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Enrichment of a microbial consortium capable of reductive deiodination of 2,4,6-triiodophenol

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An anaerobic microbial consortium capable of reductively dehalogenating 2,4,6-triiodophenol (2,4,6-TIP) to 4-iodophenol was enriched from sediments collected from an iodine-producing industry, Chiba, Japan. In the presence of lactate, the enrichment reductively deiodinated 2,4,6-TIP, 2,4-diiodophenol and 2-iodophenol, suggesting preferential removal of *ortho*-substituted iodines. PCR-denaturing gradient gel electrophoresis and 16S rRNA gene sequence analysis showed that at least 4 bacteria, including *Pseudomonas stutzeri*, *Clostridium* sp., *Sedimentibacter* sp., and unidentified *Chloroflexi* bacterium, were predominant in the enrichment. Interestingly, the DGGE band corresponding to the *Chloroflexi* bacterium disappeared when the enrichment was grown in the absence of 2,4,6-triiodophenol. In addition, the DGGE band with a nearly identical gene sequence was detected in another enrichment that consumed 3-amino-2,4,6-triiodobenzoic acid (ATBA). Phylogenetic analysis of 1416 bp of 16S rRNA gene sequence for this putative deiodinating bacterium revealed that it was closely related (93% sequence similarity) with an anaerobic bacterium MO-CFX2 belonging to the class *Anaerolineae*, which was recently isolated from subseafloor sediments. The sequence similarities with other known reductive dehalogenating bacteria such as *Dehalococcoides mccartyi* and *Dehalobium chlorocoercia* were relatively low (78%–80%). Quantitative PCR analysis targeting specific 16S rRNA gene region of the putative deiodinating bacterium showed that the enrichments consuming 2,4,6-TIP or ATBA contained 18 to 1070 times much higher amounts of 16S rRNA gene copies than those in the enrichments that do not consume these iodoaromatic compounds. These results suggest that a novel anaerobic bacterium in the class *Anaerolineae* is capable of reductively deiodinating 2,4,6-iodobenzene derivatives.

[**Key words:** 2,4,6-Triiodophenol; Reductive dehalogenation; Iodine; Enrichment; PCR-DGGE; *Anaerolineae*; *Chloroflexi*]

Japan is the second largest iodine producer in the world, and Chiba Prefecture accounts for about 90% of all production in Japan. Annual production of iodine in this area accounts for 6000 tons, which constitutes approximately one third of the world iodine production (1). Iodine is manufactured from natural gas brine water, which often contains 2000 times higher iodine (about 1 mM) than that in natural seawater. Natural gas, mainly methane, is associated with brine water in this area, and brine is pumped up from the late Pliocene to early Pleistocene Kazusa Group (1). Iodine has a wide range of uses such as medicated gargle, X-ray contrast media and antimicrobial agents (2). Recently, demand for iodine has grown in high tech fields such as liquid crystal display polarizing film and etching agents for semiconductors. However, construction of new wells for brine intake is now significantly restricted, since continuous pumping of brine water has caused severe ground settlement in this area. Therefore, to save iodine as a raw material, some iodine-producing companies have started their own iodine-recycling program, such as high temperature decomposition of iodine-containing compounds to recover iodine (2).

Iodinated X-ray contrast media account for 23% of demand for iodine in Japan (2). They are derivatives of 2,4,6-triiodobenzene with polar carboxylic and hydroxyl moieties in their side chains. Due to their stability and hydrophilicity, they pass wastewater treatment plants without degradation, and are expected to be released into natural environments. Significant concentrations (occasionally more than 10 μg L^{-1}) of iodinated X-ray contrast media such as iopamidol and diatrizoate have been detected in German municipal sewage treatment plant, rivers and groundwater (3). Recently, Boumura and Nakata determined iodinated X-ray contrast media in effluents of Japanese wastewater treatment plants, and found that concentrations of iopamidol and iohexole were approximately 10 μg L^{-1} and 2 μg L^{-1}, respectively (Nakata, H. and Boumura, T., Abstr. 13th Annu. Meet. Jap. Soc. Water Environ., p. 31, 2010). Although no apparent toxicity has been observed, their relatively high concentrations in the aquatic environment, highly persistent nature, and the lack of information about potential sublethal effects highlight the importance of an environmental assessment of iodinated X-ray contrast media (3). Thus, efficient mineralization or dehalogenation of iodinated X-ray contrast media is expected not only from the viewpoint of iodine recycling, but also to reduce possible environmental impact of these compounds.

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Certain anaerobic bacteria can efficiently eliminate one or more halide atoms from a wide variety of halogenated compounds including haloaromatics, haloaliphatics, polychlorinated biphenyls and polychlorinated dibenzo-*p*-dioxins/furans through reductive dehalogenation (4,5). These bacteria utilize halogenated compounds as the terminal electron acceptors for growth, a process called organohalide respiration. To date, a number of organohalide respiring bacteria such as *Desulfitobacterium* spp., *Dehalobacter* spp. and *Dehalococcoides* spp. have been enriched and isolated as pure cultures. Due to strong toxicity and persistency in the environments, studies on microbial reductive dehalogenation have largely been restricted to chlorinated and, to a lesser extent, brominated compounds. However, several observations suggest that microbial reductive deiodination also occurs in nature. Reductive deiodination of 2-, 3- and 4-iodobenzoates was observed in lake sediment under methanogenic conditions (6,7). Cupples et al. (8) reported ioxynil (3,5-diiodo-4-hydroxybenzonitrile), an iodinated herbicide, was deiodinated by *Desulfitobacterium chlororespirans*, although it was a cometabolic process. Lecouturier et al. (9,10) enriched a microbial consortium capable of reductively deiodinating 5-amino-2,4,6-triiodoisophthalic acid (ATIA), a precursor of a wide range of X-ray contrast agents. In the presence of ethanol as the electron donor, ATIA was deiodinated completely to 5-aminoisophthalic acid. Microbial community analysis revealed that bacteria closely related with *Desulfitobacterium* spp. were dominant in the enrichment. Recently, *Desulfoluna spongiiphila* was isolated from the marine sponge (11). This bacterium was able to dehalogenate bromophenols, 2- and 3-iodophenol, but not chlorinated and fluorinated phenols. Although these studies clearly indicate that reductive-deiodinating bacteria are actually present in nature, little information is still available on their phylogenetic diversity and substrate specificity.

In this study, we attempted to enrich microbial consortia capable of reductively deiodinating 2,4,6-triiodobenzene derivatives, i.e., 2,4,6-triiodophenol (2,4,6-TIP) and 3-amino-2,4,6-triiodobenzoic acid (ATBA). The former compound was chosen because its potential deiodinated intermediates such as 2,4-diiodophenol (2,4-DIP), 2-iodophenol (2-IP), 3-iodophenol (3-IP) and 4-iodophenol (4-IP) are all commercially available. Since 2,4,6-TIP is not very soluble in water, successive additions of this compound with low concentration (5 μM) were necessary for the enrichment. On the other hand, solubility of ATBA is relatively higher than 2,4,6-TIP due to its carboxylic and amino groups. Thus, ATBA was added at a high concentration (100 μM), although its potential deiodinated intermediates are not commercially available.

MATERIALS AND METHODS

Enrichment of 2,4,6-TIP and ATBA deiodinating cultures A strict anaerobic technique was used in the preparation of the minimal medium and manipulation of the enrichments (12). Sediments collected from iodine-producing industry, Chiba, Japan were used as an inoculum. The sediments were located in the bottom of a blowing-out tower, in which iodide ion (I^-) involved in brine water was mixed with oxidizing agent and sprayed toward land. Thus, the sediments have been exposed to molecular iodine (I_2), an oxidizing product of I^-. Since I_2 is known to easily react with aromatic ring of soil organic matter to form iodine-carbon covalent bond (13,14), it was expected that microorganisms capable of deiodinating the iodoaromatic compounds exist in the sediments. The sediments were diluted 10 times with sterile distilled water under N_2 atmosphere, and stored anaerobically at 4°C until use. The enrichment culture was prepared by inoculating 1 mL of the anaerobic slurry into 19 mL minimal medium. The medium contained the following (per liter): NH_4Cl (0.535 g), KH_2PO_4 (0.136 g), $MgCl_2 \cdot 6H_2O$ (0.204 g), $CaCl_2 \cdot 2H_2O$ (0.147 g), trace mineral element solution (1 mL), vitamin solution (1 mL), 1 g L^{-1} resazurin solution (1 mL), and $NaHCO_3$ (2.52 g). The medium was dispensed into 60-mL serum bottles under N_2:CO_2 (80:20) atmosphere. Bottles were sealed with thick butyl rubber stoppers and aluminum caps, and were sterilized by autoclaving at 121°C for 20 min. Lactate (final concentration of 7 mM), $Na_2S \cdot 9H_2O$ (1.5 mM), and cysteine-HCl (1.5 mM) were added separately from sterile anaerobic stock solutions. Initial and successive additions of 2,4,6-TIP (Wako Pure Chemical Industries, Osaka, Japan) as the electron acceptor were performed with a concentrated stock solution prepared in 0.1 N NaOH. The enrichment was incubated at 30°C in the dark without shaking. Subculture was prepared by transferring 1 mL of the enrichment to 19 mL fresh liquid medium. In some cases, ATBA (Alfa Aesar, Lancashire, UK), 2-, 3-, or 4-iodophenol (Wako Pure Chemical Industries) was used as the electron acceptor.

PCR-DGGE DNA was extracted from the enrichments using a FastDNA Spin kit (MP Biomedicals, Morgan Irvine, CA, USA) according to the manufacturer's instructions. PCR-DGGE was performed according to the method reported by Muyzer et al. (15) using the primers 341fGC and 534r. The PCR protocol used was as follows: initial denaturation at 94°C for 10 min; 30 cycles of (i) denaturation at 94°C for 15 s, (ii) annealing at 55°C for 45 s, and (iii) extension at 72°C for 30 s; and final extension at 72°C for 10 min. DGGE was performed using a DCode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA) as described previously (16). The major bands were excised and used for reamplification with the primers 341f and 534r. The sequences of the re-amplified DNA fragments were determined using a DNA sequencing kit (Big Dye Terminator V3.1, Applied Biosystems, Carlsbad, CA, USA) and a DNA sequencer (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems). When purity of the bands was not enough for sequencing, they were cloned into pCR4-TOPO plasmid vectors using a TOPO-TA Cloning kit (Invitrogen, Carlsbad, CA, USA), and transformed into One Shot TOP10 chemically competent *Escherichia coli* (Invitrogen) according to the manufacturer's instructions. Plasmid extraction from *E. coli* clones was performed with an illustra plasmidPrep Mini Spin kit (GE Healthcare, Little Chalfont, UK). The sequences were aligned to known sequences in the DNA Data Bank of Japan (DDBJ)/European Molecular Biology Laboratory (EMBL)/GenBank databases by using the Basic Local Alignment Search Tool (BLAST) program (http://www.ncbi.nlm.nih.gov/BLAST/).

Detection of *Dehalococcoides*-specific genes and putative dehalogenase genes *Dehalococcoides*-specific 16S rRNA genes were amplified with the primers Fp DHC 1 and 1386r as described previously (17). Putative reductive dehalogenase genes were amplified with the primers DHAR1000F and DHU1350R according to the PCR condition described by Rhee et al. (18).

Sequencing and phylogenetic analysis of 16S rRNA gene for the putative deiodinating bacterium Based on the result of BLAST search for the DGGE band corresponding to putative deiodinating bacterium, a primer 54f (5′-CATGCAAGTCGAAC-3′, *E. coli* positions 54–67) was designed. This primer consists of a region conserved in bacteria closely related with putative deiodinating bacterium, including anaerobic bacterium MO-CFX2 and *Anaerolinea thermophila*. A *Chloroflexi*-specific reverse primer, 1242r (5′-CCATTGTAGCGTG-3′, *E. coli* positions 1230–1242), was also designed based on a *Chloroflexi*-specific FISH probe (CFX1223) reported by Björnsson et al. (19). The 16S rRNA gene of the putative deiodinating bacterium was first amplified from the 2,4,6-TIP deiodinating enrichment culture by using primers 54f and 1242r. The obtained 16S rRNA gene sequences of putative deiodinating bacterium and of closely related bacteria were then aligned using the Clustal X program, version 2.0, and analyzed for regions containing conserved and variable sequences. Regions unique to putative deiodinating bacterium were identified, and 2 sets of primers (491f and 678r) specific for these regions were designed (see *Real-time PCR assays* in detail). The latter half of the 16S rRNA gene of the putative deiodinating bacterium was then amplified and sequenced by using 491f and a bacterial consensus primer 1491R (20). Finally, 1416 bp of 16S rRNA gene sequence was subjected to a BLAST search to determine sequence similarity, and the retrieved sequences were aligned using the Clustal X program. The phylogenetic tree was constructed using the neighbor-joining method (21). Bootstrap values were obtained for 1000 replicates to estimate the confidence of tree topologies.

Real-time PCR assays The following set of primers specific for putative deiodinating bacterium was designed: forward primer 491f (5′-CCGGAA TAAGTCTCGGCTAAC-3′; *E. coli* numbering, 491–511) and reverse primer 678r (5′-AATTCCACACGCCTCTACCTAC-3′; *E. coli* numbering, 657–678). The specificity of the primers was first verified using the ProbeMatch program at the Ribosomal Database Project (http://rdp.cme.msu.edu/) and the BLAST program. The primer specificity was also evaluated by cloning and sequencing of PCR products obtained from the 2,4,6-TIP deiodinating enrichments. DNA extracted from the enrichments was amplified using 491f and 678r. The PCR protocol used was as follows: initial denaturation at 94°C for 10 min; 30 cycles of (i) denaturation at 94°C for 15 s, (ii) annealing at 60°C for 45 s, and (iii) extension at 72°C for 30 s; and final extension at 72°C for 10 min. The PCR products were separated by electrophoresis through a 3% agarose gel and stained with ethidium bromide. The gel bands were excised and purified using the QIAquick PCR Purification kit (Qiagen, Hilden, Germany), and were cloned into pCR4-TOPO plasmid vectors as described above. For the 2,4,6-TIP deiodinating enrichments growing with or without 2,4,6-TIP, 10 each clone was randomly picked up.

Real-time PCR assays were performed using the Power SYBR Green PCR Master Mix (Applied Biosystems) and ABI PRISM 7000 system (Applied Biosystems). The reaction mixture (final volume, 20 μL) consisted of 10 μL of 2× Master Mix, 1 μL each of primer to give a final concentration of 0.1 pmoL μL^{-1}, and 1 μL of template. The cycling conditions were as follows: initial denaturation at 95°C for 30 s; 40 cycles of