Bioscience, Biotechnology, and Biochemistry

ISSN: 0916-8451 (Print) 1347-6947 (Online) Journal homepage: http://www.tandfonline.com/loi/tbbb20

Cloning and Functional Analysis of Aniline Dioxygenase Gene Cluster, from Frateuria Species ANA-18, That Metabolizes Aniline via an ortho-Cleavage Pathway of Catechol

Shuichiro MURAKAMI, Teruhiko HAYASHI, Tetsuya MAEDA, Shinji TAKENAKA & Kenji AOKI

To cite this article: Shuichiro MURAKAMI, Teruhiko HAYASHI, Tetsuya MAEDA, Shinji TAKENAKA & Kenji AOKI (2003) Cloning and Functional Analysis of Aniline Dioxygenase Gene Cluster, from Frateuria Species ANA-18, That Metabolizes Aniline via an ortho-Cleavage Pathway of Catechol, Bioscience, Biotechnology, and Biochemistry, 67:11, 2351-2358, DOI: 10.1271/bbb.67.2351

To link to this article: http://dx.doi.org/10.1271/bbb.67.2351

Published online: 22 May 2014.

Biosci. Biotechnol. Biochem., **67** (11), 2351–2358, 2003

Cloning and Functional Analysis of Aniline Dioxygenase Gene Cluster, from *Frateuria* Species ANA-18, That Metabolizes Aniline *via* an *ortho*-Cleavage Pathway of Catechol

Shuichiro MURAKAMI, Teruhiko HAYASHI, Tetsuya MAEDA, Shinji TAKENAKA, and Kenji AOKI†

Laboratory of Applied Microbiology, Department of Biofunctional Chemistry, Faculty of Agriculture, Kobe University, Nada, Kobe 657-8501, Japan

Received May 19, 2003; Accepted August 21, 2003

Genes encoding an aniline dioxygenase of *Frateuria* sp. ANA-18, which metabolizes aniline *via* the *ortho*-cleavage pathway of catechol, were cloned and named *tdn* genes. The *tdn* genes were located on the chromosomal DNA of this bacterium and weren't clustered with catechol-degrading gene clusters. These results show that the ANA-18 aniline-degrading gene cluster is constructionally different from *Pseudomonas tdn* and *Acinetobacter atd* gene clusters, which degrade aniline *via* the *meta*-cleavage pathway of catechol and organize catechol-metabolic genes in the gene clusters. When cloned *tdnQTA1A2B* genes were expressed in *Escherichia coli*, aniline dioxygenase activity was observed. Southern blot analysis revealed that homologues of the *tdnA1A2B* genes didn't exist in strain ANA-18. Disruption of the *tdnA1A2* genes gave the parent strain ANA-18 a defect in aniline metabolism. On the basis of these results, we concluded that only the cloned *tdn* genes function as genes encoding aniline dioxygenase in strain ANA-18 although this bacterium had two catechol-degrading gene clusters.

Key words: *tdn* genes; aniline degradation; hydroxylation; deamination; LysR-type regulator

Aniline is converted to catechol through the hydroxylation accomplishing deamination by microorganisms.[1] Catechol is metabolized to organic compounds involved in the TCA cycle *via* an *ortho*- or *meta*-cleavage pathway. Microorganisms metabolizing aniline *via* the *meta*-cleavage pathway are found in some genera.[2–5] Furthermore, genes encoding aniline dioxygenases (AD), catalyzing the conversion of aniline to catechol, have been cloned from *Pseudomonas putida* UCC22,[6] *Acinetobacter* sp. YAA,[7,8] and *Pseudomonas* sp. AW-2,[9] which metabolize aniline *via* the *meta*-cleavage pathway. AD consisted of five polypeptides encoded by *tdnQTA1A2B* in *P. putida* UCC22. The strain UCC22 *tdnA1*, *tdnA2*, and *tdnB* genes encode the large and small subunits of a terminal dioxygenase and an electron transfer protein, respectively. TdnQ and TdnT are related to the amino group transfer of aniline and the release of ammonia.[6]

Microorganisms metabolizing aniline *via* the *ortho*-cleavage pathway are found in the genera *Frateuria*,[10] *Acinetobacter*,[11] and *Rhodococcus*.[12] *Frateuria* sp. ANA-18 is an excellent aniline-degrader and metabolizes catechol by two catechol 1,2-dioxygenases (CD)[13] and *cis*,*cis*-muconate cycloisomerase isozymes[14] after the conversion of aniline to catechol. We cloned two catechol-degrading cat_1 and cat_2 gene clusters, consisting of different *catABC* genes and genes encoding LysR-type regulators ($CatR_1$ and $CatR_2$) from this bacterium.[15] The cat_2 gene cluster was localized on a catabolic plasmid,[15] and was expressed at a lower concentration of an inducer, *cis*,*cis*-muconate, than the cat_1 gene cluster.[16] Although catechol-degrading genes have been cloned in some aniline-degrading bacteria that metabolize catechol *via* the *ortho*-cleavage pathway,[11,17–19] genes encoding ADs have not yet been cloned in these bacteria. In addition, there is great interest in the number of gene clusters involved in the conversion of aniline to catechol in *Frateuria* sp. ANA-18 because two catechol-degrading gene clusters were found in this strain.[15]

In this paper, we report the cloning of genes encoding AD from *Frateuria* sp. ANA-18. Southern blot and gene disruption analyses revealed that the only AD functioned in this bacterium.

Materials and Methods

Bacterial strains and bacterial culture. Frateuria sp. ANA-18 was grown on 23 mM aniline[10] or 22 mM *p*-hydroxybenzoic acid medium at 30°C. For the 22 mM *p*-hydroxybenzoic acid medium, two solutions

† To whom correspondence should be addressed. Fax: +81-78-882-0481; E-mail: kaoki@kobe-u.ac.jp

Abbreviations: AD, aniline dioxygenase; Ap, ampicillin; CD, catechol 1,2-dioxygenase; LB, Luria-Bertani medium; ORF, open reading frame; Tc, tetracycline

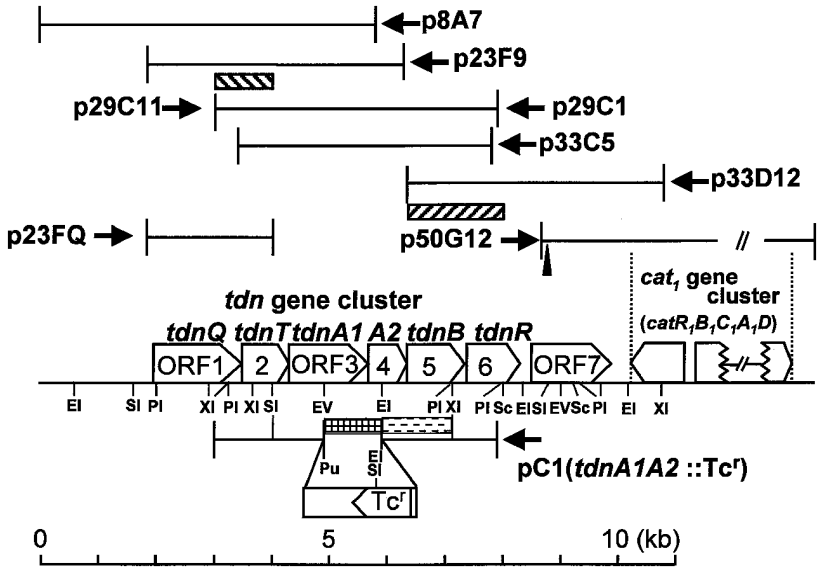

Fig. 1. Location of Cloned DNA Fragments and Genes.

Open arrows show ORFs and directions of their transcription. Probe 1 (a 1.8-kbp *Sac*I fragment from p33D12) and probe 2 (a 1.0-kbp *Sal*I fragment from p29C1) are shown by ▨ and ▧, respectively. The left *Sac*I and *Sal*I sites in probe 1 and probe 2, *Ban*III and *Sac*I sites used in the construction of the plasmid p29C11, and a *Sal*I site used in the construction of the plasmid p23FQ are located on the vectors. A 1.0-kbp *Eco*RV-*Eco*RI and 1.2-kbp *Eco*RI-*Xho*I fragments used in Southern blot analysis of *tdnA1A2* and *tdnA2B* as probes are indicated by ▦ and ▤, respectively. A solid triangle indicates a position where the CAT1 probe hybridizes. Arrows show directions of the transcription from the *lac* promoter. Abbreviations; EI, *Eco*RI site; EV, *Eco*RV site; PI, *Pst*I site; Pu, *Pvu*II site; Sc, *Sac*I site; SI, *Sal*I site; XI, *Xho*I site; Tc^r, tetracycline resistance gene.

were prepared separately. Solution A contained 0.6 g of K_2HPO_4, 0.3 g KCl and $MgSO_4 \cdot 7H_2O$, 6 mg of $FeSO_4 \cdot 7H_2O$ and $CaCl_2 \cdot 2H_2O$, and 0.12 g of yeast extract S (Nihon Seiyaku, Tokyo, Japan) in 600 ml of water (pH 7.0). Solution B contained 2.7 g of *p*-hydroxybenzoic acid and 0.7 g of ammonium sulfate in 300 ml (pH 7.0). Solution A was autoclaved, and solution B was sterilized by filtration. The two solutions were mixed at room temperature. When the medium containing agar was prepared, solution A containing 13 g agar was autoclaved and mixed with sterile solution B at 50 to 60°C. Transformants derived from strain ANA-18 were grown on 7.2 mM *p*-hydroxybenzoic acid medium, prepared by the addition of 0.9 g of *p*-hydroxybenzoic acid and 45 mg of yeast extract S in place of 2.7 g and 0.12 g of these components in the 22 mM *p*-hydroxybenzoic acid medium, supplemented with tetracycline (Tc) (6.25 μg/ml). *E. coli* XL1-Blue was cultured in Luria-Bertani medium (LB)[20] at 37°C, if necessary, supplemented with ampicillin (Ap) (100 μg/ml), Tc (12.5 μg/ml), and kanamycin (50 μg/ml).

DNA manipulations, gene cloning, and sequencing. Standard methods were used for plasmid DNA purifications, restriction enzyme digestions, and *E. coli* transformations.[20] Electroporation for *Frateuria* sp. ANA-18 was done in the following setting: 10 kV/cm, 400 Ω, and 25 μF. Subcloning experiments were done in pBluescript II vectors (Stratagene). A gene library of strain ANA-18 was constructed from 9,600 transformants, as previously described.[17] Recombinant plasmids of the transformants were fixed on a Hybond-N+ membrane (Amersham Biosciences) according to the manufacturer's instructions. The CAT1 probe (5′-CATCCTTGAGCCTTCGCGCT-3′, corresponding to the sequence of a position of 58–77 bases in p50G12) was labeled with T4 polynucleotide kinase (New England Biolabs, Beverly, USA) and [γ-^{32}P]ATP (ICN, Costa Mesa, USA). Probe 1 (a 1.8-kbp *Sac*I fragment from p33D12), and probe 2 (a 1.0-kbp *Sal*I fragment from p29C1) (Fig. 1) were labeled with a Random primer DNA labeling kit ver. 2 (Takara Bio, Otsu, Japan) and [α-^{32}P]dCTP (ICN). DNA fragments located upstream of the cat_1 gene cluster were cloned by colony hybridization using the CAT1 probe. Probes 1 and 2 were used in colony hybridization to clone DNA fragments flanking the inserts of the plasmids p33D12 and p29C1, respectively. Colony hybridization was done under standard conditions.[20]

The sequencing reactions were done 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). The computer analyses of deduced amino acid sequences were accomplished through the use of the BLAST database searching program at the DNA Data Bank of Japan. Identities of deduced amino acid sequences were calculated by using a Genetyx-win software

version 3 (Software Development, Tokyo, Japan). The DDBJ/EMBL/GenBank accession number for the sequence reported in this paper is AB089795.

Plasmids construction. Plasmid DNA for gene disruption was constructed in pBluescript II KS (+) (*ΔPst*I-*Sal*I), carrying a multiple cloning site with the deletion of a region between *Pst*I and *Sal*I sites. After digestion of the plasmid p29C1 with *Sma*I and *Xba*I, a 5.1-kb fragment was recovered and ligated with *Sma*I and *Xba*I-digested pBluescript II KS (+) (*ΔPst*I-*Sal*I). The resulting plasmid DNA was named p29C1*Δ*. The plasmid pC1(*tdnA1A2*::Tc^r) was constructed by the substitution of a 1.2-kbp *Eco*RV-*Eco*RI fragment in p29C1*Δ* with a 2.0-kbp *Eco*RI-*Pvu*II fragment containing a Tc resistance gene from pBR322.

A 5.1-kb DNA fragment, obtained by the digestion of p29C1 with *Ban*III and *Sac*I, was ligated to a pBluescript II SK (+) vector. A resulting plasmid was named p29C11, in which, the *tdnTA1A2B* genes were transcribed by the *lac* promoter. For expression of the *tdnQ* gene, a 2.3-kbp *Sac*I-*Sal*I fragment containing a *tdnQ* gene from p23F9 was ligated to the low copy vector pMW218 (Nippon Gene, Osaka, Japan) and the resulting plasmid was named p23FQ.

Expression of tdn gene cluster in E. coli and AD assay. E. coli XL1-Blue carrying various plasmid DNAs was cultivated in 6 ml of LB medium supplemented with antibiotics. When cell growth reached early log phase, isopropyl-*β*-D(−)-thiogalactopyranoside was added at a final concentration of 1 mM. After 5 h, cells were harvested and washed with 0.8% sodium chloride solution. Washed cells were mixed with 0.8% sodium chloride solution and cell suspensions with the turbidity of 10 to 18 at 660 nm were prepared. Aniline-dependent oxygen uptake showing AD activity was measured in a Clark-type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, USA) mounted in a water-jacketed reaction vessel at 25°C. The reaction mixture consisted of 2.8 ml of 0.1 M Tris-HCl buffer (pH 8.0), 0.1 ml of 10 mM aniline hydrochloride, and 0.1 ml of the cell suspension. One unit of AD activity was defined as the amount of enzyme that consumed 1 nmol of O_2 per min.

Isolation of double-crossover mutants deleting tdnA1A2. Frateuria sp. ANA-18 cells, grown on 22 mM *p*-hydroxybenzoic acid medium, were harvested from 10 ml of the culture by centrifugation, and mixed with 100 *μ*l of 10% glycerol after washing with the same solution. The cells were transformed by electroporation using 1.2 *μ*g of the plasmid pC1(*tdnA1A2*::Tc^r) that causes the deletion of *tdnA1A2* genes by homologous recombination (Fig. 1). After incubation on ice for 10 min, the pulsed cells were transferred into 7.2 mM *p*-hydroxybenzoic acid medium, and cultivated at 30°C for 8 h with shaking. Harvested cells were plated on the 7.2 mM *p*-hydroxybenzoic acid medium supplemented with Tc. Strain No. 49, grown on the plate, was isolated as a single-crossover mutant. Strain No. 49 was subcultured on the 7.2 mM *p*-hydroxybenzoic acid medium ten times, and cultivated in 6 ml of the same liquid medium. Cells harvested by centrifugation were spread on the same plates. Isolated colonies were inoculated on an aniline plate supplemented with Tc to examine their growth on aniline. Strains No. 49-1, 49-2, and 49-3, showing a defect in the aniline metabolism, were isolated as candidates for double-crossover mutants with deleted *tdnA1A2* genes, on the basis of the result that *tdnA1A2* homologues didn't exist in strain ANA-18 (Fig. 2).

Southern blot analyses. Homologues of the *tdnA1A2* genes and *tdnA2B* genes in strains No. 49, 49-1, 49-2, and 49-3, derived from strain ANA-18 by homologous recombination, were analyzed by Southern hybridization. Each 1 *μ*g of total DNA, purified as previously described,[17] was digested with restriction endonucleases, and was fixed on a Hybond-N+ membrane by a VacuGene XL vacuum blotting system (Amersham Biosciences) after 1% agarose gel electrophoresis. A 1.0-kbp *Eco*RV-*Eco*RI fragment containing partial *tdnA1A2* genes and a 1.2-kbp *Eco*RI-*Xho*I fragment containing partial *tdnA2B* genes were labeled with an AlkPhos direct labelling module (Amersham Biosciences), and used as probes. The probes were hybridized with DNA fragments fixed on the membrane according to the manufacturer's instructions. DNA fragments hybridized with the probes were detected using the CDP-*Star* detection reagent (Amersham Biosciences) according to the manufacturer's instructions.

Induction of AD and CD. A parent strain, *Frateuria* sp. ANA-18, and a strain defective in the aniline metabolism, No. 49-1, were cultivated in 900 ml of 22 and 7.2 mM *p*-hydroxybenzoic acid media, respectively, for 20 h. Cells harvested by centrifugation (13,000 × *g*, 10 min, 4°C) were washed twice with 0.8% sodium chloride solution. Reaction mixtures for the AD and CD induction and cell suspensions used for enzyme assays were prepared as previously described.[14]

cis,*cis*-Muconate, added to the reaction mixture, was enzymatically synthesized from catechol by the catalysis of CD. The CD was prepared as previously described.[14] A reaction solution consisted of 9.75 ml of 0.1 M Tris-HCl buffer (pH 8.0) and 12 ml of CD solution (80 U). The conversion of catechol to *cis*,*cis*-muconate started by the addition of 0.9 ml of 0.25 M catechol to the reaction solution, and was continued at 30°C with shaking. The remaining catechol in the

Table 1. Identity of Amino Acid Sequences Deduced from Cloned Genes

	Identity (%)	Gene	Product or function	Strain	References
ORF1	88.4	*tdnQ*	amino group transfer	*P. putida* UCC22	(6)
	63.2	*atdA1*	component of aniline dioxygenase	*Acinetobacter* sp. YAA	(7)
ORF2	62.0	*tdnT*	amino group transfer	*P. putida* UCC22	(6)
	37.7	*atdA2*	component of aniline dioxygenase	*Acinetobacter* sp. YAA	(7)
ORF3	83.4	*tdnA1*	large subunit of aniline dioxygenase	*P. putida* UCC22	(6)
	52.9	*atdA3*	α-subunit of aniline dioxygenase	*Acinetobacter* sp. YAA	(7)
ORF4	79.0	*tdnA2*	small subunit of aniline dioxygenase	*P. putida* UCC22	(6)
	47.6	*atdA4*	β-subunit of aniline dioxygenase	*Acinetobacter* sp. YAA	(7)
	81.4	ORF1	small subunit of aniline dioxygenase	*Pseudomonas* sp. AW-2	(9)
ORF5	75.7	*tdnB*	reductase component of aniline dioxygenase	*P. putida* UCC22	(6)
	31.1	*atdA5*	reductase component of aniline dioxygenase	*Acinetobacter* sp. YAA	(7)
	67.8	ORF2	reductase component of aniline dioxygenase	*Pseudomonas* sp. AW-2	(9)
ORF6	76.3	*tdnR*	LysR-type regulator	*P. putida* UCC22	(6)
	37.5	*atdR*	LysR-type regulator	*Acinetobacter* sp. YAA	(8)
	74.0	ORF3	LysR-type regulator	*Pseudomonas* sp. AW-2	(9)
ORF7	37.0	ORF9	unknown	*Alcaligenes eutrophus* SK4040	(23)
	37.0	RSp0051	probable porin signal peptide protein	*Ralstonia solanacearum* GMI1000	accession No. (AL646076) protein ID (CAD17202.1)

reaction solution was measured spectrophotometrically using 4-aminoantipyrine.[21] When catechol in the reaction solution disappeared, the pH of the reaction solution was adjusted to 8.0 with 1 N sodium hydroxide. By the addition of 0.9 ml of 0.25 M catechol to the reaction solution, the synthesis of *cis*,*cis*-muconate was restarted. The cycle converting catechol to *cis*,*cis*-muconate, described above, was repeated six times. After centrifugation (13,000 × *g*, 10 min, 4°C) of the reaction solution, the supernatant was filtered by a Collodion bag (Sartorius AG, Göttingen, Germany) to remove the enzyme. The pH of the filtrate was adjusted to 2.0; the white precipitate was collected by filtration, and then dissolved in ethyl acetate. Water was removed from the sample by the addition of sodium sulfate and the ethyl acetate layer was evaporated to dryness. The white residue was analyzed by TLC as previously described[1] and identified as *cis*,*cis*-muconate on the basis of the *Rf* values.

CD activity was assayed as previously described.[22] One unit of CD activity was defined as the amount of enzyme that converted 1 μmol of catechol to *cis*,*cis*-muconate per min. AD activity was assayed using cell suspensions with the turbidity of 45 at 660 nm as described above.

Results

Gene cloning and sequence analysis

To clone DNA fragments containing the upstream region of the cat_I gene cluster, we screened the gene library of *Frateuria* sp. ANA-18 by colony hybridization using the CAT1 probe, and isolated the plasmid p33D12 from a transformant showing a positive signal (Fig. 1). Preliminary sequence analysis revealed that the insert DNA of p33D12 carried a *tdnB* homologue, encoding a reductase component of AD from *P. putida* UCC22.[6] Colony hybridization using probe 1 was done to select recombinant plasmids with a region flanking the insert DNA of p33D12. Two plasmids were isolated from two transformants selected by the colony hybridization, and named p29C1 and p33C5, respectively (Fig. 1). Furthermore, we screened transformants carrying recombinant plasmids hybridized with probe 2 and isolated two plasmids, p8A7 and p23F9 (Fig. 1).

We found the complete 7 open reading frames (ORFs) with the same orientation by sequencing cloned fragments. ORF7 was located 175 bp downstream of ORF6. Intergenic regions between other ORFs were less than 9 bp and ORF2 and 3, and ORF3 and 4 were overlapped. The identities of the amino acid sequences deduced from these ORFs are summarized in Table 1. The amino acid sequences of the products of ORF1 to 6 showed more than 62.0% identities to those of AD components encoded by *tdn* genes from *P. putida* UCC22.[6] Compared with the UCC22 AD, the amino acid sequences of these ORFs showed lower identities to those of AD encoded by *atd* genes from *Acinetobacter* sp. YAA.[7,8] The ORFs were organized as well as the alignments of the *tdn* and *atd* genes. On the basis of these results, it was concluded that ORF1 to 5 and ORF6 were *tdnQTA1A2B* and *tdnR*, respectively, encoding AD components and a LysR-type regulator. A product of ORF7 consisted of 404 amino acid residues, and had 37% identities to those of ORF9 from *Alcaligenes*

Table 2. Expression of AD in *E. coli* Carrying Various Plasmids

Plasmid	AD activity (u/mg·dry cell)
p29C11	0
p23FQ	0
p29C11 + pMW218	0
p29C11 + p23FQ	1.9 ± 0.32[1)]

[1)] Values are the means ± SD (n = 4).

eutrophus SK4040,[23)] and RSp0051, encoding a probable porin signal peptide protein, from *Ralstonia solanacearum* GMI1000 (accession no., AL646076; protein ID, CAD17202.1). An intergenic region between ORF7 and $catR_1$ was 382 bp, but contained no ORFs. The amino acid sequences deduced from a 1,959-bp sequence upstream of *tdnQ* showed no identity to those of ORFs reported previously.

Expression of AD genes in E. coli

AD activity was assayed in *E. coli* carrying plasmids with *tdn* genes (Table 2). No aniline-dependent oxygen uptake showing AD activity was observed in *E. coli* carrying p23FQ that contained only the *tdnQ* gene or carrying the plasmid p29C11 with *tdnTA1A2B* genes. On the other hand, *E. coli* carrying *tdnQTA1A2B* genes showed the aniline-dependent oxygen uptake, although the *tdnQ* gene was located on a different plasmid from that of the *tdnTA1A2B* genes, indicating that the catalysis of AD needs components encoded by *tdnQTA1A2B*.

Southern blot analysis of homologues of tdnA1A2 genes

Homologues of the *tdnA1A2* genes were analyzed by Southern blot analysis using partial *tdnA1A2* genes as a probe. Figure 2 shows the results of the Southern blot analysis. When the probe was hybridized with the *Eco*RV-*Eco*RI-digested DNA fragments from strain ANA-18, only the main band appeared (lane 5). Although there were some extra bands for digestion with other restriction endonucleases (lanes 1–4 for *Xho*I, *Pst*I, *Eco*RI, and *Eco*RV digestion), another band with a similar intensity to each main band of 3.4-, 3.9-, 5.2-, and 4.2-kbp in lanes 1–4 was not observed. These results suggest that no significant homologues with high identity to the cloned *tdnA1A2* existed in this strain.

Disruption of tdnA1A2 genes in Frateuria sp. ANA-18

Disruption of *tdnA1A2* genes was done to examine how the cloned *tdnA1A2* genes function as components of aniline dioxygenase in *Frateuria* sp. ANA-18. The plasmid pC1(*tdnA1A2*::Tc^r) was introduced into the chromosomal DNA of *Frateuria* sp. ANA-18 by homologous recombination, and

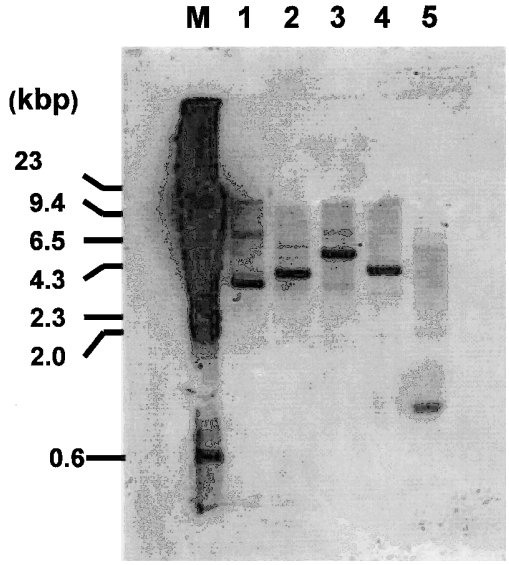

Fig. 2. Southern Blot Analysis of *tdnA1A2* in *Frateuria* sp. ANA-18.

DNA fragments, digested with *Xho*I (lane 1), *Pst*I (lane 2), *Eco*RI (lane 3), *Eco*RV (lane 4), and *Eco*RI-*Eco*RV (lane 5), were fixed on a membrane and hybridized with a 1.0-kbp *Eco*RV-*Eco*RI fragment containing partial *tdnA1A2* genes. M, size marker.

strain No. 49, showing Tc resistance, was isolated as a single crossover mutant. Furthermore, strains No. 49-1, 49-2, and 49-3, showing a defect in the aniline metabolism, were isolated from strain No. 49 as candidates for double-crossover mutants, which deleted *tdnA1A2* genes.

Deletion of *tdnA1A2* genes in strains derived from strain ANA-18 by homologous recombination was examined by Southern blot analysis. When a 1.2-kbp *Eco*RI-*Xho*I fragment containing *tdnA2B* was used as a probe, the probe was hybridized with the only 3.1-kbp *Sal*I-*Xho*I fragment carrying partial *tdnTA1A2B* genes in strain ANA-18 (lane 1 in Fig. 3). This result shows that *tdnB* homologues didn't exist in the strain in addition to *tdnA1A2* homologues. In strain No. 49, a 1.9-kbp *Sal*I-*Xho*I fragment containing partial *tdnA2B* genes on the plasmid pC1(*tdnA1A2*::Tc^r) (Fig. 1) appeared, in addition to the 3.1-kbp band (lane 2), indicating that pC1(*tdnA1A2*::Tc^r) was introduced into the chromosomal DNA of strain ANA-18 and two DNA segments containing *tdnA2B* existed in the chromosomal DNA of strain No. 49. On the other hand, the 3.1-kbp band containing the original *tdnTA1A2B* genes disappeared in strains defective in the aniline metabolism, No. 49-1, 49-2, and 49-3 (lanes 3–5). These results show that strains No. 49-1, 49-2, and 49-3 are double-crossover mutants with a deletion of the *tdnA1A2* genes.

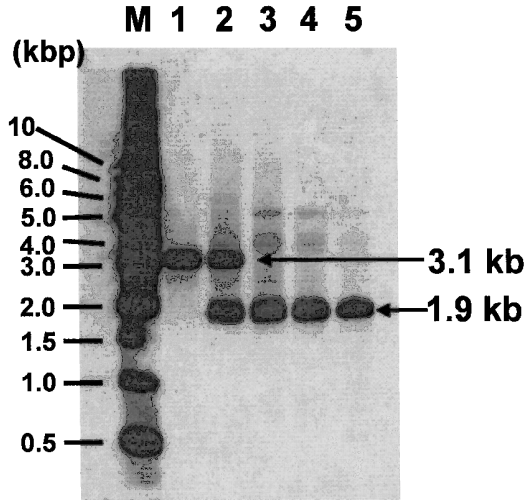

Fig. 3. Southern Blot Analysis of Strains Defective in Aniline Metabolism.

Total DNA from *Frateuria* sp. ANA-18 (lane 1), strain No. 49 (lane 2), strain No. 49-1 (lane 3), strain No. 49-2 (lane 4), and No. 49-3 (lane 5), were digested with *Sal*I-*Xho*I, and fixed on a membrane. The fixed DNA fragments were hybridized with a 1.2-kbp *Eco*RI-*Xho*I fragment containing partial *tdnA2B* genes. M, size marker.

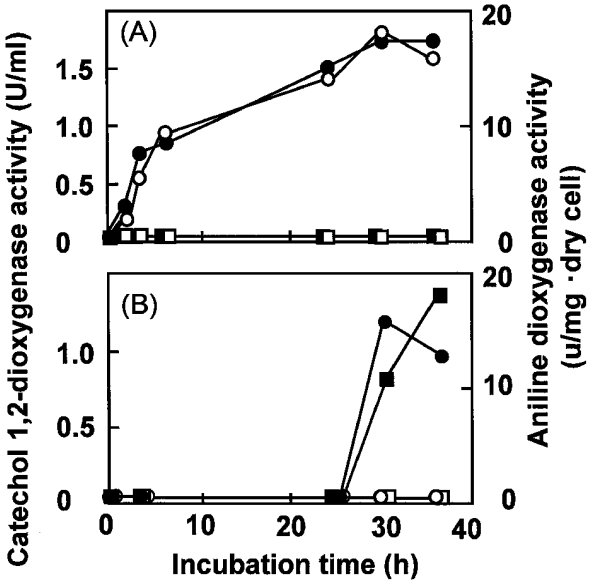

Fig. 4. Induction of AD and CD with *cis*,*cis*-Muconate and Aniline in the Parent Strain, *Frateuria* sp. ANA-18, and a Strain Defective in the Aniline Metabolism, No. 49-1.

Cells of strains ANA-18 and No. 49-1 were incubated in the reaction solution containing 10 mM *cis*,*cis*-muconate (panel A) and aniline (panel B), and AD and CD activities induced in the cells were assayed at various times. Symbols: ■ and ●, AD and CD activities in strain ANA-18, respectively.; □ and ○; AD and CD activities in No. 49-1, respectively.

Induction of aniline-degrading enzymes

To confirm that the deletion of *tdnA1A2* caused a defect in aniline metabolism, the induction of AD and CD was examined in the parent strain, *Frateuria* sp. ANA-18, and a strain defective in the aniline metabolism, No. 49-1. By the induction with 10 mM *cis*,*cis*-muconate, no production of AD was observed in strain ANA-18 and No. 49-1; CD, on the contrary, was produced in both strains (panel A in Fig. 4). These results show that catechol-degrading enzymes are functional in both strains. Panel B shows the induction of AD and CD with 10 mM aniline in both strains. The AD was induced by incubation for 30 h in strain ANA-18, and increased after 36 h. The production of CD was also induced by incubation for 30 h because *cis*,*cis*-muconate, an inducer of the expression of catechol-degrading gene clusters,[14] was generated from aniline by induced AD. On the other hand, no induction of either enzyme was observed in strain No. 49-1. These observations show that the defect in the aniline metabolism in this strain was caused by the loss of ability for the production of AD.

Discussion

We found 7 ORFs in a flanking region of the DNA fragment inserted in p50G12 (Fig. 1), and concluded that the 6 ORFs of them encode AD and a LysR-type regulator on the basis of their high identities to reported ADs (Table 1) and the same organization as *tdn* and *atd* gene clusters encoding ADs. In a recent study, a protein with an NH_2-terminal amino acid sequence showing similarity to that of the *Pseudomonas* TdnT, which is involved in the amino group transfer in the aniline degradation, is reported in the analysis of amino terminal sequences of the aniline-induced proteins in *Acinetobacter lwoffii* K24 metabolizing aniline *via* the *ortho*-cleavage pathway.[24] However, this is the first report describing the cloning of aniline-degrading *tdn* genes from bacteria metabolizing aniline *via* the *ortho*-cleavage pathway.

The deduced amino acid sequences of *tdnQTA1A2BR* cloned from *Frateuria* sp. ANA-18 showed more than 62.0% identities to those of the AD components found in *P. putida* UCC22, which metabolizes aniline *via* the *meta*-cleavage pathway (Table 1). Furthermore, we found a similar sequence to the UCC22 *tdnQ* promoter region containing possible −35 and −10 sequences and inverted repeats where a regulator in the *tdn* gene cluster, TdnR, may be bound,[6] upstream of the ANA-18 *tdnQ* gene (Fig. 5). In addition to the identities between ANA-18 and UCC22 ADs (Table 1), the similarity of nucleotide sequences in the *tdnQ* promoter regions suggests that these *tdn* genes were derived from a common ancestor. Interestingly, T-11 nt-A sequences, reported as a binding motif of LysR-type regulators,[25] weren't found in the inverted repeats of both strains and there may be unknown binding motifs of LysR-type regulators in the *tdnQ* promoter regions. The aniline-degrading genes of

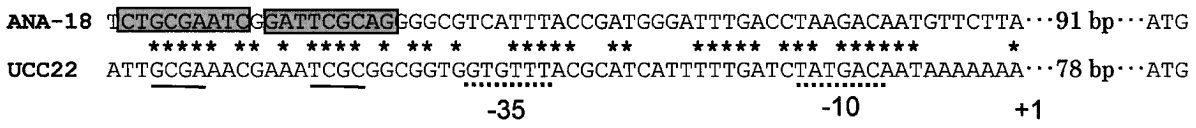

Fig. 5. Alignment of *tdn* Promoter Regions from *Frateuria* sp. ANA-18 and *P. putida* UCC22.
Identical nucleotides are indicated by asterisks. The transcriptional initiation site for *tdnQ* and putative −35 and −10 sequences in the strain UCC 22 sequence[6] are indicated by +1 and dotted lines, respectively. Inverted repeat sequences in strain UCC22[6] and ANA-18 are underlined and shadowed, respectively. The ATG codons at right sides show the initial codons of *tdnQ*.

strain UCC22 are carried on the catabolic plasmid pTDN1,[26] and organize a huge operon with genes encoding the *meta*-cleavage pathway (accession no. D85415). On the other hand, the *tdn* gene cluster of strain ANA-18 was independent of cat_1 and cat_2 gene clusters involved in the *ortho*-cleavage pathway. Furthermore, a *tnpA* gene, which encodes transposase and is located upstream of the UCC22 *tdn* gene cluster,[6] didn't exist in the same region from strain ANA-18; ORF7, on the contrary, was located downstream of *tdnR* in strain ANA-18. These differences show that the aniline-degrading operons *via* the *ortho*- and *meta*-cleavage pathways fundamentally differ in their organization in evolutionary processes although AD genes are derived from a common ancestor.

The *tdn* genes were expressed in *E. coli* and AD activity was examined. Only *E. coli* cells carrying both p23FQ with *tdnQ* cloned in the low copy vector pMW218 and p29C11 with *tdnTA1A2B* showed AD activity (Table 2). This result shows that the expression of AD activity needs all components encoded by *tdnQTA1A2B*. First, we tried to construct a gene cluster containing *tdnQTA1A2B* genes, transcribed as a single operon by the *lac* promoter of vector DNA, but were unable to obtain a recombinant molecule containing them as the single operon in spite of several trials. We also failed in the construction of plasmid DNA containing *tdnQTA1* genes, transcribed by the *lac* promoter of the vector. Excess expression of the *tdnQ* genes may be toxic for *E. coli* host cells.

Southern blot analyses suggested that no significant homologous genes for the cloned *tdnA1A2B* genes existed in strain ANA-18 (Fig. 2, 3). In addition to results of the Southern blot analyses, deletion of the *tdnA1A2* genes in strain ANA-18 by homologous recombination caused a defect in the aniline metabolism (Fig. 3). Furthermore, no AD activity was observed in aniline-induced cells of strain No. 49-1, lacking the *tdnA1A2* genes (Fig. 4). Although polar effects for *tdnBR* genes, located downstream of the deleted *tdnA1A2* genes, may influence the defect in aniline metabolism, results of the deletion experiments of the *tdnA1A2* genes show that the cloned *tdn* gene cluster was related to the conversion of aniline to catechol in strain ANA-18. On the basis of these results, we concluded that only cloned *tdn* genes function as genes encoding AD in strain ANA-18. *Frateuria* sp. ANA-18 has two catechol-degrading gene clusters, the cat_1 and cat_2 gene clusters.[15] The ANA-18 *tdn* gene cluster was found upstream not of a catabolic plasmid-born cat_2 gene cluster but of the cat_1 gene cluster, and located on chromosomal DNA. The cat_2 gene cluster was expressed at a lower concentration of an inducer, *cis*,*cis*-muconate, than the cat_1 gene cluster.[16] The location of the *tdn* and cat_1 gene clusters and characters of the cat_2 gene cluster may suggest that aniline is originally metabolized by the *tdn* and cat_1 gene clusters, and the cat_2 gene cluster was acquired as an advantage for rapid catechol metabolism.

It is reported that the LysR-type regulator BenM in *Acinetobacter* sp. ADP1 responds to *cis*,*cis*-muconate, in addition to benzoate, and activates the expression of the *ben* gene cluster, which encodes enzymes catalyzing the conversion of benzoate to catechol.[27] However, the production of AD was not induced in strain ANA-18 by *cis*,*cis*-muconate (Fig. 4). This suggests that the LysR-type regulators TdnR and BenM differ in responses to *cis*,*cis*-muconate although the *tdn* and *ben* gene clusters are involved in the hydroxylation of benzene rings. Further works on regulation systems of the expression of the *tdn* genes from strain ANA-18 are needed to understand the rapid aniline degradation in this bacterium.

References

1) Aoki, K., Shinke, R., and Nishira, H., Metabolism of aniline by *Rhodococcus erythropolis* AN-13. *Agric. Biol. Chem.*, **47**, 1611–1616 (1983).
2) Latorre, J., Reineke, W., and Knackmuss, H.-J., Microbial metabolism of chloroanilines: enhanced evolution by natural genetic exchange. *Arch. Microbiol.*, **140**, 159–165 (1984).
3) Aoki, K., Nakanishi, Y., Murakami, S., and Shinke, R., Microbial metabolism of aniline through a *meta*-cleavage pathway: isolation of strains and production of catechol 2,3-dioxygenase. *Agric. Biol. Chem.*, **54**, 205–206 (1990).
4) Fujii, T., Takeo, M., and Maeda, Y., Plasmid-encoded genes specifying aniline oxidation from *Acinetobacter* sp. strain YAA. *Microbiology*, **143**, 93–99 (1997).
5) Boon, N., Goris, J., Vos, P. D., Verstraete, W., and

Top, E. M., Bioaugmentation of activated sludge by an indigenous 3-chloroaniline-degrading *Comamonas testosteroni* strain, I2*gfp*. *Appl. Environ. Microbiol.*, **66**, 2906–2913 (2000).
6) Fukumori, F., and Saint, C. P., Nucleotide sequences and regulational analysis of genes involved in conversion of aniline to catechol in *Pseudomonas putida* UCC22 (pTDN1). *J. Bacteriol.*, **179**, 399–408 (1997).
7) Takeo, M., Fujii, T., and Maeda, Y., 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**, 17–24 (1998).
8) Takeo, M., Fujii, T., Takenaka, K., and Maeda, Y., Cloning and sequencing of a gene cluster for the *meta*-cleavage pathway of aniline degradation in *Acinetobacter* sp. strain YAA. *J. Ferment. Bioeng.*, **85**, 514–517 (1998).
9) Murakami, S., Nakanishi, Y., Kodama, N., Takenaka, S., Shinke, R., and Aoki, K., Purification, characterization, and gene analysis of catechol 2,3-dioxygenase from the aniline-assimilating bacterium *Pseudomonas* species AW-2. *Biosci. Biotechnol. Biochem.*, **62**, 747–752 (1998).
10) Aoki, K., Ohtsuka, K., Shinke, R., and Nishira, H., Rapid biodegradation of aniline by *Frateuria* species ANA-18 and its aniline metabolism. *Agric. Biol. Chem.*, **48**, 865–872 (1984).
11) Kim, S. I., Leem, S.-H., Choi, J.-S., Chung, Y. H., Kim, S., Park, Y.-M., Park, Y. K., Lee, Y. N., and Ha, K.-S., Cloning and characterization of two *catA* genes in *Acinetobacter lwoffii* K24. *J. Bacteriol.*, **179**, 5226–5231 (1997).
12) Aoki, K., Shinke, R., and Nishira, H., Identification of aniline-assimilating bacteria. *Agric. Biol. Chem.*, **46**, 2563–2570 (1982).
13) Aoki, K., Konohana, T., Shinke, R., and Nishira, H., Two catechol 1,2-dioxygenases from an aniline-assimilating bacterium, *Frateuria* species ANA-18. *Agric. Biol. Chem.*, **48**, 2097–2104 (1984).
14) Murakami, S., Takemoto, J., Takashima, A., Shinke, R., and Aoki, K., Purification and characterization of two muconate cycloisomerase isozymes from aniline-assimilating *Frateuria* species ANA-18. *Biosci. Biotechnol. Biochem.*, **62**, 1129–1133 (1998).
15) Murakami, S., Takashima, A., Takemoto, J., Takenaka, S., Shinke, R., and Aoki, K., Cloning and sequence analysis of two catechol-degrading gene clusters form the aniline-assimilating bacterium *Frateuria* species ANA-18. *Gene*, **226**, 189–198 (1999).
16) Takashima, A., Murakami, S., Takenaka, S., and Aoki, K., Regulation by two CatR proteins that differ in binding affinity to *catB* promoters expressing two *cat* gene clusters. *Biosci. Biotechnol. Biochem.*, **65**, 2146–2153 (2001).
17) Murakami, S., Kodama, N., Shinke, R., and Aoki, K., 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**, 49–54 (1997).
18) Kim, S. I., Leem, S.-H., Choi, J.-S., and Ha, K.-S., Organization and transcriptional characterization of the cat_1 gene cluster in *Acinetobacter lwoffii* K24. *Biochem. Biophys. Res. Commun.*, **243**, 289–294 (1998).
19) Kim, S. I., Ha, K.-S., and Leem, S.-H., Differential organization and transcription of the cat_2 gene cluster in aniline-assimilating *Acinetobacter lwoffii* K24. *J. Biosci. Bioeng.*, **88**, 250–257 (1999).
20) Sambrook, J., Fritsch, E. F., and Maniatis, T., Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y., (1989).
21) LaRue, T. A., and Blakley, E. R., Spectrophotometric determination of catechols with 4-aminoantipyrine. *Anal. Chim. Acta*, **31**, 400–403 (1964).
22) Aoki, K., Konohana, T., Shinke, R., and Nishira, H., Purification and characterization of catechol 1,2-dioxygenase from aniline-assimilating *Rhodococcus erythropolis* AN-13. *Agric. Biol. Chem.*, **48**, 2087–2095 (1984).
23) Valentin, H. E., Zwingmann, G., Schonebaum, A., and Steinbuchel, A., Metabolic pathway for biosynthesis of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) from 4-hydroxybutyrate by *Alcaligenes eutrophus*. *Eur. J. Biochem.*, **15**, 227, 43–60 (1995).
24) Kim, S. I., Kim, S.-J., Nam, M. H., Kim, S., Ha, K.-S., Oh, K.-H., Yoo, J.-S., and Park, Y.-M., Proteome analysis of aniline-induced proteins in *Acinetobacter lwoffii* K24. *Curr. Microbiol.*, **44**, 61–66 (2002).
25) Goethals, K., Van Montagu, M., and Holsters, M., 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**, 1646–1650 (1992).
26) McClure, N. C., and Venables, W. A., pTDN1, a catabolic plasmid involved in aromatic amine catabolism in *Pseudomonas putida* mt-2. *J. Gen. Micobiol.*, **133**, 2073–2077 (1987).
27) Collier, L., Gaines III, G. L., and Neidle, E. L., Regulation of benzoate degradation in *Acinetobacter* sp. strain ADP1 by BenM, a LysR-type transcriptional activator. *J. Bacteriol.*, **180**, 2493–2501 (1998).