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Selective Inhibition by 2-Bromoethanesulfonate of Methanogenesis from Acetate in a Thermophilic Anaerobic Digestor

S. H. ZINDER,* T. ANGUISH, AND S. C. CARDWELL

Department of Microbiology, Stocking Hall, Cornell University, Ithaca, New York 14853

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The effects of 2-bromoethanesulfonate, an inhibitor of methanogenesis, on metabolism in sludge from a thermophilic (58°C) anaerobic digestor were studied. It was found from short-term experiments that 1 μmol of 2-bromoethanesulfonate per ml completely inhibited methanogenesis from $^{14}CH_3COO^-$, whereas 50 μmol/ml was required for complete inhibition of $^{14}CO_2$ reduction. When 1 μmol of 2-bromoethanesulfonate per ml was added to actively metabolizing sludge which was then incubated for 24 h, it caused a 60% reduction in methanogenesis and a corresponding increase in acetate accumulation; at 50 μmol/ml it caused complete inhibition of methanogenesis and accumulation of acetate, H_2, and ethanol.

Gunsalus et al. (3) found that 2-bromoethanesulfonate (BES) was the coenzyme M (2-mercaptoethanesulfonate) analog most inhibitory to the reduction of methyl-coenzyme M to methane in cell extracts of *Methanobacterium thermoautotrophicum*. BES at 1 nmol/ml has been found to inhibit methanogenesis by whole cells of *Methanosarcina* (6) and *Methanothrix* spp. (9) and by a coenzyme M-requiring strain of *Methanobrevibacter ruminantium* (1). BES has also been used in ecological studies as an inhibitor of methanogenesis, usually at concentrations of 0.1 to 1 μmol/ml (2, 4, 5, 8).

Since little is known about the concentrations of BES that are inhibitory to natural methanogen populations, especially thermophiles, we examined the effect of different BES concentrations on the two primary methanogenic reactions in an anaerobic digestor, acetate cleavage and carbon dioxide reduction. Short-term (1- to 2-h) studies with ^{14}C-labeled substrates and 24-h time courses both indicated that methanogenesis from acetate in our digestor was inhibited by much lower BES concentrations than CO_2 reduction was.

The digestor, a New Brunswick Microferm 105 fermentor with a 3-liter liquid volume, was operated at 58°C and was fed a lignocellulosic waste (loading rate, 2.6 g of volatile solids per liter per day; retention time, 10 days) as described previously (10). During these studies, *Methanobacterium* sp. was apparently the dominant CO_2-reducing methanogen, and *Methanothrix* sp. was the most numerous aceticlastic methanogen (10).

Sludge samples (10 ml) from the digestor were anaerobically dispensed into 37-ml serum vials sealed with butyl rubber stoppers and incubated under a 70% N_2–30% CO_2 headspace (scrubbed by hot copper turnings; Matheson Scientific, Inc., Joliet, Ill.) at 58°C in a shaking waterbath (10). Levels of methane, CO_2, H_2, short-chain fatty acids, and alcohols were measured by gas chromatography, and the $^{14}CH_4$ activity was measured by using a gas chromatograph-proportional counter system (10). The results presented are the average for duplicate vials.

BES was obtained from Sigma Chemical Co., St. Louis, Mo. Sodium [2-^{14}C]acetate (58.9 mCi/mmol) and $NaH^{14}CO_3$ (58 mCi/mmol) were purchased from Amersham Corp., Arlington Heights, Ill., and stored as sterile anaerobic solutions.

The short-term effects of different BES concentrations on the potential rate of methanogenesis from acetate are shown in Fig. 1. Unfed sludge samples (no substrate added) were incubated with a saturating concentration of acetate (5.3 μmol/ml), 46 kdpm of $^{14}CH_3COO^-$ per ml, and different concentrations of BES. Rates were measured after 1 h of exposure to BES and the label. BES at 10^{-4} M (0.1 μmol/ml) caused partial inhibition of methanogenesis from acetate; this inhibition increased with time (data not shown). BES at 1 μmol/ml caused complete inhibition. A much higher concentration, 50 μmol/ml, was required for complete inhibition of methanogenesis from CO_2 in samples containing 121 kPa (1.2 atm) of H_2, 30 kPa of CO_2, and 395 kdpm of $NaH^{14}CO_3$ per ml. Little if any inhibition of CO_2 reduction was seen at BES concentrations as high as 10 μmol/ml.

The effects of BES on overall metabolism in sludge incubated with substrate for 24 h are shown in Fig. 2. In control vials receiving no BES (Fig. 2A), methanogenesis (total accumulation = 24 μmol/ml [liquid volume]) and acetate concentration followed patterns similar to those reported previously (10). Adding 1 μmol of BES per ml, which caused complete inhibition of methanogenesis from acetate in short-term experiments (Fig. 1), decreased methane accumulation to <10 μmol/ml (60% inhibition), whereas acetate accumulated to 12.5 μmol/ml; no H_2 (detection limit, 50 Pa) or ethanol (detection limit, 0.1 μmol/ml) was detected (Fig. 2B).

The inhibition of methanogenesis and accumulation of acetate were as expected if methanogenesis from acetate were completely inhibited, since we have found that acetate is the precursor of more than 60% of the methane formed in the digestor (10). The lack of hydrogen accumulation indicated that CO_2-reducing methanogens were apparently not significantly affected by 1 μmol of BES per ml. The sum of the CH_4 and acetate accumulations (22.5 μmol/ml) was only slightly lower than that in the control vials (24.5 μmol/ml), indicating that fermentative organisms were not significantly affected by the presence of BES at 1 μmol/ml. We have determined that acetic acid accumulation to 12.5 μmol/ml would cause the pH to drop to ca. pH 5.5; therefore, the addition of 1 μmol of BES per ml initiated classic digestor failure due to acetic acid buildup.

In vials containing BES at a concentration of 50 μmol/ml (Fig. 2C), methanogenesis was completely inhibited after 2 h of exposure, and <1.0 μmol/ml accumulated overall. Acetate accumulated to ca. 10 μmol/ml after 24 h of incubation. After 6 h of incubation, H_2 was detectable in the headspace

* Corresponding author.

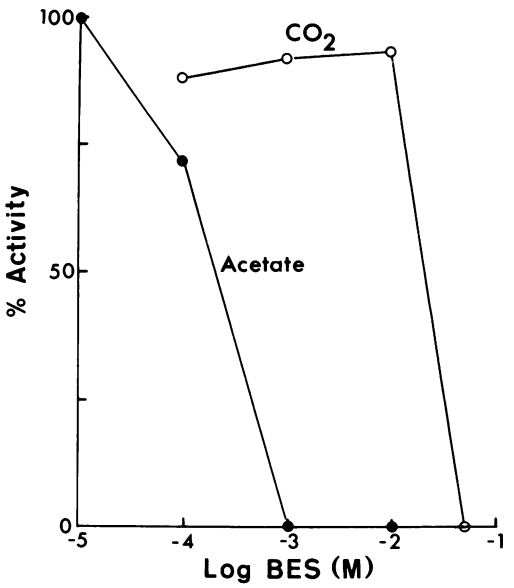

FIG. 1. Effect of different BES concentrations on the potential rates of methanogenesis from sodium [2-^{14}C]acetate (●) or $^{14}CO_2$ (○) by populations in a thermophilic anaerobic digestor.

and accumulated to 10 μmol/ml by 24 h. After 12 h of incubation, ethanol was detectable in the sludge; it was present at 2.5 μmol/ml at 24 h. The final propionate concentration was 0.5 to 0.6 μmol/ml both in the control vials and in those containing 50 μmol of BES per ml. Butyrate was not detected in the control vials or in those with 1 μmol of BES per ml, but a trace (ca. 0.1 μmol/ml) accumulated in the vials with 50 μmol of BES per ml. Nonvolatile fermentation products such as lactate were not assayed.

We have no explanation for the 6-h lag in H_2 accumulation, since acetate accumulated during this period. Perhaps some H_2 was consumed by nonmethanogens, such as homoacetogens. The amount of CH_4 which could be formed from the products detected (acetate, ethanol, H_2, and CH_4) in the vials after 24 h of exposure to BES at 50 μmol/ml was 17 μmol of CH_4 per ml. Thus, fermentative organisms were only partially inhibited, if at all, at this BES concentration.

This differential effect of BES on methanogenesis is further evidence that methanogenesis from acetate and CO_2 in digestors and other similar ecosystems are separate processes, often carried out by distinct microbial populations. This can be an important consideration in the study of methanogenesis inhibition by toxic compounds. Results showing partial inhibition of overall methanogenesis by a toxic compound may actually be explained by complete (or nearly complete) inhibition of methanogenesis from either acetate or CO_2.

Weimer and Zeikus (7) found that *Clostridium thermocellum* produced acetate, ethanol, and H_2 as the primary fermentation products when cultured alone on cellulose. When it was cocultured with the CO_2-reducing methanogen *M. thermoautotrophicum*, the primary products were acetate and CH_4. Similarly, our digestor populations produced acetate and CH_4 as primary products when CO_2 reduction was allowed to proceed in the presence of 1 μmol of BES per ml. Acetate, ethanol, and H_2 were produced when methanogenesis was completely inhibited by BES at 50 μmol/ml. It is of interest that the digestor substrate contained a significant proportion of cellulose and that high numbers of a *C. thermocellum*-like organism were found in the sludge (10).

The level of BES required to inhibit methanogenesis from acetate in our digestor was significantly higher than those reported for pure cultures of aceticlastic methanogens (6, 9). This may be partially explained by adsorption of BES to particles in the sludge and by absorption to and possibly uptake by other organisms. Also, Smith and Mah (6) found that cultures of *Methanosarcina* sp. strain 227 incubated in the presence of BES readily developed resistance to it within 10 days; a significant proportion of the population were mutants which could tolerate BES at concentrations 100-fold higher (0.1 μmol/ml) than the other members of the popula-

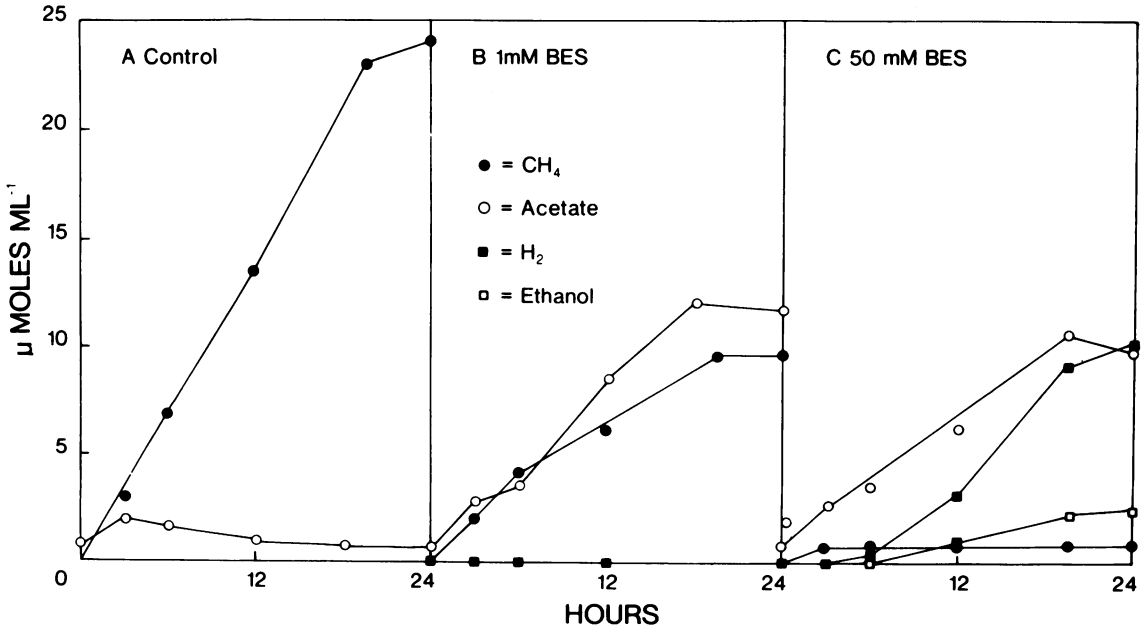

FIG. 2. Metabolism of sludge incubated with 0 (A), 1 (B), or 50 (C) μmol of BES per ml.

tion could. Natural aceticlastic methanogen populations may resemble these resistant strains.

The extremely high BES concentration (50 μmol/ml) required to halt the reduction of CO_2 to methane was surprising. However, we have found that a similar concentration was required to completely inhibit methanogenesis by a growing pure culture of a thermophilic *M. thermoautotrophicum* strain (S. H. Zinder and M. Koch, Arch. Microbiol., in press). The growth of *Acetogenium kivui*, a thermophilic homoacetogen, under H_2-CO_2 was not affected by 50 μmol of BES per ml (Zinder and Koch, in press).

It should be emphasized that these results cannot be extrapolated to other methanogenic ecosystems unless tests similar to ours are performed. Zehnder and Brock (8) studied Lake Mendota sediments and found that 1 μmol of BES per ml caused 50% inhibition of methanogenesis from CO_2 and that 7 μmol/ml was required for complete inhibition of methanogenesis from acetate. Healy et al. (4) found that BES at 1 μmol/ml caused complete inhibition of methanogenesis from both acetate and CO_2 in an anaerobic ferulate-degrading consortium. Bouwer and McCarty (2) reported that BES at 0.6 μmol/ml caused 41% inhibition of methanogenesis from acetate in an anaerobic upflow reactor that was fed acetate and traces of halogenated organic compounds. Finally, it should be noted that results from short-term studies such as ours should not be extrapolated to longer terms since adaptation to or degradation of BES may occur (2, 6).

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LITERATURE CITED

1. **Balch, W. E., and R. S. Wolfe.** 1979. Transport of coenzyme M (2-mercaptoethanesulfonic acid) in *Methanobacterium ruminantium*. J. Bacteriol. **137**:264–273.
2. **Bouwer, E. J., and P. L. McCarty.** 1983. Effects of 2-bromoethanesulfonic acid and 2-chloroethanesulfonic acid on acetate utilization in a continuous-flow methanogenic fixed-film column. Appl. Environ. Microbiol. **45**:1408–1410.
3. **Gunsalus, R. P., J. A. Romesser, and R. S. Wolfe.** 1978. Preparation of coenzyme M analogs and their activity in the methylcoenzyme M reductase system in *Methanobacterium thermoautotrophicum*. Biochemistry **17**:2374–2377.
4. **Healy, J. B., Jr., L. Y. Young, and M. Reinhard.** 1980. Methanogenic decomposition of ferulic acid, a model lignin derivative. Appl. Environ. Microbiol. **39**:436–444.
5. **Oremland, R. S., L. M. Marsh, and S. Polcin.** 1982. Methane production and simultaneous sulfate reduction in anoxic, salt marsh sediments. Nature (London) **296**:143–145.
6. **Smith, M. R., and R. A. Mah.** 1981. 2-Bromoethanesulfonate: a selective agent for isolating resistant *Methanosarcina* mutants. Curr. Microbiol. **6**:321–326.
7. **Weimer, P. J., and J. G. Zeikus.** 1977. Fermentation of cellulose and cellobiose by *Clostridium thermocellum* in the absence and presence of *Methanobacterium thermoautotrophicum*. Appl. Environ. Microbiol. **33**:289–297.
8. **Zehnder, A. J. B., and T. D. Brock.** 1980. Anaerobic methane oxidation: occurrence and ecology. Appl. Environ. Microbiol. **39**:194–204.
9. **Zehnder, A. J. B., B. A. Huser, T. D. Brock, and K. Wuhrmann.** 1980. Characterization of an acetate-decarboxylating non-hydrogen oxidizing methane bacterium. Arch. Microbiol. **124**:1–11.
10. **Zinder, S. H., S. C. Cardwell, T. Anguish, M. Lee, and M. Koch.** 1984. Methanogenesis in a thermophilic (58°C) anaerobic digestor: *Methanothrix* sp. as an important aceticlastic methanogen. Appl. Environ. Microbiol. **47**:796–807.