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Isolation and Characterization of Aniline-Degrading Bacteria

Kahng, Hyung-Yeel, Seung-il Kim, Mi-Jeong Woo, Yong-Keun Park and Yung-Nok Lee

Dept. of Biology, Korea University, Seoul, Korea

Six isolated strains degrading aniline were selected, identified and designated as *Pseudomonas putida* K6, *Pseudomonas acidovorans* K82, Achromobacter gr. D.V K24, *Achromobacter xylosoxidans* K4, *Moraxella* sp. K21 and *Moraxella* sp. K22. All of them degraded 1000 ppm aniline completely within 30 to 36 hours. Most of these strains are resistant to antibiotics more than one, but *Moraxella* sp. has not any antibiotic marker tested. Most strains except for *P. acidovorans* K82 were shown to have resistance to the heavy metal ions such as Ni, Cu, Li, Ba, Co, etc, but not to Hg to which only *P. putida* K6 was resistant. M. sp. K21 was capable of degrading aniline to a maximum concentration of 2500 ppm without any repression. The incubation of the cell in limited pH ranges (4-8) had no great effect on aniline degradation. The addition of bactopeptone to the minimal media promoted the speed of aniline degradation, but the addition of glucose rather repressed the rate of aniline degradation. Through enzyme assay, A. gr.D.V K24 was shown to degrade aniline through ortho-pathway and formed β-ketoadipate as intermediate metabolite.

KEY WORDS □ ***Pseudomonas, Achromobacter, Moraxella* sp., aniline, *bactopeptone ortho* pathway**

The necessity of studying microorganisms that utilize aniline as the sole source of carbon and energy is due to the fact that many widely used herbicides and pesticides are aniline derivatives (31). Since anilines are the primary degradation products of some important classes of herbicides (carbanilates, phenylureas, acylanilides), they are ubiquitous in the environment (17, 19, 24). Nevertheless, at present, there is a limited amount of information on micrbial degradation of anilines in soil and water (36).

There are a number of possible routes of removal of toxic substrates from a natural environment, including mineralization (e.g., dehalogenation), assimilation as nutrient into microbial biomass, polymerization, volatilization, leaching, and adsorption (Andrew,M. F. and O.H., Tuovinen, 1991). In water environment, biodegradation is the most important mechanism in removal of aniline, while mineralization of anilines in soil is very slow; less than 30% of [14 C]aniline and less than 12% of [^{14}C]3,4-dichloroaniline was converted to 14CO2 in various soils over a 10-week period (32). Only soils supplemented with particular microorganisms(37) or with aniline(34) showed a rapid mineralization of 3,4-dichloroaniline. On the other hand, rather than being mineralized by microbial attack, anilines tend to undergo various spontaneous chemical transformations (7, 9, 27) in the environment and to turn into nonextractable humic acid-like compounds (4, 29, 30) or persistent xenobiotics such as azobenzenes and triazenes (18, 23)

At the present time, the possibility of using aniline as the sole source of carbon and nitrogen has been demonstrated for *Alcaligenes faecalis* (33), *Pseudomonas putida* (35), *Rhodococcus* (2), *Frateuria* sp. (3), *Moraxella* sp. (36), and for Pseudomonas acidovorans (22).

In this study, we report the characteristics of the isolated strains degrading aniline.

MATERIALS AND METHODS

Sources for screening tests

Soil of gardens, farms, and rice fields in Chochiwon, Kapyeong districts of Korea was used for screening tests.

Isolation of the strains

Enrichment and cultivation medium contains 1 g K_2HPO_4, 1 g KH_2PO_4, 0.41 g $MgSO_4.7H_2O$, 0.02 g $CaCO_3$, $FeSO_4.7H_2O$, appropriate conc. of aniline (Katayama Chem., Osaka) in 1*l* of water (pH 6.8). Higher substrate concentrations inhibited exponential growth. Aniline utilizing

microorganisms were isolated as followed: basal medium was used throughout isolation tests. The isolates were stored on agar basal medium at 4°C. For the test of utilization of various aromatic hydrocarbons, cells were inoculated on minimal agar media containing an aromatic hydrocarbon in the place of glucose, and were incubated at 30°C for 3 days to a week. To avoid toxicity of a higher concentration of aromatic hydrocarbons, they were added on various concentration (1 mM to 5 mM). For pyrocatechol and naphtalene, 0.46% of the volume of agar medium were provided in the vapor state to the cover of petri dish (38).

Identification tests

Strains isolated were incubated at 30°C. A part of them were identified by use of API 20NE Kits, and the others on the basis of Bergey's manual of systematic bacteriology (21) and Manual for the identification of medical bacteria (8). Morphological tests were observed by the Gram stain (12). Aniline degradation on limited pH ranges was tested in minimal medium, after incubation for up to 4 days with shaking; the pH of the medium was adjusted with 1N HCl or NaOH. For catalase test, the cell was spread on disks of filter paper which were dropped into 3% H_2O_2; When catalase was present the gas was quickly formed(11). For oxidase test the organism was removed with a platinum wire and smeared across the surface of the clear filter paper. A positive reaction was shown by the development of a dark purple colour within 10-second (20). Nitrate reduction and denitrification test was performed by Pickett and Pederson's method (28), OF test was conducted by growing the bacterium in two tubes of Hugh's and Leifson's (15) medium; the medium in one tube was covered with a layer of soft paraffin petrolatum. For indole detection the cell was inoculated into nutrient broth; after incubation for 48 hours 0.5ml Kovac's reagent was added and shaked well. A red colour in the reagent layer indicated indole formation (13), gelatin liquefaction was shown by a test in which the organism grows in a nutrient medium solidified by gelatin (10). Arginine hydrolysis was indicated by the development of a brown color after dropping Nessler's reagent into culture media (25). For the test of citrate utilization, Christensen's method (6) was used; the tests of utilization of various carbon sources were conducted by the method of Holding and Collee (14).

Selection of strains

Cells were inoculated into a minimal medium containing aniline (0.7 mg/m*l*), and were incubated with shaking at 30°C. An appropriate sample uptaken every 6 hour was used to measure cell density in OD600 nm, Subsequently, after it was centrifugation at 6000 rpm the supernant was used to measure the absorbance at OD280 nm by UV spectrophotometry. As a consequence, the degradative capability of aniline for the isolated strains was investigated.

Cell growth and Aniline degradation

Cells were incubated on minimal agar media ranging from 0.5 mg/m*l* to 4 mg/m*l* at 30°C for 3 days. The strains were inoculated into the solid media containing 0.5 mg/m*l* through 4 mg/m*l* aniline and incubated for 2-3 hours. In order to examine cell growth and aniline degradation on various concentration, we precultured the cell on 5 m*l* of minimal media, and transferred and cultured on the minimal media containing various concentration of aniline. To examine range of pH capable of degrading aniline, the strain precultured on aniline basal medium was transferred to each aniline medium with various pH; While the cell being cultured for up to 3 days at 30°C with shaking, every 6 hour the sample was taken and used for examination of aniline degradation.

Resistance to antibiotics and heavy metals

The resistance of the strains to antibiotics and heavy metals was tested to use them as the genetic markers. For the antibiotics' test, Ampicillin, Kanamycin, Streptomycin, Chloramphenicol, Tetracyclin, Rifampicin were used and added to LB media on various concentrations, respectively; For heavy metal test, $HgCl_2$, $NiSO_4.6H_2O$, $CuSO_4$, LiCl, $AgNO_3$, $BaCl_2$ were used. The microorgansims to be tested were inoculated to the media containing each antibiotic and heavy metal, and incubated for 2-3 days at 30°C.

Aniline degradation in the presence of supplemented nitrogen and carbon source

Strains precultured on aniline basal medium were inoculated into each minimal medium containing 1% and 0.2% bactopeptone. During the incubation of 3-4 days with shaking, 1 m*l* sample was taken and used to measure cell density at OD600 nm and absorbance of remaining aniline at OD280 nm. The same precultured cell was transferred to each minimal medium containing 0.1%, 0.2%, and 0.05% glucose. The sample uptaken was used as in the above.

Enzyme assay

Catechol 2,3-dioxygenase activity was measured by the method of Nozaki(26). Catechol 1,2-dioxygenase was assayed by the procedure of Aoki (3). A strain was inoculated on aniline minimal media and LB, and incubated for 30 hrs; Cell-free extract was prepared after destruction of the cell with bead beator (bead 0.1 mm, BioSpec) and removing cell-debris, which was used as enzyme solution. The activities were measured immediately after preparation of the extracts. Assays were carried out at saturating levels of substrate so that the reaction rates were proportional to protein content. One unit of enzyme activity was defined as the amount of enzyme required to produce or convert 1 μmol of product or substrate per min. Specific

activities were expressed as units per gram of protein at 24°C. Protein content was determined by the method of Bradford (5). Identification of β-ketoadipate was performed by Rother reaction (14).

RESULT AND DISCUSSION

Isolation and identification of microorganisms

6 strtains were isolated and selected which grew on aniline at pH 6.8 as the sole source of carbon and nitrogen after measuring cell density at OD 600nm and absorbance of aniline remaining at OD280 nm by use of UV spectrophotometry (Hewlett Packard 8452A). They were identified as *Achromobacter xylosoxidans* K4, *Pseudomonas putida* K6, *Moraxella* sp. K21, *Moraxella* sp. K22, *Achromobacter* gr. D.V K24, and *Pseudomonas acidovorans* K82, respectively (Table 1,2).

Cell growth on limited concentration of aniline and aniline degradation

A. xylosoxidans K4, *P. putida* K6, *M.* sp. K22, and *A. gr.D.V* K24 were able to grow on solid minimal media to 2.5 mg/ml aniline; *P. acidovorans* K82 to 3 mg/ml. However, *M.* sp. K-21 did not grow on above 1 mg/ml concentration of aniline (Table 3). In liquid culture, *M.* sp. K21 and *M.* sp. K22 degraded aniline (0.7 mg/ml) completely within 30 hours; *A. gr.D.V* K24 and *P. acidovorans* K82 completely degraded aniline within 24 hours (Fig. 1), whilist *A. xylosoxidans* K4 and *P. putida* K6 could not utilize above 0.5 mg/ml aniline. *Moraxella* sp. K21 incubated on limited pH ranges (4-8) has no great difference in aniline degradation, but the rate of aniline degradation was fast induced more on pH 7-8 than on strong acid. As a consequence, pH has no greatly influenced to degradation of aniline by *M.* sp. K 21.

Growth on several other aromatic hydrocarbons

Table 4 shows that *Achromobacter xylosoxidans* K4 and *Pseudomonas putida* K6 grew on benzoate, salicyate, ρ-aminobenzoate, 3-CB, and nitroaniline; *Moraxella* sp. K21 and K22 grew on low concentration of benzoate(1 mM) and ρ-

Table 1. *Chacteristics of the species in genus Pseudomonas, Achromobacter.*

Physiological tests	*A. xylosoxidans* K4	*P. putida* K6	*A. gr.D.V* K24	*P. acidovorans* K82
NO_3	−	−	−	+
T R P	−	−	−	−
G L U	−	−	−	−
A D H	+	−	−	−
U R E	−	−	−	−
E S C	−	−	−	−
G L E	−	−	−	−
P N P G	−	−	−	−
G L U	+	+	+	−
A R A	−	−	−	−
M N E	−	+	+	−
M A N	−	−	−	−
M A G	−	−	−	−
M A L	−	−	−	−
G N T	+	+	+	+
C A P	+	+	+	+
A D I	−	−	−	+
M L T	+	+	+	+
C I T	+	+	+	−
P A C	+	+	+	−
O X	+	+	+	+
K B	+	+	+	−

NO_3: reduction of nitrates to nitrites, MAN: mannose assilila ion, TRP: indole production, NAG-acetyl-glucosamine, GLU: acidification, MAL: maltose, ADH: arginine dihydrolase, GNT: gluconate, URE: urease, CAP: caprate, ESC: hydrolysis(B-glucosidase), ADI: adipate, GEL: hydrolysis (protease), MLT: malate, PNPG: B-galactosidase, CIT: citrate, GLU: glucose assimilation, PaC: phenyl-acetate, ARA: arabinose, OX; cytochrome oxidase, MNE: mannose, KB: pigment production

Table 2. *Chracteristics of the species in genus Moraxella.*

Physiological test	*Moraxella* sp. K21	*Moraxella* sp. K22
Catalase	+	+
Oxidase	+	+
Glucose(acid)	−	−
O F test	−	−
Macconkey growth	−	+
Urease	−	+
Starch hydrolysis	−	−
Arginine hydrolysis	−	+
Gelatin hydrolysis	−	−
Indole formation	−	−
Leven formation	−	+
KCN growth	−	+
Growth on citrate	−	−
42°C growth	−	−
Denitrification	−	−
Glucose assimilation	+	+
Lactose	+	+
Maltose	+	+
Mannose	+	−
Mannitol	+	+
Sucrose	+	+
L-Arginine	−	+
L-Alanine	+	+
Fructose	+	−
Trehalose	+	+
L-Valine	+	+

Table 3. *Cell growth on various concentration of aniline*

농도(mg/ml)/균주	*A. xylosaxidans* K4	*P. putida* K6	*Moraxella sp.* K21	*Moraxella sp.* K22	*A. gr. D.V* K24	*P. acidovorans* K82
0.5	+	+	+	+	+	+
1	+	+	+	+	+	+
1.5	+	+	−	+	+	+
2	+	+	−	+	+	+
2.5	+	+	−	+	+	+
3	−	−	−	−	−	+
3.5	−	−	−	−	−	−
4	−	−	−	−	−	−

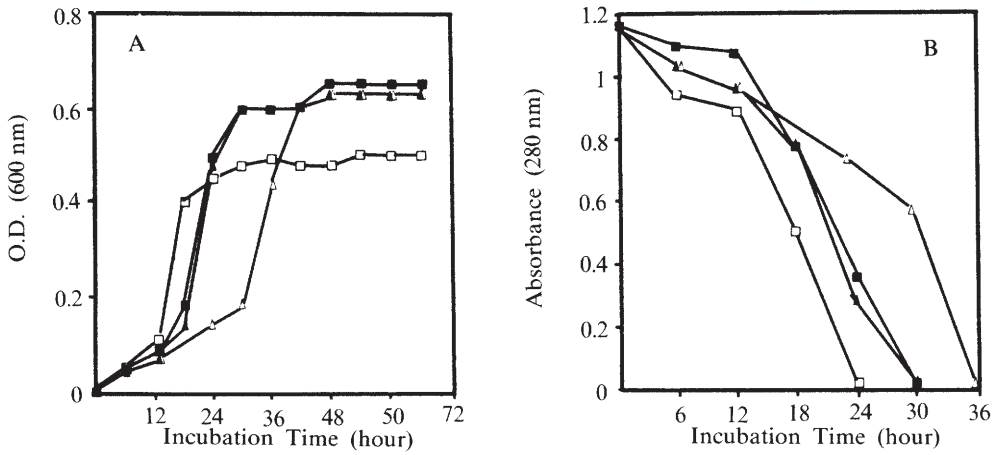

Fig. 1. *Aniline degradation and growth curve of the strains isolated in this study*
*aniline concentration: 0.7 mg/ml
A: cell density, B: remaining aniline, ▲: *Moraxella* sp. K21, □: *Pseudomonas acidovorans* K82, ■: *Moraxella* sp. K22, △: *Achromobacter gr.* D.V K24

Table 4. *Growth on several aromatic hydrocarbons.*

농도(mg/ml)/균주	*A. xylosaxidans* K4	*P. putida* K6	*Moraxella sp.* K21	*Moraxella sp.* K22	*A. gr. D.V* K24	*P. acidovorans* K82
Benzoate 1 mM	+	+	+	+	+	+
2.5 mM	+	+	+	+	+	+
5 mM	+	+	−	−	+	−
Salicylatel mM	+	+	+	+	+	+
2.5 mM	+	+	+	+	+	+
5 mM	−	+	+	+	−	−
3CB 1 mM	+	+	−	−	+	+
1 mM	+	+	−	−	+	+
2.5 mM	+	+	−	−	+	−
5 mM	+	+	−	−	−	−
p-Aminobenzoate						
1 mM	+	+	+	+	+	+
2.5 mM	−	−	+	+	+	+
5 mM	−	−	−	+	+	−
Nitroaniline 1 mM	+	+	−	−	+	−
2.5 mM	+	−	−	−	+	−
5 mM	NT*	NT	−	−	NT	−
Catechol	+	+/−	+	+	+	+
Naphtalene	−	+	−	−	+	+

*Not tested

Table 5. *Resistance to antibiotics*

Antibiotics (conc. μg/ml)		*A. xylosoxidans* K4	*P. putida* K6	*Moraxella sp.* K21	*Moraxella sp.* K22	*A. gr. D.V* K24	*P. acidovorans* K82
	200	+	−	−	−	+	+
AP	400	−	−	−	−	+	+
	800	−	−	−	−	+	+
	200	+	−	−	−	−	
Cm	400	−	−	−	−	−	−
	800	−	−	−	−	−	−
	50	+	+	−	−	−	−
Sm	100	+	+	−	−	−	−
	200	+	+	−	−	−	−
	25	−	−	−	−	+	−
Km	50	−	−	−	−	+	−
	100	−	−	−	−	+	−
Tc	100	−	−	−	−	−	+
	25	−	−	−	−	−	−
Rif	10	−	−	−	−	−	−
	25	−	−	−	−	−	−

Ap: Ampicillin, Cm: Chroamphenicol, Sm: Streptomycin, Km: Kanamycin, Tc: Tetracyclin, Rif: Rifampicin

Table 6. *Resistance to heavy metal ions*

Heavy metals (μg/ml)		*A. xylosoxidans* K4	*P. putida* K6	*Moraxella sp.* K21	*Moraxella sp.* K22	*A. gr. D.V* K24	*P. acidovorans* K82
	50	+	+	+	+	+	+
$NiSO_4.6H_2O$	100	+	+	+	+	+	+
	100	+	+	+	+	+	+
	200	+	+	+	+	+	−
$CuSO_4$	400	+	+	+	+	+	−
	800	+	+	+	+	+	−
	200	+	+	+	+	+	+
KiCl	400	+	+	+	+	+	+
	800	+	+	+	+	+	+
	200	+	+	+	+	+	−
$BaCl_2$	400	+	+	+	+	+	−
	800	+	+	+	+	+	−
	200	+	+	+	+	+	−
$Co(NO_3)_2.6H_2O$	400	+	+	−	−	−	−
	800	−	−	−	−	−	−
	50	−	+	−	−	−	−
$HgCl_2$	400	−	+	−	−	−	−
	200	−	−	−	−	−	−

aminobenzoate (2.5 mM) while they could not grow on 3-CB and nitroaniline. 6 strains all utilized catechol and salycilate which are primary metabolites of aniline so that aniline was proposed to degrade through *ortho-* and *meta-*pathway. However, the confirmable degradative pathway will be examined through an enzymatic study.

Resistance to antibiotics and heavy metals

A. xylosoxidnas K4 and *P. putida* K6 was resistant to Ap, Sm; *A. gr.D.V* K24 to Ap while *M.* sp. K21 and K22 have no any antibiotic marker tested (Table 5). Most of the strains except for *P. acidovorans* K82 were shown to have a resistance to $NiSO_4.6H_2O$, $CuSO_4$, LiCl, $Co(NO_3)_2.6H_2O$, $BaCl_2$, but not to $HgCl_2$ to which only *P. putida* K6 was resistant (Table 6).

Influence of additional nitrogen and carbon sources on aniline degradation by *M.* sp. K21

M. sp. K21 degraded aniline completely within 42 hours when only 1 mg/ml aniline was given as a sole source carbon and nitrogen source. When each 0.1% and 1% bactopeptone was added to the aniline minimal media, aniline was completely

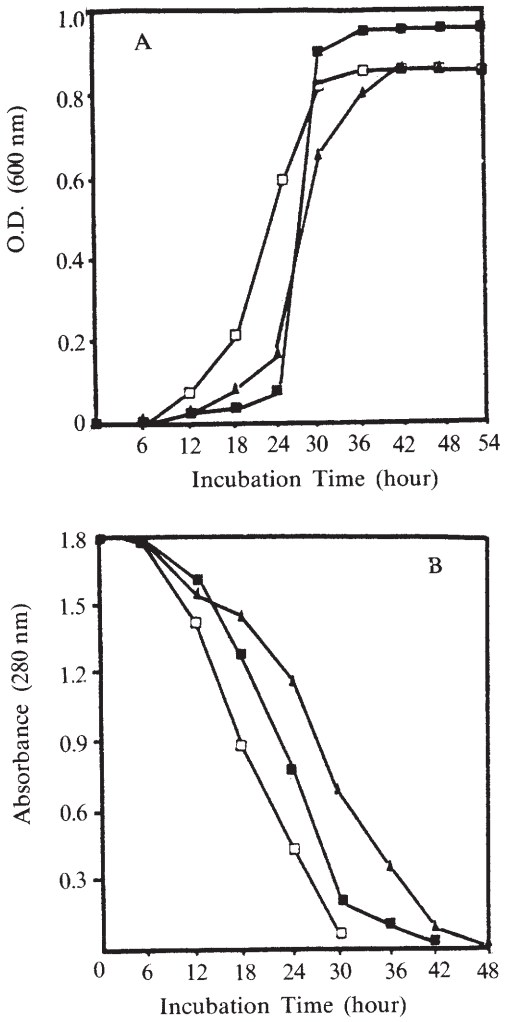

Fig. 2. *Promotion of cell growth and aniline biodegradation by additional nitrogen sources*
A: cell density, B: remaining aniline, ▲: aniline 1 mg/ml, ■: aniline 1 mg/ml+ bactopeptone 1%, □: aniline 1 mg/ml+ bactopeptone 0.2%

degraded within 30 hours. The speed of aniline degradation was acclerated more when 0.2% bactopeptone was added than when 1% bactopeptone was added (Fig. 2). However, when each 0.1 %, 0.2%, and 0.05% glucose was added to the animal media the speed of aniline degradation was reduced about twofolds at 30 hours' culture (Fig. 3). It is considered that reduction of the rate of aniline degradation by glucose was due to catabolic repression.

Activities of catabolic enzymes in cell-free extracts

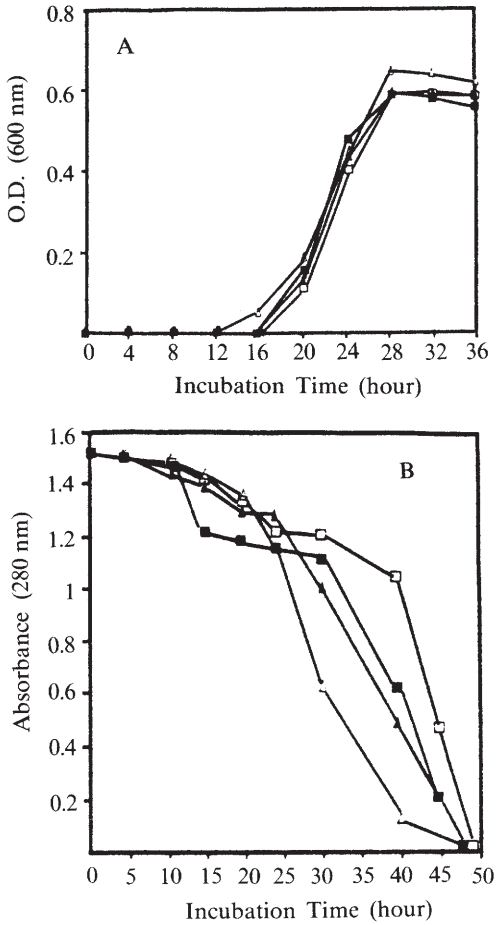

Fig. 3. *Influence of additional glucose on cell growth and aniline biodegradation*
A: cell density, B: remaining aniline, △: aniline 1 mg/ml, ■: aniline 1 mg/ml+glucose 0.1%, ▲: aniline 1 mg/ml+glucose 0.2%, □: aniline 1 mg/ml+glucose 0.5%

Table 7. *Specific activities of catechol 1,2-dioxygenase and catechol 2,3-dioxygenase in cell-free extracts of Achromobacter gr.D.V. K 24 grown on aniline.*

Substrates	Activities (U/mg)	
	Catechol 1,2-dioxygenase	Catechol 2,3-dioxygenase
Catechol	0.459	0.00
3-methylcatechol	0.324	0.00

*Specific activities was given in units per miligram of protein. 1 U represents the conversion of 1 μmol substrate per minute at 24°C.

A. gr.D.V K24 was found to have catechol 1,2-dioxygenase activity, not to have catechol 2,3-dioxygenase activity. Accordingly, aniline degradation by *A. gr. D.V* K24 was found to degrade aniline through ortho pathway (Table 7). The specific activity of catechol 1,2-dioxygenase of *A. gr.D.V* K24 was similiar to that of other aniline degrading bacteria such as *Rhodococcus erythropolis* AN-13(2) and *Pseudomonas acidovorans* (22). It was also found that β-ketoadipate was formed in media by Rother reaction.

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초 록: Aniline 분해 세균의 분리 및 특성
강형일, 김승일, 우미정, 박용근, 이영록 (고려대학교 이과대학 생물학과)

탄소 및 질소원으로 aniline을 이용하는 균주를 분리하고 6균주를 선별, 동정하여 *Pseudomonas putida* K6, *Pseudomonas acidovorans* K82, *Achromobacter gr.*D.V K24, *Achromobacter xylosoxidans* K4, *Moraxella* sp. K21, *Moraxella* sp. K22로 명명하였다. 이들 대부분의 균주는 1000 ppm의 aniline을 30-36시간안에 완전히 분해하였다. 대부분의 균주가 하나 이상의 항생제 marker를 가지고 있었으나, Moraxella속은 시험한 어떤 항생제 marker도 갖고 있지 않았다. *P. acidovorans* K82를 제외한 균주 모두 Hg를 제외한 Ni, Cu, Li, Ba, Co등과 같은 중금속에 대한 내성을 나타내었고, *P. putida* K6만이 Hg에 대한 내성을 나타내었다. 이들 균주중 *Moraxella* sp. K21의 아닐린 분해시 여러 생리적 특성을 조사한 결과 아닐린 분해는 pH4-8 사이에서 큰 영향을 받지 않았이며, 최고 2500 ppm 농도에서도 repression됨이 없이 aniline 분해능을 나타내었다. Bactopeptone과 같은 다른 질소원에 의해 그 분해속도가 가속화 되었고, glucose에 의해서는 그 분해속도가 오히려 억제되었다. 효소분석을 통해 *A. gr.*D.V K24는 aniline분해시 ortho-pathway를 거쳐 중간대사산물로 β-ketoadipate를 생성하였다.