APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1992, p. 4051–4054
0099-2240/92/124051-04$02.00/0

Vol. 58, No. 12

Subculturing of a Polychlorinated Biphenyl-Dechlorinating Anaerobic Enrichment on Solid Media

HAROLD D. MAY,* ALFRED W. BOYLE, W. ALLEN PRICE II, AND CHERYL K. BLAKE

Celgene Corp., 7 Powder Horn Drive, Warren, New Jersey 07059

Received 8 June 1992/Accepted 21 September 1992

An anaerobic culture capable of dechlorinating polychlorinated biphenyls was subcultured under strict anaerobic conditions on solid media containing sterilized river sediment. The dechlorination activity was transferred as a bacterial colony on a solid medium three times. After two transfers on solid medium, the culture was no longer methanogenic but still dechlorinated a mixture of tri- and tetrachlorobiphenyls. This demonstrates that anaerobic bacteria are responsible for the polychlorinated biphenyl dechlorination and can be grown without polychlorinated biphenyl on solid media.

Polychlorinated biphenyls (PCBs) have been a public concern for several decades because of their persistence in the environment, ability to accumulate in animal tissue, and potential carcinogenicity (22). The PCBs usually reach the environment as a complex mixture of PCB homologs and isomers. There are 209 different PCB homologs and isomers that are collectively known as congeners. Commercial PCBs (Aroclors) are mixtures of these congeners and were used in electrical transformers and capacitors. Although PCBs were thought to be recalcitrant, recent studies have shown that PCBs have been extensively dechlorinated in some environments (5, 6).

In the laboratory, many PCB congeners can be reductively dechlorinated under anaerobic conditions (18, 20, 21) or oxidatively degraded by aerobic microorganisms (2, 3). Isolation of the aerobic organisms has included the use of traditional techniques such as growth on a solid medium. Such techniques have led to the biochemical and physiological investigation of aerobic PCB transformation (1, 2, 12, 19). The successful transfer of activity from anaerobic sediments to fresh sterile sediment and the lack of activity in sterilized sediment suggest that the anaerobic activity is biologically mediated (18, 20, 21). However, the anaerobic activity has never been directly attributed to bacterial activity, and no dechlorinating organism has been cultured on solid medium. Hence, no PCB-dechlorinating anaerobe has been isolated, nor has the biochemical mechanism of reductive PCB dechlorination been elucidated.

Although dechlorination of an aryl chlorine has been shown to occur under many anaerobic conditions (4, 13–16), only one microorganism that reductively dehalogenates a chlorinated aromatic has been isolated (24). The dechlorinating organism is now classified as a sulfate-reducing anaerobe capable of aryl reductive dehalogenation (*Desulfomonile tiedjei* DCB-1) (7). *D. tiedjei* can dechlorinate chlorinated aliphatics (11) and halobenzoates (10), but it has not been shown to dehalogenate PCBs. The ability to culture *D. tiedjei* on an artificial medium has enabled researchers to characterize the reductive dechlorination of chlorobenzoate (9, 10, 17).

Using a combination of anaerobic enrichment procedures, we attempted to subculture PCB-dechlorinating anaerobes on solid media to isolate an anaerobic PCB-dechlorinating organism(s). The initial enrichments were prepared in duplicate and contained the following: Hudson River H7 sediment (15%, wt/vol) (20) as an inoculum, sterile reduced anaerobic mineral medium (RAMM; 60%, vol/vol) (23), Spier Falls sediment (33%, wt/vol), Aroclor 1242 (400 ppm), 2,3,6-trichlorobiphenyl (2,3,6-CB; 100 μM), 2,4,6-CB (100 μM), 2,2′,6,6′-CB (70 μM); cysteine-HCl (0.1%, wt/vol), and $Na_2S \cdot 9H_2O$ (0.025%, wt/vol). One set of enrichment cultures also received 20 mM Na_2SO_4. The PCBs were added as acetone solutions. The final concentration of acetone was 0.1% (vol/vol). The enrichment cultures were incubated under N_2 at 30°C in the dark in 50-ml serum bottles capped with Teflon-coated butyl rubber stoppers and aluminum crimp seals. The Hudson River H7 sediment and Hudson River Spier Falls sediment were supplied by General Electric Co. (Schenectady, N.Y.) and were collected at river miles 193.5 and 205, respectively, of the Hudson River. In later work, Raritan River sediment (Hunterdon County, N.J.) was used in place of Spier Falls sediment. Analysis of the Raritan River and Spier Falls sediments showed these sediments to be PCB free, and we observed no difference in dechlorinating activity when Raritan River sediment was used in place of Spier Falls sediment.

The sulfate-amended enrichments were transferred to medium containing a mixture of 2,3,4-CB (105 μM), 2,4,5-CB (210 μM), 2,3′,4′-CB (49 μM), 2,3′,4′,5-CB (39 μM), and no sulfate or Aroclor. These cultures were streaked onto anaerobic slants consisting of RAMM and one of the following: methanol (200 μl at the base of the slant), cellulose (0.1%, wt/vol), fatty acids (2.5 mM each sodium acetate, sodium propionate, and sodium butyrate), or PCB-free sediment (0.5%, wt/vol). The sediment-containing medium was prepared by first sterilizing a slurry of the sediment in RAMM for 3 h at 121°C and then solidifying the medium with Noble agar (1.5%, wt/vol; Difco, Detroit, Mich.). The medium was boiled, dispensed into 23-mm-diameter tubes under a stream of O_2-free N_2, and then autoclaved again for 20 min. No growth was observed on the uninoculated sediment-containing agar medium. Colonies from the streaked slants were inoculated into anaerobic slurries of sediment and RAMM containing the four congeners mentioned above. Screening for activity was done in aluminum crimp seal-type anaerobic culture tubes (18 by 150 mm; Bellco Glass, Inc., Vineland, N.J.).

The enrichment cultures were sampled anaerobically by the methods of Quensen et al. (20). The PCBs were extracted

* Corresponding author.

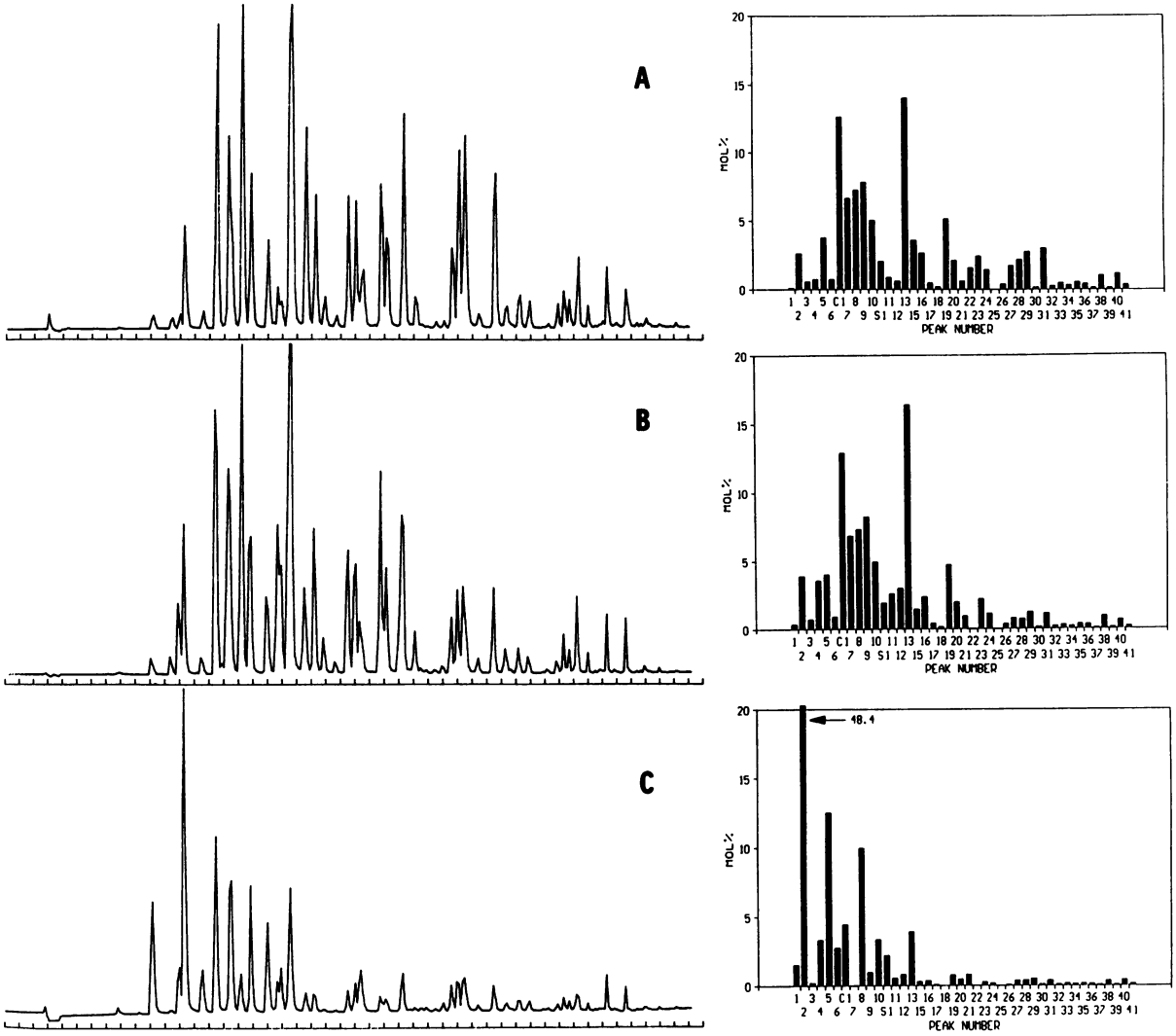

FIG. 1. Chromatograms and mole percentage plots of PCBs in enrichment cultures after 15 weeks of incubation with 2,3,6-CB, 2,4,6-CB, 2,2',6,6'-CB, and Aroclor 1242. Cultures were sterilized (A), amended with 20 mM sulfate (B), or not amended (C).

in ethyl acetate (5:1 solvent/sample volume ratio) and then passed over a Florisil-copper powder column as described by Quensen et al. (20). The PCB congeners were analyzed by passaging 1-μl samples through a Tracor (Austin, Tex.) 9000 Series gas chromatograph equipped with a J&W Scientific (Folsom, Calif.) DB-1 capillary column (30 m by 0.25 mm) and an electron capture detector. Helium was used as the carrier gas. The oven program for Aroclor analysis was as follows: a 0.25-min hold at 140°C, a 2.67-min 140 to 180°C gradient, a 15-min hold at 180°C, a 1.0-min 180 to 200°C gradient, a 10-min 200 to 280°C gradient, and finally a 3.0-min hold at 280°C. The products were identified by comparison of retention times with those of standards (Accustandard, New Haven, Conn.). For quicker analysis of single congeners, the oven program was changed to the following: a 0.25-min hold at 140°C, a 2.67-min 140 to 180°C gradient, a 15.5-min hold at 180°C, a 2.0-min 180 to 280°C gradient, and finally a 3.0-min hold at 280°C. Peak integration was performed with Nelson (Norwalk, Conn.) 2100 integrator software.

The enrichment cultures that initially received Na_2SO_4 were used for the subculturing experiments described here. The enrichment cultures expressed no activity until after the sulfate was depleted (9 weeks) (detection limit of 1.4 mM with EM Quant test strips [EM Science, Gibbstown, N.J.]). After 15 weeks of incubation, the activity observed was very different from that of a nonamended enrichment culture (Fig. 1; Table 1). The pattern of dechlorination from the sulfate enrichment cultures suggested that *para* chlorine removal occurred only with congeners containing *para* and *meta* chlorines that were adjacently positioned. The observation of different dechlorination patterns suggests that different dechlorinating organisms were enriched. There are precedents for more than one population of PCB-dechlorinating anaerobes being present in the Hudson River (6, 8).

The initial sulfate enrichment cultures were transferred with the single congener mixture (see above) three times and then streaked onto agar-containing medium. After 1 month of incubation, colonies were picked from the solid medium and screened for dechlorinating activity. The only culture that showed dechlorinating activity came from a slant containing river sediment. Analysis of the first active screen

TABLE 1. PCB congener designation corresponding to peak numbers in Fig. 1

Peak no.	Congener(s) in peak	Peak no.	Congener(s) in peak
1	2-CB	21	2,2',4,4'-CB
2	2,2'-CB, 2,6-CB	22	2,2',4,5-CB, 2,4,4',6-CB
3	2,4-CB, 2,5-CB	23	2,2',3,5-CB
4	2,3'-CB	24	2,2',3,4'-CB, 2,3,3',6-CB, 3,4,4'-CB
5	2,4'-CB, 2,3-CB	25	2,3',4',6-CB, 2,2',3,4-CB, 2,3,4',6-CB, 2,3',5,5'-CB
6	2,2',6-CB	26	2,2',3,3'-CB
C1	2,4,6-CB	27	2,4,4',5-CB, 2,2',3,5,6-CB
7	3,4-CB, 3,4'-CB	28	2,3',4',5-CB, 2,3',4',5'-CB
8	2,2',4-CB	29	2,2',3,5',6-CB, 2,2',4,5,6-CB, 2,3',4,4'-CB
9	2,3,6'-CB, 2,3,6-CB	30	2,3,3',4-CB, 2,2',3,4',6-CB
10	2,2',3-CB, 2,4',6-CB	31	2,3,3',4'-CB, 2,3,4,4'-CB
S1	2,2',6,6'-CB	32	2,2',4,4',6,6'-CB, 2,2',3,3',6-CB, 2,2',3,3',5-CB
11	2,3',5-CB	33	2,2',4,5,5'-CB, 2,2',3,4',5-CB
12	2,3',4-CB	34	2,2',4,4',5-CB
13	2,4',5-CB, 2,4,4'-CB, 2,2',4,6-CB	35	2,2',3,4',6,6'-CB 2,3,3',5,6'-CB, 2,3',4,4',6-CB
15	2',3,4-CB, 2,3,4-CB, 2,3,3'-CB 2,2',5,6'-CB	36	2,2',3',4,5-CB, 2,2',3,4,5-CB, 2,2',3,5,6,6'
16	2,3,4'-CB, 2,2',4,6'-CB	37	2,2',3,4,5'-CB, 2,3,4,4',6-CB, 2,3,3',5,5'-CB
17	2,2',3,6-CB	38	3,3',4,4'-CB, 2,3,3',4',6-CB
18	2,2',3,6'-CB	39	2,2',3,3',4-CB
19	2,2',5,5'-CB, 2,3',5',6'-CB	40	2,3',4,4',5-CB, 2,2',3,4',5',6-CB, 2,3,3',4,5-CB
20	2,2',4,5'-CB	41	2,2',3,3'4,6'-CB, 2,3,3',4,4'-CB

tube after 24 weeks indicated that the *para* and possibly *meta* chlorines of the supplied congeners were removed (Fig. 2B). This was determined by the presence of 2,3'-CB, which could only be produced if a *para* chlorine was removed from 2,3',4'-CB, and by an increase in the peak that represents 2,4- and/or 2,5-CB. This active culture was resupplied with PCBs and sterile sediment slurry; additional activity was seen after 2 more weeks of incubation. During this period the culture produced 127.4 μmol of CH_4 with sediment as its sole source of carbon.

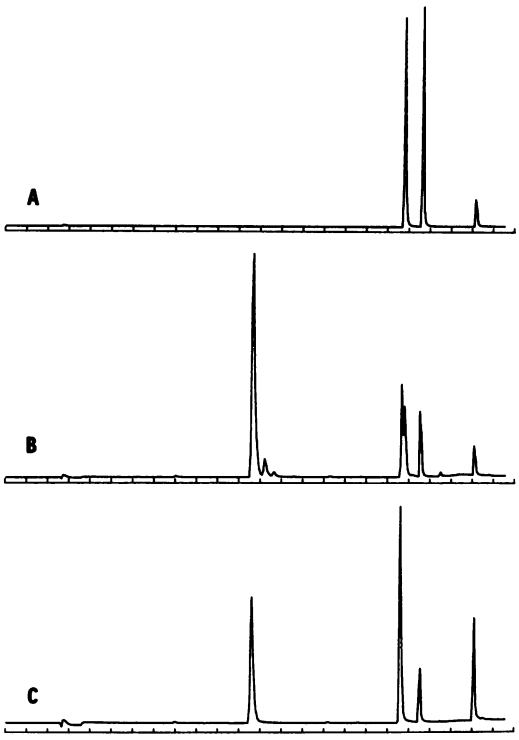

FIG. 2. Dechlorination of PCBs by the first active screen in sediment slurries after inoculation (A) and after 24 weeks of incubation on solid medium (B). Chromatogram C represents the activity in sediment slurries after 12 weeks of incubation of a colony that had been cultured twice on solid media. Retention times of congeners: 2-CB, 8.0 min; 2,4- and/or 2,5-CB, 11.8 min; 2,3- and 2,4'-CB, 12.1 min; 2,3'-CB, 12.3 min; 2, 4,5-CB, 18.9 min; 2,3,4- and 2,3',4'-CB, 19.8 min; 2,3',4',5-CB, 22.2 min.

The procedure of growth on solid medium followed by reintroduction into sediment slurry was repeated; two of the eight colonies selected expressed dechlorinating activity in the screen tubes between 4 and 8 weeks of incubation. A chromatogram demonstrating the activity from one of these colonies appears in Fig. 2C. Growth on solid medium was repeated once more; one of the four selected colonies was dechlorinating after 41 days. The PCB-dechlorinating colonies that had been grown on solid medium for a second or third time were not methanogenic in the sediment slurries. In contrast to the colonies that had been grown only once on the solid medium, the cultures that were passed two or three times on solid medium produced only 2,4-CB and/or 2,5-CB after 12 weeks of incubation in the sediment slurries. The ability of these cultures to dechlorinate other congeners is still under investigation.

This is the first report of anaerobic bacteria being removed from river sediment, grown as colonies on solid media, and then dechlorinating PCBs after being reintroduced into sediment slurries. The repetitive transfer of PCB-dechlorinating activity by the transfer of a bacterial colony proves that anaerobic dechlorination of PCBs in sediment is biologically mediated. This has been accomplished by using RAMM and 0.5% (wt/vol) river sediment with 1.5% (wt/vol) agar to solidify the medium; therefore, we can also conclude that this PCB-dechlorinating organism(s) does not require PCBs for growth. A pure culture of this dechlorinating organism(s) has not been isolated, but the ability to culture these organisms as colonies on a solid medium may have allowed for some selection of the dechlorinating organism(s). Our success rate of selecting active colonies suggests that a consortium may be required for the dechlorinating activity to occur. James Tiedje was recently able to grow anaerobic bacteria from an actively dechlorinating sediment on a heterotrophic solid medium (25). Transfer of these bacteria

from the agar surface back into sediment resulted in PCB-dechlorinating activity. This result is in agreement with our findings which show that anaerobic bacteria are responsible for the dechlorination observed and can be grown without PCB on solid media.

This work was performed under contract with the General Electric Co.

We thank Dan Abramowicz, Donna Bedard, and their colleagues for their helpful discussion and assistance. We also thank Guang-Run Chen and Nicole White for their technical assistance.

REFERENCES

1. **Bedard, D. L.** 1990. Bacterial transformation of polychlorinated biphenyls Adv. Appl. Biotechnol. Ser. **4**:369–388.
2. **Bedard, D. L., M. L. Haberl, R. J. May, and M. J. Brennan.** 1987. Evidence for novel mechanisms of polychlorinated biphenyl metabolism in *Alcaligenes eutrophus* H850. Appl. Environ. Microbiol. **53**:1103–1112.
3. **Bedard, D. L., R. Untermann, L. H. Bopp, M. J. Brennan, M. L. Haberl, and C. Johnson.** 1986. Rapid assay for screening and characterizing microorganisms for the ability to degrade polychlorinated biphenyls. Appl. Environ. Microbiol. **51**:761–768.
4. **Boyd, S. A., and D. R. Shelton.** 1984. Anaerobic biodegradation of chlorophenols in fresh and acclimated sludge. Appl. Environ. Microbiol. **47**:272–277.
5. **Brown, J. F., Jr., D. L. Bedard, M. J. Brennan, J. C. Carnahan, H. Feng, and R. E. Wagner.** 1987. Polychlorinated biphenyl dechlorination in aquatic sediments. Science **236**:709–712.
6. **Brown, J. F., Jr., R. E. Wagner, D. L. Bedard, M. J. Brennan, J. C. Carnahan, and R. J. May.** 1984. PCB transformations in upper Hudson sediments. Northeast. Environ. Sci. **3**:167–179.
7. **DeWeerd, K. A., L. Mandelco, R. S. Tanner, C. R. Woese, and J. M. Suflita.** 1990. *Desulfomonile tiedjei* gen. nov. and sp. nov., a novel anaerobic, dehalogenating, sulfate-reducing bacterium. Arch. Microbiol. **154**:23–30.
8. **Dingyi, Y., J. F. Quensen III, J. S. Tiedje, and S. A. Boyd.** 1992. Anaerobic dechlorination of polychlorobiphenyls (Aroclor 1242) by pasteurized and ethanol-treated microorganisms from sediments. Appl. Environ. Microbiol. **58**:1110–1114.
9. **Dolfing, J.** 1990. Reductive dechlorination of 3-chlorobenzoate is coupled to ATP production and growth in an anaerobic bacterium, strain DCB-1. Arch. Microbiol. **153**:264–266.
10. **Dolfing, J., and J. M. Tiedje.** 1991. Influence of substituents on reductive dehalogenation of 3-chlorobenzoate analogs. Appl. Environ. Microbiol. **57**:820–824.
11. **Fathepure, B. Z., J. P. Nengu, and S. A. Boyd.** 1987. Anaerobic bacteria that dechlorinate perchloroethene. Appl. Environ. Microbiol. **53**:2671–2674.
12. **Furukara, K., and A. M. Chakrabarty.** 1982. Involvement of plasmids in total degradation of chlorinated biphenyls. Appl. Environ. Microbiol. **44**:619–626.
13. **Genthner, B. R. S., W. A. Price II, and P. H. Pritchard.** 1989. Anaerobic degradation of chloroaromatic compounds in aquatic sediments under a variety of enrichment conditions. Appl. Environ. Microbiol. **55**:1466–1471.
14. **Horowitz, A., J. M. Suflita, and J. M. Tiedje.** 1983. Reductive dehalogenations of halobenzoates by anaerobic lake sediment microorganisms. Appl. Environ. Microbiol. **45**:1459–1465.
15. **King, G. M.** 1988. Dehalogenation in marine sediments containing natural sources of halophenols. Appl. Environ. Microbiol. **54**:3079–3085.
16. **Kohring, G. W., X. Zhang, and J. Wiegel.** 1989. Anaerobic dechlorination of 2,4-dichlorophenol in freshwater sediments in the presence of sulfate. Appl. Environ. Microbiol. **55**:2735–2737.
17. **Mohn, W. W., and J. M. Tiedje.** 1990. Strain DCB-1 conserves energy for growth from reductive dechlorination coupled to formate oxidation. Arch. Microbiol. **153**:267–271.
18. **Nies, L., and T. M. Vogel.** 1990. Effects of organic substrates on dechlorination of Aroclor 1242 in anaerobic sediments. Appl. Environ. Microbiol. **56**:2612–2617.
19. **Omari, T., K. Sugimura, H. Ishigooka, and Y. Minoda.** 1986. Purification and some properties of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid hydrolyzing enzyme from *Pseudomonas cruciviae* S93B1. Agric. Biol. Chem. **50**:931–937.
20. **Quensen, J. F., III, S. A. Boyd, and J. M. Tiedje.** 1990. Dechlorination of four commercial polychlorinated biphenyl mixtures (Aroclors) by anaerobic microorganisms. Appl. Environ. Microbiol. **56**:2360–2369.
21. **Quensen, J. F., III, J. M. Tiedje, S. A. Boyd.** 1988. Reductive dechlorination of polychlorinated biphenyls by anaerobic microorganisms from sediments. Science **242**:752–754.
22. **Safe, S.** 1989. Polychlorinated biphenyls (PCBs): mutagenicity and carcinogenicity. Mutat. Res. **220**:31–47.
23. **Shelton, D. R., and J. M. Tiedje.** 1984. General method for determining anaerobic biodegradation potential. Appl. Environ. Microbiol. **47**:850–857.
24. **Shelton, D. R., and J. M. Tiedje.** 1984. Isolation and partial characterization of bacteria in an anaerobic consortium that mineralizes 3-chlorobenzoic acid. Appl. Environ. Microbiol. **48**:840–848.
25. **Tiedje, J.** Personal communication.