Plasmid-encoded genes specifying aniline oxidation from *Acinetobacter* sp. strain YAA

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***Acinetobacter* sp. strain YAA is able to use aniline and o-toluidine as the sole carbon and energy source. This strain has several different plasmids and acridine orange curing suggested that aniline utilization in strain YAA was plasmid-encoded. The gene cluster involved in aniline oxidation was cloned in *Escherichia coli* JM109 from the total plasmid DNA of strain YAA. A recombinant *E. coli* containing an 18.5 kb insert fragment showed yellow colouration on aniline-containing plates, indicating the formation of 2-hydroxymuconic semialdehyde from aniline. In addition, subcloning of a 9.0 kb *SalI* fragment from the insert in *E. coli* resulted in the accumulation of catechol. Southern hybridization studies indicated that the aniline oxygenase gene (*atdA*) was present on one of the plasmids, pYA1. These results suggest that in strain YAA aniline is degraded via catechol through a pathway involving meta-cleavage of the benzene-ring by plasmid-encoded genes including *atdA*.**

Keywords: *Acinetobacter*, aniline oxidation, biodegradation

INTRODUCTION

Aniline and its derivatives have been widely used as materials for the synthesis of chemical products such as herbicides and dyes, and released into the environment during their use. Since they are known to be toxic and carcinogenic to living organisms (Lyons *et al.*, 1985), many researchers have paid attention to their fate in the environment (Lyons *et al.*, 1984; Amador & Alexander, 1991). The major route for the breakdown of anilines has been found to be by biodegradation (Lyons *et al.*, 1984).

Several authors have studied bacteria that are able to degrade aniline, toluidines and chloroanilines, and some catabolic pathways for the degradation of anilines by bacteria have been elucidated. Firstly, anilines are converted to the corresponding catechols via one or more oxidative steps including deamination. The resultant catechols are degraded through either the *ortho*- (Aoki *et al.*, 1983; Zeyer *et al.*, 1985; Loidl *et al.*, 1990) or the *meta*-cleavage pathway (McClure & Venables, 1986; Konopka *et al.*, 1989; Fuchs *et al.*, 1991). Like other aromatic compound degradative plasmids, TOL, NAH, SAL and so on, plasmids encoding the ability to

utilize aniline have been recognized. Plasmid pCIT1 carried in *Pseudomonas* sp. strain CIT1 is about 100 kb in size and contains the aniline oxidase and catechol 2,3-dioxygenase (C23O) genes on a 42 kb *Sau3A* fragment (Anson & Mackinnon, 1984; Meyers, 1992). *Pseudomonas putida* UCC2 bears pTDN1 which contains the catabolic genes for aniline, toluidines and *m*-toluate within a 26 kb region bounded by direct-repeat sequences (Saint *et al.*, 1990). Recently, a 9.3 kb fragment of pTDN1 has been sequenced and found to encode several ORFs (Fukumori & Saint, 1995). These ORFs were predicted to encode large and small subunits of terminal dioxygenase, an electron-transfer protein, a LysR-type regulatory protein and a glutamine synthetase-like protein. However, the detail is still unclear, because the sequence data have not been published.

In this paper, we report the cloning of the genes that convert aniline and *o*-toluidine to the corresponding catechols from plasmid DNA of *Acinetobacter* sp. strain YAA. The expression of these genes in *Escherichia coli* was confirmed by identification of the metabolites from aniline or *o*-toluidine by GC-MS.

METHODS

Media and culture conditions. A mineral salts medium (MSA) consisting of 1.73 g K₂HPO₄, 0.68 g KH₂PO₄, 1.0 g NH₄NO₃, 0.1 g MgSO₄·7H₂O, 0.03 g FeSO₄·7H₂O, 0.03 g FeCl₃,

Abbreviation: C23O, catechol 2,3-dioxygenase.

The GenBank/EMBL/DBJ accession number for the partial 7.4 kb sequence reported in this paper is D86080.

Table 1. Bacterial strains and plasmids

Strain/plasmid	Relevant characteristic
Bacterial strains	
<i>Acinetobacter</i> sp. strain YAA	Ani ⁺
<i>Acinetobacter</i> sp. strain YAA-113	Derivative of strain YAA, Ani ⁻
<i>E. coli</i> JM109	<i>thi hsdR</i>
Plasmids	
pYA1	Native plasmid encoding aniline-degrading pathway of <i>Acinetobacter</i> sp. strain YAA
pUC19	Ap ^r
pAS185	18.5 kb <i>SalI</i> fragment of pYA1 in pUC19
pAS90	9 kb <i>SalI</i> fragment of pAS185 in pUC19
pAS91	9 kb <i>SalI</i> fragment of pAS185 in opposite orientation to pAS90
pAS92	7.8 kb <i>EcoRI</i> - <i>SalI</i> fragment of pAS90 in pUC19
pAS93	7.6 kb <i>SalI</i> - <i>EcoRV</i> fragment of pAS91 in pUC19
pAS94	4 kb <i>SalI</i> - <i>PstI</i> fragment of pAS90 in pUC19
pAS60	6 kb <i>SalI</i> fragment of pAS185 in pUC19
pAS61	6 kb <i>SalI</i> fragment of pAS185 in opposite orientation to pAS60
pAS50	5 kb <i>Bam</i> HI fragment of pAS90 in pUC19
pAS51	5 kb <i>Bam</i> HI fragment of pAS90 in pUC19 in opposite orientation to pAS50
pAS16	1.5 kb <i>PstI</i> - <i>SmaI</i> fragment of pAS60 in pUC19
pAS17	0.7 kb <i>PstI</i> - <i>EcoRV</i> fragment of pAS16 in pUC19
pAS18	1.2 kb <i>Hind</i> III- <i>SmaI</i> fragment of pAS16 in pUC19

0.02 g CaCl₂·2H₂O, 0.03 g MnSO₄·H₂O and 0.3 g aniline as the sole source of carbon in 1 l distilled water, pH 7.0, was used for screening and cultivation of aniline-assimilating bacteria. MSS medium supplemented with 1.0 g disodium succinate l⁻¹, instead of aniline, was also used as a mineral salts medium. LB medium containing 10 g Bacto-peptone (Difco), 5 g Bacto-yeast extract (Difco), 10 g NaCl in 1 l distilled water, pH 7.0, was used as a rich medium. These media were solidified with 1.5% agar for plate growth. For propagation and maintenance of vector-based plasmids in the host, ampicillin was added at 50 µg ml⁻¹ to the media described above.

Isolation and identification of aniline-assimilating bacteria.

Aniline-assimilating bacteria were isolated from the activated sludge of Wakagawa final waste-water treatment facility (Wakayama, Japan), which treats waste-waters from chemical plants and textile-dyeing factories. A 300 ml Erlenmeyer flask containing 100 ml MSA medium and the activated sludge (0.5%, w/v) was shaken at 120 r.p.m. and 30 °C on a rotary shaker for 1 week. The culture was spread onto MSA agar plates and incubated at 30 °C. The bacteria able to form a colony on the plates were regarded as aniline-assimilating bacteria. The isolate was characterized by taxonomic studies based on Hasegawa (1985), and identified according to the criteria of *Bergey's Manual of Systematic Bacteriology* (Juni, 1984). The partial sequences of 16S ribosomal RNA were used for the identification as described by Rochelle *et al.* (1995). For preliminary identification at species level, the multiple-test system API 20 NE (bioMérieux-Vitek) was also employed.

Bacterial strains and plasmids. The bacterial strains and plasmids used and constructed in this study are listed in Table 1.

Curing experiment. Acridine orange was added at a final concentration of 300 µg ml⁻¹ to LB medium inoculated with about 10⁸ exponential-phase cells per ml. After 24 h incubation

at 30 °C, the culture was diluted with saline and plated out onto LB agar plates. The colonies appearing on the plates were replicated onto MSA plates to examine aniline-assimilation ability.

Methods for DNA extraction, analysis and cloning. Plasmid DNA was isolated by the alkaline extraction procedure (Birnboim & Doly, 1979), followed by CsCl/ethidium bromide equilibrium ultracentrifugation (100 000 g, 20 °C, 16 h) for purification. Restriction endonuclease digestion and DNA ligation were done according to the instructions of the enzyme suppliers (Takara Shuzo and TOYOBO). Transformation was carried out according to the methods of Sambrook *et al.* (1989).

Southern hybridization was performed according to established procedures (Southern, 1975). *EcoRI*-digested plasmid DNAs were separated by electrophoresis through 0.7% agarose gel and Southern-blotted onto Hybond N+ nylon membranes (Amersham). DNA fragments were fluorescein-labelled by using a Fluorescein Gene Images random prime module (Amersham) to prepare probes. Hybridization, stringency washes and signal detection were conducted with a Fluorescein Gene Images dioxetane detection module (Amersham) according to the manufacturer's instructions.

Oxygen uptake. Fresh cells, pre-grown in 40 ml LB medium, were harvested by centrifugation at 8000 g and 4 °C for 10 min and washed with 10 mM potassium phosphate buffer (pH 7.0). Cells were suspended in 100 ml MSA medium to obtain aniline-induced cells or MSS medium to obtain aniline-non-induced (succinate-grown) cells, and incubated at 30 °C with rotary shaking for 24 h. The cells were harvested, washed twice with phosphate buffer and oxygen uptake was measured polarographically at 30 °C using a Clark-type oxygen electrode (YSI 5750, Yellow Springs Instruments). Reaction mixtures contained 100 mg aniline l⁻¹ and washed cells (2.0–3.0 g dry wt l⁻¹) in phosphate buffer. The rate of oxygen

uptake was monitored, and the values obtained were corrected for endogenous respiration.

Preparation of cell extracts. Fresh cells, prepared in the same way as for oxygen uptake measurement, were suspended in phosphate buffer and disrupted sonically by three 2 min bursts using an ultrasonic disruptor UD-200 (TOMY). Cellular debris was removed by centrifugation at 15 000 g and 0 °C for 10 min. Acetone was added at 10% (v/v) to the clear supernatant and the mixture was used immediately for enzyme assays.

Measurement of C23O activity. C23O activity was measured spectrophotometrically by the increase in A_{375} concomitant with the formation of 2-hydroxybenzoic semialdehyde. Reaction mixtures contained 0.1 mM catechol and cell extract in phosphate buffer (pH 7.0) at 24 °C and the amount of 2-hydroxybenzoic semialdehyde formed was estimated as described by Nakazawa & Yokota (1973). Specific activity is expressed as $\mu\text{mol product formed min}^{-1} (\text{mg protein})^{-1}$. The quantity of protein in the cell extract was determined by the method of Lowry using BSA as the standard.

On agar plates, *E. coli* clones expressing C23O activity were detected by the catechol spray test using 100 mM catechol in phosphate buffer (pH 7.0).

Extraction and identification of metabolites from bacterial cultures. *E. coli* cells harbouring recombinant plasmids were incubated with 300 mg l^{-1} aniline or *o*-toluidine in 100 ml mineral salts medium at 30 °C overnight with rotary shaking. Cells were removed by centrifugation at 12 000 g and 4 °C for 10 min. The supernatant was adjusted to pH 2 with phosphoric acid and extracted with equal volumes of ethyl acetate. Extracts were dried over sodium sulfate and ethyl acetate was removed by evaporation under vacuum using a rotary evaporator. Residues were taken up in small volumes of acetone. After separation by preparative TLC using 20% (w/v) potassium chloride as the solvent system, metabolites were scraped from the silica gel plate, extracted with ethyl acetate, and stored at -20 °C prior to analysis.

The metabolites were identified by GC-MS analysis using a Hewlett Packard model 5890 II gas chromatograph fitted with a 5% diphenyl and 95% dimethylpolysiloxane capillary column HP5 (30 m $\times$ 0.32 mm) and JMS Automass model 150 mass spectrograph (JEOL) at an ionization potential of 70 eV. Helium was used as the carrier gas. The oven temperature was raised continuously from 50 to 200 °C at a rate of 20 °C min^{-1} .

RESULTS AND DISCUSSION

Isolation and identification of aniline-assimilating bacteria

During 7 d enrichment batch cultivation, the colour of the culture gradually turned yellow, which probably indicated the formation of an intermediate, 2-hydroxybenzoic semialdehyde, from aniline. Several aniline-assimilating bacteria were isolated from the culture by repeated subculture on MSA agar plates. An aniline-degradation test was performed, using six fast-growing bacteria (named YAA, YAB, YAD, YAI, YAJ and YAK, respectively), on MSA agar plates. Aniline concentration in the culture was determined using the diazo-coupling method (Snell *et al.*, 1954). As shown in Fig. 1, when the cells pre-grown on LB medium containing aniline were suspended at $\text{OD}_{600} = 1.0$ in MSA

Fig. 1. Aniline-degradation test by isolated strains. MSA was inoculated with an exponentially growing culture of each isolate, and incubated with rotary shaking at 30 °C. The initial cell concentration was 1.0 at OD_{600} . Aniline concentration was determined as described in Methods. Strains: ○, YAA; △, YAB; □, YAD; ●, YAI; ▲, YAJ; ■, YAK.

medium, these strains degraded 300 mg aniline l^{-1} within 6 h without a lag period. All the cultures turned yellow with the progress of aniline degradation as was observed in the enrichment culture. Therefore, it was presumed that aniline was metabolized via catechol through the pathway involving *meta*-cleavage of the benzene-ring.

One of the isolates, designated strain YAA, was Gram-negative, short rod shaped, oxidase-negative, catalase-positive, non-motile and showed no growth in anaerobic conditions. Acetoin, indole and H_2S were not produced. These morphological and biochemical properties are the characteristic criteria for the *Acinetobacter* genus in *Bergey's Manual of Systematic Bacteriology*. The API 20 NE identification system, which is based on 21 phenetic characters, also classified strain YAA as *Acinetobacter junii* with a 91.7% probability. The partial 16S ribosomal RNA sequences of this strain (nt 28–449 and nt 669–983 in the *E. coli* numbering system, Brosius *et al.*, 1978), the former of which contains variable regions useful for the identification of eubacteria (Rochelle *et al.*, 1995) and proteobacteria (Woese, 1987), were compared to other sequences in DNA databases such as GenBank, EMBL and DDBJ. The sequences determined showed strong homology to those of *Acinetobacter* strains.

Strain YAA is able to utilize aniline and *o*-toluidine as the sole source of carbon, but not *m*-toluidine, *p*-toluidine, chloroanilines, aminophenols or diamino-benzenes. Additionally, strain YAA is able to grow on phenol, benzoate, salicylate and naphthalene as the sole source of carbon. However, we could detect no C23O activity except during aniline or *o*-toluidine metabolism (data not shown).

Loss of aniline-assimilation activity

A culture of strain YAA treated with acridine orange was spread onto LB plates. After incubation at 30 °C for 24 h, 400 of the colonies appearing on the plates were picked out randomly and tested for growth on MSA medium. Thirty-six percent of the colonies could not grow on aniline. This high frequency of loss of aniline assimilation ability induced by acridine orange mutagenesis was observed, but no mutant strain was obtained which lost the ability to grow on benzoate or phenol. One of the mutant strains, designated YAA-113, seemed identical to the wild-type in all respects except for the loss of aniline assimilation ability.

To examine aniline oxidation activity in strains YAA and YAA-113, oxygen uptake measurement of the resting cells of both strains was carried out. The aniline- and *o*-toluidine-grown YAA cells showed apparent aniline oxidation activity [aniline-grown cells, 36.6 mg O₂ (g dry wt)⁻¹ h⁻¹; *o*-toluidine-grown cells, 70.9 mg O₂ (g dry wt)⁻¹ h⁻¹], whereas the aniline- and *o*-toluidine-grown YAA-113 cells showed no activity (below endogenous level). The endogenous respiration subtracted was about 20 mg O₂ (g dry wt)⁻¹ h⁻¹. In addition, the aniline-grown YAA cells showed strong C23O activity [14.1 U (mg protein)⁻¹], while the aniline-grown YAA-113 cells showed no C23O activity [$<10^{-3}$ U (mg protein)⁻¹]. These results demonstrated that the acridine orange treatment caused the concomitant loss of aniline oxidation activity and C23O activity. As the succinate-grown YAA cells showed little aniline oxidation activity [<6.0 mg O₂ (g dry wt)⁻¹ h⁻¹], aniline oxygenase appears to be induced in strain YAA.

Comparison of plasmids in strains YAA and YAA-113

Fig. 2 shows the results of agarose gel electrophoresis analysis of the plasmid DNA from strains YAA and YAA-113. As shown in Fig. 2(a), there is a difference in the plasmid profiles between both strains, although both strains have several similar plasmids. Strain YAA-113 seems to have lost some plasmid bands of a high molecular size (>20 kb), which are present in the YAA plasmid DNA. *Eco*RI digestion of both plasmid DNAs clarified the situation of the lost plasmid DNA (Fig. 2b; lanes 1 and 2). The results described above suggested that the aniline-degradation pathway in strain YAA was plasmid-encoded. We designated the putative plasmid encoding the aniline-degrading pathway as pYA1. If strain YAA-113 had lost only one plasmid, it could be deduced to be more than 50 kb in size, as estimated from the sum of the lost fragment sizes in this curing experiment.

To further characterize the plasmids in strain YAA, we recovered each plasmid band from agarose gel, and analysed the recovered plasmid DNA by *Eco*RI-digestion. The results confirmed that there were four different plasmids, sizes 3.9, 10.9, 14.8 and 16.0 kb, with some faint bands being the different conformational

Fig. 2. Agarose gel electrophoresis of plasmid DNA (a) and *Eco*RI-digested plasmid DNA (b) from strains YAA and YAA-113. Lanes: λ , *Hind*III-digested λ DNA; 1, YAA plasmid DNA; 2, YAA-113 plasmid DNA. Arrowheads indicate differences in the plasmid profile.

forms (probably open circular form) of these plasmids (data not shown). Unfortunately, we were unable to obtain large plasmids (>20 kb), including pYA1, because of the small amounts of DNA and the difficulty in their recovery. Moreover, the four plasmids described above interfered with the recovery, restriction analysis, and purification by ultracentrifugation of pYA1, owing to their abundant DNA content. Therefore, we decided to clone the aniline-oxidative genes from the total plasmid DNA of strain YAA.

Cloning of the genes specifying aniline oxidation

Plasmid DNA extracted from strain YAA was digested with *Sal*I, and ligated onto *Sal*I-digested pUC19. The resultant library was transformed into *E. coli* JM109. One of the transformants developed a yellowish colour on the selection plate containing aniline, but not on the plate without aniline. The colouration was considered to be due to the formation of 2-hydroxymuconic semialdehyde, as is known to occur in clones containing other aromatic compound degradative genes (Takeo *et al.*, 1995). Plasmid DNA was extracted from the clone, and digested by *Sal*I. The recombinant plasmid, designated pAS185, had an 18.5 kb *Sal*I insert fragment.

Restriction endonuclease analysis of pAS185

The recombinant plasmid pAS185 was digested with various restriction endonucleases, and a restriction map of the *Sal*I insert fragment was constructed (Fig. 3). The 18.5 kb insert consisted of three different *Sal*I fragments (3.0, 9.0 and 6.5 kb). Subsequently, the 9.0 kb *Sal*I

Fig. 3. Restriction map of plasmid pAS185 and subcloned plasmids. The approximate location of the *atdA* and *atdB* genes are shown as boxes. Expression of these genes in *E. coli* JM109 harbouring these plasmids are shown on the right-hand-side of plasmid designations. The location of ORFs (A1–A5) in the *atdA* gene and putative Tn1000 were deduced as described in the text. AO, aniline oxygenase activity; C23O, catechol 2,3-dioxygenase activity; +, activity; –, no activity.

fragment was subcloned in both orientations of pUC19 to give pAS90 and pAS91. When these plasmids were in *E. coli* JM109, both hosts showed a brownish colour on LB plates containing aniline, which probably indicates the accumulation and auto-oxidation of catechol formed from aniline. Because the expression in *E. coli* is not dependent on the direction of the fragment to the vector sequence, the aniline oxygenase gene (*atdA*) with a functional self-promoter could be presumed to be present in the 9.0 kb region. By further subcloning, the smallest region expressing the aniline oxidation activity was confirmed to be a 6.3 kb segment between the *SalI* and *EcoRV* sites in the 9.0 kb fragment. On the other hand, subcloning of the 6.5 kb *SalI* fragment into pUC19 resulted in the construction of pAS60, which gave the host *E. coli* C23O activity. Using catechol spray tests for various subclones, the gene encoding the C23O activity (*atdB*) was found to be located within the 1.5 kb *PstI*–*SmaI* region in the 6.5 kb fragment.

We have sequenced the 9.0 kb *SalI* fragment, and found five ORFs in the sequence, which were presumed to encode a glutamine synthetase-like protein, a GMP synthase-like protein, large and small subunits of dioxygenase, and a reductase component, in this order. We also found the transposase gene sequence of Tn1000 (Guyer, 1978) over 0.7 kb downstream of the last ORF. The restriction map of the region between the *atdA* and *atdB* genes coincides completely with that of Tn1000. Therefore, a functional Tn1000 may lie in the region between both genes. The interaction between Tn1000 and aromatic compound catabolic genes has never been

reported, and the detail of these gene sequences is now under investigation.

A plasmid-encoded aniline-degradation pathway was first reported by Anson & Mackinnon (1984). pCIT1 was discovered in *Pseudomonas* sp. strain CIT1. After that, Meyers (1992) cloned the aniline oxidative genes from the total DNA of strain CIT1 and showed that the genes encoded 72 and 36 kDa proteins. However, the detail of the genetic organization is still unclear. Another plasmid involved in aniline degradation, pTDN1, was found in *P. putida* UCC2 which is able to grow on aniline, *m*-toluidine and *p*-toluidine (McClure & Venables, 1986). To date, the only genetic characterization of aniline oxidation to catechol has been done using pTDN1 (McClure & Venables, 1987; Saint *et al.*, 1990; Fukumori & Saint, 1995). The toluidine oxygenase gene, whose product oxidizes aniline, *m*-toluidine and *p*-toluidine to the corresponding catechols, was located within a 9.3 kb *HindIII* fragment of pTDN1. Fukumori & Saint have found several ORFs in this fragment, three of which were presumed to encode large and small subunits of terminal dioxygenase and an electron-transfer protein, usually found in the multi-component oxygenase enzyme system. In addition, they found a glutamine synthetase gene-like sequence and an IS1071 sequence with 1 bp substitution. We also found glutamine synthetase gene-, GMP synthase gene- and Tn1000-like sequences. Since glutamine synthetase and GMP synthase mediate the addition of amido groups to glutamate and the release of amido groups from glutamine, respectively, both enzyme-like proteins could

Fig. 4. Southern hybridization of YAA and YAA-113 plasmids with a *Bam*HI fragment encoding the *atdA* gene of pAS90. (a) Agarose gel electrophoresis of YAA and YAA-113. Lanes: λ , *Hind*III-digested λ DNA; 1, *Eco*RI-digested YAA plasmid DNA; 2, *Eco*RI-digested YAA-113 plasmid DNA. (b) Detected signals of corresponding Southern blot of (a) after hybridization with a fluorescein-labelled 5 kb *Bam*HI fragment of pAS90. Arrowheads indicate positions of the DNA fragments (9.5 and 2.2 kb) to which the probe hybridized.

be involved in the recognition and release of aniline amido-groups.

At present, we are unable to compare our sequence with the sequence of the *Hind*III fragment from pTDN1 directly, because the latter has not been published or registered in any nucleotide database yet. However, the restriction maps of both sequences are quite different.

Identification of intermediates from aniline or *o*-toluidine in recombinant *E. coli*

After *E. coli* JM109 harbouring pAS90 was grown on aniline, a metabolite was extracted from the culture. It showed a TLC R_f value of 0.83, identical to that of authentic catechol. The identity of the product was confirmed by GC-MS analysis. Evidence for catechol was the molecular ion at m/z 110 (M^+ , 100%), which agreed with the molecular formula of $C_6H_6O_2$. Major fragment ions were at m/z 92 ($M^+ - H_2O$, 11%), 81 ($M^+ - CHO$, 9.6%) and 64 ($M^+ - H_2O$, $-CO$, 43%). When this strain was grown on *o*-toluidine, a purple-brownish product accumulated. The mass spectrum of the purple-brownish product showed that there was a molecular ion at m/z 124 (M^+ , 100%), which agreed with the molecular formula of 3-methylcatechol, $C_7H_8O_2$, as expected. Major fragment ions were at m/z 106 ($M^+ - H_2O$, 22%), 95 ($M^+ - CHO$, 8.3%) and 78 ($M^+ - H_2O$, $-CO$, 64%). The GC-MS analyses of the

products accumulated in recombinant *E. coli* prove that the aniline oxygenase gene (*atdA*) is encoded on the cloned 9.0 kb *Sal*I fragment.

These results indicate that anilines are degraded via catechols in strain YAA, and this pathway is consistent with previous studies (Aoki *et al.*, 1983; Lyons *et al.*, 1984). We were unable to detect the presence of *cis*-diol compounds which are found as precursors of catechol in aromatic compound oxidation (Gibson *et al.*, 1970; Axcell & Geary, 1975; Shirai, 1986; Eaton & Timmis, 1986) by GC-MS analyses.

Evidence for the existence of the *atdA* gene on plasmid pYA1

To confirm the existence of *atdA* on plasmid pYA1, Southern hybridization studies were carried out. A 5.0 kb *Bam*HI fragment was prepared from the *Sal*I insert fragment in pAS90, fluorescein-labelled, and used as a probe for the hybridization studies. As shown in Fig. 4, the probe hybridized to 2.2 and 9.5 kb *Eco*RI-digested fragments in the plasmid DNA of strain YAA, but not in that of the cured strain YAA-113 (in the case of strain YAA-113, such fragments could not be observed in the electrophoresis photograph). The sizes were similar to those expected from the restriction map (Fig. 3). This result indicates that the cloned *Sal*I fragment was derived from the plasmid DNA of *Acinetobacter* sp. strain YAA.

REFERENCES

- Amador, J. A., Alexander, M. & Zika, R. G. (1991). Degradation of aromatic compounds bound to humic acid by the combined action of sunlight and microorganisms. *Environ Toxicol Chem* 10, 475–482.
- Anson, J. G. & Mackinnon, G. (1984). Novel *Pseudomonas* plasmid involved in aniline degradation. *Appl Environ Microbiol* 48, 868–869.
- Aoki, K., Shinke, R. & Nishira, H. (1983). Metabolism of aniline by *Rhodococcus erythropolis* AN-13. *Agric Biol Chem* 47, 1611–1616.
- Axcell, B. C. & Geary, P. J. (1975). Purification and some properties of a soluble benzene-oxidizing system from a strain *Pseudomonas*. *Biochem J* 146, 173–183.
- Birnboim, H. C. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* 7, 1513–1523.
- Brosius, J., Palmer, M. L., Kennedy, P. J. & Noller, H. F. (1978). Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proc Natl Acad Sci USA* 75, 4801–4805.
- Eaton, R. W. & Timmis, K. N. (1986). Characterization of a plasmid-specified pathway for catabolism of isopropylbenzene in *Pseudomonas putida* RE204. *J Bacteriol* 168, 123–131.
- Fuchs, K., Schreiner, A. & Lingens, F. (1991). Degradation of 2-methylaniline and chlorinated isomers of 2-methylaniline by *Rhodococcus rhodochrous* strain CTM. *J Gen Microbiol* 137, 2033–2039.
- Fukumori, F. & Saint, C. P. (1995). Analysis of the aniline degradation pathway of *Pseudomonas putida* UCC2 (pTDN1). *Fifth International Symposium on Pseudomonas: Biotechnology and Molecular Biology*, abstract, p. 70.

- Gibson, D. T., Hensley, M., Yoshioka, H. & Mabry, T. J. (1970).** Formation of (+)-*cis*-2,3-dihydroxy-1-methylcyclohexa-4,6-diene from toluene by *Pseudomonas putida*. *Biochemistry* **9**, 1626–1630.
- Guyot, M. (1978).** The gamma delta sequence of F is an insertion sequence. *J Mol Biol* **126**, 135–140.
- Hasegawa, T. (1985).** *Biseibutsu no bunrui to doutei*. Tokyo: Gakkai Shuppan Center.
- Juni, E. (1984).** In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 303–307. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
- Konopka, A., Knight, D. & Turco, R. F. (1989).** Characterization of a *Pseudomonas* sp. capable of aniline degradation in the presence of secondary carbon sources. *Appl Environ Microbiol* **55**, 385–389.
- Loidl, M., Hinteregger, C., Ditzelmüller, G., Ferschl, A. & Streichsbier, F. (1990).** Degradation of aniline and mono-chlorinated anilines by soil-borne *Pseudomonas acidovorans* strain. *Arch Microbiol* **155**, 56–61.
- Lyons, C. D., Katz, S. & Bartha, R. (1984).** Mechanisms and pathways of aniline elimination from aquatic environments. *Appl Environ Microbiol* **48**, 491–496.
- Lyons, C. D., Katz, S. E. & Bartha, R. (1985).** Persistence and mutagenic potential of herbicide-derived aniline residues in pond water. *Bull Environ Contam Toxicol* **35**, 696–703.
- McClure, N. C. & Venables, W. A. (1986).** Adaptation of *Pseudomonas putida* mt-2 to growth on aromatic amines. *J Gen Microbiol* **132**, 2209–2218.
- McClure, N. C. & Venables, W. A. (1987).** pTDN1, a catabolic plasmid involved in aromatic amine catabolism in *Pseudomonas putida* mt-2. *J Gen Microbiol* **133**, 2073–2077.
- Meyers, N. L. (1992).** Molecular cloning and partial characterization of the pathway for aniline degradation in *Pseudomonas* sp. strain CIT1. *Curr Microbiol* **24**, 303–310.
- Nakazawa, T. & Yokota, T. (1973).** Benzoate metabolism in *Pseudomonas putida* (arvilla) mt-2, demonstration of two benzoate pathways. *J Microbiol* **115**, 262–267.
- Rochelle, P. A., Will, J. A. K., Fry, J. C., Jenkins, G. J. S., Parkes, R. J., Turley, C. M. & Weightman, A. J. (1995).** Extraction and amplification of 16S rRNA genes from deep marine sediments and seawater to assess bacterial community diversity. In *Nucleic Acids in the Environment*, pp. 219–239. Edited by J. T. Trevors & J. D. van Elsas. Berlin: Springer.
- Saint, C. P., McClure, N. C. & Venables, W. A. (1990).** Physical map of the aromatic amine and *m*-toluate catabolic plasmid pTDN1 in *Pseudomonas putida*: location of a unique *meta*-cleavage pathway. *J Gen Microbiol* **136**, 615–625.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989).** *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Shirai, K. (1986).** Screening of microorganisms for catechol production from benzene. *Agric Biol Chem* **50**, 2875–2880.
- Snell, F. D. (1954).** In *Colorimetric Methods of Analysis*, 3rd edn, vol. IV, pp. 198–199. Amsterdam: Van Nostrand.
- Southern, E. M. (1975).** Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* **98**, 503–517.
- Takeo, M., Maeda, Y., Okada, H., Miyama, K., Mori, K., Ike, M. & Fujita, M. (1995).** Molecular cloning and sequencing of the phenol hydroxylase gene from *Pseudomonas putida* BH. *J Ferment Bioeng* **79**, 485–488.
- Woese, C. R. (1987).** Bacterial evolution. *Microbiol Rev* **51**, 221–271.
- Zeyer, J., Wasserfallen, A. & Timmis, K. (1985).** Microbial mineralization of ring-substituted anilines through an *ortho*-cleavage pathway. *Appl Environ Microbiol* **50**, 447–453.

Received 30 April 1996; revised 5 August 1996; accepted 2 October 1996.