Biodegradation of Endocrine-Disrupting Chemical Aniline by Microorganisms

Teruo Tanaka,*,[a] Hideo Hachiyanagi,[a] Naoko Yamamoto,[a] Tomonori Iijima,[a] Yutaka Kido,[a] Masaru Uyeda,[b] and Kazuo Takahama[c]

[a]*Division of Instrumental Analysis, Institute of Resource Development and Analysis, Kumamoto University, 5–1 Oe-honmachi, Kumamoto 862–0973, Japan,* [b]*Department of Nutrition Science Facultiy of Living Science, Shokei University, 2–6–78 Kuhonji, Kumamoto 862–8678, Japan and* [c]*Department of Environmental and Molecular Health Sciences, Kumamoto University, 5–1 Oe-honmachi, Kumamoto 862–0973, Japan*

(Received January 10, 2009; Accepted May 8, 2009; Published online May 18, 2009)

Two pure cultures (strains No. A-11 and A-12) from soil sample capable of utilizing aniline as the sole source of nitrogen and energy were regarded as Achromobacter sp and Pseudomonas sp, respectively. Degradation patterns of aniline and aniline derivatives were observed on the high-performance liquid chromatogram (HPLC) of the culture filtrate of both strains and growth of both strains were measured as protein by the Kennedy and Fewson method. The growth yield of both strains were about 46.3 g and 47.4 g of protein per mole of nitrogen source of aniline and were similar to those in the case of NH_4Cl as a nitrogen source. Biodegradation of aniline was achieved (200 mg/l) in less than 3 and 4 days using strains No. A-11 and strain No. A-12, respectively. The strain No. A-11 degraded aniline more rapidly than strain No. A-12.

Key words —— aniline, biodegradation, sole nitrogen source, growth yield, *Pseudomonas*, *Achromobacter*

INTRODUCTION

Within the past decades, people have invented an enormous array of synthetic chemicals, and utilized them to make their lives convenient. However, the artificial compounds have provided not only huge benefits but also a high environmental price to all countries and people.

In recent years, aniline has attracted increasing attention owing to their widespread use, ubiquity in the environment, and endocrine-disrupting activity.[1,2] Aniline is a synthetic compound used in dyes and herbicides.[2,3] Aniline is also one of the most important chemical products in industry as synthetic intermediate.[4] Aniline is widely used in polyurethane, rubber, agricultural chemicals, and, to a limited extent, in food and medical products. Because of the global utilization of aniline and its derivatives in large quantities, they have been detected in every environment in which they have been soughed. Known as endocrine-disrupting chemicals, aniline may also interfere with the reproductive system and normal development of animals and humans.[5,6] Therefore, many researchers have warned people about the toxicity and carcinogenicity of aniline.[3,7] Recently, many studies on aniline mainly focused on biodegradability, metabolic pathway by pure culture of microorganisms and removal of aniline in wastewater treatment systems.[8–14] Studies researching into the degradation of aniline by bacteria as the sole source of carbon have been done by many researchers.[4,8] However, few studies have focused on the processes and degradation ability of bacteria to utilize aniline as the sole source of nitrogen. In the previous paper, we reported the complete degradation of phthalic acid and dimethyl phthalate ester by *Flavobacterium*.[15,16] In this paper, the biodegradation of aniline by two new isolates (strain No. A-11 and No. A-12) from soil sample is described together with the taxonomic characteristics of their strains.

MATERIALS AND METHODS

Materials —— Aniline (purity, 99%) was purchased from Nacalai Tesque Co., INC. (Kyoto, Japan) and used without further purification. All other chemicals were guaranteed to be of the best grade commercially available.

Apparatus, Analysis, and Measurement of Aniline —— Spectrophotometric analysis was done using a Hitachi 124 spectrophotometer (Tokyo, Japan), equipped with flow-through cell of 1.0 cm path length. High-performance liquid chromatography (HPLC) was done using jacketed stainless steel

*To whom correspondence should be addressed: Division of Instrumental Analysis, Institute of Resource Development and Analysis, Kumamoto University, 5–1 Oe-honmachi, Kumamoto 862–0973, Japan. Tel. & Fax: +81-96-371-4809; E-mail: tfujii@gpo.kumamoto-u.ac.jp

analytical column (4.6 × 150 mm i.d.) packed with Cosmosil $5C_{18}$-MS-II (5 μm). The mobile phase was CH_3OH-H_2O (70 : 30, v/v, pH 7.0) containing 0.05 M heptanesulfonic acid. The concentration of aniline was routinely measured by HPLC on an apparatus equipped with UV detector set at 280 nm. Quantitation of aniline was achieved through the use of a calibration curve. A set of standards (50–500 μg/ml) produced daily by serial dilution was used. In the above concentration range, a linear response was obtained (regression equation y = 2799.7x, correlation coefficient = 0.9998). Samples from bacterial cultures were filtered (0.45 μm pore diameter) before chromatography.

Growth Media and Isolation of Microorganisms —— The growth medium used for nitrogen-limited growth was 10 mM potassium phosphate buffer, pH 7.3, and contained 0.25 mM $MgSO_4$, 2% Glucose as the sole source of carbon, trace elements (0.1% NaCl, 0.1% $FeSO_4 \cdot 7H_2O$, 0.01% $CuSO_4 \cdot 5H_2O$, 0.01% $ZnSO_4 \cdot 7H_2O$, 0.01% $MnSO_4 \cdot 7H_2O$ and 0.01% $CaCl_2$; supplied at 2 ml/l) and less than 2.5 mM aniline as the sole source of nitrogen. The buffered solution of $MgSO_4$ was autoclaved, and the sterile solution of trace elements, aniline, and ammonium chloride were added aseptically. The term, buffered salts, refers to 10 mM potassium phosphate buffer, pH 7.3, containing 0.25 mM $MgSO_4$. Enrichment cultures were used to obtain isolates able to utilize aniline as a sole and limiting source of nitrogen for growth. Inocula for enrichments were prepared from soils of agricultural farm (Kumamoto, Japan). Enrichment cultures were nonsterile and contained aniline at 200 μg/ml as a nitrogen source, buffered salts, glucose and 20% of preculture. Cultures were carried out with 100 ml medium in 500-ml Erlenmeyer flasks under aerobic condition for 7d at 37°C on a rotary shaker (200 rpm). All enrichments were subcultured three times into homologous medium. Positive enrichments were streaked on nutrient agar plates and growth medium agar plates containing aniline at 200 μg/ml. A representative of each colony type was picked from the agar plates. Stock cultures of each isolate were maintained in growth medium agar slants containing aniline at 200 μg/ml.

Taxonomic Characteristics of Strain No. A-11 and No. A-12 —— The morphology of strain No. A-11 and No. A-12 were studied by phase-contrast microscopy, and the Gram reaction was examined by using the Gram stain. Roch Oxi-Ferm and Enterotubes were used in combination with the method of Stanier *et al.*[17]

Quantification of Growth and Aniline Utilization —— Bacterial growth yields with limiting nitrogen source were determined at 37°C with 50 ml cultures in 200-ml Erlenmeyer flasks on a rotary shaker (200 rpm). Media containing nitrogen source at five concentrations between 0 and 2.5 mM nitrogen were inoculated (4% v/v) with cell suspension of bacterial culture induced to grow in a limiting amount of aniline. The carbon source was 2% (final concentration) glucose for microorganism. Aniline in cultures was quantified by HPLC. Growth of bacteria was measured routinely as protein (rather than turbidity or dry weight) to eliminate trivial complication arising from the accumulation of lipid or carbohydrate storage polymers, which include factors interfering with bacterial growth measurement. Bacteria were treated with a final concentration of 0.5 M trichloroacetic acid and collected by centrifugation at 12000 rpm at 0°C for 20 min. The supernatant fluid was discarded. The resultant precipitate was suspended in 0.66 M NaOH, and protein was assayed according to the method of Kennedy and Fewson.[18]

Table 1. Taxonomic Characterization of Strains No. A-11 and A-12

Properties or tests	Strain No. A-11	Strain No. A-12
Bacterial type	Rods	Rods
Mobility	Motile	Motile
Size	0.5 by 1.2 μm	0.6 by 1.0 μm
Optimal temperature	37°C	37°C
Optimal pH	8.0	8.0
Gram stain	−	−
Oxidase	+	+
Catalase	+	+
Arginine dihydrolase	+	−
Lysine decarboxylase	−	−
Indole	−	−
Urease	+	+
Citrate	+	+
Acid production from carbon source		
Ana-Glc[a)]	−	−
Aer-Glc[b)]	+	−
Xylose	+	−
Maltose	−	−
Mannitol	−	−
Sucrose	−	−

a) Glucose under anaerobic conditions. *b*) Glucose under aerobic conditions.

RESULTS AND DISCUSSION

Enrichment and Isolate of Microorganisms Utilizing Aniline

Cultures were enriched under aerobic condition. Two pure cultures of bacteria were obtained from the aerobic enrichment culture, and no fungi were isolated.

Taxonomic Characteristics of Strain No. A-11 and No. A-12

Taxonomic characteristics of strain No. A-11 and No. A-12 are summarized in Table 1. The strain No. A-11 was regarded as *Achromobacter* sp. because it was Gram-negative, strictly aerobic, oxidase-positive, arginine dihydrolase-positive, production acid from glucose as carbon source, and characterized as motile rods which grew aerobically with citrate as a carbon source. Urease reaction was strongly positive, whereas lysine decarboxylase reaction was negative. No production of H_2S or indole was observed. The strain No. A-12 was regarded as *Pseudomonas* sp. because it was Gram-negative, strictly aerobic, oxidase-positive, and characterized as motile rods which grew without requirement of growth factors in the pH range of 6–8 and used both citrate and glucose as carbon sources. Urease reaction was positive, whereas arginine dihydrolase and lysine decarboxylase reactions were negative. No production of H_2S or indole was observed.

Aniline-Degrading Activity of Isolates

The growth curves of the two isolates were examined using aniline as a sole source of nitrogen. Strains No. A-11 and No. A-12 could utilize aniline as a sole source of nitrogen or degrade it. The HPLC chromatograms of aniline in the culture filtrate of strains No. A-11 and No. A-12 are shown in Fig. 1. Photolysis products of aniline were observed on

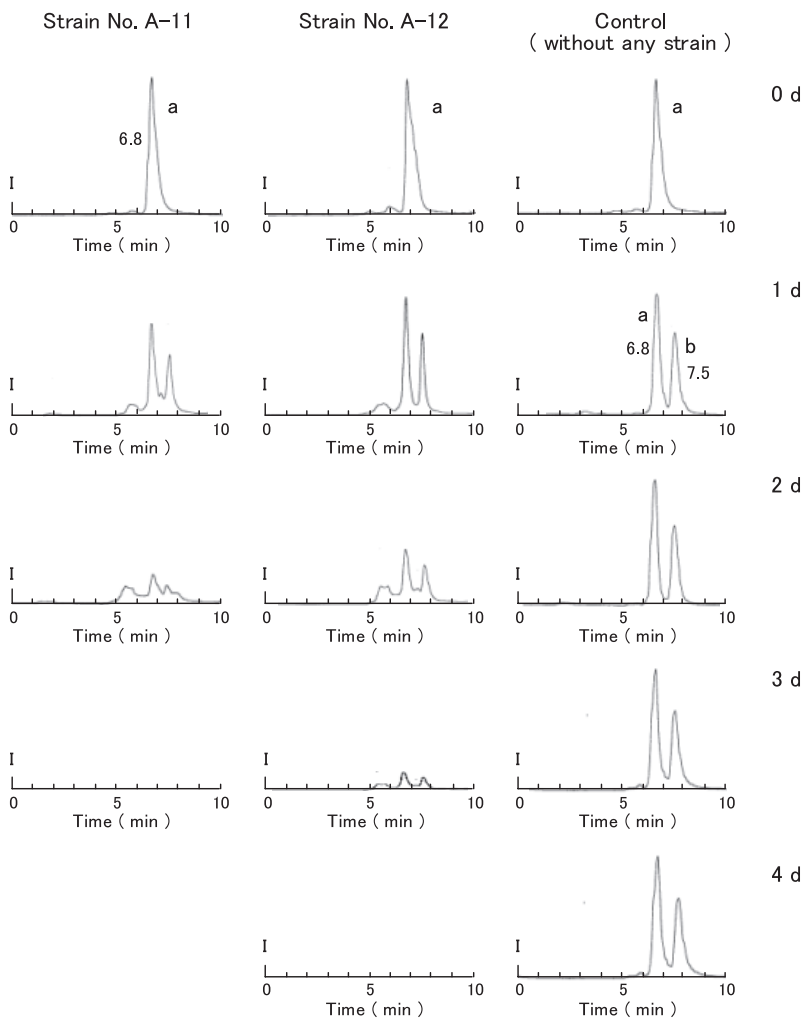

Fig. 1. HPLC Chromatograms of the Culture Filtrate of Strains No. A-11 and A-12
Grown on Aniline as a Source of Nitrogen for 4 d., Detector = 280 nm; sensitivity, 0.1 a.u.f.s; I = injection; a = aniline, b = aniline derivatives.

the HPLC of both strains' culture and control culture without bacteria, but aniline and its derivatives were not observed on HPLC of both strains' culture in less than 3 and 4 days, respectively. Biodegradation of aniline and its derivatives was achieved (200 mg/l) in 3 and 4 days using strain No. A-11 and No. A-12, respectively. The adsorption of aniline on both strains was not observed. Strain No. A-11 degraded aniline and its derivatives more rapidly than strain No. A-12. Figure 2 shows that the concentration of aniline decreases inversely with respect to the increase of the growth yield of the strain No. A-11. The cultures without bacteria showed no change in the concentration of aniline and its derivatives, and negligible growth in the medium. The pH of the pure culture (strain No. A-11) was nearly constant throughout the cultivation time. These results suggest that the decrease of aniline is not due to precipitation by formation of salts or to decomposition by acid. The increase of growth yield was considered to be due to utilization of aniline as a nitrogen source. The degradation pattern of strain No. A-12 was similar to that of strain No. A-11 (not shown). As described above, strain No. A-11 and No. A-12 were regarded as aniline-degrading bacteria.

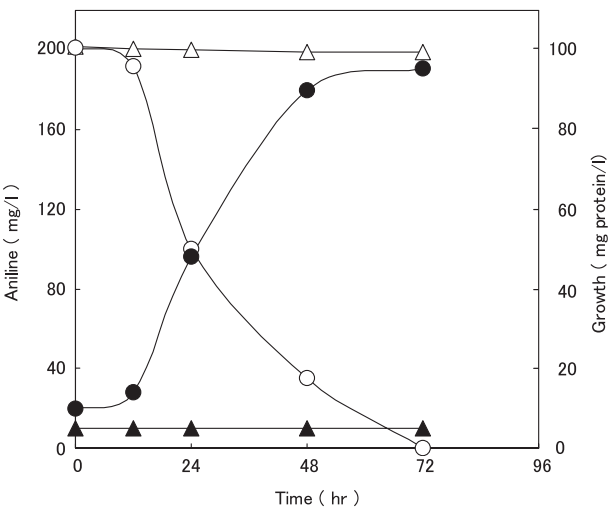

Fig. 2. Microbial Degradation of Aniline by Strain No. A-11
○, △, concentration of Aniline and its derivatives in medium; ●, ▲, growth measured as protein; ○, ●, with Strain No. A-11, △, ▲, control (without any strain) $n = 5$, standard derivation within 2%.

Quantification of Aniline Utilization for Growth

Preliminary evidence for the utilization of aniline as the sole source of nitrogen was the growth yield of organisms (measured as protein) per mole of supplied nitrogen (Table 2). In both strains the yield of cells per mole of aniline nitrogen was similar to the yield of this strain per mole of ammonium ions (*e.g.*, 46 and 47 g of protein, respectively, for strain No. A-11). This implies that the aniline nitrogen was quantitatively used by the bacteria for growth. In all cases, the measurable nitrogen-containing substrates were quantitatively removed from the growth medium (Table 2). The growth yields were the gradients of lines obtained by plotting cell yield *vs.* initial concentration of nitrogen source, and the cultures without combined nitrogen showed negligible growth. Additional controls were the following : Aniline and its derivatives were stable throughout the cultivation time and no ammonium ion was taken up from the atmosphere. A typical growth curve of strain No. A-11 and No. A-12 is shown in Fig. 3 and Fig. 4, respectively. The strains No. A-11 and No. A-12 grew exponentially with aniline as a sole source of nitrogen. The differential plot of substrate concentration *vs.* protein concentration (Fig. 3 and Fig. 4, insert) was linear, demonstrating that aniline utilization was concomitant with growth. Similar data was obtained for strain No. A-11 and No. A-12 growing with ammonium ion as the sole and limiting nitrogen source (not shown). The growth yields in our experiment (Table 2) are consistent with the quantitative utilization of aniline nitrogen as a nitrogen source of bacteria because the molar growth yields are similar to those in a review of bacterial cell composition.[19] The yield is independent of the nature of the nitro-

Table 2. Growth Yields of Strains No. A-11 and No. A-12 with Aniline as a Sole Source of Nitrogen

Substrate	Atom of N per mole of substrate	Bacterial strain used	N-source remaining after growth	Growth yield (g of protein per mole of N)[a)]
Aniline	1	A-11	None	46.3 ± 0.5
NH_4^+	1	A-11	None	48.0 ± 0.5
Aniline	1	A-12	None	47.4 ± 0.5
NH_4^+	1	A-12	None	49.1 ± 0.5

a) nitrogen: Aniline, NH_4Cl.

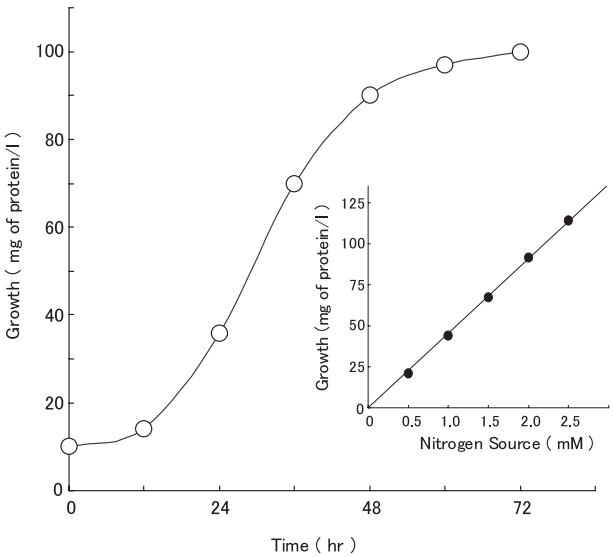

Fig. 3. Growth Curve of Strain No. A-11 with Aniline as the Sole Source of Nitrogen

The insert is a plot of substrate concentration *vs.* the corresponding protein concentration. $n = 5$, standard derivation within 2%.

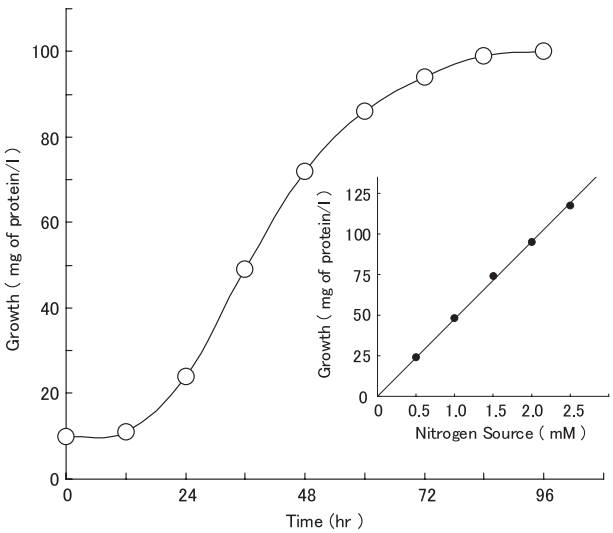

Fig. 4. Growth Curve of Strain No. A-12 with Aniline as the Sole Source of Nitrogen

The insert is a plot of substrate concentration *vs.* the corresponding protein concentration. $n = 5$, standard derivation within 2%.

gen source without nitrogen fixations. This indirect proof of quantitative aniline utilization was supported by substrate disappearance (Table 2), which was usually concomitant with growth (*e.g.*, Fig. 2). In summary, the biodegradation of aniline has been achieved (200 mg/l) in less than 3 and 4 days using strain No. A-11 and No. A-12, respectively, which was isolated from soil sample of agricultural farm. The strain No. A-11 degraded aniline and its derivatives more rapidly than strain No. A-12.

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