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SHORT CONTRIBUTION

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Degradation of aniline by newly isolated, extremely aniline-tolerant *Delftia* sp. AN3

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Abstract A bacterial strain, AN3, which was able to use aniline or acetanilide as sole carbon, nitrogen and energy sources was isolated from activated sludge and identified as *Delftia* sp. AN3. This strain was capable of growing on concentrations of aniline up to 53.8 mM (5000 mg/l). Substituted anilines such as *N*-methylaniline, *N*,*N*-dimethylaniline, 2-methylaniline, 4-methylaniline, 2-chloroaniline, 3-chloroaniline, *o*-aminoaniline, *m*-aminoaniline, *p*-aminoaniline, and sulfanilic acid did not support the growth of strain AN3. The optimal temperature and pH for growth and degradation of aniline were 30 °C and 7.0, respectively. The activities of aniline dioxygenase, catechol 2,3-dioxygenase and other enzymes involved in aniline degradation were determined, and results indicated that all of them were inducible. The K_m and V_{max} of aniline dioxygenase were 0.29 mM and 0.043 mmol/mg protein/min, respectively. The K_m and V_{max} of catechol 2, 3-dioxygenase for catechol were 0.016 mM and 0.015 mmol/mg protein/min, respectively. Based on the results obtained, a pathway for the degradation of aniline by *Delftia* sp. AN3 was proposed. The importance of the strain to the operation of municipal wastewater treatment plants is discussed.

Introduction

Aniline is a widely distributed environmental pollutant resulting from the manufacture of dye materials (Meyer 1981) and agricultural chemicals such as herbicides (Kearney and Kaufmann 1975). Because of its toxic and recalcitrant nature and the wide application of aniline-containing chemicals, aniline is considered to be an increasing threat both to the environment and to human health. Thus, the fate of aniline in the environments is of great concern.

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Microbial transformation and degradation are major mechanisms to eliminate aniline from the environment. Bacterial species of *Pseudomonas* (Hinteregger et al. 1992), *Comamonas* (Parales et al. 1997), *Acinetobacter* (Kim et al. 1997), *Rhodococcus* (Aoki et al. 1983), *Frateuria* (Murakumi et al. 1999), *Moraxella* (Zeyer et al. 1985) and *Nocardia* (Bachofer et al. 1975) have been shown to be able to degrade aniline and/or its derivatives. However, there has been no report that species belonging to the genus *Delftia* are capable of degrading aniline. Highly aniline-tolerant bacteria are desirable for environmental applications as well as for the biotransformation of aniline and its analogues into useful chemical products. *Pseudomonas* sp. is considered to be the most aniline-tolerant bacterial strain, utilizing concentrations of up to 32 mM (Konopka et al. 1989). In this report, we describe the isolation of a novel bacterial strain that efficiently utilizes up to 53.8 mM (5,000 mg/l) aniline. This strain was characterized and tentatively named *Delftia* sp. AN3. Enzymes involved in aniline degradation were analyzed and their catalytic parameters were determined. Based on the results, a complete route of aniline degradation of this strain was proposed.

Materials and methods

Strain, media and cultural conditions

Delftia sp. AN3 was isolated from a municipal wastewater treatment plant in the city of Dalian, in northern China. The strain was deposited in the China General Microbiological Collection Center (Beijing) under accession number AS1.2774.

Cultures were grown in mineral medium MMN (mineral medium without nitrogen and carbon) which contained the following ingredients (in 1 l distilled water): $Na_2HPO_4 \cdot 12H_2O$, 2.0 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.03 g; trace elements (Konopka 1993) 5 ml. The pH was adjusted to 7.0. Aniline was added at 2 g/l or as indicated in the text. For preparation of aniline plates, 1.5% agar was added to the MMN medium.

Cultures were incubated on a rotary shaker at 30 °C. Growth was monitored by measuring the turbidity at 460 nm with a 721 photometer (Shanghai No. 3 Optical Equipment, P. R. China).

Enrichment and isolation

Activated sludge samples from the municipal wastewater plant were inoculated into MMN media and incubated at 30 °C for 3 days. After three consecutive transfers, the cultures were diluted and spread onto aniline plates. Single colonies that developed on the plates were picked and inoculated into MMN media. The process was repeated until a pure culture was obtained.

Characterization and identification of strain AN3

Strain AN3 was identified using the Biolog MicroStation Identification System (Biolog Microstation, USA) and the software which accompanied the instrument. GN (for gram-negative bacteria) plates were used to obtain the metabolic fingerprint of the isolate. This fingerprint was transformed into computer-readable data which were then compared to the information provided by the supplier regarding standard bacterial strains.

Sequencing of 16S rRNA gene of strain AN3 and GenBank accession number

DNA was extracted according to the method of Sambrook et al. (1989). The 16S rRNA gene was amplified by PCR with primers [Pf 5′-AGAGTTTGATCCTGGCTCAG-3′ and Pr 5′-ACGGCTACCTTGTTACGACT-3′, corresponding to positions 8–27 and 1495–1514 of the *Escherichia coli* 16S rDNA sequence, respectively (Brosius et al. 1978)] using a Biometra T-gradient (Whatman, Germany). The sequence of the 16S rRNA gene of strain AN3 is available under the GenBank accession number AY052781.

Assays of enzymatic activities

Aniline dioxygenase

Cells were harvested by centrifugation at 5,000×*g*, washed twice with 20 mM 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 were used in a parallel experiment.

Catechol 2,3-dioxygenase, catechol 1,2-dioxygenase and other enzymes

Cells (the same cell suspension as used in the aniline dioxygenase assay) were disrupted by passing them through a French press twice. The cellular lysates were centrifuged at 19,000×*g* for 20 min, and the supernatant was used for enzymatic assays. The reaction mixture (total 3.0 ml) contained 2.0 ml phosphate buffer, 0.6 ml 1 mM catechol, 0.2 ml deionized water and 0.2 ml cellular lysates. The reaction proceeded at 22 °C. Catechol 2,3-dioxygenase and catechol 1,2-dioxygenase activities were determined by measuring the production of either 2-hydroxymuconic semialdehyde at 375 nm (Sala-Trepat and Evans 1971) or muconic acid at 260 nm (Hayaishi et al. 1957) using a DU-7 spectrophotometer (Beckman, USA). The absorption coefficients of 2-hydroxymuconic semialdehyde and muconic acid were 12,000 $mol^{-1}cm^{-1}$ and 16,000 $mol^{-1}cm^{-1}$, respectively.

2-Hydroxymuconic semialdehyde dehydrogenase, 4-oxalocrotonic acid decarboxylase and 4-hydroxy-2-oxovalerate aldolase activities were assayed according to the methods used by Sala-Trepat and Evans (1971).

Chemical analysis

Protein concentration was determined according to the method of Bradford (1976). 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 eluent 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. Ammonia was measured by the method of Weatherburn (1967). Intact cells (0.5 ml) were added to the reaction mixture to give a final volume of 1.0 ml with 5 mM aniline. After shaking for 1 h at 30 °C, supernatants were recovered by centrifugation (10,000×*g*, 1 min, room temperature): 0.5 ml of the supernatant was treated once with 0.3 ml chloroform. A parallel experiment was run without aniline.

Results

Isolation and identification of aniline-degrading strains

With aniline as sole carbon and nitrogen source, six isolates from the activated sludge samples were obtained. Among them, strain AN3 was the most active in degrading aniline; therefore, further studies focused on this strain.

Cells of strain AN3 were gram-negative rods and motile. The strain showed a similarity index of 0.82 to *Delftia acidovorans* (Wen et al. 1999) (previously *Comamonas acidovorans* (Tamaoka et al. 1987)), as revealed with the Biolog MicroStation Identification System. The sequence (1,472 bp) of the 16S rRNA gene of strain AN3 had similarities of 99.58 and 98.54% to *Delftia* sp. EK3 (Katsivela et al. 1999) (AJ237966) and to *Delftia acidovorans* (AF078774), respectively. Thus, a tentative name, *Delftia* sp. AN3, was assigned to this isolate.

Aniline or acetanilide was used as sole carbon and nitrogen source by strain AN3. Other substituted anilines tested (at concentrations of 500 mg/l and 1,000 mg/l in MMN media) but not supporting the growth of strain AN3 included *N*-methylaniline, *N*,*N*-dimethylaniline, 2-methylaniline, 4-methylaniline, 2-chloroaniline, 3-chloroaniline, *o*-aminoaniline, *m*-aminoaniline, *p*-aminoaniline, and sulfanilic acid.

Degradation of aniline by *Delftia* sp. AN3

The results indicated that aniline degradation was simultaneous with cell growth (data not shown). When adapted cells (previously incubated in MMN medium containing aniline) were used, aniline was degraded much faster. Anilines at high concentrations are toxic to cells, and 64.5 mM of aniline completely inhibited the growth of strain AN3. Concentrations of aniline between 43.0 and 53.8 mM resulted in slow growth of strain AN3, as indicated by the final biomass after 3 days of cultivation (Fig. 1). However, degradation still proceeded at aniline concentrations between 43.0 and 53.8 mM, and complete removal of 53.8 mM aniline was obtained after 7 days of incubation (inoculum was 0.01% v/v). Thus, to our knowledge, this is the most aniline-tolerant strain that has been described.

Table 1 Activities of aniline dioxygenases and catechol dioxygenases of *Delftia* sp. AN3 grown on different substrates. One unit of aniline dioxygenase was defined as the amount consuming 1 μmol of oxygen per min under the conditions described in Materials and methods. One unit of catechol oxygenase was defined as the amount of protein needed for oxidation of 1 μmol of catechol per min at 22 °C under the conditions described in Materials and methods

Growth substrates	Aniline dioxygenase	Catechol 1,2-dioxygenase (U/mg wet cell)	Catehol 2,3-dioxygenase (U/mg protein)
Aniline	1.094	0.068	5.224
Lactate	0	0	0
LB medium	0	0	0

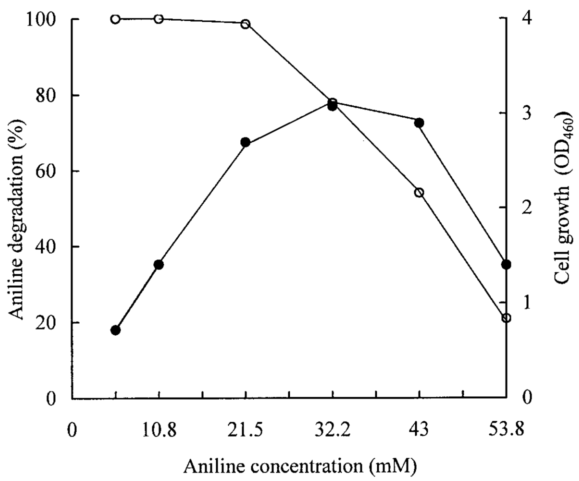

Fig. 1 Effect of aniline concentration on its degradation and on the growth of *Delftia* sp. AN3.. Experiments were conducted in 500-ml conical flasks containing 200 ml MMN media containing up to 53.8 mM aniline. Each flask was inoculated with 20 μl of LB-cultured cells and incubated for 3 days at 30 °C with rotary shaking at 180 rpm. –○– Aniline degradation, –●– Cell growth

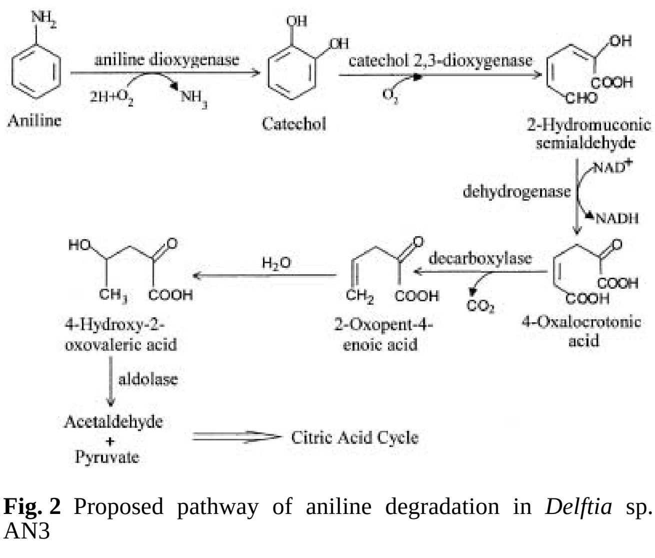

Fig. 2 Proposed pathway of aniline degradation in *Delftia* sp. AN3

Effects of pH, temperature, and heavy-metal ions on the degradation of aniline

With aniline as sole carbon and nitrogen sources the growth and degradation of aniline by strain AN3 occurred at a narrow pH range. When the pH was lower than 6.0 or higher than 8.0, neither the growth of cells nor the degradation of aniline was significant. The optimal temperature for cell growth and aniline degradation was 30 °C. The efficiency of aniline degradation increased gradually when the temperature was increased from 10 to 30 °C. The heavy-metal ions Hg^{2+} and Ag^{+} are toxic to growing cells, and completely inhibition of growth occurred at 0.02 mM Hg^{2+} and 0.1 mM Ag^{+}.

Release of ammonia and activities of aniline dioxygenase

Intact cells cultivated with aniline as sole carbon and nitrogen source released ammonia (Zeyer et al. 1985) in phosphate buffer containing aniline. The amounts of ammonia released were stoichiometric to the amounts of aniline added (data not shown). The dioxygenase activities in *Delftia* sp. AN3 cells were exclusively dependent on cultivation with aniline (Table 1), indicating the inducibility of this enzyme. The *Km* and *Vmax* values of aniline-activated cells were determined to be 0.29 mM and 0.043 mmol/mg protein/min, respectively.

Catechol dioxygenase and other enzymatic activities

The catechol 1,2-dioxygenase and catechol 2,3-dioxygenase activities of cellular lysates obtained from aniline-cultivated *Delftia* sp. AN3 were determined. Results showed that the activity of catechol 2,3-dioxygenase was much higher than that of catechol 1,2-dioxygenase (Table 1), indicating cleavage of the benzene ring mainly at the *meta*- position by catechol 2,3-dioxygenase in *Delftia* sp. AN3. The K_m and V_{max} values of the catechol 2,3-dioxygenase were determined to be 0.016 mM and 0.015 mmol/mg protein/min, respectively.

Significant activities of 2-hydroxymuconic semialdehyde dehydrogenase, 4-oxalocrotonic acid decarboxylase and 4-hydroxy-2-oxovalerate aldolase were also detected in the cellular lysates of *Delftia* sp. AN3. These results show that *Delftia* sp. AN3 uses a *meta*-cleavage pathway to degrade aniline (Fig. 2).

Discussion

Delftia sp. AN3 is a novel aniline-degrading strain isolated from a municipal wastewater treatment plant which grows significantly at aniline concentrations as high as 53.8 mM. This is much higher than the 32 mM reported for *Pseudomonas* sp. (Konopka et al. 1989). Aniline-cultivated cells of strain AN3 contained aniline dioxygenase, catechol 2,3-dioxygenase, 2-hydroxymuconic semialdehyde dehydrogenase, 4-oxalocrotonic acid decarboxylase and 4-hydroxy-2-oxovaleric acid aldolase activities, indicating that a complete *meta*-cleavage pathway exists in *Delftia* sp. AN3 cells (Fig. 2). The fact that only aniline-cultivated cells contained the activities of these enzymes indicated that those enzymes were inducible. The specific activity of aniline dioxygenase of strain AN3 (Table 1, 1.094 U/mg wet weight) was higher than that of *Pseudomonas putida* mt-2 (0.037 U/mg dry weight) (McClure and Venables 1986) and *Pseudomonas acidovorans* CA28 (0.375 U/mg wet weight) (Loidl et al. 1990). These results suggest that *Delftia* sp. AN3 degrades aniline with high efficiency. This property as well as the resistance to heavy-metal ions shows that strain AN3 is important to the performance of municipal wastewater treatment plants, which often receive pollutants from the organic chemical and machine industries containing heavy metal ions.

As mentioned previously, *Delftia* sp. AN3 uses a *meta*-cleavage pathway for aniline degradation. The genes encoding the enzymes for the *meta*-cleavage pathway are often located on plasmids (Fukumori and Saint 1997; Hinteregger et al. 1992; Saint et al. 1990). Since there was no plasmid detected in strain AN3, the pathway of aniline degradation might be encoded by genes on the chromosome of *Deftia* sp. AN3.

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