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
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'-TGGATTTTATGGGTTTCAAG-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 A600 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 10000g for 10 min, and the supernatant was used for determining the activities of catechol dioxygenases. Enzyme activities of catechol 1,2-dioxidogenases (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 A500 or the increase in 2-hydroxymuconic semialdehyde at A375, 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 Sau3AI 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 BamHI 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'-TGGATTTTATGGGTTTCAAG-3') and a reverse primer (5'-CTTC-CCAGGTTCGAG-3'), based on the method of Cladera et al. (19). The PCR product was labeled with 32P-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-loxP 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 pDCA51 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.
suggested 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 dioil 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 dehydrogenase 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 × 10^10 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.

### Table 2. Homology of Catechol-Degrading Gene (nah) Cluster Cloned from Pseudomonas sp. KB35B

<table>
<thead>
<tr>
<th>ORF (gene name)</th>
<th>position (no. of nt)</th>
<th>putative function</th>
<th>homologous protein (sequence identity)</th>
<th>source (accession no.)</th>
<th>E value^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>ORF 1 (nahR)</td>
<td>1–903 (903)</td>
<td>LysR type transcriotional regulator</td>
<td>NahR (99%)</td>
<td>P. fluorescens (AAM15444)</td>
<td>3 × 10^{-168}</td>
</tr>
<tr>
<td>ORF 2 (nahG)</td>
<td>1050–2354 (1305)</td>
<td>salicylate hydroxylase</td>
<td>NagG (94%)</td>
<td>P. putida G7 NAH7 (YP_534831)</td>
<td>0.0</td>
</tr>
<tr>
<td>ORF 3 (nahT)</td>
<td>2851–3177 (327)</td>
<td>chloroplast type ferredoxin</td>
<td>NahT (98%)</td>
<td>P. putida G7 NAH7 (YP_534832)</td>
<td>1 × 10^{-42}</td>
</tr>
<tr>
<td>ORF 4 (nahH)</td>
<td>3186–4109 (924)</td>
<td>CD-2,3</td>
<td>NahH (98%)</td>
<td>P. putida G7 NAH7 (YP_534833)</td>
<td>0.0</td>
</tr>
<tr>
<td>ORF 5 (nahI)</td>
<td>4144–5604 (1461)</td>
<td>2-hydroxyxuconic semialdehyde dehydrogenase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ORF 6 (nahM)</td>
<td>5612–6493 (882)</td>
<td>2-hydroxyxuconic semialdehyde hydrolase</td>
<td>NahN (92%)</td>
<td>P. putida G7 NAH7 (YP_534835)</td>
<td>8 × 10^{-147}</td>
</tr>
<tr>
<td>ORF 7 (nahL)</td>
<td>6505–7290 (786)</td>
<td>ODH</td>
<td>NahL (97%)</td>
<td>P. putida G7 NAH7 (YP_534836)</td>
<td>1 × 10^{-140}</td>
</tr>
<tr>
<td>ORF 8 (nahO)</td>
<td>7310–8233 (924)</td>
<td>acetaldehyde dehydrogenase</td>
<td>NahO (99%)</td>
<td>Pseudomonas sp. PD6 pPD6-1</td>
<td>1 × 10^{-168}</td>
</tr>
<tr>
<td>ORF 9 (nahM)</td>
<td>8245–9285 (1041)</td>
<td>HOA</td>
<td>NahM (98%)</td>
<td>Pseudomonas sp. PD6 pPD6-1</td>
<td>0.0</td>
</tr>
<tr>
<td>ORF 10 (nahK)</td>
<td>9282–10076 (795)</td>
<td>4-oxalocrotonate decarboxylase</td>
<td>NahK (97%)</td>
<td>P. putida G7 NAH7 (YP_534839)</td>
<td>3 × 10^{-144}</td>
</tr>
<tr>
<td>ORF 11 (nahJ)</td>
<td>10131–10322 (192)</td>
<td>4-oxalocrotonate tautomerase</td>
<td>NahJ (96%)</td>
<td>P. putida G7 NAH7 (YP_534840)</td>
<td>9 × 10^{-23}</td>
</tr>
<tr>
<td>ORF 12 (nahX)</td>
<td>10330–10752 (423)</td>
<td>unknown protein</td>
<td>NahX (97%)</td>
<td>P. putida G7 NAH7 (YP_534841)</td>
<td>1 × 10^{-60}</td>
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</table>

^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.
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-oxopent-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 mpa gene (29), which is a transposase, was inserted between nahY 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.

**LITERATURE CITED**


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