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Genes Involved in Aniline Degradation by *Delftia acidovorans* Strain 7N and Its Distribution in the Natural Environment

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Aniline-degraders were isolated from activated sludge and environmental samples and classified into eight phylogenetic groups. Seven groups were classified into Gram-negative bacteria, such as *Acidovorax* sp., *Acinetobacter* sp., *Delftia* sp., *Comamonas* sp., and *Pseudomonas* sp., suggesting the possible dominance of Gram-negative aniline-degraders in the environment. Aniline degradative genes were cloned from *D. acidovorans* strain 7N, and the nucleotide sequence of the 8,039-bp fragment containing eight open reading frames was determined. Their deduced amino acid sequences showed homologies to glutamine synthetase (GS)-like protein, glutamine amidotransferase (GA)-like protein, large and small subunits of aniline dioxygenase, reductase, LysR-type regulator, small ferredoxin-like protein, and catechol 2,3-dioxygenase, suggesting a high similarity of this gene cluster to those in *P. putida* strain UCC22 and *Acinetobacter* sp. strain YAA. Polymerase chain reaction (PCR) and sequencing analyses of GS-like protein gene segments of other Gram-negative bacteria suggested that Gram-negative bacteria have aniline degradative gene that can be divided into two distinctive groups.

Key words: aniline; biodegradation; *Delftia acidovorans*; Gram-negative bacteria

The OECD test guideline[1)] is used for evaluating the safety of chemical compounds. In this guideline, six kinds of tests are adopted to evaluate whether a chemical compound is easily degraded in an aerobic liquid culture. One of these tests, 301C, is authorized by the Ministry of International Trade and Industry of Japan (MITI). In the 301C test, activated sludge, prepared by culturing composite sewage obtained by mixing environmental water gathered from rivers, lakes, and seas in Japan with seed culture from activated sludge obtained at a sewage disposal plant, is used as the resource for microorganisms. In this test, aniline is used as a standard material to confirm the activity of the sludge. Hence a speedy method is needed to quantify the aniline degradative activity of the activated sludge in this test.

Aniline has been widely used as a material for the synthesis of chemical products such as herbicides and dyes, and has been released into the environment during the past century. Since it is known to be toxic and carcinogenic to living organisms,[2)] many researchers have paid attention to the fate of aniline and its derivatives in the environment.[3,4)] A major way to remove aniline is biodegradation.[3,5)] Several researchers have been searching for bacteria which can degrade aniline, and the distribution of these bacteria, the catabolic pathways of aniline, and consequently the aniline degradative genes have been reported.[6–14)] The first step in the aerobic degradation pathway is oxidative deamination, resulting in the formation of catechol, which is then further degraded to *cis*,*cis*-muconic acid by the catechol 1,2-dioxygenase (*ortho*-cleavage pathway),[6–8)] or to a yellow compound, 2-hydroxymuconic semialdehyde by catechol 2,3-dioxygenase (the *meta*-cleavage pathway).[9–13)] The aniline degradative plasmids isolated from *Pseudomonas putida* strain UCC22 and *Acinetobacter* sp. strain YAA have been found to contain *tdnQTA1A2BR*[10)] and *atdA1A2A3A4A5*[11,12)] respectively, which are arranged in the same direction and involved in the conversion of aniline to catechol. On the basis of functional analyses using gene-specific deletion clones, it was found that the first five gene products were indispensable for the oxidative deamination of aniline to form catechol as a glutamine synthetase (GS)-like protein (*tdnQ* and *atdA1*), a glutamine amidotransferase (GA)-like protein (*tdnT* and *atdA2*), large and small

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Abbreviations: a.a., amino acid; BOD, biological oxygen demand; CE, crude cell extract; C23O, catechol 2,3-dioxygenase; GA, glutamine amidotransferase; GS, glutamine synthetase; HPLC, high-performance liquid chromatography; IPTG, isopropyl-β-D-thiogalactopyranoside; LB, Luria–Bertani; ORF, open reading frame; PCR, polymerase chain reaction

subunits of aniline dioxygenase (*tdnA1A2* and *atdA3A4*), and reductase of aniline dioxygenase (*tdnB* and *atdA5*), and that the LysR-type regulator TdnR was involved in the transcription of the *tdn* operon.[10,11] In the case with strains UCC22 and YAA, catechol formed is degraded by *meta*-cleavage by catechol 2,3-dioxygenase (C23O encoded by *tdnC* or *atdB*, which are located downstream of the *tdn* and *atd* gene clusters).[13] On the other hand, another aniline degradative gene cluster was isolated from the chromosome of *Frateuria* sp. ANA-18, which degrades aniline *via ortho*-cleavage of catechol by catechol 1,2-dioxygenase.[8] These chromosomal aniline degradative genes were named *tdn* genes (*tdnQTA1A2BR*), and their gene products have been proposed to have the same function as those of strain UCC22 in aniline degradation.[8] It has also been reported that the *tdn* gene cluster of strain ANA-18 is not flanked by the genes encoding *ortho*-cleavage pathway enzymes.[8]

In this study, we isolated several aniline-degrading bacteria from activated sludge and environmental samples. The basic information at the nucleotide sequence level of aniline degrader is expected to be helpful to establish a method for easy and rapid detection of such bacteria in the environment. Hence we also cloned and sequenced an aniline degradative gene from one of the isolated strains, *Delftia acidovorans* strain 7N, and genetically analyzed aniline degradative genes of other aniline degraders.

Materials and Methods

Inocula from environmental samples and media. A activated sludge, river water, and lake water were collected from the Ochiai sewage disposal plant (Shinjuku, Tokyo, Japan), the Kashima sewage disposal plant (Kashima, Ibaragi, Japan), the Fushiko river sewage disposal plant (Sapporo, Hokkaido, Japan), the Nakahama sewage disposal plant (Osaka, Japan), the Shinano River (Nishikambara, Niigata, Japan), and Lake Biwa (Otsu, Shiga, Japan). The collections were carried out in July, December of 1997, and December of 1998. A sample of lake water was also collected at Lake Biwa in September of 1997. Surface sediment was also collected from the rivers and the lake.

For screening and cultivation of aniline-degrading bacteria, Basal Medium (BM) supplemented with proper volumes of aniline, protease peptone, and yeast extract was used according to the 301C test.[1] We also employed M-A medium containing nutrient broth (500 mg per liter) and aniline (500 mg per liter, 5.37 mM) for screening and cultivation of aniline-degrading bacteria. We also used an AM medium, which contained 500 mg protease peptone, 100 mg yeast extract, and 150 mg aniline per liter (1.61 mM aniline) when screening for aniline degrading strains. M-A, AM, and Luria–Bertani (LB) media[15] were solidified with 1.5% (wt/vol) agar for plate culture. When necessary, ampicillin (Ap; 50–100 μg/ml), isopropyl-β-D-thiogalactopyranoside (IPTG; 1.0 mM), and 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (20 μg/ml) were also added to selective media.

Isolation of aniline-degrading bacteria. After precipitation for several minutes, 0.5 ml of supernatant of collected samples was added to BM supplemented with aniline (100 mg/l, 1.07 mM) and then cultured for the 301C test. Biological oxygen demand (BOD) was measured using an automatic electrolytic BOD meter. At the log phase of the BOD curve, 0.5 ml of culture was collected with a syringe, and stored as glycerol stock at $-70\,^{\circ}$C. A small volume of glycerol stock was suspended in 1 ml of sterile distilled water, and 20 μl of the resultant suspension was spread on an M-A plate. After incubation at 25 °C for 2–4 d, the bacterial colonies were transferred to 2 ml of the AM in a test tube. After 2 d of cultivation at 25 °C, the amount of aniline remaining in the culture was measured using high-performance liquid chromatography (HPLC). From the sample in which aniline degradation could be detected, single colonies were isolated using the AM plate to isolate aniline-degrading bacteria.

Quantification of aniline by HPLC. We added an equal volume of acetonitrile to the culture, and mixed for ten seconds with a vortex mixer. The resultant mixture was centrifuged at 12,000 $\times$ *g* for 3 min, and the supernatant was directly analyzed by HPLC. HPLC analysis was carried out on Develosil ODS-UG-3 (50 mm by 4.6 mm i.d., Nomura Chemical, Aichi, Japan) with the mobile phase composition (acetonitrile:water = 1:1, vol/vol), at a flow rate of 1 ml/min. The aniline eluted was detected by the absorption at 230 nm.

Grouping of aniline-degrading bacteria. Aniline-degrading bacteria were cultured on the M-A plate at 25 °C for 1–4 d. The bacterial colonies formed were suspended in 100 μl of distilled water in a 1.5 ml microtube. After incubation at 65 °C for 10 min, the cell suspensions were directly used as templates for polymerase chain reaction (PCR). For PCR, primer sets of 16SF (5′-AGAGTTTGATCCTGGCTCAG-3′) and 16SR (5′-GGCTACCTTGTTACGACTT-3′), both of which were designed to locate within the conserved region in the 16S rRNA gene, and InsertCheck -Ready- (TOYOBO, Tokyo, Japan) were used. Amplification was performed under the following conditions: 98 °C for 5 min, followed by cycle reactions of 65 °C for 5 sec and 72 °C for 90 sec, followed by 72 °C for 7 min. After PCR amplification, the amplified DNA fragments were digested with *Hae*III, *Hha*I, or *Msp*I, and the resultant restriction fragments were analyzed by electrophoresis on an agarose gel.

Identification of aniline-degrading bacteria. The selected strains were identified by morphological and

physiological characteristics (Hucker–Conn method, OF test, catalase test, oxidase test, and so on). In addition, for preliminary identification of Gram-negative bacteria at the species level, the multiple-test system API20NE (bioMérieux-Vitek, Tokyo, Japan) was employed. To confirm the conventional identification, the nucleotide sequences of the amplified 16S rRNA genes of the selected strains were determined.

Construction of the total DNA library of strain 7N. Strain 7N was cultivated in 50 ml nutrient broth supplemented with 500 mg/l aniline (5.37 mM) at 30 °C overnight with reciprocal shaking at 105 strokes per min. The cells were harvested by centrifugation at 1,000 × *g* for 15 min, and then washed using STE buffer (100 mM NaCl, 10 mM Tris–HCl, 1 mM EDTA, and pH 8.0). The cells harvested from 25 ml culture were suspended into 1 ml of solution I of Flexi Prep Kit (Amersham Biosciences, Tokyo, Japan). After 30 minutes of incubation at 37 °C, 40 μl of 10 mg/l proteinase K was added, and the mixture was incubated at 37 °C for 10 min. Then 20 mg of sodium *N*-lauroyl-sarcosine was added, and the mixture was incubated at 37 °C until it became clear. After phenol–chloroform (1:1, vol/vol) extraction, the upper layer was transferred to a 50-ml centrifuging tube. Five molar NaCl was added until the final concentration of NaCl reached 0.1 M. Two volumes of ethanol were then added. The precipitated DNA was harvested using a glass stick. The harvested DNA was washed using 70% ethanol, dried up, and then dissolved in TE buffer.

To construct the total DNA library carrying the small genomic DNA fragment, the extracted DNA from strain 7N was completely digested with restriction enzyme (*Eco*RI, *Hinc*II, *Pst*I, *Sac*I, *Sma*I, or *Sph*I), and the resultant restriction fragments were separated by electrophoresis on an agarose gel. DNA fragments 4–7 kb in length were selected and extracted from the agarose gel using RECOCHIP (Takara Shuzo, Kyoto, Japan). The extracted DNA fragments were ligated to the corresponding site of pUC18. *E. coli* strain JM109 was transformed with the plasmids formed, resulting in the total DNA library. *E. coli* transformants carrying this library were cultured on LB plate supplemented with Ap (50 μg/ml) and IPTG (1.0 mM), and shot-gun cloning by catechol 2,3-dioxygenase (C23O) activity was performed. The detection of C23O activity was assayed by spraying catechol dissolved in acetone to the colonies onto the LB plate.

To construct the DNA library carrying the large fragment, the extracted DNA was partially digested with *Sac*I, and the resultant DNA fragments were directly ligated to the *Sac*I-digested pUC18. *E. coli* strain JM109 was transformed with the resultant plasmids, and the resultant total DNA library was screened by Southern hybridization using PCR product containing part of the aniline degradative gene as a probe. The probe for Southern hybridization was obtained by PCR using the total DNA of strain 7N prepared as described above and a primer set designed on the reported aniline degradative gene clusters of *P. putida* strain UCC22 and *Acinetobacter* sp. strain YAA.[10,11] The nucleotide sequences of the forward and reverse primers were 5′-ACCGGCTGGATGCTGGCAGACCT-3′ and 5′-TCACGCATTCGCCGTGGCTTT-3′ respectively. For PCR, PfuTurbo DNA polymerase (Stratagene, La Jolla, CA, U.S.A.) was used according to the manufacturer's recommendations. Temperature conditions were as follows: 98 °C for 5 min, followed by 25 cycle reactions of 98 °C for 30 sec, 60 °C for 1 min, and 72 °C for 2 min, followed by 72 °C for 7 min. Labeling reaction of the PCR-amplified fragment, hybridization, washing, and detection of probe were performed using the DIG-DNA Labeling and Detection KIT and the DIG Wash and Block Buffer Set (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's recommendations.

General DNA manipulations. Plasmid DNA was extracted from *E. coli* transformants using a Flexi Prep Kit (Amersham Biosciences) or the Wizard Plus SV Minipreps DNA Purification Systems (Promega, Tokyo, Japan) according to the manufacturer's instructions. *E. coli* cells were transformed as described by Sambrook and Russell.[15] Southern hybridization was performed as described by Southern.[16] Restriction fragments of plasmid were separated on an agarose gel and transferred to Hybond N^+ nylon membrane (Amersham). Determination of the nucleotide sequences of the DNA fragments was done as described previously.[17]

Measurement of aniline degradation activity of E. coli *transformants. E. coli* strain JM109 carrying the target DNA fragment was cultured in 2 ml of LB media containing Ap (50 μg/ml) at 37 °C overnight with reciprocal shaking at 150 strokes per min. Twenty μl of the culture was transferred to 2 ml of LB media containing Ap. After 3 h of cultivation, 1.0 mM IPTG was added and the mixture was further incubated for 6 h at 37 °C. The resultant cells were harvested by centrifuging at 12,000 × *g* for 30 sec, washed twice with 50 mM sodium phosphate buffer (pH 7.5), and finally suspended into 200 μl of the same buffer. To this cell suspension, aniline was added at the final concentration of 50 mg/l (0.537 mM), and the resultant mixture was incubated for 15 h at 25 °C with reciprocal shaking at 150 strokes per min. The aniline remaining after the incubation was quantified by HPLC as described above.

Measurement of the meta-cleavage (C23O) activity of E. coli *transformants.* Appropriate *E. coli* transformants were cultured at 30 °C in 3 ml of LB supplemented with both Ap (50 μg/ml) and IPTG (1.0 mM), until the optical density at 660 nm reached approximately 1.0–1.5. The cells were harvested by centrifugation at 4,000 × *g* for 10 min, and washed with 1 ml of 50 mM sodium phosphate buffer (pH 7.5). The washed cells were

suspended with 600 μl of 50 mM sodium phosphate buffer (pH 7.5) containing 10% ethanol. The crude cell extract (CE) was prepared by sonication and subsequent centrifugation (15,000 $\times$ *g*, 10 min). The protein concentration was determined by using the Protein Assay Kit II (Bio-Rad Laboratories, Richmond, CA, U.S.A.) with bovine serum albumin as a standard. Ten to 500 μg of freshly prepared CE were added to 2 ml of reaction mixture and incubated (25 °C, 1 min) in a cell set in the spectrophotometer. The reaction mixture consisted of 50 mM sodium phosphate buffer (pH 7.5) containing 100 μM catechol, 3-methylcatechol, 4-methylcatechol, 4-chlorocatechol, or 2,3-dihydroxybiphenyl. The wavelength measured and the molar extinction coefficient of ring-cleavage products of catechol derivatives were taken to be 375 nm and 33,000 cm^{-1} M^{-1} (catechol), 388 nm and 13,400 cm^{-1} M^{-1} (3-methylcatechol), 379 nm and 28,100 cm^{-1} M^{-1} (4-methylcatechol), 379 nm and 36,100 cm^{-1} M^{-1} (4-chlorocatechol), and 434 nm and 13,200 cm^{-1} M^{-1} (2,3-dihydroxybiphenyl) at pH 7.5 respectively.[18] One unit of activity was defined as the amount of enzyme required to produce 1 μmol of *meta*-cleavage compounds per min.

PCR amplification and sequencing analysis of parts of the glutamine synthetase (GS)-like protein gene from several aniline-degraders. To monitor the distribution of the aniline degradative gene isolated in this study and its homologues, two PCR primer sets (primer1-primer2R and primer4-primer5R) were designed based on the GS-like protein genes of *P. putida* strain UCC22, *Acinetobacter* sp. strain YAA, and *D. acidovorans* strain 7N. Using the above primer sets, 428-bp (corresponding to 307–734 bp in $ORF_{7N}A$) and 370-bp (corresponding to 357–728 bp in $ORF_{7N}A$) internal DNA fragments of GS-like protein gene were amplified, respectively, and the entire 370-bp fragment was contained by the 428-bp fragment. The nucleotide sequences of the primers were as follows: primer1, 5′-ATGGT(ACGT)CC(ACGT)GA-(CT)CC(ACGT)AC(ACGT)AC-3′; primer2R, 5′-AC-(AG)TC(AG)AA(AGCT)GT(AGCT)GT(CT)TCCAT-3′; primer4, 5′-GGCTGGATGCTGGCAGATCT-3′; and primer5R, 5′-AAGGTGGTTTCCATCTGGCT-3′. For PCR amplification, Z-Taq DNA polymerase (Takara Shuzo) and total DNAs prepared from the respective aniline degraders were used. The amplification reaction was performed according to the following conditions: 98 °C for 5 min, followed by 30 cycle reactions of 98 °C for 5 sec, 60 °C for 5 sec, and 72 °C for 10 sec, followed by 72 °C for 7 min. The 428-bp DNA fragments amplified using the primer sets, primer1 and primer2R, were ligated to pGEM-T easy vector (Promega, Tokyo, Japan), and sequenced as described above.

Nucleotide sequence accession numbers. The nucleotide sequences of the region containing the aniline degradation gene cluster of *D. acidovorans* strain 7N have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession no. AB177545. The accession numbers for the PCR-amplified partial segment of GS-like protein gene are as follows: 7B, AB177863; 6K1, AB177864; 7K, AB177865; 6K2, AB177866; and 12F, AB177867.

Results and Discussion

Aniline degradation of the environmental and activated sludge samples and isolation of aniline-degraders

Although the length of the lag time differed with the each sample, the BOD curves suggested that all samples collected in July, September, and December 1997 and June 1998 showed significant aniline degradative capacities except for the Lake Biwa sample in July 1997. Concomitant to the aniline degradation, all the samples showed the accumulation of a yellow-colored compound. The yellow color was thought to indicate the production of 2-hydroxymuconic semialdehyde, an intermediate in the aniline degradation pathway *via meta*-cleavage of catechol.

From each sample showing aniline degradation (Table 1), we have succeeded in isolating one or two aniline degrading strains.

Identification of aniline-degrading bacteria

The restriction patterns of 16S rRNA genes amplified from total DNAs of 19 isolated strains were compared (data not shown), and then 19 strains were classified into 8 groups (Groups 7N, 7K, N1, 7B, 12F, 6K1, 6K2, and 12-0), as summarized in Table 1. Representative strains were selected from respective groups, strains 7N, 7K,

Table 1. Groups of Aniline-Degrading Bacteria Isolated from Various Sources

Sampling place of inocula	July 1997	September 1997	December 1997	June 1998
Ochiai sewage disposal plant	7N[a]	None[b]	12F[a]	N1
Kashima sewage disposal plant	7K[a]	None[b]	12F	6K1[a] and 6K2[a]
Fushio sewage disposal plant	N1[a]	None[b]	12F	12-0[a]
Nakahama sewage disposal plant	7N	None[b]	7N	N1
Shinano River	7B[a]	None[b]	7B	7B
Lake Biwa	ND[c]	7B	7B	7B

[a] Strain selected as representative strain of each group.
[b] Sample not collected.
[c] No degradation of aniline was observed.

Table 2. Characteristics of Aniline-Degrading Strains Isolated in This Study

	7N	7K	N1	7B	12F	6K1	6K2	12-0
Morphology	rod	coccus∼ short rod	rod	rod	short rod	rod	short rod	rod
Gram stain	−	−	+	−	−	−	−	−
Spore formation	−	−	ND[a]	−	−	−	−	−
Motility	+	−	ND[a]	+	−	+	−	+
Aerobe or anaerobe	aerobe	aerobe	ND[a]	aerobe	aerobe	aerobe	aerobe	aerobe
Oxidase test	+	−	−	+	−	+	−	+
Catalase test	+	+	−	+	+	+	+	+
OF test	−	−	−	ND[a]	−	−	−	−
Pigment	−	−	−	−	−	−	−	−
16S rRNA sequence comparison	*Deltfia acidovorans* (99% [AF538930])[b]	*Acinetobacter* sp. (98% [AY177359])[b]	*Pimelobacter simplex* (99% [AY509240])[b]	*Pseudomonas* sp. (96% [AF015487])[b]	*Acinetobacter* sp. (100% [AF321029])[b]	*Comamonas testosteroni* (98 % [AB007996])[b]	*Acinetobacter junii* (97% [AF417863])[b]	*Acidovorax* sp. (99% [AF235010])[b]

[a]Not determined.
[b]Most closely related bacteria (% identity [accession number]).

N1, 7B, 12F, 6K1, 6K2, and 12-0 respectively, and then were identified by conventional procedures (for all strains), the API20NE kit (for Gram-negative strains), the result of quinone analysis and reference data (for strain 7B only), and comparison of the nucleotide sequences of 16S rRNA genes. They were identified as *Delftia acidovorans* (strain 7N), *Acinetobacter lwoffii* (strain 7K), *Pimelobacter simplex* (strain N1), *Pseudomonas* sp. (strain 7B), *Acinetobacter* sp. (strain 12F), *Comamonas testosteroni* (strain 6K1), and *A. junii* (strain 6K2) respectively. The characteristics of representative strains are summarized in Table 2. Although the results of conventional procedures were identified as *Pseudomonas fluorescens* (strain 12-0), the 16S rRNA sequence comparison suggested that strain 12-0 was classified as *Acidovorax* sp. (data not shown). Seven of the eight representative aniline-degrading bacteria were classified as Gram-negative bacteria, indicating that Gram-negative aniline-degrading bacteria are widely distributed in activated sludges of sewage disposal plants and natural water from rivers and lakes. As shown in Table 1, Group 7B strains identified as *Pseudomonas* sp. were isolated from river and lake water throughout the year, implying that related (or similar) *Pseudomonas* aniline-degraders were dominant in natural conditions.

Cloning and sequencing of DNA region involved in aniline degradation by D. acidovorans *strain 7N*

Since the aniline degradative gene clusters previously reported have been cloned from *P. putida*, *Acinetobacter* sp., and *Frateuria* sp.,[8,10,11] to obtain information on the diversity of aniline degradative gene clusters, we analyzed the aniline-degradation system of *D. acidovorans* strain 7N.

When strain 7N was grown on aniline, catechol was identified as a metabolite by Mass analysis, and it was found to be converted to a yellow compound by mixing with CE of aniline-grown strain 7N cells (data not shown). From these results, it was predicted that in strain 7N, aniline is degraded by the *meta*-cleavage pathway. Therefore, to obtain the aniline degradative genes from this strain, we prepared the total DNA libraries from strain 7N and selected a clone showing C23O activity, pS3, which was a pUC18-based plasmid having a 2,523-bp insert DNA. The nucleotide sequence of the pS3 insert showed that the pS3 clone contained the gene encoding C23O, but not the gene responsible for the initial deamination of aniline (data not shown).

To identify the genes involved in aniline deamination, we designed one primer set for PCR from the DNA region spanning from the gene encoding the GS-like protein to the gene encoding the glutamine amidotransferase (GA)-like protein, which is conserved in aniline degradative gene clusters of *P. putida* strain UCC22[10] and *Acinetobacter* sp. strain YAA.[11] PCR using this primer set and total DNA of strain 7N as a template amplified approximately a 1.6-kb DNA fragment. Homology search analysis indicated that the deduced amino acid (a.a.) sequences of 5′- and 3′-terminal sequences of this amplified fragment had 84% identity with part of the GS-like protein of strain UCC22, and 58% identity with part of the GA-like protein of strain UCC22 respectively. Then this PCR-amplified fragment was labeled with DIG, and the library prepared from partially *Sac*I-digested total DNA of strain 7N was screened using the probe. As a result, a plasmid pSacI, pUC18, carrying appromately a 28-kb insert DNA, was obtained. When *E. coli* strain JM109 harboring pSacI was cultivated on an LB plate containing Ap, IPTG, and aniline, the colonies showed a yellow color, indicating the accumulation of 2-hydroxymuconic semialdehyde, which is an intermediate of the aniline-degradation pathway. In addition, resting cells of the *E. coli* strain harboring pSacI showed aniline-degradation activity, and the 50 mg/l (0.537 mM) of aniline was decreased to 15% after 15 h of incubation. This observation suggested the possibility that aniline was converted to 2-hydroxymuconic semialdehyde *via* oxidative deamination and subsequent *meta*-cleavage by *E. coli* strains harboring pSacI. The above results also imply that the pSacI insert

contained the *meta*-cleavage enzyme gene located on the pS3 insert. In fact, Southern hybridization analysis using the pSacI insert and the DIG-labeled probe prepared from the pS3 insert resulted in clear hybridization (data not shown), indicating that the 28-kb insert of pSacI actually contained the whole or at least a part of the pS3 insert.

Restriction analysis of the pSacI insert

When the above approximately 28-kb pSacI insert was digested with *Sma*I, five restriction fragments (about 11, 7, 4, 3, and 2.5 kb) were detected. Southern hybridization using the probes prepared from the pS3 insert and the above-mentioned 1.6-kb PCR amplicon showed clear hybridization to about a 11-kb fragment, suggesting that the aniline degradative genes (at least the genes encoding *meta*-cleavage enzyme, the GS-like protein, and the GA-like protein respectively) were contained in this fragment. Hence it was ligated to the corresponding sites of pUC18 and pBluescript SK+ and designated p1-7 and p1-7X respectively. We constructed a restriction map of p1-7 and p1-7X. The 11-kb restriction fragment was found to be divergently inserted downstream of the *lac* promoter of the respective plasmids. In addition, the insert of p1-7X (or p1-7) was digested with *Eco*RI or *Sal*I and ligated to the corresponding sites of pUC18, to give pS3-2′, pE2-2, and pS5-4′ (Fig. 1). When *E. coli* strain JM109 harboring each of these plasmids was cultured on an aniline and IPTG-containing LB plate, only the colonies of cells harboring p1-7X became yellow. This result indicates that only the cells harboring p1-7X converted aniline to *meta*-cleavage compound *via* oxidative deamination followed by *meta*-cleavage (Fig. 1). When *E. coli* transformants carrying each of these plasmids were sprayed with catechol, only the cells harboring p1-7X, pE2-2, or pS5-4′ showed a yellow color, which was due to the formation of *meta*-cleavage compound. Considering the proposed transcriptional direction and relative locations of the genes involved in oxidative deamination and *meta*-cleavage, it was implied that the p1-7X insert contained the aniline degradative gene cluster, whose gene structure is similar to those of the *atd* and *tdn* genes.[10,11]

Genes involved in aniline degradation in strain 7N

Based on the proposed structure of aniline degradative genes in strain 7N, we determined the nucleotide sequence of the upstream region of pS3 insert by sequencing using designed primers. Consequently, the nucleotide sequence of the 8,039-bp-long DNA region was determined (Fig. 1). In this region, there were 8 open reading frames (ORFs; $ORF_{7N}A$ to $ORF_{7N}H$) in the same transcriptional direction. As summarized in Table 3, the deduced a.a. sequences of $ORF_{7N}A$, $ORF_{7N}B$, $ORF_{7N}C$, $ORF_{7N}D$, $ORF_{7N}E$, $ORF_{7N}F$, $ORF_{7N}G$, and $ORF_{7N}H$ showed significant homology with GS-like protein, GA-like protein, a large (α) subunit of aniline dioxygenase, a small (β) subunit of aniline dioxygenase, reductase, LysR-type regulator protein, small ferredoxin-like protein, and catechol 2,3-dioxygenase respectively. These 8 enzymes of strain

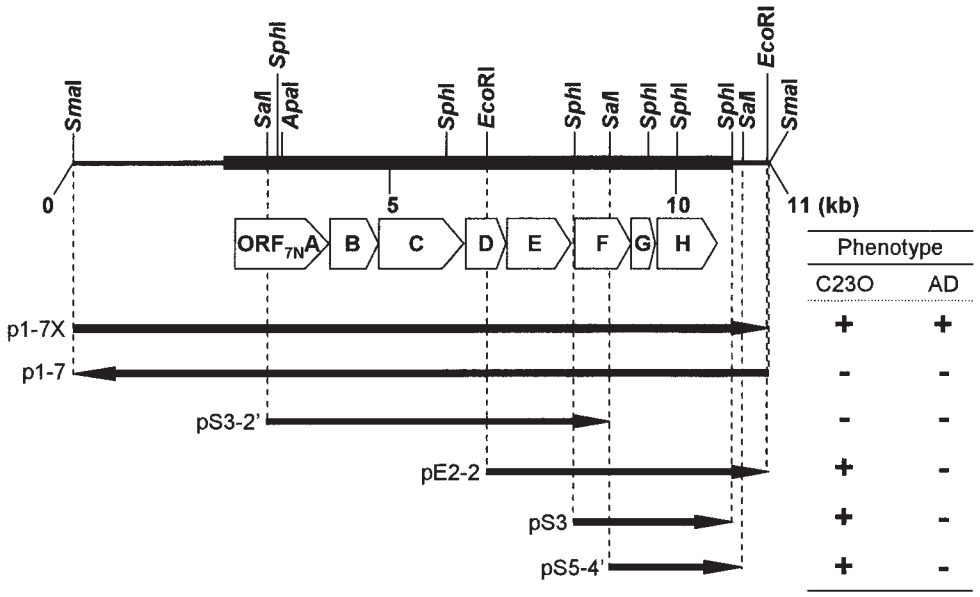

Fig. 1. Physical Map of the 11-kb *Sma*I Fragment Containing the Aniline Degradative Gene Cluster of *D. acidovorans* Strain 7N.

The black box at the top shows the 8,039-bp-long DNA region sequenced in this study. The pentagons in the physical map indicate the sizes, location, and transcriptional directions of the ORFs derived from DNA sequence analysis. Only restriction sites of the relevant enzymes are shown. The location of each insert and the orientation of transcription from the *lac* promoter are indicated by arrows. AD (aniline dioxygenase [oxidative deamination] activity) was monitored by yellow color product formation from aniline *via* oxidative deamination followed by *meta*-cleavage on an aniline-containing plate. C23O activity was monitored by spraying catechol onto the colonies of *E. coli* transformants. Phenotypes are: +, positive; −, negative.

Table 3. Homology Search Analysis of 8 ORFs Found in 8,038-bp-Long DNA Region of *D. acidovorans* Strain 7N

ORF	G+C content (%)	Probable function	Homology			Accession no.
			Amino acid identity (%)	Name(s) of protein(s)	Source	
$ORF_{7N}A$	64.4	Amino group transfer component of aniline dioxygenase (GS-like protein)	84.2	TdnQ	*P. putida* strain UCC22	BAA12805
			83.4	TdnQ	*Frateuria* sp. strain ANA-18	BAC82524
			63.0	AtdA1	*Acinetobacter* sp. strain YAA	BAA13010
$ORF_{7N}B$	65.8	Amino group transfer component of aniline dioxygenase (GA-like protein)	60.8	TdnT	*P. putida* strain UCC22	BAA12806
			59.0	TdnT	*Frateuria* sp. strain ANA-18	BAC82525
			34.2	AtdA2	*Acinetobacter* sp. strain YAA	BAA13011
$ORF_{7N}C$	64.4	Large (α) subunit of aniline dioxygenase	80.8	TdnA1	*P. putida* strain UCC22	BAA12807
			78.0	TdnA1	*Frateuria* sp. strain ANA-18	BAC82526
			51.8	AtdA3	*Acinetobacter* sp. strain YAA	BAA23550
$ORF_{7N}D$	67.6	Small (β) subunit of aniline dioxygenase	69.3	TdnA2	*P. putida* strain UCC22	BAA12808
			69.3	TdnA2	*Frateuria* sp. strain ANA-18	BAC82527
			38.1	AtdA4	*Acinetobacter* sp. strain YAA	BAA23551
$ORF_{7N}E$	68.4	Reductase	73.6	TdnB	*P. putida* strain UCC22	BAA12809
			67.8	TdnB	*Frateuria* sp. strain ANA-18	BAC82528
			32.2	AtdA5	*Acinetobacter* sp. strain YAA	BAA23552
$ORF_{7N}F$	64.0	Putative LysR-type regulatory gene	74.6	TdnR	*P. putida* strain UCC22	BAA12810
			70.1	TdnR	*Frateuria* sp. strain ANA-18	BAC82529
			31.0	AtdR	*Acinetobacter* sp. strain YAA	BAA23553
$ORF_{7N}G$	65.6	Small ferredoxin-like protein	55.8	TdnD1	*P. putida* strain UCC22	BAB62044
			25.6	AtdS	*Acinetobacter* sp. strain YAA	BAA23554
$ORF_{7N}H$	64.4	Catechol 2,3-dioxygenase	85.7	TdnC	*P. putida* strain UCC22	BAB62045
			41.5	AtdB	*Acinetobacter* sp. strain YAA	BAA23555

7N showed 55.8–85.7% identity with those of *P. putida* strain UCC22,[10] but lower (25.6–63.0%) with those of *Acinetobacter* sp. strain YAA.[11]

Substrate specificity of ring-cleavage dioxygenase

CE of *E. coli* strain JM109 harboring pS5-4′ expressing the $ORF_{7N}H$ product (ring-cleavage dioxygenase) was used to determine the substrate specificity of this enzyme. As substrates, catechol, 3-methylcatechol, 4-methylcatechol, 4-chlorocatechol, and 2,3-dihydroxybiphenyl were used, and the results obtained are summarized in Table 4. For the $ORF_{7N}H$ product, monocyclic catecholic compounds were preferable as a substrate to the bicyclic compound, 2,3-dihydroxybiphenyl. Among the monocyclic catecholic compounds, the $ORF_{7N}H$ product showed the highest activity to catechol, the intermediate of the aniline degradation pathway.

Table 4. Substrate Specificity of Ring-Cleavage Dioxygenase, $ORF_{7N}H$ Product

Substrate	Activity (units/mg protein)
Catechol	2.2
3-Methylcatechol	1.0
4-Methylcatechol	1.1
4-Chlorocatechol	0.42
2,3-Dihydroxybiphenyl	0.18

Distribution of the aniline degradative genes among the aniline degraders

As described above, the genes specifying aniline degradation by strain 7N showed high structural similarity and sequence homology with those of the aniline-degrading *P. putida*,[10] *Frateuria* sp.,[8] and *Acinetobacter* sp.[11] strains previously reported. Especially, the gene encoding the GS-like protein of strain 7N ($ORF_{7N}A$) showed 84.2%, 83.4%, and 63.0% identity with those of strains UCC22, ANA-18, and YAA respectively. As shown in Table 3, the homology observed in the GS-like protein is highest among the proteins whose genes are contained in the aniline degradative gene cluster. Previously, Boon *et al.*[14] used PCR amplification within the GS-like protein gene to access the distribution of the *tdnQ* gene homolog in five aniline-degrading strains. To access the distribution of the aniline degradative gene in the aniline degraders isolated from activated sludges, river water, and lake water in this study, we also investigated the sequence variations of part of the GS-like protein gene.

Two primer sets were designed from the sequence conserved among the GS-like protein genes of strains 7N, UCC22, and YAA. Total DNAs were extracted and used as a template for PCR amplification. As a result, DNA fragments of expected sizes could be amplified from all Gram-negative strains using both primer sets (data not shown). The nucleotide sequences of the 428-

Table 5. Comparison of the Deduced Amino Acid Sequences of Parts of GS-Like Proteins Isolated from Various Aniline Degraders

Aniline degraders	% identity among the parts of GS-like protein from								
	7N	7B	6K1	UCC22	ANA-18	7K	6K2	12F	YAA
D. acidovorans strain 7N	100	99.2	87.6	89.1	86.3	62.8	62.8	62.8	63.6
Pseudomonas sp. strain 7B		100	86.8	89.9	87.1	63.6	63.6	63.6	62.8
C. testosteroni strain 6K1			100	90.7	91.4	60.5	60.5	60.5	61.2
P. putida strain UCC22				100	90.6	62.0	62.0	62.0	61.2
Frateuria sp. ANA-18					100	65.5	65.5	65.5	64.7
A. lwoffii strain 7K						100	100	100	98.4
A. junii strain 6K2							100	100	98.4
Acinetobacter sp. strain 12F								100	98.4
Acinetobacter sp. strain YAA									100

bp-long fragments (corresponding to 307–734 bp in $ORF_{7N}A$) amplified by primer1 and primer2R showed high similarity with the corresponding parts of the genes encoding GS-like proteins previously reported. Comparison of the deduced a.a. sequences of the amplified GS-like protein genes with those of strains UCC22, ANA-18, YAA, and our isolates suggested that at least the GS-like protein genes in aniline degradative gene clusters of Gram-negative bacteria could be divided into two distinct groups (Table 5). Group I contained those of strains classified into the genus *Acinetobacter*, *viz.*, strains 7K (*A. lwoffii*), 6K2 (*A. junii*), 12F (*Acinetobacter* sp.), and YAA (*Acinetobacter* sp.), showing 98.4–100% a.a. sequence identities with each other. On the other hand, group II contained those of other Gram-negative strains, 7N (*D. acidovorans*), 7B (*Pseudomonas* sp.), 6K1 (*C. testosteroni*), ANA-18 (*Frateuria* sp.), and UCC22 (*P. putida*), showing 86.3–99.2% a.a. sequence identities with each other. The a.a. sequences of the strains in group I showed 60.5–65.5% identities with those in group II. Boon *et al.* amplified the 384-bp region of the *tdnQ* gene (corresponding to 523–896 in $ORF_{7N}A$) to access the presence of similar genes in the genome of several aniline-degrading *Comamonadaceae* strains (*C. testosteroni* and *D. acidovorans*).[14] Comparison of the overlapped regions between our 428-bp amplified fragments and the 384-bp amplified fragments by Boon *et al.*[14] indicated that the deduced a.a. sequences of the parts of the *Comamonadaceae tdnQ* genes showed higher homology (>91.8% identity) to those of group II than to those of group I. This observation is in accordance with our proposal that aniline degradative genes can be divided into the *Acinetobacter* group (group I) and the other group (group II).

The above results suggest that Gram-negative aniline-degrading bacteria are widely spread in activated sludges of sewage disposal plants, rivers, and lakes and have similar aniline degradative genes that can be divided into at least two groups. By PCR of the conserved region of aniline degradative genes, it may be possible to quantify Gram-negative aniline-degrading bacteria in the environment and in activated sludge rapidly and easily. In addition, we might be able to distinguish the two groups of Gram-negative bacteria (*Acinetobacter* sp. and others) in natural samples using the optimized primer sets designed for the GS-like protein genes of the respective groups. On the other hand, in the case of Gram-positive *Pimelobacter simplex* strain N1, no DNA fragment of the expected size was detected by PCR using the above-designed primer set (data not shown). This result suggests that Gram-positive bacteria may have aniline degradative genes different from those of Gram-negative bacteria.

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References

1) Paris, OECD, OECD Guidelines for the Testing of Chemicals/Section 3: Degradation and Accumulation Test No. 301: Ready Biodegradability (1992).
2) Lyons, C. D., Katz, S., and Bartha, R., Persistence and mutagenic potential of herbicide-derived aniline residues in pond water. *Bull. Environ. Contam. Toxicol.*, **35**, 696–703 (1985).
3) Lyons, C. D., Katz, S., and Bartha, R., Mechanisms and pathways of aniline elimination from aquatic environments. *Appl. Environ. Microbiol.*, **48**, 491–496 (1984).
4) Amador, J. A., Alexander, M., and Zika, R. G., Degradation of aromatic compounds bound to humic acid by the combined action of sunlight and microorganisms. *Environ. Toxicol. Chem.*, **10**, 475–482 (1991).
5) Gheewala, S. H., and Annachhatre, A. P., Biodegradation of aniline. *Water Sci. Technol.*, **36**, 53–63 (1997).
6) Aoki, K., Ohtsuka, K., Shinke, R., and Nishina, H., Rapid biodegradation of aniline by *Frateuria* species ANA-18 and its aniline metabolism. *Agric. Biol. Chem.*, **48**, 865–872 (1984).
7) Loidl, M., Hinteregger, C., Ditzelmueller, G., Ferschl, A., and Streichsbier, F., Degradation of aniline and monochlorinated anilines by soil-borne *Pseudomonas acidovorans* strains. *Arch. Microbiol.*, **155**, 56–61 (1990).
8) Murakami, S., Hayashi, T., Maeda, T., Takenaka, S., and Aoki, K., Cloning and functional analysis of aniline

dioxygenase gene cluster, from *Frateuria* species ANA-18, that metabolizes aniline *via* an *ortho*-cleavage pathway of catechol. *Biosci. Biotechnol. Biochem.*, **67**, 2351–2358 (2003).

9) Konopka, A., Knight, D., and Turco, R. F., Characterization of a *Pseudomonas* sp. capable of aniline degradation in the presence of secondary carbon sources. *Appl. Environ. Microbiol.*, **48**, 491–496 (1989).

10) 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).

11) Fujii, T., Takeo, M., and Maeda, Y., Plasmid-encoded genes specifying aniline oxidation from *Acinetobacter* sp. strain YAA. *Microbiology*, **143**, 93–99 (1997).

12) 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).

13) 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).

14) Boon, N., Goris, J., Vos, P. D., Verstraete, W., and Top, E. M., Genetic diversity among 3-chloroaniline- and aniline-degrading strains of the *Comamonadaceae*. *Appl. Environ. Microbiol.*, **67**, 1107–1115 (2001).

15) Sambrook, J., and Russell, D. W., Molecular cloning: a laboratory manual, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (2001).

16) Southern, E. M., Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.*, **9**, 503–517 (1975).

17) Ouchiyama, N., Miyachi, S., and Omori, T., Cloning and nucleotide sequence of carbazole catabolic genes from *Pseudomonas stutzeri* strain OM1, isolated from activated sludge. *J. Gen. Appl. Microbiol.*, **44**, 57–63 (1998).

18) Hirose, J., Kimura, N., Suyama, A., Kobayashi, A., Hayashida, S., and Furukawa, K., Functional and structural relationship of various extradiol aromatic ring-cleavage dioxygenases of *Pseudomonas* origin. *FEMS Microbiol. Lett.*, **118**, 273–278 (1994).