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Isolation and Identification of *Pseudomonas* Utilizing Hydrocarbon*

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탄화수소를 자화하는 *Pseudomonas*의 분리동정

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ABSTRACT

238 strains of bacteria were isolated from sewage and soil samples collected mainly in Seoul and its suburbs by enrichment culture on crude oil or hydrocarbon minimal medium.

Of the isolates, 68 strains were tentatively identified as the genus *Pseudomonas*, 11 strains as *Alcaligenus*, and 10 strains as *Acinetobacter*. Of the 68 strains of *Pseudomonas sp.*, 35 strains were identified as *P. aeruginosa*, 5 strains as *P. fluorescence*, 10 strains as *P. putida*, and 2 strains as *P. mendocina*.

INTRCDUCTICN

The genus *Pseudomonas* is notable for the large number and variety of compounds that serve as carbon and energy sources for its member(Stanier *et al.*,1966). Recent work in a number of laboratories has indicated that, in certain strains, the genes coding for the enzymes responsible for catabolism of some of the less common substrates are carried on transmissible plasmid. Salicylate(Chakrabarty, 1972), naphthalene(Dunn and Gunsalus, 1973), camphor(Rheinwald *et al.*, 1973), octane(Chakrabarty *et al.*, 1973), benzoate and toluates(Williams and Murray, 1974), xylene(Friello *et al.*, 1976), nicotine(Thacker *et al.*, 1978), toluene and ethylbenzene(Kanemitsu *et al.*, 1980) are all compounds the breakdown of which appears to be plasmid coded in certain *Pseudomonas* strains.

But, in the case of naphthalene catabolism, it has been reported that the same pathway can apparently be determined by plasmid or chromosomal genes in different strains(Dunn and Gunsalus, 1973),

Meanwhile, it has been reported that a strain of oil-degrading *Pseudomonas* isolated from the marine environment has a degradative plasmid (Devereux and Sizemore, 1980).

In this research, as the first step of an experiment to study the degradative plasmids in *Pseudomonas*, 68 strains of hydrocarbon-utilizing pseudomonads were isolated and identified.

MATERIALS AND METHODS

Sampling

Sampling was done from May to July in 1982 and April to May in 1983. Sewage and soil samples were taken from the areas thought to have been polluted severely with oil mainly in Seoul and its suburbs.

Isolation of bacteria

For the enrichment and isolation of bacteria utilizing a hydrocarbon as sole carbon source, the following compositions of media and the procedures were employed.

1) Media

Medium A(Jobson *et al.*, 1672), pH 7.0

It contains K_2HPO_4, 0.5g/*l*; NH_4Cl,1.0g/*l*;

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Na_2SO_4, 2.0g/l; KNO_3, 2.0g/l; $CaCl_2 \cdot 6H_2O$, 0.001g/l; $MgSo_4 \cdot 7H_2O$, 1.0g/l; $FeSO_4$, trace amount; crude oil (carbon source), 1ml/l.

Medium B(Murray *et al.*, 1972) pH 7.0

It contains $(NH_4)_2SO_4$, 1.0g/l; KH_2PO_4, 5.0 g/l; $MgSO_4 \cdot 7H_2O$, 0.1mg/l; $FeSO_4 \cdot 7H_2O$, 5mg/l; nitrilotriacetic acid, 0.5g/l; 1ml/l of stock salts solution and carbon source (naphthalene or sodium salicylate).

Naphthalene was added separately to the medium in crystalline form at a concentration of 0.46%(wt/vol). Minimal agar plates were solidified with 1.5% agar and naphthalene was provided in the vapor phase by adding crystals to petri dish lids at a concentration equivalent to 0.46%(wt/vol) of the plate volume(Zuniga *et al.*, 1981).

Sodium salicylate was added to the medium at 10 mM concentration(Chakrabarty, 1972).

*The stock salts solution

MgO, 10.75g/l; $CaCO_3$, 2.0g/l; $FeSO_4 \cdot 7H_2O$, 4.5 g/l; $ZnSO_4 \cdot 7H_2O$, 1.44g/l; $MnSO_4 \cdot 4H_2O$, 1.12g/l; $CuSO_4 \cdot 5H_2O$, 0.25g/l; $CoSO_4 \cdot 7H_2O$, 0.28g/l; H_3BO_3, 0.06g/l; conc HCl, 51.3ml/l.

Medium C(Rheinwald *et al.*, 1973)

It contains potassium phosphate buffer, pH 6.8, 37mM; NH_4Cl, 40mM; $MgCl_2$, 1.6mM; $MnCl_2$, 0.3mM; and trace amounts of Fe^{++}, Ca^{++}, NaCl and $NaMoO_4$. Camphor, carbon source, was added to the medium at 10mM concentration.

Medium D(Charkrabarty *et al.*, 1973)

It contains 25ml/l of 1M K_2HPO_4, 12.5ml/l of 1M KH_2PO_4, 40ml/l of 1M NH_4Cl, and 10ml/l of a salt soution. The salt soultion contained per liter: 19.5g of $MgSO_4$ 5g of $MnSO_4 \cdot H_2O$, 5g of $FeSO_4 \cdot 7H_2O$, 0.3g of $CaCl_2 \cdot 2H_2O$, and 1g of ascorbic acid. The salt solution was sterilized by filtration through 0.22-μm Millipore filters and was added to the autoclaved and cooled mineral salts medium along with other supplements.

For growth on minimal octane, plates were poured and allowed to solidify; a 1-cm strip of agar was removed from one side of the plate and 1~2ml of octane was added. Octane positive cells grew under these conditions within 48hr by uptake of octane vapor.

2) Procedure

(1) 1ml of sewage sample was inoculated in a 250ml flask containing 50ml of medium A, and then incubated, with shaking, at 30°C. Following the appearance of turbid growth, usually within 3 to 4 days, 1ml of culture was transferred to second flask containing the crude oil medium and incubated an additional 3 to 4 days. By streaking the resulting cultures onto the nutrient agar plates, pure cultures were obtained.

(2) 1g of soil was suspended in 100ml of NaCl solution(0.9%), and then it was shaked thoroughly for 60 min. 1ml of the upper aqueous part of the solution was inoculated in a 250ml flask containing 50ml of above each medium, and then incubated, with shaking, at 30°C. Following the appearance of turbid growth, usually within 3 to 7 d, pure cultures were obtained bv streaking the resulting cultures onto the each minimal hydrocarbon agar plate.

Maintenance

Isolated crude oil-degrading bacteria were maintained on nutrient agar slopes and stored at 4°C. Isolated bacteria that utilized a hydrocarbon as sole carbon source were maintained on each minimal hydrobon agar slope and nutrient agar slopes and stored at 4°C.

Identification

Identification was based on Gram negative aerobic rods and cocci in Bergey's Manual of Determinative Bacteriology(Buchanan and Gibbons, 1974) and The Prokaryotes(Starr *et al.*, 1981). The characteristics examined for the identification are as follows:

1) Morphological characteristics

Isolates, grown on nutrient agar slopes, were examined after 18 h. Gram reaction and cell morphology were recorded from observations of heat-fixed smears stained by the Gram reaction.

Motility was determined by examination of wet-mount preparations under a light microscope. The number and type of flagella were determined by the method of Mayfield and Inniss(1977). Spores were examined by Schaeffer-Fulton method after 48 h(Gerhardt *et al.*, 1981). The presence of pyocyanin(a blue phenazine pigment) and fluorescent pigments was determined by examining 24- to 48-h cultures in daylight and under ultraviolet light(King *et al.*, 1954).

2) Physiological and biochemical characteristics

Catalase activity was determined by the addition of a 3% solution of hydrogen peroxide to 24-h cultures, and a positive response was recorded when effervescence of oxygen resulted (Gerhardt *et al.*, 1981). Oxidase activity was determined by smearing 24-h cultures on the filter paper moistened with a 1% solution of tetramethyl-*p*-phenylenediamine dihydrochloride, and a positive response was recorded when a violet or purple color was developed in 10 s (Kovacs, 1956). Oxidative and fermentative metabolism of glucose was determined by using the method of Hugh and Leifson(1953). Nitrate reduction was recorded from nitrate broth using the method of Cowan and Steel(Cowan, 1975). The resistance to penicillin G was determined by using the gradient plate technique(Szybalski, 1952). Arginine dihydrolase activity was detected by the method of Thornley(Gerhardt *et al.*, 1981). The ability to perform denitrification was examined by the method described by Stanier *et al.* (1966). The ability to grow at 41°C was determined by examining the growth in liquid media such as nutrient broth-yeast extract (Starr *et al.*, 1981). Levan formation from sucrose was detected on the nutrient agar plate that contains 4% sucrose(Klinge, 1960).

For gelatin hydrolysis(Starr *et al*, 1981) test, nutrient broth-agar supplied with 1% gelatin is streaked with the strain to be tested. After growth of the bacteria, the agar plate was flooded with a solution of saturated ammonium sulfate in 1N sulfuric acid. A hydrolysed zone became visible by precipitation of gelatin in the nonhydrolysed area. For starch hydrolysis(Starr *et al.*, 1981) test, a suitable medium was nutrient agar supplied with 0.5% soluble starch. The agar was streak-inoculated and incubated at 37°C for 2-3 d. After growth of the bacteria, the plate was flooded with Lugol's solution. Areas in which starch had not been hydrolyzed was stained blue; areas in which starch had been hydrolyzed remained colorless. The ability to utilize glucose, trehalose, *meso*-inositol, L-valine, β-alanine, DL-arginine, xylose was tested, using basal medium to which the carbon sources were added to a final concentration of 1%(wt/vol) (Palleroni and Doudoroff, 1972).

RESULTS AND DISCUSSION

181 crude oil-degrading bacterial strains and 57 bacterial strains that utilized a hydrocarbon as sole corbon source were isolated. Some of the isolated strains were tentatively identified as the genus *Pseudomonas*, *Alcaligenes*, and *Acinetobacter*(Table 1). 68 strains belong to *Pseudomonas*, 11 strains to *Alcaligenes* and 10 strains to *Acinetobacter*. The 10 strains of *Acinetobacter* were resistent to penicillin G at about 200-400 i.u./ml.

The 68 strains of *Pseudomonas* were also identified at the species level(Table 2). Of the 68 strains, 35 strains were identified as *P. aeruginosa*, 5 strains as *P. fluorescence*, 10 strains as *P. putida*, and 2 strains as *P. mendocina* and the remaining 16 strains couldn't be identified as any species of *Pseudomonas*. Their characteristics were indicated in Table 3. All of them didn't produce diffusible fluorescent pigments and a soluble phenazine pigment, pyocyanin. Meanwhile, 4 strains of 5 *P. fluorescence* could denitrify but the remaining one strain not.

Of the 57 bacterial strains utilizing a hydrocarbon as sole carbon source, 35 strains were

Table 1. Characteristics of Genus *Pseudomons*, *Alcaligenes*, and *Acinetobacter*.

Characteristics \ Genus	*Pseudomonas*	*Alcaligenes*	*Acinetobacter*
No. of strains	68	11	10
Shape	rod	rod	coccobacillus
Gram stain	−	−	−
Oxidase	+	+	−
Catalase	+	+	+
OF(Glucose)	oxidative	alkaline	oxidative
Motility	+	+	−
Flagella	polar, mono or multitrichous	peritrichous	NT
Spore	−	NT	NT
Nitrate reduction	NT	+	NT
Xylose	NT	−	NT
Resistance to penicillin G	NT	NT	+

*NT, not tested. *+, positive response; −, negative response.

Table 2. Characteristics of the species of the genus *Pseudomonas*

Characteristics \ Species	*P. aeruginosa*	*P. fluorescence*	*P. putida*	*P. mendocina*
No. of flagella	1	>1	>1	1
Fluorescent pigment	+	+	+	−
Pyocyanin	+	−	−	−
Growth at 41°C	+	−	−	+
Levan formation from sucrose	−	−	−	−
Arginine dihydrolase	+	+	+	+
Denitrification	+	d	−	+
Hydrolysis of gelatin	+	+	−	−
Hydrolysis of starch	−	−	−	−
Carbon sources for growth:				
Glucose	+	+	+	+
Trehalose	−	+	−	−
meso-Inositol	−	+	−	−
L-Valine	+	+	+	+
β-Alanine	+	+	+	+
DL-Arginine	+	+	+	+

*d: denitrification positive for 4 of 5 *P. fluorescence*, but negative for the remainder

isolated by the salicylate enrichment, 14 strains by the naphthalene enrichment, 7 strains by the *n*-octane enrichment, and only one strain by the camphor enrichment (Table 4). There were more bacteria from the salicylate enrichment than those from the other enrichments.

Williams and Worsey(1976) isolated thirteen bacteria from nine different soil samples by selective enrichment culture on *m*-toluate(*m*-methylbenzoate) minimal medium. Eight of these were classified as ***Pseudomonas putida***, one as a fluorescent ***Pseudomonas sp.***, and four as nonfluorescent ***Pseudomonas sp.*** Although different carbon sources were used in this research, 57 bacterial strains were isolated from 28 soil samples and nine of these were classified as *P-*

Table 3. Characteristics of unidentified *Pseudomonas sp.*

Strain No.	182	184 186	192	198 201 219 223	199	202 207 208	203	216	234	235
No. of flagella	1	1	1	1	1	>1	>1	1	1	1
Fluorescent pigment	−	−	−	−	−	−	−	−	−	−
Pyocyanin	−	−	−	−	−	−	−	−	−	−
Growth at 41°C	−	−	−	−	−	−	−	−	−	−
Levan formation from sucrose	−	−	−	−	−	−	−	+	+	+
Arginine dihydrolase	+	+	+	−	+	−	−	−	+	+
Denitrification	−	−	+	−	−	−	−	−	−	−
Hydrolysis of gelatin	+	−	−	−	−	−	+	−	−	+
Hydrolysis of starch	−	−	−	−	−	−	−	−	−	+

Table 4. Degradation of the carbon sources used for the enrichment culture

Carbon source	No. of strains	No. of *Pseudomonas*	No. of *P. putida*
Crude oill	181	43	1
Sodium salicylate	35	16	6
Naphthalene	14	4	1
Camphor	1	1	1
n-Octane	7	4	1
Total	238	68	10

Table 5. Pseudomonas species from different sources

Source	Sewage	Soil	Total
Total isolates	181	57	238
Species:			
P. aeruginosa	35	0	35
P. fluorescence	5	0	5
P. mendocina	2	0	2
P. putida	1	9	10
Psedomonas. sp	0	16	16

seudomonas putida and sixteen as nonfluorescent *Pseudomonas sp.* From the sewage samples, meanwhile, four species of *Pseudomonas* were isolated and *Pseudomonas aeruginosa* was predominant among them (Table 5).

摘 要

서울 근교에서 채취한 河川水 및 土壤標品으로 부터 238菌株의 탄화수소資化細菌을 分離하였다. 分離된 菌株중 68菌株는 *Pseudomonas*屬으로 同定되었고, 11菌株는 *Alcaligenes*屬으로, 10菌株는 *Acinetobacter* 屬으로 同定되었다. 68菌株의 *Pseudomonas*중, 35菌株는 *P.aeruginosa*, 5菌株는 *P. fluorescence*, 10菌株는 *P. putida*, 그리고 2菌株는 *P. mendocina*로 각각 同定되었다.

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