Characterization of a Pseudomonad 2-Nitrobenzoate Nitroreductase and its Catabolic Pathway Associated 2-Hydroxylaminobenzoate Mutase and a Chemoreceptor Involved in 2-Nitrobenzoate Chemotaxis†

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Running title: 2-NITROBENZOATE METABOLISM AND CHEMOTAXIS

1

1   *Pseudomonas fluorescens* strain KU-7 is a prototype microorganism that

2   metabolizes 2-nitrobenzoate (2-NBA) via the formation of 3-hydroxyanthranilate

3   (3-HAA), a known antioxidant and reductant. The initial two steps leading to the

4   sequential formation of 2-hydroxyaminobenzoic acid (2-HABA) and 3-HAA are

5   catalyzed by a NADPH-dependent 2-nitrobenzoate nitroreductase (NbaA) and

6   2-hydroxylaminobenzoate mutase (NbaB), respectively. The 216-amino acid

7   protein NbaA is 78% identical to a plasmid-encoded hypothetical conserved

8   protein of *Polaromonas* strain JS666; structurally it belongs to the homodimeric

9   NADH:FMN oxidoreductase-like fold family. Structural modeling of complexes

10   with the flavin, coenzyme and substrate suggested specific residues contributing

11   to the NbaA catalytic activity, assuming a ping-pong reaction mechanism.

12   Mutational analysis supports the roles of Asn40, Asp76 and Glu113 predicted to

13   form the binding site for a divalent metal ion implicated in FMN binding, and a

14   role in NADPH binding for the 10-residue insertion in the $\beta5$-$\alpha2$ loop. The

15   181-amino acid sequence of NbaB is 35% identical to the

16   4-hydroxylaminobenzoate lyases (PnbBs) of various 4-nitrobenzoate assimilating

17   bacteria, e.g., *P. putida* strain TW3. Co-expression of *nbaB* with *nbaA* in

18   *Escherichia coli* produced a small amount of 3-HAA from 2-NBA, supporting the

19   functionality of the *nbaB* gene. We also showed by gene knockout and chemotaxis

20   assay that *nbaY*, a chemoreceptor NahY homolog located downstream of the *nbaA*

21   gene, is responsible for strain KU-7 to be attracted toward 2-NBA. NbaY is the

22   first identified chemoreceptor in nitroaromatic metabolism, and this study

23   completes the gene elucidation of 2-NBA metabolism that is localized within a

24   24-kb chromosomal locus of strain KU-7.

Nitroreduction, catalyzed by the NAD(P)H-dependent nitroreductases, is the quintessential first step in the catabolism of a variety of structurally diverse nitroaromatic compounds. The commonly known oxygen-insensitive and type I nitroreductases are flavoproteins that mediate the sequential transfer of two electrons from NAD(P)H to the nitro group to produce nitroso and hydroxylamine derivatives (9). From the hydroxylamine intermediate, ammonium ions are subsequently removed through two possible routes: a lyase-mediated reaction with the direct elimination of ammonia to produce the corresponding catechol (e.g. degradation of 4-nitrobenzoate, 4-nitrotoluene, and 3-nitrophenol); and a mutase-mediated Bamberger-type rearrangement to produce 2-aminophenols or benzoates resulting in a much later release of ammonia (e.g., metabolism of nitrobenzene, 3-nitrophenol, 4-chloronitrobenzene, 4-nitrotoluene, and 2,4,6-trinitrotoluene). For recent reviews see Esteve-Nunez et al (20) and Nishino and Spain (51).

The metabolism of 2-nitrobenzoate (2-NBA) by *Pseudomonas fluorescens* strain KU-7 afforded the first example in a prokaryotic organism for the formation of 3-hydroxyanthranilate (3-HAA), a metabolite that is otherwise well known in the kynurenine pathway of tryptophan metabolism, and the biosynthesis of nicotinic acid in yeast and mammalian systems (27, 49, 53). This organism is unable to grow on 3-nitrobenzoate or nitrophenols as sole carbon or nitrogen source (27). Recently, Colabroy and Begley (15) identified a new tryptophan catabolic pathway in *Burkholderia cepacia* J2315 that includes the formation of 3-HAA. Crude extracts of strain KU-7 cells converted 2-NBA to 3-HAA with oxidation of 2 mol of NADPH. In this pathway, 2-NBA is first reduced to 2-hydroxyaminobenzoic acid (2-HABA) by an uncharacterized NADPH-dependent reductase, followed by a mutase-catalyzed conversion of 2-HABA to 3-HAA via a Bamberger-type rearrangement (27). Previously, we have described the genetic locus, consisting of *nbaEXHJIGFCDR*, that

3

1   is responsible for the conversion of 3-HAA to Krebs cycle intermediate in strain KU-7

2   (49). A highlight of this pathway is establishment of the novel identity of *nbaC*

3   encoding 3-hydroxyanthranilate 3,4-dioxygenase, and *nbaD* encoding

4   2-amino-3-carboxymuconate 6-semialdehyde decarboxylase, that catalyze respectively,

5   the ring-opening of 3-HAA to form 2-amino-3-carboxymuconate-6-semialdehyde and

6   a decarboxylation step to form 2-aminomuconate-6-semialdehyde (49). The latter

7   compound is a convergent metabolite in the metabolism of nitrobenzene,

8   2-aminophenol, 3-HAA and 4-amino-3-hydroxybenzoic acid (49, 52).

9   To complete the full gene complement for 2-NBA metabolism in strain KU-7, we

10   describe here the gene localization and characterization by nucleotide sequencing and

11   functional analysis of initial genes *nbaA* and *nbaB* encoding 2-NBA nitroreductase

12   and 2-HABA mutase, respectively. In particular, a homology model of NbaA was built

13   to probe the structure-function relationship of this protein. Additionally, we

14   determined the chemotactic property of a methyl-accepting chemotaxis transducer-like

15   open-reading frame (*nbaY*) that is located downstream of *nbaA*. This study has also

16   led to the discovery of a related gene locus in the *Polaromonas* sp. JS666 genome

17   (Joint Genome Institute [JGI] database), an organism known to degrade

18   *cis*-dichloroethene. The possible mobility of the *nba* gene locus is discussed.

19   (A portion of this work was presented at the American Society for Microbiology 9th

20   International Conference on *Pseudomonas*, Quebec City, Canada, 6 to10 September,

21   2003).

22

23   **MATERIALS AND METHODS**

24   **Chemicals.** 2-HABA were synthesized chemically as described by Bauer and

25   Rosenthal (5). All other chemicals were of the highest purity commercially available.

26   **Bacterial strains, culture conditions, and plasmids.** The bacterial strains and

4

plasmids used in this study are listed in Table 1. The culture conditions for the *Pseudomonas* and *E. coli* strains, their growth media except for MS-S medium (MY medium without the yeast extract and 2-NBA but replaced with 0.3% succinate) and antibiotic selection, are as previously described (27, 49).

DNA manipulations, sequencing and analysis. Chromosomal and plasmid DNA isolation used the methods of Wilson, and Birnboim and Doly, respectively, as referenced in Muraki et al (49). Purification of DNA fragment, labelling with digoxigenin (DIG)-11-dUTP using the DIG DNA Labeling Kit, and visualization with a DIG Luminescent Detection kit (Roche Diagnostics K.K., Tokyo) were as previously described (27, 49). Southern blotting used a positively charged nylon membrane (Hybond-N+; Amersham Biosciences Corp., Piscataway, NJ) and other DNA manipulations such as DNA ligation are as described in Sambrook et al. (61). Restriction endonucleases and T4 DNA ligase were from New England Biolabs (Beverly, Mass). Automated and BigDye terminator nucleotide sequencing were used as before (49). DNA and protein sequence analyses were carried out using the GENETYX-Mac software (Software Development Co., Ltd., Tokyo) as well as the BLAST programs at the National Center for Biotechnology Information (NCBI) server. (2).

Transposon mutagenesis and screening for 2-NBA-negative mutant strains. Transposon mutagenesis was carried out in order to gain access to the 2-NBA catabolic pathway gene locus by the generation of mutant derivatives of strain KU-7 that are unable to grow on 2-NBA as a sole carbon source. As previously described (49), pKN31 was used as a delivery vehicle for transposon Tn*5-31*Tp and *E. coli* strain S17-1 as a donor in conjugation with the wildtype KU-7 for the construction of strains KUM-4 etc (Table 1). Kanamycin (Km, 50 µg/ml) was used as a selective marker for the transposon. Transconjugants of strain KU-7 were identified following incubation at

5

1   30°C for 2 days. Mutants that were not able to grow on 2-NBA were identified by

2   replica plating on MS medium containing 0.2% 2-NBA (12 mM) and Km as previously

3   described (49).

4   Amplification of Tn*5-31*Tp flanking fragments by inverse PCR (IPCR) was

5   performed using the method of Huang et al. (30). Total DNA prepared from Tn*5-31*Tp

6   mutant of strain KU-7 was digested with *Bam*HI to cut the middle of the Tn*5-31*Tp

7   and the *Bam*HI recognition sequence flanking the transposon insertion site. Following

8   phenol:chloroform extraction and ethanol precipitation, the digested DNA was

9   self-ligated in a final volume of 1 ml at a concentration of 0.3-0.5 μg in the presence

10   of 3 U T4 DNA ligase (New England Biolabs) overnight at 16°C. The ligation mixture

11   was extracted with phenol:chloroform, precipitated with ethanol, and resuspended in

12   sterile distilled H_2O to a concentration of 20 ng μl^{-1}. To amplify the flanking

13   sequences of Tn*5-31*Tp insertion, a set of two parallel IPCR reactions using the

14   ligation mixture as template were performed using primers BL and IR1, and BR and

15   IR1. The BL and BR primers (5'-GGGGACCTTGCACAGATAGC and 5'-

16   CATTCCTGTAGCGGATGGAGATC, respectively) are sequences on both sides of the

17   unique *Bam*HI site of the transposon; IR1 primer

18   (5'-GAGCAGAAGTTATCATGAACG) is based on the terminal inverted repeats (IR)

19   of Tn*5-31*Tp. IPCR was performed in a volume of 25 μl with 25 ng DNA of ligation

20   mixture, 7.5 pmol of each primer, 200 μM of each dNTP, 2.5 μl of GeneAmp High

21   Fidelity 10X PCR buffer with $MgCl_2$, and 0.25 μl of GeneAmp High Fidelity enzyme

22   mix (Applied Biosystems). For amplification, a 2 min initial denaturation step at 94°C

23   was followed by 30 thermal cycles of denaturation at 94°C for 15s, annealing at 50°C

24   for 1 min and extension at 68°C for 8 min. The PCR product was purified from a 1.0%

25   agarose gel and sequence reaction was performed with IR1 primer.

6

1   **Construction of *nbaY* deletion mutant.** A crossover PCR deletion product of *nbaY*

2   was constructed as follows, based on the same strategy as PCR overlap extension

3   mutagenesis according to Link et al (47). Supplemental Fig. S1 depicts the strategy. At

4   first, two different asymmetric PCRs were used to generate fragments to the left and

5   right of *nbaY* with the following pairs of PCR primers: nbaY-Co,

6   5'-CG<u>GAATTC</u>GCGAACTGCTGGACGTGCCT-3' and nbaY-Ci,

7   5'-<u>TGTTTAAGTTTAGTGGATGGG</u>GACCTGAGTCAGCTGTTAGG-3', and

8   nbaY-Ni, 5'-<u>CCCATCCACTAAACTTAAACA</u>ATTACGTAAACTAGTCACCG-3',

9   and nbaY-No, 5'-CG<u>GAATTC</u>TATCTCAGAAGCCGAAGCCG-3', respectively. The

10   left and right fragments were then annealed at their overlapping region (double

11   underlined complementary sequences) and amplified by PCR as a single fragment,

12   using the primers nbaY-Co and nbaY-No. The crossover PCR deletion product was

13   subcloned into the *Eco*RI site of pK18*mobsacB*, generating pNbaΔY. To facilitate this

14   cloning, *Eco*RI recognition sites were introduced in the primer design of nbaY-Co and

15   nbaY-No as underlined above.

16   Plasmid pNbaΔY was transferred into strain KU-7 by conjugation with *E. coli*

17   S17-1 as described earlier. Transconjugants were first selected on MS-S agar

18   containing Km. These transconjugants were not able to grow on MS-S agar containing

19   10% sucrose since the levansucrase-encoding gene (*sacB*) on the pK18*mobsacB* vector

20   when expressed would provide a lethal phenotype to the cells growing on sucrose (18,

21   23). To select for a double cross-over event, a single colony was grown for 18 hrs in a

22   non-selective MS-S medium at 30°C. The cells were plated onto MS-S agar containing

23   10% sucrose and incubated for 24 hrs at 30°C. The resulting colonies were sensitive to

24   Km, indicating the excision of the plasmid by a second cross-over event. This second

25   cross-over either restores the wild type situation or leads to a mutant with the desired

26   deletion. The deletion mutants were analyzed by PCR and designated strain KU-ΔY.

7

1   **Subcloning of *nbaA* and *nbaB* and expresssion in *E. coli*.** The DNA fragment

2   carrying *nbaA* and *nbaB* were amplified from KU-7 genomic DNA by using

3   KOD-Plus-DNA polymerase (Toyobo) with the following pairs of PCR primers, and

4   the desired restriction sites (*Eco*RI and *Pst*I [underlined sequences]) to facilitate

5   subsequent cloning: *nbaA,* 5'-CGGAATTCATGACGCACATTGCAATGTCA and

6   5'-AAAACTGCAGTCAGGGAGTAATCGGAAAGA.   *nbaB,*

7   5'-CGGAATTCATGTCCAATGCCCCGAATGCA   and

8   5'-AAAACTGCAGTGAGTATTGTCAGGAAGT.

9   In each case, the amplified DNA fragment was purified from an agarose gel,

10   digested with the appropriate restriction enzymes, and cloned in the linearized pSD80

11   *tac*-inducible expression vector (66). The resultant recombinant plasmids were

12   designated pSD*nbaA* and pSD*nbaB* (Table 1). DNA sequencing was performed to

13   exclude the possibility of mutations of the amplified genes. The plasmid carrying *E.*

14   *coli* BL21 cells were cultivated in 100 ml of LB medium containing 100 µg of Ap/ml

15   at   30°C.   When   the   culture   reached   an A_{600} of   0.3   to   0.4,

16   isopropyl-ß-thio-D-galactoside (IPTG) was added to a final concentration of 1 mM in

17   the medium. The cells were further cultured for 3 h, harvested by centrifugation,

18   washed twice with 50 mM potassium phosphate buffer (pH 7.0), resuspended with the

19   same buffer, and sonicated by two 30-s bursts with a Braun-Sonifier 250 apparatus.

20   After centrifugation for 30 min at 18,000 x *g* at 4°C, the supernatant was used for the

21   determination of enzyme activity.

22   **Co-expression of *nbaA* and *nbaB* in *E. coli*.** Two strategies were used. First, tandem

23   expression of the two genes on a single plasmid, and second, expression of *nbaA* and

24   *nbaB* on two compatible plasmids. In the tandem gene construct, the strategy was to

25   have a single transcript producing two independently translated polypeptides with the

26   use of a strong ribosomal binding site located between the two genes. Toward this

8

1   construction, a synthetic ribosomal binding site was introduced after NbaA into the

2   original pSD80.NbaA clone at the HindIII and NheI sites, using the primers

3   pSD80PC5'

4   5'-<u>AGCTT</u>TGCACATATCGAGGTGAACATCACG<u>CCCGGG</u>**TAAGGAGGT**GGCGC

5   <u>GCCCTCGAGG</u>   and   pSD80PC3'

6   5'-<u>CTAGCCTCGAGGGCGCGCC</u>**ACCTCCTTA**<u>CCCGGG</u>CGTGATGTTCACCTCG

7   ATATGTGCAA, producing pSD80.NbaA.SD. The synthetic region contains the

8   ribosomal binding sequence as well as the restriction endonuclease sites for HindIII,

9   SmaI, AscI, XhoI and NheI. NbaB was amplified from the original pSD80.NbaB

10   clone   using   the   primers: NbaB5'-AscI

11   5'-TT<u>GGCGCGCC</u>ATGTCCAATGCCCCGAATG   and   pSD80R

12   5'–GTTTTATCAGACCGCTTCTGCG.   The   product   was   ligated   into

13   pSD80.NbaA.SD   at   AscI   and   NheI   creating   the   single   plasmid   clone

14   pSD80.NabA.SD.NbaB. The final plasmid was transformed into *E. coli* BL21(DE3)

15   Rosetta (Novagen) cells containing the pRARE plasmid which encodes rare *E. coli*

16   tRNA codons resulting in enhanced recombinant protein expression otherwise limited

17   by codon usage. Ampicillin and chloramphenicol were used for plasmid selection and

18   maintenance in the same cell. Cells were grown overnight in LB Amp/Cm, subcultured

19   (1:100) and induced with 1mM IPTG at OD 0.5 for 5 hours.

20   The compatible plasmid system consists of pREP1.SD.NbaA and pT7-5.SD.NbaB

21   derived from pREP1 and pT7-5, respectively, according to Parales et al (57). As

22   described above, the synthetic ribosomal binding site was introduced into the pSD80

23   vector producing pSD80.SD. NbaB was amplified as described above. NbaA was

24   amplified from the original pSD80.NbaA clone using the primers: NbaA5'-AscI   5'-

25   TT<u>GGCGCGCC</u>ATGACGCACATTGCAATGTCAG and pSD80R. Both NbaA and

26   NbaB products were introduced into pSD80.SD at the AscI and NheI sites. Both

9

1   SD.NbaA and SD.NbaB were amplified using the primers pSD80pc5'-SmaI 5'-

2   CACG<u>CCCGGG</u>TAAGGAGG and pSD80 R and cloned into pREP1 and pT7-5

3   respectively, at the SmaI and PstI sites. The plasmids were transformed in

4   JM109(DE3) that has the IPTG-inducible T7 RNA polymerase on the chromosome.

5   Ampicillin and chloramphenicol were used for plasmid selection and maintenance in

6   the same cell. Cells were grown overnight in LB Amp/Cm, subcultured (1:100) and

7   induced with 1mM IPTG at OD 0.5 for 5 hours.

8   1 mM final concentration of 2-NBA was added to the single and double plasmid

9   cultures and grown overnight. The cells were centrifuged and an aliquot (20 μl) of the

10   supernatant was spotted on TLC plates (Sigma T-6145). Authentic standards of 2-NBA

11   and 3-HAA from Sigma were used to match the Rf values of the substrate and product.

12   TLC was developed with ethyl acetate:hexane (1:1), dried and observed under UV

13   light. 2-NBA appeared purplish and 3-HAA appeared yellow.

14   **Enzyme activities. (i) 2-NBA nitroreductase activity.** 2-NBA nitroreductase

15   activity was measured by monitoring the decrease in absorbance at 340 nm with the

16   conversion of NADPH to NADP$^+$ in a spectrophotometer (27). Reaction mixtures

17   contained 2-NBA (0.2 μmol), NADPH (0.2 μmol), potassium phosphate buffer (50

18   μmol, pH 7.0), and appropriate amount of cell-free extracts in a final volume of 1 ml.

19   To identify 2-HABA as a reaction product, 0.25 μmol of 2-NBA and 0.96 μmol of

20   NADPH were incubated with a cell-free extract of *E. coli* BL21(pSD*nbaA*) in 30 mM

21   potassium phosphate buffer (pH7.0) in a total volume of 500 μl. After 10 min

22   incubation at 30 °C, the reaction mixture was analyzed by HPLC. Control experiments

23   were carried out without the addition of heterologous proteins. One unit (U) of

24   enzyme activity was defined as the quantity of enzyme required to oxidize 1 μmol

25   NADPH per min. at 25°C. Protein concentrations were determined using the Bradford

26   assay and bovine serum albumin (Pierce, Rockford, Ill) as the protein standard.

10

1   **(ii) 2-HABA mutase activity.** 2-Hydroxyaminobenzoic acid mutase activity was

2   identified by measuring the conversion of 2-HABA to 3-HAA using a preassay

3   mixture that consisted of 0.25 μmol of 2-NBA and 0.96 μmol of NADPH, and

4   cell-free extract of *E. coli* BL21(pSD*nbaA*) in 30 mM potassium phosphate buffer (pH

5   7.0) in a total volume of 500 μl. The reaction mixture was incubated for 10 min at

6   30°C; an aliquot of *E. coli* BL21(pSD*nbaB*) cell-free extract was then added and the

7   reaction mixture further incubated for 10 min before analysis by HPLC. One unit (U)

8   of activity is defined as the amount of enzyme required to convert 1 µmol of substrate

9   in 1 min.

10   **HPLC analysis.** A Shimadzu instrument (model LC-6AD) equipped with an UV

11   detector operating at a wavelength of 254 nm was used. Separations were performed

12   on a CAPCELL PAK C18 UG120 column (column size, 4.6×250 mm; particle size, 5

13   μm; Shiseido, Tokyo) with a methanol-10 mM KH_2PO_4 mixture [30:70 (v/v)] at a flow

14   rate of 1.0 ml min^{-1} as a solvent.

15   **Site-directed mutagenesis.** PCR overlap extension mutagenesis (29) was used to

16   generate *nbaA* mutants using pSD*nbaA* as template (Table 1). The following 27-mer

17   primers were used and in each case, its complementary mutagenic primer (not shown);

18   the underlined codon represents the alanine (A) change.

19   N40A: 5'-GAGGGGGTTGTGC<u>GCG</u>GCCGCGCCTTAT;

20   H63A: 5'-ATCGCCGTGGAT<u>GCG</u>TATGGTGAAGAA;

21   H69A: 5'-GGTGAAGAAAGC<u>GCG</u>CGTCCTGGCGAG;

22   D76A: 5'-GGCGAGCAAAAA<u>GCG</u>ACGCTAAAAAAC;

23   H110A: 5'-GATTTCCCCTCT<u>GCG</u>ATCTCAGAAGCC;

24   E113A: 5'-TCTCATATCTCA<u>GCG</u>GCCGAAGCCGTT.

25   After cloning, the sequences of the cloned fragments were confirmed by DNA

26   sequencing and their expression verified.

11

1   **Partial purification of 2-NBA nitroreductase.** The cell extract of strain KU-7

2   harvested from 1.25 litres of culture was used as the starting material for the

3   purification of 2-NBA nitroreductase. All procedures were carried out at 4°C. The cell

4   extract was fractionated using ammonium sulfate at 30 to 50% saturation. The

5   resulting precipitate was dissolved in 50 mM potassium phosphate buffer (pH 7.0).

6   This enzyme solution was dialyzed against the same buffer for 12 hrs. The dialyzed

7   solution was put on a DEAE-Cellulose DE52 column. The column was washed with

8   50 mM potassium phosphate buffer (pH 7.0) until no protein could be detected in the

9   flow-through, and the enzyme was subsequently eluted with a linear gradient of 0 to

10   0.5 M KCl in the same buffer. Active fractions were collected, pooled, and used as a

11   partially purified 2-NBA nitroreductase.

12   **Protein analysis.** The partially purified 2-NBA nitroreductase was subjected to

13   (12%) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

14   carried out by conventional method on a Mini-PROTEAN II Electrophoresis cell

15   (Nippon Bio-Rad Laboratories, Tokyo). The gel was transferred to a polyvinylidene

16   difluoride membrane (MiniProBlott; Applied Biosystems Japan Ltd.) with a Mini

17   Trans-Blot electrophoretic transfer cell (Nippon Bio-Rad laboratories) as described by

18   the manufacturer. The area on the membrane containing 2-NBA nitroreductase was cut

19   out and subjected to N-terminal amino acid sequencing with a model PPSQ-21 protein

20   sequencer (Shimadzu Co., Kyoto, Japan).

21   **Molecular modeling of NbaA and its complexes with Ni^{2+}, FMN and NADPH.**

22   Structural bioinformatics analysis of the NbaA sequence was carried out at the

23   BioInfoBank MetaServer (http://bioinfo.pl/meta), which assembles state-of-the-art

24   fold recognition methods and provides a consensus sequence-to-structure

25   hyper-scoring with the 3D-JURY meta-predictor (24).

26   Structural manipulations were done with the SYBYL 6.6 software (Tripos, Inc., St.

12

1   Louis, MO). The homology modeling program COMPOSER (7) in SYBYL was

2   employed to delineate six structurally conserved regions (SCRs) and five intervening

3   structurally variable loop regions (SVRs) between NbaA and its template structures.

4   The NbaA residues in the SCRs were modeled based on the structures of the two

5   closest homologs (an FMN-binding protein and a styrene monooxygenase, PDB codes

6   1EJE and 1USF, respectively), while the SVRs were initially built using the PROTEIN

7   LOOPS structure database search module in SYBYL. Based on the crystal structure of

8   the closest NbaA homolog identified (PDB code 1EJE), the NbaA homodimer was

9   then assembled, together with one FMN molecule, one divalent metal ion (Ni^{2+}) and

10 seven adjacent structured water molecules per each monomer. Side chain repacking

11 and re-orientation of polar hydrogens were performed to alleviate minor steric clashes

12 and improve hydrogen bonding. The NbaA-Ni^{2+}-FMN dimeric model was refined by

13 sequential runs of molecular mechanics force field energy minimization that allowed

14 gradual relaxation of (i) the SVRs, (ii) the 23-residue N-terminal segments, and (iii)

15 all side chains.

16   In order to dock NADPH into the modeled NbaA-Ni^{2+}-FMN complex, the

17 nicotinamide mononucleotide (NMN) half of NADPH was manually positioned into

18 its putative binding cleft on NbaA and facing the isoalloxazine ring of FMN, in an

19 orientation and conformation observed in one of the identified NbaA templates (a

20 ferric reductase) complexed with FMN and $NADP^+$ (PDB code 1IOS). The initial

21 conformation of the 2'-P-AMP half of the NADPH was also built by analogy as

22 extending outward from the pocket. The resulting NADPH binding mode was then

23 relaxed by energy minimization. Feasible bound conformations of the solvent exposed

24 2'-P-AMP moiety were subsequently explored using the Monte Carlo-minimization

25 (MCM) conformational sampling procedure (44) adapted to flexible ligand docking

26 (13, 46, 65). The NMN half of NADPH was kept as an anchor from which random

13

1 dihedral angle perturbations were introduced in the 2'-P-AMP half of NADPH,

2 followed by energy minimizations, for a total of 10,000 MCM cycles.

3 **Docking of 2-NBA to the modeled NbaA-Ni^{2+}-FMN complex**. This was

4 performed manually, first by positioning its aromatic ring similarly with the

5 nicotinamide ring in the modeled NADPH complex (resulting in parallel stacking

6 between 2-NBA and the isoalloxazine ring of FMN). Several alternate orientations of

7 2-NBA with respect to its two *ortho* groups were then generated and refined

8 independently by energy minimization.

9 All conjugate gradient energy minimizations were carried using the AMBER

10 all-atom molecular mechanics force field (17, 73). A distance dependent (4R_{ij})

11 dielectric, an 8 Å non-bonded cutoff and a root-mean-square gradient of 0.05

12 kcal/(mol·Å) were used. The protonation state at physiological pH was adopted.

13 Published AMBER-compatible atomic parameters were used for FMN (46) and

14 NADPH (61). Partial atomic charges for 2-NBA were obtained by a two-stage

15 restrained fitting procedure to the single-point HF 6-31G* electrostatic potential (6)

16 calculated in GAMESS (64). A charge-delocalized model with octahedral geometry

17 was used for Ni^{2+} (55, 65). During all energy minimizations, the FMN and Ni^{2+}, as

18 well as the SCR backbone atoms of NbaA were constrained to their initial positions

19 using harmonic potentials with force constants of 20 kcal/(mol·Å^2) and 1

20 kcal/(mol·Å^2), respectively.

21 **Chemotaxis assay**. The chemotactic behavior of strain KU-7 and its *nbaY* deletion

22 mutant *nbaΔY* was studied by swarm plate assay (26), drop assay (21) and agarose

23 plug assay (41, 75). In the swarm plate assay, soft agar swarm plates consisted of MS

24 medium that contains 1 mM of chemicals (2-NBA, 3-HAA, benzoate, succinate) and

25 0.3% agar. Cells (OD$_{600}$ 1.0) were inoculated at the center of the plates and these

26 plates were incubated at 30°C overnight. The medium used for the drop assay

14

1   consisted of 50 mM phosphate buffer (pH 7.0) that contains 20 μM EDTA and 0.05%

2   glycerol. Cells were grown in MS medium, pelleted, washed and suspended in drop

3   assay medium, and finally poured into petri plates. The chemotactic response was

4   determined by placing the paper disk containing 100 mM attractant at the center of the

5   plate. Agarose plug assays were carried out with slight modifications as previously

6   described (41, 75). Plugs contained 2% agarose in chemotaxis buffer (50 mM

7   phosphate buffer (pH 7.0) that contains 20 mM EDTA and 0.05% glycerol), and 10

8   mM 2-NBA. A drop (100 μl) of the melted agarose mixture was placed on a Petri dish.

9   Cells were grown in MS medium, pelleted, washed and suspended in chemotaxis

10   buffer, and finally poured into Petri plates with agarose plug. The chemotactic

11   response was determined by the ring appearance after 20 min incubation at room

12   temperature.

13   **Nucleotide sequence accession number.** The nucleotide sequence determined in this

14   study has been deposited in the DDBJ under accession number AB263093.

15

RESULTS AND DISCUSSION

Isolation of the *nbaA* disrupted mutant strain by transposon mutagenesis. In our previous study, analysis of a 19-kb DNA region of strain KU-7 that encompasses the 3-HAA *meta*-cleavage and regulatory gene cluster, designated *nbaEXHJIGFCDR*, was found to be devoid of the two initial degradative genes, *nbaA* and *nbaB* (49). We screened additional Tn*5-31*Tp transformants and obtained apparently new candidates that were unable to grow on 2-NBA as a sole carbon source. Some 500-bp sequences were determined from each IPCR product and used to search the nonredundant protein sequence database of the NCBI for the presence of possible target sequences. However, the majority of the transposon insertion sites, except KUM-9, -19 and -33 (Fig. 1), appeared to bombard the known 3-HAA *meta*-cleavage and regulatory gene cluster (49).

Insertion site of Tn*5-31*Tp in strain KUM-9 was found to be within a 386-codon ORF (Orf16) predicted to encode an ABC transporter substrate binding protein. Its closest homolog (81.8% sequence identity) is a putative extracellular ligand-binding receptor of *Polaromonas* sp. JS666 [CP000317 (ABE46993)]. Tn*5-31*Tp insertion site in strain KUM-19 was found within a sequence, designated Orf23, and subsequently identified as NbaA, that is similar to a FMN-binding protein of *Methanobacterium thermoautotrophicum* ΔH (14). Resting cells of strain KUM-19, grown on succinate as the carbon source that were non-induced with the substrate, did not degrade 2-NBA.

Insertion of the Tn*5-31*Tp transposon into strain KUM-19 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 7.4-kb *Eco*RI fragment and a 4.1-kb *Bam*HI fragment) was observed in each digest (Fig. 1).

Cloning and sequencing of the Tn*5-31*Tp flanking regions. Cloning of the 7.4-kb *Eco*RI fragment or the 4.1-kb *Bam*HI fragment was carried out by using a pUC19 as a

16

1 vector. In the former case, transformants were selected on trimethoprim- and

2 ampicillin-containing plates. In the latter case, kanamycin and ampicillin were used as

3 the selection markers. The resulting plasmids were designated pNBA5 and pNBA6,

4 respectively. The pNBA5 insert contained a 4.5-kb Tn*5-31*Tp segment and 2.9-kb of

5 KU-7 DNA. Plasmid pNBA6 was found to contain a 3-kb Tn*5-31*Tp insert and 1.1 kb

6 of KU-7 DNA.

7   In parallel with the transposon mutagenesis experiment, DNA sequence of the

8 flanking region of the 9.3-kb *Hin*dIII fragment was determined by direct sequencing

9 of a IPCR product. The template for IPCR was prepared from self-ligated 5.5-kb *Pst*I

10 fragment. The sequence of IPCR product revealed that there is a 13-bp gap between

11 KU-7 DNA in pNBA6 and 9.3-kb *Hin*dIII fragment (pNBA3).

12   Sequencing of the Tn*5-31*Tp flanking regions in pNBA5 and pNBA6 and data

13 analysis revealed three ORFs, two divergently transcribed *orf22* and *orf23*, separated

14 by a 419-bp intergenic space, and 160-bp downstream of *orf23* and on the same DNA

15 strand, the presence of *orf24* (Fig. 1). Various evidence establishing these ORFs as

16 NbaA-, NbaB- and a chemotactic receptor NbaY-encoding genes are provided in the

17 following sections.

18   **Identification of NbaA as 2-NBA nitroreductase.** Sequence determination of the

19 N-terminal 15-amino acid peptide (THIAMSGLTNMQKYW) derived from a partially

20 purified 2-NBA nitroreductase of strain KU-7 confirmed the gene assignment of the

21 651-bp *orf23* that encodes a 216-residue polypeptide that is preceded by an

22 appropriate ribosome-binding sequence (GGAG) 6-bp from the methionine start codon.

23 The predicted molecular size of the protein, M_r of 24,410 matched well with the

24 experimental value of 28 kDa that was derived from a SDS-PAGE of the

25 IPTG-inducible expression of the NbaA protein in pSD*nbaA* (Fig. 2). By Superdex[TM]

26 200 gel chromatography, NbaA was found in an active fraction that eluted at a

17

1 molecular mass that is slighter greater than 44-kDa of molecular standard chicken

2 albumin, indicating NbaA functions as a dimer (not shown).

3   The BLAST search retrieved a number of homologs of microbial origin displaying

4 40-78% sequence identity, but all are hypothetical or uncharacterized proteins. Two

5 such sequences are derived from pollutant-degrading bacteria: a hypothetical

6 conserved protein (77.7% identity), designated [CP000317 (ABE46991)], derived

7 from a large plasmid of *Polaromonas* strain JS666, a chlorinated-alkene-degrading

8 organism; the other, Bcep 4121 (39.7%) derived from a well-known PCB-degrader

9 *Burkholderia xenovorans (*formerly *fungorum)* strain LB400. However, the most

10 useful information comes from a number of structural homologs (presented in a

11 following section), notably an FMN-binding protein (MTH152; accession no.

12 NP_275295, 31 % identity), derived from *Methanobacterium thermoautotrophicum*

13 ΔH (14).

14 **Biochemical properties of NbaA**. The specific activity of NbaA toward 2-NBA was

15 found to be 10.26 U/mg. Crude cell extracts of *E. coli* BL21(pSD*nbaA*) were able to

16 oxidize NADPH only in the presence of 2-NBA. An investigation of the reaction

17 stoichiometry demonstrated that complete disappearance of 2-NBA by the cell extracts

18 required 2 mol of NADPH per mol of 2-NBA. By HPLC analysis of the NbaA reaction

19 product with 2-NBA and NADPH as substrates, only one product was formed in

20 significant amount with a retention time at 4.96 min. The expected product 2-HABA

21 was identified by comparison to an authentic standard (Fig. 3).

22   Supplements of flavins (FMN, FAD and riboflavin) to crude extracts of *E. coli*

23 BL21(pSD*nbaA*) at the final concentration of 1.0 mM resulted in an increase of

24 specific activities by 3.8-fold and 5.2-fold in the case of FAD and FMN, respectively.

25 The addition of riboflavin gave the same basal level as the case without the addition of

26 FAD or FMN. Evidently, FMN was more effective than FAD suggesting that FMN

18

1   may be a physiological cofactor of NbaA.

2   The effect of divalent cations on NbaA activity were examined by preincubating the

3   cell-free extracts containing NbaA with the chloride salts of the divalent cations: Ca,

4   Co, Cu, Fe, Mg, Mn, Ni and Zn, at 0.1 mM and 0.3 mM. Fe, Mg and Mn appeared to

5   have a potentiating effect at 0.1 mM providing a 23%, 16% and 51% increase in

6   activity, respectively. The addition of 0.3 mM Fe^{2+} and Mn^{2+} gave the most increase,

7   66% and 89%, respectively, whereas, Ni^{2+} showed an increase of 17%. Ca^{2+} and Zn^{2+}

8   seem to inhibit the enzyme to some extent. Under these experimental conditions, Mn^{2+}

9   rendered the best activity to NbaA.

10   **Modeled NbaA structure and interactions**. Structural bioinformatics searches

11   unambiguously assigned the NbaA protein to the FMN-binding split barrel fold, and

12   specifically to the NADH:FMN oxidoreductase-like structural family (accession

13   numbers   b.45.1.2   or   50482)   according   to   the   SCOP   database

14   (http://scop.berkeley.edu/) (3), also classified as the flavin reductase-like domain

15   (accession   number   PF01613)   in   the   Pfam   database

16   (http://www.sanger.ac.uk/Software/Pfam/) (4). Currently, the solved structures

17   adopting   this   fold   include   the   FMN-binding   protein   MTH152   from   *M.*

18   *thermoautotrophicum* (PDB code 1EJE) (14), a ferric reductase from *Archeoglobus*

19   *fulgidus* (PDB codes 1I0R and 1I0S) (12), the flavin reductase PheA2 from *Bacillus*

20   *thermoglucosidasius* (PDB codes 1RZ0 and 1RZ1) (70), a styrene monooxygenase

21   from *Thermus thermophilus* (PDB codes 1USC and 1USF) (unpublished), and a

22   probable flavoprotein from the same organism (PDB codes 1WGB and 1YOA)

23   (unpublished). The sequence alignment of NbaA and these five structurally similar

24   enzymes is given in Fig. 4A.

25   This alignment formed the basis for the three-dimensional homology modeling of

26   the NbaA homodimeric structure (Fig. 4B). The core of each modeled NbaA subunit is

1 proposed to be organized around a six-stranded antiparallel β-barrel with a capping

2 α-helix, a fold recognized as a circular permutation of the flavin binding domain of

3 the ferredoxin reductase superfamily (34, 45, 50). However, the ferredoxin

4 reductase-type proteins utilize a second, Rossmann fold domain to bind the NAD(P)H

5 (34). The homology to the NADH:FMN oxidoreductase-like fold family strongly

6 suggests that the predicted single-domain structure of NbaA would provide both the

7 flavin and NAD(P)H binding sites (12, 70).

8 The modeled binding mode of FMN to NbaA (Fig. 4C) is similar to that observed in

9 other members of the NADH:FMN oxidoreductase-like fold family (12, 70). FMN fits

10 into a well-shaped groove near the homodimer interface of NbaA. Based on this

11 proposed model, the *si* face of the isoalloxazine ring is buried against the β-barrel and

12 directly contacts the main-chain atoms of residues Ser45, Ala46, Ala60, Asp62, and

13 side-chain atoms of Ser45 and Lys75 *via* hydrogen bonds, as well as Ile27, Pro43,

14 Tyr44, Met100, Val101, Arg187 and Tyr193 by non-polar contacts. The 3'-hydroxyl

15 group of the ribityl moiety is modeled in hydrogen bond contact with the Lys75 side

16 chain. The FMN phosphate group is predicted to be anchored to the β-barrel-capping

17 α-helix of NbaA *via* hydrogen bonds to the Asn40, Tyr44 and Thr77 side chains and to

18 Asp76 and Thr77 main-chain atoms.

19 A divalent metal ion, Ni^{2+}, was modeled as mediating the interaction between the

20 FMN phosphate and NbaA. This interaction has been observed in the crystal structure

21 of the FMN-binding protein MTH152 (PDB code 1EJE) (14), the structural template

22 most similar in primary sequence to NbaA. In the more distant structural homologs of

23 NbaA, this phosphate-bridging role is fulfilled by protein side chains, e.g., the

24 ammonium group of Lys69 in ferric reductase (PDB codes 1I0R and 1I0S) (12). In the

25 modeled NbaA-FMN complex, the octahedral coordination sphere of the Ni^{2+} metal

26 ion consists of side-chain oxygen atoms from residues Asn40, Asp76 and Glu113

20

1 (Asn36, His62 and Glu99, respectively, in the FMN-binding protein MTH152), two

2 water molecules, and an oxygen atom from the FMN phosphate (Fig. 4C).

3   The NADPH binding mode to NbaA (Fig. 4D) was inferred from the NADP$^+$

4 binding mode observed in some of the structural homologs (styrene monooxygenase,

5 ferric reductase and flavin reductase PheA2, PDB codes 1USF, 1IOS and 1RZ1,

6 respectively), primarily for the more buried NMN half of NADPH. In this binding

7 mode, the nicotinamide ring stacks against the available *re* face of the FMN

8 isoalloxazine ring. The modeled C4(nicotinamide)-N5(isoalloxazine) distance of

9 ~3.5 Å is compatible with a direct hydride transfer from NADPH to FMN (12, 70).

10 The amide group of nicotinamide is predicted to form hydrogen bonds with the side

11 chains of Asp62, Arg153, as well as Tyr49 from the second subunit in the NbaA

12 homodimer. In this plausible model, the NMN phosphate would be stabilized by a

13 salt-bridge with Arg187.

14   Multiple sterically feasible binding modes may be expected for the solvent exposed

15 2'-P-AMP fragment of NADPH, due to the fairly wide opening at the top of the FMN

16 and NMN binding cleft of NbaA. Although the low-energy conformations obtained

17 from conformational sampling indeed revealed variability in the binding mode of the

18 adenosine portion of NADPH, it also appeared that the adenine moiety has a tendency

19 to interact with the loop β5-α2 of NbaA (Fig.4B, 4D). The resolution of our modeled

20 structure in this solvent exposed region does not allow speculations on specific

21 intermolecular interactions. Interestingly, the β5-α2 loop of NbaA includes a

22 10-residue insertion relative to the other members of the NADH:FMN

23 oxidoreductase-like structural family (Fig. 4A). Thus, it resembles the corresponding

24 loop of the ferredoxin reductase superfamily members, that has been shown to be able

25 to interact *via* one of its aromatic residues with the adenine moiety of FAD (8, 19).

26 The β5-α2 loop of NbaA does include an aromatic residue, His69, which might

21

1 therefore represent a potential site for interaction with the adenine moiety of NADPH.

2 **Proposed NbaA reaction mechanism.** A structural model for the 2-NBA substrate

3 binding mode to NbaA assumes the ping-pong mechanism in which the oxidized

4 coenzyme leaves its binding site and is replaced by the substrate that contacts the

5 enzyme-bound reduced flavin. This assumption is supported by the available data for

6 NbaA structural homologs such as ferric reductase (12) and flavin reductase PheA2

7 (71), where steric constraints impede simultaneous binding of the substrate and

8 nicotinamide, thus suggesting a ping-pong kinetic mechanism. Indeed, biochemical

9 data have recently shown that the flavin reductase PheA2 catalyzes the

10 NADH-dependent reduction of free flavins by a ping-pong bisubstrate-biproduct

11 mechanism (36). Other nitroaromatic reductases, although not displaying significant

12 sequence or structural similarities to NbaA, have been shown to act according to a

13 ping-pong catalytic mechanism. For example, the structures of nitroaromatic

14 reductases from *E. coli* and *Enterobacter cloacoe* complexed with substrate analogs,

15 inhibitors and dinitrobenzoate prodrugs show that these ligands replace the NAD(P)H

16 nicotinamide in order to form parallel stacking with the isoalloxazine moiety of

17 enzyme-bound FMN or $FMNH_2$ (28, 33, 48). Furthermore, a recent kinetic study of *E.*

18 *coli* nitroaromatic reductase demonstrates that the nitroso-intermediate becomes the

19 substrate in a subsequent enzyme-catalyzed reduction reaction leading to the

20 hydroxylamine product (59). A plausible ping-pong mechanism for reaction catalyzed

21 by NbaA is depicted in Fig. 5.

22 Our model of 2-NBA bound to NbaA (Fig. 4E) is consistent with the observed

23 orientation of the substrate nitrophenyl moiety against the nitroreductase-bound flavin

24 (33). According to this putative binding mode, the aromatic substrate forms a parallel

25 stacking with the isoalloxazine ring. The nitro group of 2-NBA would contact the

26 isoalloxazine rings above the N5 nitrogen atom, where it is predicted to be anchored

by polar interactions with the Arg25 and Arg187 positively charged side chains of NbaA. These interactions remain compatible with the binding of the 2-nitrosobenzoate intermediate, but not with the 2-hydroxyaminobenzoate product, due to electrostatic repulsions between positively charged hydrogen atoms. In the proposed model, the *ortho* carboxylate substituent of 2-NBA is complementary to its putative binding site on NbaA by forming a salt-bridge with the side chain of Arg153, and hydrogen bonds with Thr19 and Tyr49 (from the second monomer), which may account for the NbaA specificity toward 2-NBA. For example, a similar carboxylate binding motif (i.e., Arg, Ser, Tyr) is present in *p*-hydroxybenzoate hydroxylase (65).

Site directed mutagenesis of predicted NbaA active site residues. Structural modeling analysis of relevant NbaA complexes suggests how specific residues may contribute to the catalytic activity of the enzyme, and thus forms the basis for mutational analyses. We first probed by site-directed mutagenesis the predicted metal binding residues Asn40, Asp76 and Glu113. We also mutated residue His69 from the 10-residue insertion loop β5-α2 predicted to interact with the adenosine portion of NAD(P)H. Mutations of two other histidine residues, His63 and His110, predicted to be solvent exposed and not to contribute to NbaA activity (neither *via* ligand binding nor dimerization), were also tested. Hence, we generated six NbaA variants that in each case resulted in an alanine substitution. Individual mutations of the three predicted divalent metal ion binding site residues (N40A, D76A, and E113A) resulted in a complete loss of the NbaA activity (Table 2). These results are in agreement with our model showing that the metal ion would act as a bridge between the FMN phosphate and the protein, thus being essential for FMN binding (Fig. 4C). The H69A substitution resulted in an enzyme that retained only 2-3% of the wild-type NbaA activity, but nonetheless still active (Table 2). In a recent study of another NADH:flavin oxidoreductase, a similar drop in activity has been observed for a

23

1 histidine residue that represents an NADH interacting site (60). Taken together, these

2 observations are in agreement with the predicted NAD(P)H interacting role of His69

3 in NbaA. On the other hand, the H63A and H110A substitutions retained 72-73% and

4 33-44% of the original activity, respectively, indicating that these residues do not

5 contribute substantially toward NbaA activity. For all active mutants, it was shown

6 that the addition of exogenous FMN potentiated the various enzyme activities about 3

7 to 5 fold. We also noted that the amounts of the variant NbaA proteins produced, as

8 evident in a Coomassie-stained SDS-PAGE analysis, are comparable to the native

9 NbaA (results not shown). Certainly, additional biochemical data from purified

10 enzyme and direct experimental structural evidence will be required for a refined

11 characterization of the NbaA catalytic residues and mechanism.

12 **Identification of NbaB as 2-hydroxylaminobenzoate mutase.** The deduced

13 181-amino acid sequence of NbaB showed 35% identity to the

14 4-hydroxylaminobenzoate lyase (PnbB) sequences of three known degradative

15 organisms - *P. putida* TW3 degrading 4-nitrotoluene (31), and *Pseudomonas* sp.

16 YH102 and *Ralstonia pickettii* YH105, degrading 4-nitrobenzoate (76). Higher

17 identities were observed with four hypothetical proteins in the genomes of *Rhodoferas*

18 *ferrireducens* DSM 15236 (38%); *Azoarcus* sp. EbN1 (39.3%), *Burkholderia*

19 *fungorum* LB400 (40%), and *Polaromonas* sp. JS666 (66.5%). A multiple sequence

20 alignment of the PnbB lyases with NbaB (Supplemental Fig. S2) revealed that the

21 conserved sequences are distributed evenly throughout the polypeptide with the

22 exception that NbaB (and its JS666 homolog) appears to have an N-terminal extension

23 of 14-20 residues and two sequence gaps (amino acids 110-111 and 142-143 [NbaB

24 numbering]) are needed for an optimal sequence alignment. The sequence similarity

25 between PnbB and NbaB provides a plausible basis for the second action of PnbB in

26 the 4-nitrobenzoate pathway of *P. putida* strain TW3 in the production of

24

1 4-amino-3-hydroxybenzoate from 4-nitrobenzoate besides the better known product,

2 protocatechuate (31). Evidently, 4-amino-3-hydroxybenzoate is an analog of

3 2-amino-3-hydroxybenzoate, i.e. 3-HAA.

4   The expression of a 22-kDa protein band, although weak, derived from plasmid

5 pSD*nbaB* confirmed the expected molecular mass of NbaB, calculated to be 19,554

6 (Fig. 2). No enhanced protein band of this size was detectable in the control cells

7 containing the pSD80 vector only. However, a better expression of NbaB was seen in

8 the pT7-5SD.NbaB construct transformed in *E. coli* JM109(DE3) (Fig. 2). This level

9 was just as good as the expression of NbaA in pSD*nbaA*. On the other hand, NbaA was

10 not readily expressed in the pREP1.NbaA construct as in pSD*nbaA*.

11   By HPLC analysis of the enzyme reaction product of NbaB with 2-NBA and

12 NADPH in the presence of NbaA, the reaction product, a peak at retention time 4.40

13 min, was identified to be 3-HAA, by comparison to an authentic standard (Fig. 3).

14 3-HAA production by the appearance of a yellow spot under UV illumination was also

15 observed by thin layer chromatography (not shown).

16   **NbaY (Orf24) gene disruption and chemotaxis.** This Orf24 consists of sequence

17 coding for 544 amino acid residues. The primary structure showed 29% sequence

18 identity to NahY from *P. putida* G7, a known methyl-accepting chemotactic transducer

19 that recognizes naphthalene (25). An updated BLAST search showed 62-63%

20 sequence homology to a putative chemotaxis sensory transducer, [CP000094

21 (ABA74342)] and [CP000076 (AAY92385)], each derived from *P. fluorescens* strains

22 PfO-1 and Pf-5, respectively. A multiple sequence alignment is shown in

23 Supplemental Fig. S3. The greatest sequence conservation occurs within a region

24 designated the HCD, highly conserved domain, typical of chemoreceptor proteins (39).

25 In NbaY, two possible transmembrane segments (amino acids 13-35 and 190-212 are

26 predicted.

25

1   To test whether NbaY serves as a possible chemoreceptor for 2-NBA, we

2   constructed a *nbaY* deletion mutant, designated strain KU-ΔY. The use of three

3   independent assays by their ring appearance in the case of the wildtype compared to

4   the mutant supported the involvement of NbaY in 2-NBA chemotaxis (Fig. 6). In

5   liquid culture, strain KU-ΔY grew just as readily as the wildtype when 2-NBA was

6   used as the sole carbon source. At this time, we have not tested whether any of the

7   2-NBA metabolite would serve as a chemoattractant. A detailed chemotaxis study is

8   outside our present scope.

9   To the best of available knowledge, NahY and PcaK (a transporter and

10   chemoreceptor protein from *P. putida* PRS2000 that is encoded as part of the

11   beta-ketoadipate pathway regulon for aromatic acid degradation) are two of the

12   existing characterized chemoreceptor genes for aromatic metabolism (reviewed in

13   54, 58). On the other hand, there have been several studies illustrating chemotaxis

14   of a variety of nitroaromatic degradative organisms toward compounds such as

15   nitrotoluene, dinitrotoluene, and TNT etc. (43, 54, 56, 58). But in all cases, no

16   pathway-associated gene, like NahY in napthalene degradation, PcaK in benzoate

17   metabolism or NbaY as in this study, has been ascribed to the observed chemotaxis

18   phenomena. Hence, NbaY is a prototype chemoreceptor for nitroaromatics. The

19   relevance of chemotaxis in pollutant-degrading bacteria to bioremediation strategy

20   has been reviewed (54, 58).

21   **Concluding remarks**. For the first time, a homology model of 2-nitrobenzoate

22   nitroreductase is available that adds to our limited knowledge on structure and

23   function of nitroreductases in nitroaromatic metabolism of which a limited number of

24   genetic and/or biochemical studies are available (11, 35, 40, 51, 67). NbaA is a new

25   representative of a family of homodimeric NADH:FMN oxidoreductase-like fold

26   proteins that is different from the nitro/flavin reductase family. The latter comprises of

26

1   the major oxygen-insensitive nitroreductase (NfsA) from *E. coli* NfsA (37), flavin

2   reductase (FRP) of *Vibrio harveyi* (42), PnrA of a TNT-degrading *P. putida* JLR11 (11),

3   and NitA and NitB of another TNT-degrading *Clostridium acetobutylicum* ATCC 824

4   (40). It remains to be seen whether the 10-residue insertion loop between strand β5

5   and helix α2 (amino acids 65-74) is unique to NbaA that attacks an *ortho*-substituted

6   nitroaromatic substrate. It is unfortunate that we are as yet unable to assign a function

7   or substrate to the MTH152 FMN-binding protein of *M. thermoautotrophicum* (14).

8   However, the structure relationship of MTH152 to NbaA suggests that aromatic

9   compounds should be tested as possible substrates.

10   The availability of NbaA and NbaB-encoding genes paves a plausible

11   biotechnological route for the production of 3-HAA, a known antioxidant and

12   reductant. However, for an efficient substrate uptake of aromatic acid such as

13   2-NBA, this recombinant route might require involvement of the numerous

14   ABC-transporter permease and outer membrane porin sequences, etc., that are

15   possibly encoded by Orfs 16, 14, 13, 12, and 11 (immediately downstream of *nbaB*),

16   and Orf17 that is downstream of the *nbaE* gene cluster (49; DDBJ database accession

17   number AB088043).

18   This study completes the gene elucidation of 2-NBA metabolism in strain KU-7 in a

19   24-kb chromosomal locus, that includes a chemoreceptor gene (*nbaY*) on the 5'-end

20   flanking the core biodegradative gene cluster

21   (*nbaAB*[*orf*]$_{16,15,14,13,12,11}$*nbaEXHJIGFCD*), and a putative transcriptional regulator

22   *(nbaR)* of the LysR-type on the 3'-end that is followed by an unknown *orf*17 (49; Fig.

23   7). Interestingly, in the current microbial genome sequence database (JGI database),

24   we have identified the presence of a very similar gene cluster, exhibiting high

25   sequence identities (in the range 63% - 87%), that is encoded on a plasmid of

26   360,405-bp, in the draft genome sequence of *Polaromonas* sp. JS666, a strain studied

27

1 for its degradation of chlorinated alkenes such as *cis*-dichloroethene, but no other

2 compounds (16). Evidently, the gene organization of the core *nba* biodegradative gene

3 cluster (i.e, from *nbaA* to *nbaR*) is the same in the two strains. Notable differences are

4 in the lengths of the various intergenic spaces and especially the presence of a putative

5 integrase and insertion sequence IS*3* and IS*911* found at both ends of the JS666 gene

6 locus, in the regions otherwise occupied by *nbaY* and *orf*17 in strain KU-7 (Fig. 6).

7 This observation leads to the possibility that the *nba* gene locus constitutes a

8 transferable or transposable "genomic island" or integrative and conjugative elements

9 as frequently found among catabolic pathways of pollutant-degrading bacteria (72).

10

11

12   **ACKNOWLEDGMENTS**

20

21

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1 **Legends:**

2

3   Fig. 1. The two initial steps of 2-nitrobenzoate (2-NBA) metabolism by *P. fluorescens*

4   strain KU-7 catalyzed by 2-NBA nitroreductase (NbaA) and 2-HABA mutase (NbaB)

5   to produce 3-hydroxyanthranilate (3-HAA) via the formation of

6   2-hydroxylaminobenzoate (2-HABA). The orientation and localization of *nbaA and*

7   *nbaB* genes and flanking open reading frames (*orf*) are indicated by large open arrows

8   beneath a restriction map. Sites of transposon Tn*5-31*Tp insertions producing the

9   various KUM mutant strains are pinpointed. The numbering of *orfs* is according to

10 Muraki et al (49). Orf11, 12, 13, 14, and 16 likely constitute an ABC transporter (22)

11 for 2-NBA uptake. Orf15 is a putative FMN reductase. *nbaE* encodes a semialdehyde

12 dehydrogenase as previously described (49). The cloned DNA fragments and probe

13 regions are as indicated. The broken lines indicate the Tn*5-31*Tp DNA. *dhfrII* and *neo*

14 are trimethoprim resistance- and kanamycin resistance-encoding genes, respectively.

15 The DNA probes, labeled with DIG-11-dUTP are: a 1.8-kb HindIII-BamHI fragment

16 (Probe 1) and a PCR-amplified DNA that is a *neo* gene sequence in Tn*5-31*Tp (Probe

17 2). The PCR primers for Probe 2 are: 5'-GATCAAGAGACAGGATG-3' and

18 5'-CACTCCTGCAGTTCG-3'.

19

20 Fig. 2. Coomassie blue-stained protein profiles of recombinant *E. coli* crude extracts

21 separated on SDS-12% PAGE. Lane 1: molecular size markers as indicated alongside

22 in kilodaltons. Lane 2, NbaA expressed in pSD*nbaA* lane 3, NbaB expressed in

23 pSDn*baB*; lane 4, *E. coli* cells containing the pSD80 vector only. Lane 5, NbaB

24 expressed in pT7-5SD.NbaB. Lane 6, control *E. coli* JM109(DE3) containing the

25 vector only. The positions of the expressed protein are indicated by circles.

26

1 Fig. 3. HPLC profiles of the reaction products generated by cell-free extracts

2 containing NbaA and/or NbaB. (A) Authentic standards 2-NBA, 2-HABA, 3-HAA,

3 and NADPH. (B) Identification of 2-HABA as a reaction product produced by

4 cell-free extracts containing NbaA. 0.25 µmol of 2-NBA and 0.96 µmol of NADPH

5 were incubated with NbaA in 30 mM potassium phosphate buffer (pH7.0) at the total

6 volume of 500 µl. Reaction mixture was analyzed by HPLC after 10 min incubation at

7 30°C. (C) Identification of 3-HAA as a reaction product produced by cell-free extracts

8 containing NbaA and NbaB. The reaction mixture, same as described in (B), was

9 incubated for 10 min at 30°C, and then aliquot of cell-free extracts containing NbaB

10 were added and further incubated for 10 min before the assay. In all cases, no product

11 formation was generated in the absence of the added heterologous enzymes.

12

13 Fig. 4. Modeled NbaA structure and interactions with FMN, NADPH and 2-NBA. (A)

14 Fold recognition based sequence alignment between NbaA and the currently

15 structurally characterized members (identified here by their PDB accession numbers at

16 http://www.rcsb.org/) of the NADH:FMN oxidoreductase-like structural family from

17 the FMN-binding split barrel fold. 1EJE – *Methanobacterium thermoautotrophicum*

18 FMN-binding protein MTH152; 1USF – *Thermus thermophilus* styrene

19 monooxygenase; 1IOS – *Archeoglobus fulgidus* ferric reductase; 1RZ1 – *Bacillus*

20 *thermoglucosidasius* flavin reductase PheA2; and 1WGB – *Thermus thermophilus*

21 probable flavoprotein. Secondary structure elements (α-helices, β-strands) of NbaA,

22 corresponding to its 3D homology model built based on this alignment, are indicated

23 above its sequence. The NbaA residues individually mutagenized to alanine in this

24 study are marked by closed circles color-coded according to the activity of the

25 corresponding mutants: red – inactive, magenta – residual activity, black – activity

26 comparable to wild-type. (B) Stereo view displaying the structural model of the NbaA

39

1   dimer. The trace of the NbaA main-chain, rendered schematically by its secondary

2   structure, is colored differently for the two monomers (green and cyan, respectively),

3   with the 10-residue insertion in the β5-α2 loop relative to the other members of the

4   fold family highlighted in red in one of the monomers. Ligands docked to NbaA are

5   shown only in one of the two equivalent binding sites of the homodimer, and colored

6   as: FMN – blue, NADPH – red, and Ni^{2+} – magenta. (C) Stereo view zooming in the

7   FMN binding site of NbaA. (D) Stereo view showing the modeled interactions of

8   NADPH with the FMN-bound NbaA. (E) Stereo view detailing the putative binding

9   mode of the 2-NBA substrate. In the latter three panels, the protein main-chain is

10   rendered as in panel (B), select protein residues and side chains are shown explicitly,

11   and the following color scheme is applied to the C atoms belonging to the various

12   displayed molecules: FMN – yellow, NADPH in panel (D) – light blue, 2-NBA in

13   panel (E) – magenta, protein – green or cyan. The other atom types are colored as: N –

14   blue, O – red, S – yellow, P – orange. Hydrogen bonds are shown with yellow dashed

15   lines, and metal ion hexa-coordination with black solid lines. The Ni^{2+} ion is

16   represented as a magenta sphere, and its two coordinating water molecules as red

17   spheres in panel (C).

18

19   Fig. 5. Proposed ping-pong mechanistic action of 2-nitrobenzoate nitroreductase in the

20   formation of 2-hydroxyaminobenzoic acid (2-HABA).

21

22   Fig. 6. NbaY is necessary for 2-NBA chemotaxis, as determined by A) swarm plate

23   assay, B) drop assay, and C) modified agarose-plug assay.

24

25   Fig. 7. Comparative organization and homology between the *nba* gene locus in *P.*

26   *fluorescens* strain KU-7 and the uncharacterized open reading frames encoded on a

40

1   large plasmid in *Polaromonas* sp. JS666. Major differences of the coding capacity are

2   notably at the extremities of the core biodegradative genes and in the spacing of the

3   intergenic regions.

4

5

6   Supplemental Fig. S1. The creation of in-frame deletion constructs of *nbaY*. The top

7   and second lines represent the two PCRs used to generate fragments (PCR1 and

8   PCR2) which will form an in-frame deletion of *nbaY* when fused. The PCR primers

9   nbaY-Ni and nbaY-Ci are complementary over 21 nucleotides (represented by the

10   broken lines) so that when two PCR products are mixed, the complementary regions

11   anneal and prime at the 3' overlapping region for a 3' extension of the complementary

12   strand. In the third line, the fused nucleotide molecule is amplified by PCR with

13   primers nbaY-No and nbaY-Co. Primers nbaY-No and nbaY-Co have *Eco*RI sites

14   incorporated into the 5' ends of both oligonucleotides (represented by the gray broken

15   lines) so that fusion product can be restriction digested and cloned into pK18*mobsacB*.

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17   Supplemental Fig. S2. Multiple sequence alignment of NbaB. PoJS666 [*Polaromonas*

18   sp. JS666 acc. number: CP000317 (ABE46992)]; BxLB400 [*B. xenovorans* LB400 acc

19   number CP000270 (ABE31534)]; AzEbN1 [*Azoarcus* sp. EbN1 acc. number:

20   NC_006513 (YP_158576)]; RfDSM15236 [*Rhodoferax ferrireducens* DSM 15236 acc.

21   number: CP000267 (ABD67975)]; PnbBTW3 [*P. putida* TW3 acc. number: AF292094

22   (AAG01543)]; PbnBYH102 [*Pseudomonas* sp. YH102 acc. number: AF187880

23   (AAF01448)); and PnbBYH105 [*Ralstonia pickettii* acc. number: AF187879

24   (AAF01444)].

25

26   Supplemental Fig. S3. Multiple sequence alignment of NbaY. PfPf-5 [*P. fluorescens*

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TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
E. coli		
BL21	F⁻ *ompT hsdSB* (r$_B^-$m$_B^-$) *gal dcm*	Novagen
DH5α	*supE44 thi-1 recA1 hsdR17 endA1 gyrA* (Nalr) Δ(*lacIZYA -argF*) U169 *deoR* [φ80*dlac* Δ(*lacZ*)M15]	62
S17-1	*recA pro thi hsdR* RP4-2-Tc::Mu-Km::Tn7 Tra⁻ Tp^i Sm^i	66
XL1-blue	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* [F' *lacI*^{q} ZM15 Tn*10* (Tet^r)]	10
P. fluorescens		
KU-7	Wild type, 2-NBA⁻, 4-HBA⁺	27
KUM-9	*orf16* ::Tn*5 -31* Tp, 2-NBA⁻, 4-HBA⁺	This study
KUM-19	*nbaA* ::Tn*5 -31* Tp, 2-NBA⁻, 4-HBA⁺	This study
KUM-33	*nbaB* ::Tn*5 -31* Tp, 2-NBA⁻, 4-HBA⁺	This study
KU7Δ7	*nbaY* deletion mutant	This study
Plasmids		
pKN31	Tn*5-31* Tp transposon delivery plasmid; *mob* RP4 Km^r Tp^r	1
pK18*mobsacB*	Negative selection vector, Km^r	63
pREP1	Expression vector with T7 promoter, p15A origin, Cm^r	57
pSD80	Expression vector with *tac* promoter, ColE1 origin, Ap^r	68
pT7-5	Expression vector with T7 promoter, ColE1 origin, Ap^r	69
pUC19	Cloning vector with *lac* promoter, ColE1 origin, Ap^r	74
pNBA3	9.3-kb *Hin* dIII fragment from *P. fluorescens* strain KU-7 in pUC19; Ap^r	49
pNBA5	6.2-kb *Eco* RI fragment from *P. fluorescens* strain KUM-19 in pUC19; Ap^r ,Tp^r	This study
pNBA6	3.8-kb *Bam* HI fragment from *P. fluorescens* strain KUM-19 in pUC19; Ap^r ,Tp^r	This study
pSD*nbaA*	*Eco* RI*-*Pst* I* fragment containing *nbaA* in pSD80	This study
pSD*nbaA* -N40A	Replacement of Asn40 by Ala in NbaA in pSD*nbaA*	This study
pSD*nbaA* -H63A	Replacement of His63 by Ala in NbaA in pSD*nbaA*	This study
pSD*nbaA* -H69A	Replacement of His69 by Ala in NbaA in pSD*nbaA*	This study
pSD*nbaA* -D76A	Replacement of Asp76 by Ala in NbaA in pSD*nbaA*	This study
pSD*nbaA* -H110A	Replacement of His110 by Ala in NbaA in pSD*nbaA*	This study
pSD*nbaA* -E113A	Replacement of Glu113 by Ala in NbaA in pSD*nbaA*	This study
pSD*nbaB*	*Eco* RI*-*Pst* I* fragment containing *nbaB* in pSD80	This study
pSD*nbaAnbaB*	pSD*nbaA* plus ribosome binding site at HindIII and NheI plus nbaB cloned at AscI and NheI	This study
pNba∆Y	*nbaY* deletion cassette in pK18*mobsacB*	This study

[a]*Eco* RI* and *Pst* I* are restriction endonucleases introduced by PCR design.
2-NBA+, growth on 2-NBA; 4-HBA+ , growth on 4-hydroxybenzoate; 2-NBA-, no growth on 2-NBA

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TABLE 2. NbaA activities in the cell-free extracts contining NbaA or its mutant proteins and the effect of FMN on their activities[a]

NbaA	Specific activity			
	Without FMN		With FMN	
	U/mg[b]	%	U/mg	%
Wild-type	10.26 ± 0.23	100	31.73 ± 0.37	100
N40A	ND[c]	-	ND	-
H63A	7.44 ± 0.17	73	22.85 ± 0.33	72
H69A	0.20 ± 0.01	2	0.96 ± 0.03	3
D76A	ND	-	ND	-
H110A	3.38 ± 0.01	33	13.99 ± 0.38	44
E113A	ND	-	ND	-

[a] FMN was added to the cell-free extracts containing NbaA and its mutants at the concentration of 5.0 mM, and it was preincubated for 5 min on ice prior to the assay.

[b] One unit is the amount (in micromoles) of NADPH oxidized per minute. Results are the mean of three independent experiments with standard deviations noted.

[c] Not detected

Fig. 1:

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Fig. 2:

Fig. 3:

Fig. 4:

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Fig. 5:

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Fig. 6:

Fig 7: