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Evidence for *para* Dechlorination of Polychlorobiphenyls by Methanogenic Bacteria

DINGYI YE,[1]† JOHN F. QUENSEN III,[1] JAMES M. TIEDJE,[1,2] AND STEPHEN A. BOYD[1]*

Department of Crop and Soil Sciences[1] and Center for Microbial Ecology,[2] Michigan State University, East Lansing, Michigan 48824-1325

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When microorganisms eluted from upper Hudson River sediment were cultured without any substrate except polychlorobiphenyl (PCB)-free Hudson River sediment, methane formation was the terminal step of the anaerobic food chain. In sediments containing Aroclor 1242, addition of eubacterium-inhibiting antibiotics, which should have directly inhibited fermentative bacteria and thereby should have indirectly inhibited methanogens, resulted in no dechlorination activity or methane production. However, when substrates for methanogenic bacteria were provided along with the antibiotics (to free the methanogens from dependence on eubacteria), concomitant methane production and dechlorination of PCBs were observed. The dechlorination of Aroclor 1242 was from the *para* positions, a pattern distinctly different from, and more limited than, the pattern observed with untreated or pasteurized inocula. Both methane production and dechlorination in cultures amended with antibiotics plus methanogenic substrates were inhibited by 2-bromoethanesulfonic acid. These results suggest that the methanogenic bacteria are among the physiological groups capable of anaerobic dechlorination of PCBs, but that the dechlorination observed with methanogenic bacteria is less extensive than the dechlorination observed with more complex anaerobic consortia.

Methanogenic bacteria play an important role in the anaerobic carbon cycle (4, 10). There have been many observations of reductive dechlorination of chlorinated compounds either under methanogenic conditions (12, 13, 23) or by pure methanogenic strains (11, 15, 18). It has been proposed that methanogens, which are among the anaerobes that are rich in reduced transition metal cofactors (such as F_{430}), may catalyze reductive dechlorination in anaerobic habitats (22).

When dechlorination of PCBs has been observed in sediment slurries, concomitant methane production has usually been observed (19, 20, 25). However, in these studies workers could not determine whether methanogenic bacteria were among the microorganisms capable of PCB dechlorination or whether they functioned indirectly by maintaining a continuing electron flow through the food chain so that the dechlorination could proceed. Heat and ethanol treatments of mixed PCB-dechlorinating populations eliminated non-spore-forming bacteria, including methanogens, but a *meta*-preferential dechlorination activity was maintained (25), suggesting that involvement of methanogenic bacteria was not necessary for dechlorination. However, heat and ethanol treatments resulted in a loss of *para* dechlorination activity, perhaps because of elimination of some microorganisms. Methanogenic bacteria were among the microorganisms eliminated by the heat and ethanol treatments, but the relationship between methanogens and the activities lost from the heat and ethanol treatments was not determined. It is important activity to identify the role that methanogenic bacteria play in anaerobic reductive dechlorination of PCBs, so that comprehensive physiological and ecological understanding of the microbial community involved in anaerobic PCB dechlorination can be obtained. Such an understanding should improve PCB bioremediation strategies and facilitate isolation of microorganisms capable of PCB dechlorination.

* Corresponding author. Phone: (517) 252-2992.

† Present address: U.S. EPA Environmental Research Laboratory, Athens, GA 30613.

MATERIALS AND METHODS

Abbreviations. The following abbreviations are used in this paper: PCB, polychlorobiphenyl; BESA, 2-bromoethanesulfonic acid; CB, chlorobiphenyl; 3,4-2-CB, 2′,3,4-trichlorobiphenyl.

Approach. Methanogenic bacteria depend on fermentative bacteria for substrates. Addition of eubacterium-inhibiting antibiotics (penicillin G plus D-cycloserine) directly inhibits eubacteria and thus indirectly inhibits methanogens. However, when substrates for methanogenic bacteria are provided along with the antibiotics to free methanogens from dependence on eubacteria, methanogens should be enriched. There are three kinds of archaebacteria, extreme halophiles, extreme thermophiles, and methanogens. Only methanogens are enriched if the cultural conditions are not favorable for the other two groups. Therefore, if such a culture has any PCB dechlorination capacity, the responsible microorganisms are most likely methanogenic bacteria. The role that methanogens play in dechlorination can also be examined by determining the effect of BESA, a specific inhibitor of methanogens (2), on PCB dechlorination by methanogenic cultures. It is possible that BESA could inhibit dechlorination of PCBs by nonmethanogens by serving as a preferred electron acceptor. But if BESA inhibits both dechlorination and methane production in cultures that are treated with antibiotics and are given methanogenic substrates (such cultures are referred to as methanogenic cultures below) and does not inhibit dechlorination in untreated cultures inoculated from the same source, then the results support the hypothesis that methanogens are responsible for the dechlorination.

Inoculum preparation. Inoculum was prepared by eluting microorganisms from upper Hudson River sediment that had been collected near Hudson River Falls, N.Y. (site H7 in reference 8) as previously described (21).

Incubation conditions. Barker's medium (16) modified by adding $NaHCO_3$ (2.4 g/liter) and $Na_2S \cdot 9H_2O$ (0.28 g/liter) was used for enrichment of methanogenic bacteria.

For the initial methanogenic enrichment cultures and subsequent transfers, 160-ml serum bottles were used. The vessels were preincubated as described below to ensure that the conditions were strictly anaerobic (21). The serum bottles each contained 25 g of PCB-free air-dried Hudson River sediment and 15 ml of modified Barker's medium which contained 5% inoculum and 0.1% ethanol. After methane was detected, all vessels were autoclaved at 121°C for 1 h on 3 consecutive days with incubation at 37°C between each autoclaving treatment.

After the third autoclaving, the following preparations were added to each bottle while the bottle was being flushed with filter-sterilized O_2-free N_2-CO_2 (80:20, vol/vol) with a Hungate apparatus: 25 ml of Barker's medium, 25 ml of inoculum, 25 ml of sediment extract, and 100 μl of 10% (wt/wt) Aroclor 1242 (Monsanto Co., St. Louis, Mo.) in acetone. The sediment extract was prepared by autoclaving PCB-free Hudson River sediment with deionized water (1:1, wt/vol) for 1 h; the mixture was then shaken for 1 h, centrifuged, and filtered. The

final concentration of PCBs was 400 μg/g of dry sediment. In addition to the reductant in the Barker's medium (sodium sulfide), titanium(III) citrate (26) was also added to a final concentration of 0.2 mM. Stock solutions of penicillin G (Sigma Chemical Co., St. Louis, Mo.) and D-cycloserine (Sigma) were prepared, bubbled with O_2-free N_2-CO_2 (80:20, vol/vol) to drive out the dissolved oxygen, and then filter sterilized with 0.22-μm filters (Millipore, St. Louis, Mo.). Penicillin G and D-cycloserine were added to final concentrations of 3,340 U/ml and 0.1 g/liter of liquid, respectively. Methanogenic substrates (methanol, formate, and acetate) were also added each at a final concentration of 7 g/liter of liquid; these substrates were autoclaved. The bottles were then sealed with Teflon-coated butyl rubber stoppers and aluminum crimps and shaken thoroughly to completely disperse the PCBs. The controls were autoclaved twice at 121°C for 1 h and then kept for 5 h before PCBs were added.

For the BESA experiment we used the preparation procedure described above, with the following modifications: the experimental vessels were 60-ml serum bottles containing 10 g of PCB-free dry sediment; the liquid portion was proportionally reduced on the basis of the weight of sediment; antibiotics were added at zero time at the concentrations described above; and after 8 weeks antibiotics were added again at one-half the concentrations described above. The PCB used was 3,4-2-CB, and the methanogenic substrate provided was methanol (7 g/liter of liquid). BESA (Aldrich, Inc., Milwaukee, Wis.) was filter sterilized and added to a final concentration of 2 mM; the same amount of BESA was added again after 3 and 6 weeks of incubation.

Transfer of inocula. Cultures to be transferred were shaken for 30 min, and the slurry was then allowed to settle for about 30 min. A 25-ml portion of supernatant was transferred into each 160-ml serum bottle containing 25 ml of Barker's medium, 25 ml of sediment extract, and 25 g of preincubated PCB-free Hudson River sediment. PCB, titanium(III) citrate, and the antibiotics were then added as described above.

Analyses. Methane production was determined by gas chromatography with a flame ionization detector. The headspace gas was analyzed to determine methane production after a culture was shaken and before the slurry was sampled for PCB analysis. Cultures were incubated in the dark at 25°C without shaking. Samples were taken periodically, extracted, and analyzed for PCBs by capillary gas chromatography with electron capture detection as previously described (21).

RESULTS

Aroclor 1242 dechlorination by methanogenic cultures. Dechlorination of Aroclor 1242 occurred only in the cultures amended with both antibiotics and methanogenic substrates (the methanogenic cultures). Neither dechlorination nor methane production was observed in the autoclaved controls or the cultures amended with antibiotics but not substrates (referred to as the negative controls below). After 8 weeks of incubation the methanogenic cultures exhibited decreases in peaks 19 (3,4-2-CB; 2,3,4-CB; 2,3-3-CB; 2,5-2,6-CB), 35 (mainly 2,4,5-4-CB), 36 (2,5-3,4-CB; 3,4,5-2-CB), 37 (2,4-3,4-CB; 2,3,6-2,5-CB; 2,4,5-2,6-CB), 39 (mainly 2,3-3,4-CB and 2,3,4-4-CB), and 40 (2,4,5-2,5-CB; 2,3,5-2,4-CB), increases mainly in peaks 5 (2-3-CB), 9 (2,5-2-CB; 4-4-CB), 15 (2,5-3-CB), and 16 (2,4-3-CB), and smaller increases in peaks 3 (2-2-CB; 2,6-CB), 6 (2-4-CB; 2,3-CB), 17 (2,5-4-CB), and 18 (2,4-4-CB) (Fig. 1).

The extent of Aroclor 1242 dechlorination in the methanogenic cultures was assessed in two ways. In terms of the average number of chlorines removed from each biphenyl molecule, the dechlorination activity of the methanogenic cultures was low. An average of only 0.22 chlorine per biphenyl was removed during the 8 weeks of incubation. However, marked decreases of several congeners did occur as a result of dechlorination. As shown in Fig. 1, after 8 weeks of incubation, little change in peak 28 (2,3-2,5-CB) was observed, and therefore this peak was used as an internal reference. The molar ratios of several selected peaks to peak 28 were plotted (Fig. 2) to show differences among treatments in the concentrations of the congeners after 8 weeks of incubation. Changes in the concentrations of these congeners occurred only in the cultures amended with both antibiotics and methanogenic substrates (the methanogenic cultures). The ratios obtained for the methanogenic cultures after 8 weeks of incubation were compared with the ratios obtained at zero time (defined as 100%). After 8 weeks of incubation, peaks 19, 35, 36, 37, and 39 had decreased 53, 70, 78, 65, and 64%, respectively, while peaks 5, 15, and 16 had increased 263, 156, and 234%, respectively.

Several dechlorination pathways could be inferred from these decreases and increases in chromatographic peaks. The observed decreases in peaks 36 and 37 with concomitant increases in peaks 15 and 16 (Fig. 3A and B) suggested that 2,5-3-CB (peak 15) and 2,4-3-CB (peak 16) were the dechlorination products of 2,5-3,4-CB (in peak 36) and 2,4-3,4-CB (in peak 37), respectively (Fig. 3C). Similarly, 3,4-2-CB (peak 19) was probably dechlorinated to 2-3-CB (peak 5). All three of these transformations involve the removal of the *para* chlorines from 3,4-chlorophenyl groups.

BESA experiment. BESA was used to examine the role of methanogens in the dechlorination of 3,4-2-CB. BESA has been widely used as a specific inhibitor of methanogens, but it might inhibit PCB dechlorination by nonmethanogens through a different mechanism. PCB-dechlorinating microorganisms could use the PCBs as terminal electron acceptors in the absence of a more preferred electron acceptor. Considering that BESA is also a potential electron acceptor for some bacteria, it is possible that it could inhibit PCB dechlorination by nonmethanogens by competing with PCBs for electrons. Our experiment included a control group (Table 1, group 3) to distinguish whether inhibition of dechlorination by BESA was due to competition for electrons (which should also have inhibited dechlorination by the group 3 preparation) or due to the specific inhibition of methanogens.

Cultures not amended with BESA, antibiotics, or methanol (Table 1, group 5) produced methane and dechlorinated 3,4-2-CB from both the *meta* and *para* positions to produce 2-CB (Table 1). Addition of only BESA (2 mM BESA at 0, 3, and 6 weeks) (Table 1, group 3) prevented methanogenesis but not dechlorination (Table 1 and Fig. 4). Dechlorination from the *meta* position apparently was not affected, but *para* dechlorination was partially inhibited as both 2-CB and 2-4-CB accumulated as major dechlorination products (Table 1, group 3). No methanogenesis or dechlorination was observed in the negative control cultures receiving only antibiotics (Table 1 [group 4] and Fig. 4). Both methanogenesis and dechlorination were observed in cultures amended with both antibiotics and a methanogenic substrate (methanol) (Table 1 and Fig. 4), as in the Aroclor 1242 experiments. Furthermore, 2-3-CB was the major dechlorination product in these latter cultures (Table 1 [group 1]), indicating that the *para* chlorine was preferentially removed. Inclusion of BESA with the antibiotics and methanol prevented both methanogenesis and dechlorination (Table 1 and Fig. 4).

Dechlorination activity of the transferred cultures. Initially, two groups of cultures were prepared. The cultures in the first group were amended with both methanogenic substrates and antibiotics and were used in the experiments described above. The cultures in the second group were amended with the same methanogenic substrates but not antibiotics. Dechlorination of added Aroclor 1242 was observed in both of these groups (Fig. 5), and inocula from both groups were then transferred to Aroclor 1242-amended cultures. Dechlorination activity increased in the second serial cultures of the first group, while little dechlorination was observed in the second serial cultures of the group without antibiotics (Fig. 5).

DISCUSSION

In previously reported work methane production and PCB dechlorination always occurred simultaneously in PCB dechlorination assays performed with PCB-free Hudson River sediments and untreated microorganisms (no heat or ethanol

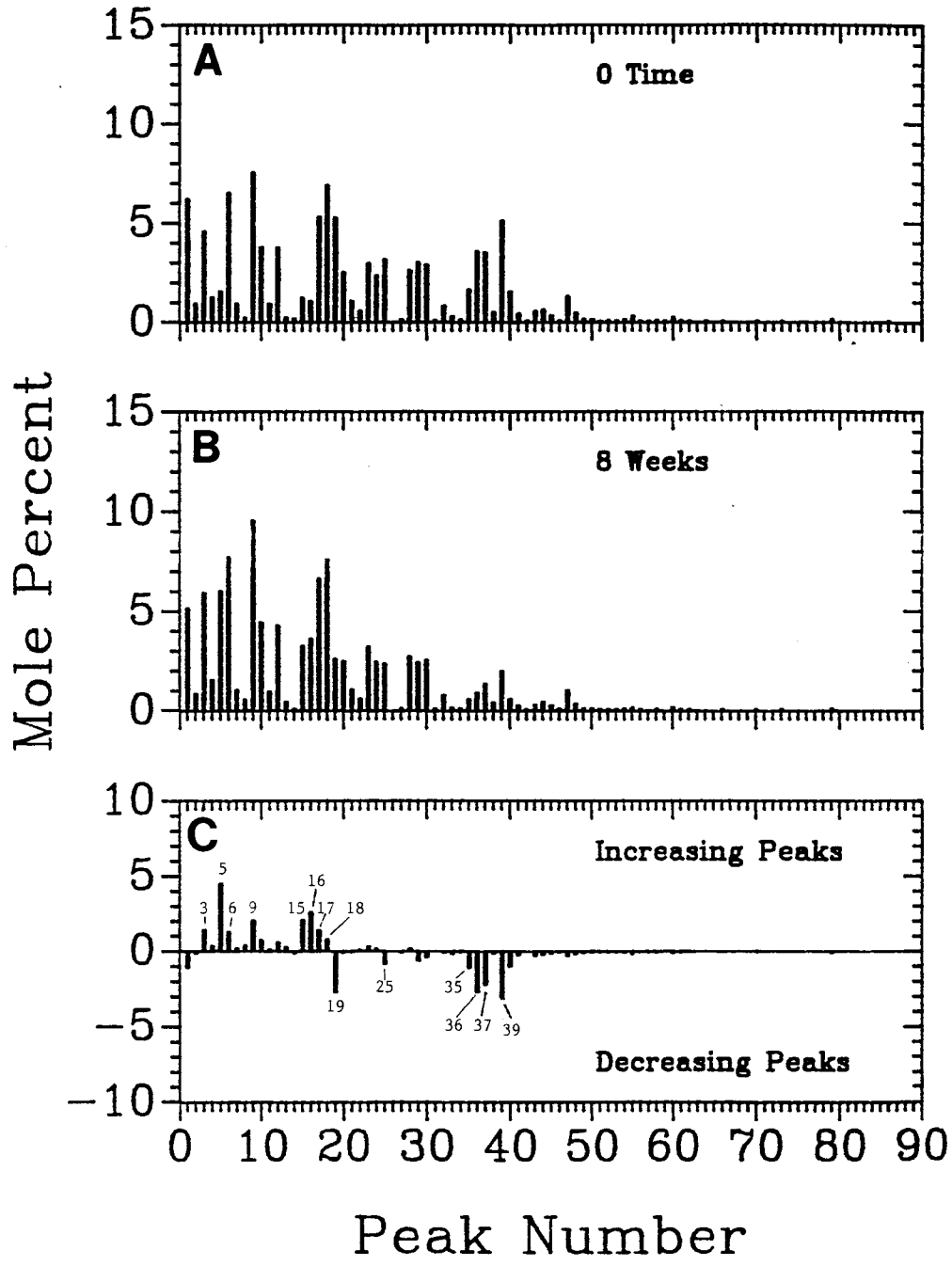

FIG. 1. Mole percentage of Aroclor 1242 represented by each chromatographic peak after 8 weeks of incubation in a methanogenic culture. (A) Zero time. (B) After 8 weeks. (C) Difference between the zero-time and 8-week values. For a complete list of the PCB congeners associated with each peak see reference 23.

treatment) eluted from PCB-contaminated sediments (19, 20, 25). In these assays, PCB dechlorination normally occurred from both the *meta* and *para* positions. But when inoculum from the sediments was pasteurized or treated with ethanol, methane was not produced and dechlorination from the *para* positions did not occur (25). These results suggested that methanogens might somehow be involved in dechlorination of PCBs from the *para* positions.

In order to investigate the role of methanogens in the dechlorination of PCBs, we sought a way to enrich for the methanogens in mixed cultures of Hudson River microorganisms. We used the approach of Zinder and Mah (27), who used a combination of penicillin G (3,340 U/ml) and D-cycloserine (0.1 g/liter) to inhibit eubacteria and thus aid in the isolation of a thermophilic *Methanosarcina* strain; in the experiments of Zinder and Mah the antibiotics successfully eliminated all eubacteria. Penicillin G and D-cycloserine specifically inhibit cell wall synthesis of eubacteria (24), and neither antibiotic inhibits archaebacteria, except that *Methanococcus vannielii* is sensitive to D-cycloserine (5). In our experiments we used the same concentrations of these antibiotics that Zinder and Mah used, although the incubation temperature that they used for enrich-

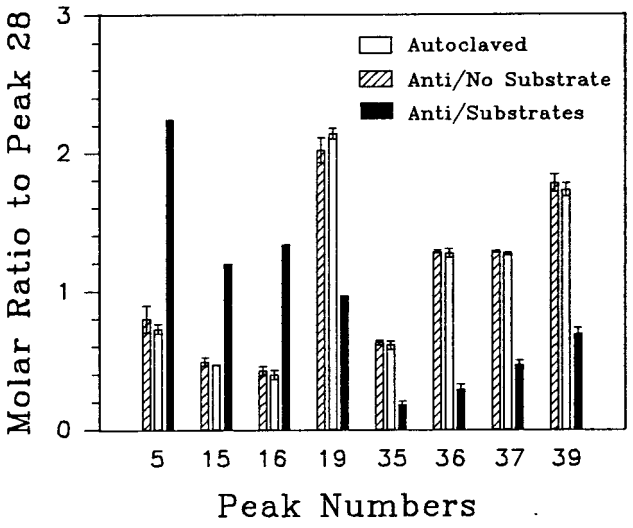

FIG. 2. Molar ratios of several selected peaks to peak 28 after 8 weeks of incubation with different amendments. The error bars indicate the standard deviations of duplicate samples; where error bars are not shown, they are smaller than can be shown. Anti, penicillin G and D-cycloserine. The substrates used were methanol, formate, and acetate.

ment was 55°C, while we used 25°C in our experiments. The antibiotics should have been effective longer in our cultures than in the culture of Zinder and Mah because the antibiotics are more labile at higher temperatures. The direct inhibition of eubacteria by penicillin G and D-cycloserine might be expected to indirectly inhibit methanogens by preventing the formation of methanogenic substrates by the eubacteria. For this reason it was necessary to provide methanogenic substrates in addition to the antibiotics in order to enrich for methanogens. Under these culture conditions, only archaebacteria should have been able to grow. Since our culture conditions were favorable for only methanogenic archaebacteria, the enriched microorganisms were probably methanogenic bacteria. Thus, we deduced that the dechlorination activity that we observed in the presence of the antibiotics and methanogenic substrates was due to methanogens.

The effects of the antibiotics on PCB dechlorination were evidenced in three ways. First, addition of antibiotics in the absence of methanogenic substrates (the negative controls) resulted in no dechlorination activity or methane production, indicating that the eubacteria were effectively inhibited. Second, the original cultures in Barker's medium containing methanogenic substrates exhibited dechlorination activity both in the presence and in the absence of antibiotics (Fig. 5). However, the dechlorination activity of the cultures containing antibiotics increased after subsequent transfer, while the dechlorination activity of the original cultures that lacked antibiotics decreased with time (data not shown) and was almost lost after subsequent transfer (Fig. 5). This may have occurred because in the absence of antibiotics the microorganisms responsible for the dechlorination activity were outcompeted by some nondechlorinating eubacteria stimulated by the methanogenic substrates. Third, the methanogenic cultures (i.e., the cultures amended with both antibiotics and methanogenic substrates) gave a different dechlorination pattern than the pattern typically found in dechlorination assays conducted with Hudson River microorganisms without antibiotics and methanogenic substrates; fewer congeners were dechlorinated, and dechlorination appeared to be limited to the *para* positions.

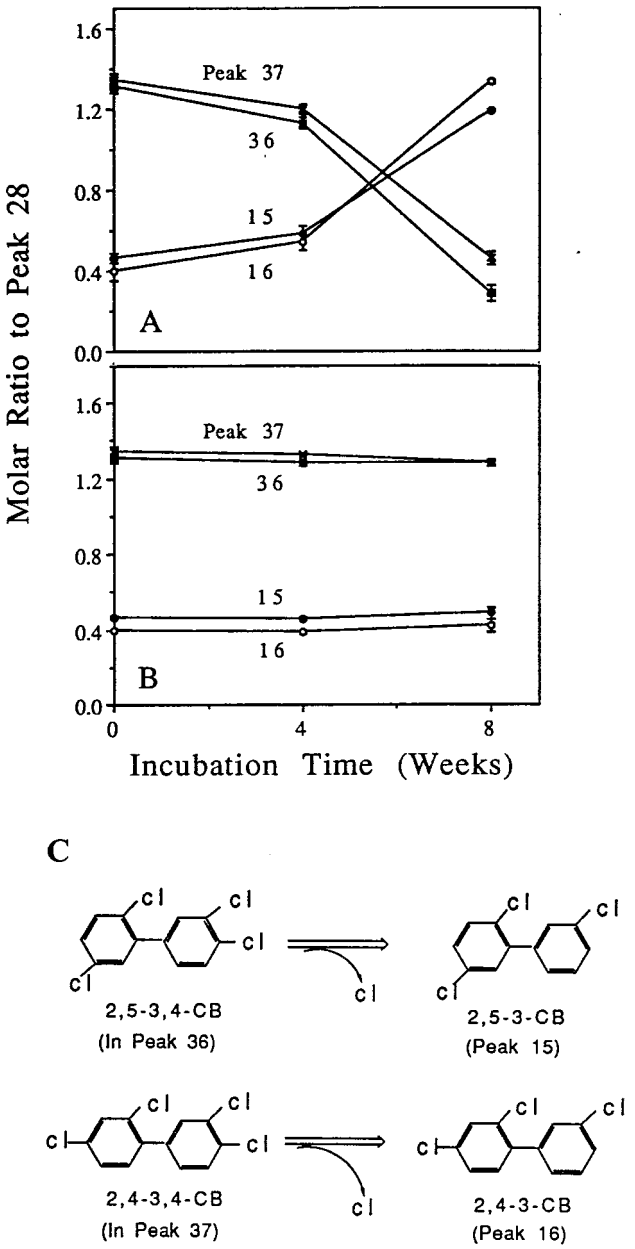

FIG. 3. Changes in peaks 15, 16, 36, and 37 after 8 weeks of incubation with a methanogenic culture (A) and a negative control group (with antibiotics but without methanogenic substrates (B). (C) Proposed dechlorination pathway for congeners present in peaks 36 and 37. The error bars indicate the standard deviations of duplicate samples; where error bars are not shown, they are smaller than the symbols.

Several different environmental PCB dechlorination patterns have been reported (6–9), and such patterns have also been observed in laboratory experiments (1, 25). These different dechlorination patterns have been explained by Abramowicz et al. (1), Brown et al. (8), and Quensen et al. (21) as due to the activities of different populations of PCB-dechlorinating microorganisms, each with its characteristic congener specificity. Processes M, N, Q, H, and H′ have all been observed in experiments performed with upper Hudson River sediments (3). Process M is capable of removing flanked and unflanked *meta* chlorines from 2,3-, 2,5-, 3,4-, 2,3,4-, 2,3,6-, and possibly

TABLE 1. Effects of BESA on dechlorination of 3,4-2-CB and methane production

Group	Amendments	Dechlorination	CH_4 production	Predominant product(s)
Methanogenic cultures				
1	Antibiotics, substrate	+[a]	+	2-3-CB
2	Antibiotics, substrate, BESA	−	−	
Controls				
3	BESA only, no antibiotics, no substrate	+	−	2-CB, 2-4-CB
4	Antibiotics only, no substrate	−	−	
5	No BESA, no antibiotics, no substrate	+	+	2-CB[b]

[a] +, positive; −, negative.
[b] Both 2-3-CB and 2-4-CB were intermediate products.

3-chlorophenyl groups. Pattern M dechlorination was observed in assays performed with pasteurized and ethanol-treated inocula prepared from Hudson River sediments (25). Process N is capable of removing flanked and doubly flanked *meta* chlorines from 3,4-, 2,3,4-, 2,3,6-, 2,4,5-, 2,3,4,5-, 2,3,4,6-, and 2,3,4,5,6-chlorophenyl groups. Process Q is capable of removing flanked and unflanked *para* chlorines from 4-, 2,4-, 3,4-, 2,4,5-, and possibly 2,3,4-chlorophenyl groups and of removing *meta* chlorines from 2,3- and possibly 2,3,4-chlorophenyl groups. Process H is capable of removing flanked and doubly flanked *para* chlorines from 3,4-, 2,4,5-, 2,3,4,5-, 2,3,4,5,6-, and possibly 2,3,4,6-chlorophenyl groups and of removing the *meta* chlorine from a 2,3,4-substituted ring. Process H′ is similar but can also remove the *meta* chlorine from a 2,3-substituted ring. Thus, only processes Q, H, and H′ are capable of removing *para* chlorines.

Of the latter three dechlorination processes, the dechlorination activity of our methanogenic enrichment cultures is most similar to process H. If process Q were active in the methanogenic enrichment cultures, unflanked *para* chlorines would also be removed, so that peaks 6 (2-4-CB), 10 (2,4-2-CB), and 18 (2,4-4-CB) would decrease, but they do not. If process H′ were active, peaks 12 (2,3-2-CB) and 20 (2,3-4-CB) would decrease, but they do not. Because of the pattern of Aroclor 1242 dechlorination observed in the methanogenic enrichment cultures, we propose that methanogens are at least among the microorganisms responsible for process H dechlorination.

The results of the BESA experiment strengthen the interpretation described above. First, the addition of penicillin, D-cycloserine, and methanol selected against *meta* dechlorination and for *para* dechlorination of 3,4-2-CB. Both *meta* and *para* dechlorination of 3,4-2-CB occurred in unamended cultures (Table 1 and Fig. 4), with the proportion of 2-4-CB peaking at 4 weeks (data not shown). However, in the cultures amended with antibiotics and methanol, only *para* dechlorination of 3,4-2-CB to 2-3-CB occurred (Table 1). Thus, the addition of antibiotics and methanol selected for a dechlorination pathway consistent with process H. Second, both this specific dechlorination activity and methanogenesis were inhibited when BESA was also added to cultures amended with antibiotics and methanol (Table 1 and Fig. 4). It is also noteworthy that addition of BESA alone apparently had little effect on *meta* dechlorination but partially inhibited *para* dechlorination as less 2-3-CB accumulated and much more 2-4-CB persisted (Table 1).

While it is possible that BESA can inhibit the dechlorination of PCBs by non-methanogenic bacteria, probably by serving as a preferential electron acceptor, we believe that the action of

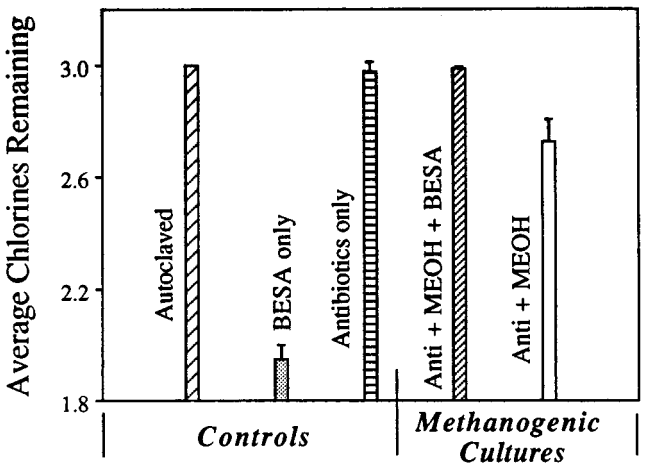

FIG. 4. Effects of BESA on dechlorination of 3,4-2-CB. The error bars indicate the standard deviations of triplicate samples; where error bars are not shown, they are smaller than can be shown. Anti, penicillin G and D-cycloserine; MEOH, methanol.

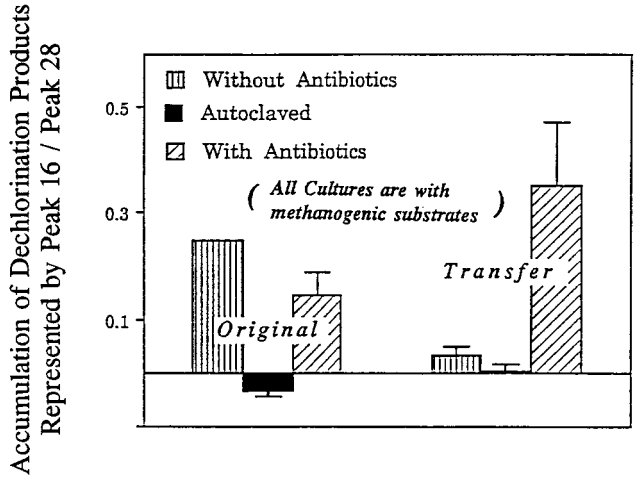

FIG. 5. Dechlorination of Aroclor 1242 by initial cultures and after subsequent transfers of both methanogenic cultures (containing both methanogenic substrates and antibiotics) and cultures amended with methanogenic substrates but not antibiotics. The dechlorination activities are represented by the accumulation of the dechlorination product, peak 16 (normalized to peak 28), after 4 weeks of incubation. The error bars indicate the standard deviations of duplicate samples; where error bars are not shown, they are smaller than can be shown.

BESA on the dechlorination of 3,4-2-CB in these experiments was due to the specific inhibition of methanogens. We have previously observed that BESA inhibits dechlorination of PCBs by pasteurized cultures containing no methanogens. In this case, cultures that received high concentrations of BESA (50 mM at zero time and 25 mM at 2, 4, 6, and 8 weeks) turned black, indicating that metal sulfide(s) was formed, and gave off a sulfide smell when they were sampled while being flushed with N_2-CO_2 (data not shown). These observations suggested that BESA was probably used as an electron acceptor and that its inhibitory effect on PCB dechlorination might be due to the competition of BESA with PCBs for electrons. Similar inhibitory effects of BESA on PCB dechlorination (19) and on anaerobic dechlorination of other compounds (14) have been reported previously. For this reason in our experiments a control group was used to determine whether the inhibitory effect of BESA on dechlorination by the methanogenic cultures was due to the inhibition of methanogens or due to the possible competition for electrons. As shown in Table 1, in the control group (group 3) no methane production was observed, while PCB dechlorination did occur. This meant that BESA at the concentration used effectively inhibited methanogenesis but allowed dechlorination by some other microorganisms. It is also significant that the *para* dechlorination activity that we have ascribed to methanogens was inhibited in the control group. The dechlorination that occurred was primarily from the *meta* position. Thus, the results obtained with the control group indicate that inhibition of dechlorination by BESA was due to direct inhibition of methanogens instead of competition with PCB for electrons. The high concentration of added methanol (218 mM) should also rule out the possibility that BESA competed for electrons, because this concentration was high enough to effectively scavenge BESA in case BESA was used as an electron acceptor. These results support the hypothesis that methanogens are responsible for *para* dechlorination (process H).

The results of May et al. (17) are consistent with our results which implicate methanogens in process H dechlorination. May et al. found that cultures repeatedly plated on solid medium simultaneously lost the ability to produce methane and the ability to dechlorinate (2,5-3,4-CB and 3,4-2-CB to 2,5-3-CB and 2-3-CB, respectively. Both of these transformations are expected from process H activity.

Direct proof of the ability of methanogens to dechlorinate PCB would preferably come from studies performed with pure cultures of methanogens. However, we have not been able to demonstrate such activity either in methanogens obtained from sediment or in other pure cultures of methanogens (19). It may, however, be difficult to recreate the conditions that are necessary for PCB dechlorination in pure culture. Thus, studies such as this study in which community activities are dissected provide the best insight into the microbial groups responsible.

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