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Prokaryotic Homologs of the Eukaryotic 3-Hydroxyanthranilate 3,4-Dioxygenase and 2-Amino-3-Carboxymuconate-6-Semialdehyde Decarboxylase in the 2-Nitrobenzoate Degradation Pathway of *Pseudomonas fluorescens* Strain KU-7†

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The 2-nitrobenzoic acid degradation pathway of *Pseudomonas fluorescens* strain KU-7 proceeds via a novel 3-hydroxyanthranilate intermediate. In this study, we cloned and sequenced a 19-kb DNA locus of strain KU-7 that encompasses the 3-hydroxyanthranilate *meta*-cleavage pathway genes. The gene cluster, designated *nbaEX HJIGFCDR,* is organized tightly and in the same direction. The *nbaC* and *nbaD* gene products were found to be novel homologs of the eukaryotic 3-hydroxyanthranilate 3,4-dioxygenase and 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase, respectively. The NbaC enzyme carries out the oxidation of 3-hydroxyanthranilate to 2-amino-3-carboxymuconate-6-semialdehyde, while the NbaD enzyme catalyzes the decarboxylation of the latter compound to 2-aminomuconate-6-semialdehyde. The NbaC and NbaD proteins were overexpressed in *Escherichia coli* and characterized. The substrate specificity of the 23.8-kDa NbaC protein was found to be restricted to 3-hydroxyanthranilate. In *E. coli*, this enzyme oxidizes 3-hydroxyanthranilate with a specific activity of 8 U/mg of protein. Site-directed mutagenesis experiments revealed the essential role of two conserved histidine residues (His52 and His96) in the NbaC sequence. The NbaC activity is also dependent on the presence of Fe^{2+} but is inhibited by other metal ions, such as Zn^{2+}, Cu^{2+}, and Cd^{2+}. The NbaD protein was overproduced as a 38.7-kDa protein, and its specific activity towards 2-amino-3-carboxymuconate-6-semialdehyde was 195 U/mg of protein. Further processing of 2-aminomuconate-6-semialdehyde to pyruvic acid and acetyl coenzyme A was predicted to proceed via the activities of NbaE, NbaF, NbaG, NbaH, NbaI, and NbaJ. The predicted amino acid sequences of these proteins are highly homologous to those of the corresponding proteins involved in the metabolism of 2-aminophenol (e.g., AmnCDEFGH in *Pseudomonas* sp. strain AP-3). The NbaR-encoding gene is predicted to have a regulatory function of the LysR family type. The function of the product of the small open reading frame, NbaX, like the homologous sequences in the nitrobenzene or 2-aminophenol metabolic pathway, remains elusive.

There is a continuing need to isolate new microorganisms from the environment, not only because we want to enrich the little that we know about microbial diversity or community structure (41) but also because new microorganisms may have interesting new metabolic or biocatalytic properties. As this study illustrated, a soil pseudomonad can continue to amaze us because of its metabolic potential and can be a source for discovering new biocatalysts. Furthermore, the strain described here provides new insight into the possible evolutionary process of a catabolic pathway.

Pseudomonas fluorescens strain KU-7 was isolated from a petrochemical-contaminated site and is able to utilize 2-nitrobenzoic acid (2-NBA) as a sole source of carbon, nitrogen, and energy (17). A novel feature of the 2-NBA degradation pathway of strain KU-7 is the formation of 3-hydroxyanthranilate (3-HAA) as an intermediate (Fig. 1). Until now, 3-HAA was only known to be an intermediate in the kynurenine pathway for the degradation of tryptophan and the biosynthesis of nicotinic acid in yeast and mammalian systems (23). In *Arthrobacter protophormiae* strain RKJ100, there is an alternative route of 2-NBA metabolism (9). Although the initial reaction is also reductive, the conversion of 2-NBA to 2-hydroxylaminobenzoate was found to proceed via the formation of 2-aminobenzoate, which is also known as anthranilate. Subsequently, anthranilic acid is believed to go through deamination and β-ketoadipic acid formation before entering the Krebs cycle (9).

To gain a better understanding of the metabolism of 2-NBA in bacteria, in this study we performed the first analysis of a genetic locus of strain KU-7 that is responsible for the conversion of 3-HAA to Krebs cycle intermediates. We concentrated on the characterization of two novel encoding genes, *nbaC* and *nbaD*, and the evolution of the new pathway is discussed below in relation to convergence or divergence of the 2-aminophenol (2-AP) and nitrobenzene metabolic pathways in other bacteria.

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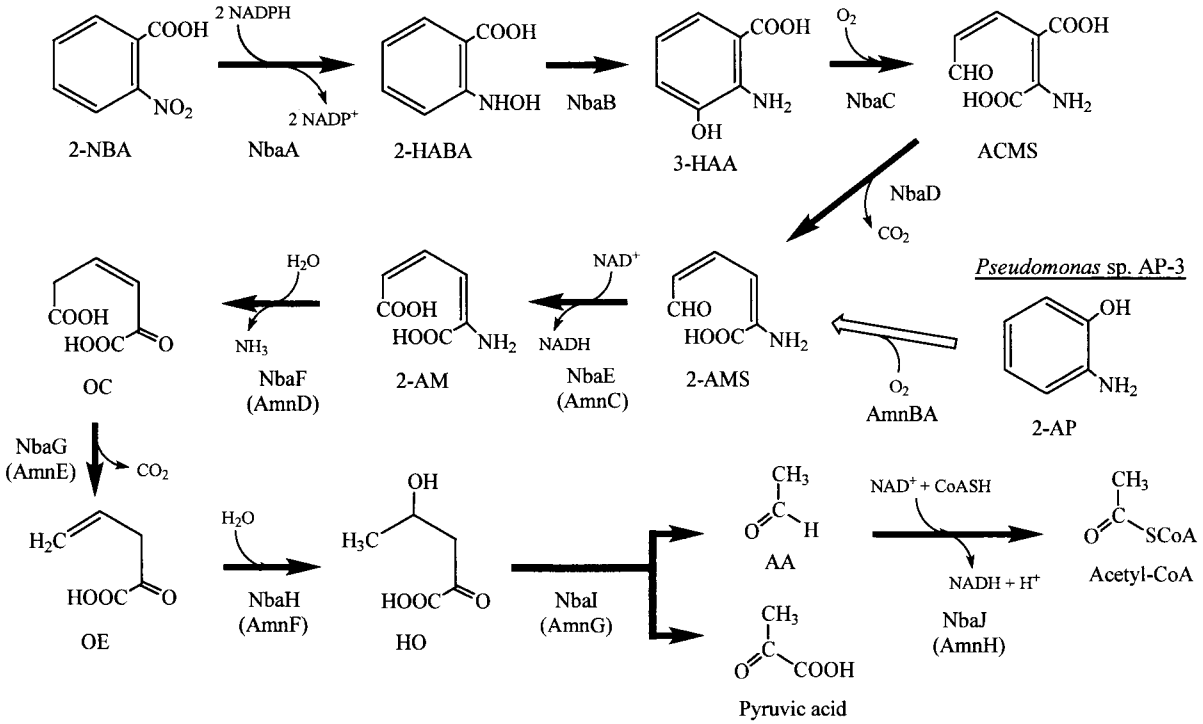

FIG. 1. Proposed pathway for degradation of 2-NBA by *P. fluorescens* strain KU-7. The responsible Nba enzymes are as follows: NbaA, 2-NBA nitroreductase; NbaB, 2-hydroxylaminobenzoate mutase; NbaC, HAO; NbaD, ACMSD; NbaE, 2-AMS dehydrogenase; NbaF, 2-aminomuconate deaminase; NbaG, 4-oxalocrotonate decarboxylase; NbaH, 2-oxopent-4-dienoate hydratase; NbaI, 4-hydroxy-2-oxovalerate aldolase; and NbaJ, acylating aldehyde dehydrogenase. For comparison, the 2-AP degradation pathway and enzymes of *Pseudomonas* sp. strain AP-3 are shown (37). AmnBA, 2-aminophenol 1,6-dioxygenase. Abbreviations: 2-HABA, 2-hydroxylaminobenzoate; 2-AM, 2-aminomuconate; OC, 4-oxalocrotonate; OE, 2-oxopent-4-dienoate; HO, 4-hydroxy-2-oxovalerate; AA, acetaldehyde; CoA, coenzyme A.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *Pseudomonas* strains were grown in Luria-Bertani (LB) broth, MY medium (17), or MS medium (MY medium without the yeast extract). *Escherichia coli* strains were routinely cultured in LB media. Cultures were incubated at 30°C for the *Pseudomonas* strains and at 37°C for the *E. coli* strains. Antibiotics were added at the following final concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; and trimethoprim 200 μg/ml.

Transposon mutagenesis and screening for 2-NBA-negative mutant strains. *E. coli* strain S17-1 carrying the Tn*5-31*Tp transposon on pKN31 (Table 1) was grown overnight with shaking at 37°C in LB medium containing kanamycin. Recipient strain KU-7 was grown at 30°C in LB medium. Donor and recipient cultures were mixed at different ratios and filtered onto a 0.45-μm-pore-size membrane (Nihon, Millipore Ltd., Tokyo, Japan). The membrane was placed with the bacterium side up on an LB medium plate. After overnight incubation at 30°C, the bacteria were resuspended in 10 ml of 0.85% NaCl and plated (200 μl/plate) on MS medium containing 0.2% 4-hydroxybenzoate, a growth substrate utilized by strain KU-7 but not by *E. coli* S17-1. Kanamycin was used as a selective marker for the transposon. Transconjugants of strain KU-7 were identified following incubation at 30°C for 2 days. Mutants that were not able to grow on 2-NBA were identified by replica plating on MS medium containing 0.2% 2-NBA and kanamycin. The production of ammonia, detected colorimetrically by the method of Fawcett and Scott (14), was used as a marker for the deamination step after ring cleavage of 3-HAA (Fig. 1).

DNA manipulations. Unless otherwise stated, standard methods were used for DNA manipulations (32). Total DNA was prepared by the method of Wilson (43), and plasmid DNA was isolated by the method of Birnboim and Doly (4). Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs (Beverly, Mass). Purified DNA fragments were labeled with digoxigenin (DIG)-11-dUTP by using a DIG DNA labeling kit (Roche Diagnostics K.K., Tokyo, Japan). For Southern blot experiments, DNA was transferred onto a positively charged nylon membrane (Hybond-N; Amersham Biosciences Corp., Piscataway, N.J.), and the DIG labels were visualized by using a DIG luminescent detection kit (Roche Diagnostics K.K.) according to the supplier's instructions.

DNA sequencing and sequence analysis methods. The nucleotide sequence was determined with an Applied Biosystems automated sequencer (model 310) by using a BigDye terminator cycle sequencing FS Ready Reaction kit (Applied Biosystems Japan Ltd., Tokyo, Japan). Synthetic primers were used for sequencing reactions. DNA sequences were analyzed with GENETYX-Mac software (Software Development Co., Ltd., Tokyo, Japan). Nucleotide and protein sequence similarity searches were done with the BLAST program (2) via the National Center for Biotechnology Information server. Amino acid sequences were analyzed with the Proteomics and Sequence Analysis Tools available at the Swiss Institute of Bioinformatics ExPASy web site. Multiple-protein-sequence alignments were constructed with the CLUSTAL W program (40) at the DNA Data Bank of Japan server.

Expression of *nbaC* and *nbaD* in *E. coli*. The *nbaC* gene, including its potential Shine-Dalgarno (SD) sequence, was amplified by PCR with the following pairs of primers having built-in *Eco*RI and *Pst*I restriction sites (underlined sequences): 5′-CGGAATTCAAGCAGTTGCCTACAAACC and 5′-AAAACTGCAGAAG TGCGAGTGCATATC. PCR amplifications were carried out in 50-μl reaction mixtures containing 0.1 μg of template DNA, 50 pmol of each primer, each deoxynucleoside triphosphate at a concentration of 200 μM, 5 μl of *Pfu* DNA polymerase 10× buffer with $MgSO_4$, and 1.25 U of *Pfu* DNA polymerase (Promega K.K., Tokyo, Japan). After a 3-min hot start at 94°C, each reaction mixture was subjected to 30 cycles of 30 s at 94°C, 30 s at 55°C, and 2 min at 72°C. The PCR product was purified from a 1.2% agarose gel, digested with *Eco*RI and *Pst*I, and ligated into the equivalent sites of pSD80, placing the genes of interest in frame with the *tac* promoter to form plasmid pHAO (Table 1). The insert was sequenced to ensure that mutations had not been incorporated during the PCR. A 100-ml culture of *E. coli* DH5α(pHAO) in LB broth containing 100 μg of ampicillin per ml was grown at 30°C to an A_{660} of 0.4 to 0.5, and then 1.0 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added and growth was continued for a further 3 h prior to harvesting.

For expression of *nbaD1* (the first possible ATG start codon of *nbaD*) in *E.*

TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Characteristics[a]	Source or reference
E. coli strains		
BL21	F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm*	Novagen
DH5α	*supE44 thi-1 recA1 hsdR17 endA1 gyrA* (Nal^r) Δ(*lacIZYA-argF*)*U169 deoR* [ϕ80*dlac*Δ(*lacZ*)M15]	32
S17-1	*recA pro thi hsdR* RP4-2-Tc::Mu-Km::Tn*7* Tra^+ Tp^r Sm^r	34
XL1-blue	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* [F′ *lacI*^{q} ZM15 Tn*10* (Tet^r)]	6
P. fluorescens strains		
KU-7	Wild type, grows on 2-NBA and 4-hydroxybenzoate	17
KUM-1	KU-7, *nbaJ*::Tn*5-31*Tp	This study
KUM-2	KU-7, *nbaR*::Tn*5-31*Tp	This study
KUM-3	KU-7, *nbaD*::Tn*5-31*Tp	This study
KUM-4	KU-7, *nbaF*::Tn*5-31*Tp	This study
Plasmids		
pHAO	*Eco*RI*-*Pst*I* fragment containing *nbaC* in pSD80	This study
pHAO-H52A	His52 (NbaC) is replaced by Ala by site-directed mutagenesis in pHAO	This study
pHAO-H93A	His93 (NbaC) is replaced by Ala by site-directed mutagenesis in pHAO	This study
pHAO-H96A	His96 (NbaC) is replaced by Ala by site-directed mutagenesis in pHAO	This study
pACMS1	*Eco*RI*-*Pst*I* fragment containing *nbaD1* (the first potential ATG codon) in pSD80	This study
pACMS2	*Eco*RI*-*Pst*I* fragment containing *nbaD2* (the second potential ATG codon) in pSD80	This study
pKN31	Km^r Tp^r; *mob* RP4 delivery plasmid for Tn*5-31*Tp	1
pNBA1	Ap^r Tp^r; pUC19 with 6.1-kb *Eco*RI fragment from *P. fluorescens* strain KUM-1	This study
pNBA2	Ap^r Km^r; pUC19 with 6.1-kb *Bam*H1 fragment from *P. fluorescens* strain KUM-1	This study
pNBA3	Ap^r; pUC19 with 9.3-kb *Hin*dIII fragment (1-9312) from *P. fluorescens* strain KU-7	This study
pNBA4	Ap^r; pUC19 with 8.4-kb *Hin*dIII fragment (10705-19065) from *P. fluorescens* strain KU-7	This study
pSD80	Ap^r; expression vector with *tac* promoter	35
pUC19	Ap^r; high-copy-number cloning vector	44

[a] The numbers in parentheses are the positions in the sequence deposited in the databases (DDBJ/GenBank/EMBL accession no. AB088043). *Eco*RI* and *Pst*I* are restriction endonucleases introduced by PCR design.

coli, the gene was amplified by PCR with forward primer 5′-CGGAATTCATG AAAAAACCGCGGATTGATA and reverse primer 5′-AAAACTGCAGACC ATTAAACATTGATATTG (the built-in *Eco*RI and *Pst*I sites are underlined). The *Eco*RI site was introduced into the forward primer sequence to optimize the space between the vector SD sequence and the possible ATG codon of *nbaD1*. For expression of *nbaD2* (the second possible ATG start codon of *nbaD*), the gene was amplified by using forward primer 5′-CGGAATTCATGCACTCGC ACTTCTTCC and reverse primer 5′-AAAACTGCAGACCATTAAACATT GATATTG. Again, the *Eco*RI site was introduced to optimize the space between the vector SD sequence and the possible ATG codon of *nbaD2*. PCR amplification was carried out as described above for *nbaC*. The resulting plasmids containing *nbaD1* and *nbaD2* were designated pACMS1 and pACMS2, respectively. Both inserts were sequenced to ensure that mutations had not been incorporated during the PCR. *E. coli* BL21 cells harboring pACMS1 or pACMS2 were grown and induced by using the same procedure that was used for *E. coli* DH5α(pHAO).

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by using the method of Laemmli (24) and a Mini-PROTEAN II electrophoresis cell (Nippon Bio-Rad Laboratories, Tokyo, Japan).

Determination of NH_2-terminal amino acid sequences. The cell extracts derived from *E. coli* DH5α(pHAO) were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane (MiniProBlott; Applied Biosystems Japan Ltd.) with a Mini Trans-Blot electrophoretic transfer cell (Nippon Bio-Rad Laboratories) as described by the manufacturer. The area on the membrane containing NbaC was cut out and subjected to N-terminal amino acid sequencing with a model PPSQ-21 protein sequencer (Shimadzu Co., Kyoto, Japan).

Preparation of cell extracts. Cells were harvested by centrifugation and washed twice with 50 mM MOPS (3-morpholinopropanesulfonic acid)–NaOH buffer (pH 6.5) for preparation of the NbaC protein or with 50 mM potassium phosphate buffer (pH 7.0) for preparation of the NbaD protein. They were resuspended in the same buffer at a concentration of approximately 0.2 g (wet weight)/ml. Cells were disrupted by sonication with a model 250 Sonifier (Branson, Danbury, Conn.) by using 40-s bursts on ice, and particulates were removed by centrifugation at 18,000 × *g* and 4°C for 30 min.

Enzyme activities. (i) HAO activity. 3-Hydroxyanthranilate 3,4-dioxygenase (HAO) activity was measured spectrophotometrically by monitoring the formation of 2-amino-3-carboxymuconate-6-semialdehyde (ACMS) at 360 nm by the method of Walsh et al. (42). The assay mixture (final volume, 1 ml) contained 3-HAA (0.1 μmol), $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (0.1 μmol), MOPS buffer (50 μmol, pH 6.5), and an aliquot of cell extract. The assay mixture was preincubated without substrate for 1 min at 25°C, and the reaction was started by adding 3-HAA and monitoring the increase in A_{360} for 20 s at 25°C. One unit of activity was defined as the amount of enzyme that produced 1 μmol of ACMS per min under the assay conditions. A molar extinction coefficient of 47,500 M^{-1} cm^{-1} for ACMS at 360 nm was used (22).

Relative rates for alternate substrates of NbaC were determined by using a Clarke-type oxygen electrode (YSI model 5300 biological oxygen monitor; YSI Inc., Yellow Springs, Ohio). The test compounds used were 3-amino-4-hydroxybenzoic acid, 4-aminoresorcinol, and 2-amino-*m*-cresol (purchased from Sigma-Aldrich Japan K.K., Tokyo, Japan); 4-amino-3-hydroxybenzoic acid, 3-aminosalicylic acid, 6-amino-*m*-cresol, 3-methylcatechol, 4-methylcatechol, 1,2,4-trihydroxybenzene, 2,3-dihydroxybenzoic acid, 4-amino-*m*-cresol, 5-aminosalicylic acid, gentisic acid, and homogentisic acid (obtained from Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan); and 2-AP, 2-amino-4-chlorophenol, 2-amino-*p*-cresol, catechol, 1,2,3-trihydroxybenzene, protocatechuic acid, and hydroquinone (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Each reaction mixture (3.0 ml) contained MOPS buffer (50 mM; pH 6.5), $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (0.1 mM), substrate (0.1 mM), and the appropriate amount of cell extract. The assay was started by adding the substrate, and the reaction mixture was incubated at 30°C. One unit of enzyme activity was defined as the amount of enzyme which catalyzed utilization of 1 μmol of O_2 per min under the conditions specified above. The rate of O_2 consumption caused by NbaC was calculated by subtracting the value for the control reaction mixture without NbaC.

(ii) ACMSD activity. 2-Amino-3-carboxymuconate-6-semialdehyde decarboxylase (ACMSD) activity was determined as described by Nishizuka et al. (28) by measuring the conversion of ACMS to 2-aminomuconate-6-semialdehyde (2-AMS) by using a preassay mixture that consisted of 25 μM 3-HAA, 0.1 mM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, and a solution of NbaC (39 mU/ml) prepared as described above in 50 mM MOPS buffer (pH 6.5). The reaction mixture was incubated at 25°C, and the increase in A_{360} due to the formation of ACMS from 3-HAA was monitored. After the reaction was completed, a solution of NbaD extract was added, and the decrease in A_{360} was monitored at 20-s intervals. One unit of ACMSD activity was defined as the amount of enzyme that converted 1 μmol of ACMS per min under the assay conditions. For calculation of activity,

a molar extinction coefficient of 47,500 M^{-1} cm^{-1} for ACMS at 360 nm was used (22). The rate of the decrease in absorbance caused by NbaD was calculated by subtracting the value for the control reaction mixture without NbaD.

Protein concentrations were determined by the Bradford assay (5) with bovine serum albumin (Pierce, Rockford, Ill.) as the protein standard.

Site-directed mutagenesis. PCR overlap extension mutagenesis (19) was used to generate *nbaC* mutants (Table 1). The following mutagenic primers were used (the base changes are underlined): for H52A, 5′-TCGCACAGATTTTGCG GATGATCCGATGGA and 5′-TCCATCGGATCATCCGCAAAATCTGT GCGA; for H93A, 5′-TTCTTCTGCCACCGGCGTTGCGCCACTCGC and 5′-GCGAGTGGCGCAACGCCGGTGGCAGAAGAA; and for H96A, 5′-CACC GCATTTGCGCGCGTCGCCACAACGAC and 5′-GTCGTTGTGGCGACG CGCGCAAATGCGGTG. The insert in pSD80 was sequenced to ensure that base changes had been introduced correctly.

Nucleotide sequence accession number. The DNA sequence encompassing the *nba EXHJIGFCDR* gene cluster (8.7 kb) and additional open reading frames (ORFs) (data not shown) on either side of the *nba* cluster (total, 19,065 bp) has been deposited in the DDBJ database under accession number AB088043.

RESULTS AND DISCUSSION

Isolation of 2-NBA-negative mutant strains. To provide a handle on the 2-NBA catabolic pathway gene locus, transposon mutagenesis was carried out to generate mutant derivatives of wild-type strain KU-7 that are unable to grow on 2-NBA as a sole carbon source. As a result, screening of approximately 3,000 transconjugants produced four candidates, designated strains KUM-1, -2, -3, and -4 (Table 1). Strain KUM-1 was analyzed first and was found to accumulate ammonia in the culture fluid (about two-thirds of the available nitrogen), indicating that the insertion of the transposon had inactivated the gene or genes involved after the deamination step. Subsequent DNA sequencing indicated that the *nbaJ* ORF had been disrupted (Fig. 1). The remaining three mutations were located in the predicted *nbaD*, *nbaF*, and *nbaR* ORFs as described below.

Cloning of the Tn*5-31*Tp disrupted regions. Insertion of the Tn*5-31*Tp transposon into strain KUM-1 was verified by Southern blot analysis of the total DNA digested with *Eco*RI and *Bam*HI. As a result, only one hybridization band (a 6.1-kb *Eco*RI fragment and a 6.1-kb *Bam*HI fragment), which was probed with a DIG-11-dUTP-labeled DNA probe (1.8-kb *Hin*dIII-*Bam*HI probe 1 or PCR-amplified probe 2 [Fig. 2]), was observed in each digest, indicating that a single insertion event had occurred (data not shown).

Cloning of the 6.1-kb *Eco*RI fragment or the 6.1-kb *Bam*HI fragment was carried out by using a pUC19 plasmid transformed in *E. coli* XL1-blue. In the former case, transformants were selected on trimethoprim- and ampicillin-containing plates. In the latter case, kanamycin and ampicillin were used as the selection markers. The resulting plasmids were designated pNBA1 and pNBA2, respectively. The pNBA1 insert contained a 4.5-kb Tn*5-31*Tp segment and 1.6 kb of KUM-1 DNA. Plasmid pNBA2 was found to contain a 3-kb Tn*5-31*Tp insert and 3.1 kb of KUM-1 DNA (Fig. 2). Further cloning of the DNA upstream of the pNBA1 insert resulted in plasmid pNBA3, which contained a 9.3-kb *Hin*dIII fragment. Cloning of DNA downstream of the pNBA2 insert resulted in plasmid pNBA4, which contained a 8.4-kb *Hin*dIII fragment (Fig. 2).

Identification of the gene disrupted by the transposon and structural analysis of the *nba* genes. We sequenced the Tn*5-31*Tp flanking regions in pNBA1 and pNBA2 with two oligonucleotides derived from IS*50*R and IS*50*L of the transposon, respectively (Fig. 2). Data analysis of the initial sequences revealed that the point of transposon disruption was in a locus, subsequently designated *nbaJ*, that had a high level of sequence similarity to the loci encoding the acylating acetaldehyde dehydrogenases in the database.

Further nucleotide sequencing and analyses of the four overlapping clones revealed 10 complete ORFs, which we designated *nbaEXHJIGFCDR*, all of which are oriented in the same direction and are organized in a tight manner. Either the intergenic spaces are short or the stop and potential start codons overlap, which is indicative of translational coupling (16) and an operon structure (Fig. 2).

Table 2 shows the location of each ORF or gene and the predicted molecular masses of the products, along with details for proteins in the National Center for Biotechnology Information nonredundant databases exhibiting significant similarity to the predicted gene products. Below we describe the novel characteristics of the *nba* gene cluster in the order of the predicted biochemical reaction (Fig. 1).

Identification of NbaC as HAO and its overexpression in *E. coli*. The deduced amino acid sequence of NbaC does not show any similarity to the amino acid sequences of the widely known extradiol dioxygenases and aminophenol dioxygenases, but instead it exhibits 33 to 37% identity to the amino acid sequences of the HAOs (EC 1.13.11.6), all of which have eukaryotic origins (Table 2). Among the human liver, rat, mouse, sponge, worm, and yeast proteins (20, 23, 26, 27, 39), only the human and yeast proteins have been characterized genetically and biochemically. The human HAO is a 286-amino-acid enzyme that is involved in the synthesis of quinolinic acid from 3-HAA, an intermediate in the kynurenine pathway of tryptophan metabolism (26). On the other hand, the yeast HAO (Had1) is shorter; it is 177 amino acids long, which is similar to the length of the 185-amino-acid NbaC protein. A multiple-sequence alignment of these sequences revealed that the N terminus for the first 120 amino acids was more conserved than the C terminus, although the sequences of the mouse (accession no. AK002608), rat (D44494), human (Z29481), sponge (AJ298053), and worm (Z70751 and Z70755) proteins are more closely related to each other (86 to 93% identity among the mouse, rat, and human sequences; 38 to 48% identity between the sponge and worm sequences).

The DNA sequence predicts that there are two consecutive ATG codons that are possible *nbaC* start codons. As a result of overexpression of the *nbaC* gene in pHAO, we established that the first 20 amino acids of NbaC are MFTFGKPLNFQRWLD DHSDL, a sequence that confirms the DNA prediction and the notion that *nbaC* is translated from the first ATG. By using SDS-PAGE, the molecular mass of NbaC was estimated to be 23.8 kDa, which is in good agreement with the predicted molecular mass (21,245 Da) (Fig. 3, lane 2).

Substrate specificity of NbaC. The specific activity of NbaC toward 3-HAA was found to be 7.9 U/mg of protein. No activity was observed with the extract from *E. coli* DH5α carrying pSD80. We next examined the substrate specificity of NbaC using a variety of aminophenol and catechol analogs as described in Materials and Methods. Briefly, NbaC was found to be specific for the substrate 3-HAA, which gave an oxygen consumption rate of 10.9 U/mg, whereas all other activities were not detectable.

Requirement of histidine residues 52 and 96 and ferrous

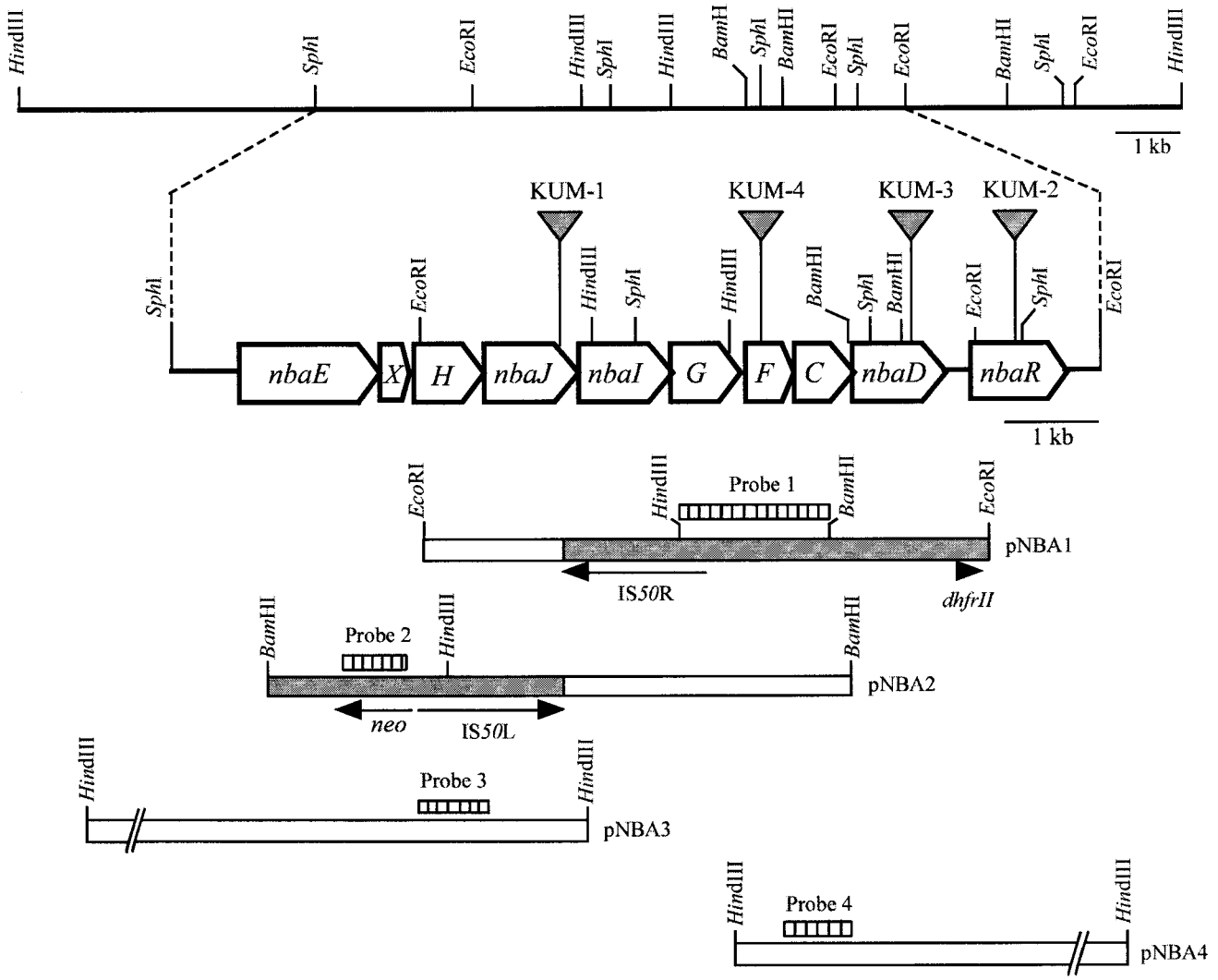

FIG. 2. Physical map and genetic organization of the *P. fluorescens* strain KU-7 *nba* gene cluster. The large open arrows indicate the unidirectional arrangement of the genes or ORFs described in Table 2. The following gene pairs have overlapping stop and potential start codons: *nbaJ* and *nbaI* (8-bp overlap); *nbaI* and *nbaG* (4-bp overlap); *nbaF* and *nbaC* (1-bp overlap); and *nbaC* and *nbaD* (4-bp overlap). Otherwise, there is a short intergenic space, as follows: *nbaE* and *nbaX* (11-bp space); *nbaX* and *nbaH* (10-bp space); *nbaH* and *nbaJ* (6-bp space); *nbaG* and *nbaF* (21-bp space); and *nbaD* and *nbaR* (284-bp space). A potential promoter consensus sequence (−10 sequence, TAAAAT; −35 sequence, TTGAAT or TTTGCT) is predicted immediately upstream (35 bp) of the start codon of *nbaE*. Following *nbaD*, two inverted repeat sequences may act as transcriptional terminators of a potential operon. The gray triangles indicate the sites of transposon insertion (*nbaJ* codon 286, *nbaR* codons 181 and 182, *nbaD* codon 228, and between codons 80 and 81 of *nbaF* in mutant strains KUM-1, KUM-2, KUM-3, and KUM-4, respectively). The open boxes in pNBA1 and pNBA2 were derived from KUM-1 DNA; pNBA3 and pNBA4 were derived from KU-7 DNA. The grey region indicates the Tn*5-31*Tp DNA. Probes 1, 2, 3, and 4 were used in the hybridization experiments. *dhfrII* and *neo* are trimethoprim resistance- and kanamycin resistance-encoding genes, respectively.

iron for NbaC activity. A large number of *meta*-ring cleavage dioxygenases have been characterized, and these enzymes have conserved histidine residues that function as ferrous iron ligands for catalytic activity (12, 36). An alignment of the NbaC sequence with the sequences of eukaryotic HAOs revealed that two histidine residues (His52 and His96 in NbaC) are conserved in all sequences (data not shown). To test whether these residues are required for activity, we generated *nbaC* mutants that resulted in His-to-Ala substitutions (H52A and H96A). In addition, we carried out mutagenesis of His93 since this residue is adjacent to His96. As a result, even though the amounts of the desired proteins produced were equivalent to the amount of unmodified NbaC produced (data not shown), both mutations resulted in no detectable NbaC activity. On the other hand, HAO with the H93A substitution was found to exhibit 24.8% of the activity of the native HAO, indicating that His93 is not as critical as His96 or His52.

We also tested the effects of chelating agents, chemical modification reagents, and metal ions on the HAO activity (Table 3). The activities of NbaC were completely eliminated by the presence of 2,2′-dipyridyl and 1,10-phenanthroline, which are Fe^{2+} chelators. This indicated that NbaC is dependent upon Fe^{2+} for catalytic activity. Diethyl pyrocarbonate, which has previously been shown to modify histidine residues of catechol

TABLE 2. Predicted functions of the proteins encoded by the genes located in the *nba* gene cluster

Gene	Nucleotide positions[a]	Predicted function	Deduced molecular mass (Da)	Homology			
				% Amino acid identity	Protein(s)	Source	Accession no.
nbaE	5394-6896	2-Aminomuconate 6-semi-aldehyde dehydrogenase	53,678 (498)[b]	59	DmpC	*Pseudomonas* sp. strain CF600	X52805
				57	AmnC	*Pseudomonas* sp. strain AP-3	AB020521
nbaX	6908-7300	Unknown	13,839 (130)	50	ORF1 (YjgF-like protein)	*P. pseudoalcaligenes* JS45	AF036343
				50	NbzI (putative ferredoxin)	*P. putida* HS12	AF319593
nbaH	7311-8102	2-Oxopent-4-dienoate hydratase	27,928 (263)	61	AmnF	*Pseudomonas* sp. strain AP-3	AB020521
				60	NbzG	*P. putida* HS12	AF319593
nbaJ	8109-9071	Acetaldehyde dehydrog-enase	33,394 (320)	71	NbzI	*P. putida* HS12	AF319593
				68	AmnH	*Pseudomonas* sp. strain AP-3	AB020521
				68	CarF	*Pseudomonas* sp. strain CA10	AB047548
nbaI	9064-10092	4-Hydroxy-2-oxovalerate aldolase	36,715 (342)	82	MhpE	*Comamonas testosteroni* TA441	AB024335
				81	TobH	*P. putida* PB4071	AF180147
nbaG	10089-10844	4-Oxalocrotonate decarboxylase	27,011 (251)	50	NbzF	*P. putida* HS12	AF319593
				49	XylI	*Sphingomona aromaticivorans* F199	AF079317
nbaF	10866-11297	2-Aminomuconate deaminase	15,549 (143)	72	AmnD	*Pseudomonas* sp. strain AP-3	AB020521
				70	NbzE	*P. putida* HS12	AF319593
				70	AmnD	*P. pseudoalcallgenes* JS45	P81593
nbaC	11297-11854	HAO	21,245 (185)	37	Possible HAO	*Caenorhabditis elegans*	Z70751 and Z70755
				35	HADI	*Saccharomyces cerevisiae*	Z49525
				33	HAO	*Rattus norvegicus*	D44494
nbaD	11851-12855	ACMSD	37,140 (334)	40	ACMSD	*Rattus norvegicus*	AB069781
				40	ACMSD	*Homo sapiens*	AB071418
nbaR	13140-14099	Regulatory protein	35,117 (319)	31	Probable transcriptional regulatory protein	*P. aeruginosa* PAO1	AE004647
				31	FldY	*Xanthomonas campestris* ATCC 33913	AE012524

[a] The numbers are the positions in sequence deposited in the databases (DDBI/GenBank/EMBL accession no. AB088043).
[b] The values in parentheses are the numbers of amino acid residues.

dioxygenases (36), also inhibited the HAO activity (Table 3). Complete inactivation by dithionitrobenzoic acid, a cysteine-directed reagent, indicated that there is a cysteine residue at or near the active site(s) of the enzyme.

The effects of metal ions on HAO activity were investigated by introducing the metals into the assay mixture following addition of the divalent iron cofactor, which initiated catalytic activity. Table 3 shows that complete or nearly complete inhibition of HAO activity was caused by the presence of 0.1 mM Cu^{2+}, 0.1 mM Zn^{2+}, 0.1 mM Cd^{2+}, or 0.1 mM Fe^{3+}. At a concentration of 1 mM, Ni^{2+} and Co^{2+} were also quite inhibitory, although Ca^{2+}, Mg^{2+}, Mo^{2+}, and Pb^{2+} had little or no effect. Presumably, some of these metals can bind to the active site or block the access of iron to the active site.

Inhibition of human HAO by Zn^{2+} and Cd^{2+} has been reported recently, and the former effect was interpreted to indicate the possible physiological relevance of zinc in the metabolism of human cerebral cells (8).

Identification of NbaD as ACMSD and its expression in *E. coli*. Like the NbaC sequence, the sequence of NbaD has no prokaryotic counterpart in the available databases except for a few eukaryotic homologs. The deduced amino acid sequence of NbaD exhibits 31 to 40% overall identity to the rat, mouse, and human ACMSD (EC 4.1.1.45) sequences (15, 38). *Caenorhabditis elegans* has two ACMSD sequences; one (Y71D11A.3b; accession no. AC006816) consists of 401 amino acids, while the other (Y71D11A.3a; accession no. AC006816) has 352 amino acid residues, a size that is more consistent with the sizes of the other sequences (304 to 336 amino acids). We confirmed the finding of Fukuoka et al. (15) that no typical consensus sequences for nucleotide, metal, or any other cofactor binding sites were detectable with motif search programs. However, a multiple-sequence alignment of these sequences with the NbaD sequence revealed the following two conserved peptides that are at least 6 amino acids long: VHPWDM (amino acids 176 to 181 [NbaD numbering]) and LG(S/T)DYPFP

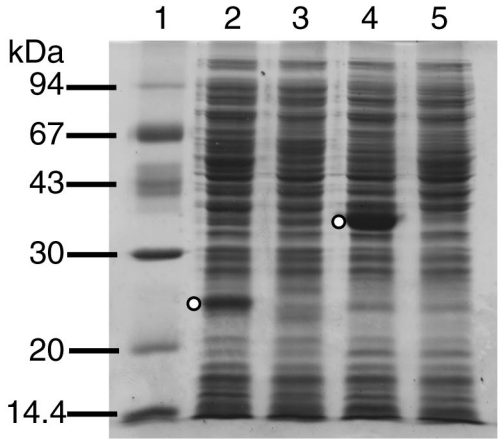

FIG. 3. Expression of the *nbaC* and *nbaD* gene products of *P. fluorescens* strain KU-7 by recombinant *E. coli* strains by using P_{tac} induction. An SDS–12% PAGE gel was used and stained with Brilliant blue R (Sigma). The open circles in lanes 2 and 4 indicate the positions of the overexpressed NbaC and NbaD proteins, which had estimated molecular masses of 23.8 and 38.7 kDa, respectively. Lane 1, LMW marker (Amersham Biosciences); lane 2, cell extracts of *E. coli* DH5α (pHAO); lane 4, cell extracts of *E. coli* BL21(pACMS1); lanes 3 and 5, controls consisting of *E. coli* DH5α and BL21 cells containing only the pSD80 vector.

TABLE 3. Effects of chelating agents, chemical modification reagents, and metal ions on NbaC[a]

Reagent added	Concn (mM)	Remaining activity (%)
None		100
Fe^{2+} chelators		
2,2′-Dipyridyl	1.0	0
1,10-Phenanthroline	1.0	0
Nonspecific chelator EDTA	1.0	1
Cysteine-directed reagents		
DTNB[b]	1.0	0
Sodium iodoacetate	1.0	58
Histidine-directed reagent diethyl pyrocarbonate	1.0	30
Carboxyl-directed reagent EDC[c]	1.0	74
Metal ions		
Mo^{2+}	1.0	85
Mg^{2+}	1.0	98
Ca^{2+}	1.0	105
Fe^{3+}	0.1	18
Ni^{2+}	1.0	18
Co^{2+}	1.0	8
Mn^{2+}	1.0	54
Cu^{2+}	0.1	0
Zn^{2+}	0.1	1
Cd^{2+}	0.1	<1
Pb^{2+}	1.0	76

[a] Each reagent was added to the assay mixture after catalytic activity was initiated by the introduction of Fe^{2+}, and it was preincubated for 30 min at 25°C prior to the assay.
[b] DTNB, dithionitrobenzoic acid.
[c] EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide methiodide.

LGE (amino acids 291 to 301). These peptides may be regarded as signature peptides for this group of proteins.

There are two potential ATG start codons for *nbaD*, both of which are preceded by a consensus ribosome binding sequence (GGAGA for *nbaD1* and GGA for *nbaD2*). The two methionines are 6 amino acids apart. To examine which of the two methionines could be translated into protein, PCR-amplified *nbaD* was expressed in *E. coli* in two possible constructs, pACMS1 and pACMS2, as described in Materials and Methods. The cellular protein extracts were analyzed by SDS-PAGE. As a result, only pACMS1 yielded a polypeptide (M_r, 38,700) of the expected size (Fig. 3, lane 4), indicating that the first methionine is the start codon.

The cellular extract of *E. coli* BL21(pACMS1) gave an ACMSD activity of 194.9 U/mg of protein. For comparison, the specific activities of ACMSD prepared from crude rat liver and kidney homogenate were 1.8 and 4.6 mU/mg, respectively (38).

In the course of transposon tagging experiments, *nbaD*::Tn*5-31*Tp (strain KUM-3) was obtained. Sequencing revealed that the transposon was inserted at codon 228 of *nbaD* (data not shown). This indicated that ACMS was not utilized because of the absence of a functional NbaD in the KUM-3 cells.

2-AMS is a convergent point in nitrobenzene metabolism, 2-AP metabolism, and 3-HAA metabolism. 2-AMS is emerging as a common intermediate compound in the nitrobenzene pathway in *Pseudomonas putida* HS12 (30) and *Pseudomonas pseudoalcaligenes* JS45 (18), the 2-AP pathway in *Pseudomonas* sp. strain AP-3 (37), and the 2-NBA pathway in *P. fluorescens* strain KU-7 (17) (Fig. 1). This is reminiscent of the formation of 2-hydroxymuconate semialdehyde as a result of *meta*-cleavage of catechols (e.g., from the metabolism of xylenes, phenol, and related aromatic hydrocarbons) (21).

In a previous study a particular route for the 2-NBA degradation pathway in strain KU-7 beyond the ACMS formation step was not established (17). However, based on the extensive homology between the predicted gene products of *nbaEFGHIJ* and a familiar set of homologs in the databases (Table 2) and the inherent sequence motifs (data not shown), one could predict that further metabolism of 2-AMS in strain KU-7 proceeds in the same manner as the metabolism encoded by the nitrobenzene catabolic genes (*nbz*) and the 2-AP *meta*-cleavage genes (*nbzDEFGHI*) in *P. putida* HS12 (30). In *Pseudomonas* sp. strain AP-3, the corresponding genes of the 2-AP pathway are *amnCDEFGH* (37).

Elusive gene X and a potential regulatory gene, *nbaR*. The small ORF between *nbaE* and *nbaH* was designated *nbaX*. The predicted amino acid sequence has 50% identity with the amino acid sequence of a YjgF-like protein (ORF1) from *Pseudomonas pseudoalcaligenes* JS45 and with the amino acid sequence of NbzJ from *P. putida* HS12 (Table 2 and Fig. 4). Kim et al. (30) proposed that the latter compound is a possible ferredoxin. We were unable to confirm this by motif searches. Instead, the sequence of NbaX (residues 103 to 121) was found to match the consensus sequence pattern of the YjgF-like family protein (Prosite accession no. PS01094; pfam01042) (3, 13). Hence, the possible function of NbaX and its counterparts in the nitrobenzene or aminophenol pathways remains unclear.

One of the transposon mutant strains generated in this study was KUM-2 (Table 1). Like the other mutant strains, KUM-2 was deficient in terms of utilizing 2-NBA as a sole source of carbon and energy. By using DNA sequencing, the site of transposon insertion was found to be located between codons 181 and 182 of an ORF designated *nbaR* (Fig. 2). The predicted amino acid sequence of NbaR indicates that the molecular mass is 35,117 Da (319 amino acids), which is similar to the molecular masses of the LysR-type transcriptional regulators (33). Moreover, the N-terminal NbaR sequence (residues 31 to 61) possesses sequence features of the LysR-type transcriptional regulator helix-turn-helix motif (Prosite accession no. PS00044). In addition, it is possible to locate the consensus binding motif of LysR-type regulators (viz., the T-N_{11}-A sequence) upstream of the regulated promoter. Specifically, this sequence (TCTGGTATT-N_9-AATAACTGA [inverted repeat sequences are underlined]) is located 78 bp upstream of the possible ATG start of *nbaE*, presumably the first gene of the *nba* operon (Fig. 2). Because of the numerous characteristics that have been determined and the transposon mutagenesis data, NbaR is expected to be a bona fide positive regulator of the *nba* pathway. A detailed study of NbaR regulation was outside the scope of this study.

Concluding remarks. A molecular view of 2-NBA metabolism in bacteria is emerging for the first time. Although the initial reductase- and 2-hydroxylaminobenzoate mutase-encoding genes in the 2-NBA pathway of strain KU-7 have not been characterized yet (they were not found in the 19-kb region sequenced), this study nonetheless filled a void in our knowledge of microbial degradation of mononitrobenzoates or nitroaromatics in general (for a review see reference 45). Besides the two known routes of 2-NBA metabolism, one via the formation of anthranilic acid in *A. protophormiae* (9) and the

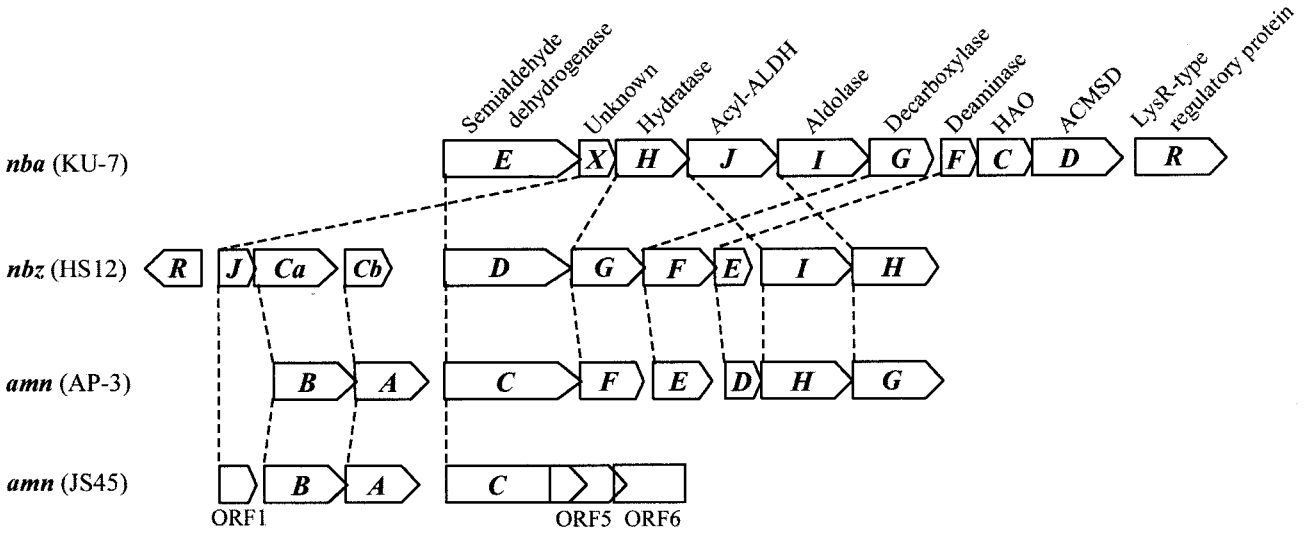

FIG. 4. Comparison of the *nba* gene cluster of strain KU-7 with the nitrobenzene degradative genes (*nbz*) of *P. putida* HS12 (30) and the 2-AP degradative genes (*amn*) of *Pseudomonas* sp. strain AP-3 (37) and *P. pseudoalcaligenes* JS45 (11). Homologous genes are connected by dashed lines. Gene designations: *nbaE*, *nbzD*, and *amnC*, 2-aminomuconate 6-semialdehyde dehydrogenase genes; *nbaX* and ORF1, YjgF-like protein genes; *nbzJ*, putative ferredoxin gene; *nbaH*, *nbzG*, and *amnF*, 2-oxopent-4-dienoate hydratase genes; *nbaJ*, *nbzI*, and *amnH*, acetaldehyde dehydrogenase genes; *nbaI*, *nbzH*, and *amnG*, 4-hydroxy-2-oxovalerate aldolase genes; *nbaG*, *nbzF*, and *amnE*, 4-oxalocrotonate decarboxylase genes; *nbaF*, *nbzE*, and *amnD*, 2-aminomuconate deaminase genes; *nbaC*, HAO gene; *nbaD*, ACMSD gene; *nbaR*, LysR-type transcriptional regulatory gene; *nbzR*, MarR-type transcriptional regulatory gene; *nbzCa* and *amnB*, genes encoding the β-subunit of 2-aminophenol 1,6-dioxygenase; *nbzCb*, and *amnA*, genes encoding the α-subunit of 2-aminophenol 1,6-dioxygenase; ORF5 and ORF6 (incomplete ORF), genes similar to the genes encoding the ATP-dependent RNA helicases.

other via the formation of 3-HAA (this study), it has been suggested that 2-NBA degradation in *Nocardia* could occur through an oxidative route via catechol formation, following an initial release of nitrite from 2-NBA (7). However, there has been no confirmatory data for this putative Cain pathway. Nevertheless, it is clear that bacteria can evolve multiple ways of degrading 2-NBA, just as there are at least five ways in which aerobic bacteria can metabolize toluene. Importantly, different gene families may be involved in the regulation of these pathways (25). In this study, we propose a LysR-type transcriptional regulation for 2-NBA metabolism in strain KU-7. The lack of molecular data for the anthranilate pathway of *A. protophormiae* prevents a comparative analysis at this time. However, given the convergence of 2-AP metabolism as shown by the formation of the common 2-AMS intermediate (Fig. 1), it is worth noting that it has been proposed that the regulatory unit for 2-AP metabolism in *P. putida* HS12 (*nbzR*) was derived from the MarR family of regulators (30).

The genes of the nitrobenzene and 2-AP pathways provide valuable insight into both convergence and divergence pertaining to the intermediate steps (Fig. 1). The steps in the AP-3 and HS12 strains are rather similar (Fig. 4). In terms of pathway convergence, one step is at the level of 2-AMS formation, the result of decarboxylation of ACMS by ACMSD (NbaD) in strain KU-7 and dioxygenation of 2-AP by 2-aminophenol 1,6-dioxygenase in strain AP-3 (37). In the case of nitrobenzene degradation in strain JS45, 2-AP is formed after the action of a nitrobenzene nitroreductase and a hydroxylaminobenzene mutase(s). Although the genes encoding the mutases in strain JS45 have been cloned, other genes are not yet available (10). A second level of convergence is at the formation of 2-oxopent-4-dienoic acid or its isomer, 2-hydroxypenta-2,4-dienoate. Indeed, degradation of these isomers is known to proceed by means of a rather universal and common set of enzymes, including a hydratase, an aldolase, and an acylating dehydrogenase that are present in the pathways for degradation of aromatic hydrocarbons in general (31).

It has been proposed that the 2-AP operon of *P. putida* HS12 has evolved in a modular fashion involving transfer and fusion of regulatory and structural units of *meta*-cleavage genes (30). A similar mode of gene acquisition may be responsible for the nature of the *nba* gene organization in strain KU-7.

Once again, a soil pseudomonad has been found to display diversity in its catabolic potential and to provide a unique link to tryptophan metabolism in eukaryotes insofar as HAO- and ACMSD-encoding genes are concerned. These enzymes have been studied because they may have relevance to the progression of Huntington's disease and related diseases according to the so-called quinolinate hypothesis (15). Briefly, the connection is as follows. In mammalian cells, quinolinate is a potent endogenous excitotoxin of neuronal cells, and it can be nonenzymatically derived from ACMS. As in bacterial 2-NBA metabolism, ACMS is generated from 3-HAA via the action of HAO. The HAO activity in the brains of Huntington's disease patients has been found to be elevated and to be 3.5-fold higher than the activity in normal controls, suggesting that there is an association between the formation of quinolinate and the disease. Furthermore, ACMSD has been postulated to play an important role in preventing progression of the disease by diverting ACMS to a nonbenign metabolite, which is 2-AMS (15). The possible contribution of NbaD as a prokaryotic enzyme supplement is exciting.

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