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NOTE

Anaerobic 4-chlorophenol mineralization in an enriched culture under iron-reducing conditions

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We enriched an anaerobic, soil-free 4-chlorophenol (4-CP)-degrading culture under iron-reducing conditions. The [ring-^{14}C(U)]4-CP tracer experiment showed that 65 μM 4-CP mineralized to CO_2 and CH_4 through phenol, 4-hydroxybenzoate, and benzoate intermediates over 60 days. 16S rRNA gene analyses suggested the involvement of *Dehalobacterium* in the 4-CP dechlorination in the culture.

[**Key words:** Anaerobic mineralization; 4-Chlorophenol; 16S rRNA gene library analysis; ^{14}C-tracer experiment; *Dehalobacterium*]

Biological technologies have been developed to remediate soil and groundwater contaminated with highly toxic halogenated aromatic compounds (1). Reductive dehalogenation, the initial step of the degradation process, preferably occurs under anaerobic conditions utilizing H_2 as the electron donor. Previously, we proposed an anaerobic–anaerobic method that combines a reductive dechlorinator and an anaerobic oxidative degrader to achieve efficient mineralization of halogenated aromatic compounds, such as pentachlorophenol, under anaerobic conditions (2,3). However, the reductive dehalogenation rate was slower for relatively less halogenated compounds. The rate-limiting step for biodegradation is frequently terminated on di-/mono-halogenated compounds (4). Among the less halogenated phenols, chlorine at the *para* position is the most difficult to remove (4), including 4-chlorophenol (4-CP). To date, 4-CP anaerobic dissipation has been reported in methanogenic sludge/sediment-containing cultures (5–7) and bioreactors (8,9), as well as in sulfate-reducing (10,11) and iron-reducing cultures (12). However, in these studies, the end metabolites of 4-CP were not identified. Although some studies using [ring-^{14}C(U)]4-CP confirmed 4-CP mineralization to CO_2 and CH_4 in methanogenic sediment cultures (6,13), the specific microorganisms involved in the mineralization process were not identified. In order to develop an anaerobic biomineralization technique for less halogenated aromatic compounds, it is important to first understand the relationship between the degradation process and the responsible microbial population. In this study, we enriched an anaerobic soil-free culture with stable decomposition activity of 4-CP in the presence of amorphous FeOOH. The 4-CP mineralization process was monitored using a [ring-^{14}C(U)]4-CP tracer experiment, and the involved bacteria were characterized based on analysis of the 16S rRNA gene library. An iron-reducing condition was selected in this study to assess the suitability of this method for future applications. Fe (III) is abundant in water sediments and a variety of subsurface environments (12), and compared with other electron acceptors such as NO_3^- and SO_4^{2-}, Fe (III) has low toxicity and does not generate secondary contamination to the applied sites (12).

The culture was enriched from soil samples collected from an uncontaminated paddy field in Yatomi city (Aichi prefecture, Japan). The chemical properties of the soil have been described previously (3). A 100-mL volume of paddy soil slurry was acclimated to phenol by periodic spiking with 100 μM of phenol for more than one year, using a sealed 600-mL bottle with flushing of N_2 gas (>99.99% purity). The total amount of phenol added was approximately 240 μmol. After acclimatization, the soil culture stably degraded 100 μM of phenol within 10 days. Ten milliliters of the soil slurry was taken out and immediately introduced to a 60-mL serum bottle by using a sterilized syringe. The serum bottle contained 20 mL of sterilized anaerobic mineral medium and was sealed with a butyl rubber cap as described previously (14). Then, the bottle headspace was changed to a mixed gas of N_2:CO_2 (80:20), and each 0.2 mL of 100-times-diluted vitamin solution mix (14), 0.01 g/L yeast extract stock solution, and 2.5 mM titanium (III)-trinitriloacetic acid (Ti-NTA) solution (the reducing reagent) were added through sterilized 0.2-μm membrane filters. The aqueous solution of 4-CP and the amorphous FeOOH were further spiked to yield final concentrations of 65 μM and 5 mM, respectively. This FeOOH concentration (5 mM) greatly exceeds the amount required to confirm degradation activity. FeOOH solution was prepared by mixing iron (III) salt ($FeCl_3$) and a NaOH

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solution (2). The culture was incubated statically at 30°C in the dark. Once most of the 4-CP was degraded, 10% of the culture was transferred and subcultured repeatedly to remove the soil from the culture. Concentrations of halogenated phenols and phenol were determined with a gas chromatograph-mass spectrometer (QP5050A; Shimadzu, Kyoto, Japan) (3). The concentrations of benzoate and 4-hydroxybenzoate (OHB) were determined using a high-performance liquid chromatograph (CTO-10A; Shimadzu) equipped with an ultraviolet detector (UV 280 nm) and a Puresil C18 column (4.6 mm inner diameter, 250 mm length; Waters, Milford, MA, USA), with a mobile phase of CH_3CN:H_2O:CH_3COOH (65:34:1). The concentration of Fe^{2+} was analyzed using the ferrozine coloration method in an ultraviolet–visible spectrophotometer at 562 nm (2). All the measurements were recorded in triplicate. Metabolites of 4-CP were determined on the 17th culture transfer using [ring-^{14}C(U)]4-CP (specific activity of 52 mCi/mmol; Moravek Co., Brea, CA, USA), which was introduced as 0.5 mL of ethanol solution into a bottle containing 25 mL of medium to achieve an approximate final concentration of 2×10^4 dpm/mL. After 60-day incubation, the culture was acidified, and $^{14}CO_2$ and $^{14}CH_4$ in the headspace were determined as described previously (3). Then, the whole culture was extracted with a mixture of acetonitrile and ethylacetate (1:1), and the extracts were combined and concentrated by flushing with N_2 gas to 1 mL. Next, 10 μL of the concentrate was mixed with a cold standard mixture containing 4-CP, phenol, benzoate, and 4-OHB (5 mM each), and subjected to thin layer chromatographic analysis using a 20-cm-long silica gel plate (Silica Gel 70 Plate; Wako Pure Chemical Industries, Osaka, Japan) with a developing solvent of *n*-hexane-ethyl acetate (80:20). The retention factor values were determined by dividing the migration distances of the standards by the migration distance of the solvent in the plate, and were calculated as 0.25, 0.34, 0.33, and 0.37 for 4-CP, phenol, benzoate, and 4-OHB, respectively. The ^{14}C radioactivity values were determined with a liquid scintillation counter (LSC 5100; Aloka, Tokyo, Japan). DNA of the culture was extracted from the 17th transferred culture with a DNA extraction Isoplant kit (Nippon Gene Co., Tokyo, Japan). The extracted DNAs were amplified with a pair of bacterial consensus primers, 27f and 1492r (14), with the KOD FX Neo PCR kit (Toyobo Co. Ltd., Osaka, Japan) and cloned into One Shot TOP10 *E. coli* competent cells using cloning kits (PCR8/GW/TOPO; Invitrogen, Carlsbad, CA, USA). The methods for clone library analysis of 16S rRNA genes as well as phylogenetic tree construction have been described previously (3). The representative sequences obtained from the clones were deposited in the DNA Data Bank of Japan under accession numbers AB755779 to AB755789.

After four transfers, the soil was almost completely removed from the enriched culture, and the 4-CP degradation rate became stable. As shown in Fig. 1A, 65 μM of 4-CP was degraded within 60 days upon the 17th culture transfer, whereas the abiotic control (without culture inoculation) showed no 4-CP loss. Phenol was occasionally detected as an intermediate product at a relatively low concentration. Fe^{2+} production accompanied 4-CP decomposition and was regarded as the metabolite of FeOOH reduction. A total of approximately 0.68 mM Fe^{2+} was generated when 65 μM of 4-CP was completely degraded after 60 days (Fig. 1B). There was no degradation of 4-CP observed without the addition of FeOOH (data not shown), indicating that FeOOH is required to maintain 4-CP decomposition activity, and that the degradation occurred under iron-reducing conditions. The decomposition metabolites of ^{14}C-4-CP after 60 days of incubation are shown in Table 1. In addition to the 5.9% of ^{14}C-4-CP remaining in the culture, 25.1% and 37.2% of ^{14}C were recovered as $^{14}CO_2$ and $^{14}CH_4$, respectively, and 30.3% was converted to ^{14}C-aromatic compounds (^{14}C-phenol, ^{14}C-benzoate, and ^{14}C-4-OHB). The total recovery rate was 98.5%. In the abiotic

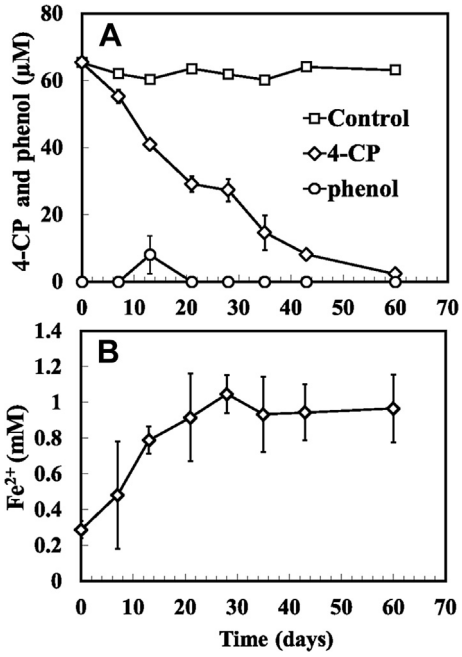

FIG. 1. 4-CP anaerobic degradation in an enriched FeOOH-reducing culture: (A) changes in concentrations of 4-CP and phenol; (B) changes in concentration of ferrous iron (Fe^{2+}). Data were obtained from triplicate cultures, and error bars represent standard deviations. The abiotic control (squares) represents 4-CP concentrations in abiotic samples without culture inoculation.

control culture, the major ^{14}C-compound recovered was ^{14}C-4-CP (95.5%). None of the metabolites was detectable in abiotic samples, except for negligible amounts of ^{14}C-phenol and ^{14}C-benzoate (1.9%). The results confirmed the anaerobic microbial mineralization of 4-CP.

The clones of nearly full-length 16S rRNA genes, as determined from the library, were classified into 11 operational taxonomic units (OTUs) in the phylogenetic tree (Fig. 2). Five of the OTUs were assigned to Firmicutes, three to Proteobacteria, two to Bacteroidetes, and one to Actinobacteria. OTU4 was the most abundant (23.9%), sharing 97% similarity with *Dehalobacterium formicoaceticum* strain DMC, which was reported as the organism responsible for the dechlorination of dichloromethane (15). The denaturing gradient gel electrophoresis analysis of 16S rRNA genes (Supplementary data Fig. S1 and Table S1) showed that *Dehalobacterium* was present only in the culture spiked with 4-CP, but not in those with phenol or with phenol but no 4-CP. This suggested the involvement of *Dehalobacterium* in the dechlorination of 4-CP in the culture. OTU11 (1.1%) was also identified as a possible dechlorinator, which is highly related to *Sulfurospirillum halorespirans* strain PCE-M2, an anaerobic halorespiring bacterium that dechlorinates tetrachloroethene to *cis*-dichloroethene (16). Two *Geobacter* spp. were found, OTU9 (11.4%) and OTU10 (6.8%); they are considered as possible degraders of aromatic rings. Their closest relatives are *Geobacter pelophilus* Dfr2, a dissimilatory iron-reducing bacterium (17), and *Geobacter metallireducens* strain GS-15, which degrades monoaromatic compounds such as phenol, benzoate, and 4-OHB by utilizing Fe (III) as the sole electron acceptor (18).

In summary, we successfully developed an anaerobic, 4-CP-mineralizing, soil-free culture under FeOOH-reducing conditions, and its mineralization ability was demonstrated using ^{14}C-4-CP tracer experiments (Fig. 1, Table 1). The method presented herein provides a potential approach for the complete removal of relatively less halogenated phenols by using a mixed culture with Fe (III) as an additional electron acceptor. Application of Fe (III) as the additional electron acceptor increases its *in situ* application