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Effect of Fluoroacetates on Methanogenesis in Samples from Selected Methanogenic Environments

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Trifluoroacetic acid (TFA) is an atmospheric decomposition product of a new generation of refrigerants that will replace current ozone-depleting CFCs. The effect of trifluoroacetic acid on microbial methanogenesis was assessed using environmental samples from four distinct methanogenic systems: the anaerobic digestor, the rumen, freshwater sediments, and marine sediments. TFA exhibited no toxcity, as evidenced by the rate of methanogenesis, at concentrations up to 10 mM. [1-^{14}C]TFA was used to test for biodegradation (release of $^{14}CO_2$) in methanogenic marine sediments. No significant release of $^{14}CO_2$ was observed. As a control, the toxic monofluoroacetate (MFA), which is not an atmospheric degradation product of CFC replacements, was found to inhibit methanogenesis in freshwater and anaerobic digestor samples at or above concentrations of 0.1 mM. We conclude that TFA is inert in these methanogenic systems and there is no evident toxicity to either the methanogenic or fermentative populations.

Introduction

Trifluoroacetatic acid (TFA) will enter the environment, on a global scale, due to atmospheric degradation of CFC replacements such as HFC 134a (1,1,1,2-tetrafluoroethane). It is anticipated that the average global concentration of TFA in rainwater would be unlikely to exceed 0.1−0.2 ppb (1−2 nM) (*1*). However, due to the relative paucity of available data on any biological effects of TFA, the chemical industry, under the auspices of the Alternative Fluorocarbons Environmental Acceptability Study (AFEAS), has been funding research to investigate any biological or ecological effects of TFA (*2*).

Methanogenic bacteria are ubiquitous in nature and play an important role in the carbon cycle. This process occurs in a number of distinct ecological settings including freshwater sediments, marine sediments, the rumen, anaerobic digestors, and hydrothermal vents and in some eukaryotic organisms where prokaryotic endosymbionts exist (*3*). The ecological niche occupied by methanogens is typically at the end of the food chain where acetate, carbon dioxide, and hydrogen are made available for their growth by the fermentation of higher organic acids and alcohols. Acetate is a major metabolite in anaerobic food chains and a principal source of carbon and energy for many methanogens particularly those of the *Methanosarcina* sp.

Several reports have demonstrated inhibition of methanogenesis in lake sediments by 0.1−1 mM monofluoroacetate (MFA) (*4*−*7*); however, little is known about the effect of TFA on methanogenic systems. Visscher et al. (*8*) reported TFA to be toxic to methanogenesis in marine sediments from San Francisco Bay sediments at concentrations above 1 μM. Furthermore, these sediments were shown to mineralize TFA with intermediary production of MFA and difluoroacetate (DFA) resulting in methane and presumably carbon dioxide as final end products.

This paper is a further investigation of the comparative effects of TFA and MFA on selected methanogenic samples from distinct natural environments. Surprisingly, in contrast to the report of Visscher et al. (*8*), we find that TFA (up to 10 mM) exhibited no discernible toxicity to methanogenesis. In addition, no evidence for the biodegradation of TFA was observed as determined by the release of carbon dioxide from [1-^{14}C]TFA. TFA appeared to be inert in the methanogenic systems we tested. MFA, however, inhibited methanogenesis in two out of five methanogenic systems tested.

Materials and Methods

Microbial Sources. Environmental samples were obtained from the following locations. Rumen sample: Dr. Limin Kung, Department of Animal Nutrition and Biochemistry, University of Delaware, Newark, DE. Anaerobic digestor sample: Wilmington Wastewater Treatment Facility, 12th Street, Wilmington, DE. Marine sediment sample: Intertidal zone located at the University of Delaware Marine Science Department, Lewes, DE. Creek sediment sample: White Clay Creek, Avondale, PA. San Francisco Bay sediment (collected near Palo Alto) was provided by Dr. Ron Oremland, U.S. Geological Survey. An isolate, *Methanosarcina* sp. 7792a, was enriched and isolated on trimethylamine using the Wilmington anaerobic digestor sludge as a source. Marine sediments, freshwater sediments, anaerobic digestor sludge, and rumen fluid were collected by filling a glass jar to the top with material and then capping the jar for transportation to the laboratory where the containers were opened under 1 atm of nitrogen. Rumen samples were maintained at 37 °C during transportation. San Francisco Bay sediments were collected as described by Visscher et al. (*8*).

Incubation Conditions and [1-^{14}C]TFA Incubations. For assessing the effect of fluoroacetates on methanogenesis, all sediments except San Francisco Bay sediments were suspended 1:3 (v:v) in a basal salts medium designated BTZ-3, which was devoid of organic carbon, sulfate, or vitamins (*9*). This medium was buffered at pH 7 and contained trace metals, nitrogen as ammonium ion and phosphorus as orthophosphate. For experiments on biodegradation, San Francisco Bay sediments were suspended 1:3 (v:v) in a basal salts medium as described by Visscher et al. (*8*). Static incubations were performed at 30 °C in 130-mL Wheaton bottles with a total liquid−solid volume of 20 mL. All incubations were set up by adding all components to the Wheaton bottles within an anaerobic glovebox. The bottles were then withdrawn from the chamber and sparged with pure argon by inserting gasing needles through the Teflon-lined butyl rubber septa.

For determination of TFA degradation to carbon dioxide, duplicate incubations were prepared and spiked with 0.1, 1, or 10 μCi of [1-^{14}C]TFA (Amersham Research Products) corresponding to 0.1, 1, 10, and 10 μM TFA. Cold TFA was added to the second set of 10 μCi TFA incubations to achieve 100 μM total TFA. Killed controls were autoclaved prior to testing.

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TABLE 1. Degradation of [1-^{14}C]TFA in Anaerobic San Francisco Bay Sediment Incubations

sample	TFA [μM][c]	^{14}C (dpm/2 mL)[a] 20 days	% TFA captured live-killed	^{14}C (dpm/2 mL) 82 days	% TFA captured live-killed
killed control	0.1	12		15	
live	0.1	18	0.02	20	0.02
live + molybdate[b]	0.1	15	0.01	24	0.04
killed control	1.0	95		113	
live	1.0	155	0.02	188	0.03
live + molybdate	1.0	165	0.02	188	0.02
killed control	10	253		377	
live	10	1609	0.04	2008	0.06
live + molybdate	10	1652	0.04	1501	0.04
killed control	100	548		306	
live	100	2510	0.05	2203	0.06
live + molybdate	100	2077	0.04	1815	0.04

[a] Total counts recoverd in base from acidified 2 mL slurry of sediment. [b] 2.5 mM sodium molybdate. [c] Total molar TFA concentration is equivalent to [1-^{14}C]TFA except at 100 μM TFA where 10 μM labeled TFA was used and the remainder was cold TFA.

Methane Analysis. Methane was determined by gas chromatography using a PoraPak Q column linked to a thermal conductivity detector. The carrier gas was argon. The analysis was run at ambient temperature using a Hewlett-Packard 5880A gas chromatograph The detection limit for methane, using a 100-μL injection, was 5 nmol.

Determination of $^{14}CO_2$ Formation from [1-^{14}C]TFA. The 2-mL aliquots of the incubation slurry were transferred to a Warburg flask (15 mL volume). A total of 0.2 mL of 0.5 M NaOH was added to the centerwell of the flask, and the flask was sealed with a butyl rubber stopper. A 0.05-mL sample of glacial acetic acid was then injected into the main compartment of the flask such that a pH of about 3.5 was obtained. The Warburg flasks were placed on a slow speed shaker for 20 h. After distillation and capture of the CO_2, the centerwell contents were diluted into 10 mL of Formula 989 liquid scintillation counting solution (NEN Research Products) and counted in a Packard Tricarb 4640 scintillation counter. Control experiments with known amounts of $NaH^{14}CO_3$ added to samples indicated that >70% of the $^{14}CO_2$ can be recovered by this method. To verify that the low level of counts measured came from CO_2, barium nitrate was added to a filtered aliquot of the incubation slurry to form insoluble barium carbonate. To remove contaminating [1-^{14}C]TFA, the precipitate was washed several times with 1 mM NaOH until the counts in the supernatant were at background. The pellet was then dissolved with 10 mM tetrasodium EDTA and counted as described above. The total counts in the orginal samples were determined from filtered 0.1-mL aliquots of the incubation slurry.

TFA Analysis. In some samples, TFA was analyzed on a Waters HPLC using a Bio-Rad Aminex fast acid analysis column (100 mm × 7.8 mm). The column was run isocratically with 0.02 N H_2SO_4 as the solvent at a flow rate of 0.6 mL/min. The elution of organic acids was monitored by absorption at 210 nm. Under these conditions, TFA elutes at 7.0 min, difluoroacetate at 8.8 min, MFA at 14.5 min, and acetate at 16.6 min. For radioactive samples, fractions were collected and counted by liquid scintillation as described above.

Results and Discussion

The results presented in Figure 1 and Table 1 address two distinct but related aspects of the possible impact of TFA on methanogenesis. First is the issue of TFA and MFA toxicity toward methanogenic samples from different environments. Second is the question of TFA biodegradation. Table 1 deals specifically with San Francisco Bay sediments as likely sources of methanogenic, TFA-degrading, microorganisms based on the work of Visscher et al. (*8*).

In selecting environmental samples for investigating toxicity, the main criterion was diversity in methanogenic ecosystems so as to screen a larger cross-section of methanogenic populations. For example, methanogenesis in the rumen is thought to proceed via hydrogen/CO_2 as principal methanogenic substrates, whereas in the anaerobic digestor, acetate is thought to be the principal methanogenic substrate (*3*). In marine sediments, the methanogens cannot compete effectively with sulfate-reducing bacteria for hydrogen or acetate whereas in freshwater sediments sulfate-reducers and methanogens may grow symbiotically. In either system, however, multiple groups of fermentative as well as methanogenic organisms are likely to be involved in methanogenesis from endogenous organic carbon in the sediment, rumen or digestor.

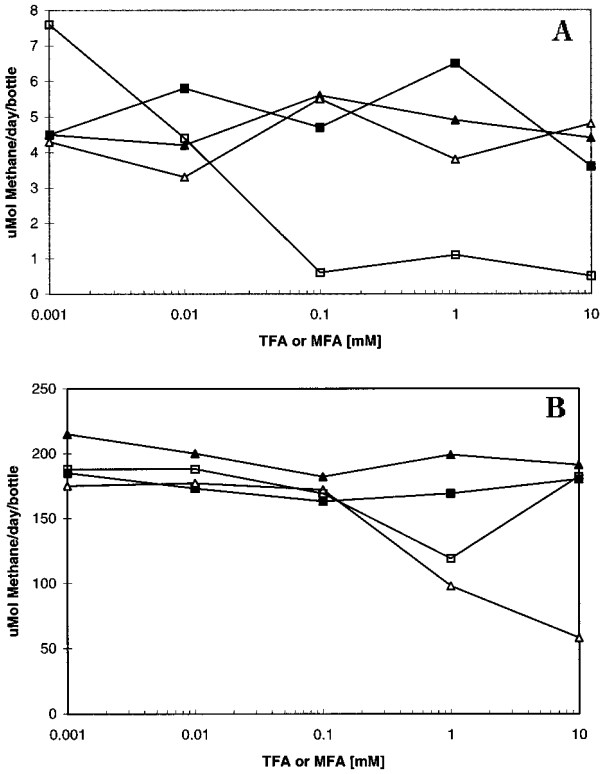

FIGURE 1. Effect of TFA or MFA (0.001−10 mM) on methane production from incubations of environmental sediments and samples. (A) Marine sediment: ▲, TFA; △, MFA. Freshwater sediment: ■, TFA; □, MFA. (B) Digestor sludge: ▲, TFA; △, MFA. Rumen sample: ■, TFA; □, MFA. Rates were determined from primary plots of methane vs time over the first 48 h of incubation.

While it seems unlikely from a chemical perspective that reductive dehalogenation of TFA to MFA can occur readily in biological systems because of the fluorine−carbon bond

strength (*10*), we chose to test MFA in our study as a positive control compound likely to cause inhibition based on its known toxicity and results from previous reports (*4*−*7*). Little is known about the toxicity of TFA to methanogenic systems with the exception of the report from Visscher et al. (*8*) where >1 μM concentrations were reported to inhibit methanogenesis from marine sediments from San Francisco Bay.

Four different environmental samples, one *Methanosarcina* isolate, and the San Francisco bay sediment were tested for sensitivity to TFA or MFA over a concentration range from 0 to 10 mM by measuring the rate of methane production. Rates of methane production varied by almost 2 orders of magnitude between sediment samples in which microbial populations are relatively low and rumen or digestor samples where microbial populations and the organic content are high. Figure 1 shows that TFA was not inhibitory to methanogenesis in either high or low rate systems at concentrations up to 10 mM, which is more than 1 million-fold higher concentration of TFA than that predicted to occur environmentally in precipitation (*1*). This concentration is 1000−10 000 times higher than the 1−10 μM inhibitory concentration range reported by Visscher et al. (*8*). In contrast to the reported results, we saw no inhibition of methanogenesis for San Francisco Bay sediments at concentrations of TFA up to 1 mM (data not shown). This is 100−1000 times higher than the level reported for inhibition by Visscher et al. MFA, as expected, displayed toxicity to a *Methanosarcina* isolate at 10 mM (data not shown) as well as the digestor sludge and the freshwater creek sediment (Figure 1). Thus, MFA toxicity was not observed with all samples but was evident in both high and low rate incubations. There was no evidence for TFA toxicity in any of the incubations.

Evidence for biodegradation of TFA was sought using San Francisco Bay sediments generously provided by Ron Oremland (USGS, Menolo Park, CA) (Table 1). Since we were not equipped to count radioactive methane in the gas phase, we used [1-^{14}C]TFA and looked for the release of $^{14}CO_2$ as evidence of TFA biodegradation. This approach differs from that of Visscher et al. (*8*) where [2-^{14}C]TFA was used and the incubations were assayed for labeled methane and other volatile products. Sodium molybdate was added to one set of samples to inhibit growth of the sulfate-reducing bacteria and thus to help promote the growth of methanogens (*8*). [1-^{14}C]TFA incubations with live sediments plus or minus molybdate did consistently generate higher levels of $^{14}CO_2$ than did the killed controls; in some cases a 5−6-fold difference was observed. However, this difference accounted for less than 0.1% of the total counts present in the sample, and even after 82 days of incubation, the total counts appearing as CO_2 did not increase. The total counts in the filtered supernatant of the incubation samples remained constant relative to the killed controls over this same time period. Some of the samples were analyzed by HPLC to determine if the TFA had been converted to other water-soluble products (data not shown). All of the counts in the samples tested eluded at the same retention time as authentic TFA. Another set of incubations over a shorter time period was performed with a second sample of San Francisco Bay sediment with the same results (data not shown). The low level release of labeled CO_2 by metabolism may be due to a <0.1% labeled non-TFA contaminant in the stock [1-^{14}C]TFA.

We conclude that TFA is rather inert in the methanogenic ecosystems we studied. This is evidenced by the lack of toxicity at extremely high concentrations and the absence of biodegradation in the two methanogenic sediment samples that were tested. However, the work of Visscher et al. describes a sediment that is both sensitive to 1−10 μM TFA and capable of complete mineralization of TFA. The difference in results could be due to site-specific or seasonal differences in the samples that were obtained.

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