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Introduction of Anaerobic Dechlorinating Bacteria into Soil Slurry Microcosms and Nested-PCR Monitoring

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***Desulfomonile tiedjei* and *Desulfitobacterium dehalogenans* were chosen as model bacteria to demonstrate the introduction of an anaerobic microbia reductive dechlorination activity into nonsterile soil slurry microcosms by inoculation. De novo 3-chlorobenzoate dechlorination activity was established with the bacterium *D. tiedjei* in microcosms normally devoid of this dechlorination capacity. The addition of *D. tiedjei* to microcosms supplemented with 20 mM pyruvate as the cosubstrate resulted in total biotransformation of 1.5 mM 3-chlorobenzoate within 7 days. The introduction of the bacterium *Desulfitobacterium dehalogenans* into nonsterile microcosms resulted in a shortening of the period required for dechlorination activity to be established. In microcosms inoculated with *Desulfitobacterium dehalogenans*, total degradation of 6 mM 3-chloro-4-hydroxyphenoxyacetic acid (3-Cl-4-OHPA) was observed after 4 days in contrast to the result in noninoculated microcosms, where the total degradation of 3-Cl-4-OHPA by indigenous microorganisms was observed after 11 days. Both externally introduced bacterial strains were detected in soil slurry microcosms by a nested-PCR methodology.**

Toxic pollutants, such as chlorinated organic compounds, cause environmental problems due to their recalcitrance and toxicity. Bioremediation, which can be defined as a means of encouraging the natural process of pollutant biodegradation by competent microorganisms, is a promising solution for the rehabilitation of contaminated soil. The degradation potential of indigenous soil microorganisms can be increased either by stimulating their nutritional environment or by introducing a new metabolic function with pure or consortial bacterial inocula. In this study, we report the inoculation of anaerobic bacterial strains capable of reductive dechlorination in nonsterile soil slurry microcosms. *Desulfomonile tiedjei* and *Desulfitobacterium dehalogenans* were used as model microorganisms because of their well-established dechlorination activities. *D. tiedjei* is the first anaerobic bacterium isolated with the capacity to dechlorinate chloroaromatics (8) and also chloroethylenes by cell extracts (26). It is a fastidious sulfate-reducing species capable of maintaining a syntrophic relationship with a methanogenic consortium (10). *Desulfitobacterium dehalogenans* is a recently described anaerobic bacterium capable of dechlorinating 3-chloro-4-hydroxyphenoxyacetic acid (3-Cl-4-OHPA) and several chlorophenols (29). It is important to be able to detect and monitor the fates of microorganisms introduced within microbial communities in order to know whether the inoculated organisms will disappear quickly if they are unable to compete with a natural microbial community or, conversely, if they will proliferate and become temporarily or permanently established in the area (13). Several procedures for detecting specific microbial populations in environmental samples exist. Molecular techniques for recovering specific chemical fragments of the microorganisms (DNA, RNA, antigens, enzymes, or specific fatty acids) have significant potential for detecting and monitoring the frequency maintenance and dispersal of microorganisms released in the environment. A major advantage of this technology is that it is capable of enumerating nonculturable microorganisms (2, 31) as well as fastidious microorganisms like *D. tiedjei*. Molecular probes already developed for detecting *D. tiedjei* in a natural environment include an immunological probe for recovering this organism in granular sludge (1) and a specific fatty acid signature for detecting it in environmental matrices (21). To date there are no reports of nucleotide-based probes for the detection of *D. tiedjei*, nor are there any published molecular techniques for the detection of *Desulfitobacterium dehalogenans*. We report here the first development of a methodology based on nested-PCR amplification of the 16S rRNA gene (rDNA) sequence for specific monitoring of both *D. tiedjei* and *Desulfitobacterium dehalogenans* in soil slurry microcosms. Nested PCR has previously been successfully applied to detect genetically engineered microorganisms in paddy soil (28) and to recover *Vibrio vulnificus* from sediment and water (3) and consists of using different primers which are internal to the first-amplified DNA fragment.

Bacteria and growth conditions. *D. tiedjei* DCB-1 (ATCC 49306) was obtained from the American Type Culture Collection, Bethesda, Md. The bacterium was maintained by periodic transfer in a defined medium (8) with 2 mM 3-chlorobenzoate as an electron acceptor and 20 mM pyruvate as an electron donor. *Desulfitobacterium dehalogenans* (DSM 9161) was obtained from the Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany). It was maintained by periodic transfer in the base medium (29) supplemented with 0.1% yeast extract, 20 mM pyruvate, and 10 mM 3-Cl-4-OHPA.

DNA extraction. Total DNA from a pure culture of *D. tiedjei* was isolated by a modified method of Jansson (14) as follows. To harvest the cells, NaCl was added to a final concentration of 1 M, and cultures were centrifuged for 10 min at 8,000 × *g*. The cells were washed once with TES (3 mM Tris [pH 8.0], 5 mM EDTA, 50 mM NaCl) and were pelleted in a preweighed centrifuge tube. TES (1 ml/g of cells) was added, and the cells were resuspended. This suspension was frozen at −70°C for at

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TABLE 1. Oligonucleotide primers designed for PCR amplification of 16S rDNA genes of *D. tiedjei* and *Desulfitobacterium dehalogenans*

Microorganism	Primer	Primer sequence	T_m[a] (°C)	16S rRNA gene position
Desulfomonile tiedjei	Dt1	5′CAAGTCGTACGAGAAACATATC3′	62	59–79
	Dt2	5′GAAGAGGATCGTGTTTCCACGA3′	66	1032–1054
	Dt3	5′GGGTCAAAGTCGGCCTCTCGACG3′	76	205–227
	Dt4	5′GCTTTCACATTCGACTTATCG3′	60	608–628
	Dt5	5′TTTCACGGACTATTCGTCCG3′	60	476–495
Desulfitobacterium dehalogenans	Dd1	5′AATACCGNATAAGCTTATCCC3′	59	174–194
	Dd2	5′TAGCGATTCCGACTTCATGTTC3′	64	1353–1373
	Dd3	5′TCTTCAGGGACGAACGGCAG3′	64	460–479
	Dd4	5′CATGCACCACCTGTCTCAT3′	58	1066–1084

[a] T_m, annealing temperature.

least 12 h. To isolate DNA, the cells were thawed and lysed as follows. TES (5 ml/g of cells) was added, followed by addition of lysozyme (3 mg/ml in TES) in a proportion of 1/10 volume. The cells were kept at 37°C for 15 min, and 1/15 volume of proteinase K was added (10 mg/ml in TES, which had been predigested for 30 min at 37°C). Sarkosyl was added (1/9 volume), and the solution was mixed well and incubated for 3 h at 37°C. Purification was performed by phenol extraction followed by chloroform-isoamyl alcohol extraction (23). Total DNA from *Desulfitobacterium dehalogenans* was isolated as follows. Cells were harvested by centrifugation for 20 min at 14,000 × *g*. The pellet, suspended in 500 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8]), was transferred to a 2-ml Eppendorf tube. Bacterial cells were lysed with lysozyme (20 mg/ml) for 15 min at 37°C. Fifty microliters of sodium dodecyl sulfate (SDS) (10%) was subsequently added, and the tube was kept at room temperature for 5 min. DNA purification was carried out with two rounds of phenol extraction and one round of chloroform-isoamyl alcohol extraction, followed by overnight precipitation with 2.5 volumes of ethanol.

PCR detection of the 16S rDNA gene with DNAs extracted from pure cultures. Oligonucleotide primers targeting the 16S rDNA gene were designed from both of the published *D. tiedjei* and *Desulfitobacterium dehalogenans* RNA sequences with GenBank accession numbers M26635 and L28946, respectively. The primers (Table 1) were selected on the basis of comparisons of 16S rDNA sequences with the most closely related species in the GenBank database with the aid of OLIGO primer analysis software (Eurogentec, Liège, Belgium) and synthesized with a DNA synthesizer (Eurogentec). One to two microliters of extracted DNA was amplified by PCR with a 9600 thermal cycler (Perkin-Elmer). PCR experiments were carried out in a 50-μl final volume with 5 μl of Ampli *Taq* 10× reaction buffer (Dyna Zym; Eurogentec), 2.5 pmol of primers, 0.2 mM deoxynucleoside triphosphate, and 0.2 μl of Ampli *Taq* polymerase (2 U/μl; Dyna Zym). Samples were amplified by 30 cycles under the following conditions: 92°C for 1 min, 55°C for 1 min, and 72°C for 1 min. A final extension was carried out at 72°C for 10 min. Nested PCR was carried out under the same conditions with 1 to 2 μl of the one-round PCR product as the template, except for the concentration of the polymerase, which was diluted approximately 10-fold in sterile water before use. Ethidium bromide-stained bands were visualized with the UVP Transilluminator and Imagestore 5000 system (Ultra Violet Products, San Gabriel, Calif.). Phage ϕX174 DNA digested with *Hae*III (Eurogentec) was used as the size marker.

PCR methodology was first applied to DNAs of the two target bacteria extracted from pure cultures. To avoid false positives that might result from contamination with free DNA, the PCR mixture including *Taq* polymerase was exposed to UV irradiation (302 nm) for 10 min before addition of the target DNA. The detection level after one-round PCR was 550 pg of DNA with the *D. tiedjei* primer set Dt1 and Dt2 and 76 pg with the *Desulfitobacterium dehalogenans* primer set Dd1 and Dd2 (Fig. 1a and c).

In order to improve these detection levels, nested-PCR amplifications were carried out under the same conditions as those of the one-round PCR except for the concentration of *Taq* polymerase, which was diluted 10-fold, corresponding to approximately 0.04 U/50 μl of the final PCR volume (the usual concentration employed in the one-round PCR had been 0.4 U/50 μl). Under these conditions, the detection limits for *D. tiedjei* were improved by 10-fold, both with the primer set Dt3 and Dt4 (Fig. 1b) and with the primer set Dt3 and Dt5. However, with the latter set, nonspecific fragments were generated (data not shown). For *Desulfitobacterium dehalogenans*, the signal of detection was improved 10,000-fold with diluted *Taq* DNA polymerase with the primer set Dd3 and Dd4 (Fig. 1d).

Sensitivity of PCR detection in nonsterile soil. To determine the sensitivities of the different primer sets for detecting both *D. tiedjei* and *Desulfitobacterium dehalogenans* in a heterogeneous soil DNA background, a known number of these bacteria were inoculated into 0.25 g of an agricultural nonsterile soil originating from Michamps, Belgium, which consisted of 60% silt, 30% clay, and 10% sand (11). The soil contained 2% organic matter, as determined by the Walkley-Black method (18). Its cation-exchange capacity was 8 to 10 meq/100 g (20), and its pH in water was 6. The samples were incubated at room temperature for 1 h to allow adsorption to soil particles before DNA extraction and PCR amplification. Total DNA from soil was isolated by two different methods. In the first approach (30), which is rapid and simple, 0.25 g of soil slurry was frozen with liquid nitrogen, followed by addition of milk powder (0.5 ml of 0.1 g per 25 ml) and SDS extraction buffer (2 ml of 0.3% SDS in 0.14 M NaCl–50 mM sodium acetate [pH 5.1]). The extract was purified once with phenol-saturated water, and DNA was precipitated overnight with 2.5 volumes of ethanol at −20°C. The DNA extract was diluted before being subjected to PCR amplification. In the second method (9), cells were lysed by lysozyme-SDS treatment. Extraction of DNA was followed by purification by ammonium acetate precipitation and filtration through NICK Spin columns prepacked with special DNA-grade Sephadex G-50 (Pharmacia, Roosendaal, The Netherlands). With *Desulfitobacterium dehalogenans*, the method of Volossiouk et al. (30) was successfully used for the one-round PCR after the 20-fold dilution of crude, extracted DNA. The limit of detection was about 10^4 cells/g of soil. The

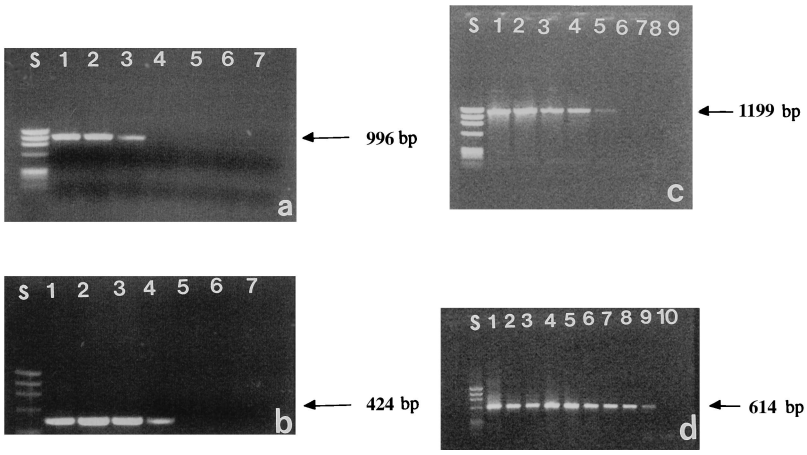

FIG. 1. Sensitivity of PCR detection of *D. tiedjei* and *Desulfitobacterium dehalogenans*. (a) Agarose gel analysis of one-round PCR products of 10-fold serial dilutions of extracted DNA from *D. tiedjei*. Lanes: 1 to 6, decreasing amounts (55 ng to 55 fg) of extracted DNA; 7, no template DNA. (b) Agarose gel analysis of nested-PCR products amplified from those used in panel a with the internal *D. tiedjei* primer set Dt3 and Dt4. (c) Agarose gel analysis of one-round PCR products of 10-fold serial dilutions of extracted DNA from *Desulfitobacterium dehalogenans*. Lanes: 1 to 9, decreasing amounts (760 ng to 7.6 fg) of extracted DNA. (d) Agarose gel analysis of nested-PCR products amplified from those used in panel c with the internal *Desulfitobacterium dehalogenans* primer set Dd3 and Dd4; lane 10, no DNA. Standards from *Hae*III-digested ϕX174 (top to bottom, 1,353, 1,078, 872, and 603 bp) are shown in lane S.

signal of detection was weak, and there was no difference in the signal intensities with higher concentrations of bacteria, such as 10^5 and 10^6 cells/g of soil. This result indicates that the one-round PCR was partially inhibited independently of the quantity of target DNA. By application, instead, of the nested PCR, the limit of detection was about 10^3 cells/g of soil (Fig. 2, lane 3). The primers permitted the detection of *Desulfitobacterium dehalogenans* (Fig. 2, lane 4), whereas no signal was observed in samples seeded with *D. tiedjei* at 10^5 cells/g of soil (Fig. 2, lane 5) and in the noninoculated sample (Fig. 2, lane 6).

With *D. tiedjei* inoculation, the methodology of Volossiouk et al. (30) failed to detect this bacterium in soil, probably because the bacterial lysis step was inefficient. In contrast, the methodology of Dijkmans et al. (9) was successful for the detection of *D. tiedjei* and the detection limit was about 10^6 cells/g of soil. As before, in this case the signal of detection was not improved in the one-round PCR when 10- or 100-fold more cells were used. Application of the nested PCR improved the level of detection by 10-fold, to about 10^5 cells/g of soil (data not shown). The results show the abilities of the primers chosen to detect both bacterial strains in soil slurry by different methods.

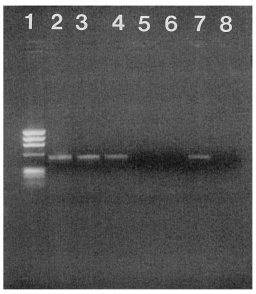

FIG. 2. Sensitivities and specificities of detection of *Desulfitobacterium dehalogenans* by nested PCR in nonsterile soil slurry microcosms. Lanes: 1, ϕX174 *Hae*III markers; 2 and 3, soil amended with *Desulfitobacterium dehalogenans* at 10^4 and 10^3 cells/g of soil, respectively; 4, amended soil mixture of 10^4 cells of *Desulfitobacterium dehalogenans*/g of soil and 10^5 cells of *D. tiedjei*/g of soil; 5, soil amended with 10^5 cells of *D. tiedjei*/g of soil; 6, nonamended soil; 7, positive control with DNA extracted from pure *Desulfitobacterium dehalogenans* culture; 8, no template DNA.

Introduction of anaerobic dechlorination activities in nonsterile soil microcosms. In order to test the feasibility of introducing an anaerobic dechlorination activity in natural soil, microcosms of soil slurry were inoculated with these anaerobic bacterial strains. Fifty grams (dry weight) of soil was placed in a 250-ml glass flask to which 80 ml of the basal medium containing 0.1% yeast extract, mineral solution, and vitamins (8) was added. To this, 20 mM pyruvate was added as a supplemental cosubstrate. The mixture was flushed under 100% N_2, and the pH was adjusted to 7.3 with $NaHCO_3$. The bottles were sealed under an atmosphere of 20% CO_2 plus 80% N_2. One millimolar titanium(III) nitrilotriacetate (17) was added as a reducing agent. 3-Chlorobenzoate or 3-Cl-4-OHPA was added from a concentrated solution. Cultures of either *D. tiedjei* or *Desulfitobacterium dehalogenans* growing exponentially in the above-described nutrient solutions, containing, respectively, 3-chlorobenzoate or 3-Cl-4-OHPA, were added to bottles at 10% (vol/vol) of the soil slurry mixture (inoculum, 10^7 cells/ml). Final chloroaromatic concentrations in the test bottles were 1.5 mM 3-chlorobenzoate or 6 mM 3-Cl-4-OHPA. The bacterial cell concentration was estimated by direct microscopic count by phase-contrast illumination. Samples for counting were fixed with 5% formalin. The inoculated bottles were incubated at 37°C in the dark, and all experiments were performed in duplicate. Samples for quantification of 3-chlorobenzoate, benzoate, 3-Cl-4-OHPA, and 4-*ortho*-hydroxyphenylacetate were analyzed by reverse-phase high-performance liquid chromatography with a Chromosphere C_{18} column (Chrompack, Middelburg, The Netherlands). The flow rate was 1 ml/min. The mobile phase consisted of CH_3CN-H_2O-H_3PO_4 (35:65:0.01). A UV detector (VWM 2141; Pharmacia LKB, Uppsala, Sweden) was set to 276 nm. With *Desulfitobacterium dehalogenans*, the results showed that dechlorination activity was rapidly manifested in the inoculated microcosms. As shown in Fig. 3a, in the inoculated microcosms total degradation of 3-Cl-4-OHPA was observed after 4 days, in contrast to that in the noninoculated microcosms, where the total degradation of 3-Cl-4-OHPA by indigenous microorganisms was observed only after 11 days. The results indicate that

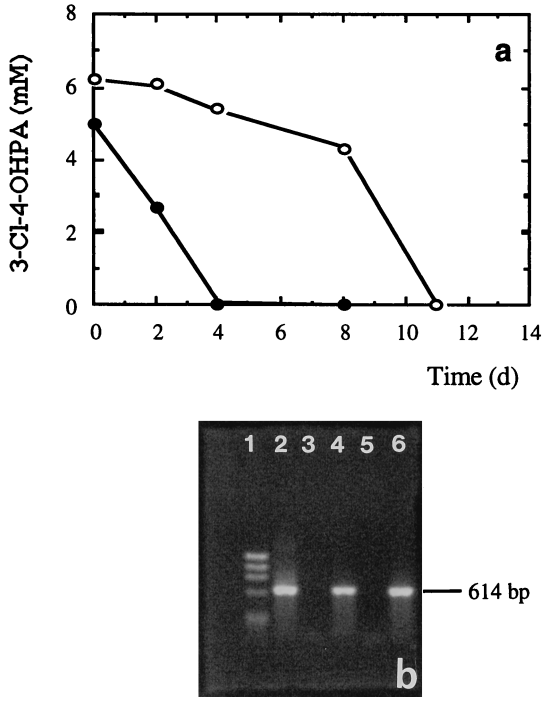

FIG. 3. (a) Biotransformation of 3-Cl-4-OHPA in soil slurry microcosms inoculated with *Desulfitobacterium dehalogenans* (●) and in noninoculated microcosms (○). The experiment was performed in duplicate. (b) Nested-PCR detection of *Desulfitobacterium dehalogenans* in soil slurry microcosms. Lanes: 1, ϕX174 *Hae*III markers; 2, positive control with pure extracted DNA of *Desulfitobacterium dehalogenans*; 3 and 4, noninoculated and inoculated microcosms, respectively, 1 day after inoculation; 5 and 6, noninoculated and inoculated microcosms, respectively, 4 days after inoculation.

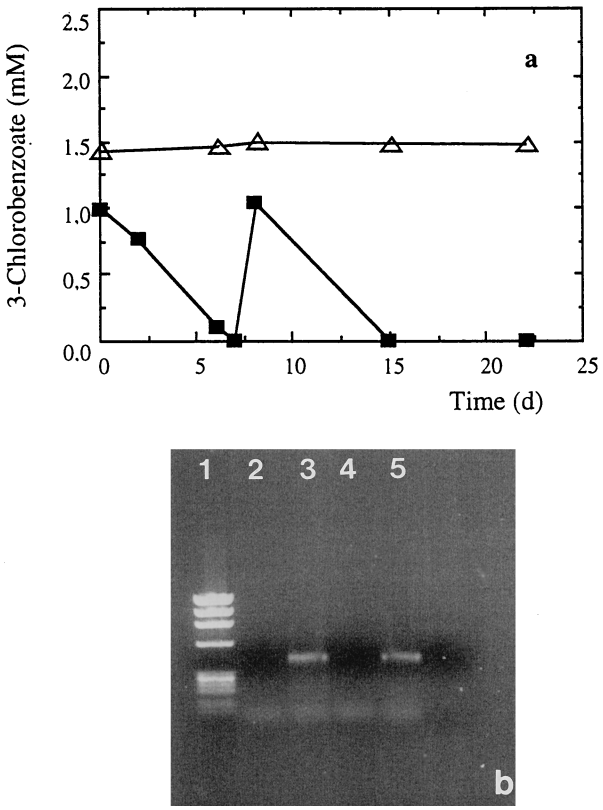

FIG. 4. (a) Biotransformation of 3-chlorobenzoate in soil slurry microcosms inoculated with *D. tiedjei* (■) and in noninoculated microcosms (△) in the presence of 20 mM pyruvate as the supplemental cosubstrate. The experiment was performed in duplicate. (b) Nested-PCR detection of *D. tiedjei* in soil slurry microcosms 1 day (lane 3) and 20 days (lane 5) after inoculation. Lane 1, ϕX174 *Hae*III markers; lanes 2 and 4, noninoculated microcosms, after 1 and 20 days, respectively. The PCR mixture was irradiated by UV (10 min at 302 nm) before addition of DNA samples.

inoculation can improve substantially the rate of dechlorination. The application of the DNA extraction and nested-PCR methodologies described above showed that this bacterium was detected in soil microcosms on both the first day following the inoculation and after the fourth day (Fig. 3b).

For *D. tiedjei*, the results show that dechlorination activity on 3-chlorobenzoate was expressed in the microcosms inoculated with *D. tiedjei* in the basal medium cited above but that no dechlorination activity was observed in the noninoculated microcosms, even upon prolonged incubation (Fig. 4a). We conclude that dechlorination activity was due to the presence of *D. tiedjei*. The 3-chlorobenzoate was first transformed by *D. tiedjei* to benzoate, which was in turn completely mineralized by the indigenous consortium to CH_4 and CO_2. Indeed, CH_4 production was enhanced in the microcosms inoculated with *D. tiedjei*. The indigenous microbial community was able to mineralize the benzoate to CH_4 and CO_2 when the benzoate was supplied as the sole source of carbon and energy (data not shown). The PCR methodology developed as described above was applied to detect *D. tiedjei*. One day after the inoculation as well as 20 days after the inoculation (which corresponded to the complete degradation of 3-chlorobenzoate) (Fig. 4a), 2 g of soil slurry from each bottle (inoculated and noninoculated microcosms) served as a source for soil DNA extraction. The samples were divided into 0.5-g aliquots, and DNAs were extracted by the method of Djikmans et al. (9). The crude DNAs of each extraction were mixed, and this mixture served as the template for PCR amplification. The results show that nested PCR was capable of detecting *D. tiedjei* both 1 day and 20 days following the inoculation (Fig. 4b). The signals were very weak in the one-round PCR. They were, however, very clear by nested PCR, and no signal was observed in noninoculated microcosms.

The fact that the detection signal was better in the nested PCR than in the one-round PCR was not only due to the sufficient quantities of DNA which resulted from the first-round PCR but also due to the dilution of the inhibitory substances in the soil slurry, such as clay substances, which were present at a high percentage (30%) in the soil used. Although PCR is an attractive method for detecting bacteria in the environment, its routine use is hampered by false negatives due, in soil extraction, to soil contaminants, such as humic acid substances, which inhibit the polymerase (25). Another problem is the appearance of false positives (22). In this study, false positives were avoided by decreasing the concentration of *Taq* polymerase and by irradiating the PCR mixture with UV as suggested by other authors (19). To our knowledge this is the first study where a difference in *Taq* DNA polymerase concentrations between one-round PCR and nested PCR has been reported. It is important that the nested PCR was able to improve detection without altering the threshold of sensitivity with a very low concentration of DNA polymerase (data not shown). This concentration was approximately 62-fold lower than the standard concentration (2.5 U/50 μl) often used for nested PCR (6, 16, 28). This is significant because DNA polymerase is the most expensive reagent of the PCR mixture. It

appears that the success of PCR, for DNA extraction from soil, depends on both the type of methodology of soil extraction and the target microorganisms, since the results are different when different methods of bacterial lysis, DNA extraction, and DNA purification are applied. Recently, several protocols for DNA extraction and purification have been proposed in order to avoid the inhibition of PCR by phenols and humic acids (25, 27). Zhou et al. (32) have recently shown that DNA recovery varies with different extraction methods and that the success of PCR varies with different purification methods. The improvements of all these parameters, in addition to PCR conditions, like addition of T4 gene 32 protein or bovine serum albumin as suggested recently (15), will allow an improvement in the sensitivity of the detection.

The sensitivity of detection of bacteria varied largely depending on the methodology used. Indeed some authors were capable of detecting 1 cell of *Pseudomonas cepacia* 1100 per g of soil by the combination of PCR amplification and DNA hybridization (4). *Pseudomonas fluorescens* R2f has been detected at a sensitivity of 10^3 inoculant cells/g of soil (24), and *Nitrobacter* spp. have been detected at 10 cells/g of soil (7). Recently Briglia et al. (5) showed that the method based on 16S rRNA gene-target PCR allowed the detection of 3×10^2 cells of *Mycobacterium chlorophenolicum* PCP-1 per g of soil. Although the methodology developed through this study is not more sensitive than the previously cited example, its comparison with other techniques proposed for detecting *D. tiedjei* shows that PCR methodology holds promise for detecting these bacteria in the environment. Until now, only two techniques have been applied to detect *D. tiedjei* in natural environments (1, 21). Ahring et al. (1) have applied a fluorescent-antibody technique to detect this bacterium in granular sludge, giving, however, no quantitative indication of detection efficiency. This methodology is hampered in soil matrices by the following major drawbacks: insufficient or excessive specificities of the antisera for the organisms studied, autofluorescence of some soil particles and organisms, and the ability of some soil particles to adsorb antisera and mask the stained organisms (12). Ringelberg et al. (21) showed the feasibility of the detection of *D. tiedjei* with its signature lipopolysaccharide branched-long-chain hydroxy fatty acids. They reported that it was possible to consistently detect the presence of more than 2×10^8 cells of *D. tiedjei* per bottle of inoculated sediment (approximately 4×10^6 cells per g of sediment) based on the presence of signature long-chain lipopolysaccharide branched-long-chain hydroxy fatty acids. To our knowledge, no methods are yet available for detecting *Desulfitobacterium dehalogenans* in natural environments that allow a comparison with the nested-PCR methodology results reported in this study.

Conclusion. The success of inoculation is a crucial parameter for bioremediating contaminated sites when the indigenous microorganisms have failed, and it may sometimes shorten the period of acclimation to the onset of biodegradation. This was clearly shown in our study with soil microcosms inoculated with *Desulfitobacterium dehalogenans*, where a shorter acclimation period was observed since the dechlorination activity was rapidly expressed in the inoculated microcosms. This study also reported the introduction of de novo biodegradation ability into previously nondechlorinating soil with *D. tiedjei*. Our attempt to detect the inoculated bacteria by nested PCR proved successful. These results are very encouraging for the future of anaerobic biodegradation of toxic aromatics in contaminated soils.

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REFERENCES

1. **Ahring, B. K., N. Christiansen, I. Mathrani, H. V. Hendriksen, A. J. L. Macario, and E. Conway de Macario.** 1992. Introduction of a de novo bioremediation ability, aryl reductive dechlorination, into anaerobic granular sludge by inoculation of sludge with *Desulfomonile tiedjei*. Appl. Environ. Microbiol. **58:**3677–3682.
2. **Amann, R. I., W. Ludwig, and K. H. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143–169.
3. **Arias, C. R., E. Gary, and R. Aznar.** 1995. Nested-PCR method for rapid and sensitive detection of *Vibrio vulnificus* in fish, sediments, and water. Appl. Environ. Microbiol. **61:**3476–3478.
4. **Atlas, R. M., A. K. Bej, R. J. Stefan, and M. H. Perlin.** 1989. Approaches for monitoring and containing genetically engineered microorganisms released into the environment. Hazard. Waste Hazard. Mater. **6:**135–144.
5. **Briglia, M., R. I. L. Eugen, W. M. De Vos, and J. D. Van Elsas.** 1996. Rapid and sensitive method for the detection of *Mycobacterium chlorophenolicum* PCP-1 in soil based on 16S rRNA gene-targeted PCR. Appl. Environ. Microbiol. **62:**1478–1480.
6. **Cassinotti, P., M. Weitz, and G. Siegel.** 1993. Human parvovirus B19 infections: routine diagnosis by a new nested polymerase chain reaction assay. J. Med. Virol. **40:**228–234.
7. **Degrange, V., and R. Bardin.** 1995. Detection and counting *Nitrobacter* populations in soil by PCR. Appl. Environ. Microbiol. **61:**2093–2098.
8. **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.
9. **Dijkmans, R., A. Jagers, S. Kreps, J. M. Collard, and M. Mergeay.** 1993. Rapid method for purification of soil DNA for hybridization and PCR analysis. Microb. Rel. **2:**29–34.
10. **Dolfing, J., and J. M. Tiedje.** 1986. Hydrogen cycling in a three-tiered food web growing on the methanogenic conversion of 3-chlorobenzoate. FEMS Microbiol. Ecol. **38:**293–298.
11. **Gee, G. W., and J. W. Bauder.** 1982. Particle size analysis, p. 283–341. *In* A. Klut (ed.), Methods of soil analysis, 2nd ed., part 1. American Society of Agronomy, Madison, Wis.
12. **Gray, T. R. G.** 1990. Methods for studying the microbial ecology of soil, p. 309–342. *In* R. Grigorova and J. R. Norris (ed.), Methods in microbiology, vol. 22. Academic Press Limited, London, United Kingdom.
13. **Jain, R. K., and G. S. Sayler.** 1987. Problems and potential for *in situ* treatment of environmental pollutants by engineered microorganisms. Microbiol. Sci. **4:**59–63.
14. **Jansson, J. K.** 1988. Development of gene probe methodology for detection of specific bacteria in environmental samples. Ph.D. thesis. Michigan State University, East Lansing.
15. **Kraeder, C. A.** 1996. Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. Appl. Environ. Microbiol. **62:**1102–1106.
16. **McManus, P. S., and A. L. Jones.** 1995. Detection of *Erwinia amylovora* by nested PCR and PCR-dot-blot and reverse-blot hybridizations. Phytopathology **85:**618–623.
17. **Moench, T. T., and J. G. Zeikus.** 1983. An improved preparation method for a titanium (III) media reductant. J. Microbiol. Methods **1:**199–202.
18. **Nelson, D. W., and L. E. Sommers.** 1982. Total carbon, organic carbon, and organic matter, p. 539–579. *In* A. L. Page, R. H. Miller, and D. R. Keeney (ed.), Methods of soil analysis, 2nd ed., part 2. American Society of Agronomy, Madison, Wis.
19. **Nesme, X., C. Picard, and P. Simonet.** 1995. Specific DNA sequences for detection of soil bacteria, p. 111–139. *In* J. T. Trevors and J. D. van Elsas (ed.), Nucleic acids in the environment: methods and applications. Springer-Verlag, Berlin, Germany.
20. **Rhoades, J. D.** 1982. Cation exchange capacity, p. 149–157. *In* A. L. Page, R. H. Miller, and D. R. Keeney (ed.), Methods of soil analysis, 2nd ed., part 2. American Society of Agronomy, Madison, Wis.
21. **Ringelberg, D. B., G. T. Townsend, K. A. DeWeerd, J. M. Suflita, and D. C. White.** 1994. Detection of the anaerobic dechlorinating microorganism *Desulfomonile tiedjei* in environmental matrices by its signature lipopolysaccharide branched-long-chain hydroxy fatty acids. FEMS Microbiol. Ecol. **14:**9–18.
22. **Rochelle, P. A., J. A. K. Will, J. C. Fry, G. J. S. Jenkins, R. J. Parkes, C. M. Turley, and A. J. Weightman.** 1995. Extraction and amplification of 16S rRNA genes from deep marine sediments and seawater to assess bacterial community diversity, p. 219–239. *In* J. T. Trevors and J. D. van Elsas (ed.), Nucleic acids in the environment: methods and applications. Springer-Verlag, Berlin, Germany.
23. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a

laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
24. **Smalla, K., N. Cresswell, L. C. Mendonca-Hagler, A. Wolters, and J. D. van Elsas.** 1993. Rapid DNA extraction protocol from soil for polymerase chain reaction-mediated amplification. J. Appl. Bacteriol. **74:**78–85.
25. **Straub, T. M., I. L. Pepper, and C. P. Gerba.** 1995. Removal of PCR inhibiting substances in sewage sludge amended soil. Water Sci. Technol. **31:**311–315.
26. **Townsend, G., and J. M. Suflita.** 1996. Characterization of chloroethylene dehalogenation by cell extracts of *Desulfomonile tiedjei* and its relationship to chlorobenzoate dehalogenation. Appl. Environ. Microbiol. **62:**2850–2853.
27. **Trevors, J. T., and J. D. van Elsas (ed).** 1995. Nucleic acids in the environment: methods and applications. Springer-Verlag, Berlin, Germany.
28. **Tsushima, S., A. Hasebe, Y. Komoto, J. P. Carter, K. Miyashita, K. Yokoyama, and R. W. Pickup.** 1995. Detection of genetically engineered microorganisms in paddy soil using a simple and rapid "nested" polymerase chain reaction method. Soil Biol. Biochem. **27:**219–227.
29. **Utkin, I., D. D. Dalton, and J. Wiegel.** 1995. Specificity of reductive dehalogenation of substituted *ortho*-chlorophenols by *Desulfitobacterium dehalogenans* JW/IU-DC1. Appl. Environ. Microbiol. **61:**346–351.
30. **Volossiouk, T., I. J. Robb, and R. N. Nazar.** 1995. Direct DNA extraction for PCR-mediated assays of soil organisms. Appl. Environ. Microbiol. **61:**3972–3976.
31. **Ward, N., F. A. Rainey, B. Goebel, and E. Stackebrandt.** 1995. Identifying and culturing the "unculturables": a challenge for microbiologists, p. 89–110. *In* D. Allsopp, R. R. Colwell, and D. L. Hawksworth (ed.), Microbial diversity and ecosystem function. CAB International, Wallingford, United Kingdom.
32. **Zhou, J., A. M. Bruns, and J. M. Tiedje.** 1996. DNA recovery from soils of diverse composition. Appl. Environ. Microbiol. **62:**316–322.