Reductive Dehalogenation of Brominated Phenolic Compounds by Microorganisms Associated with the Marine Sponge *Aplysina aerophoba*

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Marine sponges are natural sources of brominated organic compounds, including bromoindoles, bromophenols, and bromopyrroles, that may comprise up to 12% of the sponge dry weight. *Aplysina aerophoba* sponges harbor large numbers of bacteria that can amount to 40% of the biomass of the animal. We postulated that there might be mechanisms for microbially mediated degradation of these halogenated chemicals within the sponges. The capability of anaerobic microorganisms associated with the marine sponge to transform haloaromatic compounds was tested under different electron-accepting conditions (i.e., denitrifying, sulfidogenic, and methanogenic). We observed dehalogenation activity of sponge-associated microorganisms with various haloaromatics. 2-Bromo-, 3-bromo-, 4-bromo-, 2,6-dibromo-, and 2,4,6-tribromophenol, and 3,5-dibromo-4-hydroxybenzoate were reductively debrominated under methanogenic and sulfidogenic conditions with no activity observed in the presence of nitrate. Monochlorinated phenols were not transformed over a period of 1 year. Debromination of 2,4,6-tribromophenol, and 2,6-dibromophenol to 2-bromophenol was more rapid than the debromination of the monobrominated phenols. Ampicillin and chloramphenicol inhibited activity, suggesting that dehalogenation was mediated by bacteria. Characterization of the debrominating methanogenic consortia by using terminal restriction fragment length polymorphism (TRFLP) and denaturing gradient gel electrophoresis analysis indicated that different 16S ribosomal DNA (rDNA) phylotypes were enriched on the different halogenated substrates. Sponge-associated microorganisms enriched on organobromine compounds had distinct 16S rDNA TRFLP patterns and were most closely related to the δ subgroup of the proteobacteria. The presence of homologous reductive dehalogenase gene motifs in the sponge-associated microorganisms suggested that reductive dehalogenation might be coupled to dehalorespiration.

Halogenated compounds constitute one of the largest groups of environmental pollutants. Contamination of marine and freshwater sediments by anthropogenic halogenated organic compounds is common (8, 14). The marine environment is also a rich source of naturally occurring halogenated compounds produced by mollusks (3), algae (25), polychaetes (2), jellyfish (43), and sponges (29). A number of sponge species, such as *Psammopemma* sp., *Psammaphysilla purpurea*, *Aplysina aerophoba*, and *Dysidea herbacea*, have been shown to produce brominated aromatic metabolites, including bromoindoles, bromophenol (BP), polybrominated diphenyl ethers, and dibromodibenzo-*p*-dioxins (5, 7, 23, 38).

The bright yellow sponges of the *Aplysinidae* family are abundant in subtropical and tropical waters of the Mediterranean Sea and the Pacific and Atlantic oceans (6, 12, 13). Major secondary metabolites of the sponge *A. aerophoba* are bromophenolic metabolites derived from dibromotyrosine (23, 24).

Bromine-containing metabolites can account for 7 to 12% of the sponge dry weight (37). In the natural environment, these compounds may serve as a chemical defense against predators and biofouling. *Aplysina* sponges harbor large numbers of bacteria that can amount to 40% of the biomass of the animal. The largest fraction of bacteria is found in the extracellular matrix but is enclosed within the mesohyl tissue of the animal (6, 13, 30). An investigation of the microbial diversity in *Aplysina cavernico* that used fluorescence in situ hybridization revealed that δ -proteobacteria, including anaerobic sulfate reducers such as *Desulfovibrio* spp., were the predominant bacteria (6). Eight percent of all sponge-derived 16S ribosomal DNA (rDNA) sequences from *A. aerophoba* and *Theonella swinhoei* were related to the δ -proteobacteria (12).

The abundance of halogenated compounds in sponge tissue and the high bacterial biomass imply that sponge-associated microorganisms might have the ability to dehalogenate and degrade brominated compounds. The objectives of the study were to determine whether anaerobic dehalogenating microorganisms are present in the sponge *A. aerophoba* by using dehalogenase activity assays under different anaerobic conditions. We used specific PCR primers designed for the detection of reductive dehalogenase genes (26) to demonstrate the presence of putative dehalogenase gene motifs in sponge tissue and associated bacteria. To determine the microbial community

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enriched on each substrate and to identify sponge-associated microorganisms involved in dehalogenation, two community analysis techniques, denaturing gradient gel electrophoresis (DGGE) (28, 39) and terminal restriction fragment length polymorphism (TRFLP) (15, 27), were used (41).

MATERIALS AND METHODS

Sponge collection. *A. (syn. Verongia) aerophoba* (phylum Porifera, order Verongidae, family Aplysinidae) sponges were collected by scuba diving at the Marine Biological Station, Banyuls sur Mer, France, in April 2001. Freshly collected sponge material was immediately frozen and was stored at -70°C for later molecular analysis of the native bacterial community.

Enrichment cultures. For preparation of anaerobic cultures, freshly collected sponge material was aseptically homogenized with mortar and pestle in filtered (0.2- μm pore size) seawater. The homogenized sponge material was sparged with N_2 , and 1 ml was used as inoculum for 50 ml of anaerobic medium in serum flasks, capped with rubber stoppers, and crimped with aluminum seals. Methanogenic (20), sulfidogenic (20), and denitrifying (19) media were used for different enrichments. The inoculated cultures were stored at 4°C , transported back to the laboratory, and spiked with halogenated substrates within 3 weeks. Initial enrichment culture sets of four replicate bottles were supplemented with 200 μM lactate and the following halogenated substrates: a mixture of 2-BP, 3-BP, and 4-BP (100 μM each); a mixture of 2-chlorophenol (2-CP), 3-CP, and 4-CP (100 μM each); 100 μM 2,6-di-BP (2,6-DBP); 100 μM 2,4,6-tri-BP (2,4,6-TBP); and 100 μM 3,5-dibromo-4-hydroxy-benzoate (3,5-DB-4-HB). Sterile medium spiked with halogenated substrates and cultures with 200 μM lactate only served as controls. Substrates (2-BP, 3-BP, 4-BP, 2,6-DBP, 2,4,6-TBP, and 3,5-DB-4-HB [Aldrich Chemical Co., Milwaukee, Wis.]) were added from deoxygenated stock solutions (50 mM) in 0.1 N NaOH. The dehalogenation of halophenols was monitored over time, and halophenols were refed when consumed. To study the dehalogenating microorganisms associated with the sponges in more detail, the primary enrichment cultures maintained on 200 μM lactate alone or with 200 μM lactate and 2-BP, 3-BP, 4-BP, 2,6-DBP, 2,4,6-TBP, or 3,5-DB-4-HB (100 μM) or with a mixture of 2-BP, 3-BP, and 4-BP (100 μM each) were transferred to (sulfate-free) anaerobic medium (1/10 dilution). The cultures were incubated without shaking at 28°C in the dark.

Effect of antibiotics on microorganisms associated with the sponges. To investigate the effect of antibiotics, the primary methanogenic cultures enriched on brominated compounds were transferred to sulfate-free anaerobic medium (1/10 dilution). Enrichment cultures were maintained on 200 μM lactate and either 100 μM 2-BP, 3-BP, 4-BP, 2,6-DBP, 2,4,6-TBP, or 3,5-DB-4-HB with ampicillin or chloramphenicol (Boehringer Mannheim, Mannheim, Germany) (200 or 100 mg liter $^{-1}$, respectively). The effect of antibiotics was determined by the observation of the presence or absence of debromination activity.

Analytical methods. For sampling, the cultures were thoroughly mixed and 1 ml of culture was withdrawn with a sterile syringe flushed with oxygen-free N_2 . Halophenols, halobenzoates, phenol, and benzoate were analyzed by high-performance liquid chromatography (LC-10AS; Shimadzu Corp., Kyoto, Japan) as described previously (4, 20).

PCR amplification. DNA was extracted from whole sponge tissues and enrichment cultures by using a UltraClean Soil DNA isolation kit (MO BIO, Solana Beach, Calif.). For 16S rDNA gene amplification, genomic DNA was amplified with bacterial universal primers 27F and 1525R (16), as described previously (27). PCR amplification parameters were as follows: 94°C and 5 min of initial melt; 30 amplification cycles of 94°C , 30 s; 55°C , 30 s; and 72°C , 1.3 min; and a final extension at 72°C for 10 min in a Perkin-Elmer Gene-AMP PCR system 2400 thermal cycler (Perkin-Elmer, Foster City, Calif.). For DGGE analysis, amplification was done with the GC clamp primer 344F(GC) and 518R under the conditions described by Vetriani et al. (39).

For analysis of dehalogenase genes, PCR amplification was performed in a total volume of 20 μl containing the appropriate reaction buffer and reagents (26). Primers, i.e., 18-bp forward (5'-GWAGCAGGTYTRGGASAA-3') and 20-bp reverse (5'-TAGCCSAAGKGCWYCWTCCAT-3'), designed for the specific amplification of putative dehalogenase-expressing gene motifs were taken from recently developed gene sequences (26). This primer set, designated DHAR 1000 F/1350 R, was first tested on a 2-BP enrichment consortium from Arthur Kill sediments (26). The PCR mixtures for dehalogenase gene amplification were subjected to 94°C for 3 min; followed by 30 cycles of 94°C for 1 min, 46°C for 1 min, and 72°C for 1 min; and one cycle of 72°C for 7 min.

TRFLP analysis. For TRFLP analysis, a fluorescent dye was incorporated on the 5' primer for 16S rRNA genes (27). Fluorescently labeled PCR products (20

μl) were purified with the GeneClean kit II (Qbiogene, Inc., Carlsbad, Calif.) and were digested for 6 h at 37°C with *MnII* (New England Biolabs, Beverly, Mass.). Twenty-five nanograms of labeled PCR product was run on a ABI 310 genetic analyzer (Perkin-Elmer) with Genescan software and internal standards (15, 27).

DGGE analysis. DGGE was performed with a D Gene System (Bio-Rad Laboratories, Hercules, Calif.) as described previously (39). To check the purity of the bands, a small plug of the acrylamide gel was removed from each DGGE band, added to a PCR mixture, and reamplified. The PCR products were then loaded onto a DGGE gel, and band purity was checked. A small plug was removed from pure, single bands, and reamplification was performed with the same primer set with no GC clamp on the forward primer. The resulting PCR product was used as a template for sequencing. The sequence was determined for both strands. Sequence similarity searches and alignments were performed as described below.

Cloning, sequencing and phylogenetic analysis of PCR products from dehalogenase primers. The PCR amplification product was purified by using the GeneClean kit II (Qbiogene, Inc.). The recovered fragment was cloned by using a TOPO TA cloning kit (Invitrogen, Carlsbad, Calif.) as recommended by the manufacturer. Plasmid DNA of the 47 representative clones was isolated and sequenced with an ABI 310 genetic analyzer by using M13 universal primers. Sequence similarity searches and alignments were performed by using the BLAST 2.1 program (1) (National Center for Biotechnology Information, Bethesda, Md.) and the programs Clustal X, GeneDoc (36), and DNASTAR package (DNASTAR Inc., Madison, Wis.), respectively.

RESULTS

Dehalogenation of haloaromatic compounds by sponge-associated anaerobic microorganisms. The anaerobic biotransformation of haloaromatic compounds under denitrifying, sulfidogenic, and methanogenic conditions is summarized below. Debromination of 2-BP, 3-BP, 4-BP, 2,6-DBP, 3,5-DB-4-HB, and 2,4,6-TBP was observed within 30 days in the presence or absence of sulfate. No transformation of bromoaromatic compounds was observed under denitrifying conditions. A mixture of monochlorophenols was not dechlorinated over a period of 1 year under any set of conditions tested. The halogenated substrates were stable in sterile medium. Generally, the rates of dehalogenation in the presence of sulfate were lower than under methanogenic conditions. This result suggests that sulfate affected the dehalogenating microbial community but did not completely inhibit dehalogenation.

We examined dehalogenation of brominated phenolic compounds in sulfate-free anaerobic medium in more detail (Fig. 1). All mono-BPs added as a mixture were dehalogenated, but transformation of 3-BP to phenol took much longer than for 2-BP and 4-BP. 2-BP and 4-BP were dehalogenated to phenol within 30 and 120 days, respectively (Fig. 1A). After 1 year, 3-BP was almost completely debrominated to phenol ($21.5 \pm 12.3 \mu\text{M}$). 2,6-DBP was completely dehalogenated to phenol within 60 days, with 2-BP detected as a transient intermediate (Fig. 1B). 2,4,6-TBP was almost completely dehalogenated to mono-BP and phenol within 60 days. 4-BP and 2-BP accumulated as intermediates during 2,4,6-TBP dehalogenation, but only 2-BP was further dehalogenated to phenol, while 4-BP persisted over the entire 120-day period (Fig. 1C). The accumulation of 4-BP suggests that the removal of *ortho*-substituted bromines was preferential to *para* debromination. Phenol accumulated and was not degraded further by the sponge-associated microbial community. Similar results were observed in the presence of sulfate (data not shown). The differences in substrate specificities and relative rates of transformation suggest that specific dehalogenating populations are present in the sponges.

FIG. 1. Dehalogenation of brominated compounds in sponge enrichment cultures under methanogenic conditions. The results are means of four replicate cultures: 2-, 3-, and 4-BP (A); 2,6-DBP (B); and 2,4,6-TBP (C).

Effects of antibiotics on dehalogenation by sponge-associated microorganisms. To determine whether dehalogenation of brominated phenolics was mediated by bacteria (and not sponge cells), the effect of ampicillin and chloramphenicol (inhibitors of bacterial cell wall synthesis and protein synthesis, respectively) was tested. In the absence of antibiotics, debromination of 2-BP, 3-BP, 4-BP, 2,6-DBP, 2,4,6-TBP, and 3,5-DB-4-HB was observed within 7 to 21 days after transfer (1/10 dilution) to fresh anaerobic medium with brominated compounds (data not shown). However, no transformation of bromoaromatic compounds was observed over a period of 30 days in the presence of either ampicillin or chloramphenicol. Com-

plete inhibition of dehalogenation by 200 or 100 mg of ampicillin liter⁻¹ and 200 or 100 mg of chloramphenicol liter⁻¹ suggests that dehalogenation activity is mediated by bacteria associated with the sponges.

Effects of electron acceptors on community structure. TRFLP of 16S rDNA of sponge-associated microorganisms enriched on halophenols is shown in Fig. 2. The sponge tissue (Fig. 2A) and control culture with only 200 μM lactate (Fig. 2B) showed 10 major and 5 minor terminal fragments. After enrichment on BP compounds, the complexity of the microbial community was reduced. In the mono-BP (Fig. 2C to F)-, 2,6-DBP (Fig. 2H)-, and 2,4,6-TBP (Fig. 2I)-enriched cultures, two to six terminal fragments were predominant. The TRFLP fingerprint from the 2-BP enrichment (Fig. 2D) was quite different from the 3- or 4-BP enrichments. However, the enrichment that received a combination of the mono-BP (Fig. 2C) contained peaks common to the individual mono-BP enrichments yet exhibited these in very different peak intensities. The 3,5-DB-4-HB, 2,6-DBP, and 2,4,6-TBP enrichments (Fig. 2G to I) also showed remarkable similarity to each other, with the exception of different band intensities. The complexity of the microbial community was reduced after enrichment on BPs compared to the original inoculum. This degree of similarity between the different enrichments suggests that the microbial community structure changed only slightly in response to different halophenol electron acceptors.

Analysis of the microbial community by using DGGE revealed two or three major bands in each enrichment and showed one band with the same relative migration distance in most of the cultures (Fig. 3). To confirm that 16S rRNA gene fragments at the same relative migration distance in different cultures represented the same organism, a total of 17 bands from the cultures were excised and sequenced. Most of the sequences were highly related to *Desulfovibrio* sp. strain TBP-1 (GenBank accession no. AF090830) and *Desulfovibrio acrylicus* (GenBank accession no. U32578) according to a BLAST search. Phylogenetic tree reconstruction confirmed the relationship of these sequences to those of δ-proteobacteria, with six diverse lineages represented by all the 17 sequences (Fig. 4). Full 16S rRNA gene sequences of bacterial clones previously obtained from *A. aerophoba* (12) (designated *Aphysina*) are also shown on the phylogenetic tree. DGGE bands 1 and 2 from native sponge material were closely related to several of the *Aphysina* clones. The bands in the DGGE profiles corresponded to the δ-proteobacteria (bands 4, 7, 10, 12, 14, and 16), *Bacteroidetes* (bands 3, 6, and 9), *Chloroflexi* (bands 1 and 8), and unidentified bacteria (bands 5, 11, 13, and 15). The most predominant group of sequences (bands 4, 7, 10, 12, 14, and 16) had similarity to *Desulfovibrio* sp. strain TBP-1 (GenBank accession no. AF090830), a 2,4,6-TBP-dehalogenating bacterium (4). Band number 1 had similarity to *Dehalococcoides ethenogenes* (GenBank accession no. AF004928). Also, some bands (bands 3, 5, 11, 13, and 15) were closely related to uncultured dehalogenating bacteria from a TCE-dechlorinating consortium and a reductive DCE-dehalogenating enrichment. Other bands (bands 6, 9, and 17) were similar to an unidentified bacterium from coral (GenBank accession no. AY038512) and an uncultured bacterium from subseafloor habitats associated with a deep-sea volcanic eruption (GenBank accession no. AF469404).

FIG. 2. TRFLP profiles of *Mn*II-digested 16S rDNAs amplified from different anaerobic cultures enriched on brominated phenolic compounds. (A) Native sponge-associated microorganisms; (B) control with only 200 μ M lactate; (C) a mixture of 2-, 3- and 4-BP (100 μ M each); (D) 100 μ M 2-BP; (E) 100 μ M 3-BP; (F) 100 μ M 4-BP; (G) 100 μ M 3,5-DB-4-HB; (H) 100 μ M 2,6-DBP; and (I) 100 μ M 2,4,6-TBP.

Amplification of reductive dehalogenase genes from sponge-associated microorganisms. A conserved reductive dehalogenase gene motif has been found in the dehalorespiring bacteria *D. ethenogenes*, *Dehalospirillum multivorans*, and *Desulfitobacterium dehalogenans* (17, 22, 31, 32). Since reductive dehalogenation was the mechanism of halophenol transformation, we examined the sponge material and the anaerobic cultures for presence of conserved reductive dehalogenase gene motifs. By using degenerate PCR primers specific for reductive dehalogenase genes, we could amplify a PCR product of the predicted size (450 bp) from genomic DNA extracted directly from sponge tissue and from the enrichment cultures of sponge tissue maintained on 200 μ M lactate and a mixture of 100 μ M 2-BP, 3-BP, and 4-BP.

PCR products were cloned into the vector pCR2.1-TOPO,

and a total of 48 clones were randomly selected and sequenced. Among these, 21 sequences were related to reported reductive dehalogenase genes. The result of BLASTP analysis indicated that PCR products were related to the putative reductive dehalogenase of *Desulfitobacterium* sp. strain PCE-1 (GenBank accession no. AY013361) and the trichloroethene reductive dehalogenase of *D. ethenogenes* (GenBank accession no. AF228507). Clustal_X pairwise alignments of the C-terminal amino acid sequences of the reported four reductive dehalogenases and sponge clones showed that all deduced amino acid sequences of sponge clones (145 amino acids [aa]) contained two iron-sulfur cluster binding motifs (CXXCXXCXXCXXCP and CXX[₂₋₁₂]CXXCXXCXXCXXCP) and 20 identical residues (Fig. 5). The conserved sequence of these proteins contain the twin Fe_4S_4 cluster binding motif (17, 34), suggesting that our novel

FIG. 3. DGGE profiles of 16S rDNA genes from different enrichment cultures on brominated phenolic compounds. The arrow highlights a band with the same relative migration distance found in most of the cultures. Lanes: 1, native sponge-associated microorganisms; 2, control with only 200 μ M lactate; 3, mixture of 2-, 3-, and 4-BP (100 μ M each); 4, 100 μ M 2-BP; 5, 100 μ M 3-BP; 6, 100 μ M 4-BP; 7, 100 μ M 2,6-DBP; and 8, 100 μ M 3,5-DB-4-HB. Numbers indicate sequenced 16S rDNA fragments.

primers amplify genes or gene motifs encoding putative reductive dehalogenases.

The relationship between sponge clones and the four reported reductive dehalogenases of dehalorespiring bacteria is shown in Fig. 6 (35). Deduced amino acid sequences of sponge clones (145 aa) fell into two main groups. The E11, E17, and E47 clones fell outside the two groups and previously reported reductive dehalogenase genes. Nucleotide sequences varied at 1 to 9 positions (resulting in 1- to 4-aa substitutions) within each group. Groups I and II each shared approximately 41 to 44% identity with PCE dehalogenase from *D. multivorans* (GenBank accession no. AF022812) and 27% identity with *tceA* reductase from *D. ethenogenes* (GenBank accession no. AF228507), respectively. The other sponge clones (E11, E17, and E47) shared 36 to 45% identity with PCE dehalogenase and 26% identity with *tceA* reductase. Phylogenetic tree reconstruction showed that the putative dehalogenases from the sponges formed a distinct group separated from all other known reductive dehalogenase genes.

DISCUSSION

Marine sponges are natural sources of brominated organic compounds (23, 24, 37) and also support a large amount of microbial biomass (6, 13). It is thus conceivable that the abundance of brominated aromatic compounds and other complex secondary metabolites in the sponge tissue would select for bacteria with the ability to metabolize these compounds (6). Most sponges alternate between periods of high water-pumping velocity and periods of low water circulation. It is possible that oxygen becomes limited during periods of low water cir-

culation because of active respiration by the large number of bacteria present in the mesohyl (42). This study demonstrates the anaerobic reductive dehalogenation of halogenated phenolic compounds by microorganisms associated with the organobromine-containing sponge *A. aerophoba*. The fact that the dehalogenation activity could be transferred and was inhibited by the addition of ampicillin or chloroamphenicol suggests that bacteria harbored within the sponge carried out the anaerobic dehalogenation.

Several studies have demonstrated that the anaerobic reductive dehalogenation of haloaromatic compounds in different redox zones is influenced by the availability of alternate electron acceptors such as nitrate or sulfate (9, 10, 14, 15, 20). Our results showed that bromoaromatic compounds were debrominated in enrichments of sponge-associated microorganisms both in the presence or absence of sulfate. No activity was observed in the presence of nitrate. Furthermore, CPs were not dehalogenated, despite prolonged incubation. Due to the higher electronegativity of chlorine, the carbon-chlorine bond is stronger than the carbon-bromine bond. The dehalogenation of BPs only may thus reflect the exposure of sponge-associated bacteria only to natural brominated compounds prior to the establishment of the laboratory enrichments. In fact, brominated tyrosine derivatives, including aerophobin-2, aplysinamin-1, and isofistularin-3, which are found in *A. aerophoba*, can account for 7 to 12% of the sponge dry weight (37).

To date, only three marine strains able to debrominate polybrominated aromatic compounds have been described elsewhere (4, 33, 40). *Desulfobrevibrio* sp. strain TBP-1, isolated from estuarine sediments from the Arthur Kill (4), debrominated BPs but did not dechlorinate CPs (4). *Propionigenium maris* (40) and an anaerobic 2,4,6-TBP-debrominating bacterium (strain DSL-1) (33) isolated from burrows of BP-producing marine acorn worms are both capable of debrominating 2,4,6-TBP to mono-BP. Again, no transformation of CPs was observed.

In this study, sponge enrichments debrominated 2-BP (*ortho*-) faster than 4-BP (*para*-) under methanogenic and sulfate-reducing conditions. Dehalogenation of 3-BP (*meta*-) was slow. The accumulation of 4-BP during the dehalogenation of 2,4,6-TBP indicates that removal of *ortho*-substituted bromines precedes *para* debromination. A similar preference of *ortho*-halophenol dehalogenation over *meta*- and *para*-halophenols has been reported in other studies (10, 11, 20).

Analysis of sponge-associated microorganisms by using 16S rRNA gene methods independent of culturability have been reported. Fluorescence in situ hybridization analysis revealed that δ -proteobacteria, including anaerobic sulfate-reducing bacteria (*Desulfobrevibrio* spp.), were predominant in *Aplysina cavernicola* (6). Two studies have applied 16S rRNA gene clonal library analysis to assess microbial diversity in sponges (12, 42). Webster et al. (42) reported that 2 of 70 clones from the marine sponge *Rhopaloeides odorabile* were distantly related to *Desulfobacterium anilini* and uncultured members of the δ -proteobacteria. Recently, Hentschel et al. (12) reported a uniform microbial community based on 160 sponge-derived 16S rRNA gene sequences from *A. aerophoba* and *T. swinhoei*. Twenty-two and 8% of all sponge-derived sequences were related to the *Chloroflexi* and δ -proteobacteria, respectively. In order to determine the diversity and identity of sponge-asso-

FIG. 4. Phylogenetic tree of DGGE band sequences of 16S rDNA. Bootstrap values at nodes are the percentages of 100 iterations. Values less than 50% are not included. The reference bar indicates 10 nucleotide exchanges per 100 nucleotides. The sequences are numbered as in Fig. 3.

ciated microorganisms enriched in dehalogenating cultures, we used TRFLP and DGGE as community fingerprinting methods (15, 41). TRFLP patterns may be used to assess the similarity of different enrichments. Different terminal fragments were present in the native sponge tissues and control cultures without BP compounds, compared to the enrichments amended with different BPs. The difference in terminal restriction fragments could be due to selection in response to different brominated organic compounds under anaerobic conditions. Remarkable similarity between TRFLP fingerprints from 2,6-DBP-, 2,4,6-TBP-, and 3,5-DB-4-HB-amended enrichments was observed. 2,4,6-TBP has an additional *para* substitution, which may be reflected by the presence of an additional restriction fragment at 182 bp—a fragment that dominates the pattern observed for the enrichments with *meta*- and *para*-monobrominated phenol.

We used short sequences (about 150 bp) derived from DGGE bands to determine broad phylogenetic placement of

the primary members of our dehalogenating enrichments from sponge material (21). Our studies are essentially in agreement with the previously published clonal libraries of Hentschel et al. (12) in that many native sequences obtained were related to the δ subgroup of the proteobacteria and the *Chloroflexi*. Interestingly, the sequences of six DGGE bands have similarity to those of *Desulfovibrio* sp. strain TBP-1, a 2,4,6-TBP-dehalogenating bacterium isolated from the Arthur Kill (4). This band was detected in different dehalogenating enrichment cultures (and also in the control culture that was amended with only 200 μ M lactate), but they were absent or undetectable in the native sponge tissue. Also, the sequence of one DGGE band from the sponge tissue was closely related to the *Chloroflexi*, which includes *D. ethenogenes* strain 195, a known dehalorespiring organism (18). This analysis, coupled with the demonstration of dehalogenation activity, shows that the sponge-associated microbial community could be a significant reservoir of dehalogenating bacteria in the marine environment.

FIG. 5. C-terminal amino acid sequence alignment of reductive dehalogenase from the sponge-associated microorganisms. Conserved sequence motifs (FeS clusters) are indicated by boxes. Group I, N1, N2, N5, N7, N9, E13, E32, E36, and E411; group II, N4, E29, E34, E37, E310, E41, E42, and E45 (E, DNA from enrichment cultures; N, DNA from native sponge without enrichment); PceA Y51, PceA dehalogenase from *Desulfotobacterium* sp. strain Y51 (GenBank accession no. AB070709); CprA Dd, *ortho*-chlorophenol reductase from *D. dehalogenans* (GenBank accession no. AF115542); TecA De, TCE dehalogenase from *D. ethenogenes* strain 195 (GenBank accession no. AF228507); Pce1 Ds, Pce1 dehalogenase from *Desulfotobacterium* sp. strain PCE-1 (GenBank accession no. AY013361); and PceA Dm, PCE dehalogenase from *D. multivorans* (GenBank accession no. AF022812).

Several anaerobic bacteria have been shown to couple reductive dehalogenation with energy generation in a process termed dehalorespiration. Rhee et al. (26) designed degenerate PCR primers based on the conserved motifs of reductive dehalogenase genes from dehalorespiring bacteria and detected putative reductive dehalogenase gene fragments from a 2-BP-degrading consortium. With these primers, we could detect a new group of putative reductive dehalogenase gene motif fragments in the sponge-associated microbial consortia (Fig. 6). The reductive dehalogenase genes are phylogeneti-

cally deeply branched, with 26 to 44% identity to four known reductive dehalogenase genes (17, 22, 31, 32). This result also supports the hypothesis that dehalogenation of brominated aromatic compounds is mediated by dehalorespiring sponge-associated microorganisms. The presence of putative reductive dehalogenase gene motif fragments in the sponge-associated bacteria suggest that dehalorespiration might be widely distributed in natural environments such as sponges, a reflection of the prevalence of biogenic, brominated organic compounds in the environment.

In summary, anaerobic dehalogenating microorganisms are located in the sponge *A. aerophoba* and their activity was demonstrated under anaerobic conditions. We report here for the first time the presence of dehalogenating bacteria associated with marine sponges and the putative reductive dehalogenase genes from these microorganisms. Community analysis following enrichment on organohalides showed the presence of 16S rRNA gene sequences that are closely related to known dehalogenating bacterial genera. Several putative dehalogenase gene motifs were detected in the sponge and enrichment cultures. The diversity of gene motifs detected suggests that the sponge microbial community is capable of dehalogenating a broad range of organohalide compounds, only a limited number of which were examined in this study. Sponges are thus an underexplored source of anaerobic bacteria with diverse dehalogenating capabilities.

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FIG. 6. Phylogenetic tree of deduced partial amino acid sequences of reductive dehalogenases. Multiple alignment and construction of the phylogenetic tree with the neighbor-joining method with 1,000 bootstrap resamplings were performed by using the Clustal X and TreeView programs. The reference bar indicates 10 amino acid exchanges per 100 amino acids. For names of sequences, see Fig. 5.

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