

Kinetic and phylogenetic characterization of an anaerobic dechlorinating microbial community

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The reductive dechlorination (RD) of tetrachloroethene (PCE) to vinyl chloride (VC) and, to a lesser extent, to ethene (ETH) by an anaerobic microbial community has been investigated by studying the processes and kinetics of the main physiological components of the consortium. Molecular hydrogen, produced by methanol-utilizing acetogens, was the electron donor for the PCE RD to VC and ETH without forming any appreciable amount of other chlorinated intermediates and in the near absence of methanogenic activity. The microbial community structure of the consortium was investigated by preparing a 16S rDNA clone library and by fluorescence *in situ* hybridization (FISH). The PCR primers used in the clone library allowed the harvest of 16S rDNA from both bacterial and archaeal members in the community. A total of 616 clones were screened by RFLP analysis of the clone inserts followed by the sequencing of RFLP group representatives and phylogenetic analysis. The clone library contained sequences mostly from hitherto undescribed bacteria. No sequences similar to those of the known RD bacteria like '*Dehalococcoides ethenogenes*' or *Dehalobacter restrictus* were found in the clone library, and none of these bacteria was present in the RD consortium according to FISH. Almost all clones fell into six previously described phyla of the bacterial domain, with the majority (56.6%) being deep-branching members of the *Spirochaetes* phylum. Other clones were in the *Firmicutes* phylum (18.5%), the *Chloroflexi* phylum (16.4%), the *Bacteroidetes* phylum (6.3%), the *Synergistes* genus (1.1%) and a lineage that could not be affiliated with existing phyla (1.1%). No archaeal clones were found in the clone library. Owing to the phylogenetic novelty of the microbial community with regard to previously cultured microorganisms, no specific microbial component(s) could be hypothetically affiliated with the RD phenotype. The predominance of *Spirochaetes* in the microbial consortium, the main group revealed by clone library analysis, was confirmed by FISH using a purposely developed probe.

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INTRODUCTION

The commercial introduction of organo-halogenated solvents dates back to the 1920s, but the pollution by these compounds became evident many years later in areas

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Abbreviations: DAPI, 4'-diamidino-2-phenylindole; DCE, dichloroethene; ETH, ethene; FISH, fluorescence *in situ* hybridization; OTU, operational taxonomic unit; PCE, tetrachloroethene; RD, reductive dechlorination; TSS, total suspended solids; VC, vinyl chloride.

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characterized by high levels of industrialization and urbanization. In Italy, groundwater contamination became critical especially in the northern part of the country where the economical-industrial development has been highest (Berbenni, 1994). The most common organic groundwater contaminants are chlorinated compounds such as tetrachloroethene (PCE) and trichloroethene (TCE) (Funari *et al.*, 1992). These compounds are of special concern because they are suspected carcinogens, leading to the setting of maximal admissible concentrations in drinking-water (Beccari *et al.*, 1998). They have been widely used in dry-cleaning and degreasing operations and in manufacturing electronic components.

The conventional treatment for contaminated waters is by air stripping or by adsorption on activated carbon.

However, these processes simply move the contaminant from one phase to another. Anaerobic biodegradation is an alternative treatment, and a range of different microorganisms and microbial consortia have been found to be able to dechlorinate PCE.

PCE has a high chlorine content and is therefore highly oxidized. As such, it is suitable as an electron acceptor, and anaerobic degradation occurs through a reductive dechlorination (RD) process in which H₂ is substituted for chlorine, resulting in the production of less chlorinated alkenes (DiStefano *et al.*, 1992; Mohn & Tiedje, 1992). Two mechanisms for dechlorination are possible: the first is co-metabolism where this process is a minor metabolic pathway without energetic advantages to the microorganisms. The second is metabolism in which PCE acts as an electron acceptor in a reaction that represents a novel form of anaerobic respiration (Holliger *et al.*, 1993). These mechanisms can be distinguished by the kinetics of PCE reduction and the fraction of reducing electrons diverted to dechlorination.

Several anaerobic organisms have been isolated that are capable of partial dehalogenation producing TCE (DeWeerd *et al.*, 1990; Fathepure & Boyd, 1988; Fathepure *et al.*, 1987; Gerritse *et al.*, 1996; Holliger *et al.*, 1993; Terzenbach & Blaut, 1994) or *cis*-dichloroethene (DCE) (Krumholz, 1997; Krumholz *et al.*, 1996; Miller *et al.*, 1997; Scholz-Muramatsu *et al.*, 1995; Sharma & McCarty, 1996; Wild *et al.*, 1996) from PCE. Only one strain, '*Dehalococcoides ethenogenes*' (Maymó-Gatell *et al.*, 1997), has been shown to be able to dechlorinate PCE to ethene (ETH). The phylogenetic unrelatedness of the pure cultures of methanogens, acetogens and sulfate-reducing bacteria able to dechlorinate PCE highlights the fact that this capability is quite widespread in the bacterial and archaeal domains. RD as a respiratory process (dehalorespiration) seems to be a prerogative of *Bacteria*, while *Archaea* are able only to dehalogenate chlorinated compounds co-metabolically.

In addition to dechlorinating pure cultures, several anaerobic dechlorinating microbial consortia exhibiting different mechanisms of metabolism have been reported in the literature (de Bruin *et al.*, 1992; DiStefano *et al.*, 1992; Tandoi *et al.*, 1994). Despite much knowledge about the chemical transformations and the modelling of the kinetics of the processes (Smatlak *et al.*, 1996; Tandoi *et al.*, 1994), little information is available about the identity of the microbial components of the consortia. This is mainly due to the difficulties in isolating the responsible microorganisms. For this reason, a molecular approach to study the composition of a syntrophic anaerobic microbial community, able to dechlorinate PCE mainly to vinyl chloride (VC), has been followed. The consortium has been characterized in detail by kinetic tests using different substrates for the main physiological groups such as acetoclastic, H₂- and methanol-utilizing methanogens, acetogens and dechlorinators.

METHODS

Anaerobic dechlorinating reactor. The RD reactor was a 2-l Pyrex glass vessel with a narrow neck opening, which was crimp-sealed with a Teflon-faced, butyl rubber stopper. The original inoculum for the RD reactor was anaerobic digester sludge from a wastewater treatment plant operating in the Italian province of Varese. The sludge was diluted fivefold (final volume 100 ml) with mineral salts medium containing sodium bicarbonate (pH 6.7), vitamins and yeast extract, and slowly acclimatized to PCE/methanol under anaerobic conditions (headspace N₂ 70%; CO₂ 30%) at 35 °C ('clear medium'; Tandoi *et al.*, 1994). After the acclimatization, the substrates were added weekly to a final concentration of 0.5 mM PCE and 1.9 mM methanol plus 10 mg yeast extract l⁻¹ (Beccari *et al.*, 1998).

Quantitative measurements of substrates and catabolites were carried out biweekly with GC analysis of the headspace of the reactors as reported previously (Tandoi *et al.*, 1994). To remove the VC and ETH produced from PCE, the headspace was flushed with a gas mixture of N₂ 70%/CO₂ 30% before each feeding with PCE, methanol and yeast extract.

Over a period of 3 years, supernatant from the original reactor was used as inoculum (10% v/v) for new generation cultures (four sequential transfers), confirming the sustainability of the process. A detailed description of one of these new generation cultures (named G2) was reported previously (Beccari *et al.*, 1998), where the main activities shown were RD to VC, acetogenesis and to a lesser extent, methanogenesis. Acetogenesis in particular was the main sink of the electron reducing equivalents (about 75%).

The consortium described in this research was derived from G2 (150 ml G2 + 1350 ml mineral medium) and named G4. It showed basically acetogenesis, RD and very little methanogenesis. When fast RD activity developed after inoculation, it was fed every 4–5 days, following the depletion of the main substrates, with pure PCE and methanol (0.5 and 1.9 mM as final concentrations, respectively) and 10 mg yeast extract l⁻¹. Extra doses of methanol were added if PCE was not completely depleted. The pH was also measured and corrected, when it decreased, to 6.5 ± 0.1 by addition of 10% (w/v) NaHCO₃.

Kinetic tests. Kinetic tests were carried out by transferring 37.5 ml aliquots of the N₂/CO₂ gas-flushed G4 consortium into 60 ml batch reactors, which were incubated at 35 °C in a water bath on a shaking platform (New Brunswick). The cultures were fed with various substrates (PCE, 0.5 mM; methanol, 1.9 mM; yeast extract, 10 mg l⁻¹; H₂, 3 mM) at the same concentration to which the microbial culture had been acclimatized in the period immediately preceding the test. This was done to measure the main activities of the different microbial populations of the consortium, viz. methanol- and H₂-sustained dechlorination, acetogenesis, acetoclastic and hydrogenotrophic methanogenesis. Five replicates for each metabolic activity test were carried out. During the test, sampling was done at the same time from both the gas phase and the liquid phase to ascertain the main substrates and catabolites involved (PCE, TCE, DCE, VC, ETH, CH₄, H₂, methanol and acetate).

Analytical procedures. Determination of PCE, TCE, DCE, VC, ETH and CH₄ was performed by GC using a Perkin Elmer model 8500, fitted with a 222 cm × 2 mm ID glass column packed with 60/80 Carbowax B and a flame ionization detector. The carrier gas was N₂ at 17.5 ml min⁻¹ and the oven temperature was 190 °C for 0.5 min, then ramped at 20 °C min⁻¹ to 210 °C. Calibration was performed using standards in 120 ml serum bottles, capped with Teflon-faced butyl rubber stoppers (Wheaton), to which were added the established quantities of the various compounds in the liquid

mineral medium devoid of biomass. Methanol analysis was carried out on an identical column in the same chromatograph. The carrier gas was N₂ at 17.5 ml min⁻¹ and the oven temperature was 150 °C for 2 min, then ramped at 15 °C min⁻¹ to 220 °C. H₂ in the headspace was determined by injecting 0.5 ml headspace gas into a GC Autosystem Perkin Elmer, equipped with a column molecular sieve and a Thermal Conductivity Detector. Determination of chloride and acetate was performed with ion chromatography (Dionex; model DX-100, Ionpac As9-Sc column). Total suspended solids (TSS) were determined by filtering 20 ml culture through a double filter comprising a 0.45 µm cellulose acetate (Millipore) and a GF/C filter (Whatman) and drying to constant weight at 100 °C.

DNA extraction. Microbial community nucleic acids were extracted from the anaerobic dechlorinating consortium present in one of the methanol/PCE-fed replicates at the end of the kinetic test by a bead-beating protocol (Hugenholz *et al.*, 1998b). The kinetic test results for this culture are the data shown in the first line of Table 1 (methanol and PCE as substrates). For the bead-beating method, 1 ml sample was centrifuged and the pellet resuspended in 500 µl modified 2 × buffer A (200 mM Tris, pH 8.0, 50 mM EDTA, 200 mM NaCl, 2 mM sodium citrate, 10 mM CaCl₂) and 30 µl lysozyme (100 mg ml⁻¹, freshly prepared) in a 2 ml screw-capped tube. The solution was incubated for 40 min at 37 °C. A volume of 60 µl proteinase K (final concentration 1 mg ml⁻¹) and 10 µl 20 % SDS (final concentration 0.3 %, w/v) were then added, and the solution was mixed by gentle inversion and incubated for a further 30 min at 50 °C. The sample was mixed on a Mini-Beadbeater (Biospec Products), at the low setting for 2 min in the presence of 500 µl phenol/chloroform/isoamyl alcohol (24:24:1, by vol.), 200 µl 20 % (w/v) SDS and approximately 0.3 g acid-washed zirconium beads (0.1 mm diam.). After centrifuging at approximately 12 000 g at 4 °C for 3 min to deposit the beads and sample debris, the lysate was extracted with 1 vol. phenol/chloroform/isoamyl alcohol (24:24:1, by vol.). The nucleic acids from the approximately 500 µl aqueous phase were then precipitated by adding an equal volume of 2-propanol (approx. 500 µl) and 0.1 vol. (approx. 50 µl) 3 M sodium acetate, pH 5.2, and incubating for 20 min on ice. The DNA pellet was recovered by centrifuging the solution at 12 000 g for 20 min at 4 °C. The pellet was rinsed with 500 µl 70 % ice-cold ethanol and then, after discharging the ethanol solution by inverting the tube, air-dried and resuspended in 20 µl TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0).

Amplification and cloning of the 16S rDNA. Microbial community rDNAs were PCR-amplified from the extracted DNA in 100 µl reactions containing 10–100 ng DNA, 1 × PCR buffer, 1.5 mM MgCl₂, 4 × 200 µM deoxynucleoside triphosphates, 300 nM each forward and reverse primer (530f and 1492r; Lane, 1991) and 1 U of *Tth* Plus DNA polymerase (Biotech). Reaction mixtures were then overlaid with 80 µl mineral oil and incubated in a Perkin Elmer DNA Thermal Cycler 480 for an initial denaturation step at 96 °C for 3 min followed by 28 cycles of 48 °C for 1 min, 72 °C for 2 min and 94 °C for 1 min, and by a final extension of one cycle of 48 °C for 1 min and 72 °C for 5 min. Amplified rDNAs pooled from three reactions were purified by the Wizard PCR Cleanup Kit (Promega) according to the manufacturer's instructions.

Amplicons were then used in a ligation reaction mixture (pGEM-T Vector, Promega) comprising 1 µl T4 DNA ligase (1 U µl⁻¹), 1 µl 10 × buffer (provided by the manufacturer), 1 µl pGEM-T vector (about 50 ng), 2 µl amplicons (about 150 ng) and 5 µl sterile double distilled water. Ligation occurred overnight at 4 °C. Transformation of Stratagene Epicurian Coli XL2-Blue MRF' ultracompetent cells was with 2 µl ligation mix using Promega instructions for pGEM-T vector systems.

RFLP analysis of clone inserts. The rDNA inserts from recombinant clones were amplified by PCR, using specific plasmid primers (SP6 and T7) following the same protocol as that described for the initial amplification of the rDNA. A volume of 10 µl unpurified reamplified rDNA PCR products was digested with 1 U µl⁻¹ of the 4-base-cutting specific restriction endonuclease *Hin*P1 in 1 × NEB buffer 2 (New England Biolabs) in a final volume of 20 µl. The reactions were carried out at 37 °C for 3 h. The restriction products were separated by 4 % Tris/acetate/EDTA agarose gel electrophoresis for 90 min at 80 V. Bands were visualized by staining with ethidium bromide and UV illumination. RFLP patterns were grouped visually into operational taxonomic units (OTUs), and representatives of each OTU were selected for insert sequencing and analysis.

Sequencing of rDNA clones and phylogenetic analysis. Plasmid inserts from representative clones from the PCE dechlorinating enrichment (PCEDR clone library) were sequenced with an ABI 373 automated DNA sequencer (Applied Biosystems) according to the manufacturer's instructions. For first-pass sequence analysis of clone inserts, the small subunit rDNA-primer 530f was used as a sequencing primer. Second-pass analysis of selected clones involved determination of the complete sequence of both strands of the

Table 1. Maximum utilization and formation rates in batch tests (±SD) with the G4 microbial consortium

Substrate*	Maximum utilization rate [µmol (mg TSS) ⁻¹ day ⁻¹]			Maximum formation rate [µmol (mg TSS) ⁻¹ day ⁻¹]	
	Methanol	H ₂	VC	ETH	CH ₄
Methanol and PCE	6.17 ± 2.36		1.55 ± 0.81	ND	<0.018
H ₂ and PCE		17.2 ± 5.29	4.77 ± 0.26		<0.024
H ₂ and PCE (after PCE depletion†)		5.64 ± 3.29		0.120 ± 0.008	
Methanol	10.88 ± 2.54				0.105 ± 0.020
H ₂		1.97 ± 1.12			0.091 ± 0.010
None					0.092 ± 0.023
H ₂ and PCE (with no biomass)		ND	ND		

ND, Not detected.

*The medium also contained acetate at about 25.6 ± 2.43 mmol l⁻¹ and 10 mg yeast extract l⁻¹.

†Rates were measured in the H₂ and PCE incubation but after PCE was completely depleted, and the dechlorination was only with VC as electron acceptor.

inserts, performed with a combination of rDNA primers: 803f, 1230f, 926f, 1492r, 907r (Lane, 1991). Sequences were initially compiled using the SeqEd software package (Applied Biosystems) and compared with available databases by use of the Basic Local Alignment Search Tool (BLAST; Altschul *et al.*, 1997) to determine approximate phylogenetic affiliations. Phylogenetic analysis of the sequences was by methods reported by Hugenholtz *et al.* (2001b), and the inferred phylogenetic affiliations of the sequences were confirmed using multiple outgroup analyses (Dalevi *et al.*, 2001). Where possible, we used the nomenclature from the taxonomic outline of prokaryotic genera (release 1.0) available at <http://www.cme.msu.edu/bergeys/>. Sixteen representative rDNA clone sequences obtained in this study have been deposited in GenBank under the accession numbers AF408637 (PCEDR694, *Spirochaetes*), AF408638 (PCEDR702, *Spirochaetes*), AF408639 (PCEDR13, *Spirochaetes*), AF408640 (PCEDR691, *Spirochaetes*), AF408641 (PCEDR63, *Firmicutes*), AF408642 (PCEDR467, *Synergistes*), AF408643 (PCEDR319, *Chloroflexi*), AF408644 (PCEDR463, *Bacteroidetes*), AF408645 (PCEDR683, *Spirochaetes*), AF408646 (PCEDR696, *Firmicutes*), AF408647 (PCEDR137, *Chloroflexi*), AF408648 (PCEDR658, *Bacteroidetes*), AF408649 (PCEDR92, *Bacteroidetes*), AF408650 (PCEDR98, *Chloroflexi*), AF408651 (PCEDR56, *Synergistes*), AF408652 (PCEDR218, unidentified).

Fluorescence *in situ* hybridization (FISH) and microscopy.

Probes specific for most *Bacteria* (EUB338; Amann *et al.*, 1990), *Alphaproteobacteria* (ALF1b; Manz *et al.*, 1992), *Betaproteobacteria* (BET42a; Manz *et al.*, 1992), *Gammaproteobacteria* (GAM42a; Manz *et al.*, 1992) and some lineages of the *Deltaproteobacteria* (SRB385; Amann *et al.*, 1992), for the *Cytophaga-Flavobacteria* subphylum of the *Bacteroidetes* (CF319a; Manz *et al.*, 1992), for *Actinobacteria* (HGC69a; Roller *et al.*, 1994), for *Firmicutes* (LGC354 suite; Meier *et al.*, 1999), for the *Archaea* (ARC915; Stahl & Amann, 1991) and for the *Euryarchaeota* (EURY498R; Burggraf *et al.*, 1994) were used on the G4 dechlorinating consortium fixed in ethanol and paraformaldehyde (Amann, 1995) using methods described by Amann (1995). All the bacterial phylum and subphylum probes were used simultaneously with 4',6-diamidino-2-phenylindole (DAPI) and/or the EUB338 (Amann *et al.*, 1990) probe specific for most *Bacteria*. The '*D. ethenogenes*' specific reverse primer PCEB with the sequence 5'-GGTTGGCACATCGACTTCAA-3' (S. H. Zinder, personal communication) was synthesized with a 5'-Cy3 label and used in FISH for detecting the presence of this micro-organism in the dechlorinating microbial consortium.

The probe design tool of the ARB software package (<http://www.arb-home.de/>) was used to design a probe (SPL998) from the clone library data, for the *Spirochaetes* phylum owing to its high abundance in the clone library (56.6%, detailed later). The design parameters used were as previously described (Hugenholtz *et al.*, 2001a,b). Probe sequence (SPL998 – 5'-CTCCGAAACTCCTGGG-3') was subsequently confirmed for specificity using BLAST, commercially synthesized with either 5'-Cy3 or 5'-fluorescein label (MWG Biotech) and evaluated and optimized with paraformaldehyde- and ethanol-fixed G4 dechlorinating consortium.

The G4 culture was investigated by Gram staining procedures and by FISH (Amann, 1995). FISH preparations were viewed on a Zeiss Axioskop microscope with filter sets 1, 10 and 15 (for DAPI, fluorescein and Cy3/rhodamine, respectively).

RESULTS AND DISCUSSION

The anaerobic dechlorinating microbial consortium G4 was operated as a batch system, with several sequential spikes of substrates for the purpose of producing a large newly

developed dechlorinating culture. An almost negligible amount (dilution factor higher than 10^4) of the original sludge remained in G4. The feeding pattern of the G4 anaerobic dechlorinating consortium over 140 days of operation is shown in Fig. 1. The biomass in G4 increased during the 140-day incubation from 10.6 to 112 mg TSS l⁻¹. Considering the batch mode of operation and the high organic loading rate [0.18–0.22 mg COD (mg TSS)⁻¹ day⁻¹], the cells can be considered active under these conditions. The kinetic tests, described in the following paragraphs were performed at day 140 after inoculation.

Dechlorinating activity

Table 1 shows the maximum rates of dechlorination activity and electron donor utilization obtained with the methanol/PCE-grown consortium fed with specific substrates (mean of five replicates). The maximum utilization rate and maximum formation rate were calculated by fitting the concentration data with a straight line in the first linear (zero-order) part of observed profiles and are presented for reducing substrates (methanol or H₂) and for catabolites (VC, ETH and CH₄), respectively. Fig. 2 shows the typical trend, observed in one of five replicates of VC production with methanol (Fig. 2a) or H₂ (Fig. 2b) as electron donor.

PCE RD by the G4 microbial consortium using methanol exhibited zero-order and almost complete conversion to VC with the accumulation of other more chlorinated compounds being practically negligible (Fig. 2). The VC formation rate was $1.55 \pm 0.81 \mu\text{mol (mg TSS)}^{-1} \text{ day}^{-1}$, which corresponds in terms of reducing equivalents to about 25% of the methanol utilization. This high rate and high yield are usually suggested (Holliger *et al.*, 1993) to indicate that RD is linked to an energy-yielding metabolism, not simply co-metabolism. The remaining fraction of methanol utilized was diverted to acetate formation (detailed later), with CH₄ formation being practically negligible (Table 1). With H₂ instead of methanol for RD, the microbial consortium G4 did not show any additional lag phase, and RD with molecular hydrogen was about three times faster than with methanol (Table 1). Moreover, RD was the main process occurring in the consortium, as deduced from the fact that the reducing equivalents necessary to produce VC from PCE accounted for about 83% of consumed H₂, much higher than 25% found for methanol (values calculated at 30 and 18 h in Fig. 2a and b, respectively).

It should be stressed that the microbial consortium had never been fed with molecular hydrogen before the batch test. Thus, the higher rate with no lag phase when H₂ substituted the methanol supports the hypothesis that also in the PCE/methanol-fed consortium, the actual source of reducing equivalents for RD is H₂, as reported in the literature (Ballapragada *et al.*, 1997; DiStefano *et al.*, 1992; Maymó-Gatell *et al.*, 1995; Tandoi *et al.*, 1994). Because methanol is the only electron donor given to the consortium

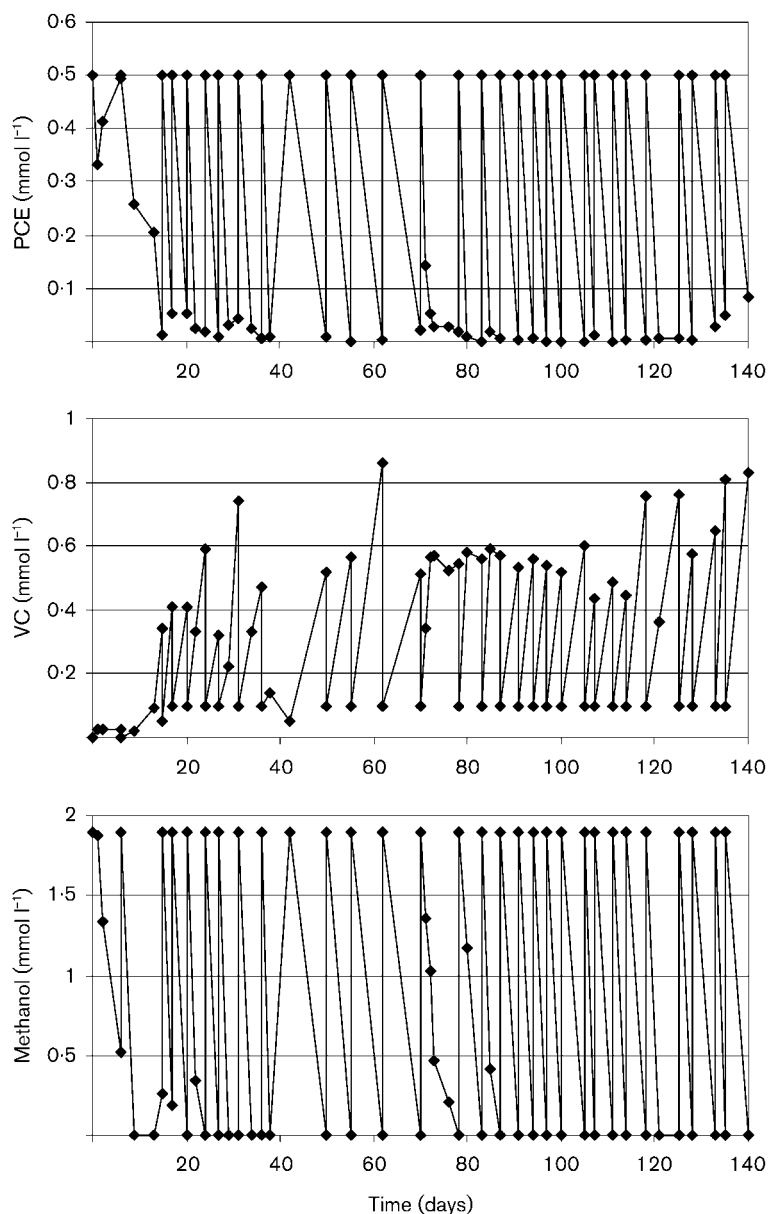


Fig. 1. Feeding pattern of PCE and methanol and VC formation by the G4 anaerobic dechlorinating consortium before the kinetic tests carried out at day 140 after inoculation (the dry wt at day 0 was 10.6 mg l^{-1} and at day 140, 112 mg l^{-1} ; pH 6.5). The methane and ETH concentrations detected over the whole period were in the range $0.001\text{--}0.003$ and $0.02\text{--}0.11 \text{ mM}$, respectively.

(with the exception of a small amount of yeast extract), the observed behaviour is also consistent with the hypothesis that H_2 is formed by methanol-utilizing micro-organisms. The presence of interspecies H_2 transfer has already been reported for methanol-utilizing acetogens coupled with hydrogenotrophic micro-organisms (Cord-Ruwisch & Olivier, 1986; Cord-Ruwisch *et al.*, 1988; Heijthuijsen & Hanson, 1986) and is consistent with the high acetogenic activity observed in the consortium (see below).

If the role of H_2 as the actual electron donor is assumed, the lower RD rate from methanol than from H_2 [$1.55 \text{ } \mu\text{mol (mg TSS)}^{-1} \text{ day}^{-1}$ vs $4.77 \text{ } \mu\text{mol (mg TSS)}^{-1} \text{ day}^{-1}$; Table 1] indicates that in the PCE/methanol-fed consortium, the RD activity is far from optimal and the H_2 formation is the rate-determining step of the overall reaction.

In the presence of an excess of molecular H_2 , no formation

of ETH was observed until all PCE had been converted to VC. However, the ETH formation rate after PCE depletion remained quite low compared with the VC formation rate (Table 1). Moreover, since only 2% of the H_2 reducing equivalents was used for ETH formation, the VC reduction was likely co-metabolic.

The dechlorination rate of PCE to VC and ETH has been reported to be equal to $69.0 \pm 10.5 \text{ nmol chloride released min}^{-1} (\text{mg protein})^{-1}$ in '*D. ethenogenes*' strain 195 (Maymó-Gatell *et al.*, 1997). If one assumes that the main conversion of PCE is to VC and the TSS is 55% protein, this dechlorination rate would correspond to the formation of $18.2 \pm 2.7 \text{ } \mu\text{mol VC (mg TSS)}^{-1} \text{ day}^{-1}$. Assuming that the dechlorinating micro-organisms in our culture have similar kinetics to strain 195, we estimated that the dechlorinators responsible for the observed PCE reduction would comprise

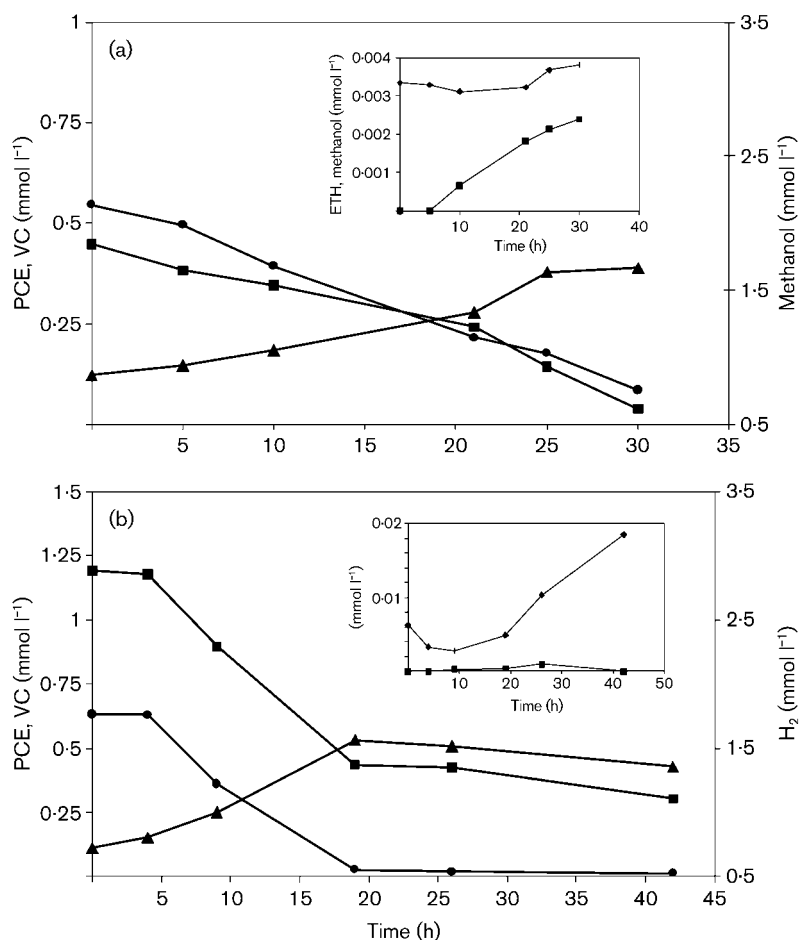


Fig. 2. Typical trend of VC production by the G4 anaerobic dechlorinating microbial consortium with (a) methanol and (b) H₂ as electron donor. Filled circles, PCE consumption; filled triangles, VC production; filled squares, methanol consumption (a) and H₂ consumption (b). Insets: filled diamonds, ETH production; filled squares, methane production.

22–28% of TSS. Even though arbitrary assumptions have been made (see above), and extrapolation of kinetic data from pure to mixed cultures is always dubious, this estimation suggests that PCE-dechlorinating micro-organisms comprise one substantial metabolic group in the G4 consortium (22–28%). However, other metabolic groups like acetogens are also abundant, and collectively, groups other than PCE dechlorinators comprise 72–78% of the microbial community.

Methanogenic activity

In all conditions tested, the microbial consortium G4 showed very little methanogenic activity (see CH₄ formation rate in Table 1). In the acetate-rich growth medium lacking PCE, methanol and H₂, the CH₄ formation rate owing to acetoclastic methanogenesis was $0.092 \pm 0.023 \mu\text{mol (mg TSS)}^{-1} \text{ day}^{-1}$ (Table 1). Considering typical kinetic parameters for acetoclastic methanogenesis (Pavlostathis & Giraldo Gomez, 1991), this rate indicates that less than 0.2% of the total biomass was acetoclastic methanogens. Also, assuming that specific methanogenic activity could have been overestimated from literature data (owing to partial inhibition by the low pH of 6.5 and a high acetate content of about 25 mmol l^{-1} in the culture), it is unlikely that they would represent more than 1% of total biomass.

In contrast, the presence of H₂ did not increase the CH₄ formation rate, demonstrating that H₂ utilizing methanogenesis was negligible compared with acetoclastic methanogenesis (Table 1). In addition, the direct formation of CH₄ from methanol was negligible. Table 1 shows low CH₄ formation rates of $0.105 \mu\text{mol (mg TSS)}^{-1} \text{ day}^{-1}$ with methanol and acetate as substrate and $0.092 \mu\text{mol (mg TSS)}^{-1} \text{ day}^{-1}$ with acetate alone.

CH₄ formation was inhibited more (about fourfold) in the presence of PCE (or related VC formation) than in its absence (Table 1). Owing to the very small methanogenic activity shown by the G4 microbial consortium, the specific source of inhibition (PCE or VC) was not investigated any further.

Acetogenic activity

A previous study (Beccari *et al.*, 1998) showed that in the initial inoculum for the G4 consortium, methanol was mostly converted to acetate by methylotrophic acetogens. Indeed, kinetic tests had been performed on the G4 parent consortium (the consortium G2 in Beccari *et al.*, 1998), showing that about 75% of methanol was converted to acetate (based on reducing electron equivalents). From the start of the enrichment, G4 maintained this intense acetogenic activity, as indicated by progressive acetate

accumulation in the medium. The acetate concentration in the medium increased from 2.2 to 25.4 mM after 33 sequential additions of 1.9 mM methanol during 140 days of enrichment. Methanol conversion to acetate has been estimated from these data and also by taking into account the dilution caused by periodic renewal of the medium (see Methods), the possible acetate formation from yeast extract (given at about 10% of methanol in terms of reducing equivalents) and from the acetate consumption for acetoclastic methanogenesis (as estimated from the data in Table 1). We concluded that the observed concentration increase corresponded to at least 62% of methanol conversion into acetate (on a reducing electron equivalents basis). This confirmed that the main mode of methanol utilization in the consortium G4 was acetogenesis. However, the high acetate concentration prevented the measurement of acetate formation rate resulting from a single spike of methanol, as the increase in acetate concentration was within the range of experimental error and was therefore not performed during the batch tests reported in Table 1.

The methanol-supported acetogenesis was like acetoclastic methanogenesis, inhibited by the presence of PCE since the methanol utilization rate was about 40% lower than the rate in the absence of PCE [$6.17 \mu\text{mol} (\text{mg TSS})^{-1} \text{day}^{-1}$ vs $10.88 \mu\text{mol} (\text{mg TSS})^{-1} \text{day}^{-1}$; Table 1].

Tests with only H_2 , in the absence of PCE, have shown a consumption that cannot be explained in terms of hydrogenotrophic methanogenesis (see previous section). This indicates the presence of homoacetogenic activity, although quite low, because the H_2 consumption rate was only 11% of the overall consumption rate obtained with the simultaneous presence of RD [$1.97 \pm 1.12 \mu\text{mol} (\text{mg TSS})^{-1} \text{day}^{-1}$ vs $17.2 \pm 5.29 \mu\text{mol} (\text{mg TSS})^{-1} \text{day}^{-1}$; Table 1].

In summary, the main metabolic activities of the consortium were methanol conversion to acetate and H_2 by methanol-utilizing acetogens and H_2 consumption for the RD of PCE to VC by dechlorinating organisms.

Microbial morphology of the RD consortium

The culture G4 consisted of Gram-negative cells comprising thin and fat rods, short chains of rods, filaments, and cocci in pairs and short chains and of Gram-positive cocci as individuals, pairs and small groups. There were no large clusters or flocs of micro-organisms.

Molecular analysis of the RD consortium

A total of 616 clones containing inserts of the expected size (approximately 1 kb) were selected from the total library for further analysis. 16S rRNA gene-containing clones were screened by RFLP analysis to group those with similar banding profiles following agarose gel electrophoresis. Representatives of OTUs were chosen for insert sequence determination. Typically, three to six bands resulted from each rDNA restriction enzyme digest, in the discernible fragment size range of approximately 100–650 bp.

In the library, four major and nine minor RFLP profiles were distinguished visually, and 16S rDNA inserts from 120 clones representing all the RFLP types were partially sequenced using primer 530f and sequence identities determined. Sequences differing by <2% were considered a single relatedness group and representatives of most of these groups were fully sequenced on both strands with additional rDNA sequencing primers (see Methods).

The clustering by RFLP analysis of the 616 clones produced a total of six OTUs. The main RFLP profile of OTU1.1 (55.5% of the total clones) typically comprised three distinct bands (500, 400 and 300 bp), as shown in Fig. 3 (profile B). The second cluster (OTU1.2) comprised 1.1% of the library. OTU2 produced two bands at about 650 and 500 bp, as shown in Fig. 3 (profile C). The main cluster within OTU3 showed a RFLP profile comprising four bands (500, 400 and two bands between 200 and 100 bp) (Fig. 3, profile D). This main cluster, along with two minor clusters of OTU3, represented 16.4% of the total screened clones. The remainder of the clone library comprised OTUs 4, 5 and 6 that together represented 8.5% of the clones. The main profile within OTU 4 showed four bands (500, ~280, 250 and ~180), as shown in Fig. 3 (profile A).

Fig. 4 shows the maximum-likelihood dendrogram of representative sequences in the context of currently recognized bacterial phyla. The analysis of the sequences showed that all are in one of five phyla in the bacterial domain. The dominant clone group representing 55.5% of all clones analysed (represented by four clones PCEDR13, PCEDR702, PCEDR691 and PCEDR694 in Fig. 4; OTU1.1) formed a cluster of sequences in the *Spirochaetes* phylum. A smaller cluster of seven clones (represented by PCEDR683 in Fig. 4; 1.1% of the library; OTU1.2) was also in the *Spirochaetes* phylum but with a low percentage identity to the large RD *Spirochaetes* cluster. Other bacterial phyla in the clone library, in decreasing abundance, were the *Firmicutes* (18.5%, but loosely affiliated; OTU2), the *Chloroflexi* (16.4%, OTU3) and the *Bacteroidetes* (6.3%, OTU4). The *Synergistes* genus was also present in the clone library (1.1%, OTU5) (Fig. 4). A sixth cluster was not affiliated with any division (1.1%, OTU 6; see sequence PCEDR218 in Fig. 4). Groupings of clones by RFLP were similar to groups determined by phylogenetic analysis of clone DNA sequences. This demonstrates the utility of this simple procedure of RFLP analysis for screening clone libraries.

Previously published probes for the domains *Bacteria* and *Archaea*, and for bacterial and archaeal phyla, and subphyla were used with fixed material from both methanol- and H_2 -fed reactors. The majority of FISH-positive cells were *Bacteria*, but almost 50% of the cells visualized with DAPI did not bind either the EUB338 (for most *Bacteria*) or the ARC915 (for most *Archaea*) probes. An expanded suite of bacterial probes (collectively called EUBMIX) reported by Daims *et al.* (1999) were also used, but they did not increase the amount of positive bacteria with respect to

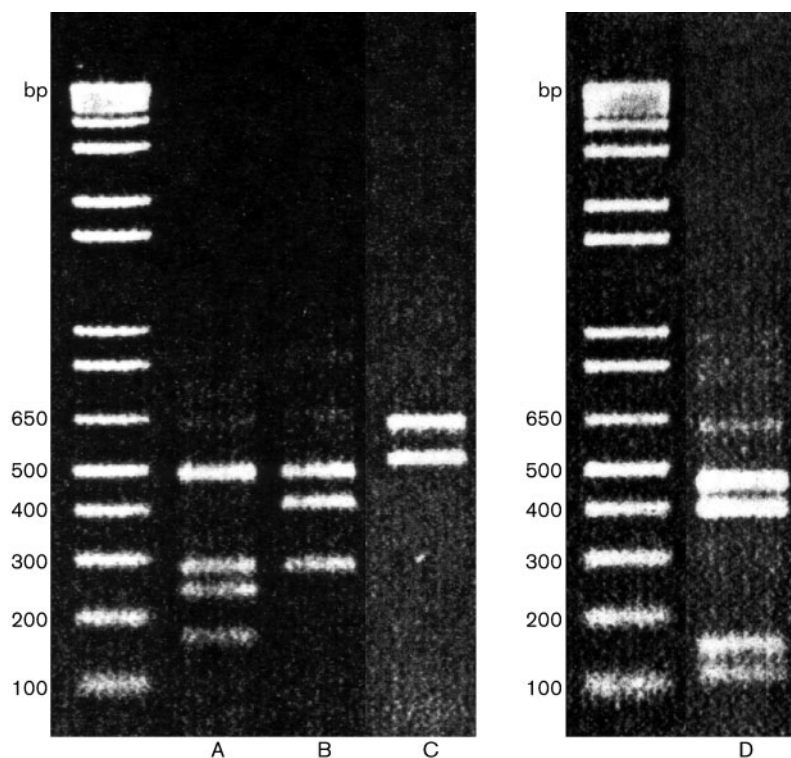


Fig. 3. Profiles of the main OTUs after RFLP analysis with *HinP1*. Profile A corresponds to cluster 1 of the *Bacteroidetes* phylum, profile B to cluster 1 of the *Spirochaetes* phylum, profile C to representatives of OTU2 and profile D to cluster 1 of the *Chloroflexi* phylum.

the total DAPI stained cells. Therefore, the FISH-negative cells were likely not permeabilized, poorly active or dead. Essentially, none of the published probes we used (see Methods) bound to the cells in the PCE-dechlorinating cultures. In each case, EUB338 was used simultaneously with group-specific probes.

The clone library comprised a high proportion of sequences that were deep-branching in the *Spirochaetes* phylum (55.5%; Fig. 4). To investigate the *in situ* abundance of these organisms in the RD consortium more intensively, a specific FISH probe (SPL998) was designed. The specificity of this probe is shown in Fig. 4, and we found the optimum formamide concentration for its use to be 10%. Probe SPL998 specifically highlighted small and longer bacilli in the G4 consortium. About half of the EUBMIX-binding cells hybridized with SPL998 probe, thus confirming the high abundance of these *Spirochaetes* in the G4 culture, as was shown by the clonal analysis (55.5%).

We chose primers 530f and 1492r to prepare the clone library as we wanted to harvest the widest spectrum of sequence types from the RD community, including those from the bacterial and archaeal domains, and we knew that both primers have broad matches in both domains (Lane, 1991). Nevertheless, bacterial but no archaeal sequences were found in the clone library. The most obvious conclusion from this finding is that the quantity of *Archaea* in the microbial consortium is too low to be found by this method. Another conclusion is that the primers did not amplify archaeal templates. The *Archaea* most likely to be found in the PCE RD consortium are methanogens, but the physiological results demonstrated that the methanogens

are unlikely to represent even 1% of the total microbial community. Therefore, it is most likely that *Archaea* were below the detection limit of the PCR cloning procedure. In this dispersed cell system, 1% of the microbial community should have been detectable by FISH. However, no cells bound the ARC915 or the EURY498R probes, but this could be due to low ribosome abundance in the archaeal cells.

A recent study has shown the presence of *Spirochaetes* in a PCE-to-ETH RD consortium (Löffler *et al.*, 2000). Two *Spirochaetes* populations were isolated, but they were unable to dechlorinate PCE. An isolate named *Dehalococcoides* sp. strain FL2 was also obtained, which had 96.6% 16S rDNA sequence identity with '*D. ethenogenes*', the strain that has been shown to be able to dechlorinate PCE to ETH (Maymó-Gatell *et al.*, 1997). Strain FL2 was deemed responsible for RD in the consortium. Our *Spirochaetes* sequences showed a very low percentage identity with those from this previous study, and the possible phenotypic traits of our novel *Spirochaetes* could not be deduced.

One goal achieved in our study was the analysis of the PCE RD microbial community in terms of its physiology (products formed from PCE and kinetics) and structure (morphology, clonal and FISH analysis). We hoped to correlate the microbial structure and the source environment as suggested in the review by Hugenholtz *et al.* (1998a). However, the novelty of most of our sequences harvested from the microbial community and analysed in the clone library prevented this goal from being completely achieved. Nevertheless, many clones have as close relatives strictly anaerobic organisms or clones from anaerobic environments. For example, clone PCEDR467 in the

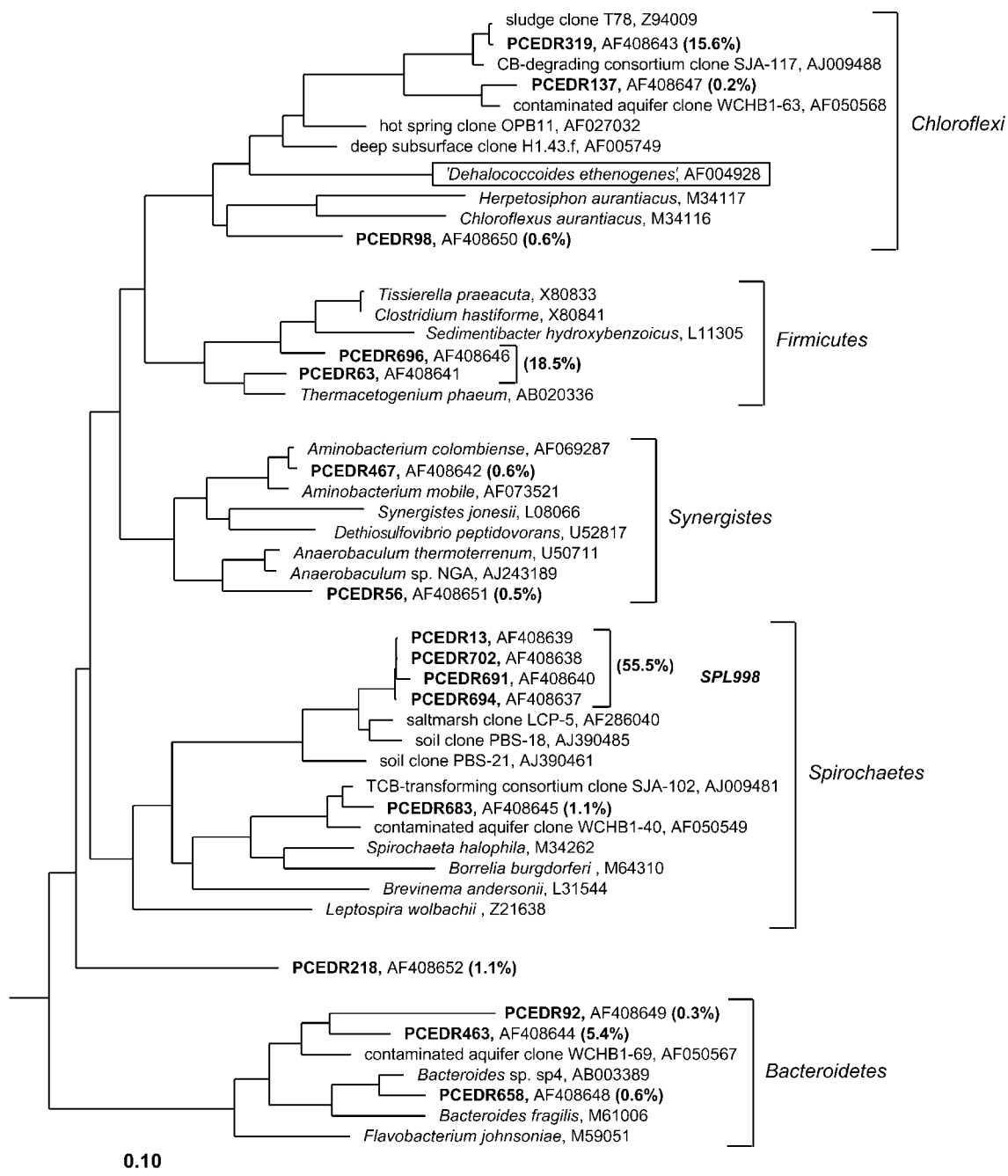


Fig. 4. Maximum-likelihood dendrogram of the bacterial domain based on comparative analyses of 16S rDNA data. Phylum designations are indicated by brackets on the right. The specificities of the SPL998 probe designed to target the *Spirochaetes* phylum are indicated in bold italics outside the brackets. Clones sequenced in the present study are shown in bold. The percentages in parentheses refer to the clone frequencies determined by RFLP analysis. '*D. ethenogenes*' (boxed) is currently the only described isolate capable of complete PCE dechlorination. The archaeal outgroups (not shown) were *Methanococcus vannielii* (M36507) and *Sulfolobus acidocaldarius* (D14876). The bar represents 10% estimated sequence divergence.

Synergistes genus (Fig. 4) is 99% identical to *Aminobacterium colombiense*, a strictly anaerobic, aminolytic organism; PCEDR63 in *Firmicutes* (Fig. 4) is 94% identical to *Thermacetogenium phaeum*, a strictly anaerobic

acetogen; and some PCEDR clones have close relatives from PCR-clonal analyses of anaerobic trichlorobenzene- (PCEDR319) and anaerobic chlorobenzene-transforming (PCEDR683) consortia.

A cluster of novel, deep-branching *Spirochaetes* sequences were obtained and dominated the clonal and FISH analyses, but we could not conclude anything about the phenotype. '*D. ethenogenes*' from the *Chloroflexi* bacterial phylum is a known dechlorinator of PCE to VC and ETH (the function of our community). No organisms like '*D. ethenogenes*' were present in our RD community according to clonal analysis. We attempted to explore this further by using a previously reported '*D. ethenogenes*' PCR primer in FISH (S. H. Zinder, personal communication) but obtained no positive results. Organisms able to dechlorinate PCE to TCE or DCE by dehalorespiration like *Desulfotobacterium* spp., *Dehalobacter restrictus*, *Desulfuromonas chloroethenica* and *Dehalospirillum multivorans* were also not present in our RD consortium on the basis of clonal analysis. The clonal analysis showed that OTUs 1, 2 and 3 (*Spirochaetes*, *Firmicutes* and *Chloroflexi*) constituted 91.5% of the screened clones, and most likely the main phenotypes of the culture (fermenters and dechlorinators) belong to these bacterial phyla. We can conclude therefore that our PCE dechlorinator(s) are hitherto undiscovered bacteria.

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