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Aerobic Mineralization of Hexachlorobenzene by Newly Isolated Pentachloronitrobenzene-Degrading *Nocardioides* sp. Strain PD653▽

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A novel aerobic pentachloronitrobenzene-degrading bacterium, *Nocardioides* sp. strain PD653, was isolated from an enrichment culture in a soil-charcoal perfusion system. The bacterium also degraded hexachlorobenzene, a highly recalcitrant environmental pollutant, accompanying the generation of chloride ions. Liberation of $^{14}CO_2$ from [U-ring-^{14}C]hexachlorobenzene was detected in a culture of the bacterium and indicates that strain PD653 is able to mineralize hexachlorobenzene under aerobic conditions. The metabolic pathway of hexachlorobenzene is initiated by oxidative dechlorination to produce pentachlorophenol. As further intermediate metabolites, tetrachlorohydroquinone and 2,6-dichlorohydroquinone have been detected. Strain PD653 is the first naturally occurring aerobic bacteria capable of mineralizing hexachlorobenzene.

Hexachlorobenzene (C_6Cl_6; HCB) is one of the most persistent environmental pollutants. Its average half-life in soil is approximately 9 years (2). When HCB is liberated in environment, it is bioaccumulated in plants, zooplankton, and shellfish. Finally, HCB is accumulated in the human body via the food chain, whereupon its possible toxicity adversely affects human health as a result of long-term exposure and accumulation. Therefore, HCB was listed as one of the 12 persistent organic pollutants in the Stockholm Convention.

A number of studies have been attempted to develop cleanup technology for environmental pollutants. Microbial degradation is a promising effective way to remediate environmental pollutants, including persistent organic pollutants. However, heavily chlorinated benzenes, especially HCB, are resistant to microbial degradation. Several studies have been reported on the reductive dechlorination of HCB. Reductive dechlorination of HCB to pentachlorobenzene by cytochrome P-450 was found in rat hepatic microsomes (22). Microbial transformation of HCB to trichlorobenzene and dichlorobenzene by reductive dechlorination was observed in anaerobic sewage sludge and a mixed culture (5, 7). Yeh and Pavlostathis maintained such an HCB-dechlorinating mixed culture for more than 1 year by adding surfactants as carbon sources (30). One of the microorganisms that reductively dechlorinates HCB is "*Dehalococcoides*" sp. strain CBDB1 (12). *Dehalococcoides* sp. strain CBDB1 dechlorinated HCB and pentachlorobenzene via dehalorespiration and gave a final end product mixture comprised of 1,3,5-trichlorobenzene, 1,3-dichlorobenzene, and 1,4-dichlorobenzene. These reductive dechlorinating processes take a longer time and leave less-chlorinated compounds such as trichlorobenzene and dichlorobenzene as end products.

Strictly aerobic, naturally occurring microorganisms that degrade and completely mineralize HCB have not been found. On the other hand, a microorganism capable of mineralizing pentachlorophenol (PCP), *Sphingobium chlorophenolicum* strain ATCC 39723, was isolated, and its gene organization involved in PCP metabolism was shown (4). Conversion of HCB to PCP was reported by using the genetically engineered mutant of cytochrome P-450_{cam} (CYP101) (13). Wild-type CYP101 from *Pseudomonas putida* had low degrading activity for dichlorobenzene and trichlorobenzene but did not decompose more highly chlorinated benzenes. The F87W/Y96F/V247L mutant showed improved di- and trichlorobenzene-degrading activity, but activity toward highly chlorinated benzenes including HCB was still low. The activity upon highly chlorinated benzenes was further improved in the mutant CYP101, F87W/Y96F/L244A/V247L (6). The rate of HCB degradation was increased 200-fold in the mutant. Yan et al. introduced the mutant CYP101 gene into *S. chlorophenolicum* strain ATCC 39723 by homologous recombination, to produce a complete HCB degrader (28). This genetically engineered bacterium degraded HCB almost completely within 12 h, together with formation of PCP as an intermediate. However, the application of genetically engineered microorganisms in natural areas is strictly restricted in many countries. HCB-degrading aerobes derived from natural sources are still required for remediation of HCB-contaminated areas.

We describe here isolation and identification of a novel aerobic soil bacterial species capable of aerobically mineralizing HCB. The characterization of metabolites caused by oxi-

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dative removal of the chlorine groups from HCB is also described.

MATERIALS AND METHODS

Chemicals and growth media. Pentachloronitrobenzene (quintozene, PCNB) and PCP were purchased from Wako Pure Chemical Industries, Osaka, Japan. HCB was from Dr. Ehrenstorfer GmbH, Augsburg, Germany. [U-ring-^{14}C]HCB (^{14}C-HCB) was purchased from Sigma-Aldrich Japan Co., Ltd., Tokyo, Japan. Tetrachlorohydroquinone and 2,6-dichlorohydroquinone standards were provided from H. Kiyota, Tohoku University, Miyagi, Japan. Pyridoxamine was from Sigma-Aldrich. Difco R2A agar medium was from Becton Dickinson and Company, Franklin Lakes, NJ. Other chemicals were from Wako. The mineral salt medium (MM) used in the present study contained 1.2 g of $Na_2HPO_4 \cdot 12H_2O$ and 0.5 g of KH_2PO_4 liter^{-1}. To prepare agar plates, 15 g of agar noble (Becton Dickinson) liter^{-1} was added to the medium. The medium was autoclaved and then supplemented with 20 mg of $MgSO_4 \cdot 7H_2O$ and 10 ml of a trace element solution liter^{-1}. The trace element solution was modified from the original description by Yanze-Kontchou and Gschwind (29) and contained EDTA (500 mg), $FeSO_4 \cdot 7H_2O$ (200 mg), $ZnSO_4 \cdot 7H_2O$ (10 mg), $MnSO_4 \cdot H_2O$ (5 mg), H_3BO_3 (30 mg), $CoSO_4 \cdot 7H_2O$ (24 mg), $CuSO_4 \cdot 5H_2O$ (5 mg), $NiSO_4 \cdot 7H_2O$ (5 mg), Na_2MoO_4 (5 mg), and $Ca(OH)_2$ (50 mg) in 1 liter. MM was supplemented with 1/1,000 volume of filter-sterilized acetone or methanol solution of PCNB after autoclaving, unless otherwise noted in the text. During cultivation, all of the agar plates were maintained in plastic bags in order to prevent desiccation.

Analytical methods. Concentration of chloride ions and nitrite ions was measured by ion chromatography (IC-2001; Tosoh Corp., Tokyo, Japan). A TSKgel Super IC-Anion column (Tosoh) and a TSKguardcolumn SuperIC-A (Tosoh) with a mobile phase of 6 mM $Na_2B_4O_7$, 15 mM H_3BO_3, and 0.2 mM $NaHCO_3$ were used at a 1.0-ml min^{-1} flow rate and 40°C. PCNB was detected with an electron capture detector-gas chromatograph (ECD-GC; HP 6890; Hewlett-Packard, Palo Alto, CA). A capillary type column with an injector of the splitless type (HP-50+ with 50% Ph Me silicone gum) with an inside diameter of 0.53 mm, a length of 15 m, and film thickness of 1 μm was used. The oven, injector port, and detector were maintained at temperatures of 180, 250, and 300°C, respectively. HCB concentration was monitored by high-pressure liquid chromatography (HPLC; Hewlett-Packard series 1100) equipped with a UV detector (set at 220 nm). A Wakosil II 5C18 RS column (250 by 4.6 mm [inner diameter]; Wako) with a mobile phase of 87% acetonitrile in 0.1% phosphoric acid aqueous solution was used at a 1.0-ml min^{-1} flow rate and 40°C. In the mineralization study of HCB, the presence of ^{14}C-HCB was monitored by HPLC (Shimadzu, Kyoto, Japan) equipped with a UV detector (set at 254 nm) and a radioactivity flowthrough detector (Raytest Isotopenmessgerate GmbH, Straubenhardt, Germany). An Inertsil ODS-3 column (250 by 4.6 mm [inner diameter]; GL Sciences, Tokyo, Japan) with a linear gradient of 70 to 87% acetonitrile in 0.1% phosphoric acid aqueous solution was used at a 1.0-ml min^{-1} flow rate and 40°C. Gas chromatography-mass spectrometry (GC-MS) was performed with an HP 6890 GC system-linked HP 5973 mass selective detector and 30-m fused DB-5MS column (J&W Scientific, Folsom, CA). The oven temperature was programmed to increase from 80 to 320°C at 20°C min^{-1}.

Enrichment culture. Enrichment of PCNB-degrading bacteria was performed by using the original soil-charcoal perfusion method (11, 21). An upland soil sample, to which PCNB had been annually applied for more than 5 years, was taken from a cabbage field (Ibaraki, Japan) at a depth of 0 to 20 cm. The soil sample (40 g [dry weight]) was mixed with autoclaved Charcoal A100 (2 g, grain size 5 to 10 mm, BET specific surface area of 100 m^2 g^{-1}, pH 7.8; Toyo Denka Kogyo, Kochi, Japan) as a microhabitat and adsorbent of PCNB. The soil-charcoal mixture was washed twice with sterilized water to remove chloride ions and then set in a perfusion apparatus (1). The surface of the soil-charcoal layer was covered with a glass microfiber filter. The first enrichment culture was carried out under dark conditions at 25°C by circulating 300 ml of MM containing 5 mg liter^{-1} PCNB. The medium was circulated with air lift using an air pump through the soil-charcoal layer in the perfusion apparatus. The perfusion rate of the medium was adequately controlled by the air pump, and smooth leaching was maintained. The medium was replaced periodically. Aliquots of culture fluids were centrifuged at 19,000 × *g* at an ambient temperature for 10 min. The concentration of chloride ions in the supernatant was measured by ion chromatography. To determine the PCNB concentration with ECD-GC, 5 ml of the supernatant was passed through a Sep-Pak C18 cartridge, which was preconditioned by washing with 5 ml of acetone, 5 ml of methanol, and 8 ml of distilled water, with a Waters vacuum manifold. The "concentrated" cartridge was then dried over the vacuum manifold for ~20 min and subsequently eluted with 5 ml of acetone. The eluate was dried and dissolved again in 1 ml of acetone in preparation for ECD-GC.

After PCNB degradation in the first enrichment culture, 0.25 g of the charcoal was transferred to another apparatus with 7.5 g of new autoclaved Charcoal A100. Further enrichment and purification were performed by circulating 300 ml of MM containing 10 mg of PCNB liter^{-1}.

Isolation of PCNB-degrading bacteria. The enriched charcoal (1 g) was crushed and suspended in 50 mM phosphate buffer (pH 7.0); the same buffer was added to the suspension to formulate a 10^{-5}-fold dilution. The diluted suspension was then inoculated on an MM agar plate containing 50 mg of PCNB liter^{-1}. After successive incubation at 25°C for 3 weeks, colonies showing clear zones on the plate were isolated and further purified on R2A agar plates at 30°C. Individual colonies grown on R2A agar plate were isolated into 12-ml sterile culture tubes (no. 2246-017N; AGC Techno Glass Co., Ltd., Chiba, Japan) containing 2.5 ml of MM supplemented with 10 mg of PCNB, 6.5 mg of sodium pyruvate, 5.2 mg of NH_4NO_3, and 1 ml of a mixture of vitamins (19) liter^{-1}. The tube cultures were shaken at 30°C and 120 rpm for 14 days. Degradation of PCNB was monitored by generation of chloride ions and nitrite ions in the supernatants of the culture fluids. The residual tube culture fluids were then transferred into 20-ml portions of the medium containing 5 mg of PCNB liter^{-1} and further cultured in 100-ml Erlenmeyer flasks shaken at 120 rpm with a rotary shaker at 30°C. After 5 days of cultivation, 5 mg of PCNB liter^{-1} was added to the culture for supplementing consumed PCNB.

Characterization of bacteria. In order to characterize the PCNB-degrading bacterium, morphological characterization of the cells grown on R2A agar for 72 h at 30°C, physiological characterization, and DNA G+C content using a DNA-GC kit (Yamasa, Chiba, Japan) were examined at TechnoSuruga Laboratory Co., Ltd. (Shizuoka, Japan). Scanning electron microscopy was performed with a JEOL JSM-5610LV scanning electron microscope (JEOL, Ltd., Tokyo, Japan) at 20 kV. Scanning electron microscopy samples were prepared by fixing colonies of the bacteria in a 1.0% glutaraldehyde solution with SEMpore (JEOL). The samples were lyophilized and coated with gold.

Bacterial total DNA was purified with DNeasy Tissue Kit (Qiagen, Valencia, CA). The 16S rRNA gene was amplified from 16.1 ng of the total DNA by PCR in a working volume of 50 μl using known primers, namely, fD1, fD2, and rD1 (25). The PCR was processed on a thermal cycler GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) according to the following program: 1 cycle of 10 min at 95°C; followed by 30 cycles of 30 s at 95°C, 30 s at 60°C, and 1 min at 72°C, followed by 1 cycle of 7 min at 72°C. The PCR products were purified with QIAquick PCR Purification Kit (Qiagen) and subjected to cycle sequencing with a Thermo Sequenase primer cycle sequencing kit (Amersham, NJ). The following 5′-Texas Red-labeled primers were used: fD1 (25), 341f (17), 534r (17), 907fT (5′-AAACTCAAATGAATTGACGG-3′), 907rA (5′-CCGTC AATTCATTTGAGTTT-3′), 1223r (5′-TTGTAGCACGTGTGTAGCCC-3′), and rP1 (25). The reaction products were analyzed on an SQ5500E DNA sequencer (Hitachi, Tokyo, Japan). The obtained 16S rRNA sequence was compared to the bacterial sequences in the GenBank database using the FASTA and BLAST search engines in GENETYX-PDB version 4.0 (Genetyx, Tokyo, Japan). The 16S rRNA sequence of the PCNB-degrading bacterium was aligned with the representative sequences of the related bacteria with GENETYX-WIN version 4.0 (Genetyx). Regions of unaligned nucleotides were manually removed. A phylogenetic dendrogram was constructed by the neighbor-joining method with CLUSTAL X and njplotWIN95 (23).

Degradation of HCB. HCB was dissolved in acetone and filter sterilized to make a stock solution of 500 mg liter^{-1}. An appropriate aliquot of the HCB stock solution was then added to sterilized 100-ml glass-stoppered Erlenmeyer flasks, and the solvent was evaporated in the ambient atmosphere. Removal of the solvent was monitored with an OMX-LR handheld odor meter (Shinyei, Kobe, Japan). Next, 20 ml of MM, formulated without EDTA but supplemented with 0.1 g of $(NH_4)_2SO_4$ liter^{-1}, was added to the flasks; HCB (corresponding to 7.2 μM) was suspended by sonication. The flasks were inoculated with a suspension of surface culture from the R2A agar plate of the PCNB-degrading bacteria. The flask cultures were shaken at 210 rpm with a rotary shaker at 30°C under dark conditions. Duplicate flasks were withdrawn periodically, and cell growth was monitored by measuring optical density at 600 nm (OD_{600}) with a DU800 spectrophotometer (Beckman Coulter, Fullerton, CA). Aliquots (1.0 ml) of culture fluid were centrifuged at 19,000 × *g* for 10 min. Concentration of chloride ions in the supernatant was determined by ion chromatography. The remaining culture fluid was moved to a 50-ml volumetric flask. HCB adhered to the inside wall of the flask was rinsed with 30 ml of methanol, and the rinsed methanol solution and remaining culture fluid were combined to make 50 ml of sample solution by adding methanol. The combined solution was centrifuged at 19,000 × *g* for 10 min. HCB in the supernatant was analyzed by HPLC.

Mineralization of HCB. The PCNB-degrading bacterium grown on a R2A agar plate was inoculated into 200-ml glass jars containing 20 ml of MM (EDTA free) supplemented with ^{14}C-HCB (corresponding to 3.6 μM) and 0.1 g of NH_4NO_3 liter^{-1}. The jars were stoppered with silicone sponge stoppers and incubated in a thermostatic shaker (30°C, 100 rpm). Duplicate jars were withdrawn periodically, and the radioactivity in the culture fluid was determined by liquid scintillation counting (LSC) with an Aloka LSC-5100 scintillation counter (Tokyo, Japan) using Atomlight scintillation cocktail (Perkin-Elmer, Kanagawa, Japan). The radioactive adhered materials were washed from the inside wall of jars with acetone, and also analyzed by LSC. The other jars were separately incubated in a thermostatic chamber with agitation by a magnetic stirrer (30°C, 50 rpm) with a continuous supply of filter-sterile air. The exhaust was passed through a flexible polyurethane foam (PUF) column and a pair of 1 M NaOH traps. The PUF column and NaOH solution were exchanged after 7 days. The radioactivity collected in NaOH solution ($^{14}CO_2$) and in acetone-eluate from PUF column was quantified by LSC to determine the ^{14}C-labeled material balance. The presence of ^{14}C-HCB in the culture fluid, the acetone washings, and the PUF-eluate was monitored by HPLC analysis. As a control experiment, duplicate jars were prepared in the same manner without inoculation of the bacterium and periodically analyzed.

Biotransformation experiment by using the resting cells. Cells of the PCNB-degrading bacterium were grown in 300 ml of R2A liquid medium in 500-ml flasks shaken at 120 rpm with a rotary shaker at 25°C, until OD_{600} of the culture reached ~1.2. After the cells were harvested by centrifugation at 3,000 × *g* and 4°C for 20 min, the cell pellets were washed twice with a 20 mM phosphate buffer (pH 7.0) and suspended in the same buffer to prepare resting cells. An aliquot of the HCB stock solution was added to sterilized 50-ml glass-stoppered Erlenmeyer flasks, and the solvent was evaporated in the ambient atmosphere. In order to study metabolic pathway with the resting cells, 10-ml portions of the cell suspension (OD_{600} = 1.0) were added to the flasks containing HCB (corresponding to 21.6 μM). A cell suspension that had been heat killed by autoclaving at 120°C for 20 min was used for the control experiment. The flasks were incubated for 0, 2, 4, 6, 12, 24, and 48 h in a rotary shaker (120 rpm) at 22°C. Triplicate flasks were withdrawn at each time point, and 1 ml of 1 N HCl was added to stop the reaction. A 0.5-ml aliquot of the incubation fluid was withdrawn before the addition of HCl. Chloride ions in the aliquot were detected by ion chromatography. HCB in the flask was extracted by adding 10 ml of CH_3CN, and the extract was centrifuged at 19,000 × *g* for 10 min. HCB in the supernatant was analyzed by HPLC. For the purpose of detection of chlorohydroquinones, a whole resting cell culture that was incubated with PCP (22.2 μM) for 2 h was acetylated by adding 3 ml of 1 M K_2CO_3 and 1 ml of acetanhydride for 10 min at the ambient temperature and was subsequently extracted by ethyl acetate. The acetylated sample was analyzed by GC-MS.

Nucleotide sequence accession number. The 16S rRNA gene sequence of *Nocardioides* sp. strain PD653 was deposited in GenBank under accession number DQ673618.

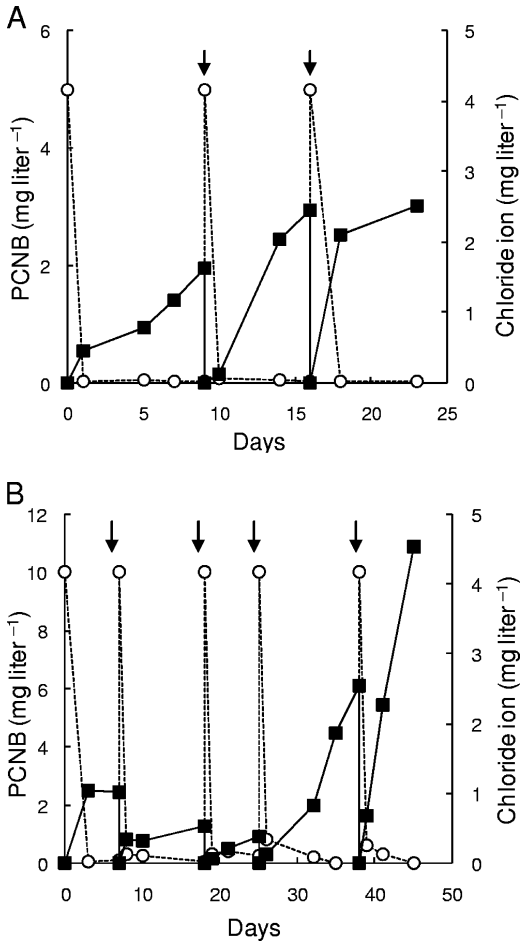

FIG. 1. (A) First enrichment culture of PCNB-degrading bacteria in Charcoal A100 using a soil-charcoal perfusion method. (B) Secondary enrichment culture using a charcoal perfusion method. Changes in concentration of PCNB (○) and chloride ions (■) in culture fluid are indicated. Down arrows indicate the time points of replacement of perfusion fluid. Concentrations of PCNB at the initial point and replacement points are calculated values of the added PCNB.

RESULTS

Enrichment and isolation of PCNB-degrading bacteria. At the beginning of enrichment, the generation of chloride ions in the perfusion apparatus was detected after 1 day of circulation. PCNB was not detected in the culture fluid. During 3 weeks of circulation and two exchanges of the medium, the generation rate of chloride ions increased (Fig. 1A). After 23 days of circulation, the charcoal was transferred to the secondary enrichment culture. The PCNB-degrading bacteria were highly enriched in the charcoal during the second enrichment. After 45 days of circulation and four exchanges of the medium, the enriched charcoal was harvested to carry out subsequent colony isolation (Fig. 1B).

Some bacterial colonies showing clear zones on MM agar containing 50 mg of PCNB liter^{-1} were isolated. Further colony purification of PCNB-degrading bacteria was performed on R2A agar. Several types of colonies having different morphology were observed on R2A agar. The PCNB-degrading abilities of the individual secondary isolates were examined in the tube cultures and subsequent flask cultures. An isolate that showed distinctive formation of chloride ions (6.6 mg liter^{-1}) and nitrite ions (1.7 mg liter^{-1}) from PCNB (total 10 mg liter^{-1}) after a 9-day cultivation in a flask culture was obtained and named strain PD653.

Morphological and physiological characterization of strain PD653. Strain PD653 was a gram-variable, non-spore-forming, nonmotile, aerobic coryneform bacterium (0.7 to 0.8 μm by 1.0 to 1.2 μm in size in rod-shaped cells). Colonies of strain PD653 on R2A agar were 1.0 mm in diameter, circular, convex, entirely margined, smooth, opaque, butterlike, and pale yellow in color. The strain also grew at 37°C. Cells of strain PD653 were positive for catalase and negative for oxidase activities. Acid and/or gas formation from glucose was negative. Acid was neither aerobically nor anaerobically produced from glucose (as determined by the oxidation-fermentation test). The DNA G+C content of strain PD653 was found to be 70.8 mol%.

16S rRNA analysis of strain PD653. The 16S rRNA sequence of strain PD653 (1,487 nucleotides) was compared to those of the bacterial sequences in the GenBank. Strain PD653

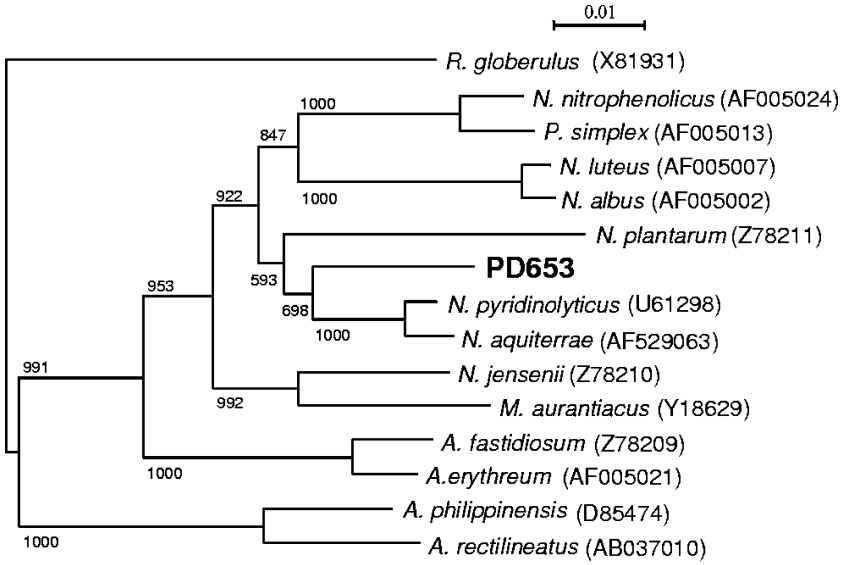

FIG. 2. Phylogenetic affiliation based on the 16S rRNA sequence data, showing the relationship of strain PD653 to the most closely related genera. The GenBank accession numbers are provided in parentheses. The dendrogram was generated by the neighbor-joining method. The numbers at the branch points are bootstrap values based on 1,000 trials; the bar indicates 10 nucleotide substitutions per 1,000 nucleotide positions. Species included in the analysis are *Actinoplanes philippinensis* (D85474), *Actinoplanes rectilineatus* (AB037010), *Aeromicrobium erythreum* (AF005021), *Aeromicrobium fastidiosum* (Z78209), *Marmoricola aurantiacus* (Y18629), *Nocardioides albus* (AF005002), *Nocardioides aquiterrae* (AF529063), *Nocardioides jensenii* (Z78210), *Nocardioides luteus* (AF005007), *Nocardioides nitrophenolicus* (AF005024), *Nocardioides plantarum* (Z78211), *Nocardioides pyridinolyticus* (U61298), *Pimelobacter* (*Nocardioides*) *simplex* (AF005013), and *Rhodococcus globerulus* (X81931) as an outlier.

exhibited a high sequence similarity to those of bacteria classified as *Nocardioides*. The highest sequence similarity (97.1%) of the 16S rRNA gene was found in *Nocardioides* sp. strain OS4 (GenBank accession no. U61298) (16). The 16S rRNA of strain PD653 was aligned with those of the representative strains of the *Actinomycetales*, and a phylogenetic dendrogram was constructed (Fig. 2). Based upon the polyphasic taxonomic data shown above, strain PD653 is a new species of the genus *Nocardioides* and designated *Nocardioides* sp. strain PD653. This strain was deposited at NIAS (National Institute of Agrobiological Sciences) GenBank, Ibaraki, Japan (MAFF 211737).

Degradation and mineralization of HCB by strain PD653. Upon aerobic culture of strain PD653 in MM containing HCB, the initial concentration of 8.0 μM HCB decreased to 1.5 μM during 9 days of cultivation, and an accumulation of chloride ions of up to 34.0 μM was observed (Fig. 3). An apparent increase of OD_{600} was not obtained after 9 days of cultivation (Fig. 3).

A study on mineralization of HCB was then performed using 3.6 μM ^{14}C-HCB (Fig. 4). The presence or absence of ^{14}C-HCB in the specimen was assigned by using HPLC equipped with a radioactivity flowthrough detector. In the 14-day culture fluid, the radioactivity decreased by 39.5% of its initial theoretical value. Radioactive HCB was not detected in the culture fluid, and the radioactivity was predominantly found in unknown water-soluble metabolites after 1 day of cultivation. The adsorbed ^{14}C-HCB residue onto the jar walls was only 2.2%. The PUF column captured 13.1% of the residue, which was ascertained as HCB volatilized during cultivation. The NaOH traps recovered 36.8%, and 84.7% of the trapped radioactivity was precipitated as $Ba^{14}CO_3$ by adding $BaCl_2$, indicating that the radioactivity was attributed to $^{14}CO_2$.

Oxidative dechlorination of HCB. In order to identify the intermediate of HCB catabolism, resting cells of strain PD653 were used in a degradation experiment. By incubating HCB with the resting cells, decreased HCB levels and increased levels of a metabolite were observed (Fig. 5). This metabolite was identified as PCP by its HPLC retention time and UV spectrum; they were found to be identical to those of an authentic sample. A stoichiometrically equal amount of chloride ions corresponding to the generated PCP was detected in the incubation fluid (data not shown). Although the only metabolite detectable by HPLC analysis was PCP, GC-MS analysis made it possible to detect other minor metabolites. To detect such minor metabolites, PCP was incubated with the culture of

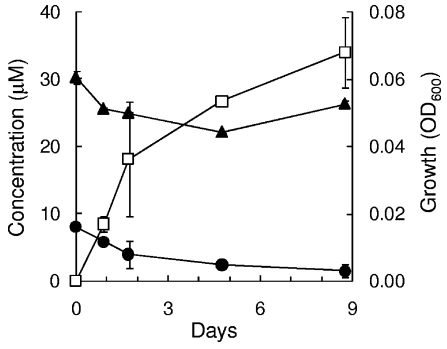

FIG. 3. Aerobic degradation of HCB by *Nocardioides* sp. strain PD653 in the MM. The changes in the OD_{600} of strain PD653 (▲) and evolution of chloride ions (□) in accordance with the HCB degradation (●) are demonstrated. OD_{600} and concentrations of the materials are mean values of the duplicate experiments. Error bars indicate standard deviations (SD).

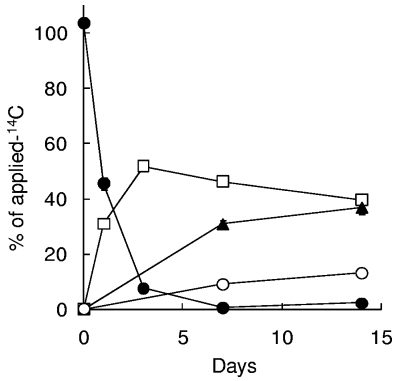

FIG. 4. Mineralization of ^{14}C-HCB by *Nocardioides* sp. strain PD653. Disappearance of ^{14}C-HCB (●) and evolution of ^{14}C-labeled unidentified water soluble metabolites (□) and $^{14}CO_2$ (▲) are demonstrated. Volatile ^{14}C-HCB (○) captured on the PUF column is also shown. Radioactivity of the materials is mean value of the duplicate experiments and is expressed as the percentage of that of the initial applied ^{14}C. Error bars indicate the SD.

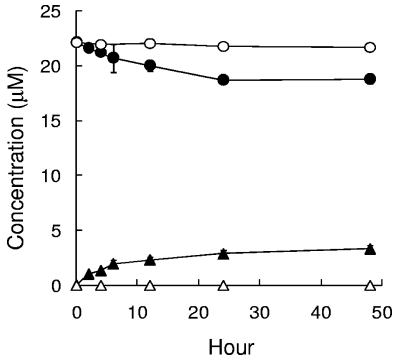

FIG. 5. Generation of PCP accompanied by degradation of HCB by resting cells of *Nocardioides* sp. strain PD653. The time courses of disappearance of HCB (●) and generation of PCP (▲) are demonstrated. The concentrations of HCB (○) and PCP (△) in heat-killed control cultures are also demonstrated. Mean values (n = 3) and the SD of concentrations of the materials are shown.

the resting cells for a short period (2 h). Although the decrease of PCP was very little, tetrachlorohydroquinone and 2,6-dichlorohydroquinone were detected as acetylated derivatives (Fig. 6). These intermediates were identified by the comparison of their GC retention times and MS spectra with authentic samples. Degradation of HCB and appearance of these metabolites were not observed in the control culture with autoclaved resting cells (Fig. 5).

DISCUSSION

Enrichment culture using a soil-charcoal perfusion system is an effective way to obtain microorganisms that degrade recalcitrant compounds (11, 21). However, the extremely low solubility of HCB in water (0.005 mg liter^{-1}) (14) makes it difficult to apply in this system. Consequently, PCNB (0.44 mg liter^{-1} soluble in water) (14) was adapted in the soil-charcoal perfusion system for enriching desired microorganisms. Thus, the PCNB-degrading bacterium was successfully enriched in Charcoal A100 (Fig. 1). Rapid disappearance of PCNB was observed at the beginning of circulation and after the perfusion medium was replaced. This rapid disappearance was supposed to be caused by adsorption of PCNB to the charcoal, so that enrichment of the degrading bacteria was indicated by the formation of chloride ions. The first isolate obtained from MM agar containing 50 mg of PCNB liter^{-1} was composed of a mixture of bacteria. The purified colony of the PCNB-degrading bacterium was obtained from the secondary isolation on the R2A agar plate. This isolate, strain PD653, is a novel species classified into the genus *Nocardioides* on the basis of its cell morphology and phylogenetic affiliation (Fig. 2). However, cells of PD653 are usually stained gram negative.

A decrease in HCB and increase in chloride ions produced by strain PD653 were observed in MM supplemented with HCB and $(NH_4)_2SO_4$ (Fig. 3). The release of at least five chloride ions per HCB molecule is estimated from the amount of the accumulated chloride ions. These results show that strain PD653 is presumed to aerobically mineralize HCB. Although the results were obtained from separate series of the jar experiments, this presumption is substantiated by the generation of $^{14}CO_2$ with degradation of ^{14}C-HCB (Fig. 4). However, this bacterium did not show apparent growth in MM containing HCB as a sole carbon source (Fig. 3). Strain PD653 is considered to require some nutritional factors for its growth. Growth occurred in MM supplemented with a small amount (0.5 g liter^{-1}) of Bacto tryptone (Becton Dickinson). PCNB-dependent increase of growth was observed in MM supplemented with 50 μg of *p*-amino benzoic acid and 8.6 mg of $(NH_4)_2SO_4$ liter^{-1} (data not shown). Although HCB is mineralized by strain PD653 in the medium used in the present study, it is still uncertain whether this strain utilizes HCB as a sole carbon source for its growth or not.

Most of the radioactivity in the medium is estimated to be attributable to unknown water-soluble metabolites (Fig. 4). Incorporation of radioactivity in the bacterial cells was negligible because growth of the strain in the medium was so small. The resting cell study revealed that PCP is the first metabolite in the HCB degradation by strain PD653 (Fig. 5). The degraded HCB (3.3 μM) was converted into a stoichiometrically equal amount of PCP and chloride ions after 48 h in the resting cell culture; therefore, strain PD653 is expected to be capable of removing chlorine groups via the oxidative pathway. Conversion of HCB to PCP was reported by using the genetically engineered mutant of CYP101 (6). However, there is no report of aerobic conversion of HCB to PCP by naturally occurring microorganisms. Aerobic microorganisms degrading chlorinated aromatic compounds via initial attack by nonheme iron dioxygenases to form *cis*-dihydrodiol are known. After rearomatization to chlorocatechols, catechol 1,2-dioxygenases cleave the aromatic ring (9, 10). Both of these dioxygenases appear to require certain ring positions to remain unsubstituted (3, 8). Aerobic degradation of aromatics through oxygenation by monooxygenase is also known. For example, alkene monooxygenase from *Xanthobacter* strain Py2 was able to catalyze aromatic monohydroxylation of benzene, toluene, and phenol (31). Ammonia monooxygenase from *Nitrosomonas europaea* also oxidized benzene, ethylbenzenes, halobenzenes, phenol, and nitrobenzene to produce phenolic compounds (15). Based on these previous reports and the data presented

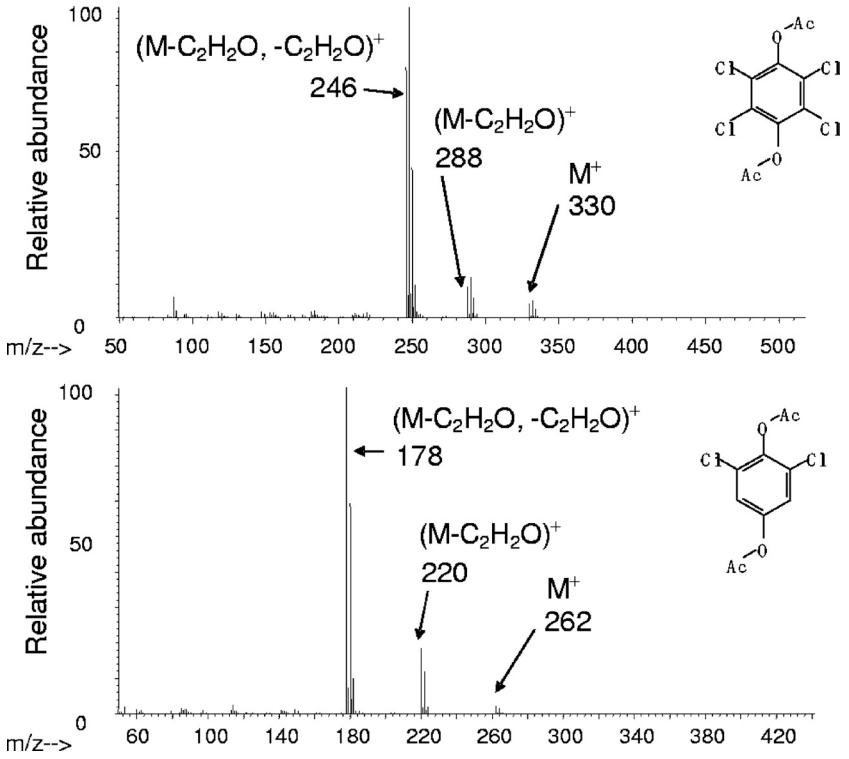

FIG. 6. GC-MS analysis of metabolites obtained from degradation of PCP by resting cells of *Nocardioides* sp. strain PD653. Acetylated derivatives of the metabolites were analyzed. The scanning was carried out at a mass range of 50 to 600 (*m*/*z*).

here, the possibility of involvement of the bacterial monooxygenase system in the conversion of HCB to PCP was suggested.

Tetrachlorohydroquinone and 2,6-dichlorohydroquinone were detected from the 2-h culture of PD653 resting cells with PCP (Fig. 6). Prior to obtaining this result, a trace amount of 2,6-dichlorohydroquinone was detected as an acetylated derivative by GC-MS analysis of the culture of the resting cells with HCB (data not shown). From these results, part of the HCB was presumed to be converted into 2,6-dichlorohydroquinone and tetrachlorohydroquinone via PCP. These metabolites have already been detected during PCP degradation by some microorganisms. A pathway for PCP degradation is known in detail for *S. chlorophenolicum* ATCC 39723 (18). PCP degradation in *S. chlorophenolicum* ATCC 39723 appears to be the *para*-hydroxylation of PCP to tetrachlorohydroquinone (26). This step is catalyzed by the enzyme PCP-4 monooxygenase and works in concert with a tetrachlorobenzoquinone reductase. However, a preliminary study on PCR detection using the primers pcpB-G and pcpB-D2 (24) indicated that the gene for PCP-4 monooxygenase (*pcpB*) was negative in strain PD653 but positive in *S. chlorophenolicum* JCM10275 (data not shown). The reactive chlorohydroquinone is then readily dehalogenated by a reductive mechanism. The reductive dehalogenation of tetrachlorohydroquinone to 2,6-dichlorohydroquinone via trichlorohydroquinone occurs by tetrachlorohydroquinone dehalogenase in the pathway for degradation of PCP (27). The putative metabolic pathway of HCB in strain PD653 is proposed in Fig. 7. HCB was initially converted to PCP, and then PCP presumed to be further metabolized through the conventional PCP degradation pathway by strain PD653. Since considerable amounts of water-soluble substances were detected in the mineralization study (Fig. 4) in contrast to small amounts of detected tetrachlorohydroquinone and 2,6-dichlorohydroquinone, the catabolic pathway of HCB degradation may be composed of complex catabolic reactions and the pathway proposed in Fig. 7 may be one of them.

Strain PD653 degraded PCNB as well as HCB, and liberated ~5 chloride ions and 1 nitrite ion per PCNB molecule. Reports of isolated aerobic microorganisms capable of degrading PCNB are rare (20), as are HCB-degrading bacteria. Detailed study on the degradation profiles of PCNB by strain PD653 is currently in progress and will be reported later.

In conclusion, a novel aerobic bacterium, *Nocardioides* sp. strain PD653, is the first aerobic bacterium capable of mineralizing HCB via PCP. This natural soil bacterium is potentially applicable to the bioremediation of contaminated sites con-

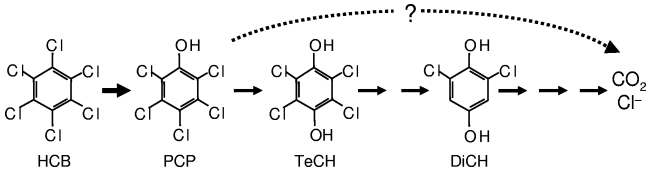

FIG. 7. Possible metabolic pathway of HCB by *Nocardioides* sp. strain PD653 under strict aerobic conditions according to the results of resting-cell treatment of spiked HCB and PCP. PCP, tetrachlorohydroquinone (TeCH), and dichlorohydroquinone (DiCH) are shown as intermediates of mineralization of HCB. All structures of the compounds were identified by GC-MS comparison with authentic compounds.

taining anthropogenic HCB. Contaminated sites with highly chlorinated aromatic compounds, such as PCP or PCNB, are also targets of the bioremediation using strain PD653.

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