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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 (CnbCab), 2-amino-5-chloromuconic semialdehyde dehydrogenase (CnbD), 2-hydroxy-5-chloromuconic acid (2H5CM) tautomerase, and 2-amino-5-chloromuconic acid (2A5CM) deaminase (CnbH). In particular, the 2A5CM deaminase showed significant identities (31 to 38%) to subunit A of Asp-tRNA^{Asn}/Glu-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 analogs. 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 monooxygenases (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.

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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-Mansi 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-positive 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 *cnbH*, which was cloned into pET-28a(+). The resulting plasmids (Table 1) were used to transform *E. coli* cells for expression of the

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

Strain, plasmid, or oligonucleotide	Description or primer sequence (restriction enzymes)[a]	Source or use and reference
Strains		
Comamonas sp. strain CNB-1	Isolated from activated sludge, assimilating *p*-chloronitrobenzene	CGMCC 1028, Wu et al. (38, 39)
E. coli BL21(DE3)	Expression host	Stratagene
Plasmids		
pET-21a(+)	Expression vector	Novagen
pET-28a(+)	Expression vector	Novagen
pBG-2	Plasmid carrying 2-aminophenol 1,6-dioxygenase genes	Wu et al. (39)
pCG-13	Plasmid carrying 2-aminophenol 1,6-dioxygenase genes	Wu et al. (39)
pET*cnbA*	Constructed for expression of 4-chloronitrobenzene nitroreductase	This study
pET*cnbAB*	Constructed for expression of nitroreductase and mutase	This study
pET*cnbCab*	Constructed for expression of 2-amino-5-chlorophenol 1,6-dioxygenase	Wu et al. (39)
pET*cnbD*	Constructed for expression of 2-amino-5-chloromuconic semialdehyde dehydrogenase	This study
pET*cnbG*	Constructed for expression of 2-hydroxy-5-chloromuconate tautomerase	This study
pET*cnbH*	Constructed for expression of 2-amino-5-chloromuconic deaminase	This study
Oligonucleotides		
NBPf	GACGTTTCA**TATG**CCGACCAGCCCGTTC (NdeI)	Amplification for *cnbA*; this study
NBPr	TGGGATCC**CTA**TTCGTGGACGAAGGTGG (BamHI)	
HabPf	GTCCGAATTCAAGGAGACCCCTTC**ATG**C (EcoRI)	Amplification for *cnbB*; this study
HabPr	GTCAAAGCTTTGCGGGAAGTC**TCA**TGGT (HindIII)	
Ps	**ATG**CAAGGTGAAATCATCG	Amplification for *cnbCab*; this study
Pat	CCGGAATTC**TCA**GAGTCGGAACTCGA**T**C (EcoRI)	
DehPf	GGAAACCCAT**ATG**AAGCAATACCGAAATTACATCAACG (NdeI site)	Amplification for *cnbD*; this study
DehPr	GGCCAAGCTTAGACACTGCCTCTTGATCAATTCGG (HindIII site)	
cnbGPr	GGAATTCCAT**ATG**CCGTTCGCACAGATCTACAT (NdeI site)	Amplification for *cnbG*; this study
cnbGPr	CCCCAAGCTT**TCA**GCGGCCGAGGTCTTT (HindIII site)	
AmdPf	CACGCGCAT**ATG**GAACCCCGGCTCAACGCCTACAAG (NdeI site)	Amplification for *cnbH*; this study
AmdPr	GGCGAAGCTT**CTA**TGGCAGGTCGGGCGCGCCCAG (HindIII site)	

[a] The restriction enzyme sites are underlined. The start and stop codons are in bold.

genes. Expression of the genes in cells of *E. coli* strains was induced with 1 mM isopropylthiogalactopyranoside (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 sonification of cell suspensions in 10 mM phosphate buffer (pH 8). Sonification 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-aminophenol 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 controlled by running electrophoresis of samples collected from each step, with a 12% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS). To visualize protein bands, the gel was stained with Coomassie brilliant blue. All purified enzymes were stored at −70°C.

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 (A_{340}). 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 (A_{235}). The reaction mixture contained the same ingredients as above, except that cellular lysate with 4-chloronitrobenzene nitroreductase was replaced with cellular lysate containing hydroxylaminobenzene mutase. The increase in A_{235}, due to the formation of 2-aminophenol, was used for estimation of the activity of hydroxylaminobenzene mutase. The activity of 1-hydroxylamino-4-chlorobenzene mutase was determined similarly.

The 2-aminophenol 1,6-dioxygenase (39), 2-aminomuconic/2-amino-5-chloromuconic semialdehyde dehydrogenase (13), and 2-aminomuconate deaminase (10, 11) activities were determined according to the methods cited. The 2-amino-5-chloromuconate deaminase activity was determined similarly to that of 2-aminomuconate deaminase (10, 11), except that the wavelength was set at 340 nm (A_{340}).

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-tRNA^{Gln} 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.

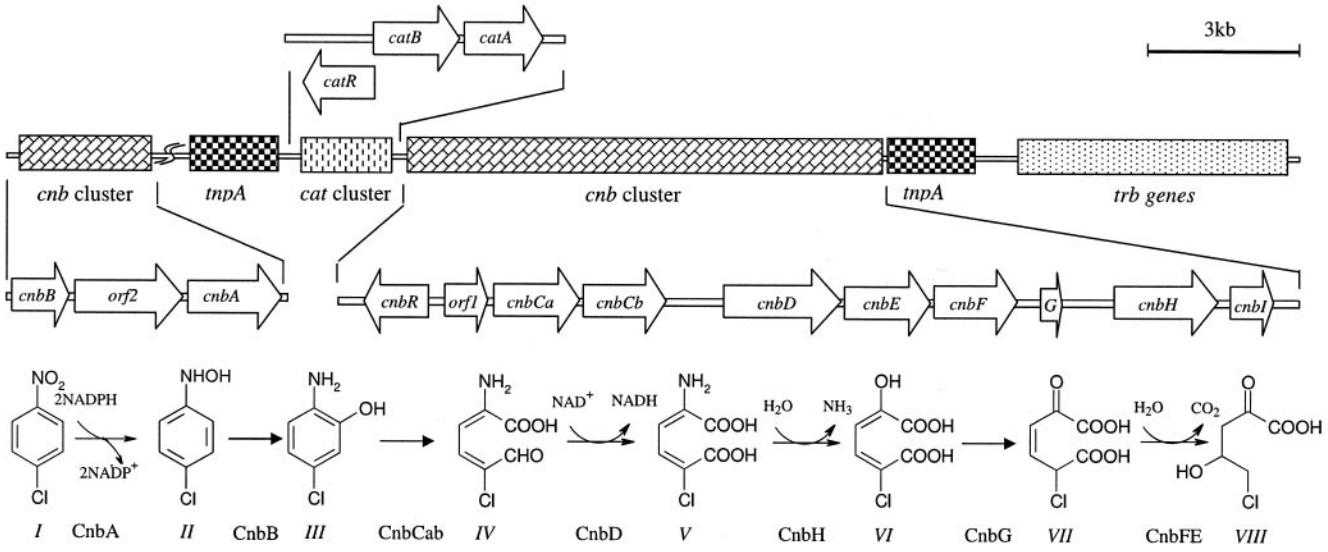

FIG. 1. Genetic organization of *cnb* genes involved in 4-chloronitrobenzene (4-CNB) and nitrobenzene degradation and the modified partial reductive pathway for 4-chloronitrobenzene and nitrobenzene degradation in *Comamonas* sp. strain CNB-1. The genes involved in 4-chloronitrobenzene and nitrobenzene degradation in *Comamonas* sp. strain CNB-1 are organized in two clusters: a larger cluster (*cnbR-orf1-cnbCaCbDEFGHI*) includes the putative regulator gene (*cnbR*), the ring cleavage gene (*cnbCab*), and several genes of the lower pathway; and a smaller cluster (*cnbB-orf2-cnbA*) includes genes for the upper pathway that converts 4-chloronitrobenzene to 2-amino-5-chlorophenol and nitrobenzene into 2-aminophenol. The decarboxylation and hydration (from compound VII to compound VIII) was catalyzed by CnbE and CnbF, which was confirmed in recombinant *E. coli* by simultaneous expression of the two genes (data not shown). Upstream of the genetic cluster *cnbR-orf1-cnbCaCbDEFGHI* is a genetic cluster, *catRBA*, that putatively encodes the catechol pathway, and the function of *catA*, encoding catechol 1,2-dioxygenase, was confirmed by expression in *E. coli* (data not shown). Between the two *cnb* genetic clusters, there are some putative genes relating to gene transposition and plasmid conjugation. Arrows indicate the direction of transcription. Symbols: I, 4-chloronitrobenzene; II, 1-hydroxylamino-4-chlorobenzene; III, 2-amino-5-chlorophenol; IV, 2-amino-5-chloromuconic semialdehyde; V, 2-amino-5-chloromuconic acid; VI, 2-oxohex-4-ene-5-chloro-1,6-dioate; VII, 2-oxopent-5-chloro-3-enoate; VIII, 5-chloro-4-hydroxy-2-oxovaletate.

Preparations of 2-amino-5-chloromuconic semialdehyde and 2-amino-5-chloromuconic acid. 2-Amino-5-chloromuconic semialdehyde was prepared by enzymatic cleavage of 2-amino-5-chlorophenol (Sigma). The reaction mixture contained 50 μl of 2-amino-5-chlorophenol (10 mM), 10 ng of partially purified 2-aminophenol 1,6-dioxygenase, and 2.9 ml of phosphate buffer (10 mM, pH 8). After reaction for 5 min, this mixture was used as the substrate without further purification.

The 2-amino-5-chloromuconate was prepared by further enzymatic oxidation of 2-amino-5-chloromuconic semialdehyde (prepared as above), by addition of 4 ng of partially purified 2-amino-5-chloromuconic semialdehyde dehydrogenase to the above reaction mixture. The product, 2-amino-5-chloromuconate, was partially purified according to He and Spain (11).

Preparation, purification, and identification of 2-hydroxy-5-chloromuconic acid with gas chromatography-mass spectroscopy. The 2-hydroxy-5-chloromuconic acid was prepared by sequential catalysis with partially purified 2-aminophenol 1,6-dioxygenase (0.2 mg/ml), 2-amino-5-chloromuconic semialdehyde dehydrogenase (0.08 mg/ml), and 2-amino-5-chloromuconic acid deaminase (0.13 mg/ml), in phosphate buffer (10 mM, pH 8). After 5 min, the reaction mixture (total 10 ml in volume, initially containing 10 mM 2-amino-5-chlorophenol and 10 mM NAD^+) was adjusted to 2.0 with HCl and centrifuged at 12,000 × *g* for 10 min. The supernatant was extracted with an equal volume of ethyl acetate. After another centrifugation at 12,000 × *g* for 10 min, the organic phase that contained 2-hydroxyl-5-chloromuconate was pooled and concentrated by evaporation of the organic solvent under a vacuum and then analyzed by UV spectrophotometer and gas chromatography-mass spectroscopy.

2-Hydroxymuconate and 2-hydroxy-5-chloromuconate were identified by liquid chromatography-mass spectrometry (LC-MS) and spectrophotometry. LC-MS analysis was performed on a Finnigan LCQ ion trap mass spectrometer (San Jose, Calif.) equipped with an atmospheric pressure ionization interface. The instrument was operated in a negative electrospray ionization mode. The capillary voltage was fixed at 16 V, and its temperature was maintained at 200°C. The spray voltage was set at 4.25 kV. Liquid chromatography was carried out with an Agilent 1100 system. The sample was separated on a ZORBAX SB-C18 column (particle size, 5 μm; inside diameter, 2.1 by 150 mm; Agilent) and detected by a diode array detector (DAD UV6000). A mobile phase of 40% methanol and 60% water was used with a flow rate of 0.2 ml/min.

Restriction enzymes and chemicals. All restriction enzymes, *Taq* and *Pfu* polymerases for PCR amplification, and T4 DNA ligase were purchased from Promega or TaKaRa. Nitrobenzene, 4-chloronitrobenzene, 2-aminophenol, 2-amino-5-chlorophenol, and other chemicals were purchased from Sigma or Fluka.

Nucleotide sequence accession numbers. The DNA sequences reported here are available in GenBank under accession numbers AY731710 and DQ207951.

RESULTS

Genetic organization for 4-chloronitrobenzene degradation. A previous study showed that genes encoding a 2-aminophenol 1,6-dioxygenase and a putative 2-aminohydroxymuconic semialdehyde dehydrogenase were located on a 15-kb fragment of plasmid pBG-2 (39). To identify other genes for the degradation of 4-chloronitrobenzene and nitrobenzene, this plasmid (pBG-2) and a second plasmid, pCG-13 (39), were sequenced with the shotgun method in this study. The results indicated that a ca. 34-kb DNA fragment of pCG-13 covered the 15-kb fragment of pBG-2. This 34-kb DNA fragment contained a genetic cluster (*cnbR-orf1-cnbCaCbDEFGHI*) that was putatively involved in 4-chloronitrobenzene and nitrobenzene degradation. Two other genes (*cnbB-orf2-cnbA*) were located on a separate DNA fragment of pBG-2 and were deduced to also be involved in the conversion of 4-chloronitrobenzene and nitrobenzene (Fig. 1). Based on BLAST searches and homology

TABLE 2. Annotation of genes involved in 4-chloronitrobenzene and nitrobenzene degradation and some properties of their encoded proteins

Gene	Position in sequence (bp)	Gene product	Calculated mass, Da (no. of residues)	Homologous protein	Source	% Identity/no. of residues	Accession no.
cnbR	330–845	Regulatory protein	18,761 (171)	NbzR	*P. putida* HS12/pNB1	64/114	AAK26517
				MarR	*Polaromonas* sp. strain JS666	38/100	EAM40420
orf1	1131–1550	Putative ferredoxin	15,130 (139)	ORF1	*P. pseudoalcaligenes* JS45	57/125	AAF03490
				NbzJ	*P. putida* HS12/pNB1	56/125	AAK26518
				NbzCa	*P. putida* HS12/pNB1	79/300	AAK26519
cnbCa	1623–2561	2-Aminophenol-1,6-dioxygenase beta subunit	35,037 (312)	AmnB	*P. pseudoalcaligenes* JS45	78/300	AAB71524
				AmnB	*Pseudomonas* sp. strain AP-3	80/295	BAB03531
				AmnA	*P. pseudoalcaligenes* JS45	60/271	AAB71525
cnbCb	2574–3389	2-Aminophenol-1,6-dioxygenase alpha subunit	29,264 (271)	NbzCb	*P. putida* HS12/pNB1	60/271	AAK26520
				AmnA	*Pseudomonas* sp. strain AP-3	58/271	BAB03532
				AmnC	*Pseudomonas* sp. strain AP-3	75/488	BAB03533
cnbD	4581–6053	2-Aminomuconic 6-semialdehyde dehydrogenase	54,026 (490)	NbzD	*P. putida* HS12/pNB1	75/487	AAK26521
				AmnC	*P. pseudoalcaligenes* JS45	74/320	AAC33839
cnbE	6197–7036	2-Keto-4-pentenoate hydratase	29,781 (279)	TdnG	*P. putida* UCC22	65/261	BAB62054
cnbF	7056–7844	4-Oxalocrotonate decarboxylase	28,077 (262)	CdoK	*Comamonas* sp. strain JS765	69/227	AAG17138
				AphH	*Comamonas testosteroni* TA441	69/227	BAA88505
cnbG	8097–8288	4-Oxalocrotonate tautomerase	7,158 (63)	AphI	*Comamonas testosteroni* TA441	76/63	BAA88507
cnbH	8728–10011	2-Amino-5-chloromuconic acid deaminase	44,966 (427)	GatA	*Agrobacterium tumefaciens* C58	38/371	NP_533743
cnbI	10054–10569	2-Oxopent-4-dienoate hydratase	18,459 (271)	CbzJ	*P. putida* GJ31	98/165	AAX38586
				TdnG	*P. putida* UCC22	37/166	BAB62054
cnbA	1869–2552	4-Chloronitrobenzene nitroreductase	25,967 (227)	NbzA	*P. putida* HS12/pNB1	92/227	AAK26512
				NbzA	*P. pseudoalcaligenes* JS45	88/227	AAT71308
cnbB	281–733	Hydroxylaminobenzene mutase	15,951 (150)	HabB	*P. pseudoalcaligenes* JS45	51/115	AAB94123
				NbzB	*P. putida* HS12/pNB2	50/115	AAK26516

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 *cnb* genes of strain CNB-1 indicated that these *cnb* 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 pET21a, 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*, *cnbCab*, 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-aminomuconic 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-$tRNA^{Gln}$ 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, pET*cnbH*, 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-aminomuconic 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

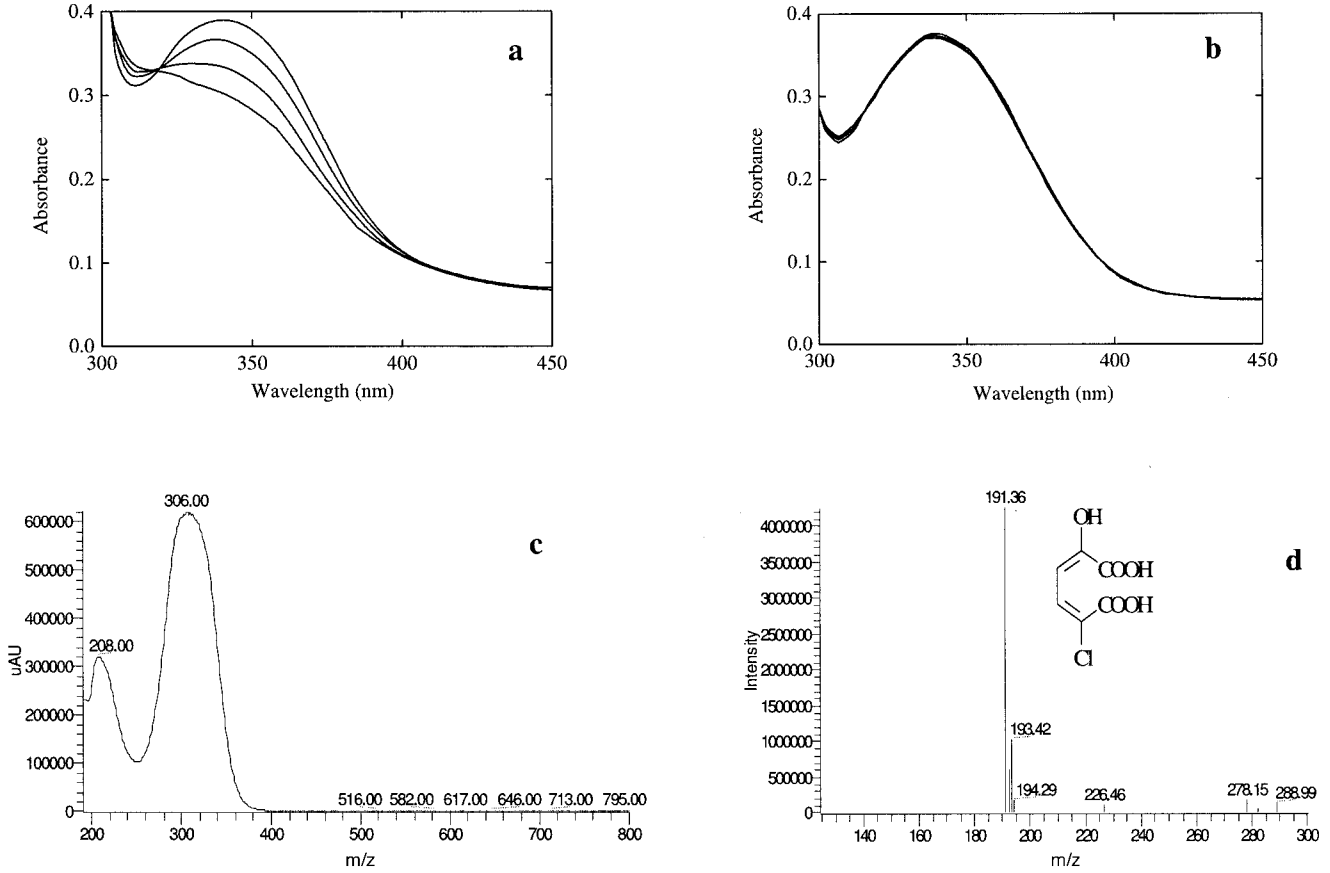

FIG. 2. Conversion of 2-amino-5-chloromuconic acid (2A5CM) into 2-hydroxy-5-chloromuconic acid (2H5CM), catalyzed by recombinant *E. coli*/pET*cnbH* that expressed 2-amino-5-chloromuconic acid deaminase (CnbH) (a) and by *E. coli*/pET28a as a control (b), and photospectrometry of 2-hydroxy-5-chloromuconic acid (c) and mass spectrometry of 2-hydroxy-5-chloromuconic acid (d). The photospectrum in a and b was recorded at 0 to 90 min after addition of cellular lysate. The specific activities for 2-aminomuconic acid were calculated to be 7 nmol min^{-1} (mg of protein)$^{-1}$ for recombinant *E. coli* expressing CnbH and 120 nmol min^{-1} (mg of protein)$^{-1}$ for *Comamonas* sp. strain CNB-1.

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

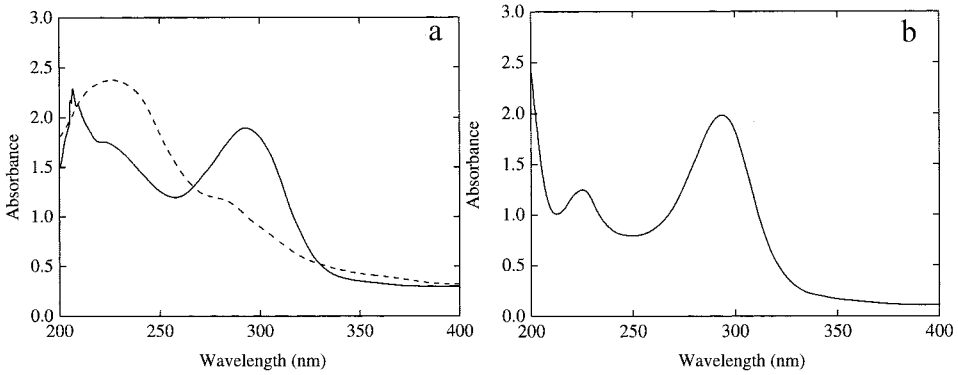

FIG. 3. Conversion of 2-hydroxymuconic acid into 2-oxalocrotonic acid catalyzed by recombinant *E. coli*/pET*cnbG* (a) and by *E. coli*/pET21a as a control (b). The photospectrum was recorded at time zero and after addition of cellular lysate for 5 seconds.

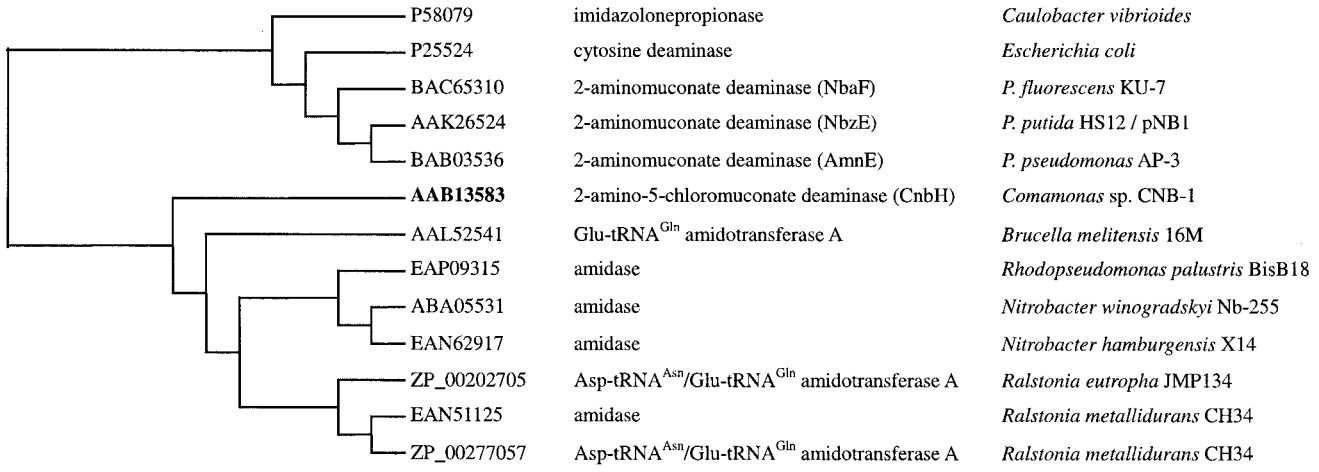

FIG. 4. Evolutionary dendrogram of deaminases involved in nitrobenzene and chloronitrobenzene degradation and homologs selected from results of a BLASTP search of GenBank, performed according to Altschul et al. (1). The phylogenetic tree was generated using the neighbor joining method of Saitou and Nei (26) with the AlignX software (Informax, Maryland), and multiple sequence alignment was done using ClustalX (37). The length of each branch pair represents the distance between the sequences.

via a partial reductive pathway that is similar to but different from that of the nitrobenzene degradative pathways in *Pseudomonas* sp. strains JS45 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 JS45 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^{Asn}/Glu-tRNA^{Gln} amidotransferase subunit A (31 to 38%).

Evolutionary analysis indicated that CnbH was more related to Asp-tRNA^{Asn}/Glu-tRNA^{Gln} amidotransferase 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 *nbzE*/*amnD* and amidotransferase genes are under investigation.

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