

Identification of a microorganism that links its growth to the reductive dechlorination of 2,3,5,6-chlorobiphenyl

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Summary

Anaerobic bacteria reductively dechlorinate polychlorinated biphenyls (PCBs) in aquatic sediments, but these microorganisms remain uncultured and, until now, unidentified. Through denaturing gradient gel electrophoresis (DGGE) of 16S rDNA from a highly enriched *ortho*-PCB dechlorinating culture, the growth of a single microorganism was shown to be dependent upon the presence and dechlorination of 2,3,5,6-tetrachlorobiphenyl. This is the first identification of a microorganism that catalyses the reductive dechlorination of a PCB. The organism, bacterium *o*-17, has high sequence similarity with the green non-sulphur bacteria and with a group that includes *Dehalococcoides ethenogenes*. Bacterium *o*-17 required acetate for dechlorination and growth. H₂:CO₂ (80:20 at 101 kPa) did not support dechlorination or growth of the dechlorinator. Archaeal 16S rDNA was not detected in actively dechlorinating bromoethanesulphonate-treated non-methanogenic cultures, which indicated that methanogenic Archaea were not required for dechlorination. The consistent association with dechlorinating activity combined with high similarity to other known dechlorinating microorganisms indicates that bacterium *o*-17 catalyses the reductive *ortho*-dechlorination of 2,3,5,6-tetrachlorobiphenyl.

Introduction

Polychlorinated biphenyls (PCBs) are found globally (Wania and Mackay, 1996; Blais *et al.*, 1998) especially in aquatic sediments, in which they accumulate near the

site of their release. The co-planar, so-called dioxin-like, PCB congeners have often been the focus of toxicological studies. However, several of the non-co-planar congeners, which are *ortho*-substituted, are now under scrutiny for their potential neurological and developmental toxicological properties (Hanson, 1999). The microbial transformation of PCBs may alter or even reduce such toxicity. Furthermore, microbial anaerobic dechlorination of more extensively chlorinated PCB congeners compliments the degradation by aerobic microorganisms, which are restricted to attacking lesser-chlorinated congeners. Environmental evidence for microbially mediated reductive dechlorination of PCBs in anaerobic sediments was first reported in 1987 (Brown *et al.*, 1987) and was later confirmed in sediment microcosms (Quensen *et al.*, 1988). However, the microorganisms responsible for PCB dechlorination have not been identified.

The aim of the present investigation was to identify an anaerobic microorganism that catalyses the reductive dechlorination of a PCB. Until recently, few bacteria had been described that reductively dechlorinate chlorinated organic molecules of any kind. Today, several such microorganisms are known including those that couple growth to the dechlorination of alkyl and aryl compounds through a process called dehalorespiration (Holliger *et al.*, 1998). The isolation and characterization of dechlorinating bacteria is important in assessing how to enhance biodegradation and for developing tools (e.g. molecular probes) to monitor or study the dechlorination *in situ*. As is the case with the majority of environmental microorganisms, conventional isolation methods have been unsuccessful in identifying a PCB dechlorinating species. In the present study, 16S rDNA of a PCB dechlorinating enrichment culture was monitored by denaturing gradient gel electrophoresis (DGGE) and the dechlorinating organism was identified by reductive analysis of the microbial community in response to different culture treatments. In a highly selective minimal medium, a culture that reductively dechlorinates a PCB congener by a discrete pathway was enriched from estuarine sediments of Baltimore Harbor (USA) under sediment-free conditions (Cutter *et al.*, 1998). The enriched culture, referred to as the *ortho*-dechlorinating culture, sequentially *ortho* dechlorinates 2,3,5,6-tetrachlorobiphenyl to 2,3,5-trichlorobiphenyl and 3,5-dichlorobiphenyl when supplied with a mixture of short chain fatty acids. Several potential electron donors could drive the dechlorination of

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the PCB under these conditions. The oxidation of acetate or hydrogen coupled to the reduction of 2,3,5,6-CB to 2,3,5-CB and 3,5-CB was examined in this study. Herein we present the first identification of a PCB dechlorinating anaerobic bacterium that links its growth to the utilization of acetate and the dechlorination of 2,3,5,6-tetrachlorobiphenyl.

Results

Effect of acetate and hydrogen on dechlorination of 2,3,5,6-CB

The *ortho*-dechlorinating culture was originally established in the absence of sediment with a mixture of three fatty acids as potential electron donors (2.5 mM each of sodium acetate, sodium propionate and sodium butyrate). Cysteine-HCl at 1.4 mM was supplied as a reductant in the medium but could also serve potentially as a source of carbon, sulphur and energy. Carbon dioxide was made available through the medium's buffer and headspace gas. PCB (2,3,5,6-CB) was supplied as the only chlorinated substrate and as a potential electron acceptor, and acetone (13.6 mM) was used as a carrier for the PCB. As the culture was maintained in the presence of several potential electron donors and acceptors, each of these components was examined for its ability to support dechlorination of 2,3,5,6-CB.

Acetate was found to support a more rapid rate of PCB dechlorination than the combination of short chain fatty acids (Fig. 1A). The addition of 20 mM acetate was optimal (30 or 40 mM acetate did not enhance the activity further). Acetone, which was added to the cultures as a carrier of the PCBs, has been shown to serve as a carbon and energy source in a methanogenic enrichment culture (Platen and Schink, 1987). However the combination of acetone, cysteine, CO₂ and PCB did not support PCB dechlorination by the *ortho*-dechlorinating culture in the absence of acetate, thus demonstrating that acetate was required for PCB dechlorination by this enrichment culture.

The *ortho*-dechlorinating culture remained methanogenic. Therefore, it was possible that the dechlorinating microorganisms and the methanogens might compete for the acetate or the methanogens might dechlorinate the PCB. Previously it was demonstrated that sediment-containing precursors to the *ortho*-dechlorinating culture would dechlorinate 2,3,5,6-CB in the absence of methanogenesis when 3 mM bromoethanesulphonate (BES) and 5 mM sodium acetate were added (Pulliam Holoman *et al.*, 1998). Here, in contrast, amendment of the sediment-free *ortho*-dechlorinating culture with 1–3 mM BES and 5 mM acetate resulted in a reduction of dechlorinating activity below detectable levels. However, dechlorination was detected when the acetate concentration was increased

to 20 mM in the presence of 1–3 mM BES (Fig. 1B). No methane was detected in any of the BES-treated cultures. The dechlorinating activity that was observed in the cultures containing 20 mM acetate and 3 mM BES continued to be the *ortho* dechlorination of 2,3,5,6-CB to 3,5-CB. Although the sediment-free cultures were more sensitive to the effects of BES, these results suggested that the methanogens in the culture were not responsible for dechlorination under these defined, sediment-free conditions. The results also confirmed the findings of Pulliam Holoman *et al.* (1998), who reported no effect by BES on *ortho* PCB dechlorination in cultures maintained with sediment.

A common electron donor for microbial reductive dechlorination is hydrogen. It is possible that the acetate required for PCB dechlorination was oxidized to carbon dioxide and the hydrogen generated was then used for reductive dechlorination. This was tested by incubating the *ortho*-dechlorinating culture with 101 kPa of H₂:CO₂ with and without acetate. Initially, all the cultures dechlorinated 2,3,5,6-CB, but eventually dechlorination rates decreased (Fig. 1C). However, no dechlorination was observed in any sequential transfers of these cultures (Fig. 1D) indicating that hydrogen did not support sustained dechlorination at this concentration and prevented acetate-driven dechlorination. Sequential transfer of the *ortho*-dechlorinating culture with 10 mM formate also resulted in a loss of dechlorinating activity (data not shown).

DGGE examination of the ortho-dechlorinating culture grown with acetate and hydrogen

The 16S rDNA gene population in each culture was amplified by PCR using universal and *Bacteria* domain-specific primers. DGGE analysis of the *ortho*-dechlorinating culture revealed 6 predominant DNA fragments (Fig. 2A). Cultures amended with 5 mM and 20 mM acetate exhibited bands A and E more intensely. Other bands from these cultures varied in intensity. The most dramatic changes were a decrease in the detection of bands C and F and the detection of band B when the culture was supplied with 20 mM acetate. In relation to bands A and E, bands B, C and F were nearly undetectable or undetected following subsequent transfer of the culture with 20 mM acetate and under additional conditions (see below). For example, treatment with BES resulted in a pattern dominated by bands A and E with little to no detection of the other bands.

The most dramatic shift in the microbial community was observed when the *ortho*-dechlorinating culture was grown without acetate. Transfer of the culture in the absence of acetate resulted in only a nearly undetectable band at position A on the DGGE gel (Fig. 2A). This experiment was repeated and the same result was

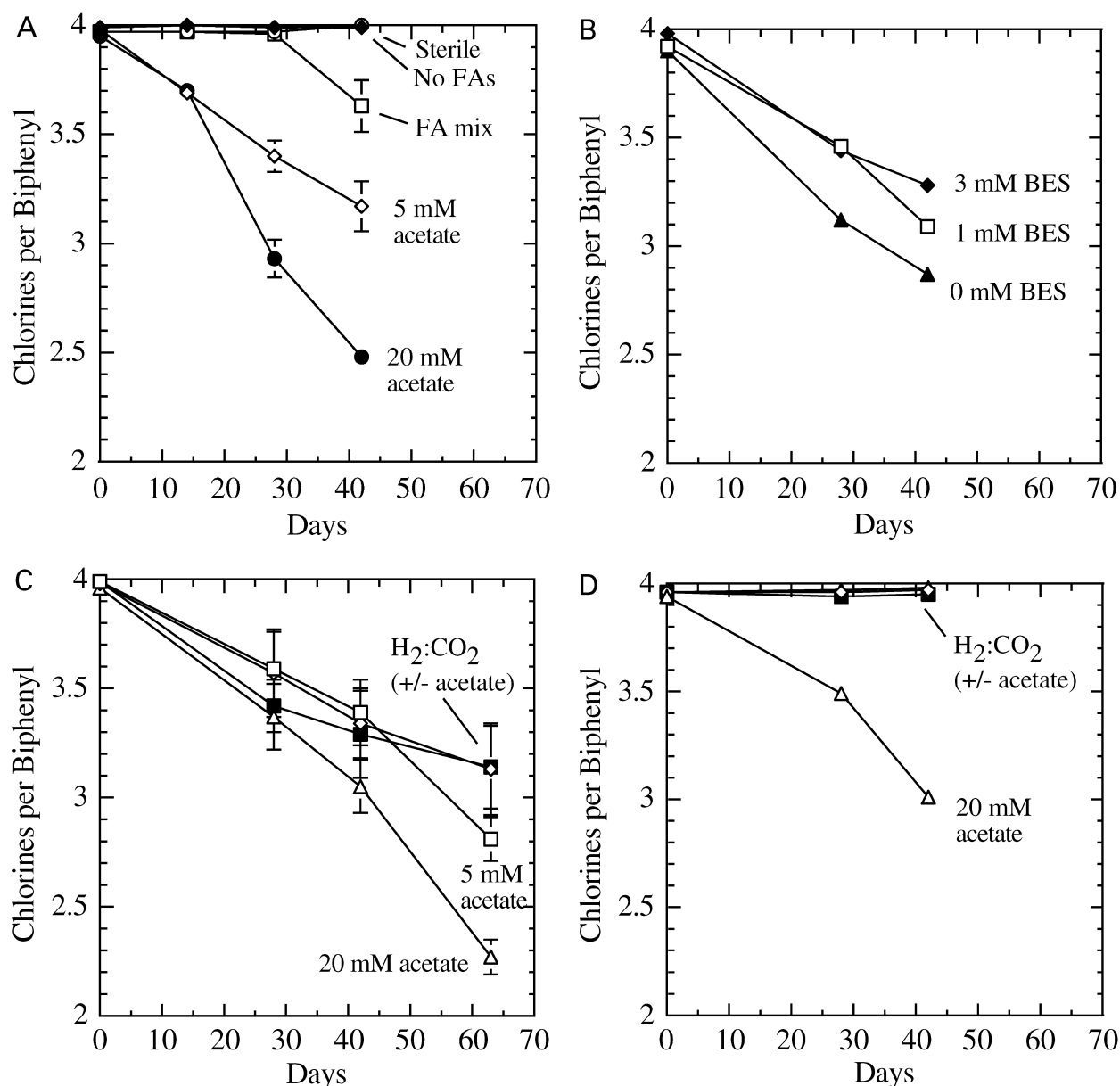


Fig. 1. Dechlorination of 2,3,5,6-CB by the *ortho*-dechlorinating culture.

A. Effect of acetate. Results are from cultures transferred from a fatty acid-fed sediment-free culture into E-CI medium with 173 μ M 2,3,5,6-CB and a mixture of sodium acetate, sodium propionate and sodium butyrate (2.5 mM each) (□), 5 mM sodium acetate (◇), 20 mM sodium acetate (●), or no additional amendment (No FAs, ◆). Sterile controls (autoclaved cultures) are marked as ○. The data are the average from triplicate cultures. The error bars represent the standard deviation.

B. Treatment with bromoethanesulphonate (BES). Results are from initial transfers from a methanogenic 20 mM acetate-fed culture into E-CI medium with 20 mM acetate, 173 μ M 2,3,5,6-CB and 0 mM (▲), 1 mM (□) and 3 mM (◆) BES. The data are the average from duplicate cultures.

C. Initial effect of hydrogen versus acetate. A 5 mM acetate-fed *ortho*-dechlorinating culture was transferred into E-CI medium with 173 μ M 2,3,5,6-CB and 5 mM acetate (101 kPa N₂:CO₂, 80:20) (□), 20 mM acetate (101 kPa N₂:CO₂, 80:20) (△), H₂:CO₂ (101 kPa, 80:20) (◇) or H₂:CO₂ (101 kPa, 80:20) plus 5 mM acetate (■). The data are the average from triplicate cultures. The error bars represent the standard deviation.

D. Effect of hydrogen after sequential transfer. The *ortho*-dechlorinating culture was sequentially transferred twice in E-CI medium with 173 μ M 2,3,5,6-CB and 20 mM acetate (101 kPa N₂:CO₂, 80:20) (△), H₂:CO₂ (101 kPa, 80:20) (■), H₂:CO₂ (101 kPa, 80:20) plus 20 mM acetate (◇). The data are the average from duplicate cultures.

obtained. As reported above, transfers in the absence of acetate, with only acetone and PCB, resulted in a complete loss of detectable growth (optical density) and dechlorinating activity.

A change in the community profile of the *ortho*-dechlorinating culture was also observed in cultures amended with 101 kPa H₂:CO₂. DGGE analysis of the microbial community showed that band A was nearly

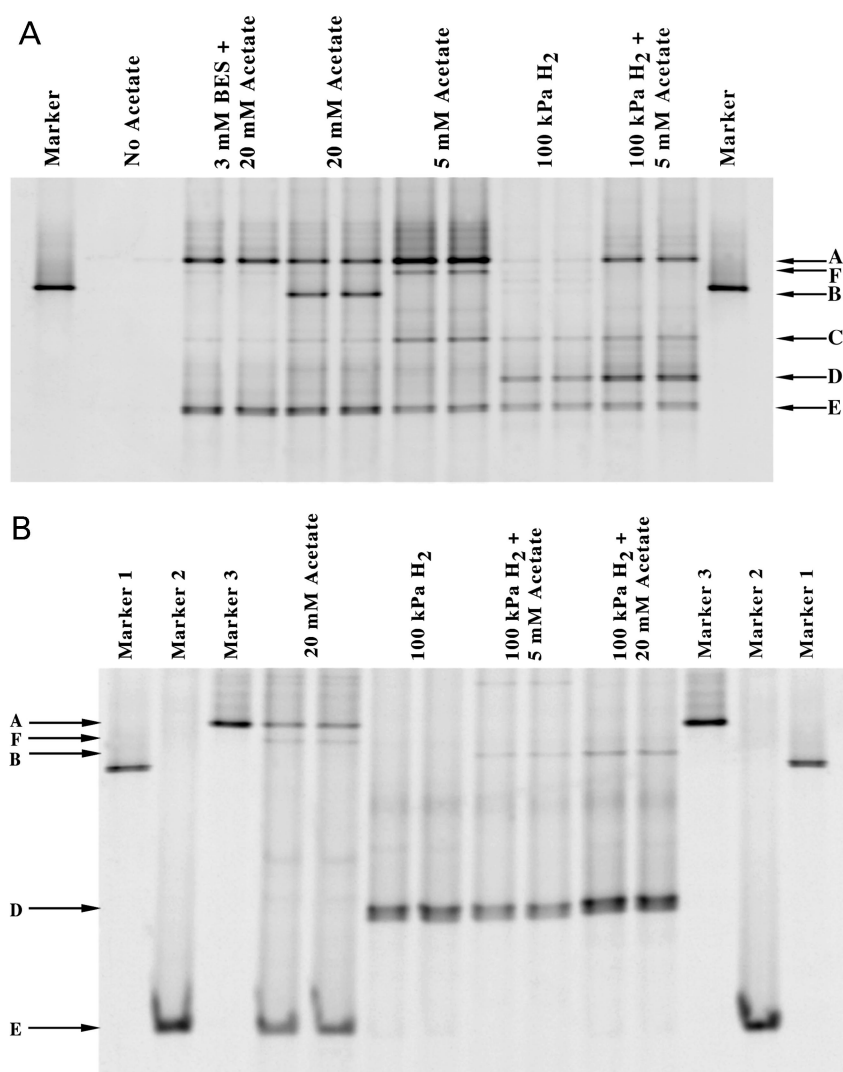


Fig. 2. DGGE analysis of the 16S rDNA of the *ortho*-dechlorinating culture grown with hydrogen, acetate and BES.

A. Samples were taken from duplicates of cultures described in Fig. 1B and C. All cultures were maintained with $173 \mu\text{M}$ 2,3,5,6-CB and the acetone used to deliver the PCB. Bands were labelled based on their migration distance and excised from positions A, B, C, D, E and F for comparative sequence analysis. Using the GC-clamped primers described in the *Experimental procedures*, 16S rDNA from *Burkholderia cepacia* was amplified for use as a standard marker.

B. DGGE analysis of the 16S rDNA from the *ortho*-dechlorinating culture sequentially transferred (2X) in E-CI medium with 20 mM acetate (101 kPa $\text{N}_2:\text{CO}_2$, 80:20), $\text{H}_2:\text{CO}_2$ (101 kPa, 80:20), $\text{H}_2:\text{CO}_2$ (101 kPa, 80:20) plus 5 mM acetate, or $\text{H}_2:\text{CO}_2$ (101 kPa, 80:20) plus 20 mM acetate (dechlorination shown in Fig. 1D). All cultures were maintained with $173 \mu\text{M}$ 2,3,5,6-CB and 0.1% (v/v) acetone. Using the GC-clamped primers described in the *Experimental procedures*, 16S rDNA from *Burkholderia cepacia* (Marker 1), OTU 5 (Marker 2) and OTU 1 (Marker 3) was amplified for use as standard markers. Bands were labelled based on their migration distance.

undetectable and that a DNA fragment at position D appeared when the *ortho*-dechlorinating culture was incubated with $\text{H}_2:\text{CO}_2$. Bands A and D were clearly present when the culture was grown with a combination of hydrogen and acetate. Figure 2B shows that the DNA fragments at position A and E were undetected following sequential transfer under hydrogen (with or without acetate) while the relative intensity of band D was enhanced. This corresponds with the loss of dechlorinating activity described above.

DNA bands (A through F) excised from the DGGE gels

for each of the culture conditions tested were sequenced. Sequence similarity was determined by comparative analysis of the partial 16S rDNA from the excised fragments and an operational taxonomic unit (OTU) designation was assigned to each DNA fragment that could be sequenced (Table 1). Phylogenetic affiliation for band F was inconclusive because DNA could not be purified sufficiently for sequencing. The five OTUs identified from the cultures were all members of the domain *Bacteria*. Archaeal rDNA was detected by polymerase chain reaction (PCR) analysis in the methanogenic *ortho*-dechlorinating culture but not in

Table 1. Phylogenetic affiliations of predominant DNA fragments from the *ortho*-dechlorinating culture based on partial bacterial 16S rRNA gene sequence from bands excised from the DGGE gel shown in Fig. 2.

Band position	Fragment designation	Closest phylogenetic relative	% similarity to closest relative
A	OTU 1 (AF294958)	Unidentified eubacterium RFLP 17 (AF058005)	100%
B	OTU 2 (AF294959)	<i>Pseudomonas grimontii</i> (AF268029)	98%
C	OTU 3 (AF294960)	<i>Aminobacterium columbiense</i> (AF069287)	96%
D	OTU 4 (AF294961)	Uncultured <i>spirochaeta</i> (AF211319)	94%
E	OTU 5 (AF294962)	<i>Desulfovibrio aminophilus</i> (AF067964)	97%

the non-methanogenic (BES-treated) culture when *Archaea* specific primers were used for DNA amplification (data not shown). This result and the lack of methanogenesis in the dechlorinating, BES-treated cultures confirmed that methanogenic bacteria in the *ortho*-dechlorinating culture do not catalyse PCB dechlorination. Examination of the data overall for the cultures grown with acetate or hydrogen did not clearly identify the PCB dechlorinating microorganism, but it did show that OTUs 1 and 5 (bands A and E) were present when dechlorination occurred and were not detected when there was no dechlorination. The other OTUs (or bands) were not always detected in

dechlorinating cultures and were at times detected in the absence of dechlorination.

Identification of the dechlorinating microorganism

A series of experiments were performed with the *ortho*-dechlorinating culture grown with and without the addition of 2,3,5,6-CB. Following incubation without PCB the culture was transferred back into medium containing 2,3,5,6-CB. This extended the lag preceding dechlorination but eventually dechlorinating activity was observed (Fig. 3). The amplified 16S rDNA of these cultures was then examined using DGGE. Four bands were detected in the PCB-amended cultures, two of which (OTUs 1 and 5) dominated the DGGE profile (Fig. 4A). The microbial community remained the same in the absence of PCB except that OTU 1 was no longer detected. The intensity of all the other DNA fragments remained the same or increased slightly in the absence of PCB. The loss of OTU 1 in the absence of the PCB indicates that the growth of this organism is dependent on the presence of 2,3,5,6-CB under these defined conditions. OTU 1 was again detected when cultures grown without PCB were transferred and incubated in media with 2,3,5,6-CB (Fig. 4A). This set of experiments was repeated with non-methanogenic cultures that were treated with 3 mM BES and the same results were observed. The detection of OTU 1 after transfer back to medium with PCB corresponds to the restoration of dechlorination activity and confirms that the growth of this microorganism is dependent on the presence of 2,3,5,6-CB. The analysis was done at the end-point sampling given in Fig. 3. At that time the initial PCB-amended cultures converted 43.6 ± 8.4 mol% of the 2,3,5,6-CB to 7.4 ± 1.4 mol% 2,3,5-CB and 36.2 ± 14.0 mol% 3,5-CB. The cultures re-introduced into PCB-containing media transformed 20.5 ± 2.3 mol% of the 2,3,5,6-CB to 6.3 ± 1.0 mol% 2,3,5-CB and 14.2 ± 1.3 mol% 3,5-CB. Although DGGE is not quantitative, a relative increase in band intensity indicates a relative increase in the amount of corresponding 16S rDNA, which is concurrent with a relative increase in cellular growth. The band intensities are consistent with the respective extent and lag in dechlorination in the

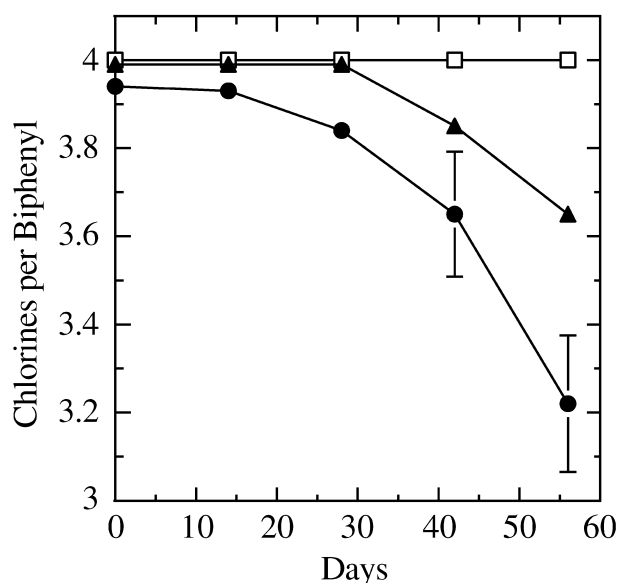


Fig. 3. Dechlorination of 2,3,5,6-CB by the initial PCB-Plus *ortho*-dechlorinating cultures (●), by cultures re-supplied with 2,3,5,6-CB following one transfer without added PCB (▲), and by cultures after three sequential transfers without added PCB (□). The initial PCB-Plus *ortho*-dechlorinating culture was maintained in triplicate in E-CI media with 20 mM sodium acetate and 173 μ M 2,3,5,6-CB. Transfers (10% v/v) from each replicate culture were made from each of the initial *ortho*-dechlorinating cultures into E-CI media amended with 20 mM acetate, 13.6 mM acetone and no added PCB (no-PCB). Transfers were subsequently made from each of the three no-PCB cultures into 10 ml of E-CI media amended with 20 mM acetate and 173 μ M 2,3,5,6-CB (in acetone). The same procedure was used to obtain the cultures that were sequentially transferred three times without added PCB before re-supply with 2,3,5,6-CB. The data are the average from the triplicate cultures \pm the standard deviation.

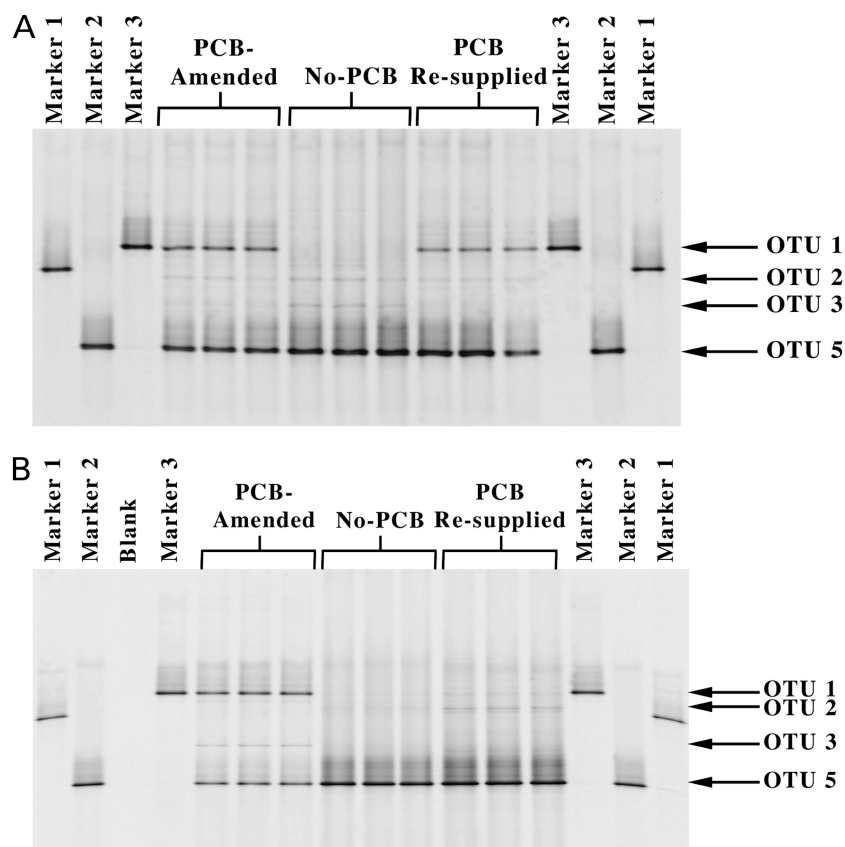


Fig. 4. DGGE analysis of the 16S rDNA from the *ortho*-dechlorinating culture after one (A) and three sequential transfers (B) without 2,3,5,6-CB. The initial PCB-Plus, no-PCB and re-supplied-PCB *ortho*-dechlorinating cultures were prepared in triplicate with 2,3,5,6-CB as described for Fig. 3. Samples were drawn from each replicate culture for subsequent polymerase chain reaction and denaturing gradient gel electrophoresis. The 16S rDNAs from *Burkholderia cepacia* (Marker 1), OTU 5 (Marker 2) and OTU 1 (Marker 3) were used as standards. All PCR products were electrophoresed in a polyacrylamide gel with a 40–70% linear denaturing gradient. Bands were labelled based on their migration distance.

re-amended cultures versus the original PCB-amended cultures.

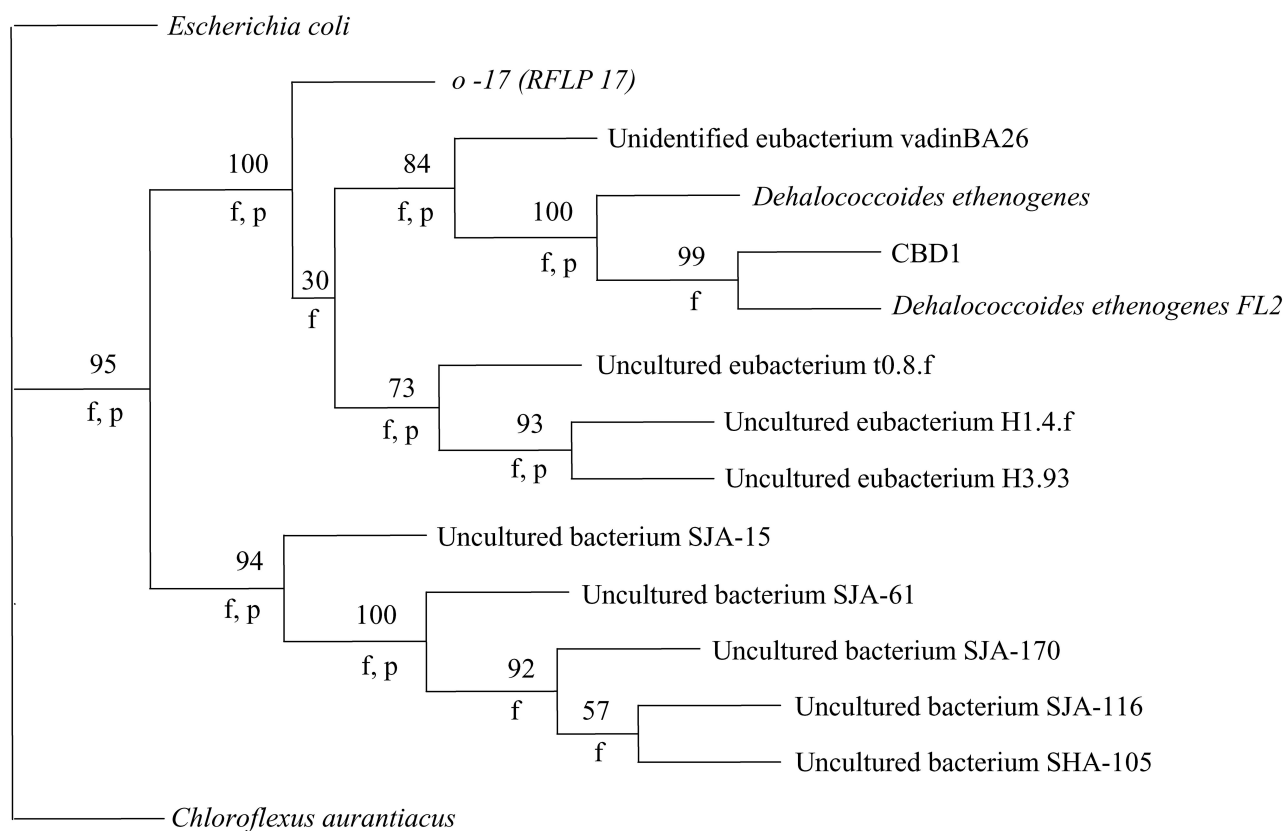
The response of OTU 1 to 2,3,5,6-CB strongly indicates that it is a PCB dechlorinator. To determine whether other organisms in the community were capable of dechlorinating 2,3,5,6-CB, the *ortho*-dechlorinating culture was sequentially transferred without PCB until dechlorinating activity could no longer be recovered following transfer back into E-CI media amended with the PCB (Fig. 3). DGGE analysis revealed that OTU 5 dominated this non-dechlorinating culture and that OTU 1 was not detected (Fig. 4B). This result shows that the microorganism represented by OTU 5 does not dechlorinate 2,3,5,6-CB and it further confirms that the microorganism represented by OTU 1 is the dechlorinator of 2,3,5,6-CB in the *ortho*-dechlorinating culture.

A 1100 bp sequence of 16S rDNA for OTU 1, which had 100% similarity to the smaller 351 bp fragment, was amplified by PCR from the *ortho*-dechlorinating culture using primers for flanking regions. Based on comparative sequence analysis, this 16S rDNA was identical to that of

unidentified eubacterium RFLP 17. RFLP 17 was first cloned with 16S rDNA from a 2,3,5,6-CB *ortho*-dechlorinating, enrichment culture maintained with sediment (Pulliam Holoman *et al.*, 1998). As this rDNA sequence and PCB dechlorination can now be assigned to the same specific microorganism, it is proposed that the dechlorinating bacterium within the *ortho*-dechlorinating culture be referred to as bacterium *o*-17. A phylogenetic tree with bacterium *o*-17 is presented in Fig. 5.

Discussion

This is the first identification of a PCB-dechlorinating anaerobic microorganism, bacterium *o*-17, and it was achieved without isolation of the microorganism. The conclusion that *o*-17 is capable of PCB dechlorination is based on three lines of evidence. First, the 16S rDNA of *o*-17 is always detected during PCB dechlorination and is only detected when PCB (2,3,5,6-CB in this case) is included in the medium. Second, dechlorination cannot be recovered when *o*-17 is systematically eliminated from the



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Fig. 5. Neighbour-joining phylogenetic tree generated from analysis of 1100 bp of 16S rDNA sequence. The symbols f and p indicate branches that were also found using the Fitch–Margoliash or maximum parsimony methods respectively. The numbers at the nodes are percentages indicating the levels of bootstrap support, based on a neighbour-joining analysis of a 1000 resampled data sets. Scale bar indicates 10 substitutions per 100 nucleotide position. *Escherichia coli* was used as an outgroup.

culture by sequential transfer without the PCB. The remaining organisms are not capable of PCB dechlorination in the absence of *o*-17. Third, the 16S rDNA of *o*-17 is most similar to that of *Dehalococcoides* spp., which reductively dechlorinate organochlorines (Maymo *et al.*, 1997; Adrian *et al.*, 2000). Furthermore, methanogens do not catalyse PCB dechlorination in the *ortho*-dechlorinating culture as methanogenesis and archaeal DNA were not detected in BES-treated cultures that dechlorinated PCB. Although PCB dechlorination was partially inhibited by BES it still continued in the absence of methanogenesis and archaeal DNA following sequential transfer (> 5 times) after BES treatment of the culture. The possibility remains that BES is also partially inhibitory to PCB dechlorination, which is consistent with the observation that BES is inhibitory to chloroethene dechlorination by anaerobic microorganisms (Löffler *et al.*, 1997). These combined lines of evidence clearly indicate that *o*-17 catalyses the reductive dechlorination of PCB. Efforts continue to isolate *o*-17, but to date the organism has not survived more than one transfer in or on solid anaerobic

media. In addition, efforts to isolate *o*-17 by serial dilution or incubation with ampicillin, the antibiotic used to successfully isolate *Dehalococcoides* spp., have been unsuccessful (Maymo *et al.*, 1997; Adrian *et al.*, 2000).

Bacterium *o*-17 came from estuarine sediment sampled from the North-west Branch of Baltimore Harbor in the Chesapeake Bay, in which PCBs have historically had an impact (Baker *et al.*, 1997). Marine and estuarine sediments are well suited to support dehalogenating bacteria owing to the natural production of organohalides in such an environment (Faulkner, 1991; 2001). In addition, coastal harbours are commonly contaminated with chlorinated organic compounds of anthropogenic origin, which could serve as electron acceptors for the growth of dehalogenating bacteria. Indeed, evidence of PCB dechlorination with marine sediments has been reported (Alder *et al.*, 1993; Øfjord *et al.*, 1994; Wu *et al.*, 1998). The PCB-dependent growth of bacterium *o*-17 under defined and minimal conditions indicates that the dechlorination of the tetrachlorobiphenyl is directly linked to the growth metabolism of the organism. A previous

study examining the growth of microorganisms in response to PCBs in anaerobic sediments demonstrated that members of a dechlorinating microbial community required PCBs for growth (Kim and Rhee, 1997). No dehalogenating organisms were identified in that study, but the results from that investigation and those presented here have demonstrated that PCBs are linked to anaerobic growth, possibly through dehalorespiration.

Bacterium *o*-17 has the highest sequence similarity (89% of an 1100 bp sequence comparison) to *Dehalococcoides ethenogenes*, which has been described as a hydrogenotrophic, tetrachloroethene-dechlorinating bacterium (Maymo-Gatell *et al.*, 1997; 1999). In addition, the sequence of the PCB dechlorinator is similar to that of the recently isolated strain CBDB1, a small irregular coccus that links its growth to the dechlorination of chlorobenzenes (Adrian *et al.*, 2000). These microorganisms and bacterium *o*-17 are distinct in terms of their phylogenetic relationship to dehalogenating microorganisms. Most known dehalogenators belong to the δ - and ϵ -subgroups of the Proteobacteria and the low G + C Gram-positive group (Holliger *et al.*, 1998). However, bacterium *o*-17, strain CBDB1 and *D. ethenogenes* are members of a deep branch that is most closely related to the green non-sulphur bacteria. This phylogenetic branch is distinct from the green non-sulphur bacteria represented by *Chloroflexus aurantiacus* and is dominated by uncultured microorganisms (Fig. 5). Dehalogenation has not been attributed to any member of this group other than bacterium *o*-17, strain CBDB1, and *D. ethenogenes*. However, the SJA strains shown in Fig. 5 were among 51 uncultured bacteria discovered (identified by cloning and sequencing of 16S rDNA) in a fluidized bed reactor that was fed with chlorobenzenes (von Wintzingerode *et al.*, 1999). In addition, uncultured bacterium SHA-105 was discovered in a microbial community that was dechlorinating 1,2-dichloropropane (Schlotelburg *et al.*, 2000). The dechlorinating organism(s) within this reactor was not identified. Although the 16S rDNA of bacterium *o*-17 is most similar to the rDNA of this group of organisms, the PCB dechlorinator has 89% similarity to the nearest reported 16S rDNA sequence, which suggests that it differs at least at the genus level.

Bacterium *o*-17 links its growth to the dechlorination of 2,3,5,6-CB, possibly by dehalorespiration. Acetate oxidation coupled to the reduction of 2,3,5,6-CB to 2,3,5-CB and 3,5-CB is an exergonic reaction according to the Gibbs free energies of formation published by Holmes *et al.* (1993). During the enrichment and analysis of the *ortho*-dechlorinating culture it was determined that acetate was required by bacterium *o*-17 for PCB dechlorination and growth, and that hydrogen was inhibitory. These results demonstrate a growth requirement for acetate and suggest that it is the electron donor for PCB dechlorination by bacterium *o*-17.

Hydrogen more commonly serves as the electron donor for the dechlorination of chlorinated organic compounds

(Freedman and Gossett, 1989; Zhang and Wiegel, 1990; DeWeerd *et al.*, 1991; Maymo-Gatell *et al.*, 1997; Middeldorp *et al.*, 1997; Löffler *et al.*, 1999). Hydrogen has been shown to support the dechlorination of 2,3,4-trichlorobiphenyl in microcosms with freshwater sediments (Sokol *et al.*, 1994). Hydrogen has also been shown to support anaerobic dechlorination of Aroclor in sediment-containing mixed microbial cultures (Morris *et al.*, 1992). In contrast, hydrogen at 101 kPa (80:20 with CO₂) would not support dechlorination of 2,3,5,6-CB by bacterium *o*-17. The presence of hydrogen actually led to a cessation of PCB dechlorination and a dramatic shift in the microbial community including the loss of bacterium *o*-17. Previous reports have indicated that hydrogen can stimulate or inhibit reductive dechlorination of some halogenated compounds (Wiegel and Wu, 2000). Wu (1996) has reported on the effect of hydrogen on PCB dechlorination in sediment microcosms. In that study, it was shown that low levels of hydrogen (1% at 1 atm) had no effect on PCB dechlorination (Aroclor 1260 and 2,3,4,6-CB). Higher levels of hydrogen (10% at 1 atm) slowed dechlorination or altered the dechlorinating activity with different dechlorination products being detected compared with controls. Relatively low levels of hydrogen may not support the growth of many hydrogenotrophic bacteria and may not influence the growth and dechlorination of bacterium *o*-17. However, it is clear that higher amounts of hydrogen do influence the growth of bacterium *o*-17. Although acetate is required for the dechlorination and growth of bacterium *o*-17, it is still possible that the dechlorinator uses hydrogen generated during the oxidation of acetate. If so, low levels of hydrogen may still support dechlorination by bacterium *o*-17 if continually supplied over a long period of time along with acetate. Overall, these results indicate the need to be aware of which electron donors are needed to stimulate or prevent specific dechlorinations when developing a biotechnological approach to PCB remediation.

PCB contamination in sediments usually consists of many different PCB congeners. Bacterium *o*-17's ability to *ortho* dechlorinate a PCB suggests that its specificity for PCBs is different from that of other PCB-dechlorinating bacteria. There are many different types of microbial PCB dechlorination (Bedard and Quensen, 1995; Wiegel and Wu, 2000). As it is clear that PCB dechlorination is linked to the growth of bacterium *o*-17, it is possible that other PCB dechlorinators will have this capacity and this type of growth can be exploited for the bioremediation of PCBs. Now that bacterium *o*-17 has been identified as a dechlorinator of PCBs it can be studied more fully either in these highly defined microbial communities or in pure culture, which is currently being pursued. The powerful combination of chemical and nucleic acid analysis has resulted in the first identification of a PCB dechlorinator.

This approach is now being used to rapidly identify and characterize similar microorganisms. Such advancement is needed for the identification, characterization and monitoring of bacteria that dechlorinate and detoxify PCBs in the environment and contribute to the global cycling of halogenated organic compounds.

Experimental procedures

Culture conditions

An estuarine-salts medium (E-CI) was prepared anaerobically in an atmosphere that contained $N_2:CO_2$ as described previously, with the exclusion of $Na_2S \cdot 9H_2O$ and sediment (Berkaw *et al.*, 1996). L-cysteine-HCl- H_2O (Sigma) was added to the medium as a reductant (1.4 mM final concentration). An anaerobic enrichment culture capable of *ortho* dechlorination of 2,3,5,6-tetrachlorobiphenyl (2,3,5,6-CB) in the absence of sediment was used in this study (Cutter *et al.*, 1998). The culture had been sequentially transferred (10% [v/v]) 8 times in E-CI media with a mixture of fatty acids [final concentration of 2.5 mM each sodium acetate, propionate and butyrate and 173 μM (50 p.p.m.) of 2,3,5,6-CB; AccuStandard]. The 2,3,5,6-CB was delivered in acetone with the final concentration of acetone at 13.6 mM.

Transfers (10% [v/v]) were made from the fatty acid-amended *ortho*-dechlorinating culture into E-CI media amended with 5 mM sodium acetate (Sigma) and 173 μM (50 p.p.m.) of 2,3,5,6-CB. Sequential transfers (10% [v/v]) were then made from 5 mM acetate-amended *ortho*-dechlorinating cultures into E-CI media amended with 0, 5, 10, 20, 30 and 40 mM sodium acetate plus 13.6 mM acetone and 173 μM (50 p.p.m.) of 2,3,5,6-CB. Bromoethanesulphonic acid (BES) (Sigma) was dissolved in sterile deionized water and filter sterilized before being tested with the *ortho*-dechlorinating culture. Sequential transfers (10% [v/v]) were also made from 5 mM acetate-amended *ortho*-dechlorinating cultures into E-CI media under an atmosphere of $H_2:CO_2$ to a final headspace pressure of 100 kPa or 250 kPa without the addition of acetate. For analysis of the *ortho*-dechlorinating cultures grown with versus without PCB the cultures were maintained with 20 mM sodium acetate. Inoculum for these experiments were grown with 173 μM (50 p.p.m.) 2,3,5,6-CB and then transferred (10% [v/v]) to media with and without 173 μM (50 p.p.m.) 2,3,5,6-CB. Cultures without 2,3,5,6-CB added are referred to as no-PCB cultures. Analysis of no-PCB cultures at the time of inoculation revealed less than 2.8 $\mu g ml^{-1}$ total PCB. Unless stated otherwise, transfers of cultures were generally made after 2–3 months of incubation. All cultures were incubated statically under strict anaerobic conditions in the dark at 30°C. Sterile controls were prepared by autoclaving at 121°C for 30 min. Optical density was monitored at 600 nm with a Spectronic 20D spectrophotometer (Milton Roy).

Culture sampling and PCB analysis

Cultures were shaken immediately before aliquots were withdrawn under anaerobic conditions using a 5 ml glass pipette. PCBs were extracted from samples with ethyl acetate and subsequently eluted from Florisil-copper columns

according to Berkaw *et al.* (1996). PCB analysis was conducted with a Hewlett-Packard 5890A gas chromatograph (Agilent) equipped with an electron capture detector (ECD) and an RTX-1 capillary column as described previously (Berkaw *et al.*, 1996). PCB analysis was also conducted with a Hewlett-Packard 6890A gas chromatograph (Agilent) equipped with an ECD and an RTX-1 capillary column as described previously (Berkaw *et al.*, 1996) with the following modifications to the oven programme: a 0.25 min hold at 140°C, a 2.67 min 140°C to 180°C gradient, a 10 min hold at 180°C, a 2 min 180°C to 280°C gradient, and a final 3 min hold at 280°C. PCB congeners were identified by matching their retention times with those of authentic standards and quantified with a 16-point calibration curve for each congener as described (Berkaw *et al.*, 1996). Standards for 2,3,5,6-CB and the possible dechlorination products were purchased from AccuStandard.

Methane determination

The culture headspace was sampled with a glass gas-tight, volume-locking syringe (Hamilton Co., NV, USA). Methane was then analysed with a Hewlett-Packard 5890A gas chromatograph equipped with a flame ionization detector (FID) and an RTX-624 capillary column (0.53 mm by 30 m; Restek Corp.) with helium as the carrier gas at a flow rate of 100 $ml min^{-1}$. The injector and detector temperatures were set at 250°C and the oven temperature was maintained in an isocratic mode at 35°C.

Extraction of total genomic DNA

Samples (1 ml) from individual cultures were withdrawn under anaerobic conditions. Genomic DNA was extracted using the Bio-Rad InstaGene Matrix (Bio-Rad) according to the manufacturers' instructions. Briefly, the sample was centrifuged at 11 750 *g* for 3 min, the supernatant decanted, and the pellet resuspended in 200 μl of the matrix. The sample was sequentially incubated at 56°C for 30 min and 100°C for 8 min, shaken by vortexing for 10 s after each incubation, and then centrifuged. The supernatant was used for PCR amplification. In order to confirm the results obtained with InstaGene and to examine Archaeal 16S rDNA, samples (1 ml) from individual cultures were bead beaten and the DNA prepared according to the procedures described by Pulliam Holoman *et al.* (1998).

Amplification of 16S rDNA

The polymerase chain reaction (PCR) was used to amplify 16S rDNA from the purified microbial community DNA. Bacterial 16S rDNAs were amplified with primer 1055–1070 forward (5'-ATGGCTGTCGTCAGCT-3') and primer 1406–1392 reverse (5'-ACGGGCGGTGTGTAC-3') (Ferris *et al.*, 1996). For DGGE analysis, the reverse primer was synthesized with a 40 base GC clamp at the 5' end yielding a 392 bp PCR fragment (Muyzer *et al.*, 1993). Archaeal 16S rDNAs were amplified with primer Arch21F (5'-TTCCGGT TGATCCYGCCGGA-3') and primer Arch958R (5'-YCCGG CGTTGAMTCCAATT-3') (DeLong, 1992). PCR was

performed using the GeneAmp PCR kit (Applied Biosystems) with *Ampli-Taq* DNA polymerase in a PE System 2400 thermocycler (Perkin Elmer) according to the manufacturers' instructions with the following modifications per 50 μ l reaction: 0.5 Units *Ampli-Taq* DNA polymerase, 1.25 μ l deionized formamide (Sigma) and 20 μ g non-acetylated bovine serum albumin (New England BioLabs, Inc.). The PCR cycle parameters were as follows: an initial denaturation step of 5 min at 94°C, 9 amplification cycles of denaturation (30 s at 94°C), annealing (30 s at 62°C minus 1°C each cycle until touchdown at 54°C) and elongation (30 s at 72°C), 30 additional amplification cycles of denaturation (30 s at 94°C), annealing (30 s at 54°C) and elongation (30 s at 72°C) and a final elongation step of 72°C for 5 min. Amplification products were checked for correct size and yield on a 1% TAE agarose gel (Bio-Rad). The amplified rDNA was examined using DGGE analysis. To obtain larger fragments of bacterial 16S rDNA for phylogenetic analysis, universal primers located on the flanking regions were used, 29–47 forward (5'-GAGTTTGATCCTGGCTCAG-3') and 1541–1525 reverse (5'-AGAAAGGAGGTGATCAGCC-3') (Rainey *et al.*, 1992).

Denaturing gradient gel electrophoresis (DGGE) analysis

Electrophoresis of the amplified 16S rDNAs was performed as previously described (Muyzer *et al.*, 1993) using the Bio-Rad DCode™ Universal Mutation Detection System (Bio-Rad). Gels contained a 40–70% linear denaturing gradient formed with urea and deionized formamide. The PCR products (20 μ l) were electrophoresed in 1X TAE buffer (0.04 M Tris base, 0.02 M glacial acetic acid, 1 mM EDTA) at 60°C for 20 h at 45 V. The gel was stained with 1X SYBR Green I nucleic acid gel stain (Molecular Bio-Probes) according to the manufacturers' instructions. A Fluorimager 575 (Molecular Dynamics) was used to visualize the DNA fragments. A 16S rDNA fragment generated from a pure culture of *Burkholderia cepacia* (ATCC 17765) was used as a standard to determine the relative migration distances of DNA fragments in DGGE gels. Purified and sequenced DNA representing OTUs 1 and 5 were also used as standards. The image contrast was adjusted using Adobe PhotoShop 5.5 (Adobe Systems, Inc.). The same DGGE profile of Bacterial 16S rDNA was observed with samples prepared with InstaGene or by bead beating with the methods of Pulliam Holoman *et al.* (1998).

Sequence analysis

DNA fragments at each unique migration position were sequenced according to the methods of Ferris *et al.* (Ferris *et al.*, 1996) using an ABI 373 Automated Sequencer (Applied Biosystems). Sequence obtained was submitted to the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990) and the Ribosomal Database Project (Maidak *et al.*, 2000) to determine similarity with other 16S rDNA molecules.

Partial 16S rDNA sequences were manually compiled and aligned using PHYLIP software (Chun, 1995). Evolutionary trees were generated using the neighbour-joining (Saitou and Nei, 1987), Fitch–Margoliash (Fitch and Margoliash, 1967)

and maximum parsimony (Kluge and Farris, 1969) algorithms in the PHYLIP software (Felsenstein, 1993). Evolutionary distance matrices for the neighbour-joining and Fitch–Margoliash methods were generated as described by Jukes and Cantor (1969). The robustness of the inferred tree topologies was evaluated after a 1000 bootstrap resamplings of neighbour-joining data.

Nucleotide sequence accession numbers

Sequences of the partial 16S rRNA of the predominant DNA fragments were submitted to GenBank as OTU 1, 2, 3, 4, and 5 under accession nos. AF294958–AF294962 respectively.

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