

Chromosome-encoded gene cluster for the metabolic pathway that converts aniline to TCA-cycle intermediates in *Delftia tsuruhatensis* AD9

Quanfeng Liang,¹ Masahiro Takeo,² Ming Chen,¹ Wei Zhang,¹ Yuquan Xu¹ and Min Lin¹

Correspondence

Min Lin

linmin57@vip.163.com

¹Department of Microbiology, Biotechnology Research Institute, Chinese Academy of Agricultural Sciences, 12 zhongguancun Nandajie, Beijing 100081, P. R. China

²Department of Materials Science and Chemistry, Graduate School of Engineering, University of Hyogo, 2167 Shosha, Himeji, Hyogo 671-2201, Japan

Delftia tsuruhatensis AD9 was isolated as an aniline-degrading bacterium from the soil surrounding a textile dyeing plant. The gene cluster involved in aniline degradation was cloned from the total DNA of strain AD9 into *Escherichia coli* JM109. After shotgun cloning, two recombinant *E. coli* strains showing aniline oxidation activity or catechol *meta*-cleavage activity were obtained by simple plate assays. These strains contained 9.3 kb and 15.4 kb DNA fragments, respectively. Sequence analysis of the total 24.7 kb region revealed that this region contains a gene cluster (consisting of at least 17 genes, named *tadQTA1A2BRD1C1D2C2EFGIJKL*) responsible for the complete metabolism of aniline to TCA-cycle intermediates. In the gene cluster, the first five genes (*tadQTA1A2B*) and the subsequent gene (*tadR*) were predicted to encode a multi-component aniline dioxygenase and a LysR-type regulator, respectively, while the others (*tadD1C1D2C2EFGIJKL*) were expected to encode *meta*-cleavage pathway enzymes for catechol degradation. In addition, it was found that the gene cluster is surrounded by two IS1071 sequences, indicating that it has a class I transposon-like structure. PFGE and Southern hybridization analyses confirmed that the *tad* gene cluster is encoded on the chromosome of strain AD9 in a single copy. These results suggest that, in strain AD9, aniline is degraded via catechol through a *meta*-cleavage pathway by the chromosome-encoded *tad* gene cluster. The *tad* gene cluster showed significant similarity in nucleotide sequence and genetic organization to the plasmid-encoded aniline degradation gene cluster of *Pseudomonas putida* UCC22.

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INTRODUCTION

A large quantity of anilines are used in the manufacture of pesticides, herbicides, dyes, plastics and pharmaceuticals, and are released into the environment through industrial wastewaters and their direct application to soils (Meyer, 1981). They are also formed in the environment during the microbial transformation of nitroaromatic compounds and aniline-based pesticides (Kearney & Kaufman, 1975). Since many of them are known to be toxic, mutagenic and carcinogenic (Crabtree *et al.*, 1991; Chung *et al.*, 1997;

Bhunja *et al.*, 2003), their fate in the environment and biodegradation have been extensively studied (Bollag *et al.*, 1978; Lyons *et al.*, 1984, 1985).

In order to determine biodegradability and mechanisms of biodegradation of anilines, many aniline-degrading bacteria have been isolated. Species of *Alcaligenes* (Rhodes, 1970), *Pseudomonas* (Anson & Mackinnon, 1984; Aoki *et al.*, 1997; Travkin *et al.*, 2003), *Acinetobacter* (Kim *et al.*, 1997), *Rhodococcus* (Aoki *et al.*, 1983; Fuchs *et al.*, 1991), *Frateruia* (Aoki *et al.*, 1984), *Moraxella* (Zeyer *et al.*, 1985), *Nocardia* (Bachofner *et al.*, 1975) and *Delftia* (Kahng *et al.*, 2000; Boon *et al.*, 2001; Liu *et al.*, 2002) are able to degrade aniline and/or its derivatives. Moreover, plasmid-dependent aniline degradation has been reported in several bacterial strains (Anson & Mackinnon, 1984; Latorre *et al.*, 1984; McClure & Venables, 1987; Fujii *et al.*, 1997; Boon *et al.*, 2001). However, gene clusters responsible for the complete conversion of aniline to TCA-cycle intermediates have been cloned only from the aniline-degradative plasmids

Abbreviations: AD, aniline dioxygenase; Ap, ampicillin; C230, catechol 2,3-dioxygenase.

The GenBank/EMBL/DDBJ accession numbers of the sequences of the aniline degradation gene cluster and the 16S rDNA cloned from strain AD9 are AY940090 and AY89912, respectively.

A supplementary table summarizing the morphological, physiological and biochemical characteristics of strain AD9, *Delftia acidovorans* and *Delftia tsuruhatensis* is available with the online version of this paper.

pTDN1 of *P. putida* UCC22 (Fukumori & Saint, 1997) and pYA1 of *Acinetobacter* sp. YAA (Fujii *et al.*, 1997). These gene clusters are very similar in their genetic organization; both contain genes encoding a multi-component aniline dioxygenase (AD), a LysR-type regulator, and several *meta*-cleavage pathway enzymes. The ADs encoded by these gene clusters consist of five proteins, two of which are homologous to glutamine synthetase and glutamine amidotransferase, suggesting that they are involved in the transfer of the amino group of aniline. Similarities in the sequences of the other three proteins to other aromatic compound dioxygenases suggest that they function as the large and small subunits of the oxygenase component and the reductase component in the AD enzyme systems (Fukumori & Saint, 1997; Takeo *et al.*, 1998). Recently, AD genes of the same type were cloned from *Frateuria* sp. ANA-18 (Murakami *et al.*, 2003) and *Delftia acidovorans* 7N (Urata *et al.*, 2004). The AD genes of the former strain are located over 1.7 kb upstream of a catechol *ortho*-cleavage pathway gene cluster (the *cat1* gene cluster) encoded on the chromosome, while those of the latter strain are located just upstream of a catechol 2,3-dioxygenase gene. However, other *meta*-cleavage pathway enzyme genes have not been reported.

Here we report a chromosome-encoded gene cluster responsible for the complete conversion of aniline to

TCA-cycle intermediates cloned from a *Delftia* strain. Interestingly, the gene cluster, surrounded by two IS1071 sequences, is almost identical to that encoded on plasmid pTDN1 of *P. putida* UCC22 (Fukumori & Saint, 1997), which is one of the representative aniline degradation gene clusters.

METHODS

Bacterial strains, plasmids, media and growth conditions.

The bacterial strains and plasmids used in this study are listed in Table 1. The culture media used were Luria–Bertani (LB) medium (Sambrook & Russell, 2001) for bacterial growth and mineral salts (MS) medium (Saber & Crawford, 1985) for the isolation of aniline-degrading bacteria, aniline oxygenase assays and aniline degradation tests. *Delftia* spp., *Acinetobacter calcoaceticus* PHEA-2 and *Bacillus thuringiensis* A-01 were grown at 30 °C; *Escherichia coli* was grown at 37 °C. Ampicillin (Ap) was used in selective media at a final concentration of 50 mg l⁻¹.

Isolation and identification of aniline-degrading bacteria.

Soil samples were taken from the area surrounding a textile dyeing plant in Guangzhou, China. Ten grams of each soil sample were shaken at 180 r.p.m. in 100 ml 0.9% (w/v) sodium chloride solution at 30 °C for 2 h. One millilitre of the suspension was transferred into LB medium containing 300 mg aniline l⁻¹ and the culture was incubated at 180 r.p.m., at 30 °C. After the culture became turbid, serial dilutions were transferred onto solid MS medium containing

Table 1. Strains and plasmids

| Strain or plasmid | Relevant characteristics | Reference or source |
|---|---|---------------------------------|
| <i>Delftia tsuruhatensis</i> AD9 | Wild-type, aniline degradation | This study |
| <i>D. tsuruhatensis</i> T7 | ATCC BAA-544 ^T , type strain | Shigematsu <i>et al.</i> (2003) |
| <i>Acinetobacter calcoaceticus</i> PHEA-2 | Phenol degradation | Xu <i>et al.</i> (2003) |
| <i>Bacillus thuringiensis</i> A-01 | Harbours several cryptic plasmids | Authors' lab. collection |
| <i>Escherichia coli</i> DH5α | <i>supE44 ΔlacU169(φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96, thi-1 relA1</i> | Sambrook & Russell (2001) |
| <i>E. coli</i> JM109 | <i>thi Δ(lac-proAB) F'[traD36 proAB⁺ lac^f lacZΔM15]</i> | Sambrook & Russell (2001) |
| <i>E. coli</i> K-12 AS1.365 | Derivative of <i>E. coli</i> K-12 | Han <i>et al.</i> (2005) |
| pUC19 | Cloning vector, Ap ^r , 2.7 kb | Takara Shuzo |
| pVK100 | Broad-host-range vector, Ap ^r Tc ^r Km ^r | Sambrook & Russell (2001) |
| pDA1 | Ap ^r , 9.3 kb <i>Hind</i> III fragment from genomic DNA of strain AD9 in pUC19, <i>tadQTA1A2BR</i> | This study |
| pDB11 | Ap ^r , 15.4 kb <i>Hind</i> III fragment from genomic DNA of strain AD9 in pUC19, <i>tadC1D2C2EFGIJKL</i> | This study |
| pDB2 | Ap ^r , 8.2 kb <i>Eco</i> RI fragment from genomic DNA of strain AD9 in pUC19, <i>tadBRD1C1D2C2</i> | This study |
| pVD1 | Ap ^r , 9.3 kb <i>Hind</i> III fragment from pDA1 in pVK100, <i>tadQTA1A2BR</i> | This study |

300 mg aniline l⁻¹ as the sole carbon and nitrogen source, and incubated at 30 °C for several days. Rapidly growing colonies of aniline-utilizing micro-organisms were screened from the plates of MS medium. Identification of the selected strains was based mainly on their physiological and biochemical characteristics and was carried out in the Institute of Microbiology, Chinese Academy of Sciences, Beijing. Genomic DNA was extracted from strain AD9 and *Delftia tsuruhatensis* T7 (ATCC BAA-554^T) and purified according to the method of Marmur (1961) except for the addition of proteinase K in the SDS-treatment step. DNA base compositions were determined by thermal denaturation (Marmur & Doty, 1962) using a spectrophotometer (DU800, Beckman). The genomic DNA of *E. coli* K-12 was used as the standard for the calibration of *T_m* values. DNA-DNA hybridizations were carried out according to the methods of De Ley *et al.* (1970) and Huss *et al.* (1983). An almost full-length 16S rDNA sequence of strain AD9, which was obtained as described by Rochelle *et al.* (1995), was used for further identification.

Construction of the genomic library of strain AD9. The genomic DNA of strain AD9 was obtained by the method of Wilson (1987) employing SDS-proteinase K lysis and selective precipitation of cell debris and polysaccharides with cetyltrimethylammonium bromide. The genomic DNA obtained was partially digested by *Hind*III or *Eco*RI and separated in a 0.6% (w/v) agarose gel. DNA fragments of 9–20 kb were purified from the gel using a QIAEXII Gel Extraction Kit (Qiagen), ligated to pUC19 digested by *Hind*III or *Eco*RI, and introduced into *E. coli* JM109 by electroporation using a Gene pulser cell (Bio-Rad) according to the manufacturer's instructions.

DNA sequencing and computer sequence analysis. Cloned DNA fragments were sequenced by TaKaRa Bio (Kyoto), and nucleotide and amino acid sequences were analysed using DNAMAN software (Lynnon Biosoft). Sequence comparisons were made against the sequences in the GenBank using the BLAST program (Altschul *et al.*, 1990). Phylogenetic trees were constructed using GENETYX-WIN Version 5.1 (Genetex Co.).

Aniline degradation tests and aniline oxygenase assay. One loop of strain AD9 was inoculated into LB medium and the culture was incubated overnight at 30 °C. The cells were harvested by centrifugation (4000 g, 4 °C, 10 min), washed with 10 mM potassium phosphate buffer (pH 7.0) twice, and suspended in MS medium to give an OD₆₀₀ of 1.0. Aniline degradation tests were started by adding aniline to the cell suspension at various concentrations up to 5500 mg l⁻¹. The cell suspension was shaken on a rotary shaker at 180 r.p.m., at 30 °C, and samples of the suspension were taken at specific intervals. After removing the cells from the samples by centrifugation (8000 g, 4 °C, 10 min), aniline in the supernatant was detected using the diazo-coupling method (Snell, 1954).

Aniline oxygenase activity was measured using a Clark-type oxygen electrode (YSI 5100, Yellow Springs Instruments). Fresh cells, pre-grown in 40 ml LB medium, were harvested by centrifugation (4000 g, 4 °C, 10 min) and washed with the phosphate buffer (10 mM, pH 7.0). The cells were suspended in 100 ml MS medium containing aniline (600 mg l⁻¹) or succinate (10 mM), and incubated at 30 °C with rotary shaking (180 r.p.m.) for 24 h to obtain aniline-induced cells or non-aniline-induced (succinate-grown) cells. The cells were harvested, washed twice with the phosphate buffer, and suspended in the same buffer. Oxygen uptake was measured polarographically at 30 °C. The reaction mixture contained 100 mg aniline l⁻¹ and washed cells in phosphate buffer. Endogenous respiration was measured in the absence of aniline, and the oxygen uptake rates obtained were corrected for the endogenous respiration.

Expression of AD genes in *E. coli*. Recombinant *E. coli* strains were cultivated in 6 ml LB medium containing Ap. When the

cultures reached early exponential phase, IPTG was added at a final concentration of 1 mM. After 4 h incubation, the cells were harvested, washed with phosphate buffer, and suspended in 0.8% (w/v) sodium chloride solution. Finally, cell suspensions with an OD₆₀₀ of 10–18 were prepared. The aniline oxygenase activity was measured polarographically as described above.

Measurement of catechol 2,3-dioxygenase (C23O) activity.

Recombinant *E. coli* strains were cultivated at 37 °C in 3 ml LB medium containing Ap and IPTG (1.0 mM) until the OD₆₀₀ reached approximately 1.0. The cells were harvested, washed with phosphate buffer, and suspended in 1.6 ml 50 mM sodium chloride solution containing 10% (v/v) ethanol. A crude cell extract was prepared by sonication of the cell suspension using an ultrasonic disruptor (TOMY UD-200; power 6, 2 min, three times on ice). Cell debris was removed by centrifugation (8000 g, 4 °C, 10 min). C23O activity was measured spectrophotometrically by the increase in absorbance at 375 nm concomitant with the formation of 2-hydroxymuconic semialdehyde (Nakazawa & Yokota, 1973). The reaction mixture contained 0.1 mM catechol and the cell extract in 50 mM sodium phosphate buffer (pH 7.5) and the reaction was carried out at 24 °C. The quantity of protein in the cell extract was determined by the Lowry method, using bovine serum albumin as the standard. One unit of activity was defined as the amount of enzyme required to produce 1 µmol product min⁻¹.

Southern hybridization analysis. The genomic DNA of strain AD9 was digested by *Hind*III, *Eco*RI or *Pst*I. After electrophoresis in a 1.0% (w/v) agarose gel, the digested fragments were transferred onto a Hybond-N⁺ nylon membrane (Amersham). A 526 bp DNA fragment and a 580 bp DNA fragment, which correspond to parts of the AD genes (*tadQ* region, see Fig. 1) and the transposase (*TnpA*) gene of the *IS1071* region (*tnpA-L1* region, see Fig. 1), respectively, were amplified from pDA1 by PCR using the primer sets for the *tadQ* gene (*TadQF*, 5'-ACGATGGTGCTGTTCGCAA-3', and *TadQR*, 5'-TATGAGGCAGGATGGTGACG-3') and for the *TnpA* gene sequence (*TnpAF*, 5'-GCCATTGAAGGTGTCATCCG-3', and *TnpAR*, 5'-AGGTATTCCACGCCATCAGC-3'), respectively. The PCR mixture contained 5 µl 10× *ExTaq* buffer (TaKaRa), 4 µl dNTPs (2.5 mM each), 100 pmol of each primer, 1.25 U *ExTaq* (TaKaRa), 2.5 ng of the template already described, and sterile distilled water to adjust the total volume to 50 µl. PCR was carried out using a PCR Express machine (Thermo Hybaid) and the following temperature programme: 94 °C for 3 min; 30 cycles consisting of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min; and 72 °C for 5 min. The PCR products were labelled with digoxigenin using a DIG-High Prime DNA Labelling and Detection Starter Kit II (Boehringer Mannheim Biochemicals) and used as gene probes. Southern hybridization was carried out according to the protocol for the labelling and detection kit.

PFGE. DNA was prepared by the method of Kieser *et al.* (1992). PFGE was performed using CHEF DRIII PFGE systems (Bio-Rad). Electrophoresis was performed at an electric field of 6 V cm⁻¹ at 14 °C for 23 h. The pulse time was increased steadily from 60 s at the beginning to 90 s at the end or from 30 s to 60 s, and the field angle was 120°.

RESULTS

Characterization and identification of strain AD9

Several morphologically distinct isolates were obtained from the enrichment culture using plates of MS medium containing aniline as a sole carbon and nitrogen source.

One such strain, named AD9, was selected from these isolates for further detailed analyses owing to its ability to grow rapidly on this medium. The morphological, physiological and biochemical characteristics of AD9 are summarized in Supplementary Table S1 available with the online version of this paper. Based on these characteristics, the Institute of Microbiology (Chinese Academy of Sciences, Beijing) tentatively identified AD9 as *Delftia acidovorans*. To confirm this identification at the genetic level, the partial 16S rDNA of AD9 was sequenced and compared with other sequences in DNA databases. The result showed that the AD9 sequence is almost identical to those of *D. acidovorans* MBIC1305 (AB020186, identity >99.1%) and *D. tsuruhatensis* T7 (AB075017, identity >99.6%). *D. tsuruhatensis* was established as a bacterial species (Shigematsu *et al.*, 2003) after the Institute of Microbiology identified AD9 as *D. acidovorans*. (Characteristics of *D. acidovorans* and *D. tsuruhatensis* are summarized in Supplementary Table S1.) Bathe (2004) constructed a phylogenetic tree based on the 16S rRNA sequences of bacterial strains belonging to the family *Comamonadaceae*, in which *D. acidovorans* strains and *D. tsuruhatensis* strains form different branches. Our similar approach revealed that AD9 belongs to the *D. tsuruhatensis* branch and is most closely related to *D. tsuruhatensis* T7 (the type strain of *D. tsuruhatensis*) (data not shown). Strain AD9 was unable to utilize 3-aminobutyrate as a carbon source; most *D. acidovorans* strains can assimilate this compound (Shigematsu *et al.*, 2003). The G+C content of AD9 is 66.8 mol%, which is very similar to that of *D. tsuruhatensis* T7 (66.2 mol%) (Shigematsu *et al.*, 2003). In addition, the DNA–DNA hybridization value between AD9 and T7 was 83.8%. These results all support the identification by the 16S rDNA sequence. Therefore, we identified AD9 as a strain of *D. tsuruhatensis*.

Aniline degradation by strain AD9

Strain AD9 grew on and degraded aniline well at 20–37 °C (the optimum temperature was around 30 °C), and it grew well in a wide pH range from 4.0 to 9.0 (the optimum pH was around 7.0). Although it could degrade up to 5000 mg aniline l⁻¹ in MS medium, over 1000 mg aniline l⁻¹ inhibited growth. The highest concentration (5000 mg l⁻¹, 53.8 mM) at which AD9 can grow is identical to that of *Delftia* sp. AN3 (Liu *et al.*, 2002), which was reported as the most aniline-tolerant aniline degrader. When cells pre-grown in LB medium containing aniline were suspended at an OD₆₀₀ of 1.0 in MS medium, the culture degraded 1000 mg aniline l⁻¹ completely within 18 h (data not shown). This ability to degrade aniline was not lost even after repeated culturing (six times) in LB medium without aniline.

Strain AD9 was also able to utilize *m*-toluidine and *p*-toluidine as a sole source of carbon, but not *o*-toluidine, 4-chloroaniline, 2-chloroaniline, 2,4-xylydine, 3,4-dichloroaniline or 2,4-dichloroaniline.

To examine the aniline oxygenase activity of AD9, oxygen

uptake was measured using aniline-induced cells and non-aniline-induced (succinate-grown) cells. The aniline-induced cells showed apparent aniline oxygenase activity of 82 ± 6 mg O₂ (g dry wt)⁻¹ h⁻¹, while the non-aniline-induced cells showed only one-third of this activity [30 ± 2 mg O₂ (g dry wt)⁻¹ h⁻¹]. This result indicates that aniline oxidation in AD9 is inducible.

Cloning of aniline degradation genes from AD9

A genomic library was constructed with *Hind*III-digested total DNA of AD9 and introduced into competent *E. coli* JM109. When transformants were screened on LB plates containing Ap and aniline, five colonies showed a brown colour on the plates, indicating accumulation of catechol resulting from aniline oxidation. A recombinant plasmid was extracted from one of the positive colonies and analysed by restriction enzymes. The analysis revealed that the recombinant plasmid, designated pDA1, had a 9.3 kb *Hind*III insert in the vector pUC19. The transformants were screened again by spraying their colonies with 0.1 M catechol solution (in 10 mM phosphate buffer, pH 7.0). One colony showed a brilliant yellow colour on the plate, indicating C23O activity. The C23O-positive strain contained a recombinant plasmid, designated pDB11, which had a 15.4 kb *Hind*III insert fragment. Restriction analysis showed that the insert fragment of pDB11 did not overlap with that of pDA1. To confirm whether the two insert fragments were situated next to each other, the transformants obtained from another library constructed with *Eco*RI-digested AD9 DNA were screened by the catechol spray. One C23O-positive strain was obtained. The strain contained a recombinant plasmid, which had an 8.2 kb *Eco*RI insert fragment and was designated pDB2. Restriction analysis of pDB2 revealed that, as shown in Fig. 1(a), the insert fragment of pDB2 overlaps the inserts of both pDA1 and pDB11 and that the insert of pDB11 is adjacent to that of pDA1.

Sequence analysis of cloned fragments

The DNA fragments cloned in pDA1, pDB2 and pDB11 were sequenced, and the nucleotide sequence of the total 24.7 kb region was thereby determined. Homology searches were performed to identify gene function. It was found that the region contains 23 intact ORFs (ORF2–24, Table 2), at least 17 of which (*tadQTA1A2BRD1C1D2C2EFGIJKL*) were expected to be involved in the complete metabolism of aniline to TCA-cycle intermediates as shown in Fig. 1(b). The first five gene products (*TadQTA1A2B*) and the subsequent gene product (*TadR*) showed significant aa sequence identity (84–96%) to multi-component aniline dioxygenases (ADs) and LysR-type regulators, respectively, found in other aniline-degrading bacteria, *P. putida* UCC22, *D. acidovorans* 7N and *Acinetobacter* sp. YAA. The remaining 11 gene products (*TadD1C1D2C2EFGIJKL*) exhibited considerable identity (73–100%) to *meta*-cleavage pathway enzymes found in aromatic-compound-degrading and aniline-degrading bacteria (Table 2).

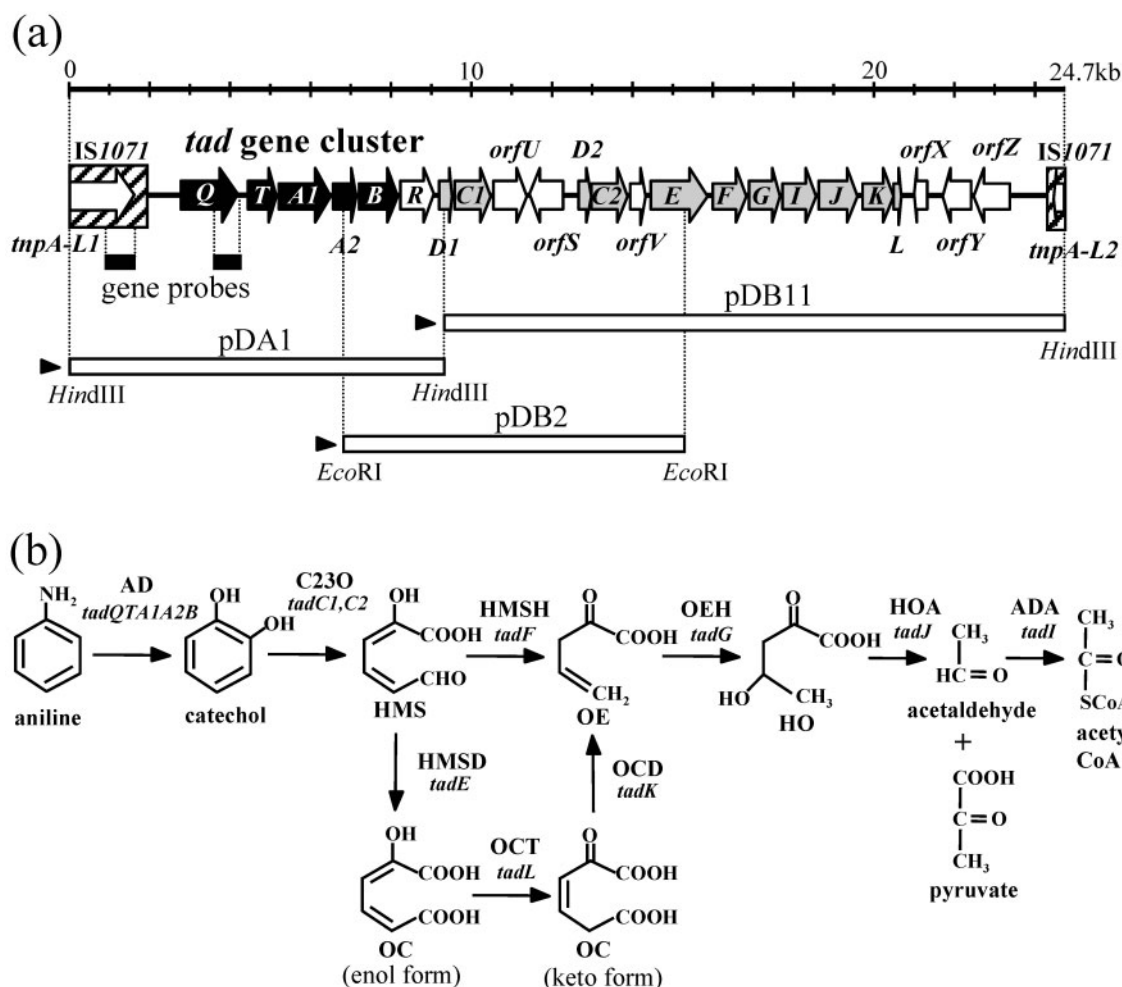


Fig. 1. Genetic organization of the aniline degradation *tad* gene cluster (a) and putative aniline degradation pathway (b) of *D. tsuruhatensis* AD9. The sequenced region of 24.7 kb is shown; the *tad* gene cluster is surrounded by two *IS1071* sequences. Open arrows indicate ORFs; black and grey arrows indicate AD genes and *meta*-cleavage pathway genes, respectively. Open bars and arrowheads show DNA fragments cloned in recombinant plasmids and *lac* promoters on the plasmids, respectively. Hatched boxes and arrows surrounded by them indicate *IS1071* regions. Black bars indicate the regions for the gene probes used in the following Southern hybridization study. Abbreviations: AD, aniline dioxygenase; C23O, catechol 2,3-dioxygenase; HMS, 2-hydroxymuconic semialdehyde; HMSD, 2-hydroxymuconic semialdehyde dehydrogenase; HMSH, 2-hydroxymuconic semialdehyde hydrolase; OE, 2-oxopent-4-dienoate; OEH, 2-oxopent-4-dienoate hydratase; OC, 4-oxalocrotonate; OCD, 4-oxalocrotonate decarboxylase; OCT, 4-oxalocrotonate tautomerase; HO, 4-hydroxy-2-oxovalerate; HOA, 4-hydroxy-2-oxovalerate aldolase; ADA, acetaldehyde dehydrogenase.

Two ORFs, *orfS* and *orfU*, whose transcriptional directions are opposite to each other, are located between *tadC1* and *tadD2*. The former was predicted to encode another LysR-type regulator, although the latter encodes an unknown protein. In addition, there is another ORF, *orfV*, between *tadC2* and *tadE*, which also encodes an unknown protein. Similar ORFs to *orfU* and *orfV* have been found in the *meta*-cleavage pathways of a phenol degrader, *Comamonas testosteroni* TA441 (Arai *et al.*, 2000), and a nitrobenzene degrader, *Comamonas* sp. JS765 (AF190463), in addition to *P. putida* UCC22 (Fukumori & Saint, 2001). However, the functions of their gene products have not been identified. In the region downstream of *tadL*, there are three ORFs,

orfXYZ, whose gene products show considerable similarity to MarR-type regulators, β -keto adipate enol-lactone hydrolases and muconate cycloisomerases, respectively (Table 2). These ORFs are transcribed in the direction opposite to those of the AD genes and the *meta*-cleavage pathway enzyme genes.

Partial sequences encoding the TnpA of *IS1071* (Nakatsu *et al.*, 1991) were found at both ends of this 24.7 kb region. Inverted repeat sequences of 110 bp (nt 1744–1853 and nt 24403–24512 in AY940090), which are completely identical to those of *IS1071*, were found near the TnpA-encoding sequences, indicating that this *tad* gene cluster is surrounded

Table 2. Analytical data on the aniline degradation genes of *D. tsuruhatensis* AD9

| ORF | Gene name | Position (no. of nt) | No. of aa | Putative function | Homologous proteins* (sequence identity) |
|-----|-----------------|-------------------------|--------------|--|--|
| 1 | <i>tnpA-L1†</i> | 1–1710 (1710) | 569 | Transposase | TnpA (96%) ¹ , TnpA (96%) ² , TnpA (96%) ³ |
| 2 | <i>tadQ</i> | 2809–4287 (1479) | 492 | Amino group transfer | TdnQ (94%) ¹ , TdnQ (82%) ⁴ , TdnQ (82%) ⁵ |
| 3 | <i>tadT</i> | 4406–5167 (762) | 253 | Amino group transfer | TdnT (84%) ¹ , TdnT (59%) ⁴ , ORF7NB (57%) ⁶ |
| 4 | <i>tadA1</i> | 5204–6550 (1347) | 448 | Large subunit of terminal dioxxygenase | TdnA1 (96%) ¹ , TdnA1 (82%) ⁴ , TdnA1 (80%) ⁵ |
| 5 | <i>tadA2</i> | 6547–7191 (645) | 214 | Small subunit of terminal dioxxygenase | TdnA2 (92%) ¹ , TdnA2 (72%) ⁴ , ORF7ND (65%) ⁶ |
| 6 | <i>tadB</i> | 7203–8216 (1014) | 337 | Electron-transfer protein | TdnB (84%) ¹ , TdnB (66%) ⁴ , ORF7NE (64%) ⁶ |
| 7 | <i>tadR</i> | 8264–9151 (888) | 295 | LysR-type regulator | TdnR (91%) ¹ , TdnR (74%) ⁴ , AtdR (33%) ⁷ |
| 8 | <i>tadD1</i> | 9163–9522 (360) | 119 | Plant-type ferredoxin | TdnD (73%) ¹ , CbzT (47%) ⁸ , CdoT (45%) ⁹ |
| 9 | <i>tadC1</i> | 9559–10503 (945) | 314 | Catechol 2,3-dioxxygenase | TdnC (97%) ¹ , ORF7NH (85%) ⁶ , CdoE (85%) ⁹ |
| 10 | <i>orfU</i> | 10536–11426 (891) | 296 | Unknown product | CdoX1 (93%) ⁹ , ORF1 (92%) ¹ , ORFX (81%) ¹⁰ |
| 11 | <i>orfS</i> | 11457–12362 (906) | 301 | LysR-type regulator | ORF2 (100%) ¹ , AphT (69%) ¹⁰ , CdoR (66%) ⁸ |
| 12 | <i>tadD2</i> | 12633–12968 (336) | 111 | Plant-type ferredoxin | TdnD2 (83%) ¹ , PhnT1 (54%) ¹¹ |
| 13 | <i>tadC2</i> | 12991–13917 (927) | 308 | Catechol 2,3-dioxxygenase | TdnC2 (93%) ¹ , AphB (42%) ¹⁰ , PhnE2 (41%) ¹¹ |
| 14 | <i>orfV</i> | 13931–14362 (432) | 143 | Unknown product | ORF4 (76%) ¹ , ORF (53%) ¹² , ORF (52%) ¹³ |
| 15 | <i>tadE</i> | 14425–15885 (1461) | 486 | HMS \ddagger dehydrogenase | TdnE (94%) ¹ , AphC (68%) ¹⁰ , CdoG (68%) ⁹ |
| 16 | <i>tadF</i> | 15959–16819 (861) | 286 | HMS \ddagger hydrolase | TdnF (89%) ¹ , DmpD (72%) ¹⁴ , XylF (70%) ¹⁵ |
| 17 | <i>tadG</i> | 16861–17649 (789) | 262 | 2-Oxopent-4-dienoate hydratase | TdnG (87%) ¹ , CdoH (83%) ⁹ , AphE (79%) ¹⁰ |
| 18 | <i>tadI</i> | 17664–18572 (909) | 302 | Acetaldehyde dehydrogenase | TdnI (93%) ¹ , CdoI (83%) ⁹ , AphF (83%) ¹⁰ |
| 19 | <i>tadJ</i> | 18594–19625 (1032) | 343 | 4-Hydroxy-2-oxovalerate aldolase | TdnE (93%) ¹ , AphG (87%) ¹⁰ , TesG (79%) ¹⁶ |
| 20 | <i>tadK</i> | 19640–20428 (789) | 262 | 4-Oxalocrotonate decarboxylase | TdnK (93%) ¹ , CdoK (81%) ⁹ , AphH (80%) ¹⁰ |
| 21 | <i>tadL</i> | 20447–20638 (192) | 63 | 4-Oxalocrotonate tautomerase | TdnL (100%) ¹ , AphI (85%) ¹⁰ |
| 22 | <i>orfX</i> | 20745–21329 (585) | 194 | MarR-type regulator | RSp0034 (60%) ¹⁷ , ORF6 (58%) ¹⁸ |
| 23 | <i>orfY</i> | 21596–22405 (810) | 269 | β -Ketoadipate enol-lactone hydrolase | COG0596 (45%) ¹⁹ |
| 24 | <i>orfZ</i> | 22402–23445 (1044) | 347 | Muconate cycloisomerase | CatB (51%) ²⁰ , CatB1 (51%) ²¹ , CatB1 (51%) ²² |
| 25 | <i>tnpA-L2†</i> | 24548–24681 (134) | 44 | Transposase | TnpA (100%) ¹ , TnpA (100%) ² , TnpA (100%) ³ |

*Sequences used were obtained from: 1, BAA12804, BAA12805, BAA12806, BAA12807, BAA12808, BAA12809, BAA12810, BAB62044, BAB62045, BAB62047, BAB62046, BAB62049, BAB62050, BAB62051, BAB62052, BAB62053, BAB62054, BAB62056, BAB62057, BAB62058, BAB62059; 2, AAT81377; 3, BAB85582; 4, BAC82524, BAC82525, BAC82526, BAC82527, BAC82528, BAC82529; 5, AAO38206, AAO38208; 6, BAD61048, BAD61050, BAD61051, BAD61054; 7, BAA23553; 8, AAP51196; 9, AAC79917, AAC79918, AAG17128, AAC79916, AAG17134, AAG17135, AAG17136, AAG17138; 10, BAA88498, BAA88500, BAA34176, BAA88501, BAA88502, BAA88503, BAA88504, BAA88505, BAA88507; 11, AAF02425, AAF02430; 12, AAR03452; 13, ZP_00167856.2; 14, CAA36993; 15, AAT09778; 16, BAC67696; 17, NP_521595; 18, AAL02068; 19, ZP_00167449; 20, BAB21460; 21, BAC16777; 22, NP_943040.

†Incomplete ORFs.

\ddagger 2-Hydroxymuconic semialdehyde.

by two IS1071 sequences (nt 1–1853 and nt 24403–24681 in AY940090).

Expression of AD and C230 genes

Aniline oxygenase activity was measured using cell suspensions of *E. coli* harbouring pDA1 to examine whether the cloned AD genes (*tadQTAIA2B*) were functional. The *E. coli* cells harbouring pDA1 showed apparent aniline oxygenase activity [32 ± 3 mg O₂ (g dry wt)⁻¹ h⁻¹]. The endogenous respiration (the oxygen uptake without the addition of aniline) subtracted was 16 ± 2 mg O₂ (g dry wt)⁻¹ h⁻¹. Catechol was detected in the cell suspension as the oxidation

product by GC/MS analysis (data not shown). These results indicate that the recombinant *E. coli* oxidized aniline to catechol. The 9.3 kb *Hind*III fragment of pDA1 was then subcloned into the broad-host-range plasmid pVK100 to make the recombinant plasmid pVD1. This plasmid was introduced by triparental mating (Winstanley *et al.*, 1989) into the phenol-degrading bacterium *A. calcoaceticus* PHEA-2 (Xu *et al.*, 2003), which can assimilate both catechol and phenol, but not aniline. The resultant strain was able to grow on aniline as a sole carbon source, indicating that the AD genes in pVD1 allowed strain PHEA-2 to convert aniline into catechol (data not shown). Furthermore, when pVD1 was introduced into the parent

strain AD9, the resultant strain accumulated a large amount of a dark brown compound, probably auto-oxidized catechol from aniline, in liquid cultures, because of the multi-copy dose effect of the AD genes. These results indicate that the cloned AD genes were functional in AD9 and also in *E. coli* and *A. calcoaceticus*.

In the catechol spray selection, the recombinant *E. coli* strain harbouring pDB2 was selected based on yellow colour formation as an index of C23O activity. To evaluate the C23O activity, the cell extract of the recombinant strain was prepared and used to measure C23O activity. The activity measured was $3.2 \text{ units (mg crude protein)}^{-1}$.

Copy number and location of the *tad* gene cluster in AD9

Southern hybridization was carried out using a 526 bp gene probe including a part of the AD genes (*tadQ* region, see Fig. 1) in order to determine the copy number of the *tad* gene cluster in strain AD9. The total DNA extracted from AD9 was digested independently by three restriction enzymes (*Hind*III, *Eco*RI or *Pst*I) for this study. As shown in Fig. 2(a, b), the gene probe hybridized to one band in each lane of the Southern blot (Fig. 2b) corresponding to the digested DNA sample lanes of the agarose gel electrophoresis (Fig. 2a), suggesting that AD9 has the *tad* gene cluster in a single copy, if it resides on the chromosome. In order to clarify the location of the *tad* gene cluster in AD9, PFGE was carried out using the AD9 cells. Fig. 2(c) clearly demonstrates that there are no detectable large plasmids in AD9, although several plasmids were detected in *Bacillus thuringiensis* A-01 (a plasmid-positive control strain), and the chromosome of AD9 could be seen together with that of *E. coli* (negative control and size marker). Southern hybridization with the same gene probe revealed that the *tad* gene cluster is located on the AD9 chromosome (Fig. 2d).

To determine the copy number of *IS1071* on the chromosome of AD9, another hybridization was carried out using a 580 bp gene probe encoding the TnpA gene of *IS1071* (*tnpA-L1* region, see Fig. 1). As shown in Fig. 2(e, f), two hybridization signals were detected in the *Hind*III-digested and *Pst*I-digested AD9 DNA samples. In the case of the *Eco*RI-digested AD9 DNA sample, two positive DNA fragments with a similar size appear to give one strong signal in the Southern blot. This result shows that there are only two copies of *IS1071* on the chromosome of AD9 and supports the conclusion that the *tad* gene cluster, surrounded by the two *IS1071*s, resides on the chromosome in a single copy.

Comparison of the chromosome-encoded *tad* gene cluster with the plasmid-encoded *tdn* gene cluster

As shown in Table 3, the putative products of the *tad* genes showed striking identity to those of the plasmid-encoded *tdn* genes of *P. putida* UCC22. Hence, the gene organization

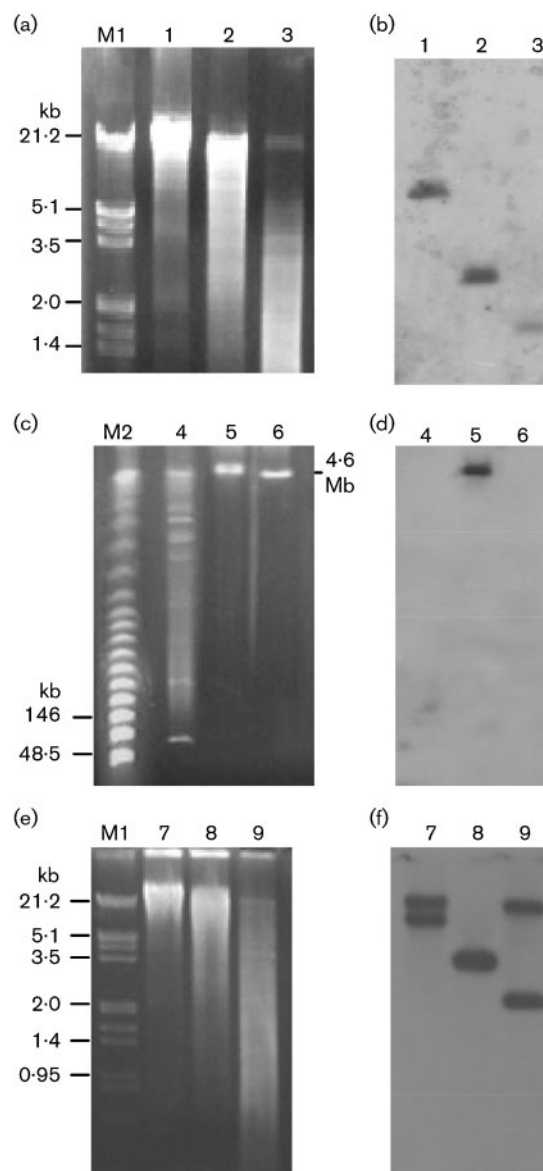
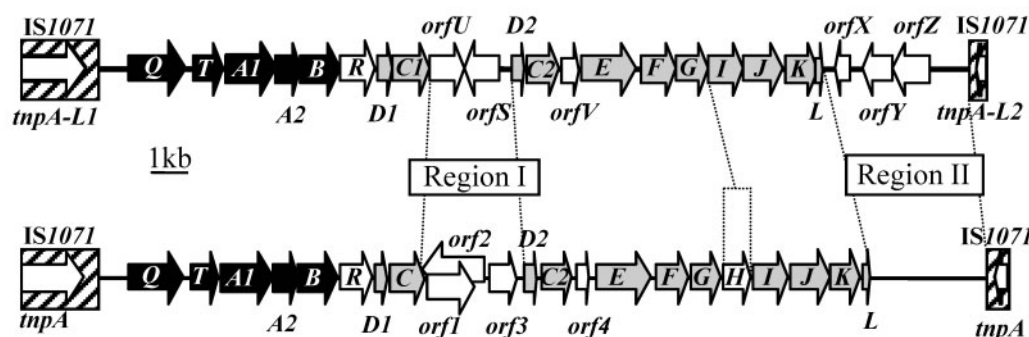


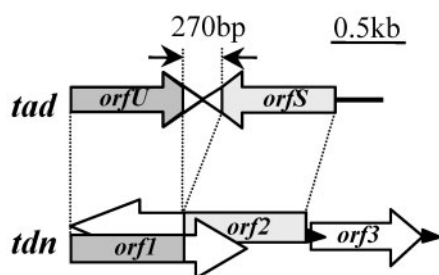
Fig. 2. Agarose gel electrophoresis profiles (a, e) and PFGE profile (c) of *D. tsuruhatensis* AD9 DNA, and the corresponding Southern blots (b, d, f) obtained in the Southern hybridization using a 526 bp AD gene probe (b, d) or a 580 bp gene probe for the transposase gene of *IS1071* (f). Lane M1, molecular size marker (lambda DNA *Eco*RI/*Hind*III digest); lane M2, molecular size marker (lambda concatemer); lanes 1 and 7, *Hind*III-digested total DNA of AD9; lanes 2 and 8, *Eco*RI-digested total DNA of AD9; lanes 3 and 9, *Pst*I-digested total DNA of AD9; lane 4, *B. thuringiensis* A-01 cells (strain harbouring plasmids, positive control); lane 5, AD9 cells; lane 6, *E. coli* K-12 cells (negative control and size marker).

of the *tad* gene cluster was compared with that of the *tdn* gene cluster in detail (Fig. 3). It was found that the gene organization of both gene clusters is quite similar and both are surrounded by two *IS1071* sequences including TnpA-encoding regions. However, there are some

(a)

Chromosome-encoded *tad* gene cluster of *D. tsuruhatensis* AD9Plasmid-encoded *tdn* gene cluster of *P. putida* UCC22

(b) Region I



(c) Region II

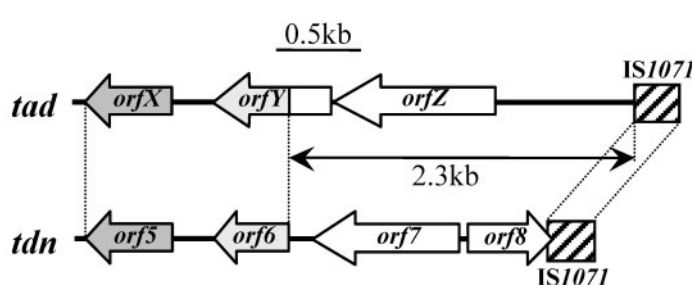


Fig. 3. Comparison of the chromosome-encoded *tad* gene cluster of *D. tsuruhatensis* AD9 with the plasmid-encoded *tdn* gene cluster of *P. putida* UCC22 (a) and regions different between the two gene clusters (b, c). Black and grey arrows in (a) indicate AD genes and meta-cleavage pathway genes, respectively. Grey regions of arrows in (b) and (c) indicate homologous regions, and hatched boxes in (a) and (c) indicate IS1071 regions. Black triangles in (b) indicate 63 bp direct repeat sequences.

differences between them. First, the *tad* gene cluster lacks the genes corresponding to *orf3* and *tdnH* of the *tdn* gene cluster. The function of the *orf3* gene product has not been characterized yet, and the *tdnH* gene product, which showed similarity to short-chain dehydrogenases (Fukumori & Saint, 2001), seems to be unnecessary for catechol metabolism, because many meta-cleavage pathways do not contain this enzyme. Secondly, in the region between *tadC1/tdnC* and *tadD2/tdnD2* (region I in Fig. 3), the *tdn* gene cluster lacks a 270 bp DNA segment of the *tad* gene cluster (nt 11266–11535 in AY940090). The loss of this small segment in the *tad* gene cluster caused the disruption of *orfU* and *orfS* and resulted in the formation of longer *orf1* and *orf2* in the *tdn* gene cluster. Thirdly, in the region downstream of *tadL/tdnL* (region II in Fig. 3), there is a substitution of a 2.3 kb DNA segment. In region II of the *tad* gene cluster, there are three ORFs, *orfXYZ*, as mentioned above. In contrast, in region II of the *tdn* gene cluster, there are four

ORFs, which have not been described so far (Fukumori & Saint, 2001). Herein, we tentatively call them *orf5678*. The putative gene products of *orf5678* showed considerable similarity to MarR-type regulators (e.g. AAL02068, identity 58 %), the C terminal half of β -ketoadipate enol-lactone hydrolases (e.g. CAD13826, identity 39 %), KfrA-like proteins (e.g. AAP22622, identity 27 %) and integrases/recombinases (e.g. CAI47894, identity 71 %), respectively. As shown in Fig. 3, a 2.3 kb DNA segment containing half of *orfY* and intact *orfZ* in the *tad* gene cluster (nt 22124–24402 in AY940090) is substituted by a smaller segment containing *orf7* and *orf8* in the *tdn* gene cluster.

Phylogenetic relationships of the AD and C23Os of AD9 with other homologous proteins

To clarify the phylogenetic relationship of the AD of strain AD9 with those of other aniline-degrading bacteria,

phylogenetic trees were constructed using the amino acid sequences of the glutamine-synthetase-like proteins and the large subunits of the oxygenase component in the AD enzyme systems. As shown in Fig. 4(a, b), the phylogenetic trees constructed consist of two major branches, the Tdn-branch and the Atd-branch, and both branches are distant from the *E. coli* glutamine synthetase, GlnA, or the *P. putida* benzoate dioxygenase large subunit, BenA (outer group members). In these trees, TadQ and TadA1 are located closest to TdnQ and TdnA1 of *P. putida* UCC22, respectively.

To locate the *meta*-cleavage pathway of AD9 among those of other aromatic-compound-degrading bacteria, another phylogenetic tree was constructed using C23O amino acid

sequences (Fig. 4c). The *meta*-cleavage pathway of AD9 contains two C23Os, TadC1 and TadC2. In the tree, TadC1 belongs to a branch including TdnC, AlnE and ORF7NH from other aniline-degrading bacteria, *P. putida* UCC22, *Pseudomonas* sp. AW-2 and *D. acidovorans* 7N, whereas TadC2 belongs to a branch including BupB and LapB from alkylphenol-degrading bacteria, *P. putida* MT4 and *P. putida* KL28. In this tree, TadC1 and TadC2 are also located closest to TdnC and TdnC2, respectively, from UCC22.

DISCUSSION

As described in the Introduction, many bacterial strains have been isolated as aniline-degraders. Urata *et al.* (2004)

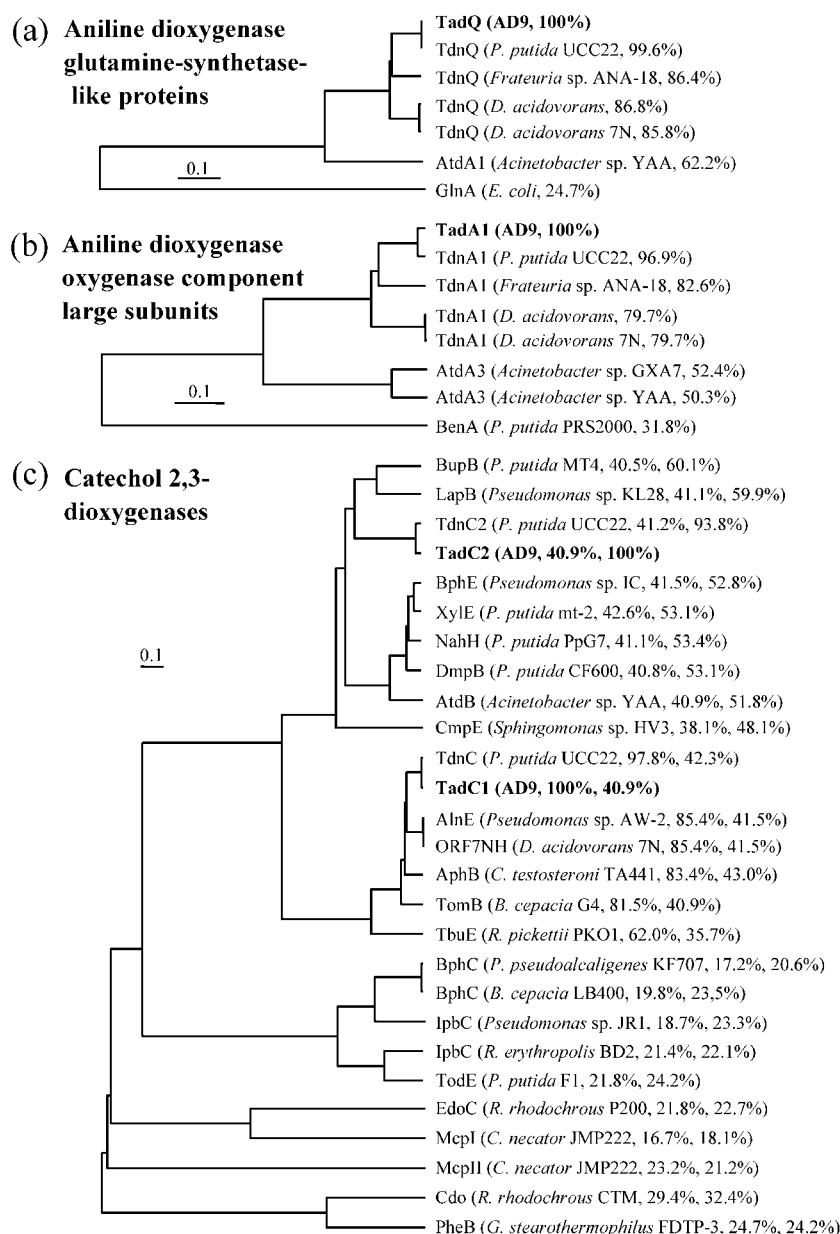


Fig. 4. Phylogenetic trees constructed based on the amino acid sequences of AD glutamine-synthetase-like proteins (a), oxygenase component large subunits (b) and C23Os (c). These trees were constructed using the UPGMA method of GENETYX-WIN version 5.1 (Genetyx). Sequences used were obtained from AY940090 (AD9), D85415 (UCC22), AB089795 (ANA-18), AY168646, AB177545 (7N), M13746, AY877266 (GXA7), D86080 (YAA), AF218267 (PRS2000), AB107791 (MT4), AY324644 (KL28), U01825 (IC), V01161 (mt-2), AJ633093 (PpG7), M33263 (CF600), Z84817 (HV3), AB004065 (AW-2), AB006479 (TA441), JC5534 (G4), U20258 (PKO1), M83673 (KF707), X66122 (LB400), U53507 (JR1), U24277 (BD2), J04996 (F1), AJ006126 (P200), X52414 (JMP222), X521415 (JMP222), X69504 (CTM), X67860 (FDTP-3), respectively. Strain names and sequence identity (to TadQ, TadA1, TadC1 or TadC2) are shown in parentheses.

isolated 19 aniline-degrading bacteria from several different places in Japan and classified them into eight groups (six genera): *Delftia acidovorans*, *Acinetobacter* sp., *Pimelobacter simplex*, *Pseudomonas* sp., *Acinetobacter* sp., *Comamonas testosteroni*, *Acinetobacter junii* and *Acidovorax* sp. This shows that aniline degradation ability is distributed among many bacterial species. However, several research groups have independently reported *Delftia* strains as aniline-degraders (Loidl *et al.*, 1990; Brunsbach & Reineke, 1993; Boon *et al.*, 2001; Liu *et al.*, 2002; Kim *et al.*, 2003; Urata *et al.*, 2004), suggesting that *Delftia* is one of the major aniline-degrading bacterial groups. Despite this, gene clusters encoding enzymes for the complete metabolism of aniline to TCA-cycle intermediates have been cloned only from the plasmids of *P. putida* UCC22 (Fukumori & Saint, 1997) and *Acinetobacter* sp. YAA (Fujii *et al.*, 1997), although AD genes have been cloned from the chromosomes of *D. acidovorans* 7N and *Frateuria* sp. ANA-18 (Urata *et al.*, 2004; Murakami *et al.*, 2003). In this study, we isolated for the first time a chromosome-encoded aniline degradation gene cluster from *D. tsuruhatensis* AD9, which is responsible for the complete metabolism of aniline to TCA-cycle intermediates via a *meta*-cleavage pathway. Interestingly, it is quite similar to the plasmid-encoded *tdn* gene cluster from UCC22, as shown in Fig. 3. Both gene clusters (*tad* and *tdn*) are surrounded by IS1071 sequences and their genetic organization is almost identical. These facts suggest that these two gene clusters have evolved from a common ancestor and they can move as a mobile element to bacterial plasmids and chromosomes. At present, we do not know whether the *tad* gene cluster can move as a mobile element, although it has a class I transposon-like structure (Tsuda *et al.*, 1999). Like these gene clusters, several catabolic gene clusters surrounded by IS1071 have been found (reviewed by Wyndham *et al.*, 1994; Tsuda *et al.*, 1999; Tan, 1999). The best-characterized of these is Tn5271, found on the plasmid pBRC60 of *Alcaligenes* sp. BR60 (now *Comamonas* sp. BR60) (Nakatsu *et al.*, 1991), which carries chlorobenzoate degradation genes, *chaABC*. This transposon could be integrated into the chromosomes of *D. acidovorans* and *C. testosteroni* (Wyndham *et al.*, 1994). However, this integration seems to have happened through the transposition of a larger transposon including the complete Tn5271, because the same larger DNA segments, including Tn5271 from pBRC60, were integrated into different sites of their chromosomes (Wyndham *et al.*, 1994). The detailed structure of the chromosome-integrated Tn5271 has not been reported. In a different study, Boon *et al.* (2001) reported five aniline/chloroaniline-degrading strains belonging to the family *Comamonadaceae*, four of which have a similar aniline degradative plasmid (~100 kb in size). In a Southern hybridization study using a *tdnQ* gene probe from *P. putida* UCC22 and an IS1071 gene probe from Tn5271, both probes hybridized to the same 1.4 kb *EcoRI*/*PstI*-digested DNA fragment from the plasmids (Boon *et al.*, 2001). This shows that AD genes are linked closely to IS1071 on the plasmids and they may form a catabolic transposon. However, the detailed structure has not been reported and

the linkage of AD genes to IS1071 has also never previously been found on bacterial chromosomes. Moreover, except for some genomic islands or conjugative transposons (Springael & Top, 2004), only a few detailed structures of catabolic transposons located on bacterial chromosomes have been reported (Hoffmann *et al.*, 2003; Shintani *et al.*, 2003). Therefore, it is quite rare that the same catabolic transposon-like structure has been found on both a plasmid of *P. putida* and a chromosome of *D. tsuruhatensis*. Consequently, it is important to analyse their sequences and gene arrangements to understand their history and transfer mechanisms.

We found some differences between the *tad* gene cluster and the *tdn* gene cluster. In region I of Fig. 3, the *tdn* gene cluster lacks a 270 bp segment of the *tad* gene cluster, resulting in the disruption of *orfS* and the formation of a longer *orf2*. *orfS* is predicted to encode a LysR-type regulator with a normal size (301 aa) compared to those of other LysR-type regulators (around 300 aa), whereas *orf2* is expected to encode a larger protein with an unusual size (532 aa) for a member of this family. The additional sequence of *orf2* compared to *orfS* encodes no known proteins. Thus, it is natural to think that the 270 bp segment was deleted from the *tad*-type gene cluster to form the *tdn*-type gene cluster. In region II, a putative transcriptional unit, *orfYZ*, is disrupted by the substitution of the 2.3 kb DNA segment. The reasons why *orfY* and *orfZ* were presumed to form a transcriptional unit are that (i) both ORFs are thought to be a part of a catechol *ortho*-cleavage pathway operon, judging from the sequence similarity of their gene products (Table 2), and (ii) the 5' terminus of *orfY* overlaps by 3 bp the 3' terminus of *orfZ*. Therefore, this substitution also might have happened after the *tad* gene cluster had been established. These sequence analyses suggest that the *tad* gene cluster is more ancestral than the *tdn* gene cluster.

Our isolate, AD9, grew on and degraded aniline at concentrations as high as those used by the most aniline-tolerant aniline degrader *Delftia* sp. AN3. In the aniline degradation pathway of AD9, the activities of the key enzymes, AD and C23O, were experimentally confirmed. The AD genes of strain AD9 were expressed in *E. coli* and more efficiently in *A. calcoaceticus* PHEA1 and the parent strain AD9. Although the function of each subunit of ADs remains unknown, catechol was detected as a metabolite of aniline after the expression of the AD genes. Moreover, cell extracts of recombinant *E. coli* harbouring pDB11 showed C23O activity. However, pDB11 contains two C23O genes, *tadC1* and *tadC2*. The phylogenetic analysis of the gene products TadC1 and TadC2 (Fig. 4) illustrates that these two C23Os belong to different phylogenetic branches. This may mean that they have come from different *meta*-cleavage pathways. The phylogenetic tree also revealed that TadC1 and TadC2 are most closely related to TdnC and TdnC2 of *P. putida* strain UCC22, respectively. Fukumori & Saint (2001) reported that TdnC and TdnC2 have distinct substrate

specificity: the *E. coli* cell extract containing TdnC showed relatively high activity on substituted catechols (catechol, 100 %; 3-methylcatechol, 93 %; 4-methylcatechol, 43 %), while that containing TdnC2 showed less activity on substituted catechols (catechol, 100 %; 3-methylcatechol, 4.6 %; 4-methylcatechol, 19 %). Strain UCC22 can assimilate *m*-toluidine (3-methylaniline) and *p*-toluidine (4-methylaniline) via 3-methylcatechol and 4-methylcatechol, respectively. Therefore, it might be necessary for cells to acquire another C23O, TdnC, for these methylcatechols, in addition to TdnC2 for unsubstituted catechol, to expand the assimilation range for toluidines. Strain AD9 can also assimilate *m*-toluidine and *p*-toluidine. In our preliminary study using recombinant *E. coli* cell extracts containing TadC1 or TadC2, C23O activity was seen on catechol (100 % and 100 %, respectively), 3-methylcatechol (56 % and 18 %) and 4-methylcatechol (28 % and <1 %). Therefore, we confirmed that both *tadC1* and *tadC2* can produce an active C23O. The activities of TadC2 towards methylcatechols were lower than those of TadC1, as reported for TdnC2 and TdnC by Fukumori & Saint (2001). However, detailed analysis should be carried out using the purified enzymes.

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