Cloning of a gene encoding 4-amino-3-hydroxybenzoate 2,3-dioxygenase from Bordetella sp. 10d

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Title: Cloning of a gene encoding 4-amino-3-hydroxybenzoate 2,3-dioxygenase from *Bordetella* sp. 10d

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Abstract

Bordetella sp. 10d produces a novel dioxygenase catalyzing the *meta*-cleavage of 4-amino-3-hydroxybenzoic acid, 4-amino-3-hydroxybenzoate 2,3-dioxgenase (4A3HBA23D). A gene encoding 4A3HBA23D was cloned and named *ahdA*. The deduced amino acid sequence of *ahdA* showed 29.2-24.2% identities to those of prokaryotic and eukaryotic 3-hydoxyenzoate 3,4-dioxygenases in reported *meta*-cleavage dioxygenases. However, no identities were observed in the amino terminal sequences of the first 29 amino acid residues. An ORF was found downstream of *ahdA*. The deduced amino acid sequence of the ORF showed identities to those of LysR family regulators involved in protocatechuate metabolism, and contained motifs conserved in the regulators. On the basis of these results, the ORF was named *ahdR* encoding a putative LysR family regulator. The transcription start point of *ahdA* was localized 414-bp upstream of the start codon of *ahdA*. Two DNA-binding motifs of LysR family regulators were found upstream of the transcription start point. These observations suggest that a LysR family regulator encoded by *ahdR* regulates the expression of *ahdA*.

Key Words:

4-Amino-3-hydroxybenzoate 2,3-dioxygenase;
4-Amino-3-hydroxybenzoate-degrading bacterium; *meta*-Cleavage pathway;
2-Aminophenol derivatives; *Bordetella*; LysR family regulator

Introduction

Dioxygenases catalyzing the fission of benzene rings are key enzymes in the microbial metabolic pathways of aromatic compounds. These previously reported dioxygenases attack aromatic compounds with two or three hydroxyl groups, such as catechol, protocatechuic acid, gentisic acid, and benzenetriol, and produce *ortho*- or *meta*-cleavage compounds [1-5]. However, it was recently reported that a few dioxygenases attack aromatic compounds with a single hydroxyl group, such as 2-aminophenol [6, 7], salicylic acid [8], 3-hydroxyanthranilic acid [9], and 4-amino-3-hydroxybenzoic acid (4A3HBA) [10].

Bordetella sp. 10d was isolated from a medium containing 4A3HBA as the sole carbon and nitrogen source [10]. This bacterium produces a novel dioxygenase, 4-amino-3-hydroxybenzoate 2,3-dioxygenase (4A3HBA23D) that catalyzes the *meta*-cleavage of a benzene ring of only 4A3HBA and shows no activities for 2-aminophenol and its derivatives with a carboxyl group. The determined amino terminal sequence of 25 amino acid residues showed no identities to those of other reported dioxygenases catalyzing the *meta*-cleavage of benzene rings. The subunit size of 4A3HBA23D was 21 kDa and differed from those of the reported dioxygenases except the 23.8 kDa subunit of 3-hydoxybenzoate 3,4-dioxygenase (HAO) from *Pseudomonas fluorescens* strain KU-7 [9]. These findings show that the 4A3HBA23D is localized as a specific enzyme in the dioxygenases of a *meta*-cleavage type.

In this study, we cloned a gene encoding the 4A3HBA23D from *Bordetella* sp. 10d, and named it *ahdA*. The deduced amino acid sequence of the cloned gene showed significant identities to only those of HAOs. Possible DNA-

binding motifs of LysR family regulators were found upstream of the transcriptional start point of *ahdA*. These results suggest that the putative LysR family regulator encoded by *ahdR*, following *ahdA*, regulates the expression of *ahdA*.

Materials and methods

Bacterial strain and cultivation. *Bordetella* sp. 10d was cultured in 4A3HBA medium [10] at 30°C with shaking. *Escherichia coli* XL1-Blue was cultured in Luria-Bertani (LB) medium [11] supplemented, if necessary, with ampicillin (100 μg/ml), tetracycline (12.5 μg/ml), isopropyl-β-D(-)-thiogalactopyranoside (IPTG) (1 mM), and X-Gal (0.04%) at 37°C with shaking. Expression of a 4A3HBA23D gene in *E. coli* XL1-Blue was performed in 50 ml of LB medium containing ampicillin (300 μg/ml) and IPTG (1 mM) at 30°C for 5 h with shaking.

Determination of amino terminal amino acid sequence of 4A3HBA23D. The amino terminal sequence of 25 amino acid residues of the 4A3HBA23D from *Bordetella* sp. 10d was determined in the previous study [10]. To design primers used in PCR amplifying a partial 4A3HBA23D gene, the 4A3HBA23D was purified and an amino terminal sequence longer than the 25 amino acid sequence was determined as described previously [10].

Gene manipulation and PCR. Standard methods were used for plasmid DNA purifications, restriction enzyme digestions, and *E. coli* transformations [11]. Subcloning experiments were performed in pBluescript II vectors

(Stratagene). Total DNA from *Bordetella* sp. 10d was purified from 4A3HBA-grown cells as described previously [12].

A partial 4A3HBA23D gene was amplified by PCR using an F primer (5'-GARAAYTTYAARATGCC) and an R primer (5'-ARIGTYTCRTCRTCCAT), corresponding to ENFKMP (at positions 5 to 10) and MDDETL (at positions 33 to 38) in the determined amino terminal sequence. The PCR cycle was performed at 95°C (30 sec), 45°C (30 sec), and 72°C (30 sec) for 38 cycles. Amplified fragments were ligated into a pGEM-T easy vector (Stratagene), and the resulting plasmids were introduced into *E. coli* cells. The plasmid pGEM-100 isolated from a transformant was sequenced to confirm that the plasmid carried a fragment encoding the amino terminal sequence of 4A3HBA23D.

DNA fragments containing a complete 4A3HBA23D gene and a ribosome-binding site were amplified by PCR to examine the expression of *ahdA* gene in *E. coli*. Primers used in the PCR were 5'-*GAAGGA*GGAGACGGCATGATCATTC (containing an additional ribosome binding site shown by italic letters) and 5'-CTCAGCCTGTACGTCGCA, corresponding to sequences at the positions of 2224-2242 and 2848-2831, respectively, in the determined nucleotide sequence. Amplified fragments were ligated into a pGEM-T easy vector, and the resulting plasmids were introduced into *E. coli* cells. The plasmid pAHDA2 isolated from a transformant was sequenced to confirm that the plasmid carried the complete 4A3HBA23D gene with the same orientation as the transcriptional direction of the *lac* promoter in the vector. A plasmid pAHDA1 carrying the 4A3HBA23D gene with the opposite orientation to the transcriptional direction of the *lac* promoter was

constructed as a negative control.

Southern blot analysis and gene cloning. DNA fragments containing the 4A3HBA23D gene were examined by Southern blot analysis. A 0.5 μg aliquot of total DNA from *Bordetella* sp. 10d was digested with restriction endonucleases, and fixed on a Hybond-N+ membrane (Amersham Biosciences) by a VacuGene XL vacuum blotting system (Amersham Biosciences) after 1% agarose gel electrophoresis. An *Eco*RI fragment (127-bp), which was cut out from the plasmid pGEM-100 by *Eco*RI digestion and contained an amplified partial 4A3HBA23D gene, was labeled with an AlkPhos direct labeling module (Amersham Biosciences), and used as a probe. The probe was hybridized with DNA fragments fixed on the membrane according to the manufacturer's instructions. DNA fragments hybridized with the probes were detected using a CDP-*Star* detection reagent according to the manufacturer's instructions. To clone the 4A3HBA23D gene, a gene library was constructed from 432 transformants carrying recombinant plasmids with 2.6-kb *Cla*I fragments appearing as a positive band in the Southern blot analysis. The recombinant plasmids were fixed on a Hybond-N+ membrane according to the manufacturer's instructions, and hybridized with the same probe. A transformant showing a positive signal was selected, and a recombinant plasmid found in the transformant was named pC1C8.

DNA fragments overlapped with a cloned 4A3HBA23D gene in the plasmid pC1C8 also were examined by Southern blot analysis using a 1.0-kb *Pst*I-*Cla*I fragment containing the gene from pC1C8 as a probe. In the Southern blot analysis, a 3.2-kb *Apa*I-*Pst*I fragment appeared as a positive band. Transformants of 699 strains carrying 3.2-kb *Apa*I-*Pst*I fragments were stored as

a gene library, and the plasmid pPA was isolated from a transformant showing a positive signal by colony hybridization using the *Pst*I-*Cla*I fragment as a probe.

Sequence analysis. The sequencing reactions were performed by using a Thermo sequenase primer cycle sequencing kit 7-deaza dGTP (Amersham Biosciences) and plasmids purified with a FlexiPrep kit (Amersham Biosciences). Reaction mixtures were run on a Shimadzu DSQ-2000L sequencer (Shimadzu, Kyoto, Japan). Computer analyses of deduced amino acid sequences were accomplished by the BLAST database searching program at the DNA Data Bank of Japan. Identities of deduced amino acid sequences were calculated by using Genetyx-win software version 3 (Software Development, Tokyo, Japan). A multiple sequence alignment was performed by CLUSTALW 1.7. The DDBJ/EMBL/GenBank accession number for the sequence reported in this paper is AB110004.

Expression of 4A3HBA23D gene in E. coli. Cells of *E. coli* XL1-Blue carrying the plasmid pPA, pAHDA1, or pAHDA2 were harvested from 50 ml culture, and a cell extract was prepared as described previously [13]. Enzyme activity of 4A3HBA23D was assayed as described previously [10]. One unit of enzyme activity was defined as the amount of enzyme that converted 1 μmol of 4A3HBA per min. Protein concentrations were measured by the method of Lowry et al. [14]. Specific activity was defined as units/mg protein. Production of 4A3HBA23D in *E. coli* was analyzed by SDS-PAGE [15].

Analysis of transcriptional start site of 4A3HBA23D gene. Bordetella sp. 10d was grown in the 4A3HBA medium containing 1% meat extract until it reached the optical density of 1.2 at 660 nm. Cells were harvested from 0.7 ml of the culture and treated with a bacterial reagent (Qiagen, Hilden, Germany) to

stabilize mRNA. Cells were disrupted by a Qiagen Mixer Mill MM203 (Qiagen), and total RNA was purified using an RNeasy Mini Kit (Qiagen) after treatment with a QIAshredder (Qiagen). Reverse transcriptional reaction was done using an Omniscript RT kit (Qiagen) and an FITC-labeled primer, 5'-TTCTGACATAGCCCCTGCCG, derived from the sequence complementary to that of 2163 to 2182 in the determined sequence. The total RNA was digested with 0.43 Kunitz units of RNase A (Sigma) at 37°C for 30 min. The reaction mixture was then extracted with phenol and chloroform. The remaining cDNA fragment was precipitated with ethanol, and dissolved in 4 μl of TE buffer (pH 8.0). After addition of 4 μl of loading dye solution supplied to a Thermo sequenase primer cycle sequencing kit 7-deaza dGTP, the sample was denatured at 95°C for 5 min. A DNA sequencing ladder was created by using the same primer and the plasmid pC1C8 purified with the FlexiPrep kit. A portion (2 μl) of the sample and sequencing products were put onto an 8% (w/v) Long Ranger (FMC, Rockland, USA) gel (26-cm length). Electrophoresis was done using a Shimadzu DSQ-2000L DNA sequencer.

Results

Cloning of 4A3HBA23D gene

We determined the amino terminal sequence of 38 amino acid residues from the purified 4A3HBA23D and designed primers to amplify a partial 4A3HBA23D gene. Sequence analysis of the plasmid pGEM-100 revealed that

the insert DNA of this plasmid was 101 bp and encoded the amino terminal sequence of 4A3HBA23D. In Southern blot analysis using the insert DNA of pGEM-100 as a probe, *Cla*I-digested total DNA of *Bordetella* sp. 10d showed a positive band at the position of 2.6-kb (data not shown). To clone the 4A3HBA23D gene, a gene library containing 2.6-kb *Cla*I fragments was constructed and screened by colony hybridization using the same probe. The plasmid pC1C8 was isolated from a transformant showing a positive signal. Preliminary sequence analysis of the insert of pC1C8 revealed that a cloned 4A3HBA23D gene lacked a carboxyl terminal region of the enzyme. Further colony hybridization was performed to obtain a 3.2-kb *Apa*I-*Pst*I fragment overlapped with a fragment containing the cloned partial 4A3HBA23D gene (data not shown). The plasmid pPA was isolated from a transformant showing a positive signal.

Sequence analysis of cloned genes

The inserts of the plasmids pC1C8 and pPA covered 4804 bp, and two ORFs were found in the determined sequence (Fig. 1). The first ORF started from the position of 2233 bp, and consisted of 528 bp encoding 175 amino acid residues. The amino terminal sequence deduced from the first ORF was identical to that determined from the purified 4A3HBA23D of *Bordetella* sp. 10d. Furthermore, a molecular mass of a polypeptide encoded by the first ORF was calculated to be 20715 Da, corresponding to the 21 kDa estimated by SDS-PAGE of a subunit of the enzyme [10]. These results show that the first ORF is a gene encoding 4A3HBA23D from *Bordetella* sp. 10d. We, therefore,

named the gene encoding 4A3HBA23D *ahdA*.

The second ORF was 154-bp downstream of the stop codon of the first ORF, and encoded 350 amino acid residues. Except for the second ORF, ORFs with a larger size than that of the first ORF weren't found in the determined sequence.

Identities of gene products

The deduced amino acid sequence of *ahdA* showed 29.9% and 27.9% identities with those of HAOs from yeast (**Z49525**) and 2-nitrobenzoate-assimilating *Pseudomonas fluorescens* strain KU-7 (**AB088043**), and shared identities with those of HAOs from rat (24.4%, **D44494**), human (24.2%, **Z29481**), and *Suberites domuncula* (22.8%, **AJ298053**), but shared no identities with those of other reported *meta*-cleavage enzymes. Fig. 2 shows an alignment of deduced amino acid sequences of *ahdA*, *nabC* encoding HAO from *P. fluorescens* strain KU-7, and an HAO gene from yeast. Two histidyl residues, which are essential for catalytic activity, are conserved in prokaryotic and eukaryotic HAOs [9]. In the sequence of AhdA, the two histidyl residues were conserved as His52 and His96. However, the amino terminal sequence of the first 29 residues of AhdA shared no identities with those of HAOs, although conserved amino acid residues were observed in comparison of yeast and *Pseudomonas* HAOs.

The deduced amino acid sequence of the second ORF showed identities with those of putative LysR family regulators involved in the protocatechuate metabolism of *P. putida* KT2440 (42.2%, **AE016783**), *Sphingomonas* sp. LB126

(33.1%, **AJ277295**), and *S. paucimobilis* SYK-6 (32.7%, **AB073227**). Fig. 3 shows an alignment of deduced amino acid sequences of the second ORF and a LysR family regulator from *P. putida* KT2440. LysR family regulators conserve three domains, an amino terminal DNA-binding domain showing a helix-turn-helix structure, a coinducer recognition/response domain, and a carboxyl-terminal domain [16]. In the second ORF sequence, a consensus sequence of the amino terminal DNA-binding domain was found at the position of 103-122 amino acid residues. Furthermore, the coinducer recognition/response domain also was found in the deduced amino acid sequence of the second ORF, but lacked both ends of the consensus sequence. On the basis of these observations, we concluded that the second ORF encoded a putative LysR family regulator involved in the 4A3HBA23D metabolism of *Bordetella* sp. 10d, and named it *ahdR*.

Expression of ahdA gene

Expression of a cloned *ahdA* was examined in *E. coli* cells. *E. coli* carrying the plasmid pPA, obtained by colony hybridization, showed no 4A3HBA23D activity although the plasmid carried an original *ahdA* gene at the same orientation as that of transcription from the *lac* promoter in the vector pBluescript II SK +. A complete *ahdA* with a ribosome-binding site was cloned by PCR to examine the expression of *ahdA* in *E. coli* because a possible ribosome-binding site recognized in *E. coli* was absent upstream of the *ahdA* in an original insert DNA of pPA. When *Bordetella* sp. 10d was grown on 4A3HBA medium containing 1% meat extract, the cell extract showed specific activity of

0.050 U/mg. The specific activity decreased, compared with data described in the previous paper [10]. However, a protein band showing 4A3HBA23D, synthesized in this strain, was observed on an SDS-polyacrylamide gel (lane 3 in Fig. 4). *E. coli* carrying pAHDA1 with an amplified *ahdA* gene at an opposite direction for the transcription of the *lac* promoter, showed no activity. On the other hand, cell extracts from *E. coli* carrying pAHDA2 expressing the *ahdA* gene by the *lac* promoter showed the specific activity of 0.64 U/mg, which was 12.8-fold that of the parent strain 10d. In SDS-PAGE, the cell extracts from *E. coli* carrying pAHDA2 contained a specifically produced protein band, which was in good agreement with the migration of the 4A3HBA23D from *Bordetella* sp. 10d.

Identification of transcriptional start site of ahdA

Total RNA was prepared from *Bordetella* sp. 10d grown on 4A3HBA medium containing 1% meat extract. A reverse transcription reaction was performed using an FITC-labeled primer and the total RNA, and a transcriptional start site of *ahdA* was examined by an 8% Long Ranger gel. As shown in Fig. 5 (A), the transcriptional start point of *ahdA* was found to be an A base 414-bp upstream of the start codon of *ahdA*.

Discussion

We cloned a gene encoding 4A3HBA23D, a novel *meta*-cleavage dioxygenase catalyzing the ring fission of 4A3HBA, and named it *ahdA*. This is

the first report describing the cloning of a gene encoding 4A3HBA23D. The deduced amino acid sequence of *ahdA* showed identities with those of prokaryotic and eukaryotic HAOs, and conserved two histidyl residues, which are essential for catalytic activity (Fig. 2). However, no identities were observed in the amino terminal sequence of the first 29 amino acid residues. The 4A3HBA23D from *Bordetella* sp. 10d shows high substrate specificity for only 4A3HBA, and does not catalyze the ring cleavage of 3-hydroxyanthranilate, which differs from 4A3HBA in the position of an amino group [10]; HAO encoded by *nbaC*, on the contrary, shows no activity for 4A3HBA [9]. Differences in the substrate specificity of these enzymes may come from differences in amino terminal sequences.

Furthermore, an ORF was found downstream of *ahdA*. The deduced amino acid sequence of the ORF showed significant identities to those of LysR family regulators involved in protocatechuate metabolism, and contained an amino terminal DNA-binding domain showing a helix-turn-helix structure conserved in LysR type regulators. On the basis of these results, we named the ORF *ahdR* encoding a putative LysR family regulator. Protocatechuic acid possesses a hydroxyl group at the 4 position in spite of an amino group at the same position in 4A3HBA, and was structurally similar to 4A3HBA. Identities between AhdR and the LysR family regulators suggest the possibility that an AhdR protein interacts with 4A3HBA and regulates the expression of *ahdA* by binding to the promoter region of *ahdA*. We examined DNA-binding motifs of LysR family regulators, T-N_{11}-A and G-N_{11}-A sequences in an inverted repeat [17, 18], upstream of the transcriptional start point of *ahdA*. As shown in Fig. 5 (B), two binding motifs were found 31-85 bp upstream of the transcriptional

start point of *ahdA*. This finding suggests that AhdR regulates the expression of *ahdA*. However, possible promoter sequences were not found in a region containing the motif sequences. Specific promoter sequences in the genus *Bordetella* may be present in the region.

The *nab* gene cluster containing a *nabC* gene encoding HAO, showing identity to the *ahdA* product, organizes all genes involved in 2-nitrobenzoic acid metabolism [9]. In general, it is known that genes encoding the *meta*-cleavage enzymes for monoaromatic rings are tightly clustered with genes responsible for lower pathways leading to the TCA cycle, and huge gene clusters are constructed [19-22]. However, we did not find other genes involved in the *meta*-cleavage pathway upstream of *ahdA* and downstream of *ahdR* in the determined 4,204-bp sequence. These observations show that the cloned *ahdAR* genes are located at a different locus from other genes responsible for the *meta*-cleavage pathway. This finding is interesting, because there are no reports of gene clusters that metabolize *meta*-cleavage compounds without genes encoding *meta*-cleavage enzymes in the monoaromatic compound-degrading pathways. Further genetic studies are needed to clarify the 4A3HBA metabolism by *Bordetella* sp. 10d.

References

[1] C. Nakai, K. Horiike, S. Kuramitsu, H. Kagamiyama, M. Nozaki, Three isozymes of catechol 1,2-dioxygenase (pyrocatechase), αα, αβ, and ββ, from *Pseudomonas arvilla* C-1, J. Biol. Chem. 265 (1990) 660-665.

[2] S. Murakami, Y, Nakanishi, N. Kodama, S. Takenaka, R. Shinke, K. Aoki, Purification, characterization, and gene analysis of catechol 2,3-dioxygenase from the aniline-assimilating bacterium *Pseudomonas* species AW-2, Biosci. Biotechnol. Biochem. 62 (1998) 747-752.

[3] H. Fujisawa, O. Hayashibara, Protocatechuate 3,4-dioxygenase. I. Crystallization and characterization, J. Biol. Chem. 243 (1968) 2673-2681.

[4] M.R. Harpel, J.D. Lipscomb, Gentisate 1,2-dioxygenase from *Pseudomonas*: purification, characterization, and comparison of the enzymes from *Pseudomonas testosteroni* and *Pseudomonas acidovorans*, J. Biol. Chem. 265 (1990) 6301-6311.

[5] L.A. Golovleva, O. Zaborina, R. Pertsova, B. Baskunov, Y. Schurukhin, S. Kuzmin, Degradation of polychlorinated phenols by *Streptomyces rochei* 303, Biodegradation 2 (1992) 201-208.

[6] U. Lendenmann, J.C. Spain, 2-Aminophenol 1,6-dioxygenase: a novel aromatic ring-cleavage enzyme purified from *Pseudomonas pseudoalcaligenes* JS45, J. Bacteriol. 178 (1996) 6227-6232.

[7] S. Takenaka, S. Murakami, R. Shinke, K. Hatakeyama, H. Yukawa, K. Aoki, Novel genes encoding 2-aminohenol 1,6-dioxygense from *Pseudomonas* sp. AP-3 growing on 2-aminophenol and catalytic properties of the purified enzyme, J. Biol. Chem. 272 (1997) 14727-14732.

[8] J.P. Hinter, C. Lechner, U. Riegert, A.E. Kuhm, T. Storm, T. Reemtsma, A. Stolz, Direct ring fission of salicylate by a salicylate 1,2-dioxygenase activity from *Pseudomonas salicylatoxidans*, J. Bacteriol. 183 (2001) 6936-6942.

[9] T. Muraki, M. Taki, Y. Hasegawa, H. Iwaki, P.C.K. Lau, 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, Appl. Environ. Microbiol. 69 (2003) 1564-1572.

[10] S. Takenaka, T. Asami, C. Orii, S. Murakami, K. Aoki, A novel *meta*-cleavage dioxygenase that cleaves a carboxyl-group-substituted 2-aminophenol, Eur. J. Biochem. 269 (2002) 5871-5877.

[11] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning. A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989.

[12] S. Murakami, N. Kodama, R. Shinke, K. Aoki, Classification of catechol 1,2-dioxygenase family: sequence analysis of a gene for the catechol 1,2-dioxygenase showing high specificity for methylcatechols from Gram^{+} aniline-assimilating *Rhodococcus erythropolis* AN-13, Gene 185 (1997) 49-54.

[13] S. Murakami, A. Takashima, J. Takemoto, S. Takenaka, R. Shinke, K. Aoki, Cloning and sequence analysis of two catechol-degrading gene clusters from the aniline-assimilating bacterium *Frateuria* sp. ANA-18, Gene 226 (1999) 189-198.

[14] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein

measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265-275.

[15] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680-685.

[16] M.A. Shell, Molecular biology of the LysR family of transcriptional regulator, Annu. Rev. Microbiol. 47 (1993) 597-626.

[17] K. Goethals, M. Van Montagu, M. Holsters, Conserved motifs in a divergent nod box of *Azorhizobium caulinodans* ORS571 reveal a common structure in promoters regulated by LysR-type proteins, Proc. Natl. Acad. Sci. USA 89 (1992) 1646-1650.

[18] M.R. Parsek, R.W. Ye, P. Pun, A.M. Chakrabarty, Critical nucleotides in the interaction of a LysR-type regulator with its target promoter region, J. Biol. Chem. 269 (1994) 11279-11284.

[19] V. Shingler, J. Powlowski, U. Marklund, Nucleotide sequence and functional analysis of the complete phenol/3,4-dimethylphenol catabolic pathway of *Pseudomonas* sp. strain CF600, J. Bacteriol. 174 (1992) 711-724.

[20] M. Takeo, T. Fujii, Y. Maeda, Sequence analysis of the genes encoding a multicomponent dioxygenase involved in oxidation of aniline and *o*-toluidine in *Acinetobacter* sp. strain YAA, J. Ferment. Bioeng. 85 (1998) 17-24.

[21] M. Takeo, T. Fujii, K. Takenaka, Y. Maeda, Cloning and sequencing of a gene cluster for the *meta*-cleavage pathway of aniline degradation in *Acinetobacter* sp. strain YAA, J. Ferment. Bioeng. 85, (1998) 514-517.

[22] S. Takenaka, S. Murakami, Y.-J. Kim, K. Aoki, Complete nucleotide

sequence and functional analysis of the genes for 2-aminohenol metabolism from *Pseudomonas* sp. AP-3, Arch. Microbiol. 174 (2000) 265-272.

Figure legends

Fig. 1. Location of cloned genes and restriction map of sequenced DNA fragments. Open arrows show ORFs and directions of their transcription. A 1.0-kb *Pst*I-*Cla*I fragment used in colony hybridization is indicated by ▨. Arrows indicates directions of the transcription of the *lac* promoters from vectors.

Fig. 2. Sequence alignment of AhdA from *Bordetella* sp. 10d, NbaC (HAO) from *P. putida* strain KU-7, and HAO from yeast. Amino acid residues conserved in all sequences are indicated by squares. Arrows show two histidyl residues, which are essential for catalytic activity [9]. Asterisks indicate amino acid residues conserved in AhdA and one of the HAOs. Amino acid residues conserved in HAOs are shown by !.

Fig. 3. Sequence alignment of the second ORF product and a putative LysR family regulator from *P. putida* KT2440. Asterisks indicate conserved amino acid residues. Consensus indicates consensus sequences, an amino terminal DNA-binding domain showing a helix-turn-helix structure and coinducer recognition/response domain conserved in LysR family regulators [16]. X, any residues; h, hydrophobic residues, p, hydrophilic residues.

Fig. 4. Synthesis of the *ahdA* product by *E. coli*. Each cell extract containing 40 μg of proteins was ran on a 12% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS at 100 V for 1.5 h. The gel was stained with 0.25% (w/v) Coomassie Brilliant Blue R-250 in a solvent of ethanol-acetic acid-H_2O (9:2:9, v/v/v). Lane 1, cell

extract from *E. coli* carrying pAHDA1; Lane 2, cell extract from *E. coli* carrying pAHDA2; lane 3, cell extract from *Bordetella* sp. 10d; M, size marker.

Fig. 5. Analysis of the *ahdA* transcriptional start site. (A), Mapping of the *ahdA* transcription start point by primer extension. The ladder sequence (lane 1) was generated using the same primer as that employed for the extension reaction (lane 2). The arrow indicates the transcriptional start point of *ahdA*. (B), The sequence upstream of the *ahdA* transcriptional start point. The primer sequence is shadowed. Asterisks indicate T, A, and G bases conserved in binding motifs of LysR family regulators, T-N_{11}-A and G-N_{11}-A sequences [17, 18]. Inverted repeat sequences involved in the motifs are underlined. The arrow indicates the transcriptional start point of *ahdA*.

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Fig. 2. Sequence alignment of AhdA from *Bordetella* sp. 10d, NbaC (HAO) from *P. putida* strain KU-7, and HAO from yeast. Amino acid residues conserved in all sequences are indicated by squares. Arrows show two histidyl residues, which are essential for catalytic activity [9]. Asterisks indicate amino acid residues conserved in AhdA and one of the HAOs. Amino acid residues conserved in HAOs are shown by !.

Fig. 3. Sequence alignment of the second ORF product and a putative LysR family regulator from *P. putida* KT2440. Asterisks indicate conserved amino acid residues. Consensus indicates consensus sequences, an amino terminal DNA-binding domain showing a helix-turn-helix structure and coinducer recognition/response domain conserved in LysR family regulators [16]. X, any residues; h, hydrophobic residues, p, hydrophilic residues.

Fig. 4. Synthesis of the *ahdA* product by *E. coli*. Each cell extract containing 40 μg of proteins was ran on a 12% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS at 100 V for 1.5 h. The gel was stained with 0.25% (w/v) Coomassie Brilliant Blue R-250 in a solvent of ethanol-acetic acid-H_2O (9:2:9, v/v/v). Lane 1, cell

extract from *E. coli* carrying pAHDA1; Lane 2, cell extract from *E. coli* carrying pAHDA2; lane 3, cell extract from *Bordetella* sp. 10d; M, size marker.

Fig. 5. Analysis of the *ahdA* transcriptional start site. (A), Mapping of the *ahdA* transcription start point by primer extension. The ladder sequence (lane 1) was generated using the same primer as that employed for the extension reaction (lane 2). The arrow indicates the transcriptional start point of *ahdA*. (B), The sequence upstream of the *ahdA* transcriptional start point. The primer sequence is shadowed. Asterisks indicate T, A, and G bases conserved in binding motifs of LysR family regulators, T-N_{11}-A and G-N_{11}-A sequences [17, 18]. Inverted repeat sequences involved in the motifs are underlined. The arrow indicates the transcriptional start point of *ahdA*.

Table 1

Expression of *ahdA* encoding 4A3HBA23D in *E. coli*

Plasmid or strain	Specific activity (U/mg)
Bordetella sp. 10d	0.050
pPA	0
pAHDA1	0.64
pAHDA2	0.0023

.

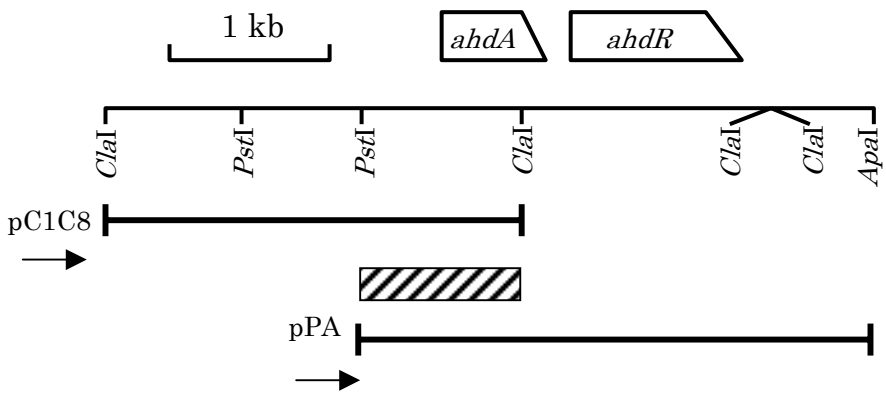

Fig. 1

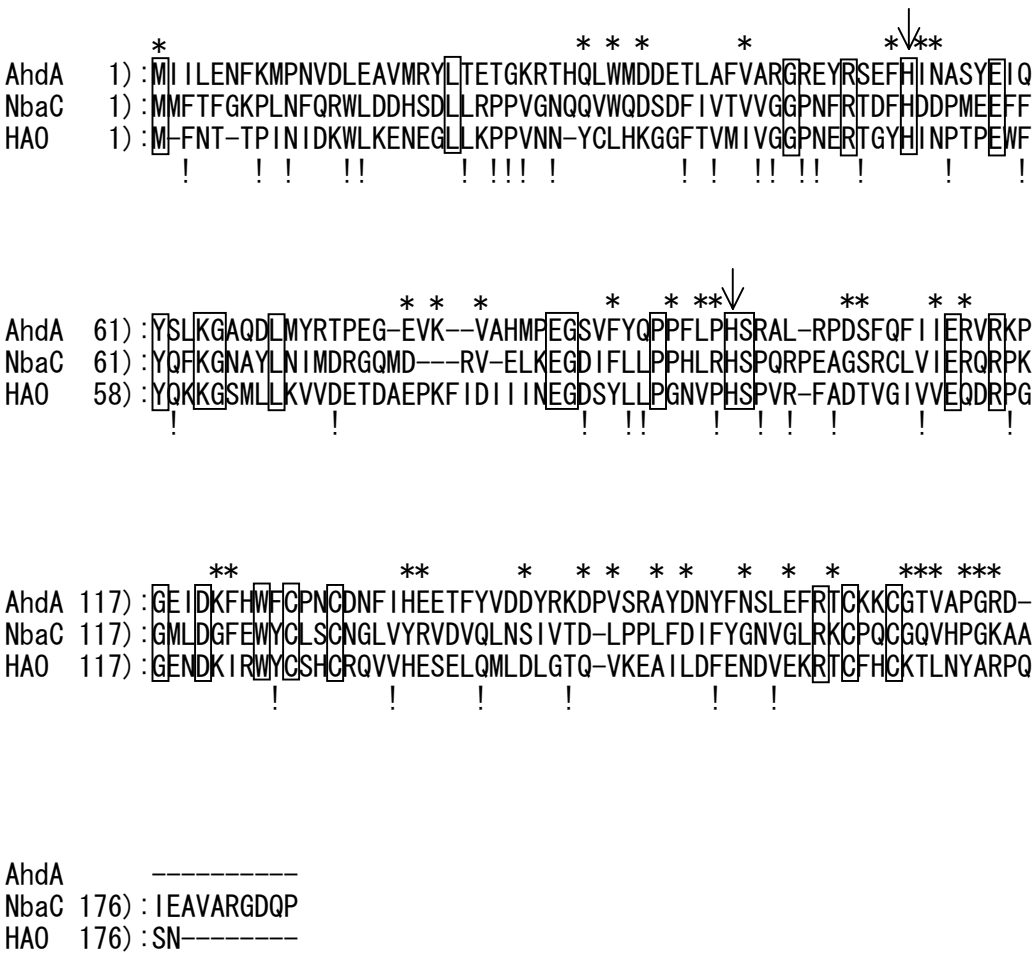
AhdA 1):MIILENFKMPNVDLEAVMRYLTETGKRTHQLWMDDETLAFVARGREYRSEFHIINASYEIQ
NbaC 1):MMFTFGKPLNFQRWLDDHSDLLRPPVGNQQVWQDSDFIVTVVGGPNFRTDFHDDPMEEFF
HAO 1):M-FNT-TPINIDKWLKENEGLLKPPVNN-YCLHKGGFTVMIVGGPNERTGYHINPTPEWF
AhdA 61):YSLKGAQDLMYRTPEG-EVK--VAHMPEGSVFYQPPFLPHSRAL-RPDSFQFIIERVRKP
NbaC 61):YQFKGNAYLNIMDRGQMD---RV-ELKEGDIFLLPPHLRHSPQRPEAGSRCLVIERQRPK
HAO 58):YQKKGSMLLKVVDETDAEPKFIDIIINEGDSYLLPGNVPHSPVR-FADTVGIVVEQDRPG
AhdA 117):GEIDKFHWFCPNCDNFIHEETFYVDDYRKDPVSRAYDNYFNSLEFRTCKKCGTVAPGRD-
NbaC 117):GMLDGFEWYCLSCNGLVYRVDVQLNSIVTD-LPPLFDIFYGNVGLRKCPQCGQVHPGKAA
HAO 117):GENDKIRWYCSHCRQVVHESELQMLDLGTQ-VKEAILDFENDVEKRTCFHCKTLNYARPQ
AhdA ----------
NbaC 176):IEAVARGDQP
HAO 176):SN--------

Fig. 2

```
10d          1:------------------------MKKAADALHRSHSVVSRAVDELEKALAVPLFERQARG
KT2440       1:MIDVELPNLMQVRAFIRVAELGSVSRATEVLFRAQSVVTRAIAELEARFAVPLFERHANG
                                         *   * *  *** **  ***   ******* * *

10d         38:ARLTVYGDILRRRVEQAFAEMQAVRTALWQLAPASGVHYANAPIYTLSIAESRLNLFLLF
KT2440      61:MRLTDYGECLLPRAQRVLAELDGVPSLLGTAQGEP----------LYLFQARRLQVFVKL
                *** **  *  *     **   *   *                          **  *

            (Amino terminal DNA-binding domain)
Consensus             hSxAApxLphSQPAhSxQhp
10d         98:AQYRHMSAVARHAGVSQPAVSMALRDLDLTIGQALFEPVSDGFRLTPAGEILIRHVKRAL
KT2440     111:CETRHMQTVARHFGLSQPAVSAALKVLEGGCGQPLFVRTSRGLQPTVASRDILFPIRRAL
                ***  **** * ****** **  *    ** **   * *   * *         ***

                                          (Coinducer recognition/response domain)
Consensus                                   LP-x10(8)-P-x5--L--x11(9)-Lpxxp
10d        158:VQLRLAGAEIAYEQGSPRGRLLVGALPYGGATILAPAVAGLLRQHPRLRVNIQEGSFSHL
KT2440     171:NELRLLDSDLSAMQGTLRGVVHVGALPLGRSRILPDAILRFTAQHPQVRVVTNESPFDLL
                ***         **  **   ***** *   **  *      ***  **   *  *  *

10d        218:ADRLAAGDLDMVVGALQPTDAYPELLSEPLFDDPIVIVARAGHPLAGKRRATLADTAREN
KT2440     231:ATELRVGDVDFVLGALRPHDYASDLVGEPLINEEMVVLARRGHPLLHT-HLTLKGVHQAR
               *  *  ** * * *** * *    *  ***    *  ** ****       **

10d        278:WVLPIEGAPTRRALSAALAERGLAAPQVVVESSNPSTISSLLQESNWVSAG---------
KT2440     290:WVLPRAGSPARQLLDNCFAAAGLTAPWPVVESADLAVIRGLLVRSDMLAAVSAHQLAYEI
               ****  * * *  *    *  ** **  ****     *  **  *   *

10d        329:----------AQACFRRVCRPGYWSPCWEGSL----------------
KT2440     350:ASGELQRLPLALPGTARAIGLMQRSGCLQSPAAVALMACIRQVITEQA
                        *    *       * *
```

Fig. 3

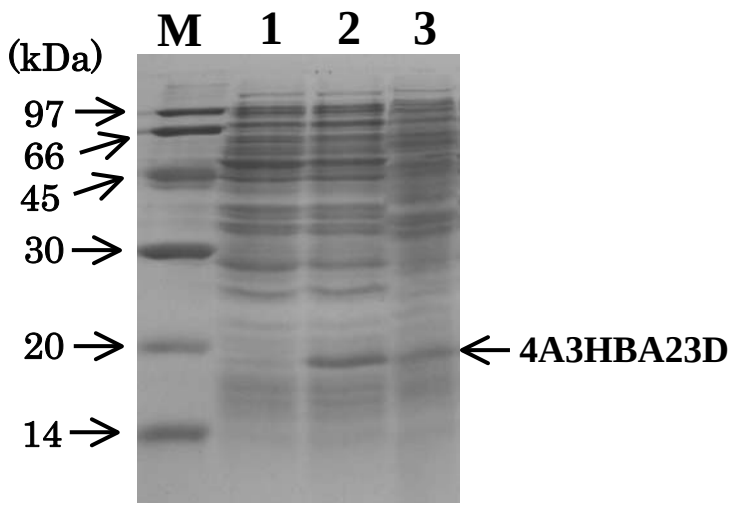

Fig. 4

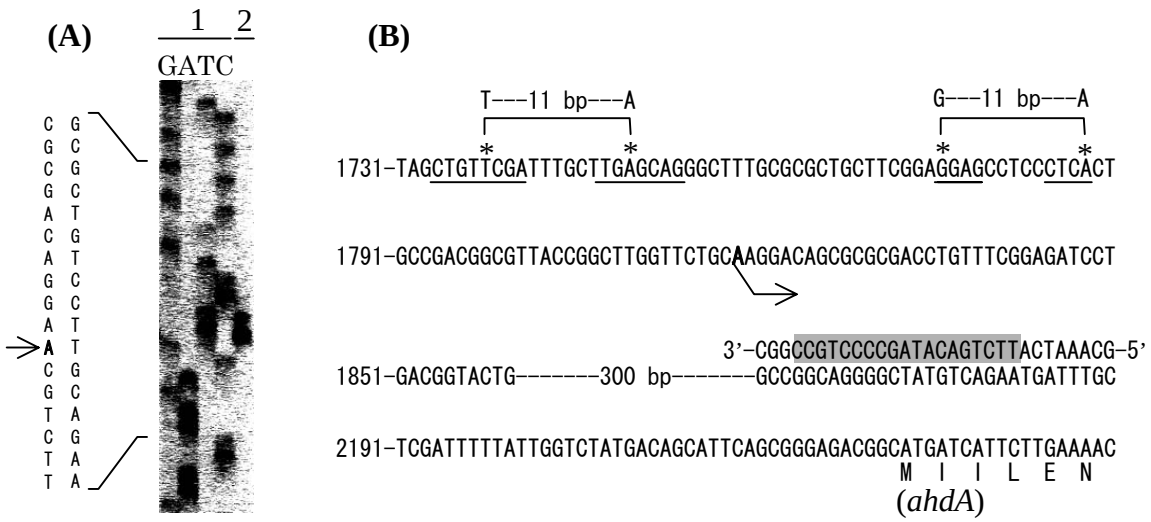
(A)
1 2
GATC
(B)
T---11 bp---A
G---11 bp---A
1731-TAGCTGTTCGATTTGCTTGAGCAGGGCTTTGCGCGCTGCTTCGGAGGAGCCTCCCTCACT
1791-GCCGACGGCGTTACCGGCTTGGTTCTGCAAGGACAGCGCGCGACCTGTTTCGGAGATCCT
3'-CGGCCGTCCCCGATACAGTCTTACTAAACG-5'
1851-GACGGTACTG-------300 bp-------GCCGGCAGGGGCTATGTCAGAATGATTTGC
2191-TCGATTTTTATTGGTCTATGACAGCATTCAGCGGGAGACGGCATGATCATTCTTGAAAAC
M I I L E N
(ahdA)

Fig. 5