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Introduction and PCR detection of *Desulfomonile tiedjei* in soil slurry microcosms

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Abstract

The aim of this work was to test the feasibility of introducing an anaerobic microbial reductive dechlorination activity into non sterile soil slurry microcosms by inoculation with the pure anaerobic bacterial strain *Desulfomonile tiedjei*, which is capable of dechlorinating 3-chlorobenzoate to benzoate. To show that the bacterium was established in the microcosms we followed the expression of the reductive dechlorination activity and a molecular probe based on PCR amplification of the 16S rDNA gene was developed. However, the success of PCR amplification of the 16S rDNA gene depends on the DNA extraction and purification methodologies applied, as shown through the use of several protocols. In this study we report a DNA extraction and purification method which generates sufficient and very clean DNA suitable for PCR amplification of the *D. tiedjei* 16S rDNA gene. The threshold of detection was about 5.10^3 bacteria per gram of soil slurry. Introduction of *D. tiedjei* in soil slurry microcosms proved successful since 3-chlorobenzoate dechlorination activity was established with this bacterium in microcosms normally devoid of this dechlorination capacity. Indeed, the addition of *D. tiedjei* to microcosms supplemented with acetate plus formate as cosubstrate, at their respective concentrations of 5 and 6 mM, led to a total biotransformation of 2.5 mM of 3-chlorobenzoate within 12 days. After complete 3-chlorobenzoate dechlorination, the 16S rDNA gene of this bacterium was specifically detected only in the inoculated microcosms as shown by PCR amplification followed by restriction mapping confirmation.

Introduction

Bioremediation is currently attracting considerable interest as a technique of enhancing the degradation of troublesome pollutants by competent microorganisms for the rehabilitation of contaminated soil. A number of authors has demonstrated the feasibility of introducing new metabolic activities into a specific microbial environment using pure bacterial strains or consortia (Oldenhuis et al. 1989; Ahring et al. 1992; Møller & Ingvorsen 1993; Brunsbach & Reineke 1993, 1995). The new biocatalytic potential can be established in the inoculated site either through the growth and metabolism of the added microorganisms or by the transfer of degradative genes (Mergeay et al. 1990; Zhou & Tiedje 1995). In this work we explored the introduction of reductive dechlorination activity into non-sterile soil microcosms by inoculation with a pure anaerobic bacterium strain and we developed reliable molecular methodology for detection of the strain over time after inoculation. *Desulfomonile tiedjei* was used as model microorganism because of its well-characterized dechlorination activity. This bacterium which can dechlorinate chloroaromatics (De Weerd et al. 1990) is a fastidious sulfate-reducing anaerobe with the capacity to grow syntrophically within a methanogenic consortium (Dolfing & Tiedje 1986). In addition to demonstrating the successful maintenance of *D. tiedjei* in the microcosms by following the reductive dechlorination of 3-chlorobenzoate, we also developed a molecular probe. Its use for detecting *D. tiedjei* in soil slurry microcosms was based on

the PCR amplification of the 16S rDNA gene. This genomic fragment is widely used as a probe for detecting bacteria in natural and polluted environments as shown by the exponential increase of recent publications (Amann et al. 1990; Britschgi & Fallon 1994; Raskin et al. 1994; Amann et al. 1995; Degrange & Bradin 1995; Briglia et al. 1996; Hales et al. 1996, Wang et al. 1996). However to obtain DNA suitable for PCR amplification different DNA extraction and purification methods had to be tested and compared (Dijkmans et al. 1993; Volossiouk et al. 1995; Zhou et al. 1996) before developing an adequate methodology for the soil and the bacterium used throughout this study.

Materials and methods

Organism and growth conditions

Desulfomonile tiedjei strain DCB-1 (ATCC 49306) was obtained from the American Type Culture Collection, Bethesda, MD, USA. The bacterium was maintained by periodic transfer in a defined medium (DeWeerd et al. 1990) with 2 mM 3-chlorobenzoate as an electron acceptor and 20 mM pyruvate as an electron donor.

Preparation of soil slurry microcosms

Throughout this study, an unacclimated agricultural soil was used originating from Michamps, Belgium. The texture of the soil was 60% silt, 30% clay and 10% sand. It contained 2% organic matter and 0.15% nitrogen. Its cation exchange capacity (CEC) was 8-10 meq/100 g and its pH in water was 6. The soil was passed through a 2.0 mm sieve, placed in airtight polyethylene bags and kept at 4 °C in the dark until use. Fifty grams (dry weight) of soil were placed in a 250 ml glass flask and 80 ml of the basal medium containing 0.1% yeast extract, mineral solution and vitamins (De Weerd et al. 1990) was added. To this, a supplemental cosubstrate consisting of 5 mM acetate plus 6 mM formate was added. The mixture was flushed under 100% N_2 and the pH was adjusted to 7.3 with $NaHCO_3$. The flasks were sealed under an atmosphere of 20% CO_2 plus 80% N_2. One mM titanium (III) nitrilotriacetate (Moench & Zeikus 1983) was added as a reducing agent. 3-chlorobenzoate was added from a concentrated solution. A *D. tiedjei* culture growing exponentially in the nutrient solution mentioned above was added to bottles at 10% (v/v) of the soil slurry mixture (inoculum of 10^7 cells/ml). The bacterial cell concentration was estimated by direct microscopic count using 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.

Bacterial DNA extraction

Total DNA from a pure culture of *D. tiedjei* was isolated using a modified method of Jansson (1988): to harvest the cells, NaCl was added to a final concentration of 1 M, and cultures were centrifuged for 10 min at 8,000 $\times$ *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 cells) was added and the cells were resuspended. This suspension was frozen at -70 °C for at least 12 h. To isolate the DNA, the cells were thawed and lysed. TES (5 ml/ g cells) was added followed by lysozyme (3 mg/ml in TES) to 1/10 volume. The cells were incubated at 37 °C for 15 min and 1/15 volume proteinase K was added (10 mg/ml in TES, predigested 30 min at 37 °C). Sarkosyl was added (1/9 volume), mixed well, and incubated for 3 h at 37 °C. The purification was performed using phenol and chloroform plus isoamylic alcohol extraction followed by overnight precipitation with 2.5 volumes of ethanol.

Total DNA from the soil microcosms was isolated using the following protocol: two grams of soil slurry were added to a 14 ml polypropylene round bottom tube (Falcon tube). To this, 4 ml of lysozyme solution (20 mg/ml in Tris, pH 9) was added. The mixture was incubated 15 min at 37 °C by horizontal shaking at 250 rpm. The samples were sonicated twice for 20 s at power level 5 and 100% active cycles using an ultrasonicator (Branson Sonifier 450, Danbury, CT, USA). To achieve complete lysis 1% SDS was added and samples were slowly mixed for 5 min, then 2.5 mM of sodium acetate was added. The supernatant was collected after centrifugation at 5,000 $\times$ *g* for 10 min at room temperature and transferred into a new tube containing an equal volume of chloroform-isoamyl alcohol (24:1, v/v). The aqueous phase was recovered by centrifugation and gently passed through Sephadex G50 (Pharmacia, Roosendaal, The Netherlands) columns. Centrifugation of the columns was performed twice in a swinging buckets centrifuge (Jouan, CR4.12, Leuven, Belgium) at 2,000 $\times$ *g* for 5 min and the eluate collected in a new tube containing 0.6 its volume of

Table 1. Comparison of different DNA extraction and purification protocols for successful PCR detection of the 16S rDNA gene of *D. tiedjei* inoculated in an agricultural non sterile soil slurry microcosm

	Zhou et al. (1996)	Volossiouk et al. (1995)	Dijkmans et al. (1993)	This study
Soil sample (g)	5	0.25	0.5	2
Bacterial cell lysis	13.5 ml DNA extraction buffer + 100 μl proteinase K (10 mg/ml) 30 min 37 °C (shaking, 225 rpm) + 1.5 ml SDS (20%) 2 h 65 °C (three cycles of extraction)	Grind with liquid nitrogen (5 min) 2 ml extraction buffer (0.3% SDS in 0.14 M NaCl, 50 mM NaAc, pH 5.1)	1 ml Tris pH 9.0 (20 mg/ml lysozyme) 5 min, 37 °C + 1% SDS, Eppendorf mixer, 5 min + 2.5 mM ammonium acetate	4 ml Tris pH 9 (20 mg/ml lysozyme) 15 min, 37 °C (shaking 250 rpm) + ultrasonication, 20 s at power level 5 and 100% active cycles + 1% SDS (5 min) + 2.5 mM ammonium acetate
DNA purification to avoid PCR inhibiting substances	i): single Wizard minicolumn* or ii): double Wizard minicolumn* or iii): gel excision plus single Wizard minicolumn*	Dilution of crude DNA extract (50-fold)	Sephadex G50 columns	Sephadex G50 columns + Single Wizard minicolumn*
PCR detection of D. tiedjei using primers (Dt60, Dt1032)**	–	–	+/–	+

* Wizard PCR preps purification (Promega, Madison, WI.).
** PCR conditions are detailed in Materials and methods.

isopropanol. The precipitation was performed for 1 h at −20 °C. The pellet of the first purification step was obtained by centrifugation at 16,000 × *g* for 20 min at room temperature, washed with cold 70% ethanol, and resuspended in deionized water, to give a final volume of 250 μl. The next purification step was performed with 100 μl of the first purification DNA suspension using Wizard™ PCR preps (Promega, Madison, WI, USA). The purified DNA was finally recovered in 100 μl deionized water. Other DNA extraction and purification protocols were also used as seen in Table 1.

PCR amplification

Oligonucleotide primers targeting the 16S rDNA gene were designed from published *D. tiedjei* RNA sequences with GenBank accession number M26635 with the aid of the Oligo Selected Program, and synthesized with a DNA synthesizer (Eurogentec, Liège, Belgium). The 16S rDNA gene was amplified with the primers Dt60 (5′-CAAGTCGTACGAGAAACATATC-forward) and Dt1032 (5′-GAAGAGGATCGTGTTTCCACGA-reverse). These primers were predicted to yield a 998 bp product. One to two μl of extracted DNA were amplified by PCR with a 9600 thermal cycler (Perkin-Elmer, Norwalk, CT, USA). Throughout this study two DNA polymerases were used. The first one was from DynaZym (Eurogentec, Liège, Belgium) and the second was the High Fidelity PCR system (Boehringer, Mannheim, Germany). PCR experiments were carried out in 50 μl final volume with 5 μl Ampli *Taq* 10x reaction buffer, 2.5 pmol of primers, 0.2 mM dNTP and 0.4 units of Ampli *Taq* polymerase (DynaZym) or 0.6 units of High Fidelity PCR system (Boehringer). 200 to 400 ng/ml of an ultrapure and molecular biology grade bovine serum albumin (BSA) (Boehringer) or 100 to 150 ng/ml of T4 gene protein 32 (Boehringer) was added to the PCR mixture for relief of amplification inhibition in PCR (Kraeder 1996). Samples were amplified with 30 cycles of the following conditions: 92 °C for 1min, 55 °C for 1 min, and 72 °C for 1 min. A final extension was carried out at 72 °C for 10 min.

PCR product confirmation by restriction enzymes

Before restrictions, the PCR product was purified using High Pure PCR Product Purification Kit (Boehringer, Mannheim, Germany). All restriction enzymes were purchased from New England BioLabs (Beverly, MA, USA) and the experimental conditions were those recommended by the manufacturers.

Detection of PCR product and digested fragments

PCR amplification products were electrophoresed on 0.8% agarose gels whereas restriction fragments were electrophoresed on 2 to 2.5% Low Melting Point Agar (Life Technologies, Gaithersburg, MD, USA). Ethidium bromide-stained bands were visualized with the UVP Transilluminator and Imagestore 5000 system (Ultra Violet Products, San Gabriel, CA, USA). Phage ΦX174 DNA digested with *Hae*III (Eurogentec, Liège, Belgium) was used as size marker.

Analytical methods

DNA concentration was estimated spectrophotometrically. Samples for quantification of 3-chlorobenzoate and benzoate were analysed by reverse-phase high performance liquid chromatography (HPLC) with a Chromosphere C18 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 LKB-VWM 2141 (Pharmacia, Uppsala, Sweden) was set to 276 nm. The gas composition was determined by gas chromatography using an Intersmat IGC model 120 MB chromatograph (Chelles-les-Coudreaux, France) equipped with a catharometer and two columns in series, filled respectively with Porapak Q 50-80 mesh and 5 A molecular sieve. All chemicals and reagents mentioned under Materials and methods were of the highest purity available and were obtained from Sigma (Saint Louis, MO, USA) or Merck (Darmstadt, Germany).

Results and discussion

Soil DNA extraction methodology

The soil DNA extraction method described in Materials and methods is not time consuming and generated a very clean DNA suitable for PCR amplifications. The method provides considerably less shearing of DNA

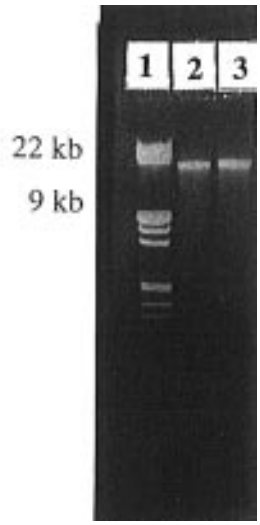

Figure 1. DNA extracted from soil slurry used throughout this study. Lanes: 1, 200 ng of lambda DNA digested with *Hin*dIII and *Eco*RI; 2 and 3, 10 μl of purified DNA from soil slurry. The DNA was resolved on a 0.8% agarose gel.

since the predominant DNA fragments obtained were between 15 and 20 Kb (Figure 1). The A_{260}/A_{280} ratio of DNA absorbance obtained after the Wizard^{TM} purification method was 2.10 ($\pm$0.02) indicating that the DNA was of good quality compared with pure culture (1.95 $\pm$0.04). Other DNA extraction methods were also applied to the agricultural soil used in this study and details of these steps are given in Table 1. Although the method of Volossiouk et al. (1995) was simple and faster than the method described above, its application for the 16S rDNA gene detection of *D. tiedjei* failed even if 2 grams of soil slurry were used instead of the 0.25 g recommended by the authors which was also used in this study. The problem encountered with this protocol is probably the inefficiency of the bacterial lysis step. The application of the protocol developed by Zhou et al. (1996) was time-consuming and the PCR amplification failed to detect *D. tiedjei* in the soil slurry used throughout this study. We observed with the latter protocol that the step which involved SDS treatment by incubating the samples at 65 °C for two hours is apparently not suitable since the liquid phase was intensely coloured with the brownish soil substances (presumably phenols, humic and fulvic acids) which are known as strong inhibitors of PCR reactions (Straub et al. 1995). Consequently the samples needed many more purification steps before PCR reactions could be initiated. The application of the protocol of Djikmans et al. (1993) proved successful for detecting

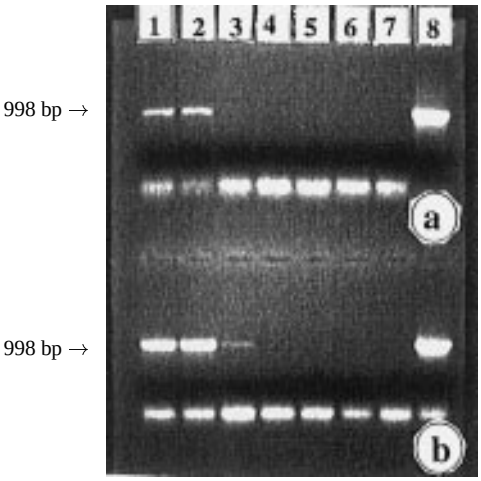

Figure 2. Sensitivity of PCR detection of *D. tiedjei* in soil slurry. (a): Sensitivity of detection using *Taq* polymerase (DynaZym). (b): Sensitivity of detection using Expand High Fidelity PCR system (Boehringer). Lanes: 1 to 5, ten-fold dilution 5.10^5 to 50 bacteria per gram of soil, respectively; 6, non inoculated microcosm; 7, negative control (no template DNA); 8, positive control (template DNA from pure culture of *D. tiedjei*).

D. tiedjei when 2 grams of soil slurry was used but the sensitivity of PCR detection was drastically lower when compared with the protocol developed for this study as detailed in Materials and methods (results not shown). The DNA extraction protocol developed here is an improvement of the protocol by Dijkmans et al. (1993). It generates a very clean DNA suitable for PCR amplification. Indeed, after the second purification step (Wizard™ purification), positive PCR reactions can be obtained with more than 10 μl of DNA sample without altering the PCR sensitivity.

PCR methodology for detecting D. tiedjei *in soil*

To determine the sensitivity of the primer set for detecting *D. tiedjei* in a heterogeneous soil DNA background, a known number of these bacteria were inoculated into 1 g of non sterile soil followed by incubation at room temperature for 1 hour to allow adsorption to soil particles before DNA extraction and PCR amplification. The bacterial soil DNA extraction and purification methodologies cited above proved successful for the detection of *D. tiedjei*. The limit of detection using set (Dt60, Dt1032), as estimated by microscopic counting, was about 5.10^4 bacteria per gram of soil slurry when *Taq* polymerase from DynaZym was used (Figure 2a) and 5.10^3 bacteria per gram of soil slurry when High Fidelity PCR system from Boehringer was used (Figure 2b). The results show that the primers chosen for detecting the bacterium strain in soil slurry were specific since no signal was detected in the non inoculated soil slurry (Lane 7, Figure 2a and 2b). There was a good correlation between detection of *D. tiedjei* in pure culture and in soil when we compared the sensitivity of the detection for these two environments using primer set (Dt60, Dt1032) and *Taq* polymerase

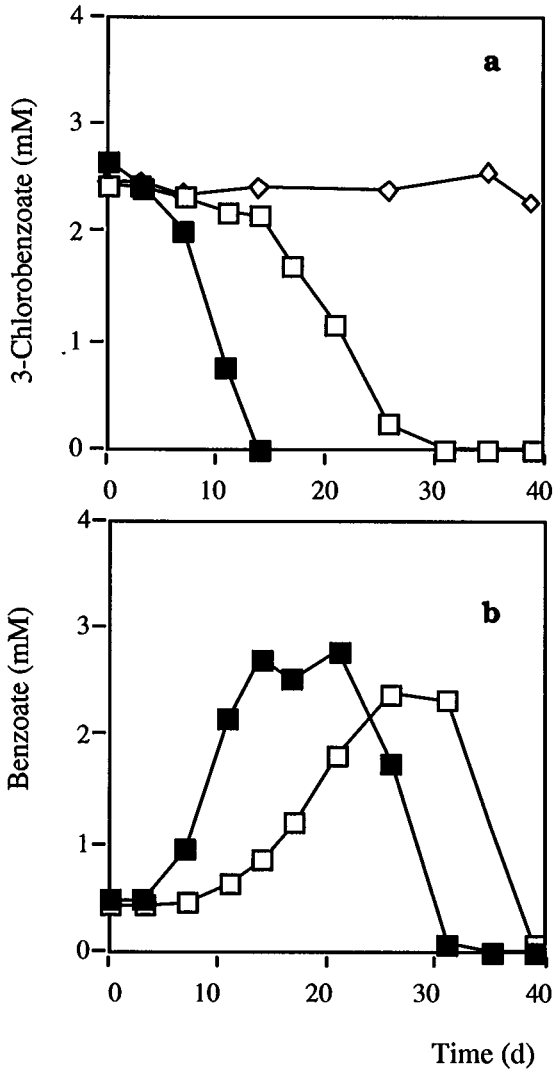

Figure 3. Biotransformation of 3-chlorobenzoate (a) to benzoate (b) in soil slurry microcosms inoculated with *D. tiedjei* in the absence (□) or presence (■) of acetate plus formate (5 and 6 mM, respectively) as supplemental cosubstrate. (◇): 3-chlorobenzoate in non inoculated microcosms.

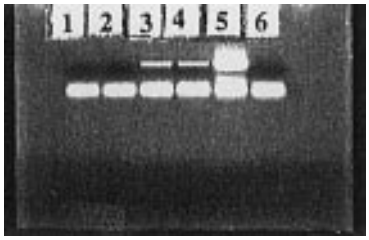

Figure 4. PCR detection of *D. tiedjei* in soil slurry microcosms after three weeks of incubation. Lanes: 1 and 2, non inoculated samples with non supplemental cosubstrate and with acetate plus formate as cosubstrate, respectively; 3 and 4, inoculated microcosms with non supplemental cosubstrate and with acetate plus formate as cosubstrate, respectively; 5, positive control (template DNA from pure culture of *D. tiedjei*); 6, negative control (no template DNA).

from DynaZym. Indeed the limit of detection with pure extracted DNA was about 550 pg which theoretically corresponds to 6.10^4 bacteria if we consider that one bacterium contains about 9.10^{-3} pg of DNA. This is similar to the sensitivity of the detection in soil slurry which was about 5.10^4 bacteria per gram of soil slurry.

Expression and monitoring of reductive dechlorination activity in non sterile soil slurry microcosms inoculated with D. tiedjei

Microcosms are very useful as a research tool for investigating the ecological fate of microorganisms and chemicals upon inoculation of soils (Daane et al. 1996; Hantshel et al. 1996). In this study we monitored the inoculated bacterium by determining the reductive dechlorination activity on 3-chlorobenzoate in appropriate soil microcosms inoculated with *D. tiedjei* and by detecting the 16S rDNA gene of this bacterium with the PCR methodology developed above. The dechlorination activity on 3-chlorobenzoate was clearly expressed in the microcosms inoculated with *D. tiedjei* in the basal medium amended with 3-chlorobenzoate whereas no dechlorination activity was observed in the non inoculated microcosms even upon prolonged incubation (Figure 3). This indicates that the dechlorination activity was the result of the introduction of *D. tiedjei*. The 3-chlorobenzoate was first transformed by this strain to benzoate which was in turn mineralized by the indigenous microbial community to CH_4 and CO_2. Evidence for benzoate mineralization can be demonstrated using carbon mass balances. Indeed after 40 days of incubation, 2.40 and 0.41 mmol of CH_4 were measured in the headspaces of microcosms inoculated with *D. tiedjei* and in non-inoculated microcosms, repectively, in the presence of acetate (5 mM) plus formate (6 mM) as supplemental cosubstrate. Taking into account only the complete transformation of acetate and formate to CH_4, the maximum expected CH_4 in the headspace is approximately 1.20 mM. On the other hand, when complete transformation of 3-chlorobenzoate (2.50 mM) to CH_4 was considered in the mass balance, the theoretical expected CH_4 in the headspace is 2.48 mmol which is closely similar to the experimentally measured CH_4 in inoculated microcosms (2.40 mmol). This increase in CH_4 production in inoculated microcosms can be attributed to benzoate mineralization.

In the inoculated microcosms, the results show that the rate of 3-chlorobenzoate biotransformation varied with the supplemental cosubstrate. Indeed, the addition of *D. tiedjei* to microcosms supplemented with acetate plus formate at their respective concentrations of 5 and 6 mM led to a total biotransformation of the chloroaromatic compound within 12 days (Figure 3). In the control microcosms which were devoid of cosubstrate, total biotransformation of 3-chlorobenzoate was observed only after 30 days (Figure 3). These results confirmed the primary role of supplemental cosubstrate on the rate of reductive dechlorination which has been previously recognized (Fathepure & Boyd 1988; Gibson & Suflita 1993; Doong & Wu 1995). The cosubstrate is a source of reducing equivalents needed for the dechlorination of 3-chlorobenzoate by the bacteria. It is no surprise then that a syntrophic association can easily occur between *D. tiedjei* and other microbial strains of a methanogenic consortium (Dolfing & Tiedje 1986). Dolfing (1990) reported previously that the doubling time of *D. tiedjei* strain DCB-1 when grown in pure culture and reductively dechlorinating 3-chlorobenzoate (8 to 10 days) was significantly longer than its doubling time within the consortium which was only 3 days.

The application of the PCR methodology developed in this study was both specific and sensitive. Figure 4 shows that the target fragment resulting from a first-round PCR using primer set (Dt60, Dt1032) was detected after three weeks in all microcosms where *D. tiedjei* was added. The PCR mixture for these results contained T4 gene 32 protein and an Expand High Fidelity PCR system (Boehringer) as the source of polymerase. Addition of these two components to the PCR mixture offers the optimum conditions for better detection of the 16S rDNA gene of *D. tiedjei* from soil slurry. The specificity of the 998 bp first-round PCR product obtained by using primer set (Dt60, Dt1032)

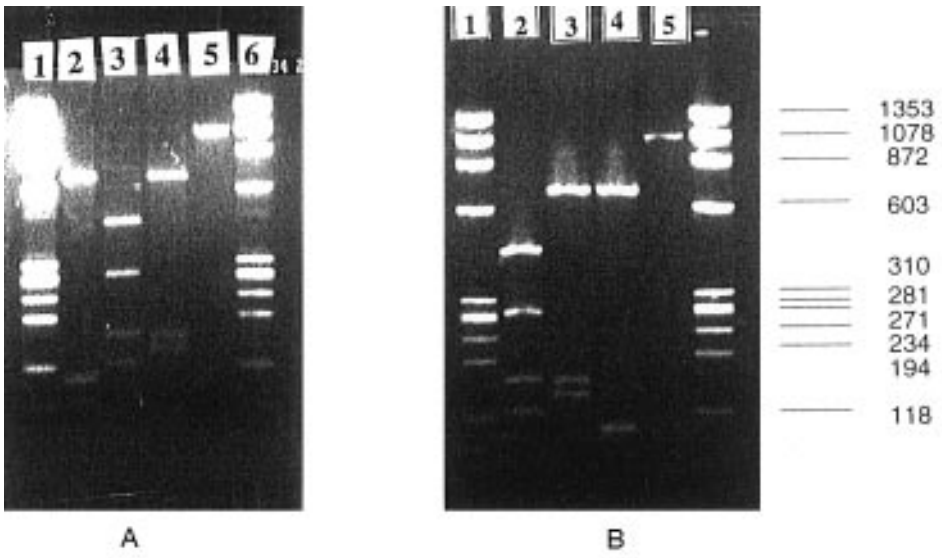

Figure 5. PCR confirmation by digestion with restriction enzymes. (A) Restriction map of sample amplified from inoculated soil; lanes: 1 and 6, ϕX174 DNA marker digested with *Hae*III; 2, 3, and 4 show digested fragments with *Hae*III, *Bsm*AI, and *Bsi*HKAI, respectively; 5, non digested fragment. (B) Restriction map using pure DNA, lanes: 1, marker; 2 , 3 and 4 show digested fragment with *Bsm*AI, *Bsi*HKAI, and *Hae*III, respectively; 5, non digested fragment.

from *D. tiedjei* was confirmed by applying appropriate restriction enzymes. The choice of enzymes was made by comparing restriction maps of the closely related species using the program of Genetic Computer Group, Inc. (Wisconsin package, version 8.1-UNIX, 1995). Three enzymes were retained since they displayed specific sites in the 16S rDNA gene of *D. tiedjei*. *Hae*III which has two sites and generated three fragments (757, 157, and 75 bp), *Bsi*HKAI which has two sites and generated three fragments (648, 208, and 134 bp) and *Bsm*AI which has three sites and generated four fragments (434, 287, 160, and 134 bp). Figure 5 shows the confirmation of the PCR results by a digestion with restriction enzymes. All PCR samples were from the inoculated microcosm containing acetate plus formate as cosubstrate.

This work reaffirms that the success of PCR, in the case of DNA extraction from soil, depends on the methodology of soil DNA extraction and target microorganisms, since the results are different by applying different methods of bacterial lysis, DNA extraction and DNA purification. Recently several protocols have been proposed for DNA extraction and purification from soil samples in order to avoid the inhibition of PCR by phenols and humic acids (Akkermans et al. 1995; Straub et al. 1995; Trevors & van Elsas 1995). It has been recently shown that different extraction methods had a strong influence on DNA recovery and that the success of PCR amplification varied with different purification methods. The results presented in this study show also that the DNA extraction and purification methodologies are crucial parameters for a successful PCR based detection in soil (Table 1). This is in line with widely differing reports in the literature. Even though 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 (Atlas et al. 1989), others after improving the temperature of annealing were able to detect 10^4 cells/g soil of *A. tumefaciens* (Picard et al. 1992). *Pseudomonas fluorescens* R2f has been detected at a sensitivity of 10^3 inoculant cells per gram of soil (Smalla et al. 1993) and *Nitrobacter* at 10 cells/g soil (Degrange & Bardin 1995).

Conclusion

In this study we have shown the successful introduction of a *de novo* biodegradation ability into previously non dechlorinating soil by inoculating *D. tiedjei*. We have shown specifically that a mineralization of 3-chlorobenzoate can occur in the soil microcosms in the presence of an anaerobic methanogenic consortium similar to the original microbial ecological niche from which *D. tiedjei* was isolated. These results are very

encouraging for developing inoculation strategies for contaminated soils especially if the strain introduced is compatible with the soil's microbiological background (Pritchard 1992). Finally we were able to successfully detect the inoculated bacterium by an improved soil DNA extraction and purification protocol well adapted to the soil/microorganism system under consideration. This demonstration augurs well for the future of anaerobic biodegradation of toxic aromatics in contaminated soils.

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