Novel Partial Reductive Pathway for 4-Chloronitrobenzene and Nitrobenzene Degradation in Comamonas sp. Strain CNB-1

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Comamonas sp. strain CNB-1 grows on 4-chloronitrobenzene (4-CNB) and nitrobenzene as sole carbon and nitrogen sources. In this study, two genetic segments, cnbB-orf2-cnbA and cnbR-orf1-cnbCaCbDEFGHI, located on a newly isolated plasmid, pCNB1 (ca. 89 kb), and involved in 4-CNB/nitrobenzene degradation, were characterized. Seven genes (cnbA, cnbB, cnbCa, cnbCb, cnbD, cnbG, and cnbH) were cloned and functionally expressed in recombinant Escherichia coli, and they were identified as encoding 4-CNB nitroreductase (CnbA), 1-hydroxylaminobenzene mutase (CnbB), 2-aminophenol 1,6-dioxygenase (Cnb-Cab), 2-amino-5-chloroammonium semialdehyde dehydrogenase (CnbD), 2-hydroxy-5-chloroammonium acid (2H5CM) tautomerase, and 2-amino-5-chloroammonium acid (2A5CM) deaminase (CnbH). In particular, the 2A5CM deaminase showed significant identities (31 to 38%) to subunit A of Asp-tRNA^Glu/Thr-tRNA^Gln amidotransferase and not to the previously identified deaminases for nitroaromatic compound degradation. Genetic cloning and expression of cnbH in Escherichia coli revealed that CnbH catalyzed the conversion of 2A5CM into 2H5CM and ammonium. Four other genes (cnbR, cnbE, cnbF, and cnbI) were tentatively identified according to their high sequence identities to other functionally identified genes. It was proposed that CnbH might represent a novel type of deaminase and be involved in a novel partial reductive pathway for chloronitrobenzene or nitrobenzene degradation.

Chlorinated nitroaromatic compounds such as chloronitrobenzenes are massively produced and are widely used as intermediates for chemical syntheses of drugs, herbicides, dyes, etc. The natural formation of chlorinated nitroaromatic compounds is rare, and most of these compounds are from industrial productions and have been introduced into the environment for a relatively short period. Apparently, their occurrence in the environment has selected microorganisms that are able to utilize chlorinated nitroaromatic compounds as carbon and/or nitrogen sources for growth. Examples of such microorganisms are bacterial strain LW1 (15), a coculture of Pseudomonas putida and a Rhodococcus sp. (25), and recently Comamonas sp. strain CNB-1 (38).

Nitroaromatic compounds and chlorinated nitroaromatic compounds are structurally analogous. The microbial degradation of nitroaromatic compounds has been extensively investigated and the removal of the nitro group(s) is carried out via oxidative pathways that initiate with monoxygenases (22, 31, 40) or dioxygenases (8, 16, 20, 19, 32) or a partial reductive pathway that initiates with nitroreductases (7–9, 17, 22, 29, 30). Although structurally related to the nitroaromatic compounds, the chlorinated nitroaromatic compounds are more resistant to microbial degradation due to the simultaneous existence of chlorine and nitro groups, and thus the knowledge of its microbial degradation is very limited.

Previous studies revealed that reductive dehalogenization (35) and partial reduction of nitro groups (15, 39) might be involved in the initial steps during chlorinated nitroaromatic compound degradation. However, these pathways have not been characterized at the genetic and enzymatic levels. This study identified the genes and pathway for 4-chloronitrobenzene degradation by previously isolated Comamonas sp. strain CNB-1.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Comamonas sp. strain CNB-1 (38, 39) was maintained in Luria-Bertani (LB) medium and in MSB (5) containing 2 mM of 4-chloronitrobenzene as the sole carbon and nitrogen source. All Escherichia coli strains were cultured and maintained in LB medium. When necessary, ampicillin at 100 μg/ml was added to the medium.

Screening for CNB-negative mutant, plasmid curing, and detection of plasmid. A mutant, Comamonas sp. strain CNB-2, that could not utilize 4-chloronitrobenzene or nitrobenzene for growth was obtained by curing the plasmid from strain CNB-1 using a modified sodium dodecyl sulfate treatment method of El-Mani et al. (6). Detection of the megaplasmid in Comamonas sp. strains CNB-1 and CNB-2 was carried out according to Barton et al. (2). Separation of chromosomal and plasmid DNAs was carried out on an agarose gel (1%) under conditions of 6 V/cm and 70 seconds for 22 h using a Bio-Rad pulsed-field gel electrophoresis apparatus (Bio-Rad). Yeast genomic DNA (catalog no. 170–3605, Bio-Rad) was used as DNA molecular weight markers.

DNA extraction and plasmid isolation. DNAs from Comamonas sp. strain CNB-1 and routine plasmid isolation were carried out following the procedures of Sambrook et al. (27). For large-plasmid isolation, a modified alkaline lysis method was used (28, 36).

DNA sequencing, sequence assembly and analysis. The 2-aminophenol 1,6-dioxygenase-positve clones pBG-2 and pCG-13, each containing a 35-kb DNA fragment from strain CNB-1, was sequenced with the shotgun method by the Beijing Genome Institute (Huada Corp., Beijing, China). Contigs were assembled using the GCG Wisconsin package.

Cloning and expression of cnb genes in E. coli. PCR primers (Table 1) were designed according to the DNA sequence obtained in this study, and entire genes were amplified by PCR from the strain CNB-1 genome. Purified PCR products were treated with restriction enzymes and then ligated into the similarly treated pET-21a (+), except for cnbHl, which was cloned into pET-28a (+). The resulting plasmids (Table 1) were used to transform E. coli cells for expression of the
genes. Expression of the genes in cells of *E. coli* strains was induced with 1 mM isopropyl thiogalactopyranoside (IPTG) when the culture reached an optical density at 600 nm of ca. 0.6.

**Preparation of cellular lysates, purification of enzymes, and SDS-PAGE.** Cellular lysates of *Comamonas* sp. strain CNB-1 or recombinant *E. coli* actively synthesizing various enzymes of 4-chloronitrobenzene and nitrobenzene degradation were prepared by sonication of cell suspensions in 10 mM phosphate buffer (pH 8). Sonication was conducted (at 200 W, 3 seconds, interval of 5 seconds, for 90 cycles) on ice bath. Cell debris was removed by centrifugation at 12,000 × g for 10 min, and the supernatant was used for purification of various enzymes and for enzymatic activity determination.

The procedures for purification of 2-amino-phenol 1,6-dioxygenase from *Comamonas* sp. strain CNB-1 were previously described (39). Purification of 2-amino-5-chloromuconic semialdehyde dehydrogenase from recombinant *E. coli* cells was performed with His Bind resin chromatography by following the instructions from the manufacturer (Novagen). The purification efficiency of each step was determined with His Bind resin chromatography by following the instructions from the manufacturer (Novagen). The purification efficiency of each step was determined by Bradford (3).

**Protein concentrations were determined according to Bradford (3).**

**Enzymatic assays.** 4-Chloronitrobenzene nitroreductase activity was determined spectrophotometrically by measuring the decrease of absorption at 340 nm (49%). The reaction mixture contained cellular lysate (4 ng), 4-chloronitrobenzene or nitrobenzene (0.1 mM), NADPH (0.2 mM), and phosphate buffer (10 mM, pH 8) in a final volume of 200 μL. The reaction was started by addition of NADPH.

Hydroxylaminobenzene mutase activities were determined by determination of increase of absorption at 235 nm (49%). The reaction mixture contained the same ingredients as above, except that cellular lysate containing 4-chloronitrobenzene nitroreductase was replaced with cellular lysate containing hydroxylaminobenzene mutase. The increase in 4-chloro-phenol 1,6-dioxygenase (39), 2-amino-5-chloromuconic/2-amino-5-chloromuconic semialdehyde dehydrogenase (13), and 2-amino-5-chloromuconate deaminase (10, 11), except that the wavelength was set at 340 nm (49%).

**Determination of molecular weight with SDS-PAGE.** The molecular weights of the recombinant proteins and enzyme subunits were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) with a 15% resolving gel and a 5% stacking gel. Protein molecular weight standards for SDS-PAGE were purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China).

**Construction of phylogenetic tree.** Peptide sequences of various deaminases and subunits of *Asp-tRNA Asn/Glu-tRNAGln amidotransferases* were extracted from NCBI (http://www.ncbi.nlm.nih.gov/). Phylogenetic trees were generated using the neighbor joining method of Saitou and Nei (26) with the AlignX software (Informax, Maryland), and multiple sequence alignment was done using ClustalX (37). The length of each branch pair represents the evolutionary distance between the sequences.

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**TABLE 1. Bacterial strains, plasmids, and oligonucleotides used in this study**

<table>
<thead>
<tr>
<th>Strain, plasmid, or oligonucleotide</th>
<th>Description or primer sequence (restriction enzymes)*</th>
<th>Source or reference</th>
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</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
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<tr>
<td><em>Comamonas</em> sp. strain CNB-1</td>
<td>Isolated from activated sludge, assimilating p-chloronitrobenzene</td>
<td>CGMCC 1028, Wu et al. (38, 39)</td>
</tr>
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<td><em>E. coli</em> BL21(DE3)</td>
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<td>Stragene</td>
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<td><strong>Plasmids</strong></td>
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<td>Novagen</td>
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<tr>
<td>pET-28a(+)</td>
<td>Expression vector</td>
<td>Novagen</td>
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<td>pBG-2</td>
<td>Plasmid carrying 2-aminophenol 1,6-dioxygenase genes</td>
<td>Wu et al. (39)</td>
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<tr>
<td>pCG-13</td>
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<td>Wu et al. (39)</td>
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<td>Constructed for expression of 4-chloronitrobenzene</td>
<td>This study</td>
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<td>nitroreductase and mutase</td>
<td>This study</td>
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<tr>
<td>pETcnb7AB</td>
<td>Constructed for expression of 2-amino-5-chlorophenol 1,6-dioxygenase</td>
<td>Wu et al. (39)</td>
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<tr>
<td>pETcnb7D</td>
<td>Constructed for expression of 2-amino-5-chloromuconic semialdehyde dehydrogenase</td>
<td>This study</td>
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<td>pETcnbH</td>
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*a The restriction enzyme sites are underlined. The start and stop codons are in bold.*
Preparations of 2-amino-5-chloromuconic semialdehyde and 2-amino-5-chloromuconic acid were carried out by extracting and purifying the organic solvent under a vacuum and then analyzed by UV spectrophotometry and gas chromatography-mass spectrometry. Preparations of 2-amino-5-chloromuconic semialdehyde and 2-amino-5-chloromuconic acid were carried out by extracting and purifying the organic solvent under a vacuum and then analyzed by UV spectrophotometry and gas chromatography-mass spectrometry.

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analyses, these genes and their putative functions were tentatively identified and are listed in Table 2.

**Functional identification of cnbA, cnbB, cnbCa, cnbCb, and cnbD, and sequential conversion of 4-chloronitrobenzene into 2-amino-5-chloromuconic acid.** cnbA, cnbB, cnbCa, cnbCb, and cnbD exhibited high identities to the previously identified nbz or amn or nba genes of Pseudomonas putida strain HS12 (23), Pseudomonas pseudoalcaligenes strain JS45 (4, 29), Pseudomonas sp. strain AP-3 (24, 33, 34), and Pseudomonas sp. strain KU-7 (18), which were involved in degradation of nitrobenzene, aminophenol, or 2-nitrobenzoate (Table 2). Assimilation of chloronitrobenzenes by those strains was not reported, but the high identities of the genes of strain CNB-1 indicated that these genes might function similarly to the nbz, nba, or amn genes of strains HS12, JS45, KU-7, and AP-3.

The cnb genes were individually PCR amplified and cloned into pET-21a, the plasmids generated containing each cnb gene (Table 1). E. coli BL21(DE3) harboring the pET derivatives was checked for synthesis of recombinant proteins and assayed for enzymatic activities. The results indicated that the cnbA, cnbB, cnbCa, and cnbD genes encoded chloronitrobenzene nitroreductase, hydroxylaminobenzene mutase, 2-aminophenol 1,6-dioxygenase, and 2-aminomuconic semialdehyde dehydrogenase, respectively (Table 2). Furthermore, when these enzymes were coupled in vitro, they sequentially catalyzed the conversions of 4-chloronitrobenzene to 2-amino-5-chloromuconic acid and nitrobenzene to 2-amino-5-chloromuconic acid.

**Gene cnbH encodes 2-amino-5-chloromuconic acid deaminase and its conversion into 2-hydroxy-5-chloromuconic acid.** The theoretical translational product of gene cnbH shows some identities to the genes encoding subunit A of glutamyl-tRNA\(^\text{Glu}\) amidotransferases (Table 2) and no significant identity to the deaminases from Pseudomonas sp. strains AP-3, HS12, and JS45. The entire cnbH was PCR amplified and cloned into pET-28a(+), and the resulting plasmid, pETcnbH, was transformed into E. coli. Recombinant E. coli cells synthesized a new protein with a molecular mass corresponding to the predicted CnbH (45 kDa).

The recombinant CnbH functioned as deaminase (Fig. 2a and b) and catalyzed the conversion of 2-amino-5-chloromuconic acid into a product that had maximal absorption spectrum at 306 nm (Fig. 2c). This product was purified and subjected to LC-MS and spectrophotometric analyses and identified as 2-hydroxy-5-chloromuconic acid (Fig. 2d). Ammonia was nearly stoichiometrically released during the reaction, and 0.18 mM of ammonia was produced from 0.2 mM of 2-amino-5-chloromuconic acid. Similarly, this CnbH catalyzed the formations of 2-hydroxymuconic acid and ammonia from 2-amino-5-chloromuconic acid. The product of 2-hydroxymuconic acid was isomerized by CnbG into 2-oxalocrotonate, as indicated by the shift of maximal absorption wavelength from 296 to 236 nm (Fig. 3), which was similarly reported by He and Spain (12). Detection of plasmid in Comamonas sp. strain CNB-1 and localization of the pathway on the plasmid. Many degradative pathways are encoded by genes on plasmids, and our previous work revealed that the gene for 2-aminophenol 1,6-dioxygenase was detected at relatively high frequency (three positive clones were obtained out of 300 clones) (39). This high recovery frequency stimulated us to consider that this gene had multicopies and was probably located on a multicopy plasmid. Plasmid curing from strain CNB-1 resulted in a mutant named strain CNB-2 that lost the ability to use 4-chloronitrobenzene and nitrobenzene for growth. Detection of a plasmid with the pulsed-field gel electrophoresis method and extraction of plasmid DNA both indicated that a plasmid of ca. 89 kb (pCNB1) existed in strain CNB-1 but not in CNB-2. The extracted plasmid DNAs were subjected to sequencing, and the results indicated that this pCNB1 was a circular plasmid of ca. 89 kb. The current data from the sequencing indicated that the genes for...
2-aminophenol 1,6-dioxygenase (cnbCab), 4-chloronitrobenzene nitroreductase (cnbA), hydroxylaminobenzene mutase (cnbB), and 2-amino-5-chloromuconic acid deaminase (cnbH) were all located on plasmid pCNB1.

DISCUSSION

In this study, we found that both 4-chloronitrobenzene and nitrobenzene were degraded in Comamonas sp. strain CNB-1.
via a partial reductive pathway that is similar to but different from that of the nitrobenzene degradative pathways in *Pseudomonas* sp. strains IS45 and HS12 (partial reductive pathway), and *Comamonas* sp. strain JS765 (oxidative pathway). The chloronitrobenzene pathway looked more like a combination of the upper pathway (nitroreduction and ring cleavage) of the *Pseudomonas* strains IS45 and HS12 and the lower pathway (after ring cleavage reactions) of *Comamonas* sp. strain JS765. Previously, Katsivela et al. (15) proposed a partial reductive pathway of bacterial strain LW1 for chloronitrobenzene degradation based on enzymatic activity assays and on identification of metabolic intermediates.

The novel pathway for chloronitrobenzene degradation in *Comamonas* sp. strain CNB-1 was identified at the genetic level in this study. The genes involved in the pathway were located on plasmid pCNB1, and two fragments related to chloronitrobenzene degradation were characterized. Among the genes located on the two fragments, seven (*cnbA, cnbB, cnbCa, cnbCb, cnbD, cnbG, and cnbH*) were functionally identified in recombinant *E. coli* and involved in chloronitrobenzene degradation. Four other genes (*cnbR, cnbE, cnbF, and cnbI*) were also tentatively identified as required for chloronitrobenzene degradation, according to the high identities to the genes whose functions are known in other bacteria. The functions of ORF1 and ORF2 were not clear. These nine genes (*cnbA, cnbB, cnbCa, cnbCb, cnbD, cnbE, cnbF, cnbG, and cnbH*) encoded enzymes that sequentially converted 4-chloronitrobenzene to 5-chloro-4-hydroxy-2-oxovalerate (*Fig. 1*). The *cnbR* gene encoded a putative regulator, but how it regulates the *cnb* genes is not clear at this stage.

The expression of *cnbH* in *E. coli* was successful in this study, but CnbH activity was much lower than that of the cellular lysate of *Comamonas* sp. strain CNB-1. This low activity raises the question of whether this CnbH could support the growth of strain CNB-1 on chloronitrobenzene or nitrobenzene. There might be two explanations for this: the expression of *cnbH* was not optimized in *E. coli*, or there was an alternative deaminase in *Comamonas* sp. strain CNB-1 that allowed this strain to grow on chloronitrobenzene or nitrobenzene. Nevertheless, the *cnbH* gene was interesting because it encoded a deaminase which is functionally similar to NbzE of strain HS12 (23) and AmnD of strain AP-3 (33) but showed no significant identity to NbzE of strain HS12 or AmnD of strain AP-3. Instead, it showed significant identities to some genes encoding Asp-tRNA^A^-Glu-tRNA^Glu^-aminotransferase A subunit A (31 to 38%).

Evolutionary analysis indicated that CnbH was more related to Asp-tRNA^A^-Glu-tRNA^Glu^-aminotransferase A subunits than to deaminases (*Fig. 4*). As far as we know, the involvement of such a gene in the biodegradation of xenobiotic compounds has not been reported. We propose that CnbH might represent a novel type of deaminase in the degradation of xenobiotic compounds. The identification of novel genes that are involved in degradation of nitroaromatic compounds of short exposure in this and previous studies (18) should stimulate studies on genetic and metabolic pathway evolution. Currently, the details of the evolutionary relationship between this gene and nbdE/amnD and aminotransferase genes are under investigation.

**ACKNOWLEDGMENTS**

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**REFERENCES**


