KOR. JOUR. MICROBIOL. March, 1987 p. 9-16 Vol. 25, No. 1

# Characterization of SAL plasmid isolated from *Pseudomonas putida*

**Kim, Hee-Yun, Young-Bock Lim, and Yung-Nok Lee**
*Department of Biology, College of Science, Korea University, Seoul, Korea*

# *Pseudomonas putida* 에서 분리한 SAL 플라스미드의 특성

김희윤 · 임영복 · 이영록
고려대학교 이과대학 생물학과

**ABSTRACT: Three strains of bacteria utilizing salicylate, KU801(pKU5, pKU8), KU803 (pKU6, pKU9), and KU806(pKU7, pKU10), were selected from the isolates and identified as *Pseudomonas putida*. By agarose gel electrophoresis, it was found that the strains had two plasmids each. All three strains were resistant to antibiotics such as ampicillin, tetracyclin, and chloramphenicol, and did not utilize other aromatic and aliphatic hydrocarbons examined except salicylate. The plasmids(pKU5, pKU6, and pKU7) of larger molecular weight were cured by treatment with mitomycin C and frequencies of curing were 0.4%, 1.67%, and 0.75%, respectively. Cured strains did not degrade salicylate and still had antibiotic resistances, which were identical with wild strains. The genes for salicylate degradation were proved to be encoded on their plasmids. The molecular weights of pKU 5 and pKU 6 were estimated as 103.5 Md, and that of pKU 7 as 101 Md. The new SAL plasmids, pKU 5, pKU 6, and pKU 7 were transferred to *P. putida* and *P. aeruginosa*, but not to *E. coli*.**
**KEY WORDS □ SAL plasmid; *Pseudomonas putida*.**

Many synthetic chemicals are added to our environment in the form of herbicides, pesticides and industrial effluents, and the problem of counteracting or neutralizing these wastes is becoming urgent. Soil microorganisms, particularly those belonging to the genus *Pseudomonas*, are able to transform or utilize a wide range of natural and synthetic organic compounds(Stanier *et al.*, 1966). Recent works have indicated that, in certain strain, the genes responsible for the degradative functions are carried not on the chromosome but are borne on plasmids. Thus the nutritional versatility can be achieved by the occurrence of broadly transmissible plasmids(Benson and Shapiro, 1978). Conjugative extrachromosomal elements conferring metabolic specificity for the degradation of salicylic acid(SAL), naphthalene(NAH), and xylene and toluene(TOL) have been isolated and studied(Chakrabarty, 1972; Dunn and Gunsalus, 1973; Worsey and Williams, 1975). Nonconjugative degradative plasmids have been also described, including one specific for toluene and xylene(XYL; Friello *et al.*, 1976).

In this report, we describe on the properties of new SAL plasmids isolated from *Pseudomonas putida*.

## MATERIALS AND METHODS

### Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1.

### Media and culture conditions

The medium L broth (Miller, 1972) was used as a complete medium, and M9 (Maniatis *et al.*, 1982) was used as a minimal medium. For growth of *Pseudomonas* strains utilizing salicylate as a sole source of carbon, the M9 medium containing 10 mM sodium salicylate instead of glucose (M9-Sal) was used. Incubations were carried out at 30℃ for *P. putida* and at 37℃ for *P. aeruginosa* and *E. coli*. For the carbon source utility test, cells were inoculated into M9 medium containing each aromatic hydrocarbon instead of glucose and incubated for one week at 30℃ as previously (Kim and Lee, 1984). Used carbon sources were salicylate, toluene, xylene, benzene, cyclohexane, naphthalene, camphor, octane, and catechol.

### Isolation and identification of bacterial strains

Sampling was done from February to June in 1985. Soil samples of stream and industrial waste were taken from the areas thought to have been polluted in Seoul and its surburbs. The enrichment and isolation of bacterial strains utilizing salicylate as a sole source of carbon were carried out as described previously (Kim and Lee, 1984). The species of selected three strains having plasmids among isolates were identified. Identification test was carried out by the method of Palleroni (1984).

### Isolation of plasmid DNA

Crude lysates of plasmid DNAs were prepared according to the method of Nakazawa *et al.* (1980). Cells were grown overnight in

**Table 1.** *Bacterial strains and their characteristics.*

| Strain/Plasmid | Relevant characteristics | Source or Reference |
|---|---|---|
| *Pseudomonas putida* | | |
| KU801 (pKU5, pKU8) | wild type/pKU5 ($Sal^+$) | This work |
| KU803 (pKU6, pKU9) | wild type/pKU6 ($Sal^+$) | This work |
| KU806 (pKU7, pKU10) | wild type/pKU7 ($Sal^+$) | This work |
| TN1032 | *trp*$^-$ *leu*$^-$ *ben1* $Str^r$ | Nakazawa and Yokota (1977) |
| TN1307 | *trp*$^-$ *leu*$^-$ *ben1*$^-$ $Str^r$ *recA*$^-$ | Nakazawa and Yokota (1977) |
| TN1126 | *met*$^-$ *trp*$^-$ | Nakazawa and Yokota (1977) |
| TN1126 (RSF1010) | *met*$^-$ *trp*$^-$/RSF1010 ($Sm^r$) | Transformation of TN1126 with RSF1010 |
| *Pseudomonas aeruginosa* | | |
| PAO303 (pMG1) | *arg*$^-$ /pMG1 ($Sm^r$) | Hansen and Olsen (1978) |
| PAO303 (Rms148) | *arg*$^-$/Rms148 ($Sm^r$) | Sagai *et al.* (1975) |
| PAO2003 | *arg*$^-$ *recA*$^-$ | Olsen *et al.* (1982) |
| PAO8 (R18) | *met*$^-$ *ilv*$^-$ $Str^r$/R18 ($Km^r$) | Issac and Holloway (1968) |
| KU815 (R18, pKU6, pKU9) | /R18 ($Km^r$) pKU6 ($Sal^+$) | Conjugation of PAO8 with KU803 |
| *Pseudomonas* sp | | |
| KU802, KU804, KU805 KU807, KU808 | wild type ($Sal^+$) | This work |
| *Escherichia coli* | | |
| HB101 | $r^-$ $m^-$ *pro*$^-$ *leu*$^-$ $Str^r$ *recA*$^-$ | Hamer *et al.* (1976) |
| HB101 (pRK290) | /pRK290 ($Tc^r$) | Corbin *et al.* (1982) |
| C600 (RP4) | /RP4 ($Ap^r$ $Tc^r$ $Km^r$) | Barth and Grinter (1977) |

L broth. Harvested cells were treated with lysozyme (1mg/m*l*) in 25% Sucrose-50 mM Tris-HCl(pH 8.0) for 5 min at 0℃, followed by incubation for another 5 min in the presence of 6.25 mM EDTA. The cells then the presence of 6.25 mM EDTA. The cells then treated with 0.5% Brji 58-0.2% sodium deoxycholate-62.5 mM EDTA in 50 mM Tris-HCl(pH 8.0) for 10 min at 0℃, and centrifuged at 30,000×g for 20 min at 2℃. Plasmid DNA contained in the supernatant was precipitated with 10% polyethylene glycol in the presence of 0.5 M NaCl. The precipitate was collected by centrifugation and dissolved in 50 mM Tris-HCl-5 mM EDTA-5 mM NaCl(pH 8.0) and then subjected to electrophoresis.

**Agarose gel electrophoresis**

Agarose gel electrophoresis was performed in a vertical slab gel of 0.7% agarose in TAE buffer composed of 0.4 M Tris, 0.2 M sodium acetate and 0.01 M EDTA at pH 8.0. Gels were run at 120 V for 2 hr. Gels were stained in a solution of ethidium bromide (1 ug/m*l*) for 20 min, rinsed and photographed under UV illumination.

**Antibiotics resistance test**

For the determination of resistance to various antibiotics, bacterial suspensions were plated on L agar containing different concentrations of various antibiotics respectively and incubated for 48 hr at 30℃.

**Curing with mitomycin C**

The method of Rheinwald *et al.* (1973) was used as follows. Cells were inoculated into 5 m*l* of L broth. After overnight growth 0.1 m*l* of the culture ($10^4$ to $10^5$) were inoculated into 5 m*l* of L broth containing mitomycin C and incubated with shaking for 48 hr. The culture was then diluted and plated on L agar. Colonies were then replica-plated to another L agar plate and also M9-Sal agar plate.

**Bacterial conjugation**

Transfer frequency was determined by mating on membrane filters as described by De Graaf *et al.* (1973). Exponentially growing donors were mixed with an equal volume of recipient culture in the exponential phase. The mixture was filtered on a membrane filter (0.2 $\mu$m pore size, 25 mm diameter Gelman Science, INC). Membrane was placed on the surface of a freshly prepared Penassay broth agar plate and incubated for 5 hr at 30℃ or 37℃. Bacteria were then suspended in 1 m*l* of saline, and 0.1 m*l* samples of suitable dilutions were spread on the selective medium. Conjugation frequency was determined after the titration of donor cells on L agar plates. When *P. aeruginosa* PAO strains were used as recipients, recipient cells were heated prior to mating for 10 min at 50℃ (Curtiss III, 1981).

## RESULTS AND DISCUSSION

**Isolation and identification of bacterial strains**

Eighty-six bacteria strains utilizing salicylate as a sole source of carbon were isolated. Three strains, KU801, KU803, and KU806, which contained plasmids respectively, were selected among them (Fig.1). From the morphological, physiological, and biochemical characteristics of the strains, they were identified as *Pseudomonas putida* respectively.

**Carbon source utility**

Selected strains, KU801, KU803, and KU806, were utilized salicylate and catechol as a sole source of carbon. Toluene, xylene, benzene, cyclohexane, naphthalene, camphor, and octane were not utilized by the selected strains.

**Resistance to antibiotics**

The resistances of the selected strains to different concentration of various antibiotics tested were very similar. All the three strains had resistance to 1600 $\mu$g/m*l* of chloramphenicol, 800 $\mu$g/m*l* of ampicillin, 50 $\mu$g/*l* of tetracyclin, but were sensitive to more than 25 $\mu$g/m*l* of gentamycin, streptomycin, and kanamycin respectively.

**Curing of cells harbouring SAL plasmid**

When KU801, KU803, and KU806 were

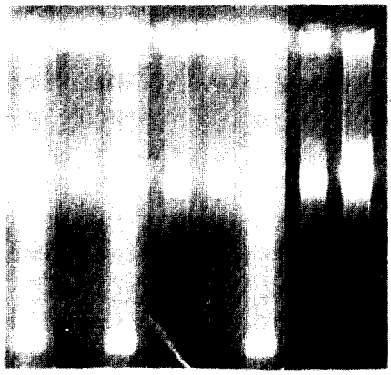


**Fig.1.** *Agarose gel electrophoresis of plasmids isolated from Pseudomonas strains.*

1. *P. putida* KU801
2. *Pseudomonas* sp. KU802
3. *P. putida* KU803
4. *Pseudomonas* sp. KU804
5. *Pseudomonas* sp. KU805
6. *P. putida* KU806
7. *Pseudomonas* sp. KU807
8. *Pseudomonas* sp. KU808

* The DNA bands between chromosome and small plasmid in lane 1, 3, and 6 are oc form of small plasmid.

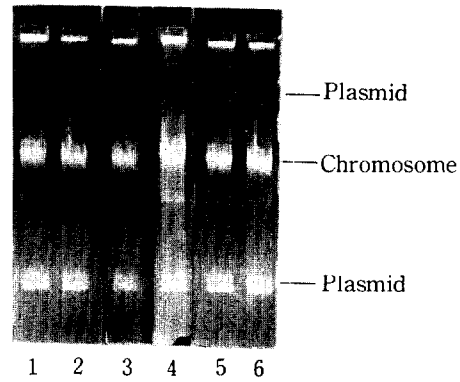


**Fig.2.** *Agarose gel electrophoresis of the cured strains.*

1. *P. putida* KU801
2. *P. putida* KU811(Cured strain of KU801)
3. *P. putida* KU803
4. *P. putida* KU813(Cured strain of KU803)
5. *P. putida* KU806
6. *P. putida* KU816(Cured strain of KU806)

treated with various concentration of mitomycin C, no growth appeared with 17.5 $\mu$g/m*l*, 12.5$\mu$g/m*l*, and 20$\mu$g/m*l*, respectively and thus 15$\mu$g/m*l*, 10$\mu$g/m*l*, and 17.5$\mu$g/m*l* were considered as sublethal concentration in KU801, KU803, and KU806, respectively. Loss of the ability to grow on salicylate at these mitomycin C concentration was varied from 0.40% to 1.67% of total viable colonies (Table 2). Agarose gel electrophoresis of the cured strains (Fig.2) also indicates that the inability to utilize salicylate is due to the loss of

**Table 2.** *Curing frequency of the selected Pseudomonas putida strains by mitomycin C.*

| Strains | Concentration of mitomycin C ($\mu$g/m*l*) | No. of colonies tested | Used marker for curing | Frequency of curing (%) |
|---|---|---|---|---|
| KU801 | 15 | 500 | Salicylate | 0.40 |
| KU803 | 10 | 900 | Salicylate | 1.67 |
| KU806 | 17.5 | 936 | Salicylate | 0.75 |

plasmid-borne genes which involves in salicylate degradation. Plasmids of the small molecular weight still exist in the cured strains and antibiotic resistances of cured strains are identical with those of the native strains. It was suggested that the samll plasmids were nothing to do with salicylate degradation, but with antibiotic resistance.

**Conjugative transfer of the ability of salicylate degradation**

The fact that the ability of salicylate degradation is plasmid-borne is also inferred from the data in Table 3. SAL plasmids can be transferred at a frequency of $10^{-5}$ to $10^{-7}$ into *P. putida* and $10^{-6}$ to $10^{-7}$ into *P. aeruginosa*. It has been reported that SAL plasmid undergoes restriction when *P. aeruginosa* PAO strains is grown at 30℃ or 37℃ (Chakrabarty, 1972; Kanemitsu, 1980). However, when *P. aeruginosa* PAO strains were heated and then used as recipients (in Methods), transfer of SAL plasmid occred without restriction. The SAL plasmid in *P. aeruginosa* KU815, conjugant formed by mating PAO8 as a recipient with KU803 as a

**Table** 3. *Conjugative transfer of the ability of salicylate degradation to various strains.*

| Donor | Recipient | Selected marker** | Frequency of transfer* |
|---|---|---|---|
| *P. putida* KU801 | *P. putida* TN1032 | *trp, leu, Sm, Sal* | $4.0\times10^{-5}$ |
| | *P. putida* TN1307 | *trp, leu, Sm, Sal* | $3.0\times10^{-6}$ |
| | *P. aeruginosa* PAO2003 | *arg, Sm, Sal* | $1.8\times10^{-6}$ |
| | *P. aeruginosa* PAO8 | *met, ilv, Sm, Sal* | $1.0\times10^{-6}$ |
| | *E. coli* HB101 | *pro, leu, Sm Sal* | 0 |
| *P. putida* KU803 | *P. putida* TN1032 | *trp, leu, Sm, Sal* | $2.0\times10^{-6}$ |
| | *P. putida* TN1307 | *trp, leu, Sm, Sal* | $3.0\times10^{-7}$ |
| | *P. aeruginosa* PAO2003 | *arg, Sm, Sal* | $5.0\times10^{-7}$ |
| | *P. aeruginosa* PAO8 | *met, ilv, Sm, Sal* | $1.6\times10^{-6}$ |
| | *E. coli* HB101 | *pro, leu, Sm, Sal* | 0 |
| *P. putida* KU806 | *P. putida* TN1032 | *trp, leu, Sm, Sal* | $2.0\times10^{-5}$ |
| | *P. putida* TN1307 | *trp, leu, Sm, Sal* | $1.0\times10^{-6}$ |
| | *P. aeruginosa* PAO2003 | *arg, Sm, Sal* | $9.0\times10^{-8}$ |
| | *P. aeruginosa* PAO8 | *met, ilv, Sm, Sal* | $2.0\times10^{-6}$ |
| | *E. coli* HB101 | *pro, leu, Sm, Sal* | 0 |
| *P. aeruginosa* KU815 | *P. putida* TN1307 | *trp, leu, Sal* | $4.1\times10^{-7}$ |

* Frequency of transfer: number of conjugants/number of donors
** The media used were M9 minimal media.

donor, could be transferred at a frequency of $4.1\times10^{-7}$ back into *P. putida*. These results indicated that SAL plasmids which isolated in this study are not restricted in *P. aeruginosa* PAO strain.

Agarose gel electrophoresis of the conjugant strains (Fig.3) shows that both of the plasmids in donor cells were transferred to the recipient cells. This seems to be a result that transmissible SAL plasmid provides the transfer to nontransmissible small plasmid (Freifelder, 1983). When SAL plasmid was transferred to *E. coli*, conjugants were not obtained. However, it is not clear whether the transmissible plasmids is not transferred to the members of other genera (Chakrabarty, 1972, Chakrabarty, 1976) or in enteric bacteria were not expressed the genes for hydrocarbon oxidation (Farrell and Chakrabarty, 1979).

**Determination of molecular weight of plasmid DNA**

The molecular weight of the plasmids was determined using covalently closed circular

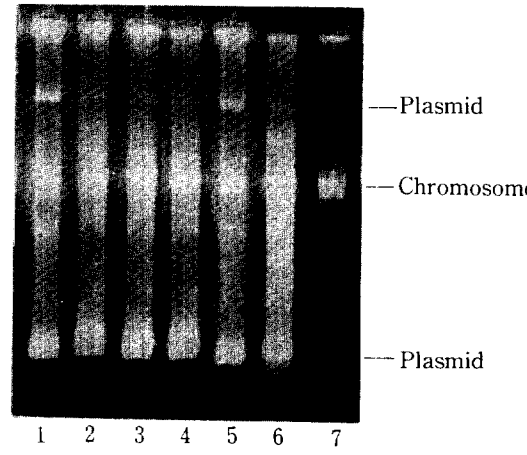


**Fig.**3. *Agarose gel electrophoresis of the conjugan's.*

1. *P. putida* KU801 (donor)
2. *P. putida* KU812 (conjugant of TN1307 with KU801)
3. *P. putida* KU803 (donor)
4. *P. putida* KU814 (conjugant of TN1307 with KU803)
5. *P. putida* KU806 (donor)
6. *P. putida* KU817 (conjugant of TN1307 with KU806)
7. *P. putida* TN1307 (recipient)

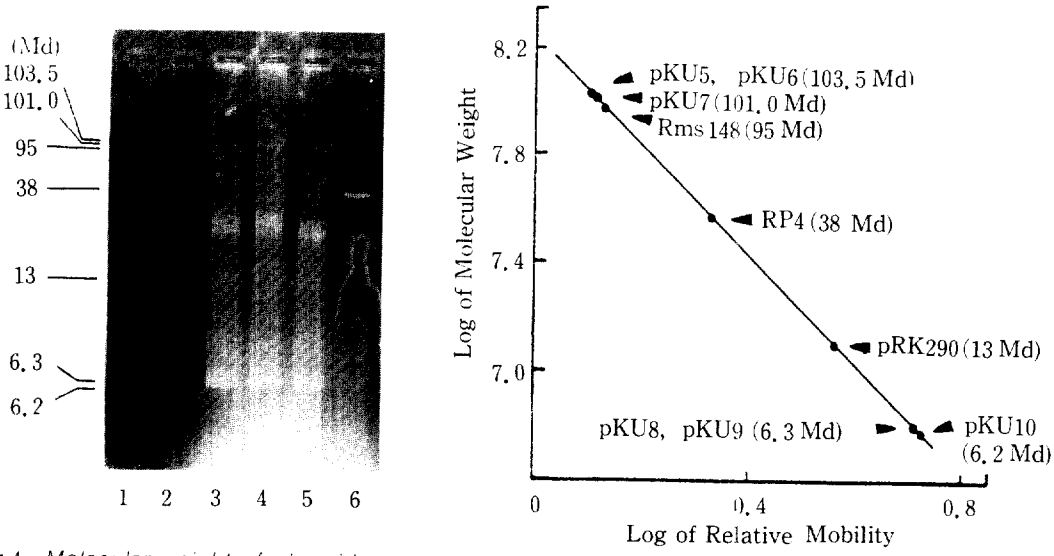


**Fig.4.** *Molecular weight of plasmids.*
1. pRK290 2. Rms148 3. pKU5, pKU8 4. pKU6, pKU9 5. pKU7, pKU10 6. RP4

forms of pRK290 (13 Md), RP4 (38 Md), Rms 148 (95 Md) as standard size markers (Fig.4). The SAL plasmids of KU801, KU803, and KU806 were named pKU5, pKU6, and pKU7, and their samll plasmids, pKU8, pKU9, and pKU10, respectively. The molecular weights of pKU5 and pKU6 were estimated as 103.5 Md, and that of pKU7 as 101 Md. Their sizes are rather large in comparison with 40 Md (Palchudhuri and Chakrabarty, 1976), 43 Md (Johnston *et al.*, 1977), 45 Md (Gunsalus *et al.*, 1981), 48 Md (Heinaru *et al.*, 1978), and 55 Md (Farrell *et al.*, 1978) which have been previously reported. Therefore, SAL plasmids examined in this research seem to be new SAL plasmids.

## 적 요

분리한 살리실산 자화세균 중 플라스미드를 갖는 세 균주를 선별하였다. 세 균주, KU801(pKU5, pKU8), KU803(pKU6, pKU9), KU806(pKU7, pKU10)는 각각 두 개씩의 플라스미드를 가지고 있음이 전기영동에 의해 밝혀졌고, *Pseudomonas putida*로 동정되었다. 세 균주들은 모두 암피실린, 테트라사이클린, 클로람페니콜등의 항생제에 대하여 내성을 지니며, 조사된 방향족과 지방족 탄화수소들 중 살리실산과 그의 중간 대사물인 카테콜만을 이용하였다. 큰 분자량의 플라스미드 (pKU5, pKU6, pKU7)는 마이토마이신 C로 처리하였을때 큐어되며 그 빈도는 각각 0.40%, 1.67%, 0.75% 이었다. 큐어된 균주는 살리실산을 분해하지 못하였으나, 여전히 야생균주와 동일한 항생제 내성을 가지고 있었다. 살리실산 분해에 관여하는 유전자가 그들 플라스미드에 있는 것으로 판명되었다. pKU5와 pKU6의 분자량은 103.5 Md, pKU7의 분자량은 101 Md으로 측정되었다. SAL 플라스미드인 pKU5, pKU6, pKU7은 접합에 의해 *P. putida*와 *P. aeruginosa*로는 전달되었으나, *E. coli*에서는 발현되지 아니하였다.

## REFERENCES

1. Barth, P.T. and N.J. Grinter, 1977. Map of plasmid RP4 derived by insertion of transposon C. *J. Mol. Biol.* **113**: 455-474.
2. Benson, S. and J. Shapiro, 1978. TOL is a broad host range plasmid. *J. Bacteriol.* **135**: 278-280.
3. Chakrabarty, A.M., 1972. Genetic basis of the biodegradation of salicylate in

*Pseudomonas. J. Bacteriol.* **112**: 815-823.
4. Chakrabarty, A.M., 1976. Plasmids in *Pseudomonas. Ann. Rev. Genet.* **10**: 7-30.
5. Corbin, D., G. Ditta and D.R. Helinski, 1982. Clustering of introgen-tixation (*nif*) genes in *Rhizobium meliloti. J. Bacteriol.* **149**: 221-228.
6. Curtiss, R. III. 1981. Manual of methods for general bacteriology. Gerhardt, Murray, Costilow, Nester, Wood, Krieg and Philliphs(eds.), American Society for Microbiology. pp.257-258.
7. De Graaf, J., P.C. Kreuning and P. Van De Putte, 1973. Host controlled restriction and modification of bacteriophage Mu and Mu-promoted chromosome mobilization in *Citrobacter freundi. Mol. Gen. Genet.* **123**: 283-288.
8. Dunn, N.W. and I.C. Gunsalus, 1973. Transmissible plasmid coding early enzymes of naphthalene oxidation in *P. putida. J. Bacteriol.* **114**: 974-979.
9. Farrell, R., I.C. Gunsalus and I.P. Grawford, 1978. Restriction endonucleases sites and aromatic metabolic plasmid structure. *Bio. Biophys. Res. Commu.* **82**: 411-416.
10. Farrell, R. and A.M. Chakrabarty, 1979. Degradative plasmids; Molecular nature and mode of evolution. Plasmids of Medical, Environmental and Commercial Importance. Timmis, K.N. and A. Pühler, ed., Elsevier/North-Holland Biochemical Press. 97-109.
11. Freifelder, D., 1983. Mechanism for rearrangement and exchange of genetic material: I. Plasmid. In Molecular Biology. Science Books International, Boston.
12. Friello, D.A., J.K. Mylroie, D.T. Gibson, J.E. Rogers and A.M. Chakrabarty, 1976. XYL, a nonconjugative xylene-degradative plasmid in *Pseudomonas* PXY. *J. Bacteriol.* **127**: 1217-1224.
13. Gunsalus, I.C. and K-M. Yen, 1981. Pathogenicity and Ecology of bacterial plasmids. In Molecular Biology. S.B. Levy, R.C. Clowes and K.E.L. Koenig(eds.), Plenum Press. N.Y. pp.499-509.
14. Hamer, D. and C. Thomas, 1976. Molecular cloning of DNA fragments produced by restriction endonuclease Sal I and BamH I. *Proc. Nat. Acad. Sci. USA.* **73**: 1537-1541.
15. Hansen, J.B. and R.H. Olsen, 1978. Isolation of large bacterial plasmids and characterization of P2 incompatibility group plasmids pMG1 and pMG5. *J. Bacteriol.* **135**: 227-238.
16. Heinaru, A.L., C.J. Duggleby and P. Broda, 1978. Molecular relationship of degradative plasmids determined by *in situ* hybridization of their endonuclease -generated fragments. *Mol. Gen. Genet.* **160**: 347-351.
17. Issac, J.H. and B.W. Holloway, 1968. Control of pyrimidine biosynthesis in *Pseudomonas aeruginosa. J. Bacteriol.* **9**: 1732-1741.
18. Johnston, J.B. and I.C. Gunsalus, 1977. Isolation of metabolic plasmid DNA from *Pseudomonas putida. Biochem. Biophys. Res. Commu.* **75**: 13-19.
19. Kanemitsu, H., M. Fukuda and K. Yano, 1980. Plasmid-borne biodegradation of toluene and ethylbenzene in a Pseudomonad. *J. Ferment. Technol.* **58**: 175-181.
20. Kim, J.K. and Y.N. Lee, 1984. Isolation and identification of *Pseudomonas* utilizing hydrocarbon. *Kor. Jour. Microbiol.* **73**: 29-34.
21. Maniatis, T., E.F. Fritsch and J. Sambrook, 1982. Molecular cloning (A laboratory manual). Cold Spring Harbor Laboratory.
22. Miller, J.H., 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory.
23. Nakazawa T. and T. Yokota, 1977. Isolation of a mutant TOL plasmid with increased activity and transmissibility from *Pseudomonas putida (arvilla)* mt-2. *J. Bacteriol.* **129**: 39-46.

24. Nakazawa, T., S. Inouye and A. Nakazawa, 1980. Physical and Functional mapping of RP4-TOL plasmid recombinants: Analysis of insertion mutants. *J. Bacteriol.* **144**: 222-231.
25. Olsen, R.H., G. DeBusscher and W.R. McCombie, 1982. Development of broad host-range-vectors and gene banks: self-cloning of the *Pseudomonas aeruginosa* PAO chromosome. *J. Bacteriol.* **150**: 60-69.
26. Palchaudhuri, S. and A.M. Chakrabarty, 1976. Isolation of plasmid DAN from *Pseudomonas*. *J. Bacteriol.* **12**: 410-416.
27. Palleroni, N.J., 1984. Bergey's manual of systematic Bacteriology. Vol.1: 141-199. William and Wilkins, Baltimore/London.
28. Rheinwald, J.G., A.M. Chakrabrty and I. C. Gunsalus, 1973. A transmissible plasmid controlling camphor oxidation in *Pseudomonas putida*. *Proc. Nat. Acad. Sci. USA.* **70**: 885-889.
29. Sagai, H., V. Krcmery, K. Hasuda, S. Iyobe, H. Knothe, and S. Mitsuhashi, 1975. R factor-mediated resistance to aminoglycoside antibiotics in *Pseudomonas aeruginosa*. *Japan. J. Microbiol.* **19**: 427.
30. Stanier, R.Y., N.J. Palleroni and M. Doudoroff, 1966. The aerobic pseudomonads: a taxonomic study. *J. Gen. Microbiol.* **43**: 159-273.
31. Worsey, M.J. and P.A. Williams, 1975. Metabolism of toluene and xylenes by *P. putida (arvilla)* mt-2; evidence for a new function of the TOL plasmid. *J. Bacteriol.* **124**: 7-13.

**(Received Nov. 9, 1986)**