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Gene cloning and in vivo characterization of a dibenzothiophene dioxygenase from *Xanthobacter polyaromaticivorans*

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Abstract *Xanthobacter polyaromaticivorans* sp. nov. 127W is a bacterial strain that is capable of degrading a wide range of cyclic aromatic compounds such as dibenzothiophene, biphenyl, naphthalene, anthracene, and phenanthrene even under extremely low oxygen [dissolved oxygen (DO)≤0.2 ppm] conditions (Hirano et al., Biosci Biotechnol Biochem 68:557–564, 2004). A major protein fraction carrying dibenzothiophene degradation activity was purified. Based on its partial amino acid sequences, *dbdCa* gene encoding alpha subunit terminal oxygenase (DbdCa) and its flanking region were cloned and sequenced. A phylogenetic analysis based on the amino acid sequence demonstrates that DbdCa is a member of a terminal oxygenase component of group IV ring-hydroxylating dioxygenases for biphenyls and monocyclic aromatic hydrocarbons, rather than group III dioxygenases for polycyclic aromatic hydrocarbons. Gene disruption in *dbdCa* abolished almost of the degradation activity against biphenyl, dibenzothiophene, and anthracene. The gene disruption also impaired degradation activity of the strain under extremely low oxygen conditions (DO≤0.2 ppm). These results indicate that Dbd from 127W represents a group IV dioxygenase that is functional even under extremely low oxygen conditions.

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Introduction

Contamination of polycyclic aromatic hydrocarbons (PAHs) such as naphthalene, anthracene, and phenanthrene and heterocyclic aromatic compounds (HACs) such as dibenzothiophene (DBT), dibenzofuran, and dibenzodioxin have been of great environmental concern because of their toxic and carcinogenic potentials (Menzie et al. 1992). It has been reported that bacterial strains that can degrade PAHs and HACs are widely distributed and their degradation activities contribute to the elimination of these compounds from contaminated sites (Kanaly and Harayama 2000). In order to promote bioremediation technology for PAH and HAC contamination, it is important to analyze and understand various degradation mechanisms of these bacteria.

Oxygen functions as an effective electron acceptor in the biological degradation pathways of extremely reduced and recalcitrant compounds including PAHs and HACs. Mono- and dioxygenases play a central part in the initial oxidative breakdown of these compounds and they require oxygen as an essential factor. One of the limiting factors for utilization of aerobic bacteria in on-site bioremediation technology is supplying enough oxygen to the contaminated site unless it is surface ground or surface water phase. Even such polluted sites soon become anoxic due to the high oxygen demand for bacterial metabolisms. Thus, high degradation activity under oxygen-depleted conditions is a preferable property for PAH- and HAC-degrading bacteria in practical use. Although several anaerobic bacteria have been reported for the degradation of mono-

cyclic aromatics, their growth rate and degradation ability are very low when compared with that of aerobic bacteria (Gibson and Harwood 2002). A PAH- and HAC-degrading bacterium, *Xanthobacter polyaromaticivorans* sp. nov. 127W, has been recently isolated from anoxic bottom sludge in a crude oil reservoir tank (Hirano et al. 2004). This strain is capable of degrading various two-ring and three-ring PAHs and HACs including DBT even under extremely low oxygen (ELO) conditions, where dissolved oxygen (DO) is less than or equal to 0.2 ppm (6 μM). Although *Pseudomonas stutzeri* T102, which is an effective PAH and HAC degrader, does not degrade them at all under ELO conditions, strain 127W degrades 82 μM (DBT), 55 μM (naphthalene), 64 μM (anthracene), and 20 μM (biphenyl) in 1 week at DO of 0.2 ppm. This excellent characteristic of strain 127W is expected to contribute to developing on-site bioremediation technology with less aeration costs.

In the present work, we adopted DBT as a substrate because strain 127W degraded it most effectively among PAHs and HACs tested and knowledge for the degradation of HACs is significantly less than for PAHs. We show that the alpha subunit terminal oxygenase component (DbdCa) of a dioxygenase is responsible for DBT degradation activities under both aerobic and ELO conditions in the strain 127W. Interestingly, DbdCa is a component of group IV dioxygenases for degradation of biphenyl and monocyclic aromatic hydrocarbons rather than group III enzymes for PAH degradation, suggesting its unique evolutionary lineage.

Materials and methods

Plasmids and bacterial strains *X. polyaromaticivorans* 127W and *P. stutzeri* T102 were previously isolated from anoxic sludge in oil reservoir tanks (Hirano et al. 2004). *Escherichia coli* JM109 [*rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17, *sup*E44, *rel*A1, delta(*lac-proAB*)/F[1]*traD36*, *proAB*[+], *lacI*[q], *lacZ delta-M15*] was used as a host strain for the gene cloning experiments. Cloning vectors pUC18, pT7Blue-T, and pSTV28 were purchased from Takara BIO Inc. (Shiga, Japan) and a broad host range vector, pBBR122, was purchased from Funakoshi Co., Ltd. (Tokyo, Japan).

Media Luria-Bertani (LB) medium was used for cultivation of *E. coli* JM109 and *X. polyaromaticivorans* 127W. LB agar containing kanamycin (5 mg/l) or ampicillin (50 mg/l) was used for selection of *dbdCa* gene disruptants and strains carrying plasmid, respectively. Carbon- and sulfur-free yeast extract (CSFY) medium contained (per liter) 4 g of K_2HPO_4, 4 g of Na_2HPO_4, 2 g of NH_4NO_3, 0.05 g of yeast extract, 0.01 g each of $MgCl_2{\cdot}6H_2O$, $CaCl_2{\cdot}2H_2O$, and $FeCl_2{\cdot}2H_2O$; the pH was adjusted to 7.0. CSFY medium supplemented with 0.1 mM DBT or a PAH (anthracene, biphenyl, or phenanthrene) was used for testing aromatic hydrocarbon degradation activity of the cells.

Preparation of cell lysate for enzyme purification Strain 127W was precultured in LB at 30°C and harvested by centrifugation at 10,000×*g* for 10 min at 4°C. Cells were washed once with water and inoculated to a new CSFY medium (final OD_{660}=5) containing 0.1 mM DBT under ELO condition. ELO condition was prepared by keeping the medium in an anaerobic chamber EAN-101 (Espec, Osaka, Japan) for longer than 12 h before inoculating the cells. DO concentration was confirmed to be lower than or equal to 0.2 ppm. Standard DO in air-saturated water was determined as 9.1 ppm at 20°C. After the cells were inoculated, the bottles were sealed with butyl rubber septa and aluminum crimps and shaken at 30°C for 3 days.

The cells were harvested by centrifugation, suspended in ice-cold 50 mM Tris–HCl buffer (pH7.5), and disrupted by sonication using a sonifier model 450 (Branson Ultrasonic Corp., Danbury, CT). Cell debris was removed by a centrifugation at 20,000×*g* for 30 min at 4°C. The resulting cell lysate was used for enzyme purification steps.

Enzymatic activity DBT degradation activity was measured by determining the amount of DBT decreased upon reaction using gas chromatography. Each degradation reaction was performed in the bottle securely sealed with butyl rubber septum and an aluminum crimp at 30°C under either aerobic (DO>7 ppm) or ELO condition unless otherwise denoted. The reaction mixture contained 50 mM 2-(cyclohexylamino)ethanesulfonic acid (CHES) buffer (pH 10), 1 mM of nicotinamide adenine dinucleotide (NADH), and an appropriate amount of the enzyme solution. The reaction product was acidified to pH 2.0 by addition of 6 M HCl and DBT was extracted with one volume of ethyl acetate containing 0.12 mM of fluorene or phenanthrene as an internal standard. In order to eliminate trace contamination of air and absorption of hydrocarbons to the butyl rubber septum, we neither used a needle to take samples from the bottle nor transferred samples to another bottle. Instead, the whole reaction mixture in the bottle was directly subjected to extraction from the original bottle at each reaction time. A portion of the ethyl acetate layer was analyzed by GC system HP6890, which was equipped with a nonpolar capillary column HP-1 (Hewlett Packard, Palo Alto, CA) and FID detector (GC/FID) or JEOL JMS-DX303 mass spectrometer (GC/MS, JEOL, Tokyo, Japan). Briefly, the operating condition was as follows: the oven temperature was linearly increased from 80 to 300°C at a rate of 10°C/min and an injection volume of the sample was 1 μl with a split ratio of 50:1. Temperature conditions for injector and detector were both set at 250°C. Every sample was prepared in triplicate together with a negative control containing no cell. One unit of enzyme activity is defined as the amount of enzyme that converts 1 μmol of substrate per minute.

Enzyme purification All purification steps were performed at 4°C with a Fast Protein Liquid Chromatography system (Amersham Biosciences, Buckinghamshire, UK). Protein concentration in each purification step was determined by the method of Bradford (protein assay kit, Bio-Rad,

Hercules, CA) with bovine serum albumin as a standard protein.

1. Precipitation by ammonium sulfate. The cell lysate was fractionated by ammonium sulfate precipitation at 30, 50, and 70% saturation. Then, precipitated proteins in each fractionation step were collected by centrifugation at 15,000 rpm for 30 min and dissolved in an appropriate volume of 50 mM Tris–HCl buffer (pH 7.5). This sample was subjected to dialysis overnight against the same buffer.
2. Anion-exchange column chromatography. An ammonium sulfate fraction (30% sup and 50% ppt) containing most of DBT degradation activity was applied to a HitrapQ column (Amersham Biosciences) that had been equilibrated with 50 mM Tris–HCl buffer (pH 7.5, buffer A). Proteins were then eluted with a linear gradient of 0 to 1 M NaCl in buffer A.
3. Hydrophobic column chromatography. Fractions that contained activity were pooled and supplemented with ammonium sulfate to a final concentration of 15%. The solution was applied to a Butyl-Sepharose column (Amersham Biosciences). The column had been equilibrated with 50 mM Tris–HCl buffer (pH 7.5) containing 15% ammonium sulfate (buffer B). Proteins were eluted by linearly decreasing ammonium sulfate concentration in buffer B.
4. Gel filtration column chromatography. Active fractions obtained by hydrophobic column chromatography were then loaded onto a Hiload 16/60 gel filtration column (Amersham Biosciences) equilibrated with 50 mM Tris–HCl buffer (pH 7.5) containing 100 mM NaCl and eluted with the same buffer. The purity of the fractions was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on a 15% polyacrylamide gel, followed by Coomassie brilliant blue R-250 staining.

N-terminal amino acid sequence analysis The protein band was transferred from SDS–polyacrylamide gel onto a polyvinylidene fluoride (PVDF) membrane by electroblotting method (Tovey and Baldo 1989). The N-terminal amino acid sequence of the protein or peptide was determined with a pulse-liquid automated sequencing system, Procise 491 (Perkin Elmer Inc., Wellesley, MA).

In-gel digestion The protein in SDS–polyacrylamide gel was digested by lysyl endopeptidase (LEP) according to Kawasaki and Suzuki (1990).

Reverse-phase high-performance liquid chromatography Reverse phase high-performance liquid chromatography (HPLC) was carried out using an HP1100 system (Hewlett Packard) and a COSMOSIL 5C18-AR column (4.6 by 150 mm, Nacalai Tesque Co., Ltd., Kyoto, Japan). This column was equilibrated with 10% acetonitrile containing 0.1% (v/v) trifluoroacetic acid (TFA), and peptides were eluted by linearly increasing acetonitrile from 10 to 100% in 30 min at a flow rate of 1 ml/min. Peptide peaks were monitored by absorbance at 220 nm. Peak fractions were collected and submitted for amino acid sequence analysis.

Gene manipulation General gene manipulation was performed by standard procedures (Sambrook and Russell 2001).

Cloning of the gene fragment encoding purified protein A part of the gene encoding the alpha subunit of terminal oxygenase (DbdCa, 48 kDa) was amplified from the chromosomal DNA of strain 127W by polymerase chain reaction (PCR). PCR was performed in a GeneAmp PCR system 2400 (Perkin Elmer) with Ex-Taq polymerase (Takara BIO Inc.) and a combination of forward (5′-ATGYTNGCNCCNCAYCAYG CNAARGG-3′) and reverse (5′-GCRTCRTCNARCATDATRTCCATRTA NGC-3′) primers whose sequences were designed from N-terminal and internal amino acid sequences of the protein. Abbreviations are Y, C or T; R, A or G; D, A or G or T; N, A or C or G or T. A DNA fragment of 555 bp in length was amplified and cloned with pT7Blue-T vector. In order to obtain the flanking region of the fragment, inverse PCR was performed with genomic DNA as a template after digestion with *Pst*I and self-ligation. The sequence of oligonucleotide primers used are 5′-GGACGAAAGTGCCC GCAACGTC-3′ and 5′-GATCTCGGGGCTGTAGCGTC CGG-3′, which are derived from the 555-bp PCR product.

DNA sequencing The nucleotide sequence of the DNA fragment was determined by the dideoxy nucleotide chain termination method with an ABI PRISM BigDye Terminator v3.0 cycle sequencing kit and an ABI PRISM 310 Genetic Analyzer (Perkin Elmer).

Phylogenetic analysis Database search was done by BLAST (blastp) program (http://www.ncbi.nlm.nih.gov/BLAST/, Altschul et al. 1990). Multiple sequence alignment was created by the neighbor-joining method in the ClustalW program (Thompson et al. 1994). A phylogenetic tree was constructed by using the TreeView program (Page 1996).

Transformation methods First, the sensitivity of strain 127W to various antibiotics was tested. The strain did not grow at 5 mg/l of ampicillin (Ap), kanamycin (Km), and chloramphenicol (Cm) and 20 mg/l of streptomycin. *E. coli* JM109 and *X. polyaromaticivorans* 127W were transformed with a plasmid by electroporation method using a Gene pulser II (Bio-Rad) under the following conditions: 25 kV/cm electric pulse, 200 Ω resistance, 25 μF capacitance, with a 0.1-cm electrode distance cuvette. Competent cells for electroporation were prepared by standard protocols (Calvin and Hanawalt 1988; Sambrook and Russell 2001). One milliliter of prewarmed SOC medium was immediately added to the cuvette after applying a pulse. The cell suspension was transferred to a test tube and incubated at 30°C for 6 h (127W) or 37°C for 2 h (JM109) and then spread on an LB agar plate containing an appropriate amount of the antibiotic for the selection of

transformants. It was found that a broad host range vector for Gram-negative bacteria, pBBR122 (Km^r, Cm^r), was useful for the transformation of strain 127W. The transformation efficiency was higher than 10^5 cfu/μg DNA. When pUC18 (Ap^r) was used for transformation, no transformant was obtained, suggesting that pUC vector does not replicate in strain 127W.

Construction of a dbdCa gene disruptant The gene encoding alpha and beta subunits of a terminal dioxygenase (*dbdCab*) was amplified by PCR using KOD polymerase (Toyobo, Osaka, Japan) and primers dbdC-F (5′-CATACG CCAAGCGGCAGGTAAATAC-3′) and dbdC-R (5′-CAT GGCATTAGGGCACGTCTCC-3′). The amplified 2-kb DNA fragment was ligated to pUC18 at the *Sma*I site. The resultant plasmid was designated as pUC-*dbdCab*. The pUC18 vector did not replicate in strain 127W and was useful for gene disruption experiment. As the 2 kb of *dbdCab* fragment had one *Nde*I site in *dbdCa*, we planned to insert the kanamycin-resistant gene cartridge into this *Nde*I site to construct a gene disruption plasmid. However, pUC18 has another *Nde*I site. Then, the *dbdCab* fragment was previously transferred to another cloning vector, pSTV28, by utilizing *Kpn*I and *Pst*I sites. We designated this plasmid pSTV-*dbdCab*. A kanamycin-resistant gene cartridge (*kan*) was prepared from pBBR122 including its original promoter by PCR with forward (5′-GATGCGT GATCATATGCTTC AACTC-3′) and reverse (5′-GCCAC GTTGTCATATGAAATCTCT) primers, where underlines represent *Nde*I sites. The PCR-amplified *kan* was inserted into the *Nde*I site of pSTV-*dbdCab*, yielding pSTV-*dbdCab::kan*. Finally, the *dbdCab::kan* fragment was transferred to pUC18 using *Kpn*I and *Pst*I sites, yielding pUC-*dbdCab::kan*. pUC-*dbdCab::kan* was introduced into strain 127W by electroporation method and kanamycin-resistant colonies (Km^r) were obtained. Construction of the *dbdCa* gene disruptant (delta-*dbdCa*) was confirmed by the size of the PCR fragment using a set of primers, dbdC-F and dbdC-R.

Assay for DBT and PAH degradation activity of strains 127W and delta-dbdCa Cells grown in LB broth for 3 days were collected, washed, and suspended with an appropriate volume of CSFY medium. An aliquot of the cell suspension was inoculated at a final OD_{660} of 5 to 10 ml of CSFY medium containing 0.1 mM substrate hydrocarbon. DO in CSFY and substrate hydrocarbons had been previously adjusted by placing for an appropriate time in an anaerobic chamber EAN-101 (Espec). The bottles were tightly sealed with butyl rubber septa with aluminum crimps and shaken at 30°C for the indicated time. The remaining DBT in the culture was quantified by ethyl acetate extraction followed by GC/FID analysis as described earlier. In order to eliminate errors caused by volatilization and absorption of substrate, a negative control experiment without bacterial cells was prepared for all experimental conditions. Each data point was an average value of three independent reactions.

Nucleotide accession number The nucleotide sequences of the *dbd* genes were deposited under the accession number AB121977.

Results

Purification of DBT-degrading activity An enzyme carrying DBT-degradation activity was purified from a 30-l culture of strain 127W (16.4 g wet weight of cells) to homogeneity by the procedure described in "Materials and methods" (Table 1). The active fraction was eluted at 170 to 330 mM NaCl condition by HitrapQ column chromatography and then at 14–10% ammonium sulfate condition by Butyl-Sepharose column chromatography. Finally, the active fraction was eluted as a single peak at the position of molecular mass over 670 kDa (Thyloglobulin) by Hiload 16/60 gel filtration column chromatography. When this fraction was analyzed by SDS-PAGE, one major protein band with a molecular mass of 48 kDa and two minor proteins with molecular masses of 40 and 23 kDa were detected (Fig. 1). The amount of these proteins purified from 30 l culture was 0.05 mg in total and was not enough for enzymatic studies.

Gene cloning of the DBT degrading enzyme The bands containing 48-, 40-, and 23-kDa proteins were excised

Table 1 Purification of the DBT-degrading activity

Steps	Volume (ml)	Total activity (unit)	Amount of total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Ammonium sulfate precipitation	215	20710	1060	19.5	100	1
Anion exchange column chromatography	11	409	55	7.4	2.0	0.4
Hydrophobic column chromatography	11	138.2	0.5	265.7	0.7	13.6
Gel filtration column chromatography	1.5	20.3	0.05	406.0	0.1	20.8

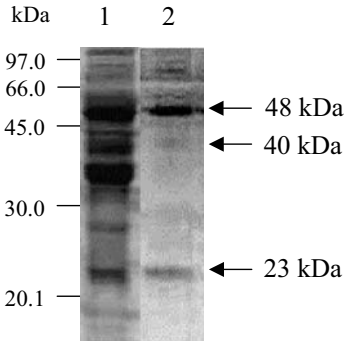

Fig. 1 SDS-PAGE analysis of DBT-degrading enzyme. Samples were subjected to electrophoresis on a 15% polyacrylamide gel in the presence of SDS. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250. *Lane 1*, after ammonium precipitation; *lane 2*, after gel filtration column chromatography. *Arrows* indicate 48, 40, and 23-kDa proteins whose amino acid sequences were analyzed

after blotting onto the PVDF membrane and the amino acid sequences were analyzed. N-terminal amino acid sequences of 48- and 23-kDa proteins were determined to be M/TLAPHT/HP/AS/KG/I (sequence A) and LDTAQ/SQA/SAFQV (sequence B), respectively. It was found that no protein in a nonredundant database (SWISS-PROT/NBRF-PIR) showed remarkable similarity to these sequences. Then, in order to determine the internal amino acid sequence of 48-kDa protein, in-gel digestion was performed using LEP and the resulting peptides were purified by reverse-phase HPLC. One of the major peptides eluted at 53% acetonitrile/0.1% TFA had an N-terminal amino acid sequence of A/FYMDIMLDD/RAE (sequence C). Based on the amino acid sequences A and C, a set of oligonucleotide primers for PCR was synthesized to obtain a part of the gene encoding 48-kDa protein. A single 555-bp DNA fragment was amplified by the PCR. This DNA fragment was cloned into pT7Blue-T vector for the nucleotide sequencing. A deduced amino acid sequence showed significant similarity to the terminal oxygenase component of aromatic ring-hydroxylating dioxygenases. Most aromatic ring-hydroxylating dioxygenases consist of four components, a ferredoxin reductase, a ferredoxin, and a terminal oxygenase composed of alpha and beta subunits, and all these components are essential for the enzyme activity (Habe and Omori 2003). Inverse PCR was performed to clone the complete gene encoding a putative alpha subunit protein and its flanking region. A 6.4-kb DNA fragment was obtained and the nucleotide sequence was determined. The fragment contained seven unidirectional open reading frames (ORFs) exhibiting significant similarity to proteins related to a degradation pathway of aromatic hydrocarbons (Fig. 2). Sequence homologies with other known proteins allowed the assignment of putative protein functions to each of the ORFs. The *dbdA* would encode a ferredoxin [109 amino acids (aa), 11,751 Da]. *dbdB* gene encodes a putative gentisate 1,2-dioxygenase (350 aa, 39,932 Da), *dbdCa* encodes the alpha subunit of terminal oxygenase (433 aa, 48, 286 Da), *dbdCb* encodes beta subunit of terminal oxygenase (187 aa, 22, 157 Da), *dbdCc* encodes another ferredoxin (110 aa, 12,114 Da), *dbdD* encodes dihydrodiol dehydrogenase (275 aa, 28,931 Da), and *dbdE* encodes a partial salicylaldehyde dehydrogenase (390 aa) with no termination codon. Organization of *dbdC* was similar to that for typical biphenyl dioxygenase (*AJ251217*, *D32142*, *M83673*) and benzene/toluene dioxygenase systems (*AF148496*, *AF006691*, *J04996*) from *Pseudomonas* and *Rhodococcus* with the exception of a missing ferredoxin reductase (putative *dbdCd*) between ferredoxin (*dbdCc*) and dihydrodiol dehydrogenase (*dbdD*). Another characteristic of the gene cluster was that it contained the genes encoding enzymes for

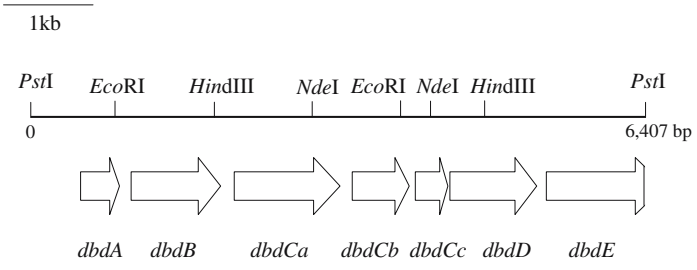

Fig. 2 Organization of the *dbd* genes in *X. polyaromaticivorans* strain 127W. *Open arrows* indicate the size and the direction of open reading frames corresponding to *dbdABCabcDE*. Results from the BLAST (blastp) search (http://www.ncbi.nlm.nih.gov/BLAST/, Altschul et al. 1990) are summarized in the table

gene name	highest homology of the a.a. sequence to	origin	homology
dbdA	ferredoxin	*Rhodopseudomonas palustris*	46.2 %
dbdB	gentisate 1,2-dioxygenase	*Pseudomonas alcaligenes*	57 %
dbdCa	alpha subunit of toluene dioxygenase	*P. putida* F1	49.3 %
dbdCb	beta subunit of chlorobenzene dioxygenase	*Ralstonia* sp. JS705	43.3 %
dbdCc	ferredoxin	*Pseudomonas sp.* P51	49.5 %
dbdD	*cis*-chlorobenzene dihydrodiol dehydrogenase	*Ralstonia* sp. JS705	47.6%
dbdE	salicylaldehyde dehydrogenase	*Burkholderia* sp. RP007	47.8%

possible lower degradation pathways, DbdB and DbdE, that would constitute a gentisate pathway (Crawford 1976).

The molecular mass of the alpha subunit (DbdCa) of terminal oxygenase was calculated to be 48.3 kDa. This value was consistent with that of the major purified enzyme estimated by SDS-PAGE (48 kDa). Moreover, the deduced N-terminal amino acid sequence of DbdCa, MTLAPHTPSG, was identical to that of the purified 48-kDa protein except for the first residue. The purified protein lacked the first methionine. Like other iron–sulfur proteins, DbdCa contained the two conserved cysteines and histidines at the N-terminal region (from position 94 to 117) as part of a motif, $CXHX_{17}CX_2H$, which constitutes a Rieske-type (2Fe-2S) cluster (Kauppi et al. 1998). The deduced N-terminal amino acid sequence of DbdCb (22.2 kDa), MLDTASQSAFQV, was also identical to that of 23-kDa protein except for the first residue. These results suggest that DbdCa and DbdCb constitute a terminal oxygenase responsible for ring hydroxylation of DBT.

Phylogenetic analysis The alpha subunit of the terminal oxygenase component has been shown to be critical for determining the substrate specificity of the ring-hydroxylating dioxygenase system (Zielinski et al. 2002). Werlen et al. proposed to classify ring-hydroxylating dioxygenases into four families based on the phylogenetic analysis of the alpha subunit of the terminal oxygenase component (Werlen et al. 1996). Recently, Nam et al.

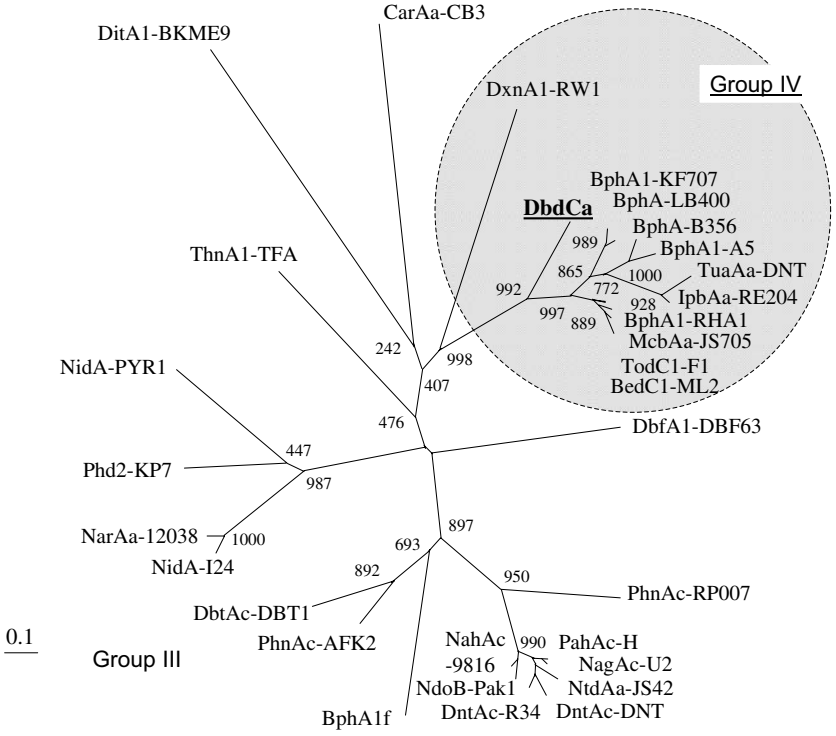

Fig. 3 A phylogenetic tree of alpha subunit oxygenase components for ring-hydroxylating dioxygenases. The tree was constructed for DbdCa and several orthologous representatives of groups III and IV by using CLUSTAL W and TreeView. The confidence levels of the branch were determined by bootstrap analysis. Bootstrap values are shown at the *internal nodes* for neighbor joining (1,000 replications). Typical group IV enzymes are shown in a *shaded circle*. Although DitA1-BKME9 and CarAa-CB3 are previously classified to groups III and IV, respectively, the tree suggests their close lineage (Nam et al. 2001). The protein abbreviations, substrate, origin, and GenBank accession numbers are as follows: BphA1-KF707, biphenyl, *P. pseudoalcaligenes* KF707 (M83673); BphA-B356, biphenyl, *Comamonas testosteroni* B356 (U47637); BphA-LB400, biphenyl, *Burkholderia cepacia* LB400 (M86348); BphA1-A5, biphenyl, *Ralstonia eutropha* A5 (X97923); TuaAa-DNT, toluene, *Thauera* sp. DNT (AB066264); IpbAa-RE204, isopropylbenzene, *P. putida* RE204 (AF006691); BphA1-RHA1, biphenyl, *Rhodococcus* sp. RHA1 (D32142); McbAa-JS705, chlorobenzene, *Ralstonia* JS705 (AJ006307); TodC1-F1, toluene, *P. putida* F1 (J04996); BedC1-ML2, benzene, *P. putida* ML2 (AF148496); DbfA1-DBF63, dibenzofuran, *Terrabacter* sp. DBF63 (AB054975); PhnAc-RP007, phenanthrene, *Burkholderia* RP007 (AF061751); PahAc-H, PAH, *C. testosteroni* strain H (AF252550); NagAc-U2, naphthalene, *Ralstonia* U2 (AF036940); NtdAa-JS42, 2-nitrotoluene, *Pseudomonas* JS42 (U49504); DntAc-DNT, 2,4-dinitrotoluene, *Burkholderia* strain DNT (U62430); DntAc-R34, 2,4-dinitrotoluene, *B. cepacia* R34 (AF169302); NahAc-9816, naphthalene, *P. putida* NCIB9816-4 (AF491307); BphA1f, naphthalene, *Novosphingobium aromaticivorans* (NC_002033); PhnAc-AFK2, phenanthrene, *Alcaligenes faecalis* AFK2 (AB024945); DbtAc-DBT1, dibenzothiophene *Burkholderia* DBT1 (AAK62353); NidA-I24, indene, *Rhodococcus* sp. I24 (AF121905); NarAa-12038, naphthalene, *Rhodococcus* sp. NCIMB 12038 (AF082663); Phd2-KP7, phenanthrene, *Nocardioides* sp. KP7 (AB017794); NdoB-Pak1, naphthalene, *P. aeruginosa* Pak1 (D84146); NidA-PYR1, naphthalene, *Mycobacterium vanbaalenii* PYR-1 (AF249301); ThnA1-TFA, tetraline, *Sphingopyxis macrogoltabida* TFA (AF157565); DitA1-BKME9, diterpenoid, *P. abietaniphila* BKME-9 (AF119621); CarAa-CB3, carbazole, *Sphingomonas* CB3 (AF060489); DxnA1-RW1, dioxin, *Sphingomonas* sp. RW1 (X72850) (DxnA1-RW1 might belong to group III)

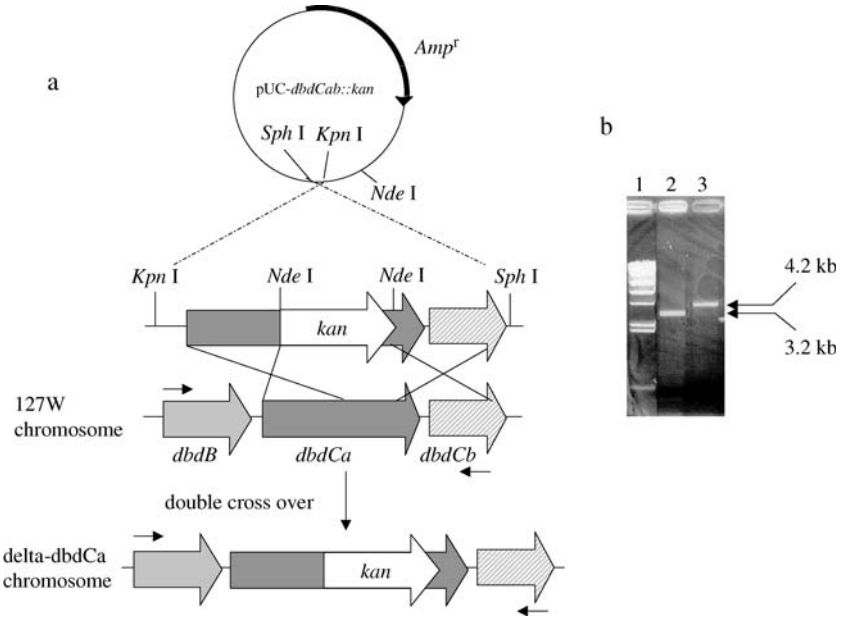

Fig. 4 The gene disruption of *dbdCa* in *X. polyaromaticivorans strain* 127W. **a** Strategy for gene disruption. Disruption of the *dbdCa* gene occurred by a double crossover event between the chromosomal *dbdCab* genes and the pUC-*dbdCab::kan* containing *dbdCab* interrupted by *kan* from pBBR122. *Small arrows* indicate primers used for confirmation of gene disruption. **b** Confirmation of the insertion of *kan* in *dbdCab* by PCR followed by agarose gel electrophoresis. *Lane 1*, *Hin*dIII size marker; *lane 2*, PCR products obtained by using 127W chromosomal DNA as a template; *lane 3*, delta-*dbdCa* chromosomal DNA was used as a template

(2001) advanced this phylogenetic classification of dioxygenases. Group I enzymes consist of broad-range dioxygenases sharing low similarity. Groups II, III, and IV contain benzoate/toluate dioxygenases, PAH dioxygenases, and benzene/toluene/biphenyl dioxygenases, respectively. Although strain 127W predominantly degraded DBT, naphthalene, and anthracene, together with biphenyl, DbdCa showed less sequence similarity to the alpha subunit of terminal oxygenases for PAHs (group III) than that for benzene/toluene/biphenyl (group IV). The amino acid sequence of DbdCa showed the highest identity of 49.4 and 48.2% to the alpha subunit terminal oxygenase of toluene and the benzene dioxygenase (group IV) from *P. putida* strains F1 (J04996) and ML2 (AF148496), respectively. On the other hand, it showed only 15 and 14% identity to the alpha subunit oxygenase of classical group III naphthalene dioxygenase from *P. putida* NCIB9816-4 (AF491307) and *P. stutzeri* AN10 (AF039533), respectively. Phylogenetic analysis for DbdCa showed that it belongs to group IV but formed a deep branch (Fig. 3).

Characterization of the gene disruptant, delta-dbdCa

In order to confirm that the *dbd* gene cluster is functional in the PAH and HAC degradation by strain 127W, the *dbdCa* gene encoding a putative terminal oxygenase alpha subunit was disrupted on the chromosome by homologous recombination technique (Fig. 4a). Replacement of *dbdCab* gene with *dbdCab::kan* gene was confirmed by PCR by using appropriate primers. The size of PCR amplified fragment from the genomic DNA of delta-*dbdCa* strain was 4.2 kb, which was larger than that from strain 127W by 1.0 kb (*kan*), suggesting that a genetic recom-

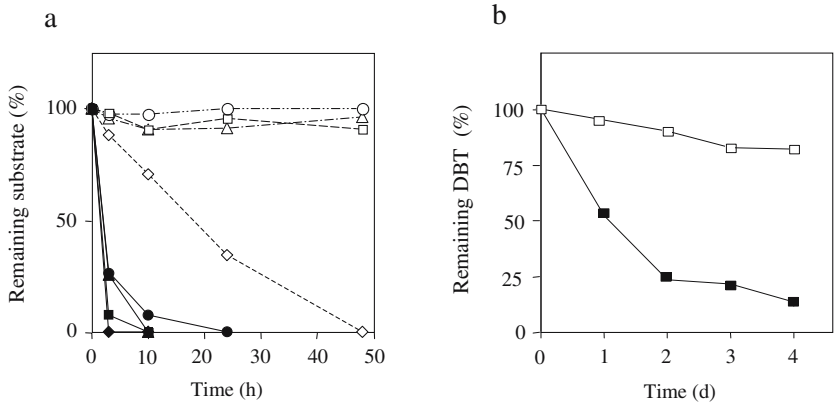

Fig. 5 Comparison of PAH degradation activities of strain127W and delta-*dbdCa*. Data are shown for strain 127W by *closed symbols* and for delta-*dbdCa* by *open symbols*. The *data points* are averages of triplicate experiments. **a** Degradation activity under aerobic condition (DO>7 ppm) was measured by using 0.1 mM of DBT (*square*), biphenyl (*circle*), phenanthrene (*diamond*), or anthracene (*triangle*) as a substrate. **b** Experiments were performed under an ELO (DO≤0.2 ppm) condition by using DBT. All the data are the difference values between the experimental flask and negative control flask with no inoculation of cells. No decrease in amount of DBT was also confirmed in the flask containing inactivated cells

bination occurred by a double crossover event as expected (Fig. 4b).

The gene disruption of *dbdCa* in strain 127W showed no significant effect on the cell growth in LB broth. Degradation activity for PAHs and HACs was compared between 127W and delta-*dbdCa* strains. Under aerobic conditions, delta-*dbdCa* lost almost of the degradation activities for PAH (anthracene), HAC (DBT), and biphenyl (Fig. 5a), whereas when phenanthrene was used as a substrate, low but significant degradation activity was observed in delta-*dbdCa*. It was previously shown that the strain 127W was capable of degrading PAHs and HACs under ELO conditions. Then, the degradation activity of delta-*dbdCa* for DBT was tested under an ELO condition. It revealed that strain 127W degraded 75% of DBT in 2 days but delta-*dbdCa* degraded only 23% of DBT in 3 days (Fig. 5b). These results indicate that the gene cluster encodes DBT dioxygenase system that functions under both aerobic and ELO conditions.

Discussion

Structure of dbd genes We have purified DBT dioxygenase in this experiment. DBT dioxygenase, which is expected to consist of four components, showed only three protein bands at 48 (DbdCa), 40, and 23 kDa (DbdCb) (Fig. 1). Assumed from its size, the 40-kDa protein could be a ferredoxin reductase. The amount of the fourth component, ferredoxin (DbdCc, 10,956 Da), was probably too low to be detected by SDS-PAGE followed by Coomassie brilliant blue staining.

The gene encoding ferredoxin reductase was unusually not found in the 6.4-kb DNA region we obtained this time. It has been demonstrated in the *Ralstonia* sp. strain U2 that a terminal oxygenase system whose operon did not contain ferredoxin reductase gene shared a ferredoxin reductase component with salicylate 5-hydroxylase system (Zhou et al. 2002). Salicylate 5-hydroxylase catalyzes the hydroxylation of salicylate to gentisate in a lower pathway of PAH degradation. A similar ferredoxin reductase may function in both DbdC and lower gentisate pathways because the *dbdC* gene cluster is adjoined to gentisate 1,2-dioxygenase, salicylaldehyde dehydrogenase, and another ferredoxin.

Gene clusters responsible for hydrocarbon degradation are often encoded on a plasmid (Denome et al. 1993; Kok et al. 1989; Maeda et al. 2003) or in a transposable element (Maeda et al. 2003; Wyndham et al. 1994) and are transmitted from strain to strain by horizontal gene transfer manner (Herrick et al. 1997). The average GC content for the *dbd* gene cluster is 52.2% (50.9% for *dbdA*, 50.7% for *dbdB*, 52.9%, 51.7% and 53.6% for *dbdCa*, *dbdCb*, and *dbdCc*, 52.3% for *dbdD*, and 51.0% for *dbdE*), which is obviously lower than that of whole chromosomal DNA (65%). The GC content for the *dbd* gene cluster, 52.2%, is rather closer to those for homologous genes from other bacteria (52% for *nah*, 58.2% for *tod*, and 60.4% for *tcb* gene clusters from *Pseudomonas* strains) than those for other genes in *Xanthobacter* (e.g., 66% for zeaxanthin biosynthetic gene cluster from *Xanthobacter* sp. Py2, AF408848; 69.5% for Rubisco gene cluster from *X. flavus*, X17252). Thus, although no cryptic plasmid was found in the cells (data not shown), *X. polyaromaticivorans* 127W would have acquired the *dbd* gene cluster by horizontal transfer from other biphenyl benzene-, or toluene-degrading bacteria and modified the substrate specificity and oxygen requirement of the encoded enzymes. No *Xanthobacter* sp. has ever been reported for PAH and HAC degradation and some of the genes closely related to *dbdCa*, such as *bedC1* from *Pseudomonas putida* ML2 (Tan et al. 1993) and *ipbA1* from *Rhodococcus erythropolis* BD2 (Kesseler et al. 1996), are encoded on a plasmid. Moreover, *bph* genes encoded in Tn4371 have similar organization to *dbd* from strain 127W (Merlin et al. 1997).

Structure of DbdCa Phylogenetic analysis clearly shows that DbdCa belongs to the group IV dioxygenase component (Fig. 3). There are dioxygenases in group IV (TcbA-P51, Bph-LB400) that show broad substrate specificity and oxidize naphthalene in part (Haddock and Gibson

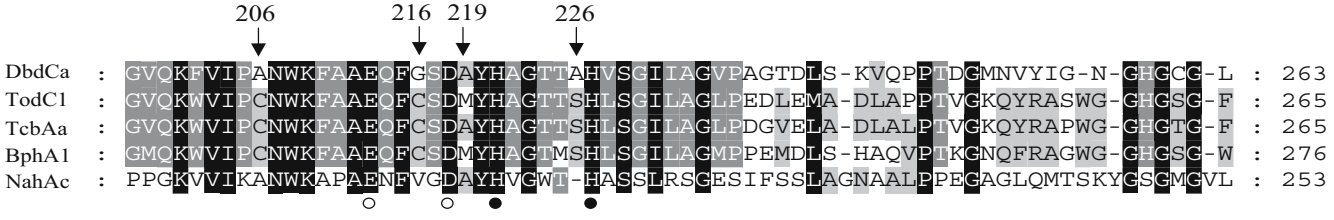

Fig. 6 Amino acid sequence of the alpha subunit of aromatic ring-hydroxylating oxygenases. Alignments are shown for regions possibly determining substrate specificity, near a putative active site. The sequence name abbreviations, origin, and GenBank accession numbers are as follows: DbdCa, DBT dioxygenase from *X. polyaromaticivorans* 127W (AB121977); TodC1, toluene dioxygenase from *P. putida* F1 (J04996); TcbAa, chlorobenzene dioxygenase from *P. putida* P51 (U15298); BphA1, biphenyl dioxygenase from *P. pseudoalcaligenes* KF707 (M83673); NahAc, naphthalene dioxygenase from *P. putida* NCIB9816-4 (AF491307). *Solid circles* (*black*) indicate 2-His-1-carboxylate binding sites of mononuclear iron at the active site and *open circles* indicate amino acid residues responsible for electron transfer and maintaining the conformation of active site (Jiang et al. 1996; Parales 2003). *Arrows* indicate the position of amino acid residues different in DbdCa among the group IV enzyme components

1995; Werlen et al. 1996). However, Bph-LB400 degraded only 3% of naphthalene at the condition in which 92% of biphenyl was degraded, and DBT was not degraded at all. Therefore, DbdCa constitutes a unique group IV terminal oxygenase that prefers larger substrates than any other enzymes in this group.

Substrate specificity of an enzyme is attributed to the structure of the active site that is ultimately determined by the amino acid sequence. The amino acid sequence of DbdCa was then compared with those of several dioxygenases in group IV and naphthalene dioxygenase, NahAc (group III) from *P. putida* NCIB9816-4 whose tertiary structure has already been elucidated (Fig. 6, Kauppi et al. 1998). Amino acid residues essential for iron binding in Rieske center and electron transfer are conserved in all enzymes (Parales 2003). Mutation studies on the alpha subunit terminal oxygenases of toluene and biphenyl dioxygenases suggested that several amino acid residues in the central core region of the active site (210–230) hinder the entrance of large substrate to the catalytic site of toluene dioxygenase (Jiang et al. 1996; Kimura et al. 1997; Zielinski et al. 2002). Replacement of the amino acid residues with smaller ones, C207→A206, C217→G216, M220→A219, S226→A226, may enlarge the substrate binding pocket of DbdCa so that it can accept bulky PAHs and HACs.

Disruption of dbdC gene Degradation activity for DBT, biphenyl, and anthracene was almost completely lost in delta-*dbdC* strain, which demonstrates that *dbdC* is essential for the activity. However, delta-*dbdC* partly maintained degradation activity for phenanthrene. The lower degradation pathway of aromatic hydrocarbon is classified into three pathways whose intermediates are catechol, gentisate, and protocatechuate. When catechol was used as a substrate, strain 127W formed *cis*, *cis*-muconic acid, suggesting the presence of catechol 1,2-dioxygenase in the strain (unpublished result). According to the sequence analysis of the *dbd* genes, another lower pathway (gentisate pathway) was also suggested to function in the present work. These facts strongly suggest that strain 127W maintains multiple catabolic pathways for aromatic hydrocarbons. It has been reported that several aromatic-hydrocarbon-degrading bacteria have multiple isozymes of ring-hydroxylating dioxygenase (McKay et al. 2003).

It was also shown that delta-*dbdC* dramatically reduced the DBT degradation activity under an ELO condition. This result suggests that Dbd dioxygenase system in strain 127W effectively utilizes oxygen in vivo and plays an important role for PAH and HAC degradation under ELO conditions. It has been reported that catechol 2,3-dioxygenases from toluene-degrading *Pseudomonas* strains have higher affinity to oxygen than those from other counterparts (Kukor and Olsen 1996). Unfortunately, ring-hydroxylating mono- or dioxygenase in the first step of aromatic hydrocarbon degradation is composed of multicomponents and reconstitution of these oxygenases has not yet been successful in vitro. This makes these enzymes difficult to be analyzed enzymatically and kinetically, including the affinity to oxygen.

Thus, we do not know whether the DBT dioxygenase is highly functional under ELO because of its high affinity to oxygen. However, it is worth noting that *X. polyaromaticivorans* 127W showed significantly higher DBT degradation activity under ELO than any other PAH-degrading *Pseudomonas* strains, such as *P. cepacia*, *P. fluorescens*, and *P. stutzeri* (Kitauchi et al. 2005).

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