets, IS1 and IS2, showed that there was no signficant difference at the 95% confidence level between incubations receiving one or two 10 μL injections of acetic

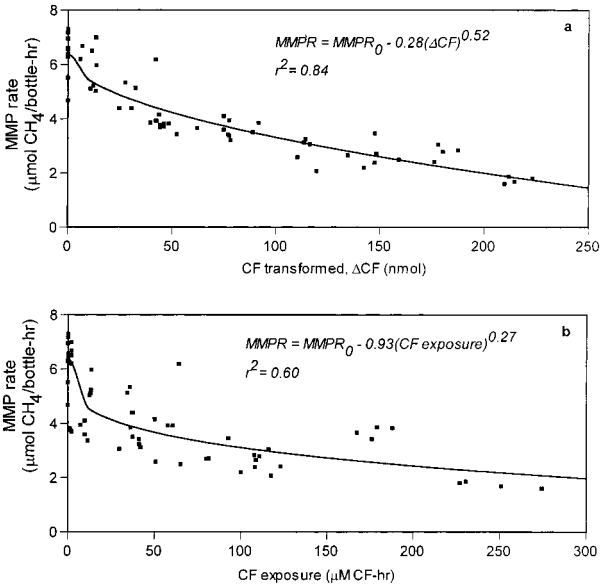

FIGURE 3. MMP rates vs (a) mass of CF transformed and (b) CF exposure from the two-phase, CF biotransformation toxicity experiment. The equation of the best-fit nonlinear equation is shown.

acid. Bicarbonate buffering in the incubations was therefore sufficient.

Two possible causes were proposed to account for depressed phase 2 MMP rates: (1) the biotransformation of CF was toxic and dependent on the mass of CF transformed or (2) exposure to CF was toxic, as proposed by Yang and Speece (*5*), and a function of the time-integrated exposure of CF that methanogens received. CF exposure was calculated as

$$\text{exposure} = \sum_{i=1}^{n} (t_{i+1} - t_i)\left(\frac{C_{i+1} + C_i}{2}\right) \qquad (1)$$

where C_i and C_{i+1} are the aqueous concentrations of CF at sampling times t_i and t_{i+1}, respectively, and n is the penultimate sample point. These hypotheses were evaluated using the data from the two-phase toxicity study by constructing graphs of the MMP rates versus the mass of CF transformed and the time-integrated CF exposure. Visual inspection of these graphs clearly revealed a nonlinear trend in each data set (Figure 3). Consequently, data sets were fit to a generalized, nonlinear equation of the form

$$\text{MMPR} = \text{MMPR}_0 - k f^n \qquad (2)$$

where MMPR is the maximum methane production rate of each treatment, $MMPR_0$ is the average of the MMP rates of the two sets of CF-free controls, 6.17 μmol CH_4 (bottle-h)$^{-1}$; f is the mass of CF transformed (nmol CF) or time-integrated CF exposure (μM CF-h); and k and n are fitting parameters. The Solver routine in Excel was employed to determine the best fit of the data to eq 2 by minimizing the sum of the squared residuals between the model fits and actual data. Values of k and n were set to 0.01 initially. The model fit for the CF exposure data set was weakly correlated, producing a coefficient of determination (r^2) value of 0.60, while the model fit for the mass of CF transformed data set produced a superior r^2 value of 0.84 (Figure 3). In addition, a comparative analysis of the data presented in Table 1 also supports the hypothesis that, among these two items, the mass of CF transformed is the toxic factor. For example, the average MMP rates for incubation set IS3 and IS4 are within 7% (3.75 vs 4.00 μmol CH_4 bottle^{-1} h^{-1}, respectively). Also,

the average mass of CF transformed in IS3 is within 5% of that for IS4 (45 vs 43 nmol CF, respectively). In contrast, the average CF exposure for IS4 was about 34× greater than that for IS3 (58.0 vs 1.7 μM CF-h, respectively). Altogether, the data clearly show that mere exposure to CF was not responsible for decreased MMP rates in phase 2.

The control experiment that examined if phase 2 methane production was inhibited by CF, possibly desorbing from the Teflon-lined rubber stoppers, showed this not to be the case. The MMP rate of the fresh cells in bottles with CF-exposed septa was 7.11 ± 0.87 μmol CH_4 bottle^{-1} h^{-1}. Based on *t*-tests at the 95% confidence level, this value was statistically equal to the average MMP values of the CF-free controls.

The control experiment examining the effect of DCM showed that exposure at a concentration of about 10 μM DCM for 24 h did not have a negative impact on methanogenesis in phase 2. DCM was not transformed during phase 1 of the experiment. Following the removal of DCM by stripping, the average MMP rate was 6.17 μmol CH_4 bottle^{-1} h^{-1}. This value falls within the range of average MMP rates for two CF-free controls, IS1 and IS2. Although DCM transformation has been reported under anaerobic conditions, these cultures have generally been enriched on DCM as a primary substrate (*22*−*24*).

The media-swapping control study showed that potential CF-nonvolatile metabolites did not inhibit methanogenesis in phase 2. In the first phase of this study, the CF incubations transformed 260 nmol CF. Statistical significance was determined using *t*-tests at the 95% confidence level. The MMP rates for incubations containing cells that were not exposed to CF were not statistically different from each other: 5.81 ± 0.31 μmol CH_4 bottle^{-1} h^{-1} ($n = 4$) for the controls (i.e., CF-unexposed cells/uncontaminated medium treatments) vs 5.66 ± 0.26 μmol CH_4 bottle^{-1} h^{-1} ($n = 3$) for CF-unexposed cells/NVM medium treatments. Also, the MMP rates for the incubations containing cells that were exposed to CF were not statistically different from each other: 1.18 ± 0.20 μmol CH_4 bottle^{-1} h^{-1} ($n = 4$) for the CF-exposed cells/ uncontaminated medium treatments vs 1.24 ± 0.26 μmol CH_4 bottle^{-1} h^{-1} ($n = 3$) for the CF-exposed cells/NVM medium treatments. However, the MMP rates for the exposed- and unexposed-cells were significantly different. Thus, it can be concluded that CF-nonvolatile metabolites, if present, did not inhibit methanogenesis.

Although we have inferred toxicity based on methane production rates during time periods of a few days or less following the removal of CF, these results are consistent with results from longer-term studies. Yang and Speece (*5*) exposed a methanogenic incubation with a biomass concentration of 925 mg VSS L^{-1} to an initial aqueous CF concentration of 418 μM. After incubation for 1 h, the bottle was centrifuged, and the supernatant was withdrawn and replaced with fresh medium. Approximately 30 days passed before methane production was restored to pre-CF exposed levels. It was concluded that the time to recover from exposure to CF increased with the time-integrated CF exposure. Due to the experimental protocol used in their study, however, it was not possible to discriminate between the CF exposure or mass of CF transformed as the cause of observed toxicity. We propose that the transformation of CF was toxic to the methanogens in this experiment and that the time required to recover reflected the time to repair or grow organisms.

In summary, our results show that the biotransformation of CF by unacclimated methanogenic bacteria, rather than simple exposure of these cells to CF, is toxic. Our results invite biochemical studies to determine the exact mechanism of the inactivation process and biochemical and molecular biology studies to resolve how methanogenic communities apparently acclimate to chloroform, issues that were beyond the scope of this research.

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