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m-Toluate를 分解하는 Pseudomonas의 分離 및 Degradative Plasmid와의 연관성에 關하여

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Studies on the m-Toluate Degradating Plasmid in Pseudomonas

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ABSTRACT

A strain able to grow on m-toluate minimal medium has been isolated after selective enrichment and given the name T81X, which was later identified as *Pseudomonas putida* according to its morphological and biochemical characteristics.

After treatment with plasmid specific curing agent, mitomycin C, followed by replica plating on m-toluate and xylene minimal agar plate, T81Xstrain has been shown to harbour a curable plasmid relating to the m-toluate and xylene metabolism. Spontaneous curing frequency of this plasmid was also greatly enhanced by growing on benzoate minimal medium. After then, it was found to be conjugally nontransmissible.

From the comparative investigation of catechol 1,2- oxygenase and catechol 2,3 -oxygenase activities in wild type and cured strain on various growth substrate, it appeared that T81X strain has both of these two enzymes while cured strain has catechol 1,2-oxygenase only. Growing on m-toluate minimal medium T81X strain has primarily catechol 2,3-oxygenase.

It could, therefore, be confirmed that T81X strain should carry the genetic information necessary for coding the catechol 1,2-oxygenase, induced by benzoate, on the chromsome and catechol 2,3-oxygenase, induced by m-toluate or benzoate, on that curable plasmid.

INTRODUCTION

The aerobic pseudomonads have been studied for many years by microbiologists who were attracted by their range of habitats, variety of biological types, and biochemical versatility(Holloway, 1969). Especially the genus *Pseudomonas* is notable for the the large number and variety of compounds that serve as sole carbon and energy sources for its members(Stanier *et al.*, 1966).

A screening of 145 compounds allowed the identification over 100 that could be utilized by a single strain of *Pseudomonas*

multivorans. Although slightly less versatile the strains used for genetic studies, principally *Pseudomonas putida* and *P. aeruginosa*, are nevertheless capable of degrading a wide variety of organic compounds, many of which are quite unusual, e.g., camphor, naphthalene, and aromatic acids (Stanier *et al*, 1969).

Rates of growth supported by some of these compounds are often faster than those supported by compounds such as glucose(Kageyama, 1977). Some pseudomonads are common soil saprophytes including the facultative pathogen, *P. aeruginosa*, and their abundance in soil is directly correlated with proximity to a source of organic carbon (Weellis, 1975). Apparently, the ubiquity of soil pseudomonads reflects their ability to utilize almost anything as a source of carbon and energy, rather than any particular to com pete successfully for readily metabolized compounds(Nakazawa, and Yokota, 1973).

Recent works in a number of laboratoratories have indicated that in certain strains, the genes coding for the enzymes responsible for catabolism of some of less common substrates are carried not on the chromosome but are borne on conjugative plasmids (Weelis, 1975; Willets, 1972; Chakrabarty, 1973). Camphor(Rheinwald *et al*, 1973; Shaham *et al*, 1973), salicylate(Chakrabarty, 1972), octane(Chakrabarty *et al*, 1973), naphthalene (Dunn and Gunsalus, 1973). benzoate and toluates(Murray *et al*, 1972; William and Murray, 1974). benzensulfonic acid(Chain and Farr, 1968), and toluene and xylenes (Worsey and Williams, 1975) are compounds the breakdown of which appears to be plasmid coded in certain *Pseudomonas* strains. Metabolic pathways and their regulation have also been studied. Among these, the degradation pathways of aromatic compounds

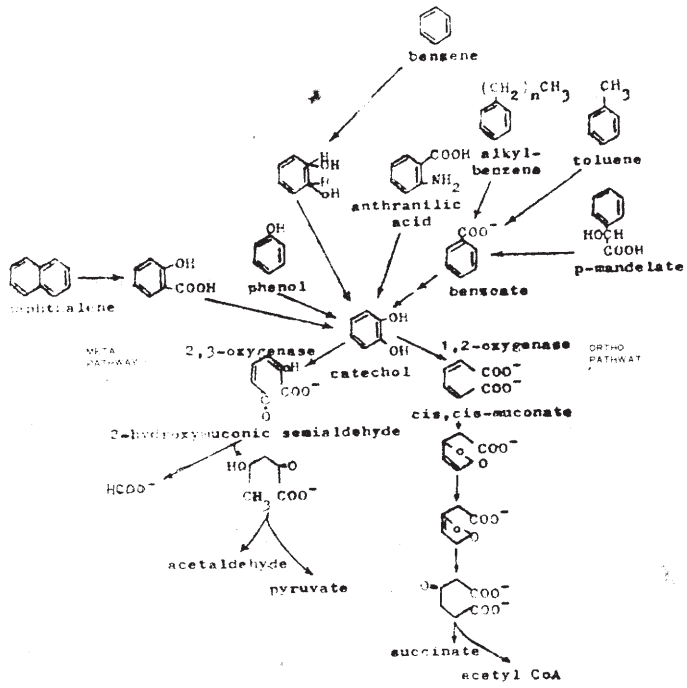

Fig 1. Degradation of Aromatic Hydrocarbon by *Pseudomonads* via Catechol

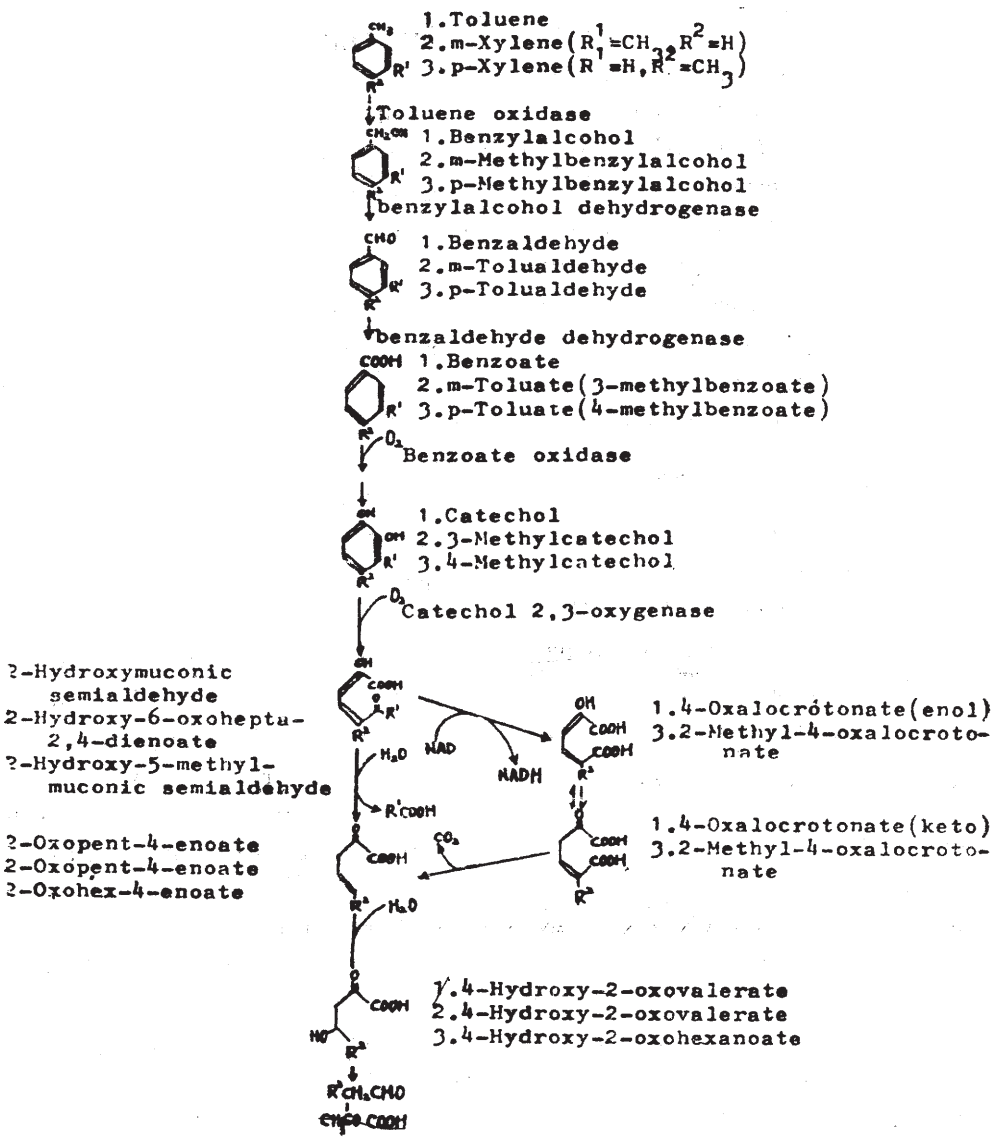

Fig 2. Proposed pathway for metabolism of toluene and m-and p-Xylene by *P. arvilla*. Metabolites of toluene are denoted (1), those of m-xylene (2), and those of p-xylene (3).

are shown in Fig. 1 (Dolle, 1975). Fig. 1 shows that not only catechol plays a major role in these metabolisms but through the catechol meta-pathway and ortho-pathway are diverged.

The genes coding for the meta-(or α-keto acid) pathway enzymes responsible for the degradation of benzoate, m-and p-tolutes in *P. putida* mt-2(Murray etal, 1972) have been shown to be carried on a transmissible plasmid, TOL (William and Murray, 1974). And several reports have indicated that bacteria grown on toluene, *m*-or *p*-xylenes degrade these compounds through benzoate, m-and p-toluates (Davey and Gibson, 1974; Davis *et al.*, 1968; Worsey and Williams, 1975) and that this occurs by a series of oxidations through the corresponding alcohols and aldehydes(Fig. 2).

Meanwhile, Williams and Worsey(1976) reported that spontaneous curing frequency of TOL plasmid related to m-toluate growth was greatly increased after growing on benzoate minimal medium(Nakazawa and Yokota, 1973; Williams and Murray, 1974)

This experiment, after isolation and identification of a *Pseudomonas* strain able to grow on m-tlouate by selective enrichment culture, was carried out to identify the existence of plasmid related to m-toluate metabolism and to study other genetic and biochemical characteristics.

MATERIALS AND METHODS

1. Isolation of bacterial strains

For the enrichment of bacterial strains capable of growing on *m*-toluate minimal medium, the following composition of medium (Clans and Walker, 1964) and the following procedure (Ornston and Stanier, 1966) were employed.

(1) m-toluate minimal medium(medium A): 250ml flask; K_2HPO_4, 0.08 g; KH_2PO_4, 0.02 g; $MgSO_4\ 7H_2O$, 0.05g; $CaSO_4$, $2H_2O$, 5.0 mg; $FeSO_4\cdot 7H_2O$, 1.0 mg; $(NH_4)_2SO_4$, 0.1 g; water to 100 ml; m-toluate (5 mM) were added and the final pH was adjusted to 7.0 with 5N NaOH.

(2) Procedure: One gram of soil was inoculated in a 250 ml flask containing 50 to 100 ml of above medium, aerated, and incubated for 2 to 3 days at 25°C-30°C. Pure cultures were then isolated by streaking from enrichment flask on the surface of above agar medium A.

2. Maintenance

(1) Pure culture and maintenance of isolates; Isolated strains were cultured

♯Stock A;	
Nitriloacetic acid	0.5 g
$MgSO_4\cdot 7H_2O$	0.1 g
$FeSO_4\cdot 7H_2O$	5 mg
$(NH_4)_2SO_4$	1.0mg
KH_2PO_4	5.0 g
Stock B	1 ml
Water to	1 liter

♯Stock B(stock salt solution)		
MgO	10.75	g
$CaCO_3$	2.0	g
$FeSO_4\cdot 7H_2O$	4.5	g
$ZnSO_4\cdot 7H_2O$	1.44	g
$MnSO_4\cdot 4H_2O$	1.12	g
$CuSO_4\cdot 5H_2O$	0.25	g
$CoSO_4\cdot 7H_2O$	0.28	g
H_3PO_4	0.06	g
conc. HCl	51.3	ml
Water to	1	l

purely by streaking and maintained on 5 mM m-toluate agar slopes of following composition Medium B (Murrayetal, 1972).

After carbon source was added, the final *p*H was adjusted to 7.0 with 5 N NaOH and 1 N HCl before sterilization.

(2) Derivatives and mutant strains were maintained on nutrient agar slopes.

(3) The ability to grow on various substrates was determined on the medium B agar plates either with the substrate incorporated at 2.5 or 5 mM, or with 10 mM in the case of succinate. In the case of toluene, p-xylene and benzylalcohol, the substrates were put in small tubes sealed at one end and placed in the lids of medium B agar plates with vapor phase (Worsey and Williams, 1975).

(4) All of the strains subcultured on appropriate agar slant were, if necessary, stored at 4°C.

(5) Luria broth used in mitomycin C curing experiment has following composition (Rheinwald *et al.*, 1973) : per litre of distilled water; Bacto tryptone, 10 g; Bacto yeast extract 5 g; NaCl, 0.5g; 1N NaOH, 2 ml; the final *p*H was adjusted to 7.0 with 1 N NaOH and then then 10 ml of 20% glucose solution was added after autoclaving.

3. Identification of Strain isolated

A strain growing very well on Medium A among isolates was selected, named T81X, and subjected to the following experiment for identification.

(1) Morphology and Staining: Gram staining, spore staining, flagella staining and microscopic examination were performed.

(2) Cultural characters: Bacterial growth on various media such as NA, NB, *Pseudomonas* selective medium, gelatine agar stab(Salle, 1973). King A and King B medium (King *et al.*, 1954) were observed.

(3) Physiological and biochemical characters: β-Galactosidase, lysine decarboxylase, tryptophan deaminase, protease (Salle, 1973), oxidase arginine dehydrolase, ornithine decarboxylase, urease, lecithinase(Narris and Ribbon, 1971), requirenemt of oxygen, citrate utilization, formation of H_2S, nitrate reduction, indole production, VP-test (Clarke and Steel, 1966), optimum temperature and optimum *p*H were investigated.

(4) Nutritional tests: Utilization by oxidatively or fermentatively of glucose, lactose, mannitol, inositol, sorbitol, rhamnose, melibiose, and arabinose was examined according to Hugh and Leifson(1953). Utilization of the other carbon sources was tested by growth on the minimal medium (Medium B) containing appropriate one.

(5) Growth on Luria broth and on m toluate minimal medium(5 ml) were examined according to Miller(1974).

4. Curing experiments

(1) Mitomycin C curing: The detailed methods, carried out according to Charkrabarty (1972, 1973) for curing the plasmid by mitomycin C were as follows. Single colony from m-toluate agar plate was inoculated into culture tubes containing 5 ml of Luria broth(LB). After overnight growth at 30°C, 0.05 ml of a 10^3 dilution of the culture(10^4—10^5 cells) was inoculated into 1 ml of L-broth containing mitomycin C in 5 (or 2.5)μg increment from 0 to 30μg/ml, and incubated with shaking for 36 to 48 hrs. Then appropriate dilutions were made and 0.1 ml of them was spread on nonselective

agar plate (L-agar plate) and replicated to the m-toluate for scoring and selecting individual clones for the *tol*⁻ phenotype.

(2) Spontaneous curing on benzoate minimal medium: Single colony from medium B agar plate was inoculated into culture tubes containing 5 ml of L broth. After overnight growth at 30°C, each of 0.05 ml and 1.0ml of a 10^3 dilution of the culture were added to 5 ml of either LB or 5 mM benzoate minimal medium, which were incubated at 30°C for 24 hrs in LB and for 1 to 2 days in benzoate medium. At time intervals of 10 hr, 20 hr, 48 hr curing frequencies were determined as above procedure.

5. Isolation of mutants

(1) Streptomycin resistant mutants were isolated spontaneously according to the following procedure(Willam and Worsey, 1976): Overnight culture in LB was collected after and resuspended in buffer solution to make appropiate cell concentration (10^9—10^{10} cells/ml). Then, 0.1ml of this was plated onto the L-agar plate containing streptomycin(100 μg/ml).

(2) Rifampin resistant mutants were ioslated as above.

6. Conjngational transfer of plasmids

According to Dunn and Gunsalus(1973), donor and recipient grown at 30°C in LB were used. The 5ml of LB in 18mm by 150mm test tubes was inoculated with actively growing cells, and then the donors were incubated in stationary culture to about 5x10^8 cells/ml. The recipients were incubated with agitation at 200 rpm in a shaker to late log phase, about 5x10^9 cells/ml. The recipipients were collected by centrifugation and suspended in equal volumes of saline, and 0.1 ml was plated together with 0.1ml of donor on the surface of appropriate selection plates, which were incubated at 30°C. when necessary the matings involved selecting against the donor by streptomycin sensitivity. In this case spontaneous streptomycin-resistant of the recipient were used, and selection plates incorporated the lowest concentration of streptomycin effective against the wild type donor. The selection against the recipient was achieved by use of m-toluate(5ml) as the sole carbon source in the selection plate.

7. Preparation of cell extracts

(1) Cell growth: A starter culture was obtained by inoculation of an enriched medium(LB) from a slant and incubation at 30°C with shaking in order to provide sufficient aeration. After approximately 10 hrs, a small aliquot from this starter culture was used to inoculate a flask of a minimal salts medium B containing appropriate carbon source. The culture was grown for 8 to 10 hrs at 30°C under conditions of maximal aeration. The culture was then diluted 1 : 10 with fresh minimal medium, and incubation was continued until the O.D. of the culture just began to deviate from logarithmic growth (Sala-Trepat and Evans, 1971).

(2) Cell harvest: The culture was centrifuged at approximately x8,000g until the supernatant fluid was clear, and the harvested wet cells were washed twice with 20 mM phosphate buffer, pH 7.0 at 4°C and used immediately or stored at 0°C until required (Sala-Trepat and Evans, 1971).

(3) Preparation of cell free extracts: 2g wet weight of cells were resuspended in 10 mM potassium EDTA, pH 7.0 and supplemented with 0.5 mg lysozyme/ml

and 10% acetone, then the suspension was swirled by means of a magnetic stirrer and incubated for 15 min at room temperature. The extracts were centrifuged at about x15,000 g for 60 min at 0°C, and the pellets discarded. The supernatant fluids were decanted and referred to as crude extracts(Kaback, 1971).

8. Determination of protein concentration

Protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

9. Enzyme assay

Catechol 1, 2-oxygenase(C120) is labile to heat(Williams and Murray, 1974). Incubation with 30 mM H_2O_2 for 10 min destroy virtually all of the catechol 2, 3 -oxygenase(C230), leaving the C120 unchanged. According to Sala-Trepat and Evans (1971) C230 is labile to oxidation. Heat treatment at 55°C for 10 min destroyed all of the C120, leaving the C230 little changed.

(1) C120 activity was determined on UV spectrophotometer(Hitachi EPS-3T) by measuring the increase in absorbance at 260 nm due to formation of cis, cis-muconate in a reaction mixture containing 10 to 50 μl (about 100 μg of protein) cell free extract, 0.2 ml 500 mM EDTA, 0.2 ml 1 mM catechol, and 100 mM phosphate buffer at pH 7.5 to 3.0 ml. The blank cell contained all except substrate. The unit of activity is defined as the amount of enzyme required to produce 1 μmole of cis, cis-muconate in 1 min equivalent to an increase in absorbance of 5.63.

(2) C230 activity was determined on UV spectrophotometer(Hitachi EPS-3T) following the increase in absorbance at 375 nm due to the accumulation of 2-hydroxymuconic semialdehyde: cuvettes contained in a total volume of 3 ml;250μmoles of phosphate buffer pH 8.0, 0.2μmole of catechol and extract equivalent to about 100 μg of protein. The unit of enzyme activity is defined as the amount of enzyme necessary to convert 1 μmole of substrate to product in one minute, under assay condition. 0.1 μmole production of 2-hydroxymuconic semialdehyde is equivalent to an increase on absorbance of 1.2. C230 activity was measured immediately after preparation of the extract as possible.

10. Bacterial Strains

P. fluorescence ATCC13525, *P. aeruginosa* PA025, *P. cepacia* 382 and *P. phaseolicola* HBY10 used as type culture were kindly gifts from Dr. Y. N. Lee, College of Medecine, Yonse University. *P. aeruginosa* ATCC15692 was obtained from H. B. Oh, National Institute of Health. All of the strains are wild type except *P. aeruginosa* PA025 and its derivatives listed in Table 6.

P. putida T81X strain from soil is wild type and harbouring m-toluate degrading plasmid. T81 strain lacking this plasmid was derived from it after mitomycin C treatment, and T81B, presumably edvoid of this plasmid, also derived after the growth on benzoate minimal medium. Antibiotic resistant mutant strains were isolated from these parental strains.

RESULTS

1. Isolation of a strain capable of growing on m-toluate minimal medium and Identification of the strain isolated

After enrichment on the m-toluate minimal medium, various types of bacteria were isolated. Among these, the bacteria whose size of colony were large were randomly selected.

Hendrie and Shewan(1966) described that in identifying *Pseudomonas* spp., the following features are considered as important.

(a) Gram-negative rods, motile by means of polar flagella

(b) Colonies generally achromogenic and may or may not produce diffusible fluorescent pigment, but chromogenic species may occur in both the fluorescent and non-fluorescent groups

(c) Positive reaction in the oxidase test (Kovacs, 1956)

(d) Reaction on carbohydrate on the medium of Hugh and Leifson(1953)

(e) Identity of diffusible pigments

(f) Ability to grow at certain temperatures

Thus, Gram staining and oxidase test were carried out as well as the shape of bacteria, motility, and polar flagella was observed. A strain which was thought to be a member of *Pseudomonas* according to these criteria was selected and named T81X. In addition to morphological and staining characters, cultural, physiological and biochemical, and nutritional characters of T81X were examined. From these results, shown in Table 1–4, T81X was identified *Pseudomonas putida* biotype B.

(1) Morphological and Cultural characteristics of T81X: As shown in Table 1, T81X strain is rod, 1.0~1.2×2.5~3.0 μ in size, non-spore forming, Gram-negative and polar flagellated. On NA plate, colonies are circular, convex and entire and yellowish green ring is formed at the surface of NB. Specific fluorescent pigmetnt appears on *Pseudomonas* selective medium, nonproduction of phe nazine pigment on King A medium and production of fluoreescnt pigment on King B medium.

Table 1. Morphology and Staining of the Strain T81X

Gram stain	negative
Spore stain	negative
No. of flagella	>2(polar)
Shape	rod
Size(micron)	1.0—1.2x2.5—3.0

Table 2. Cultural characters of the strain T81X

Culture Medium	Form	Elevation	Mrgin	Colony color	Growth	Others
N.A. plate	Circular	Convex	Entire	Pale green	Good	Pale fluorescent zone
N.A. slant	Filiform	Raised		Pale green	Good	〃
Nutrient Broth (surface growth)	Membranous			Greenish pale fluorescence	Good	Turbid
Pseudomonas selective medium	Circular	Convex	Entire	〃		
Gelatin agar stab	Dirty surface growth			White, becoming green	Scanty	No liquefaction
King A medium						Light yellow
King B medium						Fluorescence

Table 3. Physiological and Biochemical characters of the strain T81X

Test	Reaction	Test	Reaction
Requirement of oxygen	Aerobic	Oxidase	+
β-Galactosidase	−	Arginine dehydrolase	+
Lysine decarboxylase	+	Ornithine decarboxylase	−
Citrate utilization(Simmon's)	−	Urease	−
Tryptophan deaminase	+	Indole production	−
Formation of H_2S	−	VP-test	−
Protease(gelatin liquefaction)	−	Lecithinase(egg yolk reaction)	−
Nitrite reduction	−	*p*H; Minimum	5
Temperature; Minimum	4°C	Optimum	6.0—7.0
Optimum	25°C	Maximum	9.5
Maximum	37°C		

Table 4. The Utilization of various Carbon sources by T81X

Substrate	Growth	Substrate	Growth
Benzate(5ml)	+	Glucose	+
Na-succinate(10mM)	+	Mannitol	−
Phenol(2.5mM)	+	Inositol	−
p-Hydroxybenzoate (5mM)	+	Sorbitol	−
Catechol(5mM)	+	Rhamnose	−
Camphor(5mM)	+	Sacchrose	−
Naphthalene(5mM)	+	Melibiose	−
p-Xylenea	+	Arabinose	−
Toluenea	+	Lactose	−
Benzylalcohol(5mM)	+		

a; supplied by vapor phase

(2) Biochemical and nutritional characteristics: Optimum growth of aerobic T81X is obtained at 25°C −30°C and at *p*H 6.0 —7.0. The production of lysine decarboxy lase, tryptophan deaminase, arginine dehdrolase, β-galactosidase, and ornithine decarboxylase, the utilization of citrate, the formation of H_2S, indole production and VP-test are shown in Table 3. In the utilization of various carbon sources shown in Table 4, glucose seems to be used oxidatively while others such as saccharose, lactose, mannitol, inositol, sorbitol, rhammose, melibiose and arabinose used neither oxidatively nor fermentatively. Excellent growth appeared on sodium succinate and benzoate, but slightly on phenol, p-hydroxybenzoate, catechol, camphor, naphthalene, p-xylene, toluene and benzylalcohol.

(3) The growth on LB and m-toluate minimal medium: The growth on m-toluate by T81X exhibits a typical sigmoid shape, but final cell density, approximately 2.7×10^8 cells/ml, is less than that of Luria broth(Fig. 3 and Fig. 4). Comparing between the lag periods in these two media, it seemed that the former should take a more adaptation time than the latter, probably due to the induction of enzyme system required for the degrapation of m-toluate since its inoculum was obtained from mid-log phase culture in LB. The latter was inoculated with the same source.

2. Curing experiments

Table 5. shows that when T81X grew on LB the spontaneous loss of the ability to grow on m-toluate was undetectable. When treated with various concentration of mitomycin C (MC), growth appeared

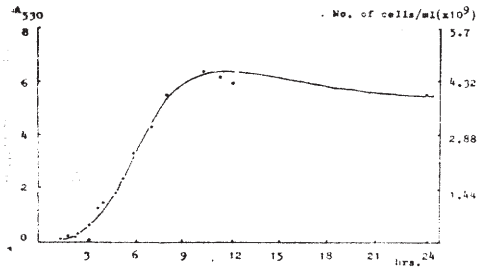

Fig 3. Growth on Luria broth by T81X

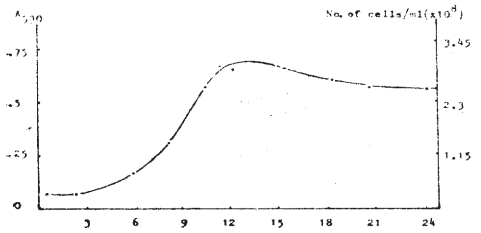

Fig 4. Growth on m-toluate by T81X

soon after incubation in the cultures containing below 7.5 μg MC/ml, but with 10 μgMC/ml growth appeared after two days and with above 15μg MC/ml no growth until 3 days after incubation. Thus 10 μgMC/ml was considered as sublethal concentration in T81X, then the loss of the ability to grow on m-toluate at this MC concentration was examined. And about 11% of total viable colonies was identified as being *m-tol*⁻ phenotype(unable to grow on m-toluate minimal medium). Such high frequency as eleven per cent implies the fact the loss of m-toluate growth phenotype must be obtained only by curing of catabolic plasmids.

m-toluate negative cells, obtained by treatment with mitomycin C, did not revert to the T81X phenotype($\langle 10^{-9}$). This also indicates that m-toluate nonutility was not derived from the mutation by mitomycin C.

One of the main criteria for determining whether a catabolic pathway is coded by plasmid-carried genes is to produce strains that have lost the ability to utilize the particular growth substrate (s). The agent frequently used to cure catabolic plasmids in genus *Pseudomonas* has been mitomycin C (William and Worsey, 1976). However, William and Worsey (1976) reported that the proportion of cells of *P. putida* MT20, cured of their TOL plasmids by growth on benzoate minimal medium (William and Worsey, 1976). is greater than that by MC. If the genetic control mechanism of m-toluate degrading pathway identified in T81X is similar to that of the TOL plasmid previously reported, the increase in spontaneous curing frequency on benzoate would also be observed in T81X strain. For the same reason, the curing frequency after growth on benzoate was investigated to the T81X strain. In fact the propor tion of cured cells gradually increased as

Table 5. Curing of m-toluate Growth Phenotype by Mitomycin C

Strain	Mitomycin C (μg/ml)	Growth	Frequency of curing (%)
T81X	0	+	ND
	2.5	+	ND
	5	+	ND
	7.5	+	ND
	10	+a	11
	11	-	

ND; Not Determined
a; Growth appeared after two days incubation

Table 6. Spontaneous Curing of m-Toluate utilizing Phenotype on Benzoate minimal medium

Strain	Inoculum size (cells/ml)	Curing frequency (%) 0hr	10hr	20hr	40hr
T81X	2.5×10^{5}	0	27	52	100
	5×10^{6}	0	25	48	71.6

inoculation time goes by, reaching approximatly 100% after 40 hrs(Seetable 6). Comparing to the effect of mitomycin C (Table 5) the benzoate curing frequency was extremely high. Also, the frequency of curing was dependent upon the size of inoculum, for example, in the case of large inoculum applied about 71.6% were cured in comparison with 100% of small inoculum. m-Toluate negative cells obtained spontaneously by growth on benzoate did not revert to the T81X phenotype either($\langle 10^{-9}$).

3. Isolation of mutants

To determine the transmissibility of m-toluate degradading plasmid between intra-strains or intra-genus, antibiotic resistant markers, either rifampicin (100 μg/ml) and/or streptomycin (100 μg/ml), were spontaneously introduced both in T81 strain and in *P. aeruginosa* PA025 strain. Each antibiotic concentration for resistant selection was obtained before experiments since minimal inhibitory concentration can be varied from strain to strain. Those mutants obtained were listed in Table 7.

4. Conjugation

In addition to the production of cured strains, the ability of the wildtype to transfer the plasmid back into the cured strain and into the same genus strain, *P. aeruginosa* by conjugation is a major criterion, which has been applied to characterize a set of genes as being plasmid carried. So the plasmid related to mtoluate degradation in T81X was named pCK1

The results of the intrastrain and intragenus matings are given in Table 8. T81X was not able to transfer its plasmid pCK1 back into its own cured strain. No measurable transfer from T81X into *P. aeruginosa* was observed either.

5. Enzyme assay

The activities of catechol 1, 2-oxygenase and catechol 2, 3-oxygenase related to aromatic to aromatic compounds catabolism as shown in Fig. 1 and Fig. 2, were measured when T81X was grown on se-

Table 7. List of *Pseudomonas* Strains used and Their origins

Strain Designation	Phenotype	Genotype Chromosome/Plasmid	Derivation/source
P. putida			
T81X	$Xyl^+Tol^+Tra^-Hg^s$	wt/pCK1	/soil
T81	Xyl^-Tol^-	wt	MC/T81X
T81—1	Rif^r	rif^r	S/T81
T81—2	Sm^r	str^r	S/T81
T81—3	Rif^rSm^r	rif^rstr^r	S/T81
T81X—1	$Sm^rXyl^+Tol^+$	str^r/pCK1	S/T81X
P. aeruginosa			
PAO25R	$Leu^-Arg^-Cb^rKm^rTc^r$	leu^-arg^-	/Dr. Y. N. Lee
PAO25	Leu^-Arg^-	leu^-arg^-	SC/PAO25R
PAO25S	$Leu^-Arg^-Sm^r$	$leu^-arg^-str^r$	S/PAO25

Abbreviations used: wt, wild type; S. spontaneous; MC, mitomycin C curing; SC, spontaneous curing Xyl, xylene degradation; Tol, m-toluate degradation; Tra, transferability; Leu, leucine; Arg, arginine; pCK1, the plasmid specifying m-toluate degrading pathway; Rif^r, rifampicin resistance(100ug/ml); Sm^r, streptomycin resistance(100ug/ml)

Table 8. Transfer of plasmid pCK1

Donor	Recipient	Counterselection	Transfer frequency
T81X[a]	T81—1	$Rif^r Tol^+$	$<10^{-8}$
	T81—2	$Sm^r Tol^+$	〃
	T81—3	$Sm^r Rif^r Tol^+$	〃
	T81—B	$Sm^r Tol^+$	〃
T81X[a]	PAo25S	$Sm^r Tol^+$ at 34°C	〃
		at 42°C	〃

a; Strain harbouring pCK1

veral carbon sources. Basal levels of these enzymes, produced by growing on succinate(Succinate is primary carbon source for *Pseudmonas*, Kageyama, 1977) were also determined.

T81, cured of pCK1, was similarly examined to find out the relationship between these enzymes and the plasmid.

Maximum absorption, measured spectrophotometrically either UV or visible range, of catechol 1, 2-oxygenase and catechol 2, 3-oxygenase appeared at about 260 nm, 380 nm respectively, as in Fig. 5 and 6.

Initial velocities of C120, C230 were measured and observed to be roughly linear until about 15 min after the reactions were started. From these preliminary experimental results, it was scheduled that the enzyme activites were assayed at 3 min afterstarting the enzyme reaction.

In summary, the results of enzyme activity assay shown on Table 9 are as follows.

(a) When T81X was grown on m-toluate the key enzyme C230 of the meta pathway, but not the key enzyme C120 of the ortho pathway, was highly induced. However normal basal level of C120 was present.

(b) When T81X was grown on benzoate, both C120 and C230 were induced.

(c) When T81X was grown on catechol and phenol, C120 was induced while only basal level of C230 was present.

(d) When T81X was grown on p-hydroxybenzoate, C230 was induced but C120 was induced slightly.

(e) When T81X was grown on succinate, the basal levels of C120 and C230 were present.

(f) When T81 was grown on benzoate, catechol, and phydroxybenzoate, C230 was undetectable, while on succinate the basal level of C230 was not present.

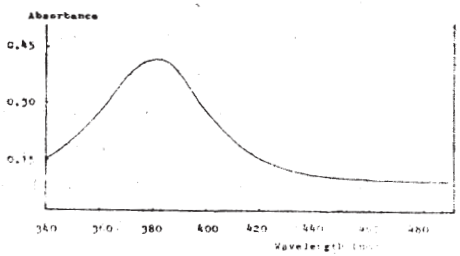

Fig 5. The meta Cleavage of Catechol by T81X strain showing Maximum absorption at 380 nm

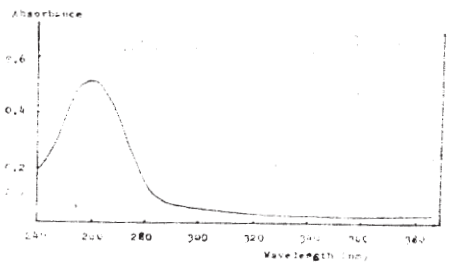

Fig 6. The ortho Cleavage of Catechol by T81X strain showing Maximum absorption at 260 nm

DISCUSSIONS

It appeared that T81X strain is a member of genus *Pseudomonas* as shown on Table 1—4 according to Stanier(1966)and that T81X is a member of fluorescent group from the fluorescent pigment pro-

Table 9. Specific activities of Aromatic Metabolism in the cell free extracts of *P. putida* T81X and T81: Assays were carried out as described in Experimental Procedure

	Sp. act. (mU of activity/mg of protein) of			
	P. putida T81X		*P. putida* T81	
	C120	C230	C120	C230
m-Toluate (5 mM)	53.3	3,500.0	—	—
Benzoate (5 mM)	799.2	2,600.0	1,509.8	n.d
Catechol (2.5mM)	242.0	16.7	532.8	n.d
p-Hydroxybenzoate (5 mM)	141.1	741.7	301.0	n.d
Phenol (2.5mM)	319.7	25.0	—	—
Succinate (10mM)	71.0	31.4	84.3	n.d

C120; Catechol 1,2-oxygenase
C230; Catechol 2,3-oxygenase
— ; Not determined because of no growth on that medium
n.d ; The activity was undetectable

duction on King B medium. Production of phenazine pigment like the typical blue or pink on King A medium was not observed, only light yellow color produced. Thus T81X seems not to be a strain of *P. aeruginosa.*

From the characteristics unable to utilize various sacchrides especially inositol and unable to produce lecithinase and protease, T81X was thought to be not *P. fluorescence* but *P. putida.*

Utilization of naphthalene, camphor, and phenol indicates that T81X is a member of biotype B, more nutritionally versatile organism than biotype A of *P. putida.* L-tryptophan deaminase activity and growth on 4°C undoubtedly confirmed that T81X is more close to *P. putida* biotype B.

The mitomycin C curing evidence, in particular, the facility with which this occurs and the simultaneous loss of its ability to grow on m-tolutate, xylene and phenol coupled with the loss of detectable C230 activity strongly suggest that it is the presence of plasmid that m-toluate metabolism is related to.

It is of interest that the cured cells are strongly counterselected against wild type during growth on benzoate. The reason why benzoate selects for cured cells of *P. putida* T81X, the host of pCK1, appears to be the same as previously described for *P. putida* MT20 (Worsey and Williams, 1977). Similar to *P. putida* MT20, *P. putida* T81X may possess another pathway different from that of plasmid for the dissimilation of benzoate, the ortho or β-ketoadipate pathway, which is presumed to be chromosomally coded. This pathway is common among saprophytic pseudomonads (Ornston, 1966). In *P. putida* MT20, the first step, the oxidation of benzoate to catechol, resembles the plasmid-coded meta pathway, but thereafter the pathway diverge.

Further utilization via orthopathway depends upon the conversion of catechol by basal level of C120 to cis, cismuconate, its probuct inducer (Ornston and Stanier, 1966). The catechol 2, 3-oxygenase initiating the meta pathway is probably induced by benzoate or m-toluate (Worsey and Williams, 1977); thus, during gro-

wth on benzoate it is fully induced and will rapidly metabolize any catechol formed, preventing induction of the ortho pathway.

In wild type, where both pathways are present, benzoate and m-toluate are degraded by the plasmid-coded meta pthway. It appears, as it were, that spontaneously cured cells have a selective advantage since they use the chromosomally coded ortho(or, β-ketoadipate) pathway for the disismilation of benzoate, by which they grow faster than wild type cells(Williams and Murray, 1974); because of differences in the regulation of the meta and ortho pathways, the ortho pathway is not normally expressed in cells containing the plasmid-coded meta pathway. Since the ortho pathway is specific for benzoate, these cured cells lose the ability to metabolize m-toluate.

The frequency of curing resulted after growing on benzoate varies from 71% to 100% with the change of inoculum size. This data leads to the fact that cured cells grow faster than wild type on benzoate.

The transfer of pCK1 plasmid was performed by using not only the same strains cured with MC or benzoate but *P. aeruginosa* PAO strain as a recipient. To the latter case, conjugation was also carried out at 42°C since the restriction system of *P. aeruginosa* PAO is temperature sensitive(Nakazawa, 1978), as well as 34°C. In both cases, transferability was not observed even at the frequency of c.a. 10^{-8}, so that the plasmid pCK1 is thought to be nontransmissible.

Considering the experimental results in Table 6, it is suggested that when wild type and the cured cell populations were mixed together in the culture on benzoate minimal medium, the relative activities of C120 and C 230 would be varied with the ratio of these two strains.

When phenol was applied as a sole carbon source, the growth appeared only in T81X strain but not in T81. Though, C120 was detected in contrast to the presence of C230 activity at the basal level. The reason why the presence of degradative plasmid pCK1 specifying the m-toluate enzyme should be necessary for the metabolism of phenol is still obscure. One possibility is such that, at first, phenol may be degraded to catechol and then catechol may goes through the ortho pathway. Namely, the plasmid pCK1 is thought to be involved in the step from phenol to batechol, but further investigation would be required to clarify this suggestion.

Consequently, it is evident that T81 strain has entirely no C230 activity for all growth substrates in comparison with T81X strain(Table 9) and, furthermore, that on succinate even the basal level of this enzyme could never be detected. The evidence, like this, could support the fact that T81X strain should carry the genetic information for degrading m-toluate on the plasmid.

적 요

부식질을 많이 포함하고 있어 갈색을 띠고 있는 토양으로 부터 m-toluate(m-methylbenzoate) 최소배지에서 enrichment하여 m-toluate를 고유의 탄소원과 에너지원으로 이용하여 생장하는 세균을 우선

적으로 분리하고 이 중에서 Pseudomonas속에 속하는 세균을 우선적으로 선택하여 이를 순수배양하고 균주의 형태적, 생리 및 생화학적 제성질을 조사하여 *Psuedomonas putida*로 동정하였다.

P. putida T81X로 명명한 이 균주는, 주로 Pseudomona속에 속하는 세균의 plasmid specific curing agent로 사용되어 온 mitomycin C를 처리한 결과, m-toluate 대사와 직접적인 관련성이 있는 degradative plasmid를 지니고 있음을 알 수 있었다, 또 sexual conjugation에 의해 이 curable plasmid는 transmissibility가 없음을 확인하였다.

T81X균주를 benzoate 최소배지에서 키울 경우에 spontaneous curing 빈도가 mitomycin C를 처리한 경우보다 훨씬 높게 증가하였는데 이는 이미 toluene-degradading plasmid에서 보고된 사실과 일치하는 것으로 생각된다.

T81X균주와 그로부터 mitomycin C curing하여 얻은 균주를 각각 benzoate 최소배지에서 키워서 benzoate 대사중간 생성물인 catechol을 intradiol fission하는 catechol 1,2-oxygenase와 extradiol fission하는 catechol 2,3-oxygenase 활성도를 측정한 결과 T81X균주는 catechol 1,2-oxygenase와 catechol 2,3-oxygenase 활성도를 다 나타내고, cured strain은 catechol 1,2-oxygenase 활성도만 나타내었다. 한편 T81X 균주가 m-toluate 최소배지에서 자랄 경우, 주로 catechol 2,3-oxygenase 활성도만 나타내었다.

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