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A novel and complete gene cluster involved in the degradation of aniline by *Delftia* sp. AN3

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Abstract

A recombinant strain, *Escherichia coli* JM109-AN1, was obtained by constructing of a genomic library of the total DNA of *Delftia* sp. AN3 in *E. coli* JM109 and screening for catechol 2,3-dioxygenase activity. This recombinant strain could grow on aniline as sole carbon, nitrogen and energy source. Enzymatic assays revealed that the exogenous genes including aniline dioxygenase (AD) and catechol 2,3-dioxygenase (C23O) genes could well express in the recombinant strain with the activities of AD and C23O up to 0.31 U/mg wet cell and 1.92 U/mg crude proteins, respectively. The AD or C23O of strain AN3 could only catalyze aniline or catechol but not any other substituted substrates. This recombinant strain contained a recombinant plasmid, pKC505-AN1, in which a 29.7-kb DNA fragment from *Delftia* sp. AN3 was inserted. Sequencing and open reading frame (orfs) analysis of this 29.7 kb fragment revealed that it contained at least 27 orfs, among them a gene cluster (consisting of at least 16 genes, named *danQTA1A2BRDCEFG1HIJKG2*) was responsible for the complete metabolism of aniline to TCA-cycle intermediates. This gene cluster could be divided into two main parts, the upper sequences consisted of 7 genes (*danQTA1A2BRD*) were predicted to encode a multi-component aniline dioxygenase and a LysR-type regulator, and the central genes (*danCEFG1HIJKG2*) were expected to encode *meta*-cleavage pathway enzymes for catechol degradation to TCA-cycle intermediates. Unlike clusters *tad* from *Delftia tsuruhatensis* AD9 and *tdn* from *Pseudomonas putida* UCC22, in this gene cluster, all the genes were in the same transcriptional direction. There was only one set of C23O gene (*danC*) and ferredoxin-like protein gene (*danD*). The presence of only one set of these two genes and specificity of AD and C23O might be the reason for strain AN3 could only degrade aniline. The products of *danQTA1A2BRD*C showed 99%–100% identity to those from *Delftia acidovorans* 7N, and 50%–85% identity to those of *tad* cluster from *D. tsuruhatensis* AD9 in amino acid residues. Besides this *dan* cluster, the 29.7 kb fragment also contained genes encoding the trans-membrane transporter and transposases which might be needed for transposition of the gene cluster. Pulsed-field gel electrophoresis (PFGE) and plasmid curing experiments suggested that the *dan* cluster might be encoded on the chromosome of strain AN3. The GenBank accession number for the *dan* cluster of *Delftia* sp. AN3 is DQ661649.

Key words: aniline; biodegradation; *Delftia* sp. AN3; genomic library; aniline degradative gene cluster

Introduction

Anilines constitute a group of xenobiotics (pollutants which resemble naturally occurring compounds), that are in use for a long time in the production of dyes, pesticides, plastics, and pharmaceuticals (Meyer, 1981; Kearney and Kaufmann, 1977). This group of refractory compounds is considered as an important environmental hazard and is subject to legislative control by the European Economic Community (EEC) directive and in the Priority Pollutant List of U.S. Environmental Protection Agency (Federal Register, 1979). Xenobiotics are released into the environment through industrial wastewaters and their direct application to soils, resulting in the introduction of carcinogenic and mutagenic chemicals to the environment. Their prevalence in the surroundings leads to bio-accumulation and can disturb the ecological balance of the microbial system in soil. Such accumulation in the long run may be hazardous to the human's health. Since it is known to be toxic and carcinogenic to living organisms (Lyons *et al*., 1985), highly aniline-tolerant bacterial strains were isolated from various environments (Meyers, 1992; Loidl *et al*., 1990; Konopka *et al*., 1989; Konopka, 1993; Boon *et al*., 2001; Liu *et al*., 2002), which are desirable for environmental applications as well as for the biotransformation of aniline and its analogues. Several aniline degradative genes or gene clusters were cloned from aniline-degraders (Meyers, 1992; Fujii *et al*., 1997; Takeo *et al*., 1998; Fukumori and Saint, 1997; Uratal *et al*., 2004; Liang *et al*., 2005). Most of them were plasmid-encoded, including pCIT1 (*Pseudomonas* sp. strain CIT1, Meyers, 1992), pYA1 (*Acinetobacter* sp. strain YAA, Takeo *et al*., 1998), pTDN1 (*Pseudomonas putita* UCC22,

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Fukumori and Saint, 1997), pNB2 (*P. putida* SM1443, Bathe, 2004) and these plasmids normally are large ones (about 100 kb). Up to date, only three gene clusters that contained complete genes involved in the pathway of conversion of aniline to intermediates of TCA-cycle have been reported, two were the plasmid-encoded *tdn* cluster (pTDN1) from *P. putita* UCC22 (Fukumori and Saint, 1997, 2001) and *atd* cluster from *Acinetobacter* sp. YAA (Fujii *et al*., 1997), and the other one was the chromosome-encoded *tad* cluster from *D. tsuruhatensis* AD9 (Liang *et al*., 2005), and clusters *tdn* and *tad* showed significant similarity in nucleotide sequence and genetic organization to each other despite of their different geographic origin (Liang *et al*., 2005). In both *tdn* and *tad* clusters, there were two genes for catechol 2,3-dioxygenase and two genes for ferredoxin-like protein, and this might be the molecular mechanism for both strains *P. putita* UCC22 and *D. tsuruhatensis* AD9 could also degrade methyl-substituted aniline in addition to aniline (Fukumori and Saint, 1997; Liang *et al*., 2005). *Delftia* sp. AN3 was a highly effective aniline-degrading strain and it could only degrade aniline (Liu *et al*., 2002). The molecular mechanism of aniline degradation and aniline degradative genes of strain AN3 might be different from those of strains *P. putita* UCC22 and *D. tsuruhatensis* AD9.

In this study, we described the cloning of a novel and complete gene cluster that converts aniline to TCA-cycle intermediates from *Delftia* sp. AN3 and the mechanism for aniline degradation by strain AN3 was also discussed in molecular level.

1 Materials and methods

1.1 Bacterial strains, plasmids, primers, and growth conditions

The bacterial strains, plasmids and primers used in this study are listed in Table 1. The culture media used were Luria-Bertani (LB) medium (Sambrook and Russell, 2001) and mineral medium (MMN) (Liu *et al*., 2002). *Delftia* sp. AN3, *E. coli* JM109 was grown at 30 and 37°C, respectively. Apramycin (Am) was used in selective media at final concentration of 133 mg/L. The growth of strains was measured by the turbidity at the wavelength of 600 nm.

1.2 Plasmid curing and pulsed-field gel electrophoresis (PFGE) detection

For curing the plasmid from *Delftia* sp. AN3, a modified sodium dodecyl sulfate treatment method of El-Mansi *et al*. (2000) was used. Detection of the mega plasmid in *Delftia* sp. AN3 was carried out according to Barton *et al*. (1995). Separation of chromosome and plasmid DNAs was carried out on an agarose gel (1%) under conditions of 6 V/cm and 70 s for 22 h using a Bio-Rad pulsed-field gel electrophoresis apparatus (PFGE, Bio-Rad, USA).

1.3 Construction of the genomic library of strain AN3

The genomic DNA of strain AN3 was obtained by the method of Sambrook and Russell (2001). The genomic DNA obtained was partially digested with *Sau*3AI or *Mbo*I and separated in a 0.6% (*W/V*) agarose gel. DNA fragments about 30 kb were recovered and purified, then ligated to pKC505 digested by *Hin*dIII and *Bam*HI and packaged by Gigapack III XL Packaging Extract Kit (Stratagene, USA), then introduced into component cells of *E. coli* JM109 by transduction.

1.4 Screening for the positive clone

Escherichia coli transformants carrying this library were cultured on LB plate supplemented with Am (133 mg/L) and screened by spraying catechol solution (10 mmol/L dissolved in phosphate buffer) onto the colonies on the LB plate for detection of catechol 2,3-dioxygenase (C23O) activity. Then the positive-candidate clones were further confirmed by C23O assay. Protein concentration was determined by the method of Bradford (1976) using Bio-Rad protein assay kit, with bovine serum albumin as a standard.

1.5 Assays of enzymatic activities

1.5.1 Aniline dioxygenase (AD)

Strain AN3 and positive clone (*E. coli* JM109-AN1) were grown on MMN (containing 300 mg/L of aniline for *E. coli* JM109-AN1) till to an OD_{600} about 0.1 and then cells were harvested by centrifugation at 5,000×*g*, washed twice with 20 mmol/L phosphate buffer (pH 7.0), and resuspended in the same buffer. This suspension was used to assay aniline dioxygenase. The activity of the enzyme was measured with an oxygen electrode (YSI, Ohio, USA), according to the method of Fukumori and Saint (1997). To estimate the endogenous respiratory rate, 0.3 ml of sterile distilled water instead of aniline solution was used in a parallel experiment.

1.5.2 Catechol 2,3-dioxygenase

Cells (the same cell suspension as used in the aniline dioxygenase assay) were disrupted by sonication using an ultrasonic disruptor (TOMY UD-200; power 6, 2 min, three times on ice). The cellular lysate were centrifuged at 19,000×*g* for 20 min, and the supernatant was used

Table 1 Strains, plasmids and primers used in this study

Strain or plasmid	Relevant characteristics	Reference
Delftia sp. AN3	Wild type that could degrade aniline	Liu *et al*., 2002
E. coli JM109	*recA*1*supE*44*endA*1*hsdR*17*gyrA*96*relA*1*Thi*Δ(*lac-proAB*)*F*' *traD*36*proAB*$^+$ *lacI*^{q}*lacZ*ΔM15	Sambrook and Russell, 2001
E. coli JM109-AN1	*E. coli* JM109 containing pKC505-AN1, growing on aniline as sole carbon, nitrogen and energy source	This study
pKC505	Shuttle vector, 18.7 kb, Am^r	Richardson *et al*., 1987
pKC505-AN1	Am^r, a 29.7-kb *Sau*3AI fragment from genomic DNA of *Delftia* sp. AN3 in pKC505	This study

for enzymatic assay. The reaction mixture (total 3.0 ml) contained 2.0 ml phosphate buffer, 0.6 ml of 1 mmol/L catechol, 0.2 ml deionized water and 0.2 ml cellular lysates. The reaction proceeded at 22°C. C23O was determined by measuring the production of 2-hydroxymuconic semialdehyde at 375 nm (Sala-Trepat and Evans, 1971) using a DU-7 spectrophotometer (Beckman, USA). The absorption coefficient of 2-hydroxymuconic semialdehyde was 12,000 $mol^{-1}cm^{-1}$.

1.6 Plasmid isolation

For isolation of recombined plasmids bearing C23O activity, pKC505-AN1 of the positive clone, a modified alkaline lysis method was used (Thomas *et al*., 1998).

1.7 DNA sequencing, sequence assembly and analysis

The recombinant plasmid pKC505-AN1 containing a DNA fragment about 30 kb from strain AN3 was sequenced with shotgun method by the Beijing Genome Institute (Huada Corp., Beijing, China). Contigs were assembled using the GCG Wisconsin package. The orfs were analyzed using Vector NTI and DNAman software (Lynnon Biosoft, USA). Sequence comparisons were made against the sequences in the GenBank using the BLASTx program (Altschul *et al*., 1990).

1.8 Construction of phylogenetic tree

Peptide sequences of various enzymes or subunits were extracted from NCBI (Altschul *et al*., 1990). Phylogenetic trees were generated using the neighbor joining method of Saitou and Nei (1987) with the AlignX software (Informax, Maryland), and multiple sequence alignment was done using ClustalX. (Thompson *et al*., 1997). The length of each branch pair represents the evolutionary distance between the sequences.

1.9 Aniline analysis

Aniline concentration was determined at 230 nm using HPLC (Waters, Millipore, USA) equipped with a C-18 reverse phase column (100 mm long) and a UV-detector. The elution was MeOH/H_2O (*V/V*)=75/25 and the elution rate was 1.5 ml/min. Under these conditions the retention time of aniline was about 3.25 min.

2 Results

2.1 Plasmid detection and curing experiment

Cells of strain AN3 for PFGE were grown on aniline (MMN) to ca. OD_{600}=0.6 and then harvested and washed twice with sucrose buffer (0.3 mol/L). The result (data not shown) showed that there was not any band except of the genomic DNA. Furthermore, this aniline-degrading ability of strain AN3 could not be cured.

2.2 Cloning of aniline degradation genes from strain AN3, and the growth of recombinant strain on aniline as sole carbon, nitrogen and energy sources

A genomic library of strain AN3 was constructed as described in Materials and methods. The transformants were spread out on LB plates containing Am (133 mg/L), and then catechol solution was sprayed onto the colonies formed on the plates. One colony turned to brilliant yellow firstly and then to brown after spraying of catechol solution, indicating that there was an accumulation of 2-hydroxymuconic semialdehyde, an intermediate in the aniline degradation pathway via *meta*-cleavage of catechol (Takeo *et al*., 1998). This recombinant strain could grow on aniline as sole carbon, nitrogen and energy source up to 0.1 of OD_{600} and degrade the added aniline completely (Fig.1). This recombinant strain and plasmid were designated as *E. coli* JM 109-AN1 and pKC505-AN1, respectively.

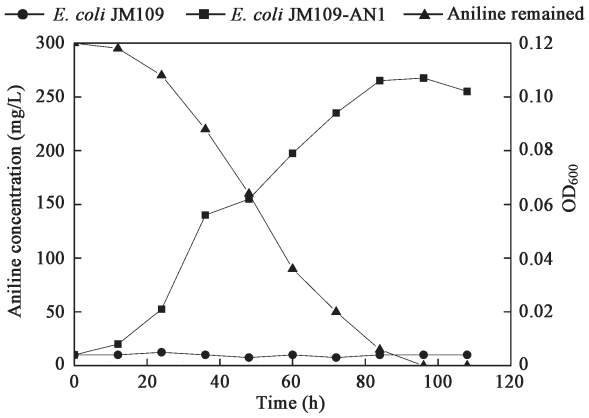

Fig. 1 Growth of recombinant strain *E. coli* JM109-AN1 in mineral medium (MMN, Liu *et al*., 2002) using aniline as sole carbon, nitrogen and energy source and the degradation of aniline. The initial aniline concentration was 300 mg/L. The cultivation was conducted out on a shaker at 180 r/min at 30°C.

2.3 Expression of AD and C23O of the recombinant strain *E. coli* JM 109-AN1 growing on aniline

The results in Table 2 show that AD and C23O expressed well in *E. coli* (recombinant strain *E. coli* JM 109-AN1). The activities of AD and C23O were as high as 0.31 U/mg wet cell and 1.92 U/mg protein, respectively, despite they were lower than those of original strain AN3 (1.09 U/mg wet cell and 5.22 U/mg protein for AD and C23O, respectively). The results (Table 2) also indicated that the AD and C23O of strain AN3 or *E. coli* JM109-AN1 were highly specific, only aniline and catechol could be the substrate for AD and C23O, respectively; not any other substituted anilines or catechols could be catalyzed.

2.4 Sequence analysis of the cloned fragment

The DNA fragment cloned in pKC505-AN1 was sequenced, and the nucleotide of the total 29.7 kb region was thereby determined. Sequence and orf analyses of this total 29.7 fragment indicated that there were at least 27 intact orfs (Fig.2a and Table 3). Homology searches for these orfs were performed to identify gene function. It was found that among these 27 intact orfs, at least 16 of which (*danQTA1A2BRDCEFG1HIJKG2*) were expected in corresponding to the complete metabolism of aniline to TCA-cycle intermediates via *meta*-cleavage pathway as shown in Fig.2b. As summarized in Table 3, the 6th–10th gene products *danQTA1A2B* and the *danR* product showed

Table 2 Activities of aniline dioxygenase (AD) and catechol 2,3-dioxygenase (C23O) of strain AN3 and recombinant strain *E. coli* JM109-AN1 growing on aniline

AD		C23O	
Substrate	Activity* (U/mg wet cell)	Substrate	Activity* (U/mg protein)
Aniline	1.09 (0.31)	Catechol	5.22 (1.92)
2-Chloroaniline	0 (0)	3-Chlorocatechol	0 (0)
3-Chloroaniline	0 (0)	4-Chlorocatechol	0 (0)
4-Chloroaniline	0 (0)	3-Methylcatechol	0 (0)
2,4-Dichloroaniline	0 (0)	4-Methylcatechol	0 (0)
2-Methylaniline	0 (0)		
3-Methylaniline	0 (0)		
4-Methylaniline	0 (0)		

* The activities in parentheses were those of enzymes expressed in recombinant strain *E. coli* JM109-AN1. One unit (U) of AD was defined as the amount consuming 1 mol O_2/min under the conditions described in Materials and methods. One unit (U) of C23O was defined as the amount of protein needed for oxidation of 1 μmol catechol/min at 22°C under the conditions described in Materials and methods.

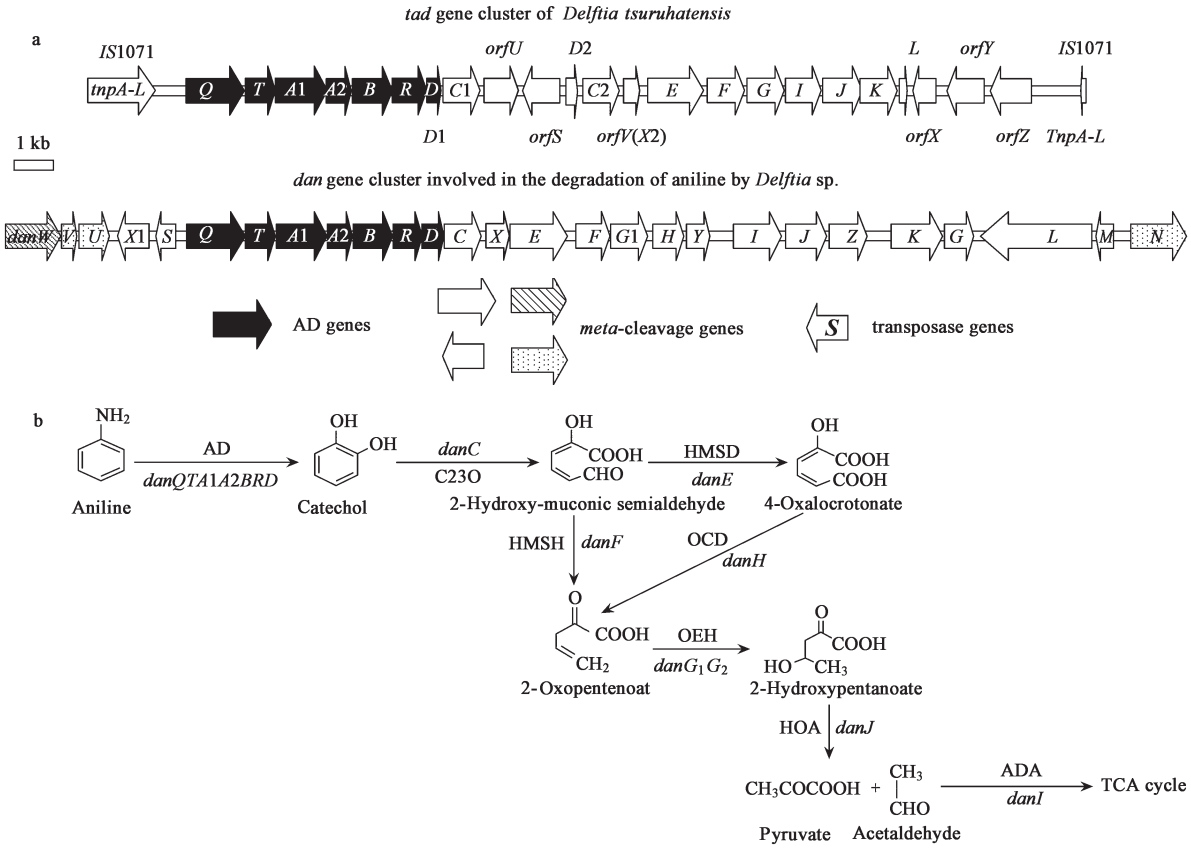

Fig. 2 Genetic organization of the aniline degradative *dan* gene cluster and the comparison of it with *tad* cluster (a) and putative aniline degradation pathway (b) of *Delftia* sp. AN3. The sequenced region of 29.7 kb is shown. The function of other genes was explained in Table 3. AD: aniline dioxygenase; C23O: catechol 2,3-dioxygenase; HMSD: 2-hydroxymuconic semialdehyde dehydrogenase; HMSH: 2-hydroxymuconic semialdehyde hydrolase; OCD: 4-oxalocrotonate decarboxylase; OEH: 2-oxopent-4-dienoate hydratase; HOA: 4-hydroxy-2-oxovalerate aldolase; ADA: acetaldehyde dehydrogenase.

significant homology (64%–100%) with multi-component aniline dioxygenases and Lys-type regulators, respectively, present in other aniline degrading strains, *D. acidovorans* 7N, *P. putida* UCC22, *Frateuria* sp. ANA-18 and *D. tsuruhatensis* AD9, especially with *D. acidovorans* 7N. The remaining 11 gene products (DanQDCEFGHI1I2JKLM) exhibited considerable amino acid identity (64%–100%) to enzymes of central catechol degrading enzymes via *meta*-cleavage pathway found in other aromatics degrading and aniline-degrading bacteria. There are also some other genes (*danWVU*) that encode proteins such as aromatic amino-transferases and transport trans-membrane proteins found in *Polaromonas naphthalenivorans* CJ2 (Copeland *et al*., unpublished, accession number ZP_01022585) and *Polaromonas* sp. JS666 (Lucas *et al*., unpublished, accession number ZP_00502922) etc., showed 72%–75% amino acid identity. Besides, on the two sides of the *dan* gene cluster are the transposases encoded by *danX*1*SN*, and *danO* potential TnpR recombinase. These gene products are believed to function in horizontal gene transfer among the strains. In addition, *orfX2YZ* were also found in the *meta*-cleavage of other aniline degraders, such as *D. tsuruhatensis* AD9 (Liang *et al*., 2005), and *Pseudomonas* sp. K82 (Kim *et al*., 2004), and a phenol phenol degrader, *Comamonas testosteroni* TA441 (Arai *et al*., 1998) in addition to *P. putida* UCC22.

Table 3 Analytical data on the aniline degradation genes and other genes of *Delftia* sp. AN3*

ORF	Gene	Position (No. of nt)	Mw/No. of aa[b]	Putative function	Homologous protein (sequence identity)	Source	Accession No.
1	*danW*	1–1392 (1392)	51 kDa/464 aa	Aromatic Amino-transferase	Aminotran 1, 2 (299/398, 75%)	*Polaromonas* sp. JS666	YP_549701
					Aminotran 1, 2 (296/397, 74%)	*P. naphthalenivorans* CJ2	ZP_01022585
2	*danV*	1408–1731 (324)	12 kDa/108 aa	4-Hydroxybenzoyl-CoA thioesterase	PnaP 1037 (78/115, 67%)	*P. naphthalenivorans* CJ2	ZP_01022584
					4HBT (80/115, 69%)	*Polaromonas* sp. JS666	ZP_00502921
3	*danU*	1734–2522 (789)	29 kDa/263 aa	Transmembrane protein	MotA ExbB (173/232, 74%)	*Polaromonas* sp. JS666	ZP_00502922
					MotA ExbB (169/232, 72%)	*P. naphthalenivorans* CJ2	ZP_01022583
4	*danX1*	2672–3490 (819)	30 kDa/273 aa	Transposase	Transposase (116/116, 100%)	*D. acidovorans*	AAO38205
					Transposase 25 (36/92, 39%)	*P. naphthalenivorans* CJ2	ZP_01023487
5	*danS*	3795–4304 (510)	18 kDa/170 aa	Transposase	Transposase 34 (22/43, 51%)	*Burkholderia fungorum* LB400	ZP_00280163
					Transposase 34 (22/43, 51%)	*Ralstonia metalli-durans* CH34	ZP_00598173
6	*danQ*	4762–6264 (1503)	56 kDa/501 aa	Glutamine synthetase	ORF7NA (493/493, 100%)	*D. acidovorans* 7N	BAD61047
					TdnQ (487/493, 98%)	*D. acidovorans*	AAO38206
7	*danT*	6287–7054 (768)	27 kDa/256 aa	Glutamine amidotransferase	ORF7NB (256/256, 100%)	*D. acidovorans* 7N	BAD61048
					TadT (155/240, 64%)	*D. tsuruhatensis* AD9	AAX47240
8	*danA1*	7167–8444 (1278)	48 kDa/426 aa	Large subunit of dioxygenase	TdnA1 (425/426, 99%)	*D. acidovorans*	AAO38208
					ORF7NC (424/426, 99%)	*D. acidovorans* 7N	BAD61049
9	*danA2*	8447–9085 (639)	24 kDa/213 aa	Small subunit of dioxygenase	ORF7ND (199/199, 100%)	*D. acidovorans* 7N	BAD61050
					TadA2 (142/200, 71%)	*D. tsuruhatensis* AD9	AAX47242
10	*danB*	9105–10109 (1005)	36 kDa/335 aa	Aniline dioxygenase reductase	ORF7NE (335/335, 100%)	*D. acidovorans* 7N	BAD61051
					TadB (251/335, 74%)	*D. tsuruhatensis* AD9	AAX47243
11	*danR*	10160–10917 (758)	28 kDa/252 aa	LysR-type regu-latory gene	ORF7NF (252/252, 100%)	*D. acidovorans* 7N	BAD61052
					TadR (190/252, 75%)	*D. tsuruhatensis* AD9	AAX47244
12	*danD*	11062–11469 (408)	14 kDa/136 aa	Small ferredoxin-like protein	ORF7NG (129/129, 100%)	*D. acidovorans* 7N	BAD61053
					CbzT (69/111, 62%)	*P. putida* UCC22	AAX38580
13	*danC*	11470–12411 (942)	35 kDa/314 aa	Catechol 2,3-dioxygenase	ORF7NH (314/314, 100%)	*D. acidovorans* 7N	BAD61054
					TdnC (269/314, 85%)	*P. putida* UCC22	CAA42452
14	*orfX2*	12559–13013 (455)	15 kDa/151 aa	Unknown product	ORF4 (83/112, 74%)	*P. putida* UCC22	BAB62051
					ORFV (83/112, 74%)	*D. tsuruhatensis* AD9	AAX47251
15	*danE*	13016–14467 (1452)	51 kDa/484 aa	2-HMSD	HMSD (426/484, 88%)	*Ralstonia eutropha* JMP134	AAZ65034
					TdnE (354/485, 72%)	*P. putida* UCC22	BAB62052
16	*danF*	14740–15603 (864)	31 kDa/288 aa	2-HMSH	DmpD (202/283, 71%)	*Pseudomonas putida*	CAA36993
					TadF (190/277, 68%)	*D. tsuruhatensis* AD9	AAX47253
17	*danG1*	15471–16412 (942)	34 kDa/314 aa	2-Keto-4-pentenoate hydratase	CnbE (261/261, 100%)	*Comamonas* sp. CNB-1	ABB13580
					ORF (226/260, 86%)	*Rubrivivax gelati-nosus* PM1	ZP_00242167

Continued

18	*danH*	16435–17220 (786)	28kDa/262 aa	4-Oxalocrotonate decarboxylase	4-OD (216/262, 82%)	*Rubrivivax gelatinosus* PM1	ZP_00242168
					CnbF (227/262, 79%)	*Comamonas* sp. CNB-1	ABB13581
19	*orfY*	17249–17848 (600)	26kDa/200 aa	Unknown product	ORF3 (135/193, 69%)	*Pseudomonas putida* UCC22	BAB62048
					ORFDR (56/139, 40%)	*Deinococcus radiodurans* R1	AAF11685
20	*danI*	18366–19595 (1230)	44kDa/410 aa	Acetaldehyde dehydrogenase	TdnI (270/300, 90%)	*P. putida* UCC22	BAB62056
					TadI (269/300, 89%)	*D. tsuruhatensis* AD9	AAX47255
21	*danJ*	19612–20649 (1038)	37kDa/346 aa	4-Hydroxy-2-oxovalerate aldolase	TadJ (291/327, 88%)	*D. tsuruhatensis* AD9	AAX47256
					TdnJ (291/327, 88%)	*P. putida* UCC22	BAB62057
22	*orfZ*	20665–21639 (975)	34kDa/325 aa	Putative exported protein	ORFJ (249/326, 76%)	*C. testosteroni* TA441	BAA88506
					CdoL (189/249, 75%)	*Comamonas* sp. JS765	AAG17139
23	*danK*	22252–23571 (1320)	46kDa/440 aa	2-Amino-5-chloromuconate deaminase	CnbH (427/427, 100%)	*Comamonas* sp. CNB-1	ABB13583
24	*danG2*	23617–24129 (513)	18kDa/171 aa	2-Keto-4-pentenoate hydratase	CnbI (171/171, 100%)	*Comamonas* sp. CNB-1	ABB13584
					TdnG (164/165, 99%)	*Pseudomonas* sp. CT14	ABA25976
25	*danL*	24410–27376 (2967)	10kDa/989 aa	Tn3 family transposase	TnpA (947/989, 95%)	*D. acidovorans* MC1	AAP88287
					TnpA (945/989, 95%)	*Ralstonia metallidurans* CH34	CAI30244
26	*danM*	27382–27851 (471)	17kDa/157 aa	TnpR recombinase	TnpR (149/172, 86%)	*Ralstonia metallidurans* CH34	NP 943467
					TnpR (147/152, 96%)	*N. europaea* ATCC 19718	CAI30245
27	*danN*	28352–29827 (1476)	53kDa/492 aa	Sulfate transporter	STAS (482/492, 97%)	*R. metallidurans* CH34	ZP_00595727
					STAS (405/491, 82%)	γ-*Proteobacterium* KT71	ZP_01101356

* The accession number for the nucleotide sequence of the *dan* cluster in GenBank is DQ661649. The amino acid sequences of the products of the genes are available kDa in GenBank under the given accession numbers. No. of nt: The number of the nucleotide position in *dan* cluster; Mw: molecular weight; No. of aa: number of amino acid.

2.5 Comparison of the *dan* gene cluster with *tad* gene cluster

As shown in Table 3, the putative products of the *dan* genes showed identity to those of *tad* genes of *D. tsuruhatensis* AD9, which was a chromosome-encoded aniline degradative gene cluster. Hence, the gene organization of the *dan* gene cluster was compared with that of the *tad* gene cluster (Fig.2a). It was found that the gene organization of both gene clusters is similar, especially the aniline dioxygenase genes share the same organization and showed significantly identify in their products. However, there are some differences between them. Firstly, in *dan* gene cluster all of the genes corresponding for the conversion of aniline to TCA intermediates were in the same transcriptional direction. Secondly, there was only one set of C23O gene (*danC*) and ferredoxin gene (*danD*) in *dan* gene cluster, while there were two sets of C23O gene (*tadC1C2*) and ferredoxin genes (*tadD1D2*) in *tad* gene cluster; there were two 2-oxopent-4-dienoate hydratase genes (*danG1G2*) in *dan* gene cluster but there was only one (*tadG*) in *tad* gene cluster. Thirdly, the *dan* gene cluster lacks the genes corresponding to *orfU*, *orfS*, *tadD2* and *tadC2* between *tadC1* and *orfV* of the *tad* cluster, and the genes corresponding to *tadL* (4-oxalocootonate tautomerase) and *orfX* (MarR-type regulator) of *tad* gene cluster. Fourthly, in contrast to *tad* gene cluster, in *dan* gene cluster there are two additional genes, *danY* and *danZ*, and their products showed considerable similarity to ORF3, a putative membrane protein, of *tdn* gene cluster of *Pseudomonas putida* UCC22 and to ORFJ, a putative exported protein, of *aph*, a phenol degradation gene cluster of *Comamonas testosteroni* TA441 (Arai *et al*., 2000), respectively.

2.6 Phylogenetic relationships of the AD, C23O, and other proteins of strain AN3 with other homologous proteins

To clarify the phylogenetic relationship of the AD of strain AN3 with those of other aniline-degrading bacteria, phylogenetic trees were constructed using the amino acid sequences of the glutamine synthetase-like proteins, the large subunit of the dioxygenase component and aniline dioxygenase reductase in the AD enzyme systems. As shown in Figs.3a, 3b and 3c, the phylogenetic trees consisted of two major branches, the Tdn-branch and

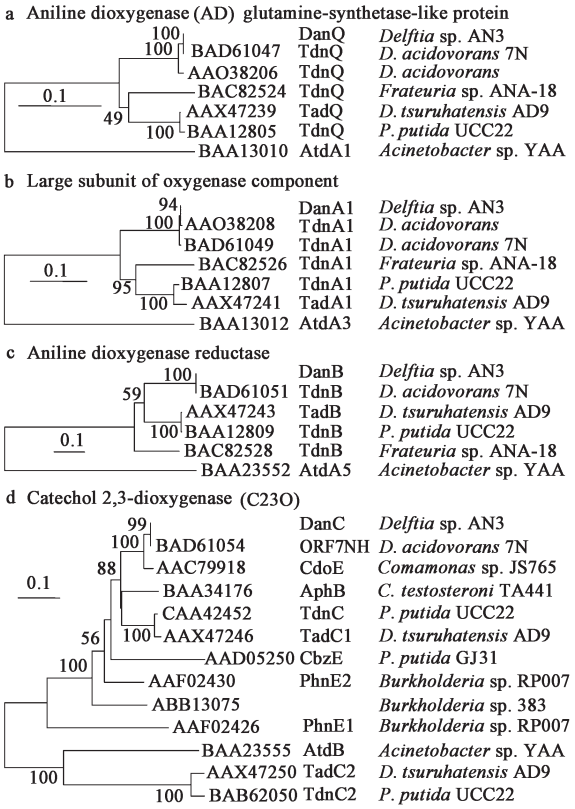

Fig. 3 Phylogenetic trees constructed based on the amino acid sequences of AD glutamine-synthetase (a), large subunit of the dioxygenase component (b), aniline dioxygenase reductase (c) and C23O (d). Sequence accession number and strain names are shown. The numbers at node indicate the bootstrap percentages of 1000 resamples.

the Atd-branch. In these trees, DanQ, DanA1 and DanB are located closest to TdnQ, TdnA1 and TdnB of *D. acidovorans* 7N, respectively.

To locate the *meta*-cleavage pathway of strain AN3 among those of other aromatic-compound-degrading bacteria, another phylogenetic tree was constructed using C23O amino acid sequence (Fig.3d). The *meta*-cleavage pathway of strain AN3 contains only one C23O, DanC. In the tree, DanC belongs to a branch including ORF7NH, TdnC and TadC1 from other aniline-degrading bacteria, *D. acidovorans* 7N, *P. putida* UCC22 and *D. tsuruhatensis* AD9, respectively. Fig.3d illustrates that DanC is closely related to TadC1 and TdnC, but not to TadC2 and TdnC2.

3 Discussion

Delftia sp. AN3 was isolated as an aniline-degrader from activated sludge from a municipal wastewater treatment plant, and it could only assimilate aniline (Liu *et al*., 2002). Up to date, the mechanism of several aniline-degraders has been illustrated in molecular level, including *D. tsuruhatensis* AD9 (Liang *et al*., 2005), *P. putida* UCC22 (Fukumori and Saint, 1997) and *Acinetobacter* sp. YAA (Fujii *et al*., 1997), due to the cloning and study of aniline-degradative gene clusters. Both plasmid-encoded and chromosome-encoded gene clusters involving the complete metabolism of aniline to TCA-cycle intermediates have been cloned from *D. tsuruhatensis* AD9 (*tad* cluster, Liang *et al*., 2005), *P. putida* UCC22 (*tdn* cluster, Fukumori and Saint, 1997) and *Acinetobacter* sp. YAA (*atd* cluster, Fujii *et al*., 1997), and *tad* cluster was chromosome-encoded and the other two were plasmid-encoded. Although *tad* cluster and *tdn* cluster were differently encoded, they showed similar gene organization and striking identity of their products, and were hypothesized to be evolved from one ancestor (Liang *et al*., 2005). In this study, we cloned a novel aniline degradation gene cluster (*dan* cluster) from *Delftia* sp. AN3, which is responsible for the complete metabolism of aniline to TCA-cycle intermediates via a *meta*-cleavage pathway. Both the negative results of the PFGE and plasmid curing experiments suggested that *dan* cluster might be chromosome-encoded. The cloning of *dan* cluster into *E. coli* enabled it to grow on aniline as sole carbon, nitrogen and energy sources, and to degrade the added aniline completely (Fig.1). This result suggested that the *dan* cluster contained all the genes needed for conversion of aniline to TCA-cycle intermediates. This was further confirmed by the analysis of the sequence and *orf*s (Table 3). Furthermore, the enzymatic assays indicated that the AD and C23O did express well in the recombinant strain, *E. coli* JM109-AN1 (Table 2).

Despite they were from very close relatives and shared significant identity of most gene products, *dan* cluster differentiated to *tad* cluster in several aspects including gene organization, gene components, and their transcriptional direction (Fig.2a and Table 3). In addition, the phylogenetic analysis revealed that the components of AD systems of *dan* cluster and *tad* cluster belonged to the two different branches (Figs.3a, 3b, and 3c). These differences might infer that they have evolved from different ancestors. In *dan* cluster of strain AN3, there was only one set of C23O gene (*dan*C), while there were two sets of C23O gene in *tad* cluster (*tad*C1 and *tad*C2) of *D. tsuruhatensis* AD9 and *tdn* cluster of *P. putida* UCC22 (*tdn*C and *tdn*C2). The phylogenetic analysis of DanC (Fig.3d) illustrates that it is closely related to TadC1 and TdnC, but not to TadC2 and TdnC2. Fukumori and Saint (2001) reported that TdnC and TdnC2 have distinct substrate specificity: TdnC showed relatively high activity on substituted catechols (3-methylcatechol and 4-methylcatechol), while TdnC2 showed less activity on these substituted catechols. Liang *et al*. (2005) reported that TadC1 and TadC2 have the similar substrate specificity to that of TdnC and TdnC2. Both strains UCC22 and AD9 can assimilate *m*-toluidine (3-methylaniline) and *p*-toluidine (4-methylaniline) in addition to aniline, and the authors assumed that it might be necessary for cells to acquire another C23O for these methylcatechols to expand the assimilation range for toluidines. But DanC has no C23O activity on those chlorocatechols and methylcatechols (Table 2) despite it is closely phylogenetic related to TdnC and TadC1. The presence of only one set of C23O gene and the specificity of AD and C23O (Table 2) of strain AN3 might be the reason for it not to assimilate any substituted anilines (Liu *et al*., 2002).

In conclusion, all of the above differences between *dan* cluster and other aniline-degradative clusters, especially

the gene organization, presence only one set of C23O gene or absence of some genes and specificity of AD and C230, indicated that *dan* cluster was a new aniline-degradative cluster, despite of they were quite similar and shared high identity of the gene products.

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