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Biodegradation of Aniline by *Pseudomonas putida* FW

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Pseudomonas putida FW에 의한 Aniline의 생물학적 분해

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The strain capable of growing on minimal medium containing aniline as a sole source of carbon was isolated from activated sludges and identified as *Pseudomonas putida* biotype A. The characterizations of the strain were determined. The optimum concentration for growth of the strain was 10-20 mM of aniline. No changes of pH were detected during cultivation. Some metabolic products of biodegradation of aniline were detected after cultivation of the strain on 10 mM aniline for 48 hours. The strain showed to be resistant to streptomycin, tetracycline, trimethoprim, and sulfanilamide. The strain was also capable of utilizing other aromatic compounds related to aniline as a sole source of carbon. One plasmid carried by this strain was detected. The properties of some of the mutant strains treated with mitomycin C were also discussed. The results suggest that separate, regulatory enzyme systems capable of degrading aniline may exist in plasmid DNA.

The genus *Pseudomonas* is an important bacteria for degradation and recycling of organic materials, in particular toxic compounds that pollute the environment (2). Recent works in certain *Pseudomonas* strains indicated that the genes coding for the enzymes responsible for catabolisms of many unusal compounds are encoded on separate transmissible plasmids (14). These include TOL (13, 20) SAL (1), CAM (17), OCT (3), and NAH (4) plasmids which determine growth on toluene, salicylate, comphor, alkane, and naphthalene, respectively.

In this paper, we present that *P. putida* FW isolated from activated sludges was the most prominent strain to reduce chemical oxygen demand (COD) of waste water resulted from the synthesis of antimicrobial agents. These findings prompted us to test the degradative properties by *P. putida* FW of aniline, one of the major recalcitrant and toxic compounds in waste water used in this study. As yet there has been little attempt to elucidate the catabolism of aromatic amines by *P. putida*. This paper describes characterization of *P. putida* FW degrading aromatic amines.

Materials and Methods

Isolation and selection of microorganisms

Waste water from chemical synthesis of antimicrobial agents was used for the isolation of microorganisms. The original waste water was adjusted to give about 2,000 ppm of final concentration of COD by mixing waste water, activated sludges, and mineral salt solution ($Na_2HPO_4 \cdot 12H_2O$ 0.0g, KH_2PO_4 1.5g, $MgSO_4 \cdot 7H_2 7H_2O$ 0.2g, and NH_4Cl 1.0g per liter). The mixture was incubated in a shaker at 30°C for 24 hr. The mixed cultures showed apparent reduction in COD were diluted, and then spreaded on nutrient agar plate for the isolation of microorganisms.

Key words: Biodegradation of aniline, *Pseudomonas putida* FW
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In order to evaluate the activity of isolated strains in reduction of COD, each isolate was inoculated to the diluted waste water (about 2,000 ppm) to give final concentration of 10^8-10^9 colony forming unit (CFU)/m*l*. The COD of the culture incubated in a shaker at 30°C for 24 hr was measured by direct permanganate digestion method (5). The most prominent strain to reduce COD of waste water was selected among the isolates.

Identification of the selected strain

The selected isolate was primarily identified by the use of selective media and Gram staining (7), and then was biochemically identified by using API 20 NE System (La Balme Les Grottes, Montalieu-Vercieu). The final identification of the selected strain was performed by the reference of Bergey's Manual of Systematic Bacteriology (12).

Media and culture conditions for *P. putida* FW

Luria broth (LB) containing 1% tryptone, 0.5% yeast extract, and 0.5% NaCl was adjusted to pH 7.2 and used as a rich medium. Brain heart infusion broth (BHI) was used for the determination of minimal inhibitory concentration (MIC) of antibiotics. M9 minimal salt medium (16) adjusted to pH 7.0 was used with a appropriate aromatic amine substrate at concentration of 5-10 mM as a sole source of carbon. Some aromatic amines dissolved in ether were sprayed on the surface of M9 minimal medium solidified with 2% agar (11). The cells were grown at 30°C with or without shaking for 24-48 hr in culture medium.

Biodegradation of aniline

P. putida FW was grown in 100 m*l* of M9 minimal medium containing 0.5-30 mM anilin with shaking. Cells were harvested at the interval of 4 hr during 48 hr incubation. The cell density and changes of pH of the culture were measured during cultivation. The biodegradation of aniline in the culture medium was monitored by measuring the decrease in ultra-violet (UV) absorbance at 229 nm and 280 nm using clear supernatant of cultures after centrifugation at 10,000 rpm for 20 min.

Detection and isolation of metabolic products

Samples cultured for 48 hours as the methods mentioned above were centrifuged at 10,000 rpm for 20 min. Clear supernatants of samples dried by vaccum were extracted by chloroform. The samples concentrated by chloroform were examined by thin-layer chromatography (TLC) using silica gel 60 F 254 (Merck, Darmstadt). Solvents used for chromatography were chloroform-methanol (2:1, vol/vol). Compounds were detected on chromatogram by viewing under UV light at 254 nm and by staining with iodine.

When definite components were shown on chromatogram, 1 liter of culture was prepared as same methods mentioned above. After removal of the solvent, the residue was directly purified by silica gel comlumn chromatography for further study using nuclear magnetic resonance (NMR). Silica gel and solvents used for column chromatography were silica gel 60 and chloroform-methanol (1.5:1, vol/vol).

Determination of minimal inhibitory concentration

Cells grown in BHI broth were diluted to approximately 10^5-10^6 CFU/m*l* into tubes containing serial two fold dilutions of antibiotics in BHI broth. After 24 hr of incubation, the MIC was determined by observing the cell growth (6).

Detection of plasmid DNA

Rapid procedure for detection of plasmid was followed by Kado *et al.* (10) except that longer lysis and ethanol precipitation of sample were used for increasing the content of plasmid DNA. The examination of the plasmid DNA was performed by agarose gel electrophoresis carried out at 10 V/cm for 1 hr using 0.7% agarose.

Curing experiments

Curing experiments were carried out by treating with mitomycin C as described by Charkrabarty (1). LB containing mitomycin C (5-100 μg/m*l*) was inoculated with overnight culture to give the cell viability of 10^5-10^6 CFU/m*l*. These cultures were further incubated on shaker at 30°C for 24-48

Table 1. Reduction of COD of waste water by isolates.

Isolate	Degree of reduction (%)
FW	25.5
FB	4.1
FTB	2.0
FTA	2.1

hours. Samples showed some growth were diluted, and then spread on L agar plate. Individual colony was transferred to both L broth containing several kinds of antibiotics (50-400 μg/ml) and M9 minimal medium containing 10 mM aniline in microtiter plate to examine for phenotypes.

Results and Discussion

Selection of isolates

The reduction of COD of the waste water by isolates designated as FW, FB, FTA, and FTB is shown in Table 1. Among the isolates the strain FW was selected in terms of reducing COD of waste water, since 25-30% of COD of waste water were reduced by strain FW itself, while almost

Table 2. Morphological, cultural, and biochemical characteristics of strain FW.

Parameter	FW	PP
Gram stain	–	–
Cell shape	rods	rods
Motility	+	+
Endospore formation	–	–
Encapsulation	–	–
Fluorescein pigment	+	+
Growth on MacConkey agar	+	+
Growth on PIA	+	+
Growth in 7% NaCl solution	+	ND
Catalase	+	+
Oxidase	+	+
Gelatin hydrolysis	–	–
Casein hydrolysis	–	ND
Starch hydrolysis	–	–
Egg-Yolk lecithinase	–	–
Lipase (Tween 80)	–	d
Arginine dihydrolase	+	+
Urease	–	–
Denitrification	–	–
MR reaction	+	ND
VP reaction	–	–
Indole formation	–	–
Citrate utilization	+	+
Acid formation from		
Glucose	+	+
Sucrose	–	–
Mannitol	–	d
Melibiose	–	d
Arabinose	+	+

Symbols: PP, *Pseudomonas putida;* PIA, *Pseudomonas* isolation agar; MR, Methyl red; VP, Voges-Proskauser; ND, Not determined; d, variable.

Table 3. Substrates utilized by *P. putida* FW.

Substrate	Utilization
Caprate, Glycerin, L-Alanine	+
L-Glutamate, phenyl acetate	
Maleate, L-Threonine	–
Mannitol	–
L-Rhamnose	–
Glucose	+
D-Galactose	+
Sucrose	–
Malonate	+
Adipate	–
Sorbitol	–
m-Inositol	–
Propylene glycol	–
Benzoate	+
p-Aminobenzoate	–
Glycine	–
L-Lysine	–
L-Tryptophan	–
Anthranilic acid	–

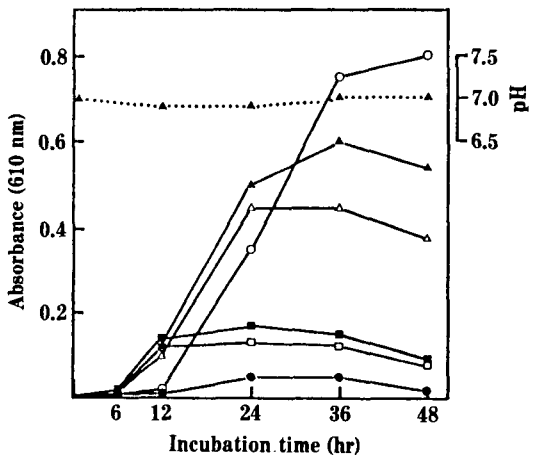

Fig. 1. Growth of *P. putida* FW on M9 minimal medium containing aniline.

Symbols: —●—, 30 mM; —○—, 20 mM: —▲—, 10 mM; —△—, 5 mM; —■—, 1 mM; —□—, 0.5 mM; --△--, pH of culture containing 10 mM aniline.

none by the other isolates.

Taxonomic characterization

Morphological, cultural, and biochemical characteristics of the strain FW are shown in Table 2. From the taxonomic properties described above, strain FW was identified as *Pseudomonas putida*. Also, nutritional characteristics described for identifying this strain as a biotype are shown in Table 3. On the basis of the properties of *P. putida* biotype described in Bergey's Manual of Systematic Bacteriology (12), this strain was closely related to *P. putida* biotype A, which is considered to be typical. However, this strain differs from biotype A in the use of D-galactose as a single soure of carbon.

Biodegradation of aniline

The growth of *P. putida* FW on aniline as a sole source of carbon is shown in Fig. 1. After 36 hr of incubation, the growth of *P. putida* FW was not continued any more due to depletion of aniline as a carbon source. The final cell density was increased as the concentration of aniline was increased from 0.5 mM to 20 mM. However, little growth was shown on very high concentration of aniline (30 mM) due to excess of aniline suspected to harmful to bacterial growth. On the cultures containing 10 mM aniline, the supernatant of cultures by the growth of *P. putida* FW was sustained to about pH 7.0 during 48 hr of incubation (Fig. 1). This phenomenon can be indicated that acid

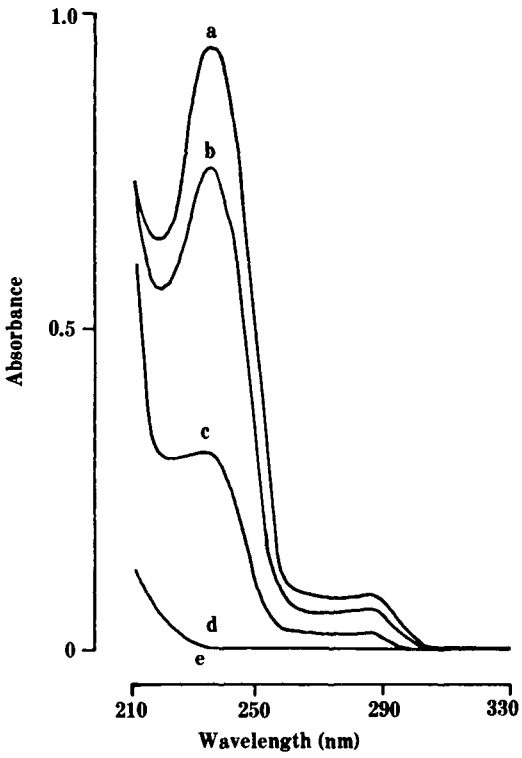

Fig. 2. UV scanning spectrum for the biodegradation of aniline by *P. putida* FW.
a, b, c, d, and e are cultures incubated for 0, 12, 24, 36, and 48 hr, respectively.

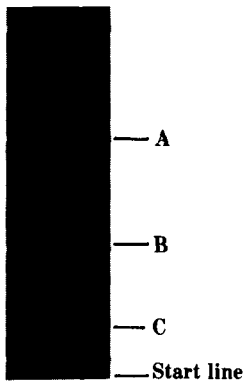

Fig. 3. Thin layer chromatogram of cultures incubated for 48 hr.
A, B, and C are compounds shown on Rf 0.89, 0.38, and 0.16, respectively. Rf: Retardation factor

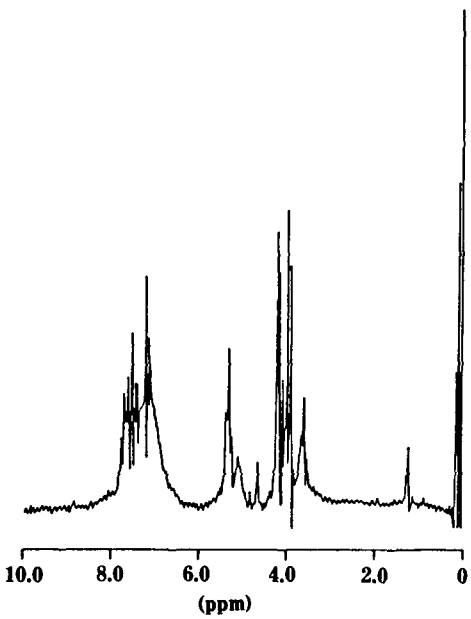

Fig. 4. H'-NMR spectrum of compound A.

metabolites are not occurred during the biodegradation of aniline by *P. putida* FW.

For demonstrating biodegradation of aniline by *P. putida* FW, further study was conducted by its UV scanning spectrum (Fig. 2). The UV scanning spectrum of aniline showed two peaks at 229 nm and 280 nm. In the cultures incubated on 10 mM aniline for 48 hr, the UV absorbance at two peaks disappeared by the degradation of aniline as the function of incubation time. These results are corresponded with the results shown in Fig. 1.

Detection and isolation of metabolic products

The pathways of degradation of aromatic compounds have been extensively analyzed in many aspects (14). In this study, the detection of any metabolic products was persuited using TLC and NMR. As shown in Fig. 2, aniline as a carbon source was fully utilized by *P. putida* FW after 36-48 hr of incubation. In a sample fully utilized by *P. putida* FW after 48 hr of incubation, several compounds were detected as biodegradation products of aniline. However, three definite compounds suspected to be metabolic products appeared on the chromatogram of TLC prepared as methods described earlier (Fig. 3). These major three compounds were designated as compound A (RF 0.89), compound B (RF 0.38), and compound C (RF 0.16). Among these three compounds, compound C was not studied by NMR because of very low concentration of it. The H'-NMR spectrum of compound A (Fig. 4) shows the existence of aromatic ring in its structure as the information of chemical shift (Fig. 5). In these results, it can be speculated that compound A and B may be main metabolites were resulted from the biodegradation of aniline by *P. putida* FW.

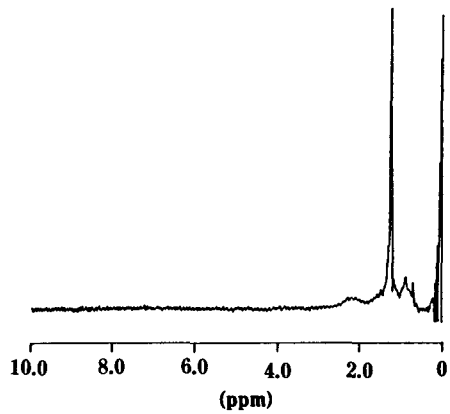

Fig. 5. H'-NMR spectrum of compound B.

Determination of antibiotic resistance

The range and mechanisms of antibiotic resistance in *Pseudomonas* may be in general determined by R factors of plasmids (8,9). By *P. putida* FW, the value of MIC of antibiotics tested are presented in Table 4. Based on the reference for MIC of An-

Table 4. Minimal inhibitory concentration to antibiotics of *P. putida* FW.

Antibiotics	MIC (g/ml)
Streptomycin	500
Tetracycline	500
Chloramphenicol	250
Ampicillin	250
Amoxicillin	250
Rifampicin	31.25
Kanamycin	1.95
Tobramycin	0.7
Gentamicin	0.49
Trimethoprim	4,000
Sulfanilamide	4,000

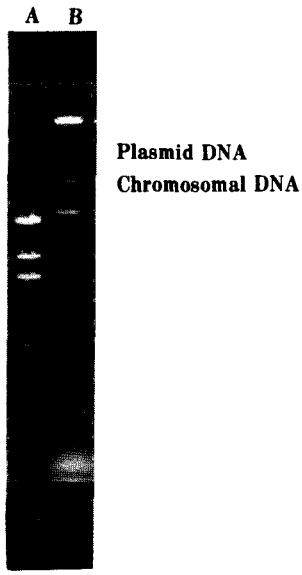

Fig. 6. Agarose gel electrophoresis of the plasmid DNA from *P. putida* FW.

Track A, λ DNA digested with Hind III; B, Plasmid DNA isolated from *P. putida* FW.

tibiotics in Laboratory Medicine (15), *P. putida* FW was shown to be resistant to streptomycin, tetracycline, trimethoprim, and sulfanilamide when compared to the value of MIC of antibiotics by *P. putida* and *P. aeruginosa*. From these results, it is presumed that *P. putida* FW may harbor plasmid genes specifying aniline utilizing or antibiotic resistance.

Detection of plasmid DNA

The agarose gel electrophoresis of plasmid DNA isolated from *P. putida* FW is shown in Fig. 6. Lane A of Fig. 6 shows DNA of bacteriophage digested by Hind III restriction endonuclease as a size marker. Lane B of Fig. 6 shows that *P. putida* FW carried one plasmid. Comparison between DNAs shown in Fig. 6 revealed that the size of plasmid isolated from *P. putida* FW would be larger than about 20 kb.

Table 5. Growth on aromatic amine substrates as a sole source of carbon.

Substrate	Growth
p-Chloroaniline	+
o-Nitroaniline	+
m-Nitroaniline	+
p-Nitroaniline	+
p-Toluidine	+
o-Toluidine	–
o-Phenylenediamine	–
Toluene	–
Salicylate	–

Utilization of compounds related to aniline

Growth of *P. putida* FW on aromatic amine substrates related to aniline as a source of carbon are presented in Table 5. Other aromatic amine compounds which supported growth as a sole source of carbon were *p*-chloroaniline, *o*-nitroaniline, *m*-nitroaniline, *p*-nitroaniline, and *p*-toluidine. Aromatic amine compounds that did not support growth as a sole source of carbon were *o*-phenylendiamine and *o*-toluidine. On salicylate and toluene as a sole source of carbon, *P. putida* FW didn't show any growth. From the properties shown in Table 3, it can be concluded that *P.*

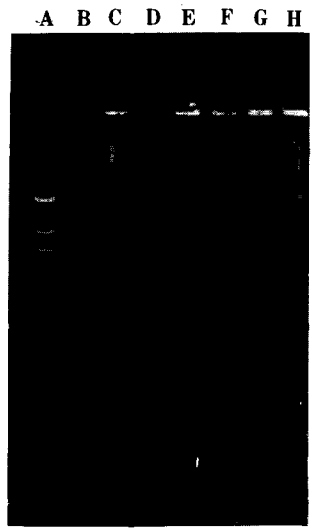

Fig. 7. Agarose gel electrophoresis of the plasmid DNA from strains treated with mitomycin C.
Lane A, λ DNA digested with Hind III; B. *P. putida* FW; C, 55a; D, 16b; E, 4a; F, 58a; G, 1a; H, 3a.

Table 6. The phenotypic characteristics of strains treated by mitomycin C.

Strain	Antibiotics (μg/ml)								
	aniline(mM)	TC		TMP		SM		SFA	
	10	100	500	400	2,000	50	250	400	2,000
1 a	+	–	–	+	–	+	–	+	+
3 a	–	–	–	–	–	–	–	+	+
4 a	+	–	–	+	–	–	–	–	–
16 b	–	–	–	–	–	–	–	–	–
55 a	–	–	–	–	–	–	–	–	–
58 a	+	–	–	+	–	–	–	+	–

Symbols; +, Growth; –, No growth; a, The strains selected from 456 colonies treated with MC (100 μg/ml) for 48 hr; b, The strain selected from 216 colonies treated with MC (7.5 μg/ml) for 24 hr; MC, Mitomycin C; TC, Tetracycline; TMP, Trimethoprim; SM, Streptomycin; SFA, Sulfanilamide.

putida FW may be a potent strain capable of degrading chemical compounds related to aromatic amines.

Curing of plasmid DNA

To determine whether the degrading aniline or antibiotic resistance are coded by plasmid DNA, following experiments were carried out. At first, the selective growth of strains treated with mitomycin C was examined for phenotypes to be cured (Table 6). Streptomycin, sulfanilamide, tetracycline, and trimethoprim shown to be resistant to *P. putida* FW in earlier results and aniline were used for these experiments. Among 672 colonies examined, only 6 colonies were shown to be cured by mitomycin C. Specially, 16 b and 55 a colonies didn't show any growth on all antibiotics tested and aniline. The electrophoresis of these 6 colonies were performed to demonstrate the loss of plasmids. As shown in Fig. 7, all 6 colonies showed the existence of plasmids. The phenotypic characteristics of 3 a, 16 b, and 55 a should be eliminated when plasmids are lost if the aniline degrading function and, or antibiotic resistance are specified by a single plasmid. However, none of 6 colonies was cured by mitomycin C even though some phenotypes speculated to be cured strains were still shown in Table 6. This phenomenon can be thought to be resulted from the mutation caused by mitomycin C as the main agent used to cure catabolic plasmids in *Pseudomonas* (19). Our experience with mitomycin C leads us to conclude that a number of strains produced cells with a much reduced growth rate or other phenotypic alteration, suggesting that mitomycin C can produce mutants at a considerable frequency. In this study, we were unable to demonstrate aniline use on extrachromosomal array in *P. putida* FW. However, we present possibility that plasmid genes in *P. putida* FW may harbor the gene cluster specifying aniline dissimilatory enzymes.

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요 약

Aniline을 유일한 탄소원으로 한 최소배지에서 성장할 수 있는 균주를 활성오니로부터 분리하여 여러가지 특성을 조사하였다. 이 균주는 분류학적인 특성에 의하여 *P. putida* biotype A로 동정되었으며 aniline을 유일한 탄소원으로 하여 배양하였을 때 10-20 mM의 농도에서 최적의 성장을 나타내었으며 배양하는 동안에 pH의 변화는 일어나지 않았다. 이 균주를 10 mM의 aniline을 유일한 탄소원으로 하여 48시간 배양한 후 UV scanning spectrum, TLC, NMR을 이용하여 분석한 결과 aniline의 생분해에 의한 대사물질이 생성되는 것으로 추정되었다. 또한 이 균주는 streptomycin, trimethoprim, tetracycline, sulfanilamide에 강한 저항성을 나타내었으며 plasmid를 1개 가지고 있는 것으로 나타났다. Mitomycin C curing을 통하여 얻어진 여러개의 변이균주의 성질을 조사하여 본 결과 이 균주의 plasmid DNA는 aniline의 분해에 관여하는 것으로 추정되었다.

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