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Hydrogen as an Electron Donor for Dechlorination of Tetrachloroethene by an Anaerobic Mixed Culture

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Hydrogen served as an electron donor in the reductive dechlorination of tetrachloroethene to vinyl chloride and ethene over periods of 14 to 40 days in anaerobic enrichment cultures; however, sustained dechlorination for more extended periods required the addition of filtered supernatant from a methanol-fed culture. This result suggests a nutritional dependency of hydrogen-utilizing dechlorinators on the metabolic products of other organisms in the more diverse, methanol-fed system. Vancomycin, an inhibitor of cell wall synthesis in eubacteria, was found to inhibit acetogenesis when added at 100 mg/liter to both methanol-fed and hydrogen-fed cultures. The effect of vancomycin on dechlorination was more complex. Methanol could not sustain dechlorination when vancomycin inhibited acetogenesis, while hydrogen could. These results are consistent with a model in which hydrogen is the electron donor directly used for dechlorination by organisms resistant to vancomycin and with the hypothesis that the role of acetogens in methanol-fed cultures is to metabolize a portion of the methanol to hydrogen. Methanol and other substrates shown to support dechlorination in pure and mixed cultures may merely serve as precursors for the formation of an intermediate hydrogen pool. This hypothesis suggests that, for bioremediation of high levels of tetrachloroethene, electron donors that cause the production of a large hydrogen pool should be selected or methods that directly use H_2 should be devised.

Chlorinated ethenes (ETHs) are widely used as solvents and chemical feedstocks. Not surprisingly, groundwater contamination by chlorinated ETHs has become a serious problem in the industrialized world. A 1984 survey of water supplies in the United States listed tetrachloroethene (PCE), trichloroethene (TCE), and the three dichloroethene (DCE) isomers as the five most frequently found contaminants, other than trihalomethanes (25). These compounds pose a public health concern and are therefore regulated by the 1986 amendments to the Safe Drinking Water Act (9).

Fully chlorinated compounds (such as PCE), which appear to be recalcitrant in aerobic environments (10, 20, 23), have been shown to be readily dechlorinated in anaerobic systems via a reductive dehalogenation mechanism (5, 12, 21). Complete, stepwise, reductive dechlorination of PCE to ETH is possible, as demonstrated in studies conducted with mixed PCE-methanol (MeOH) methanogenic enrichment cultures (12).

We recently reported the complete dechlorination of high concentrations of PCE to ETH by an MeOH-fed anaerobic enrichment culture (5). An added PCE concentration of 550 μM was routinely dechlorinated to 80% ETH and 20% vinyl chloride (VC) within 2 days at 35°C. The observed transformations occurred in the absence of methanogenesis, which was apparently inhibited by the high concentrations of PCE. An electron balance demonstrated that MeOH consumption was completely accounted for by dechlorination (31%) and acetate production (69%). The high rates of PCE dechlorination—up to 1.24 mg of PCE per mg of volatile suspended solids per day (4)—and the relatively large fraction (ca. one-third) of the supplied electron donor used for dechlorination suggest that reductive dechlorination could be exploited for the bioremediation of sites contaminated by chlorinated ETHs.

The promise of anaerobic bioremediation for chlorinated ETHs has not yet been realized, largely because of a lack of understanding of microbiological fundamentals. Despite nearly a decade of study, we still do not know the roles played by general categories of microorganisms inhabiting mixed-culture, dechlorinating enrichments (methanogens and acetogens, etc.). We know little about nutritional and process requirements, including the identities of the immediate reductants involved in the dechlorination processes. Consequently, we cannot explain the observed variability in the extent of transformation among many field and laboratory studies. PCE transformation stops sometimes at TCE (7, 14) but in other instances at DCEs (22) or VC (24); some field and laboratory studies have demonstrated complete dechlorination to ETH (5, 12, 19). It is clear that process reliability awaits a better understanding of process fundamentals. A better fundamental understanding would also facilitate modeling of the in situ fate of chlorinated ETHs.

Glucose, acetate, formate, MeOH, and hydrogen have each been shown capable of sustaining the reductive dechlorination of PCE (12), as have lactate, propionate, crotonate, butyrate, and ethanol (13). More exotic reductants, such as toluene (22) and dichloromethane (11), also have been observed to support the dechlorination of PCE. The fact that such a wide variety of electron donors sustains dechlorination suggests that it may be the simplest—hydrogen—that serves as the direct electron donor for the process; the other, more complex donors may merely serve as precursors for hydrogen formation via fermentative metabolism (13).

In this paper, we present a further characterization of our high-PCE enrichment culture—specifically, results from comparative studies with MeOH- and hydrogen-fed mixed anaerobic cultures amended with selective inhibitors (van-

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comycin, a eubacterial inhibitor, and 2-bromoethanesulfonic acid [BES], an inhibitor of methanogenesis). The results provide additional evidence that hydrogen is the direct electron donor for PCE reduction. We also report on the probable roles of major classes of organisms in PCE dechlorination.

MATERIALS AND METHODS

Chemicals. PCE, TCE, *trans*-1,2-DCE, *cis*-1,2-DCE, and 1,1-DCE were obtained in neat liquid form (1- or 5-g ampoules; Supelco, Inc.) for use as analytical standards. High-performance liquid chromatography (HPLC)-grade PCE (>99.9% pure; Aldrich Chemical Co.) and HPLC-grade MeOH (99.9% pure; Fisher Scientific) were used as culture substrates. Hydrogen (>99.95% pure) also served as a culture substrate and was purchased from Linde Specialty Gases and Equipment. VC, ETH, and methane were obtained as gases (>99% pure; Scott Specialty Gases). Vancomycin hydrochloride was purchased from Sigma Chemical Co., and BES was obtained from Aldrich.

Cultures and enrichment procedures. All experiments were conducted with 100-ml culture suspensions in duplicate 160-ml serum bottles. Duplicates always performed similarly; therefore, the figures depicted are representative. Teflon-lined butyl rubber septa were held in place with aluminum crimp caps to maintain anaerobic conditions. The integrity of the septum-seal system with regard to retaining PCE was verified with PCE-water controls, and the role of microbial (versus abiotic) activity in mediating the observed reductive dechlorination of PCE has been demonstrated (12). The bottles were incubated at 35°C with liquid in contact with the septa to minimize losses of volatile compounds. Bottles that received MeOH were incubated quiescently, whereas hydrogen-fed bottles were incubated in an orbital shaker bath to facilitate the transfer of hydrogen to the liquid phase. Neat PCE was added directly to serum bottles to achieve an initial concentration of 0.55 mM or 91 mg/liter (a nominal concentration arrived at by ignoring headspace-liquid partitioning within bottles; the actual aqueous concentration was approximately 60% of the nominal concentration [15]). The quantity of PCE added to a culture bottle in neat form was estimated directly from the delivered syringe volume.

A 6-liter PCE-MeOH enrichment culture was maintained at 35°C as an inoculum source for a variety of studies conducted with 160-ml serum bottles. The source reactor was a stirred, 9.6-liter bottle containing 6 liters of liquid and sealed with a Teflon-lined rubber gasket and a stainless steel plate (4). The inoculum for the 6-liter culture was the anaerobic (nonmethanogenic) enrichment culture that had developed the ability to dechlorinate high concentrations of PCE to ETH (5). The 6-liter culture was prepared by adding 200 ml of inoculum to 5.8 liters of basal salts medium in the 9.6-liter bottle. The basal medium contained (among other constituents) Na_2S and $FeCl_2$ as reductants and bicarbonate as a pH buffer; the recipe was modified from that described previously (12): the $NaHCO_3$ concentration was increased to 6 g/liter, and yeast extract was omitted. Every 2 days, the following procedures were performed on the 6-liter source culture: headspace analysis was completed, gas production was measured, and produced gas was vented; the culture was thoroughly purged via two cannulas with 70% N_2–30% CO_2 for 15 min to remove VC and ETH, which would otherwise accumulate to inhibitory levels; 600 ml of basal medium was anaerobically transferred to the reactor; the reactor contents were vigorously mixed; 600 ml of the mixed culture was removed; and the reactor received (via a syringe) 9.4 mmol of neat MeOH (50 mg/liter), 3.3 mmol of neat PCE (a 91-mg/liter nominal concentration), and 120 mg of yeast extract (20 mg/liter) from an anoxic, aqueous stock solution. With these procedures, a nominal hydraulic retention time of 20 days was maintained. The 6-liter culture was maintained in accordance with this protocol for well over three hydraulic retention times prior to the initiation of experiments.

Studies with hydrogen as an electron donor. The ability of hydrogen to sustain the dechlorination of PCE was investigated with four 160-ml serum bottles (100-ml liquid volume), each prepared by inoculating 90 ml of basal medium with 10 ml of the PCE-MeOH culture. Hydrogen (11.5 ml; 469 μmol) was added via a locking, gas-tight syringe to two bottles every 2 days. For comparison purposes, the other two bottles received MeOH (5 mg from a 25% aqueous stock) as the electron donor every 2 days. On a reducing-equivalent basis, hydrogen (11.5 ml; 938 μeq) and MeOH (5 mg; 938 μeq) were provided in identical doses. The four bottles also received (every 2 days) neat PCE (55 μmol) and yeast extract (0.2 mg from an anoxic, aqueous stock). Bottles were purged via a cannula with 70% N_2–30% CO_2 prior to each feeding. By day 6, each bottle had transformed its initial PCE dose; semicontinuous operation commenced at this point. Semicontinuous operation consisted of (every 2 days) gas purging, removing (via a syringe) 10 ml of the mixed culture, and adding 10 ml of basal medium. PCE, yeast extract, and hydrogen or MeOH were then added. Declining ETH production was observed in all bottles from days 0 to 15 (see Results). Micronutrient limitation was suspected; consequently, the yeast extract dose was increased to 2 mg after day 15. From days 20 to 34, culture removal was suspended because ETH production had declined in all bottles; batch additions of PCE, yeast extract, and hydrogen or MeOH were continued. After day 34, ETH production had increased to over 20 μmol per feeding in the MeOH bottles, and semicontinuous operation (every 2 days) was resumed for all bottles.

Preparation of a filtered culture supernatant. For extended studies involving the demonstration of hydrogen as an electron donor, cultures were sometimes amended with a filtered culture supernatant as a source of growth factors. Preparation of the filtered culture supernatant included the following steps: (i) 600 ml of the PCE-MeOH culture was anaerobically transferred from the 6-liter source culture to a 2-liter batch anaerobic reactor; (ii) a small volume (ca. 10%) of an aceticlastic methanogenic enrichment culture was added to consume acetate (normally present at high concentrations in the PCE-MeOH enrichment reactor; its removal was necessary to increase the precision of later results from electron balance determinations); (iii) when measurements indicated that <0.5 mM acetate remained (usually 5 to 8 days after inoculation), the culture was anaerobically transferred to centrifuge tubes inside a glove box and centrifuged (4,080 × *g*) outside the glove box, and the resulting material was subsequently filtered (0.45-μm-pore-size filter) inside the anaerobic glove box to remove organisms; and (iv) since the resulting filtrate obviously contained none of the black iron sulfide precipitate that normally helped maintain a low redox potential in our cultures, a precipitate from the basal medium was added to the filtrate.

Inhibitor studies. The inhibitors vancomycin and BES were applied to PCE-MeOH and PCE-H_2 cultures to investigate the roles of acetogens and methanogens in PCE

reduction. In an anaerobic glove box, a PCE-MeOH inoculum from the 6-liter culture was transferred to six 400-ml polypropylene bottles, which were then capped and centrifuged outside the glove box. The bottles were returned to the glove box, the supernatant was discarded, and filtered culture supernatant was added to resuspend the pellet. (Treatment with filtered culture supernatant in this manner reduced the acetate concentration of the culture, facilitating the monitoring of subsequent small changes in acetate levels during experimental runs.) The contents from all six bottles were combined, and 100-ml aliquots of the suspension were transferred to 16 160-ml serum bottles. Teflon-lined butyl rubber septa and aluminum crimp caps were applied to the bottles as before.

The bottles were removed from the glove box and purged for 5 min with 70% N_2–30% CO_2. (The glove box atmosphere was of an N_2-H_2 composition; bottle purging was done to replace the N_2-H_2 headspace with N_2-CO_2.) During purging, 10 mg (100 mg/liter) of vancomycin was added to some bottles. After purging was complete, 2 mg of yeast extract (20 mg/liter) and 156 μmol of MeOH (50 mg/liter) or 469 μmol of hydrogen (11.5 ml) were delivered to the bottles. PCE at 55 μmol (91 mg/liter) was added to some bottles; others received no PCE and served as controls. Later in the study, some bottles received 0.025 mmol of BES (0.25 mM). The bottles were maintained in batch mode; every 2 days, purging and additions of PCE, yeast extract, and MeOH or hydrogen were done.

Analysis of organic compounds and hydrogen. The total mass of each volatile organic compound (PCE, TCE, DCE isomers, VC, ETH, and methane) within a serum bottle culture was determined by a method involving a single 0.5-ml headspace gas injection into a complex network involving the simultaneous use of two gas chromatographs (GCs; Perkin Elmer model 8500), two analytical columns, a multiport air-actuated switching valve (Valco, Inc.), and two flame ionization detectors as previously described (5). Initial identification and confirmation of volatile organic compounds were described previously (12). Coefficients of variation [(standard deviation/mean) × 100] for calibration factors (relating the total mass of each volatile compound in a bottle to the GC peak area response) ranged from 0.59 to 2.8%.

Hydrogen was measured by a GC technique involving the injection of a 0.5-ml headspace sample into a stainless steel column (3.2 mm by 3.05 m) packed with 100/120 Carbosieve S-II and coupled to a thermal conductivity detector. The GC oven was kept at 150°C for 1.5 min for this analysis. Nitrogen served as the carrier gas.

MeOH was also analyzed by a GC technique with a Perkin-Elmer model 8500 GC. A 0.5-μl aqueous sample was injected into a Nukol fused-silica capillary column (0.53 mm by 1.5 m; Supelco) connected to a flame ionization detector. The oven temperature was programmed as follows: 100°C for 3 min and ramp at 20°C/min to 160°C. The sample was filtered through a 0.45-μm-pore-size syringe filter (Gelman Sciences) and acidified with 2 N HCl prior to injection. A standard calibration curve was developed for MeOH. Peak area responses from subsequent samples were compared with the standard curve to obtain MeOH concentrations.

Acetate and formate analyses were performed with a Hewlett-Packard 1090 HPLC coupled to a 300-mm HPX-87H ion-exchange column (Bio-Rad Laboratories). When only acetate was to be quantified, a Fast-Acid column (100 mm by 7.8 mm; Bio-Rad) was substituted. The Fast-Acid column, although incapable of resolving formate and acetate, was preferred because of its shorter run time when acetate was the only organic acid to be measured. Flow from the column in use was directed to a refractive index detector (Perkin-Elmer LC-25) whose response was processed by a calculating integrator (Perkin-Elmer LCI-100). Samples of 100 μl were used for all HPLC analyses. H_2SO_4 (0.013 N) served as the mobile phase and was pumped through the 300-mm column at 0.7 ml/min and through the Fast-Acid column at 0.8 ml/min. The 300-mm column was fitted with a water jacket that kept the temperature at 65°C, while the Fast-Acid column was used at an ambient temperature. Sample preparation included filtration (0.45-μm-pore-size filter), acidification, and refrigeration. Standard calibration curves were developed for formate and acetate. Peak area responses from subsequent samples were compared with the standard curves to obtain formate and acetate concentrations.

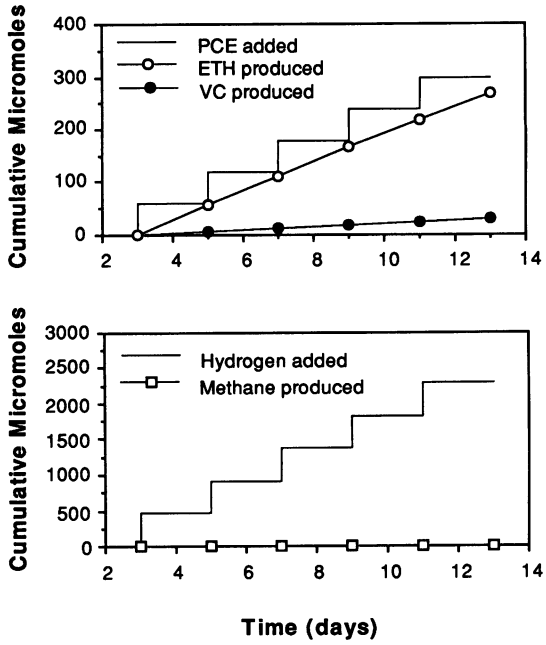

FIG. 1. Short-term study results demonstrating the ability of hydrogen to support the dechlorination of 55-μmol repetitive additions of PCE to ETH. Bottles (total volume, 160 ml) contained 100 ml of liquid.

RESULTS

Hydrogen as an electron donor for PCE reduction. We first tested whether H_2 can serve as an electron donor for PCE dechlorination in short-term studies. PCE-MeOH cultures in 160-ml serum bottles were switched from MeOH to H_2 as the electron donor and incubated in a shaking water bath. The dose of H_2 used was twofold in excess of the stoichiometric requirement for the complete dechlorination of PCE to ETH (as had been true of the MeOH doses preceding the switch). As shown in Fig. 1, such cultures immediately were able to use H_2 to convert PCE to ETH and small amounts of VC. Controls, to which H_2 was not added, did not dechlorinate PCE (data not shown).

These initial studies demonstrated that hydrogen could serve as an electron donor for PCE dechlorination—at least over the short term—in bottles started with a 100% inoculum

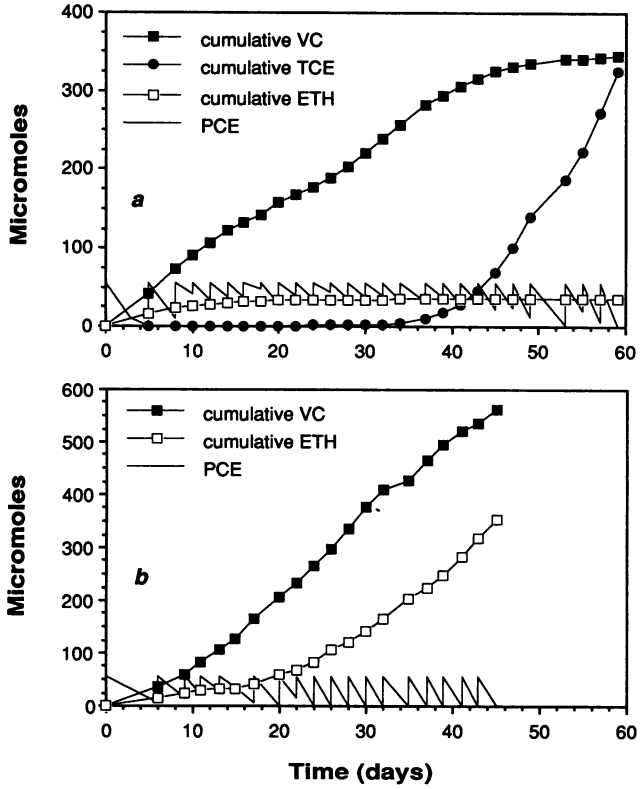

FIG. 2. Comparison of PCE reduction in a hydrogen-fed bottle (a) and an MeOH-fed bottle (b).

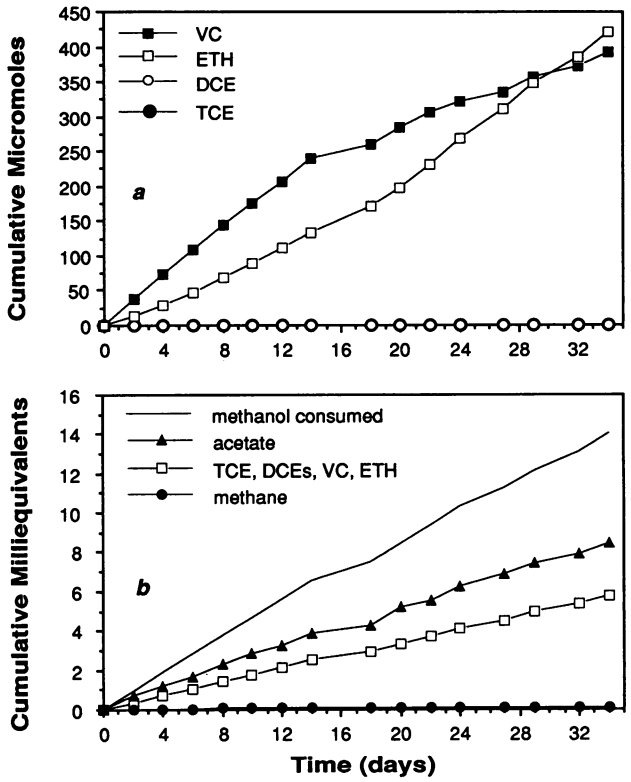

FIG. 3. PCE reduction (a) and MeOH metabolism (b) in an MeOH-fed culture.

from the PCE-MeOH culture. We next prepared a more rigorous test of hydrogen, this time starting with a 10% PCE-MeOH inoculum and extending the study over 90 days to investigate possible nutritional deficiencies that might arise from the utilization of hydrogen in place of MeOH.

Figure 2 shows the performances of hydrogen-fed and MeOH-fed bottles. VC production and ETH production in the hydrogen-fed and MeOH-fed bottles were similar for the first 10 days. ETH production in the hydrogen-fed bottles diminished to insignificant levels by day 10. VC production continued; however, residual PCE was routinely detected. By contrast, the MeOH-fed bottles transformed each PCE dose (after day 20) to VC and ETH. TCE was rarely detected, and insignificant levels of DCEs were produced. As the study progressed, the hydrogen-fed bottles experienced a progressively diminishing ability to dechlorinate. Note from Figure 2 the dramatic increase in TCE levels beginning after day 35. It seemed that hydrogen could serve as an electron donor but could not sustain PCE reduction.

The inability to sustain dechlorination to VC and ETH could have been due to a lack of growth factors in the hydrogen-fed bottles—factors that may have been produced in the MeOH-fed bottles. Serum bottles were prepared with filtered culture supernatant from a PCE-MeOH enrichment culture to test this hypothesis. The 6-liter PCE-MeOH culture provided seed organisms for this experiment. The PCE-MeOH culture (100 ml) was centrifuged, and the resultant supernatant was removed. The inoculum pellet was suspended in sufficient filtered culture supernatant to restore the 100-ml liquid volume. A batch mode of operation was adopted to avoid dilution of the presumed growth factors contributed by the supernatant diluent. Every 2 days, after headspace analysis, purging and feeding were done as described above.

The results (not shown) indicated that hydrogen-fed bottles could sustain dechlorination when treated with a filtered culture supernatant approximately every 30 days (4). To demonstrate that hydrogen (as opposed to the filtered culture supernatant) was supplying reducing equivalents for the dechlorination of PCE to ETH, we withheld hydrogen from the bottles on an every-other-feeding basis from day 96 onward. The purging procedure and PCE and yeast extract additions were done during every feeding. Incubation intervals with hydrogen resulted in the conversion of all added PCE to VC and ETH, with more ETH than VC being formed. During intervals in which hydrogen was withheld, 20 to 30 μmol of residual PCE was detected, and ETH production was insignificant.

Application of inhibitors to MeOH- and hydrogen-fed cultures. Inhibitors were applied to the PCE-MeOH or PCE-H_2 cultures to investigate the roles of different microbial populations in PCE reduction. Vancomycin is an inhibitor of cell wall synthesis in eubacteria to which methanogens are usually insensitive (1). Although methane production was low in the high-PCE culture, this fact alone does not rule out a role for methanogens in PCE reduction. BES, considered a selective inhibitor of methyl coenzyme M reductase, the enzyme that catalyzes the final step in methanogenesis (16), was therefore used to inhibit methanogenesis in the high-PCE culture.

The performance of an MeOH-fed bottle receiving no inhibitors is shown in Fig. 3. VC production and ETH

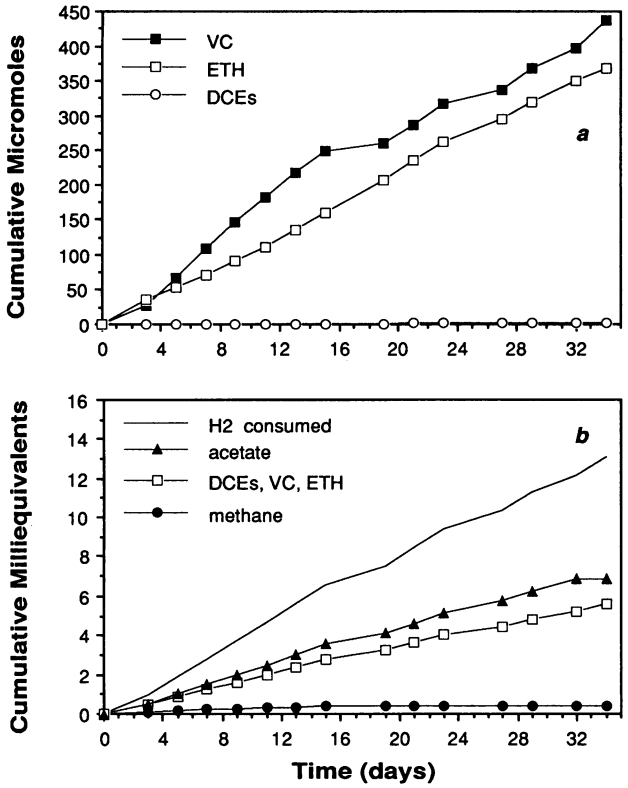

FIG. 4. PCE reduction (a) and hydrogen metabolism (b) in a hydrogen-fed culture.

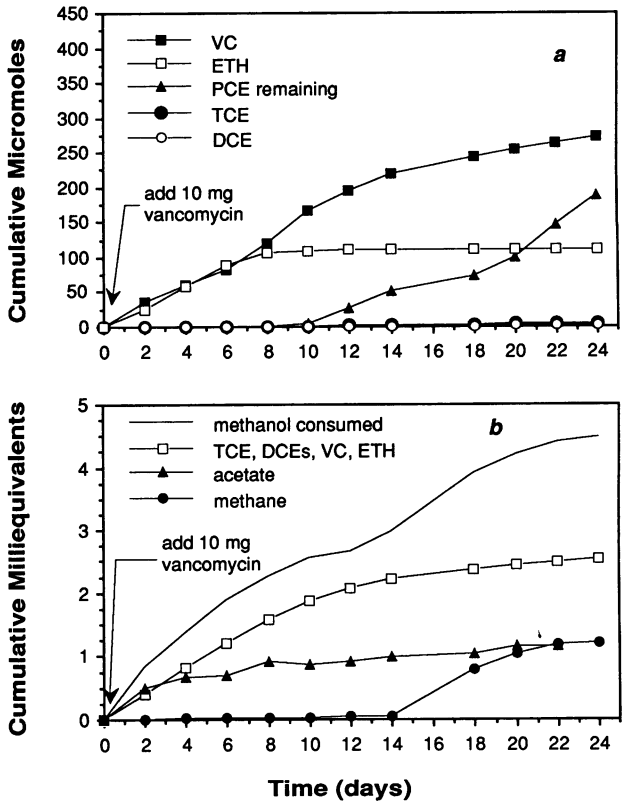

FIG. 5. PCE reduction (a) and MeOH metabolism (b) in an MeOH-fed culture amended with vancomycin.

production were steady up to day 14; afterwards, VC production decreased slightly as ETH production increased. Insignificant levels of TCE and DCEs sometimes occurred, and residual PCE was never detected. MeOH metabolism and the resultant products are shown in Fig. 3b, as is a summation of dechlorination products, to allow comparison with the usage of MeOH, the presumed electron donor. Residual MeOH was never detected, so the amount of MeOH consumed was essentially equal to the amount of MeOH added. Acetogenesis accounted for almost 60% of electron donor use, and reduced products from PCE dechlorination represented over 40%. Methane production was insignificant. These results are similar to a previously reported distribution of MeOH use (5).

PCE reduction and hydrogen metabolism in a bottle receiving no inhibitors are shown in Fig. 4. PCE reduction to VC and ETH occurred during every incubation interval. Insignificant levels of DCEs sometimes occurred; TCE or residual PCE was never detected. VC production was higher than that of ETH up to day 15; VC production and ETH production were similar thereafter. (The superiority of the dechlorination in this hydrogen-fed system to that in the system depicted in Fig. 2a was doubtless due to the 10-fold-higher inoculum level used and the consequent 10-fold-higher level of suspected growth factors from the PCE-MeOH inoculum.) Hydrogen metabolism and the resultant products are shown in Fig. 4b. Added hydrogen was used completely during each incubation interval. Acetogenesis accounted for 54% of electron donor use, and reduced products from PCE dechlorination represented nearly 45%. Methane production was low; PCE apparently inhibited methanogenesis from H_2-CO_2 as well as from acetate.

Vancomycin was added to two MeOH-fed and two hydrogen-fed bottles to observe potential effects on PCE reduction. Results for a vancomycin-amended, MeOH-fed bottle are shown in Fig. 5. Figure 5a indicates that 100 mg of vancomycin per liter had a profound effect on PCE reduction. Essentially equal amounts of VC and ETH were produced up to day 8. Thereafter, ETH production ceased and VC production declined. Increasing amounts of residual PCE were observed beginning on day 10. Figure 5b shows only slight acetate production after day 8, thus demonstrating the inhibitory effect of vancomycin on acetogens (a vertical scale smaller than that of Fig. 3b was used for clarity). From days 8 to 14, nearly all MeOH utilization could be accounted for in the products of PCE reduction. However, only slight increases in dechlorination products were evident after day 14. Methane production from MeOH became significant between days 14 and 18, when we allowed a 4-day interval between successive PCE doses. The amounts of residual PCE were probably insufficient to suppress methanogenesis over this prolonged interval. This result confirms that vancomycin, at the dose used, did not severely inhibit methanogenesis. Note that methane production decreased over ensuing 2-day intervals between feedings. Residual MeOH was routinely detected. The MeOH level was adjusted to 156 μmol per bottle (50 mg/liter) during each feeding to validate the comparison between vancomycin-amended bottles and those not amended with vancomycin.

PCE reduction and hydrogen metabolism in a vancomy-

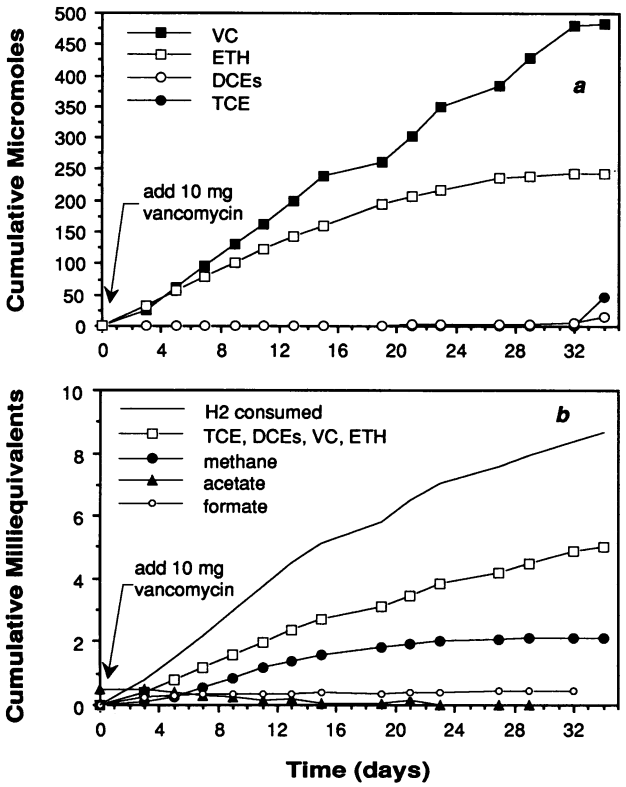

FIG. 6. PCE reduction (a) and hydrogen metabolism (b) in a hydrogen-fed culture amended with vancomycin.

cin-amended bottle are shown in Fig. 6. Figure 6a indicates that PCE reduction by this hydrogen enrichment culture continued in the presence of vancomycin. VC production was similar to that in hydrogen-fed bottles receiving no vancomycin (Fig. 4) throughout the study. ETH production was similar up to day 16 but declined to low levels by day 27. TCE and DCEs were detected on day 34; residual PCE was never observed. Inhibition of acetogenesis by vancomycin was apparent from the low levels of acetate production shown in Fig. 6b (a vertical scale smaller than that of Fig. 4b was used for clarity). Since it is possible that the low levels of acetate detected in vancomycin-amended bottles were due to consumption by aceticlastic methanogens rather than inhibition of acetogenesis, bottles containing hydrogen and vancomycin were later dosed with BES and did not accumulate acetate (results not shown), making it unlikely that aceticlastic methanogens were consuming acetate being produced in these cultures. The products of PCE reduction accounted for nearly 60% of the hydrogen use in vancomycin-amended bottles. Methane production represented a significant fraction of the remainder of hydrogen use, primarily from days 0 to 20. Methane production declined after day 19; however, the levels of PCE reduction products (primarily VC) continued to increase. Residual hydrogen was routinely detected in the vancomycin-amended bottles. Hydrogen did not accumulate, since it was removed during purging. All hydrogen-fed bottles began each incubation interval with an equivalent hydrogen dose. Thus, the dechlorinating performance of the vancomycin-treated PCE-hydrogen cultures was similar to that of cultures not treated with vancomycin, while acetogenesis was strongly inhibited and methanogenesis was stimulated.

The decline in ETH production and the detection of TCE and DCEs indicated that the hydrogen-fed bottles amended with vancomycin were slowly losing the capability to dechlorinate PCE. Treatment of the vancomycin-amended culture with filtered culture supernatant (as described earlier) plus vancomycin was done twice (days 36 and 64), with the expectation that growth factors would stimulate PCE reduction. Batch feeding and purging were done every 2 days throughout this study. After the first treatment, from days 36 to 64, VC production was steady and accounted for most of the dechlorinated PCE. Only low levels of ETH were detected throughout this cycle. Small amounts of TCE and DCEs were routinely detected. Improved dechlorination was evident after the second treatment on day 64. Increased ETH production occurred up to day 79; the production of TCE and DCEs increased thereafter. VC production remained consistent throughout each cycle. This treatment successfully restored ETH production, although to a lower rate than that observed from days 0 to 15.

The effects of BES on the dechlorination of PCE in MeOH- and hydrogen-fed systems were examined. Earlier results (Fig. 3b and 4b) had indicated that methane production was negligible in PCE-fed MeOH and hydrogen enrichment cultures, suggesting that methanogens play no significant role in dechlorination. Surprisingly, 0.25 mM BES inhibited PCE dechlorination in an MeOH-fed system (data not shown), with no apparent effect on acetogenesis. Up to day 27, this bottle performed like its replicate (Fig. 3), as expected. However, after BES addition on day 27, VC production and ETH production were low. TCE and DCEs accumulated, but residual PCE did not. A similar inhibition of dechlorination was observed with a hydrogen-fed system.

DISCUSSION

Hydrogen was able to serve as the electron donor in the reductive dechlorination of PCE to VC and ETH over periods of 14 to 40 days; however, sustained dechlorination for more extended periods required the addition of filtered culture supernatant from a presumably more diverse, MeOH-fed system. This result suggests a nutritional dependency of hydrogen-utilizing dechlorinators on the activities of other organisms.

MeOH-PCE and H_2-PCE systems without vancomycin or BES performed similarly with respect to electron donor use; acetate production accounted for a majority of reducing equivalents, with the remainder being accounted for by PCE reduction. The predominance of acetogens in the inoculum was also evident from the MeOH- and hydrogen-fed bottles from which PCE was withheld (data not shown). MeOH-utilizing methanogens were apparently present in much lower numbers than MeOH-utilizing acetogens; acetate-utilizing methanogens were virtually absent.

Vancomycin effectively inhibited acetogenesis in MeOH- and hydrogen-fed bottles of all types. Vancomycin is a eubacterial peptidoglycan synthesis inhibitor, is bacteriocidal, and is more effective against gram-positive than gram-negative eubacteria (1, 17). It is unlikely that vancomycin directly inhibited acetogenesis in the cultures but rather the inhibition was probably due to the bacteriocidal effects of vancomycin on acetogens, most of which are gram positive (18). The major MeOH-utilizing acetogen detected in the culture was a gram-positive coccus that was present in numbers exceeding 10^7/ml (26).

The effects of vancomycin on PCE dechlorination were mixed. PCE reduction in MeOH enrichment cultures was

inhibited within 1 week, like acetogenesis. In contrast to MeOH-fed bottles, hydrogen-fed bottles continued to dechlorinate well after the inhibition of acetogenesis and could do so for long periods of time when supplemented with filtered culture supernatant, like hydrogen-fed bottles lacking vancomycin. The difference in the response to vancomycin of hydrogen- and MeOH-fed bottles could be explained if hydrogen was the actual electron donor for dechlorination in both systems. It is possible that acetogens in the MeOH-fed culture were the major utilizers of MeOH and served two purposes for the PCE dechlorinators: the production of reducing equivalents in the form of hydrogen and the production of unknown nutritional factors apparently required by H_2-utilizing dechlorinators. Reductive dechlorination is considerably more thermodynamically favorable than acetogenesis (5), and the use of acetogen-produced hydrogen by organisms carrying out more thermodynamically favorable reactions has been observed (3). Moreover, interspecies nutritional codependencies are well known among anaerobes. The fact that the PCE dechlorinators were resistant to 100 mg of vancomycin per liter suggests that they were resistant eubacteria (perhaps gram negative) or methanogenic archaebacteria.

The methanogenesis inhibitor BES inhibited dechlorination beyond TCE in MeOH- and hydrogen-fed systems. This result provides circumstantial evidence for the involvement of methanogens in reductive dechlorination—in the absence of appreciable methanogenesis itself. Fathepure et al. (6–8) reported the transformation of PCE to TCE by pure methanogenic cultures but observed a stoichiometric relationship between methanogenesis and dechlorination and noted that BES inhibited dechlorination. They proposed that electrons transferred during methanogenesis are diverted to PCE by a reduced electron carrier involved in methane production. For us to conclude a dechlorinating role for methanogens in our cultures, based solely upon the observation that BES inhibited dechlorination, presumes that BES-induced inhibition is specific to methanogens because of the structural similarity of BES to coenzyme M, which is unique to methanogens. However, BES is a brominated alkane and bears structural similarity to PCE and its reduced products. It is conceivable that BES would inhibit reductive dechlorination regardless of whether the dechlorinating organism was a methanogen.

Taken together, our vancomycin and BES results with PCE-MeOH- and PCE-hydrogen-fed systems support the hypothesis that the dechlorinating organisms were hydrogen utilizers, possibly (although not necessarily) methanogens. Sustained dechlorination in the presence of vancomycin (which demonstrably inhibited acetogenesis) suggests that acetogens were probably not the dechlorinators.

Figure 7 shows the hypothetical roles of methanogens and acetogens in our PCE-MeOH enrichment cultures; high levels of PCE inhibited methanogenesis from MeOH, acetate, and hydrogen. Vancomycin inhibited acetogens, thereby stopping the source of reducing equivalents for PCE reduction. We suggest that dechlorination continued in hydrogen-fed systems amended with vancomycin because the proposed electron donor, hydrogen, was always available. In this study, PCE reduction products accounted for 60% of hydrogen use in vancomycin-amended bottles.

If borne out by subsequent studies, our hypothesis that H_2 is the actual electron donor for PCE dechlorination has practical consequences for bioremediation. It explains why a wide variety of electron donors has been observed to support PCE dechlorination in mixed-culture systems: glucose,

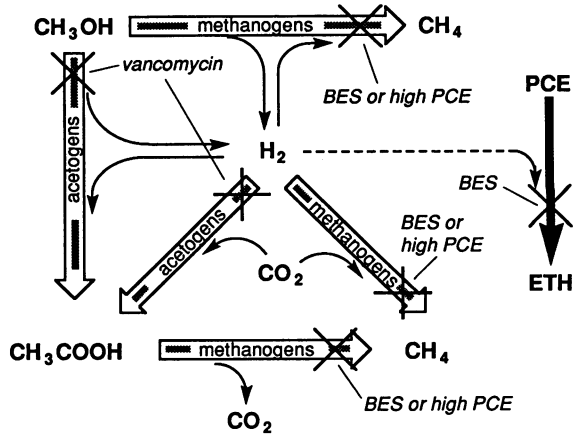

FIG. 7. Hypothetical roles of methanogens and acetogens in hydrogen- and MeOH-fed PCE-dechlorinating systems.

acetate, formate, and MeOH (12); sucrose (2); lactate, propionate, crotonate, butyrate, and ethanol (13); and even cocontaminants, such as toluene (22) or dichloromethane (11). All likely produce at least a small pool of hydrogen, and a small pool would be sufficient to transform parts-per-billion levels of PCE. For the bioremediation of relatively high PCE levels, electron donors that cause the production of a large H_2 pool should be selected or methods that directly use H_2 should be devised. One caveat is the apparent nutritional dependency of H_2-utilizing dechlorinators on other organisms that we observed. If dechlorinating organisms in other systems have the same or similar nutritional requirements, the required nutritional factor(s) must be identified and abiotically supplied, or it may be impossible to exclusively target electron donors solely to the dechlorinators.

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