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ORIGINAL PAPER

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Phylogenetic analysis of an anaerobic microbial consortium deiodinating 5-amino-2,4,6-triiodoisophthalic acid

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Abstract The dehalogenating performance of an anaerobic 5-amino-2,4,6-triiodoisophthalic acid (ATIA) fixed-bed reactor was evaluated. The reactor operating conditions were set for ATIA deiodination. A phylogenetic survey for a stable anaerobic ATIA-deiodinating microbial consortium was carried out using 16S rDNA restriction fragment length polymorphism, and unique clones were sequenced. Four phylotypes were identified. Two sequences were related to those of *Desulfitobacterium frappieri* species and another was closest to that of *Desulfitobacterium hafniense*, but may have represented a new *Desulfitobacterium* species. *Desulfitobacteria* were previously described as aryl-dechlorinating and debrominating bacteria. The new strains identified in this study were probably responsible for the ATIA deiodination. The fourth clone was related to the *Clostridium-Flavobacterium-Bacteroides* group.

Introduction

Triiodinated aromatic compounds are used as X-ray contrast agents in radiology, and so are released in hospital waste water (Erbe et al. 1998). Because of their high stability, they are not degraded in sewage treatment plants and are detected in rivers at concentrations up to 30 µg l^{-1} (Olesky-Frenzel et al. 1995; Putschew et al. 2000). Some studies have reported biological conversions of these compounds via deacetylation or partial deiodination (Kalsch 1999; Rode and Müller 1998; Steger-Hartmann et al. 2002). For example, 5-amino-2,4,6-triiodoisophthalic acid (ATIA) (Fig. 1), the core structure of a range of iodinated X-ray contrast agents, was completely reductively deiodinated by an anaerobic consortium (Lecouturier et al. 2002).

Several bacteria that reductively dehalogenate various chlorinated and brominated aromatic compounds have been isolated and characterized. They belong to various groups such as *Desulfomonile* (Shelton and Tiedje 1984), *Desulfitobacterium* (Bouchard et al. 1996; Breitenstein et al. 2001; Christiansen and Ahring 1996; Gerritse et al. 1996; Sanford et al. 1996; Utkin et al. 1994), *Desulfovibrio* (Boyle et al. 1999), and myxobacteria (Cole et al. 1994). In many cases, the bacteria use chloroaromatic compounds as terminal electron acceptors and gain energy from reductive dechlorination (Boyle et al. 1999; Christiansen and Ahring 1996; Cole et al. 1994; Dolfing 1990; Gerritse et al. 1996; Mackiewicz and Wiegel 1998; Sanford et al. 1996). As little is known about deiodinating bacteria, we set out to determine which bacteria carry out this reaction and whether they are phylogenetically related to dechlorinating organisms.

Isolation of pure cultures from anaerobic environments can often be difficult because of tight syntrophic associations between microorganisms (Dolfing and Tiedje 1986). In addition, only a small fraction of the microbial

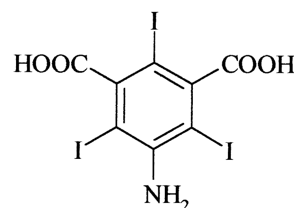

Fig. 1 Chemical structure of 5-amino-2,4,6-triiodoisophthalic acid (ATIA)

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community can be cultivated using standard techniques (Amann et al. 1995; Göbel 1995), so that isolation of xenobiotic-degrading bacteria often fails (Bedard and Quensen 1995; Phelps et al. 1998). Analysis of the 16S rRNA genes thus appears to be a good alternative method to describe community constituents of xenobiotic-degrading microbial consortia, and has already yielded interesting results (Dojka et al. 1998; Knight et al. 1999; LaPara et al. 2000; Pulliam Holoman et al. 1998; von Wintgerode et al. 1999; Wagner-Döbler et al. 2000).

Here, we first evaluated ATIA deiodination in an anaerobic continuous fixed-bed reactor, and then characterized the stable ATIA-degrading bacterial population by 16S rDNA sequence analysis.

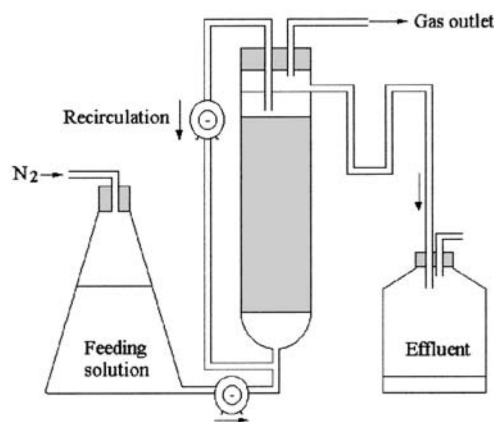

Fig. 2 Fixed-bed reactor design

Materials and methods

Source of inoculum

The microbial consortium was enriched under anaerobic conditions from sludge obtained from an industrial waste water treatment plant of the Guerbet group (Aulnay, France) that had been receiving triiodinated compounds for a long time. The anaerobic bacteria were enriched on ATIA and ethanol by repeated transfers in liquid batch cultures as already described (Lecouturier et al. 2002). After 1 year of cultivation, 40 ml of liquid culture was used as the inoculum for the anaerobic fixed-bed reactor.

Bioreactor processing

The experiments were carried out in an upflow anaerobic biofilm reactor. The set-up is shown in Fig. 2. The bacteria were cultured in a 5-cm diameter 20-cm-long glass cylinder filled with 86 g of Rashig rings (Schott Glaswerke, Mainz, Germany). These supports are hollow porous glass cylinders (8 mm long, 2 mm thick, and 8 mm in diameter) that provide a large adsorption surface for cells. In batch experiments, the cylinders had a low adsorption capacity for ATIA. To prevent oxygen diffusion, all tubing used was either viton or glass. The liquid volume was 365 ml. The liquid phase in the reactor was recirculated at a flow rate of 5 l day^{-1}. The reactor was thermostated at 30 °C.

The anaerobic mineral salt medium for reactor feeding was prepared as already described (Lecouturier et al. 2002). The feeding solution was kept sterile. One hundred ml of a solution containing phosphate buffer, ATIA, ethanol, Na_2S and Na_2CO_3 was sterilized by filtration with a 0.2-μm GH Polypro filter (Gelman). The remaining medium was sterilized by autoclaving (20 min, 121 °C). The two solutions were mixed aseptically.

Before inoculation, the reactor was flushed with N_2 and filled with reduced medium. The inoculum was introduced in the recirculation loop and the reactor was recycled for 6 h. It was then run in batch mode with 0.5 mM ATIA with a molar ethanol/ATIA ratio of 4 to allow the adhesion of cells and colonization of the glass supports. After complete ATIA deiodination, continuous feeding was started. The reactor was run with a hydraulic retention time of 8 days. The ATIA concentration in the feeding medium was 0.5 mM. The ethanol concentration was increased stepwise from 0.5 mM to 2 mM.

Analytical methods

Samples were analyzed as already described (Lecouturier et al. 2002). ATIA and metabolite concentrations were measured by C18 reverse-phase liquid chromatography. Ethanol and acetate were measured by liquid chromatography with an HI-plex column and 10^{-3} M H_2SO_4 eluent solution. Iodide was measured using a specific electrode Elit 281 (Bioblock, Illkirch, France) connected to a Consort multimeter (Bioblock). The iodide detection limit was 10 μM. Total organic carbon was measured with a Total Organic Carbon Analyzer model 1010 (IO Analytical, Bioritech, France). Gas production was detected with a Mariotte flask. Microbial biomass on glass cylinders was estimated by measuring proteins colorimetrically by a modified Lowry method (Peterson 1977). Recirculation was stopped and supports were taken from the bed under an N_2 stream to avoid oxygen diffusion in the reactor. Cells were detached from the rings by sonication (6 min, 0.8 W/ml) with a Vibracell 72405 100 W sonicator (Bioblock Scientific, France) in 10 ml of distilled water. After sonication, the ring was transferred to another tube and the operation was repeated until the protein concentration was below 2 mg l^{-1}.

Sampling and extraction of total genomic DNA

The bioreactor was run for 2 years before the bacterial community was analyzed. Eleven Rashig glass rings were sampled from the anaerobic reactor at approximately 5 cm from the top of the bed. The rings were centrifuged at 6,000 *g* for 10 min and ground in a mortar with 4 ml of 4 M guanidine thiocyanate-Tris (pH 7.5). DNA extraction was carried out as described in Godon et al. (1997) until the nucleic acids precipitation step by isopropanol. DNA was purified with a QIAamp DNA Minikit (QIAGen) according to the instructions of the manufacturer. The pellets were resuspended and pooled in 200 μl of water. Twenty μl of RNase (1 mg/ml) were added and the tube was incubated at 37 °C for 30 min. AL buffer (200 μl) was added and the tube was incubated for 10 min at 70 °C. Then, 200 μl of pure ethanol were added and the tube was vortexed. The sample was transferred to a QIAamp microcolumn and centrifuged at 14,000 *g* for 3 min. DNA was washed once with 500 μl of AW1 buffer, centrifuged for 3 min at 14,000 *g*, washed again with 500 μl AW2 buffer, centrifuged at 14,000 *g*, and then eluted with 50 μl of water by centrifugation for 1 min at 8,000 *g*.

Amplification and cloning of 16S rDNA genes

Amplification of 16S rDNA genes from purified genomic DNA was carried out with primers for conserved domains. The forward primer W018 (5′ GAGTTTCATCMTGGCTCAG 3′, [*Escherichia coli*, 9]), which targets the domain Bacteria, and the reverse primer W002 (5′ GNTACCTTGTTACGACTT 3′, [*E. coli*, 1492]), which targets all living organisms, were used to amplify bacterial 16S rDNA by PCR (Godon et al. 1997). Reaction tubes contained 1 μl of purified sample DNA, 1 U of REDTaq DNA polymerase (Sigma, Saint-Quentin-Fallavier, France), 5 μl of 10× reaction buffer containing 15 mM $MgCl_2$, 0.2 mM of each deoxyribonucleotide

triphosphate and 2 µl of each primer (100 ng/ml) in a final volume of 50 µl. Initial DNA denaturation and enzyme activation steps were done at 94 °C for 2 min in a PTC 150 thermocycler (MJ Research, Watertown, Mass., USA), followed by 25 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and elongation at 72 °C for 1 min, followed by a final elongation of 10 min at 72 °C. PCR products were purified and concentrated with a QIAquick spin PCR purification kit (QIAGen, Courtaboeuf, France). PCR fragments were eluted with 30 µl of water. Their concentration and size were estimated by agarose gel electrophoresis [0.7% (w/v) agarose and Tris borate/EDTA buffer containing 1 mg ethidium bromide ml^{-1}]. The purified products were ligated into a pCR 4-TOPO vector (Invitrogen, Groningen, The Netherlands) as specified by the manufacturer. TOP10 competent *E. coli* (Invitrogen) were transformed with ligation products by heat shock (30 s at 42 °C). Recombinant cells were selected on Luria-Bertani medium with kanamycin (50 mg l^{-1}). Colonies containing a plasmid with an insert were able to grow in the presence of kanamycin.

16S rDNA RFLP analysis and sequencing

Cloned 16S rDNA fragments were amplified directly from fresh (less than 24 h) single *E. coli* colonies using AmpliTaq DNA polymerase and plasmid-targeted T7 (5′ TAATACGACTCACTATAGGG 3′) and P13 (5′ GACCATGATTACGCCAA 3′) primers. The PCR reaction mix was prepared according to the manufacturer's instructions (Perkin Elmer Cetus). Reaction conditions were 10 min of initial denaturation to lyse the cells, then 30 cycles for 15 s at 94 °C, 15 s at 55 °C and 15 s at 72 °C. Reactions were ended by a 10 min elongation at 72 °C and cooling at 4 °C. Without purification, PCR products were digested with the restriction endonucleases *Eco*RI and *Hae*III to compare clones. Restriction digests were separated by agarose gel electrophoresis (2% agarose and Tris borate/EDTA buffer containing 1 ng ethidium bromide ml^{-1}). One clone corresponding to each group of restriction patterns was subjected to detailed sequence analysis. Relevant colony PCR products were purified as previously described. Sequencing reactions were done with a GeneAmp PCR system 9600 thermocycler (Perkin Elmer Cetus) using the Dye-Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq DNA polymerase FS kit buffer (Applied Biosystem) and the universal primer w031 (5′ TTACCGCGGCTGCTGGCAC 3′).

16S rDNA sequence analysis

Sequences were edited to exclude the PCR primer binding sites. Newly determined sequences were compared with those available in public databases in order to ascertain their closest relatives. The presence of chimeric sequences was checked with the RDP chimera check program (Michigan State University, USA). Phylogenetic analyses were carried out with AliBee software (Belozersky 1995), and construction of the phylogenetic tree by the UPGMA method.

Reference bacteria used for phylogenetic analysis and their GenBank accession numbers are listed below. *D. frappieri* PCP-1 (U40078), *D. frappieri* TCE1 (X95742), *D. frappieri* TCP-A (AJ404686), *D. hafniense* (X94975), *D. dehalogenans* (L28946), *D. chlororespirans* (U68528), *Dehalobacter restrictus* (Y10164). The sequences obtained in this study have been deposited in GenBank with the following accession numbers: ATIA-3, AY223537; ATIA-6, AY223534; ATIA-8, AY223536; ATIA-12, AY223535. Their length was respectively 451 bases for ATIA-3, 595 bases for ATIA-6, 453 bases for ATIA-8 and 355 bases for ATIA-12.

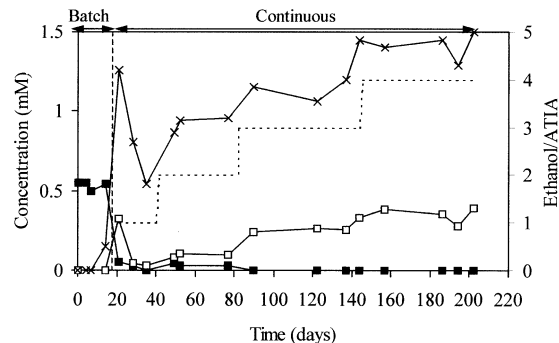

Fig. 3 Time course of the concentrations of ATIA (■), 5-aminoisophthalic acid (AIA) (❑) and iodide (×) in the anaerobic fixed-bed reactor from start-up to steady-state. Ethanol/ATIA ratio (...)

Results and discussion

Fixed-bed reactor colonization and stabilization

In previous work, we obtained the complete reductive deiodination of ATIA, the core structure of a range of iodinated X-ray contrast agents, to 5-aminoisophthalic acid (AIA) in batch liquid cultures with ethanol as an auxiliary substrate (Lecouturier et al. 2002). In this work, deiodination of ATIA was carried out in a continuous-flow anaerobic biofilm reactor. Such anaerobic bioreactors have been used for the biodegradation of a wide variety of chlorinated aromatic compounds such as chlorophenols (Hendriksen et al. 1992; Juteau et al. 1995; Tartakovsky et al. 1999; Wu et al. 1993), chlorobenzoates (Ascon and Lebeault 1999; Ahring et al. 1992), polychlorobiphenyls (Pagano et al. 1995; Tartakovsky et al. 2000). In our experiments, cultivating in a bioreactor had two advantages: (1) it was a good way to ensure conservation of the active deiodinating consortium, (2) While ATIA deiodination occurs in batch liquid cultures through a sequential process, i.e. each iodinated intermediate metabolite is degraded only after the previous compound has disappeared (Lecouturier et al. 2002), in bioreactors, operating parameters were set up so that all deiodination steps occurred simultaneously.

The biofilm reactor was driven for colonization as a batch culture for 21 days with ATIA at a concentration of 0.5 mM and ethanol at 2 mM. There was a 14-day lag period before ATIA deiodination started. ATIA was dehalogenated within 7 days. On day 21, AIA accumulated in the culture medium indicating that ATIA was completely deiodinated (Fig. 3). At this time, 77% of the expected iodide was released. Continuous feeding was then started with 0.5 mM ATIA. To optimize the load of the auxiliary substrate needed to obtain complete deiodination, the concentration of ethanol was increased gradually from 0.5 to 2 mM. During the first period, the iodide concentration dropped due to wash-out and the decrease in the ATIA deiodination rate. Diiodinated and monoiodinated intermediates were present in the effluent

(data not shown). When the ethanol/ATIA ratio was increased to 2, the iodide concentration rose to 0.92±0.05 mM. The diiodinated compound almost disappeared and the monoiodinated compound was the main intermediate in the effluent. When the ethanol/ATIA ratio was set at 3, the iodide concentration increased to 1.14±0.07 mM. Finally, when the ethanol/ATIA ratio was set at 4, the iodide concentration in the effluent was 1.45±0.05 mM and ATIA deiodination was complete. The operating parameters of the anaerobic bioreactor with an ethanol/ATIA ratio of 4 are presented in Table 1. ATIA was converted to AIA, ATIA was not detected, and the concentration of iodinated intermediates remained below 0.015 mM. The AIA concentration reached 0.37 mM in the effluent. The release of the three iodide atoms required the oxidation of four ethanol molecules to acetate. Neither AIA nor acetate were oxidized. Organic loads were the same in the inlet and the effluent flow and no gas production was observed, indicating that there was no methanisation in the fixed-bed reactor.

The specific deiodination rate of the consortium in the fixed-bed reactor was 13.7±2.3 mmol (g of volatile suspended solid, VSS)$^{-1}$ day^{-1}. This rate was higher than those reported for other chloroaromatic compounds degraded in anaerobic bioreactors. Ahring et al. (1992) reported a 3-chlorobenzoate dechlorination rate of 54 μmol (g VSS)$^{-1}$ day^{-1} in a UASB reactor. Tartakovsky et al. (1999) reported a maximal dechlorination rate of 128 μmol (g VSS)$^{-1}$ day^{-1} in a pentachlorophenol-degrading bioreactor. Both reactors were supplemented with high levels of extra carbon sources, which could explain a higher biomass content in UASB reactors. We had already found that the growth yield was quite low for the ATIA-deiodinating consortium (Lecouturier et al. 2002), and the protein concentrations remained low in our fixed-bed reactor.

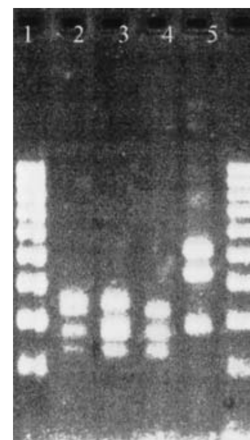

Fig. 4 Restriction fragment length polymorphism (RFLP) analysis of 16S rDNA from the ATIA-deiodinating mixed culture after digestion with *Eco*RI and *Hae*III. Only distinct clone patterns are presented. *Lanes 1, 6* 100-bp scale, *lane 2* ATIA-3, *lane 3* ATIA-6, *lane 4* ATIA-12, *lane 5* ATIA-8

The biofilm reactor was continuously fed for 2 years. Degradation parameters remained unchanged throughout.

Phylogenetic analysis

The composition of the ATIA deiodinating consortium from the stable fixed-bed reactor was explored by comparative sequence analysis of 16S rRNA genes after direct PCR amplification and cloning in *E. coli* from community DNA. Nineteen clones were grouped in four phylotypes by restriction fragment length polymorphism (RFLP) analysis (Fig. 4), and unique clones were sequenced (Table 2). One chimera was found and removed from further analysis. The ATIA-degrading culture yielded four clonal types. This low diversity

Table 1 Operational parameters of 5-amino-2,4,6-tri-iodoisophthalic acid (ATIA) deiodinating reactor with ethanol/ATIA ratio of 4 in feeding medium

Parameter	Influent concentration (mM)	Effluent concentration (mM)	Removal or release specific rate (mmol/g proteins/day)[b]
ATIA	0.48±0.03	0	0.3±0.03
AIA	0	0.37±0.03	0.25±0.03
Iodide	0	1.5±0.2	0.94±0.16
TOC[a]	103±2	95±8	None
Ethanol	1.97±0.02	0.1±0.1	1.19±0.12
Acetate	0	2±0.04	1.24±0.07

[a] In mg/l of carbon
[b] Fixed biomass value was calculated as the mean of three measurements

Table 2 Frequencies and nearest matches from the Ribosomal Data Project for clones from ATIA deiodinating anaerobic reactor

Clone	Related species	Identity level (%)	Frequency (%)[a]	Reference
ATIA-3	*Desulfitobacterium frappieri* TCE1	98	56	Gerritse et al. 1996
ATIA-6	*Desulfitobacterium frappieri* TCE1	98	5	Gerritse et al. 1996
ATIA-12	*Desulfitobacterium hafniense*	96	22	Christiansen and Ahring 1996
ATIA-8	Unknown *Eubacterium* WCHB1–69	94	17	Dojka et al. 1998

[a] 19 clones analyzed

agrees with the results of molecular community analysis of a 2-bromophenol-dehalogenating consortium (Knight et al. 1999). However, other authors have found a higher diversity in halogenated-xenobiotic-degrading anaerobic consortia (Breitenstein et al. 2001; von Wintzingerode et al. 1999). The low number of sequences in our consortium may result from the use of a mineral salt medium depleted of any complex addition and a long-term enrichment. In the reactor, ATIA deiodination represents the main energy-producing metabolism, as already shown in batch experiments (Lecouturier et al. 2002). Hence, bacteria implicated in deiodination benefit from a consistent advantage. Several parameters may also affect our ability to detect the actual species distribution, particularly cell lysis (Muyzer and Smalla 1998). Therefore, to minimize DNA extraction bias, a physical cell lysis method was used to effectively lyse all cell types. Using biomass from a continuously fed reactor enabled us to start with a good level of all the active bacterial population, because all the metabolic reactions occurred simultaneously.

Three clones were placed within the *Desulfitobacterium* genus. These bacteria usually form spores (Bouchard et al. 1996; Breitenstein et al. 2001; Madsen and Licht 1992). This result agrees with the maintenance of ATIA deiodination after pasteurization (80 °C, 40 min) and the microscopic observation of terminal spore-forming rod-shaped bacteria (data not shown). Two clones were related to the species *Desulfitobacterium frappieri.* Clones ATIA-3 and ATIA-6 corresponded to *D. frappieri* TCE1 (98% sequence identity from the GenBank for each clone) (Gerritse et al. 1999). Clone ATIA-3 represented 56% of clones analyzed. *D. frappieri* TCE1 was isolated from a soil sample from a chloroethene polluted location. It was found to grow by reductive dechlorination of tetrachloroethene and trichloroethene. This strain was able to use ethanol, hydrogen, formate, lactate and butyrate as electron donors. All of these substrates could also be used for ATIA deiodination (Lecouturier et al. 2002). However, no endospore was observed with this strain. Clone ATIA-12 resembled the strains *Desulfitobacterium hafniense* and *Desulfitobacterium frappieri* TCP-A (Breitenstein et al. 2001; Christiansen and Ahring 1996; Madsen and Licht 1992). These spore-forming bacteria are capable of dechlorinating 3-chloro-4-hydroxyphenylacetate and chlorophenols at *ortho* and sometimes *meta* positions. *D. hafniense* was isolated from waste water sludge. *D. frappieri* TCP-A was isolated from a river sediment. This strain uses pyruvate, lactate, butyrate, formate and hydrogen as electron donors but is not able to use ethanol or acetate. However, after comparison with the sequences of other aryl-dehalogenating *Desulfitobacterium* species, clone ATIA-12 was placed in the phylogenetic tree between *Desulfitobacterium chlororespirans*, a polychlorophenol *ortho*-dechlorinating bacterium (Sanford et al. 1996), and *D. dehalogenans*, which ortho-dechlorinates 2,4-dichlorophenol (Utkin et al. 1994) (Fig. 5). It may belong to a new *Desulfitobacterium* species.

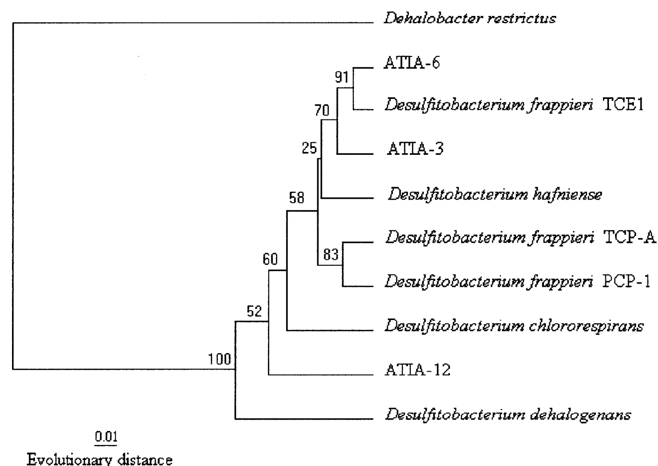

Fig. 5 Phylogenetic tree showing relationship between *Desulfitobacterium* sequences from ATIA deiodinating consortium and from known aryl-dechlorinating bacteria. The tree was constructed from 343 aligned bases. Bootstrap values were determined from 100 iterations

Desulfitobacterium species represented 83% of the clones from the ATIA degrading consortium and were probably responsible for deiodination. This genus groups several species that dehalogenate a number of chlorinated and brominated compounds such as chlorophenols, 3-chloro-4-hydroxyphenylacetic acid, chloroethenes and bromophenols. *Desulfitobacterium dehalogenans* dechlorinates 2,4-dichlorophenol and 3-chloro-4-hydroxyphenylacetate at the *ortho* position (Utkin et al. 1994). *D. frappieri* PCE-1 possesses two dehalogenating activities on chloroethenes and chlorophenols (Gerritse et al. 1996). Strain *D. frappieri* PCP-1, which was isolated from a methanogenic consortium on pentachlorophenol (Bouchard et al. 1996), is able to utilize a wide spectrum of halogenated substrates (Dennie et al. 1998). The strain has *ortho*, *meta* and *para* dechlorinating activities. In the presence of inducers, it is able to dehalogenate a variety of chlorinated aromatic compounds including pentachlorophenol and 2,3,5,6-tetrachloroaniline. It also dehalogenates 2,4,6-tribromophenol but had no activity on fluorinated phenols or aromatic compounds substituted with carboxyl groups. To date, no activity of desulfitobacteria has ever been found on iodinated compounds.

The last clone, ATIA-8, was placed in the *Cytophaga-Flavobacterium-Bacteroides* group. This clone represented 17% of the clones analyzed. Its sequence was related to those of two clone isolated from contaminated environments: clone TDC-S1:26, from an anaerobic consortium capable of degrading tetrachloroethene (Dennis et al., GenBank, unpublished data), and WCHB1-69, from a hydrocarbon- and chlorinated-solvent-contaminated aquifer (Djoka et al. 1998). However, the members of this group do not have the ability to sporulate. Since ATIA deiodination was resistant to pasteurization, this strain was not directly implicated in the deiodination. The

presence of this species may be explained by a close interrelationship with deiodinating bacteria.

We have characterized for the first time the bacterial community from a steady-state anaerobic ATIA-deiodinating bioreactor. Most of the 16S rDNA sequences amplified from this consortium belonged to the genus *Desulfitobacterium*. Interestingly, identified bacteria were closely related to known aryl-dechlorinating bacteria, but represented new strains or new species. They were highly representative of the total bacterial population and their physiological characteristics corresponded to the properties of the ATIA-degrading consortium. These results suggest that desulfitobacteria were responsible for ATIA reductive deiodination. This work confirms the large contribution of these bacteria to the elimination of a variety of chlorinated, brominated, and iodinated xenobiotic compounds.

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