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Cloning and Characterization of a Catechol-Degrading Gene Cluster from 3,4-dichloroaniline Degrading Bacterium *Pseudomonas* sp. KB35B

YOUNG-MOG KIM,† KUNBAWUI PARK,‡ WON-CHAN KIM,§ JAE-HO SHIN,§ JANG-EOK KIM,§ HEUI-DONG PARK,|| AND IN-KOO RHEE*,§

Department of Food Science and Technology, Pukyong National University, Busan, 608-737, South Sea Fisheries Research Institute, National Fisheries Research & Development Institute, Yeosu, Jeonnam, 556-823, and Departments of Agricultural Chemistry and Food Science and Technology, Kyungpook National University, Daegu, 702-701, Korea

We recently isolated a bacterium, *Pseudomonas* sp. KB35B, capable of growth on 3,4-dichloroaniline (DCA) as a sole carbon source. The isolated strain showed a high level of catechol 2,3-dioxygenase (CD-2,3) activity in the presence of 3,4-DCA. In an attempt to elucidate the relationship between biodegradation of 3,4-DCA and CD-2,3 activity, the genes encoding enzymes for the catabolic pathway of catechol were cloned and sequenced from the chromosomal DNA. The sequence analysis of the 10752 bp DNA fragment revealed 12 open reading frames in the order of *nahRGTHINLOMKJX*. Among the 12 genes, *nahHINLOMK* genes encode enzymes for the metabolism of catechol to TCA cycle intermediates. The *nahR* gene is the *LysR* type transcriptional regulator, and the *nahH* gene encodes CD-2,3 for *meta*-cleavages of catechol. 2-Hydroxymuconic semialdehyde hydrolase, 2-oxypent-4-dienoate hydratase, and 4-hydroxy-2-oxovalerate aldolase encoded by *nahLMN* genes are responsible for the three steps after *meta*-cleavages of catechol. The current results suggested that *Pseudomonas* sp. KB35B degrades 3,4-DCA via the *meta*-cleavage pathway of catechol.

KEYWORDS: Biodegradation; 3,4-dichloroaniline; *Pseudomonas* sp. KB35B

INTRODUCTION

The compound 3,4-dichloroaniline (DCA) is an aromatic amine used as an intermediate product in the synthesis of herbicides, azo-dyes, and pharmaceuticals (*1*, *2*). It is also a degradation product of some herbicides (diuron, propanil, and linuron) and of trichlorocarbanilide, a chemical used as an active agent in the cosmetic industry. Because of its toxicity toward invertebrates and vertebrates and its high production rate, however, 3,4-DCA is included in the European Union priority List 1 of chemicals. 3,4-DCA is found in the environment as a contaminant. The scope of 3,4-DCA contamination in groundwater was reported by Batista et al. (*3*). They investigated several pesticides and their metabolites regularly applied to vineyards, maize, potato, apple, pear, and rice. Among them, 3,4-DCA was detected at five sites in 79 sampled sites, and the highest detected level was 3.8 mg/L.

To remove toxic organic compounds such as pesticides, both biological and chemical treatments have been proposed. Biological treatment of the toxic organic compounds (bioremediation), using microorganisms or enzymes produced from the microorganisms, is often considered an environmentally favorable method (*4*−*8*). However, to date, there have been no unambiguous reports about the bioremediation of soil contaminated by 3,4-DCA.

We recently isolated a bacterium strain, *Pseudomonas* sp. KB35B, which is capable of growth on 3,4-DCA as a sole carbon source. It was shown that the catechol 2,3-dioxygenase (CD-2,3) activity was induced by 3,4-DCA exposure in the cells. It is well-known that the conversion of aromatic compounds and chlorine-substituted aromatics to catechol is one of the major metabolic pathways in bacterial biodegradation (*9*−*11*). Therefore, we assumed that the CD-2,3 and its flanking enzymes are involved in the biodegradation of 3,4-DCA in *Pseudomonas* sp. KB35B. In this paper, we report the cloning and characterization of a gene cluster involved in catechol metabolism in *Pseudomonas* sp. KB35B.

MATERIALS AND METHODS

Materials. 3,4-DCA was purchased from Sigma (United States) and was prepared by dissolving it in dimethyl sulfoxide. It was then added to the medium at the concentrations indicated. All other reagents were of reagent grade and were purchased commercially.

Microorganism Isolation and Identification. The bacterial strain KB35B, which is able to grow on plates containing 3,4-DCA as a sole

* To whom correspondence should be addressed. Tel: 82-53-950-5718. Fax: 82-53-953-7233. E-mail: ikrhee@knu.ac.kr.
† Pukyong National University.
‡ National Fisheries Research & Development Institute.
§ Department of Agricultural Chemistry, Kyungpook National University.
|| Department of Food Science and Technology, Kyungpook National University.

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Table 1. Bacterial Strains and Plasmids Used in This Study

strain or plasmid	relevant characteristics	ref
	strains	
Pseudomonas sp. KB35B	3,4-DCA degrader	this study
E. coli ER1647	host strain for plating libraries, amplification, *F*$^-$ *fhu*A2 Δ*(lacZ)r1 supE44 recD1014 trp31 mcrA1272*::Tn*10*(tet^r) *his*$^-$*1 rpsL104*(str^r) *xyl7 mtl2 metB1* Δ(*mcrC-mrr*)*102*::Tn10(tet^r) *hsdS*($r_{K12}^-m_{K12}^-$)	Novagen
E. coli BM25.8	host strain for automatic subcloning, *SupE thi* Δ (*lac-proAB*) [F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q}*Z* Δ*M15*] λ*imm*^{434}(kan^r)P1 (cam^r)*hsdR*($r_{K12}^-m_{K12}^-$)	Novagen
E. coli DH5α	host strain for general DNA manipulation, *F*$^-$ *Ø80dlacZ* Δ*M15* Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17* ($r_K^-m_K^-$) *supE44* λ$^-$ *thi-1 gyrA96 relA1*	Novagen
	plasmids	
pCCAD31, pDCA51, and pDCA61	about 15 kb *Sau*3AI fragment containing *chnB* derived from λBlueSTAR vector system, Ap^r	this study

carbon source, was isolated from sediment from Yeosu, Jeonnam Province, Korea. To identify the isolated strain, the culture morphology, biochemical reactions, and 16S ribosomal DNA (rDNA) sequences were investigated. Two oligonucleotides, based on the report of Dunbar et al. (*12*), were used to determine 16S rDNA of the KB35B: (forward) 5′-AGAGTTTGATCCTGGCTCAG-3′ and (reverse) 5′-TACCTTGTTACGACTT-3′. A polymerase chain reaction (PCR) was performed using intact cells, which were treated for 5 min at 95 °C, as a template. The thermal profile was 25 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C, and extension for 2 min at 72 °C. A final extension step consisting of 5 min at 72 °C was included. Amplified 16S rDNA was purified from an agarose gel and then sequenced by dideoxy-chain termination methods (*13*).

Bacterial Strains, Plasmids, and Culture Conditions. The bacterial strains and plasmids used in this study are listed in **Table 1**. *Pseudomonas* sp. KB35B was grown at 30 °C in Luria−Bertani (LB) broth or minimal medium (*14*) containing 3,4-DCA. *Escherichia coli* was routinely cultured in LB medium at 37 °C. When necessary, media were supplemented with ampicillin (100 μg/mL).

Enzyme Activity and Protein Concentration. *Pseudomonas* sp. KB35B was precultured at 30 °C in LB broth. After overnight culture, the cells were diluted 50-fold into a fresh medium and grown to A_{600} of 1.0 in the absence or presence of 50 ppm 3,4-DCA. After they were harvested, the cells were resuspended in 100 mM potassium phosphate buffer (pH 7.4) and disrupted by sonication. The unbroken cells were removed by centrifugation at 100000*g* for 10 min, and the supernatant was used for determining the activities of catechol dioxygenases. Enzyme activities of catechol 1,2-dioxygenase (CD-1,2) and CD-2,3 were measured spectrophotometrically as reported (*14*, *15*). The CD-1,2 or CD-2,3 activity was assayed by monitoring the increase in *cis,cis*-muconic acid concentration at A_{260} or the increase in 2-hydroxymuconic semialdehyde at A_{375}, respectively (*15*, *16*). The protein concentration was determined by the method of Bradford (*17*) using bovine serum albumin as the standard.

Construction of KB35B Phage Library. Chromosomal DNA from *Pseudomonas* sp. KB35B was prepared by the method of Berns and Thomas (*18*) and then partially digested with *Sau*3AI to yield fragments with an average size of 15−20 kb. These fragments were ligated in the λBlueSTAR phage (Novagen, United States), which had been completely digested with *Bam*HI and dephosphorylated with alkaline phosphatase. In vitro packaging and infection into *E. coli* ER1647 were carried out according to the manufacturer's recommendations (Novagen).

Screening of a Genomic Library of KB35B for a *nahH* Gene. To screen a *nahH* gene from the phage library of *Pseudomonas* sp. KB35B, we prepared a probe using PCR with a forward primer (5′-TGGATTT TATGGGTTTCAAG-3′) and a reverse primer (5′-CTTC-CCAGGTTTCGAG-3′), based on the method of Cladera et al. (*19*). The PCR product was labeled with ^{32}P-dCTP, using the random primer DNA labeling kit as recommended by the manufacturer (Takara, Japan). Hybridization was performed as described by Sambrook et al. (*20*), using a Hybond-N$^+$ nylon membrane (Amersham-Pharmacia Biotech., United Kingdom). Positive signal plaques, obtained from the phage library of *Pseudomonas* sp. KB35B, were automatically subcloned by the *Cre-lox*P mediated excision of the plasmids from λBlueSTAR in *E. coli* BM25.8 (Novagen). Two of the plasmids were selected and designated as pDCA31 and pDCA51.

DNA Sequence Analysis. The inserted DNA (about 15−20 kb) of pDCA31 and pCCA51 were sequenced by the out-PCR-based technique (*21*), a primer walking method with oligonucleotides constructed on the basis of a sequence known from the PCR product. Database searches were performed using the BLASTN (http://www.ncbi.nlm.nih.gov/BLAST) at the National Center for Biotechnology Information (*22*). Multiple sequence alignments were generated using the ClustalW program (http://www.ch.embnet.org/software/ClustalW.html).

Nucleotide Sequence Accession Number. The nucleotide sequence reported in this paper has been deposited in the GenBank under the accession number DQ265742.

RESULTS AND DISCUSSION

Isolation and Identification of Strain KB35B. Several morphologically distinct isolates were obtained from the enrichment culture using plates of minimal medium (*14*) containing 3,4-DCA as a sole carbon source (data not shown). One such strain, named KB35B, was selected from these isolates for further detailed analyses because of its ability to grow rapidly in this medium. The isolated strain was identified as *Pseudomonas* sp. by the morphology, biochemical reactions, and homology research based on 16S rDNA (data not shown).

Induction of CD-2,3 Activity by 3,4-DCA Exposure. The isolated strain, *Pseudomonas* sp. KB35B, was capable of growth on 3,4-DCA as a sole carbon source (data not shown),

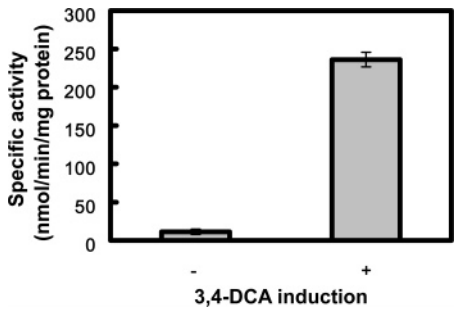

Figure 1. CD-2,3 activity of *Pseudomonas* sp. KB35B. Cells were grown in 1/10 LB for 12 h at 30 °C in the absence (–) or presence (+) of 50 ppm 3,4-DCA. The CD-2,3 activity was measured as described in the Materials and Methods.

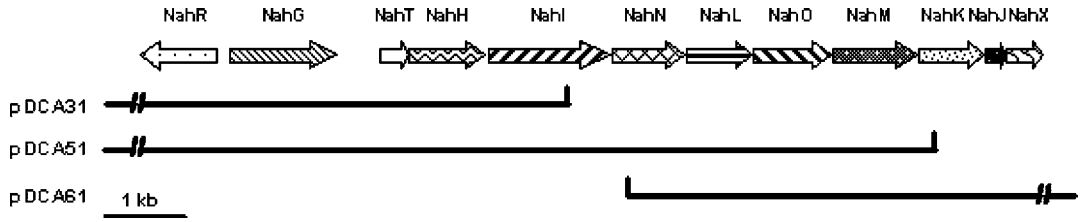

Figure 2. Genetic organization of a catechol-degrading gene (*nah*) cluster in *Pseudomonas* sp. KB35B. The locations and transcriptional directions of the genes encoding the pathway enzymes are indicated by arrows. The plasmids used for DNA sequencing are shown at the bottom.

Table 2. Homology of Catechol-Degrading Gene (*nah*) Cluster Cloned from *Pseudomonas* sp. KB35B[a]

ORF (gene name)	position (no. of nt)	putative function	homologous protein (sequence identity)	source (accession no.)	*E* value[b]
ORF 1 (*nahR*)	1–903 (903)	LysR type transcriptional regulator	NahR (99%)	*P. fluorescens* (AAM18544)	3×10^{-168}
ORF 2 (*nahG*)	1050–2354 (1305)	salicylate hydroxylase	NagG (94%)	*P. putida* G7 NAH7 (YP_534831)	0.0
ORF 3 (*nahT*)	2851–3177 (327)	chloroplast type ferredoxin	NahT (98%)	*P. putida* G7 NAH7 (YP_534832)	1×10^{-40}
ORF 4 (*nahH*)	3186–4109 (924)	CD-2,3	NahH (98%)	*P. putida* G7 NAH7 (YP_534833)	0.0
ORF 5 (*nahI*)	4144–5604 (1461)	2-hydroxymuconic semialdehyde dehydrogenase	NahI (97%)	*P. putida* G7 NAH7 (YP_534834)	0.0
ORF 6 (*nahN*)	5612–6493 (882)	2-hydroxymuconic semialdehyde hydrolase	NahN (92%)	*P. putida* G7 NAH7 (YP_534835)	8×10^{-147}
ORF 7 (*nahL*)	6505–7290 (786)	ODH	NahL (97%)	*P. putida* G7 NAH7 (YP_534836)	1×10–^{140}
ORF 8 (*nahO*)	7310–8233 (924)	acetaldehyde dehydrogenase	NahO (99%)	*Pseudomonas* sp. ND6 pND6-1 (NP_863099)	1×10^{-168}
ORF 9 (*nahM*)	8245–9285 (1041)	HOA	NahM (98%)	*Pseudomonas* sp. ND6 pND6-1 (NP_863098)	0.0
ORF 10 (*nahK*)	9282–10076 (795)	4-oxalocrotonate decarboxylase	NahK (97%)	*P. putida* G7 NAH7 (YP_534839)	3×10^{-144}
ORF 11 (*nahJ*)	10131–10322 (192)	4-oxalocrotonate tautomerase	NahJ (96%)	*P. putida* G7 NAH7 (YP_534840)	9×10^{-23}
ORF 12 (*nahX*)	10330–10752 (423)	unknown protein	NahX (97%)	*P. putida* G7 NAH7 (YP_534841)	1×10^{-60}

[a] The homology search was performed by the BLAST search provided by the National Center for Biotechnology Information (NCBI). [b] Expected value, which estimates the statistical significance of the match by specifying the number of matches, with a given score, that are expected in a search of a database of this size absolutely by chance.

suggesting that the aromatic ring of 3,4-DCA was cleaved and further biodegraded by the KB35B strain. Cleavage of the aromatic ring is a critical reaction in the multistep biodegradation of chlorine-substituted aromatic compounds and is catalyzed by dioxygenases. These enzymes use aromatic diol compounds, such as catechol, as substrates and introduce atoms of molecular oxygen into the substrate, resulting in the opening of the aromatic ring. A variety of aromatics including xylene, phenol, toluene, and naphthalene can be channeled into this pathway via conversion to catechol, which is then further degraded to *cis*,*cis*-muconic acid by the CD-1,2 (*ortho*-cleavage pathway) or 2-hydroxymuconic semialdehyde by CD-2,3 (*meta*-cleavage pathway) (*9*−*11*).

To study the possibility of the conversion of 3,4-DCA to catechol and the biodegradation pathway, we investigated the activity of the two enzymes in the isolated strain. As shown in **Figure 1**, the 3,4-DCA degrader strain, KB35B, showed a high level of CD-2,3 activity (235.98 nmol/min/mg protein) by 3,4-DCA exposure, as compared with control cells (11.35 nmol/min/mg protein). However, no CD-1,2 activity was observed (data not shown), strongly suggesting that CD-2,3 is a critical enzyme in the multistep biodegradation of 3,4-DCA by *Pseudomonas* sp. KB35B. We also hypothesized that 3,4-DCA conversion to catechol will induce the catechol-degrading enzyme in KB35B cells like aniline conversion to catechol (*23*). Therefore, we first tried to clone a CD-2,3 gene (*nahH*) to elucidate the mechanism of the biodegradation of 3,4-DCA by *Pseudomonas* sp. KB35B.

Cloning of a Gene for CD-2,3 from *Pseudomonas* sp. KB35B. To screen a *nahH* gene for catechol degradation in *Pseudomonas* sp. KB35B, primer sets were constructed on the basis of the report of Cladera et al. (*19*). PCR amplifications using each primer set with *Pseudomonas* K35B genomic DNA as template yielded an approximately 460 bp product. Sequencing of the product revealed a partial open reading frame (ORF) with 97% identity with the nahH gene of plasmid NAH7 from *Pseudomonas putida* G7 (*24*).

To clone a complete *nahH* gene and the other genes required for catechol degradation from *Pseudomonas* sp. KB35B, its genomic DNA library was constructed using λBlueSTAR phage as described in the Materials and Methods. The packaged genomic DNA library of *Pseudomonas* sp. KB35B contained a titer of 1.5×10^5 pfu/mL, as determined by transfecting of *E. coli* ER1647. Phage DNA, which was isolated from five randomly chosen *E. coli* transformants, contained large inserts of DNA (15−20 kb). On the basis of the results of plaque hybridization and automatic subcloning, two plasmids (pDCA31 and pDCA51) surrounding the putative *nahH* gene and plasmid pDCA61 including a downstream region of the *nahH* gene were obtained and further investigated. The inserted DNA fragments in both plasmids were sequenced by the primer-walking method with oligonucleotides constructed on the basis of a sequence known from the putative *nahH* gene. The 10752 bp fragment was completely sequenced.

Nucleotide Sequence of the *nahH* Gene and Its Flanking Regions. Sequence analysis of the 10752 bp DNA fragment revealed 12 ORFs in the order of *nahRGTHINLOMKJX* (**Figure 2**). The DNA sequences were translated in all reading frames, and the putative products were compared using the BLAST algorithm with all publicly available protein sequences contained

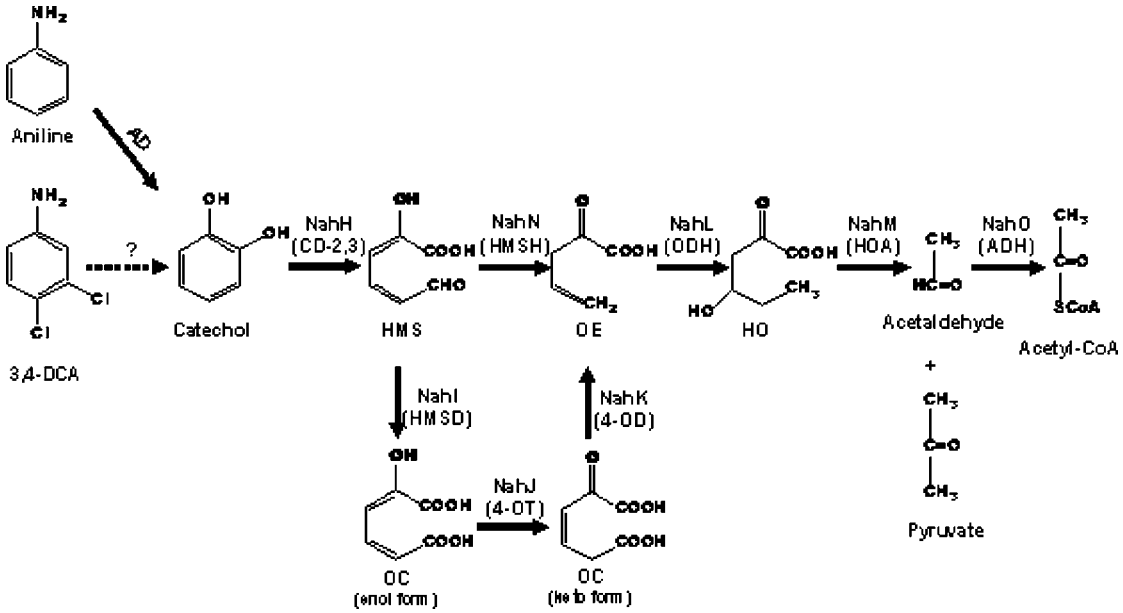

Figure 3. Proposed degradation pathway of 3,4-DCA by *Pseudomonas* sp. KB35B. HMS, 2-hydroxymuconic semialdehyde; OC, 4-oxalocrotonate; OE, 2-oxopent-4-dienoate; HO, 4-hydroxy-2-oxovalerate; AD, aniline dioxygenase; NahH, CD-2,3; NahI, 2-hydroxymuconic semialdehyde dehydrogenase (HMSD); NahJ, 4-oxalocrotonate tautomerase (4-OT); NahK, 4-oxalocrotonate decarboxylase (4-OD); NahN, HMSH; NahL, 2-oxopent-4-dienoate hydratase (ODH); NahM, HOA; and NahO, acetaldehyde dehydrogenase (ADH).

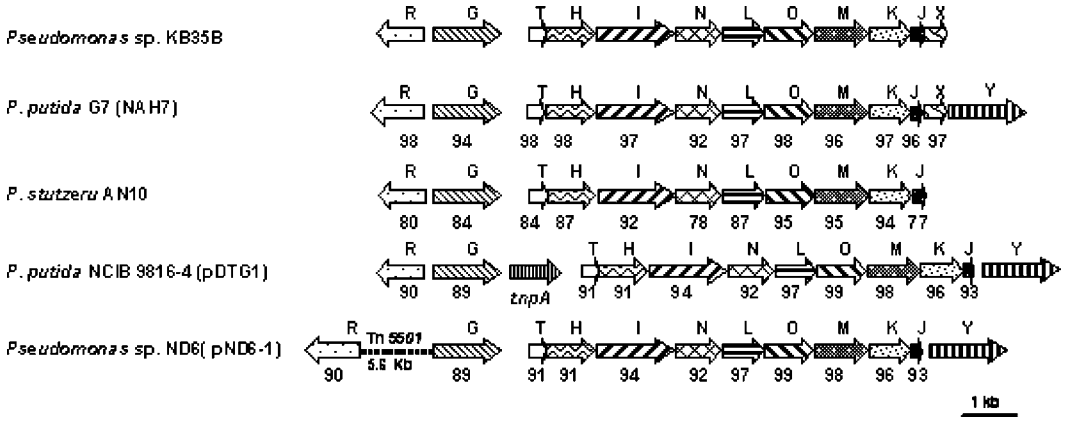

Figure 4. Comparison of catabolic genes in the *meta*-cleavage operons. The percent amino acid identity of *nah* gene cluster products from *Pseudomonas* sp. KB35B to the corresponding gene products from the other operons is shown. The *nah* is a gene for the degradation of catechol. The *tnpA* gene in pDTG1 is a putative transposase gene. Tn*5501* is a cryptic class II transposon identified in *P. putida* H (*28*). Accession numbers of the sequence data are as follows: *P. putida* G7(NAH7), NC_007926; *P. putida* NCIB9816-4(pDTG1), AF491307; *Pseudomonas* sp. ND6(pND6-1), AY208917; and *P. stutzeri* AN10, AF039534. The genes in *Pseudomonas* sp. KB35B and *P. stutzeri* AN10 are located on their chromosomes.

in the nonredundant database. The results of the homology search are shown in **Table 2**. The transcriptional direction of the first ORF, *nahR*, is opposite to the others, and its gene products (NahR) showed the highest amino acid sequence identity (99%) to LysR type regulators found in *P. fluorescens* (accession no. AAM18544; **Table 2**). As shown in **Table 2**, the remaining 11 gene products (*NahGTHINLOMKJX*) exhibited 92−99% identity in amino acid sequences homologous to the *meta*-cleavage pathway enzymes found in plasmid NAH7 (*24*) and pND6-1 (*25*). Among the 12 ORFs, seven ORFs (*nahHINLOMK*) were involved in the complete metabolism of catechol to TCA cycle intermediates, as shown in **Figure 3**. The *nahH* gene encodes CD-2,3 for *meta*-cleavages of catechol. The 2-hydroxymuconic semialdehyde hydrolase (HMSH) encoded by *nahN*, 2-oxypent-4-dienoate hydratase (ODH) by *nahL*, and 4-hydroxy-2-oxovalerate aldolase (HOA) by *nahM* are responsible for the three steps after *meta*-cleavages of catechol. From these results, we concluded that 3,4-DCA was degraded via a *meta*-cleavage pathway of catechol in *Pseudomonas* sp. KB35B.

Comparison of the *nah* Gene Cluster from KB35B with Other *nah* Gene Clusters. As shown in **Table 2**, the putative products of the *nah* genes showed striking similarity to those of the plasmid-encoded *nah* genes of *P. putida* G7 (*24*). Hence, the gene organization of the *nah* gene cluster was compared in detail with that of other *nah* gene clusters (**Figure 4**). The organization of the gene cluster in the KB35B strain was almost identical to that of the plasmid NAH7 with respect to the sequence and position of the metabolic genes. Additionally, the organizations of gene clusters found in plasmid pDTG1 (*26*), pND6-1 (*25*), and in a chromosome region in *Pseudomonas stutzeri* AN10 (*27*) are quite similar (**Figure 4**).

The gene products involved in catechol metabolism have almost 87−99% amino acid identity to their nearest counterparts in pDTG1, pND6-1, and *P. stutzeri* AN10. However, there are

some differences. First, *nahX* is absent in the *nah* cluster of pDTG1, pDN6-1, and *P. stutzeri* AN10. The function of the *nahX* product has not yet been characterized and seems to be unnecessary for catechol metabolism, because many *meta*-cleavage pathways do not contain this enzyme. Second, the *nahY* is absent in the chromosomal *nah* cluster of *Pseudomonas* sp. KB35B and *P. stutzeri* AN10 but not the plasmid-encoded *nah* cluster of NAH7, pDTG1, and pND6-1. *nahY* is not a catabolic gene but a naphthalene chemotaxis gene (*28*). Plasmid NAH7, pDTG1, and pND6-1 originated from naphthalene-degrading *Pseudomonas* sp., not from *Pseudomonas* sp. KB35B, which is a 3,4-DCA-degrading bacteria. Third, the *tnpA* gene (*29*), which is a transposase, was inserted between *nahT* and *nahG* genes in pDTG1 but not others.

The overall structures of these *nah* gene clusters were very similar to those that encode the aerobic degradation of several aromatic compounds via the *meta*-cleavage pathway in various strains, indicating that these gene clusters are distributed by horizontal transfer (*30*). We proposed that the 3,4-DCA biodegradation pathway flows via the *meta*-cleavage pathway in this *Pseudomonas* sp., based on the cloned gene cluster. However, one question remains unanswered: How is 3,4-DCA converted to catechol? We believe there is a gene involved in the conversion of 3,4-DCA to catechol in *Pseudomonas* sp. KB35B such as aniline dioxygenase, which catalyzes the conversion of aniline to catechol (*23*). To address these issues, it is necessary to clone a gene(s) involved in the conversion of 3,4-DCA to catechol.

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