

Dechlorination of Chloroethenes Is Inhibited by 2-Bromoethanesulfonate in the Absence of Methanogens

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2-Bromoethanesulfonate (BES) inhibited the reductive dechlorination of chloroethenes in several sediment-free enrichment cultures in the absence of methanogenic archaea. Archaeon-specific PCR primers confirmed the absence of methanogens in the enrichment cultures. BES should not be used to attribute dechlorination activities to methanogens.

Chlorinated ethenes are common environmental groundwater pollutants, a fact which has resulted in extensive research on the bacterial degradation of these compounds. It is known that tetrachloroethene (PCE) can be cometabolized by methanogenic archaea (7, 9, 10). Methanogens possess the methyl reductase enzyme complex which catalyzes the final step in methane formation. This enzyme contains a unique cofactor, coenzyme M (CoM) (2-mercaptoethanesulfonate), which is found only in methanogens (5). 2-Bromoethanesulfonate (BES), a structural analog and competitive inhibitor of CoM, was described as a specific inhibitor of methanogenesis (19). Several reports state that dechlorination of chloroethenes in methanogenic cultures is inhibited by BES (6–8). The common conclusion is that methanogens are directly or indirectly involved in the observed dechlorination reactions. Here we present evidence that BES is not a specific inhibitor of methanogenic archaea but also inhibits some chloroethene-dechlorinating organisms none of which are methanogens.

Chloroethene-dechlorinating enrichment cultures. Five sediment-free, methanogenic cultures that dechlorinated PCE were obtained from microcosms derived from different freshwater sediments as described previously (13, 15). All sediments were collected in spring 1995 in Michigan, except for the Parfume River sediment (Parf), which was collected in Hué, Vietnam, in March 1993. The cultures were maintained in 160-ml serum bottles containing 100 ml of a bicarbonate-buffered basal salts medium (14, 15) amended with either 1 mM acetate–0.4 mmol hydrogen or 1 mM acetate–5 mM glycerol. Acetate and hydrogen consumption was monitored as described previously (14, 15), and hydrogen was replenished upon depletion. Fresh medium was inoculated with 1% (vol/vol) samples from active cultures. Throughout this study, culture bottles were incubated upside down at 25°C without agitation. At the time this experiment was initiated, the sediment-free, methanogenic cultures had undergone three serial transfers, each of which had consumed three feedings of 15 µmol of PCE. PCE was added with a 5-µl Hamilton syringe equipped with a Chaney adapter (reproducibility was ±15%). All gases and chlorinated volatiles were measured in the headspace by gas chromatography as described previously (15). In the cultures derived from the Père Marquette River (PM), the Au Sable River (AuS), and the Red Cedar River (RC), PCE was dechlorinated to vinyl chloride (VC) and ethene (ETH).

Two cultures, one of which was derived from the Tahquamenon River sediment (TQ) and one from Parf, dechlorinated PCE to the dichloroethene (DCE) isomers *cis*- and *trans*-DCE in a ratio of 1 to 3 (±0.5). All cultures produced methane when hydrogen or glycerol were supplied as electron donors. Acetoclastic methane formation was observed in three cultures. When PCE was fed to the cultures, methane formation was reduced by up to 70% compared to cultures that did not receive PCE (data not shown). This inhibitory effect of halogenated hydrocarbons on methanogenesis is well known (3, 6).

To investigate the importance of methanogenic archaea on PCE dechlorination, we treated all cultures with 2 mM BES for four subsequent transfers. Cultures were fed twice with 15 µmol of PCE before each transfer. The addition of BES to the methanogenic cultures inhibited chloroethene dechlorination (see below). Following this treatment (no BES added), methane production was not observed in any of the cultures with a variety of different substrates including hydrogen, methanol, acetate, lactate, pyruvate, glycerol, fumarate, glucose, and yeast extract.

Evidence for the absence of methanogenic archaea. The absence of methanogenic archaea was confirmed with 16S rDNA targeted primers. BES-treated and untreated cultures were analyzed with archaeon-specific PCR primers 340f [5'-CTACGGGGCGCA(C/G)CAGG(C/G)GC-3'] and 915r [5'-GTGCTCCCCCGCCAATTCCT-3'] and bacterium-specific PCR primers FD1 (20) and 1492r (11). In order to amplify the 16S rDNA genes from the archaeal populations, DNA was extracted from 5 ml of culture fluid by bead beating. Cells harvested by centrifugation were suspended in 0.4 ml of 10× Tris-EDTA (TE) buffer in 2-ml Eppendorf tubes. Then, 0.2 ml of zirconia-silica beads (0.1 mm; catalog no. 110791012; Biospec Products, Bartlesville, Okla.), 0.5 ml of Tris-equilibrated phenol, and 0.1 ml of 10% sodium dodecyl sulfate were added. Each sample was beaten at 5,000 beats per min for 2 min in a Mini-Beadbeater (Biospec Products) and cooled on ice before a second 2-min cycle was started. After centrifugation in a microcentrifuge at 3,000 rpm for 30 s, the aqueous phase (supernatant) was removed, another 0.4 ml of 10× TE was added to the beads, and bead beating was repeated for 1 min. The phases were separated by centrifugation, and the supernatant was combined with the previous fraction. The combined supernatant was then treated with phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) and precipitated at 4°C with 0.1 volume of 3 M Na-acetate (pH 5.2) and 0.7 volume of isopropyl alcohol (1). After centrifugation the pellet was rinsed with 70% ethanol and suspended in 50 µl of sterile TE buffer. DNA yielded by this process was suitable for PCR and ranged from

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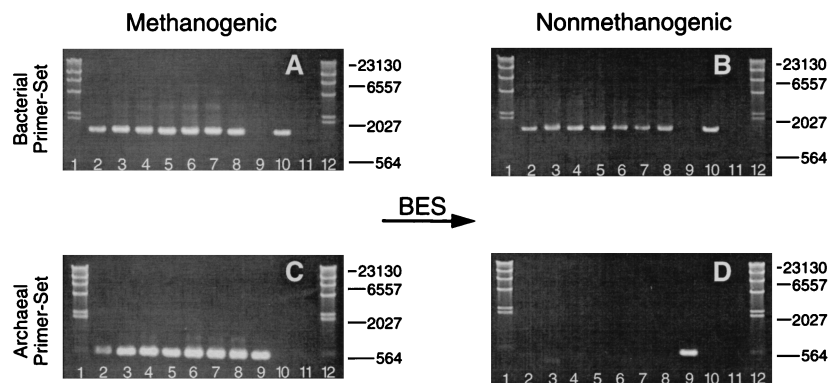


FIG. 1. Agarose gel electrophoresis of the 16S rDNA fragments amplified with bacterium-specific (A and B) and archaeon-specific (C and D) PCR primers from different dechlorinating cultures. Lanes: 1 and 12, molecular size markers (23.1, 9.4, 6.6, 4.4, 2.3, and 2.0 kb); 2, culture TQ; 3, culture Parf; 6, culture RC; 7, culture PM; 8, culture AuS; 9, archaeal control (*M. thermoautotrophicum*); 10, bacterial control (*Alcaligenes* sp.); 11, no template DNA. Lanes 4 and 5 represent two 1,2-dichloropropane-dechlorinating cultures; in both cultures dechlorination of 1,2-dichloropropane was not inhibited by BES (15).

200 to 7,500 $\mu\text{g/ml}$. This DNA (50 ng) was used in a standard PCR mixture (16), with the addition of 0.2 μl of bovine serum albumin per 30- μl reaction mixture. PCR conditions were as follows: denaturation, 94°C (2 min 10 s); 30 cycles of 94°C (30 s), 60°C (45 s), 72°C (2 min 10 s); final elongation, 72°C (6 min). Control DNA was from an *Alcaligenes* sp. for the bacterial group and *Methanobacterium thermoautotrophicum* for the archaeal group. In order to determine the detection limit for methanogens, a known quantity of *M. thermoautotrophicum* DNA was diluted and the PCR with the archaeon-specific primers was performed on both the pure DNA and DNA combined with the community DNA obtained from the BES-treated cultures.

All enrichment cultures yielded bacterial 16S rDNA PCR products with bacterium-specific primers from whole cells, freeze-thaw lysed cells, sodium dodecyl sulfate-proteinase K-lysed cells (1), and bead beater-disrupted cells. In contrast, archaeon-specific PCR products could be amplified only from cultures that were disrupted by bead beating. All methanogenic cultures (before the BES treatment) yielded amplification products with both the bacterial primer set (Fig. 1A) and the archaeon-specific primer set (Fig. 1C). In contrast, after the BES treatment for four serial transfers, only the bacterial primer set yielded amplification products (Fig. 1B). No amplification products were obtained with the archaeon-specific primer set (Fig. 1D), suggesting that the archaea, and consequently the methanogens, had been successfully removed. Three hundred femtograms of community DNA per microliter of the reaction mix was sufficient to yield a signal with the bacterium-specific primer set, whereas 1 pg of *M. thermoautotrophicum* DNA was required to get a signal with the archaeon-specific primer set. Since the same total quantity of extracted culture DNA was used for all of the reactions, the absence of a signal in Fig. 1D corresponds to a reduction of at least 2 orders of magnitude in the ratio of archaeal DNA to bacterial DNA.

Inhibition of chloroethene-dechlorinating bacteria by BES. Unexpectedly, following the BES treatment, but in the absence of BES, the addition of PCE to the nonmethanogenic enrichment cultures yielded dechlorination patterns and kinetics identical to those in the original methanogenic cultures prior to the addition of BES (13). This finding suggested that the observed dechlorination reactions were in fact not mediated by methanogenic cometabolism but rather were catalyzed by bacterial processes. Most interestingly, BES had an inhibitory

effect on chloroethene dechlorination in the cultures not containing methanogens.

To further investigate this phenomenon, the nonmethano-

TABLE 1. Influence of BES on reductive dechlorination of PCE in different nonmethanogenic cultures

| Culture ^a | Total PCE added (μmol) ^b | Amt of product formed (μmol) ^c | | | | |
|----------------------|--|--|-----------------|-------------------|------|------|
| | | TCE | <i>cis</i> -DCE | <i>trans</i> -DCE | VC | ETH |
| PM | | | | | | |
| + BES | 80 | 0 | 56.2 | 0 | 19.7 | 0 |
| - BES | 80 | 0 | 12.6 | 0 | 36.8 | 27.3 |
| AuS | | | | | | |
| + BES | 60 | 0 | 23.5 | 6.5 | 19.0 | 11.7 |
| - BES | 60 | 0 | 0 | 6.0 | 0 | 51 |
| RC | | | | | | |
| + BES | 40 | 22.7 | 10.4 | 2.2 | 0 | 0 |
| - BES | 40 | 0 | 0 | 0 | 34.3 | 9.2 |
| Parf | | | | | | |
| + BES | 40 | 38.6 | <0.5 | 0.8 | 0 | 0 |
| - BES | 40 | 13.9 | 5.7 | 19.1 | 0 | 0 |
| TQ | | | | | | |
| + BES | 20 | 17.0 | 0 | 0 | 0 | 0 |
| - BES | 20 | 1.3 | 3.3 | 10.7 | 0 | 0 |
| BB1 | | | | | | |
| + BES | 80 | 0 | 82.8 | <0.5 | <0.5 | 0 |
| - BES | 80 | 0 | 78.0 | <0.5 | <0.5 | 0 |
| Viet1 | | | | | | |
| + BES | 80 | 76.2 | <0.5 | <0.5 | 0 | 0 |
| - BES | 80 | 75.0 | <0.5 | <0.5 | 0 | 0 |

^a Bottles were inoculated (1% [vol/vol]) with nonmethanogenic cultures obtained after the BES treatment. Glycerol (5 mM) and acetate (1 mM) were supplied as electron donors, except for isolate Viet1, which was supplied with acetate (1 mM) and hydrogen (0.4 mmol).

^b Headspace analysis was performed weekly, and PCE (20 μmol) was replenished when depleted until day 65. At day 79, PCE was completely consumed in all cultures. Cultures BB1 and Viet1 were measured every 2nd day, and PCE was replenished when depleted until day 17.

^c The data are means obtained for duplicate cultures after 79 days. Similar values were observed in ± 3 days in two subsequent experiments. Dechlorination in cultures BB1 and Viet1 was monitored for 21 days.

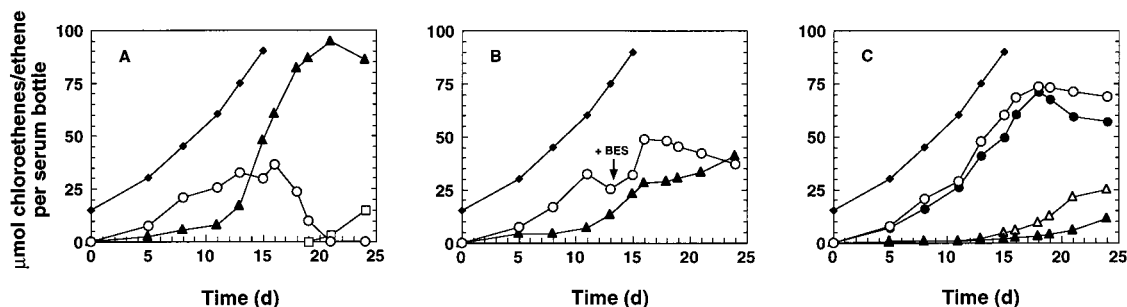


FIG. 2. Dechlorination of PCE in nonmethanogenic culture PM. Symbols: diamonds, cumulative PCE added; squares, ETH; circles, *cis*-DCE; triangles, VC. (A) No BES; (B) 1 mM BES added (arrow) to a noninhibited culture on day 13; (C) 1 mM BES (closed symbols) or 2 mM CoM and 1 mM BES (open symbols) added on day 0. Data are the mean values for duplicate cultures, and similar results were obtained in a second independent experiment. d, days.

genic cultures were transferred in quadruplicate to medium containing 1 mM acetate–0.4 mmol hydrogen or 1 mM acetate–5 mM glycerol. Hydrogen concentrations were measured by gas chromatography (15), and the cultures were fed when depleted. One bottle of each culture was amended with 1 mM BES, another bottle was amended with 2 mM BES, and two bottles did not receive any BES. Dechlorination was monitored for 79 days (Table 1). In all of the cultures capable of completely dechlorinating PCE, VC and ETH formation was severely inhibited by BES and resulted in accumulation of trichloroethene (TCE), *cis*-DCE, or *trans*-DCE. However, the extent of inhibition was not uniform in cultures PM, AuS, and RC, indicating that bacteria capable of complete dechlorination of chloroethenes exhibit differences in their susceptibilities to BES (Table 1). Two cultures, TQ and Parf, producing *cis*- and *trans*-DCE in the absence of BES, accumulated stoichiometric amounts of TCE from PCE. Culture BB1, a highly enriched subculture derived from the PM enrichment, dechlorinated PCE stoichiometrically to *cis*-DCE with acetate as electron donor (13). In this case dechlorination to *cis*-DCE was not inhibited by BES, which was also observed for PCE-to-*cis*-DCE dechlorination by *Dehalospirillum multivorans* (4). The dechlorination of PCE to TCE by Viet1, a pure culture derived from the Parf enrichment, also was not affected by BES.

Inhibition of chloroethene dechlorination in culture PM was monitored over a shorter period (Fig. 2). Duplicate cultures were amended with 1 mM BES, 2 mM CoM–1 mM BES, or no additions. In the absence of BES, stoichiometric amounts of VC accumulated (Fig. 2A). ETH formation started on day 20 after all PCE had been consumed. PCE had an inhibitory effect on VC dechlorination in the PM enrichment, as previously observed in other cultures (17). On day 13, 1 mM BES was added to one noninhibited culture (Fig. 2B). Dechlorination of PCE to *cis*-DCE was unaffected, whereas dechlorination to VC stopped and no ETH formation occurred after day 20. The addition of excess CoM has been shown to protect methanogens from the inhibitory effect of BES (18). In contrast, the addition of 2 mM CoM 12 h before addition of 1 mM BES could not prevent the inhibitory effect of BES on PCE dechlorination in the PM culture (Fig. 2C). While the inhibition mechanism of BES on methanogenesis is well understood (2, 18), the mode of BES inhibition on dechlorination of chloroethenes is unclear. Hydrogen was used as an electron donor for chloroethene reduction in all of the cultures tested except for culture BB1 (13). Chloroethene dechlorination in hydrogen-fed and glycerol-fed cultures was similarly inhibited by the addition of BES (data not shown). This suggests a direct inhibitory effect of BES on the dechlorinating organisms rather than on other organisms present in the communities which

produce hydrogen used by the dechlorinators. DiStefano et al. (6) observed complete dechlorination of PCE in a mixed culture containing methanogens. Although they observed negligible methane formation, TCE did accumulate in the presence of BES. The authors speculated as to whether methanogens were involved in dechlorination or if BES inhibited dechlorinating organisms due to the structural similarity of BES to the chlorinated ethenes. Our results show that the dechlorination of PCE to TCE (cultures Parf and TQ) or *cis*-DCE (cultures PM and BB1) is not inhibited by BES whereas the dechlorination of the DCE isomers and VC is inhibited. The addition of 10 μ mol of 1,2-dichloroethane, a compound structurally more similar to chloroethenes than BES, to cultures PM and BB1, showed no inhibitory effect on PCE dechlorination (data not shown). These observations do not support the structural-analog hypothesis; however, further research is required to determine the exact mode of how BES inhibits chloroethene dechlorination. Another possible explanation for the inhibition of dechlorination is that BES can potentially be transformed, e.g., by microbial assimilation or dissimilation of sulfonate-sulfur (12). To exclude this possibility, BES was measured in all mixed cultures by high-performance liquid chromatography as described previously (14), with H₂O-methanol-H₃PO₄ (990:10:1 [vol/vol/vol]) as an eluent at a flow rate of 1 ml min⁻¹, UV detection at 210 nm, and an Adsorbosphere C₁₈ column (250 by 4.6 mm; particle size, 5 μ m; Alltech, Deerfield, Ill.). The retention times under these conditions were as follows: BES, 3.78 min; CoM, 3.28 min; ethanesulfonate, 3.34 min. BES was neither debrominated nor desulfonated in any of the cultures tested. Also, no growth was observed in 10 mM HEPES-buffered basal salts medium containing acetate and hydrogen as electron donors and 1 mM BES as the only potential electron acceptor. In another study it was shown that the dechlorination of polychlorinated biphenyls in microcosms not containing methanogens was completely suppressed by BES. In this case, however, BES was used as an alternate electron acceptor and the sulfonic acid moiety of BES was reduced to sulfide (21). As more chloroethene-dechlorinating organisms become available, detailed studies with pure cultures will clarify the mode of BES inhibition. In summary, our findings confirm that BES is a potent inhibitor of methanogenic archaea, but its action is not as specific as previously believed. BES also inhibits chloroethene dechlorination in at least some nonmethanogenic bacterial cultures.

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