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Characterization and Incompatibility of Transmissible TOL Plasmid from *Pseudomonas cepacia*

Cho, Byung-Nam, In-Sun Cho, Soon-Young Choi, Jae-Keun Ryu* and Kyung-Hee Min

Department of Biology, Sookmyung Women's University

**National Institute of Environmental Research*

*Pseudomonas cepacia*의 전달성 TOL plasmid의 특성과 불화합성

조병남 · 조인선 · 최순영 · 유재근* · 민경희

숙명여자대학교 이과대학 생물학과

*국립환경연구원 임호연구소

ABSTRACT: Toluate degradative plasmid from *Pseudomonas cepacia* SUB37 was determined to be molecular weight as 79.3×10^6 (119 kb) by agarose gel electrophoresis. It was confirmed that TOL plasmid was transmissible into several strains of *Pseudomonas* and other genera which were unable to utilize *m*-toluate. Catechol-2, 3-oxygenase activity which plays an important role in *m*-toluate biodegradation was determined from crude extracts of *P. cepacia* SUB37 and transconjugants, suggesting that, as shown for *P. putida* mt-2, this result provides evidence that the genes coding for the catabolism of *m*-toluate via the meta-pathway are also plasmid encoded. A new and independently isolated TOL plasmid from *P. cepacia* SUB37 belongs to the IncP-4 incompatibility group and could be derivatives of TOL plasmid from *P. putida*, which is IncP-9 group of incompatibility.

KEY WORDS □ ***Pseudomonas cepacia*, Transmissible TOL plasmid.**

Recent works in a number of laboratories have indicated that, in certain strains, the genes coding for the enzymes responsible for catabolism of common substrates are not on the chromosome but are borne on conjugative plasmids (Williams, 1972; Chakrabarty, 1973; Wheelis, 1975). Aromatic hydrocarbons such as camphor, salicylate, octane, naphtalene, toluene, and xylenes were utilized by certain *Pseudomonas* strains carrying plasmids (Rheinwald *et al.*, 1973; Shaham *et al.*, 1973; Chakrabarty, 1972; Charkrabarty *et al.*, 1973; Dunn and Gunsalus, 1973; Worsey and Williams, 1975). Metabolic pathways and their regulation have also been studied. Among these, the degradative pathways of aromatic compounds show 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*-toluates in *P. putida* mt-2 (Murray *et al.*, 1972) had been shown to be carried on a transmissible TOL plasmid (Williams and Murray, 1974). Several reports indicated that bacteria grown on toluene, *m*- or *p*-xylenes degraded these compounds through benzoate, *m*- and *p*-toluates (Davey and Gibson, 1974; Davis *et al.*, 1968; Worsey and Williams, 1975) and that degradation occurred by a series of oxidation through the corresponding alcohol and aldehydes.

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This paper describes that properties of TOL plasmid from *P. cepacia* SUB37 are examined and it is confirmed that genes coding for the catabolism of *m*-toluate. via meta-pathway. are also plasmid encoded.

MATERIALS AND METHODS

Bacterial strains and plasmids

The relevant properties of the bacterial strains and plasmids used in this study are listed in Table 1.

Media and culture conditions

The medium L broth (containing Bacto-Tryptone 10g, Yeast extract 5g, NaCl 5g per liter of distilled water, pH 7.0) was used as a complete medium. And M9 (Masniatis *et al.*, 1982) was used as a minimal medium. For growth of *Pseudomonas* strains utilizing toluate as a sole source of carbon, the M9 medium containing 5 mM *m*-toluate instead of glucose was used.

The composition of the media and conditions of culture, harvesting, and preparation of cell-free extracts have been described (Williams and Worsey, 1976).

Conjugal transfer of plasmids

Donor and recipient cells were grown overnight at 30°C in 5 m*l* of 1% YE to a cell density of 10^8 to 7×10^8 cells/m*l*. About 30 min prior to the experiment, 0.2 to 0.4 m*l* of the donor culture was pipetted into 5 m*l* of fresh sterile 1% YE and shaken at 30°C. To initiate the conjugation, samples containing 0.5 m*l* of donor and recipient cultures were mixed and incubated at 30°C for 30 min without shaking, and concurrently separate tubes containing 0.5 m*l* of sterile minimal medium and either 0.5 m*l* of donor or recipient cultures were incubated as controls. The tubes with mixed donor and recipient cultures were centrifuged, washed, and suspended in 1 m*l* of minimal medium before plating 0.1 m*l* onto *m*-toluate agar plates;

Table 1. *Bacterial strains and plasmids used in this experiment*

Strain	Plasmid	Phenotype	Source & Reference
Pseudomonas cepacia			
SUB37	TOL	wild type (Tol$^+$)	This work
SUB37-1			Cured strain of SUB37
P. cepacia			ATCC 25416
Pseudomonas putida			
TN1126		trp$^-$ met$^-$	Nakazawa and Yokota (1977)
TN1032		trp$^-$ leu$^-$ benl$^-$	Nakazawa and Yokota (1977)
TN1307		trp$^-$leu$^-$benl$^-$recA	Nakazawa and Yokota (1977)
TN1126	RSF 1010	trp$^-$met$^-$/RSF 1010(Sm^r)	Transformation of TN1126 with RSF 1010
mt-2	pWWO(TOL)	Ben$^+$, Mtol$^+$, Ptol$^+$	ATCC 23973
PpG1	CAM	Ben$^+$,Cam$^+$	ATCC 17453
Pseudomonas aeruginosa			
PAO8	R 18	met$^-$liv$^-$/R 18 ($K_m{}^r$)	Issac and Holloway (1968)
PAO2003		arg$^-$Gm^{r}Rif^{r}Str^{r}RecA	Olsen *et al.* (1982)
PAO303	pMG 1	arg$^-$/pMG1 (Sm^r)	Hansen and Olsen (1978)
PAO303	Rms 148	arg$^-$/Rms 148 (Sm^r)	Sagai et al. (1975)
Pseudomonas fluorescens			ATCC 11250
Flavobacterium devorans			KCTC 1870
F. multivorum			Kon Kuk Univ.
Rhodopseudomonas sphaeroides			KCTC 1425
Acinetobacter sphaeroides			ATCC 23393
Lactobacillus casei	pPL	wild type (Lac$^+$)	

control tubes were plated directly onto *m*-toluate agar plates and diluted appropriately for plating onto 1% YE agar plates to give cell counts. In no experiments did growth appear on any of the control *m*-toluate plates (Williams and Murray, 1974; Nakazawa and Yokota, 1977).

Isolation of plasmid DNA.

Plasmid DNA was purified from cell lysates of strains of *P. cepacia* SUB37 by the methods of the previous paper. Large plasmids for molecular weight determination were served as molecular weight markers.

Agarose-gel electrophoresis

Samples of 10 to 30 μl of purified or crude plasmid DNA preparation were subjected to electrophoresis by the method of Maniatis *et al.* (1978).

Preparation of cell-free extract and enzyme assays

Cell-free extracts were prepared by passing 1 to 3g of a frozen cell paste and suspending the broken cell paste in 0.05 M Na_2HPO_4-KH_2PO_4 buffer (pH 7.0) containing 10% acetone. the suspension was centrifuged at 20,000g for 30 min at 4°C to give a supernatant referred to as crude extract.

Catechol-2, 3-oxygenase

Catechol-2, 3-oxygenase was assayed spectrophotometrically. The spectrophotometric assay consisted for measuring the rates of the respective ring fission products of catechol (375 nm) in a 3 m*l* reaction mixture, which contained a 10 mM concentration of the respective catechol, 50 mM KH_2PO_4 buffer (pH 7.5) and the cell extract. To measure catechol-2, 3-oxygenase in the cell extracts which also contained catechol-1, 2-oxygenase, the cell extracts were heated at 55°C for 10 min, as described by Murray and Williams (1974), to destory catechol-1, 2-oxygenase, which could otherwise interfere with the assay. This procedure destroyed 90 to 95% of the catechol-1, 2-oxygenase activity without affecting the catechol-2, 3-oxygenase activity. One unit of enzymes is defined as that amount which oxidizes 1 μmole of catechol per minute. Specific activity is defined as the number of enzyme units per mg of protein.

Catechol-1, 2-oxygenase

Catechol-1, 2-oxygenase was measured by following the formation of cis, cis-muconate at 260 nm (Hegeman, 1966), and cis, cis-muconate-lactonizing enzyme was assayed by following the disappearance of cis, cis-muconate as described by Ornston (1966). To measure catechol-1, 2-oxygenase in the cell extracts containing high levels of catechol-2, 3-oxygenase, the cell extracts were first treated with 50 mM H_2O_2 for 10 min (Nakazawa and Yokota, 1973), which destroyed from 90 to 95% of the catechol-2, 3-oxygenase activity. Specific activity is defined according to catechol-2, 3-oxygenase.

Incompatibility of plasmid

Determination of incompatibility group of TOL plasmid in *P. cepacia* SUB37 was carried out according to the method of Dunn *et al.* (1980).

RESULTS AND DISCUSSION

Molecular weight of TOL plasmid DNA

Fig. 1. showed the standard curve that we have constructed from agarose gel electrophoresis data for five plasmids whose range of molecular size varied from 35×10^6 for pLC plasmid to 150×10^6

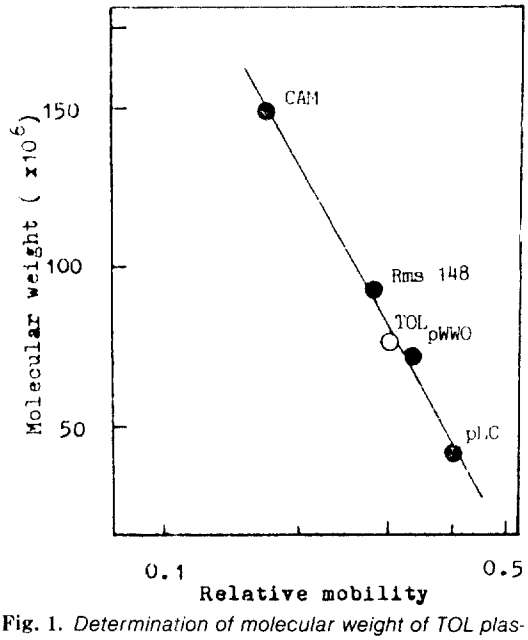

Fig. 1. *Determination of molecular weight of TOL plasmid from P. cepacia SUB37 by relative mobility on agarose gel electrophoresis.*
The reference plasmids were CAM (150×10^6), Rms 148 (95×10^6), TOL (79.3×10^6) from *P. cepacia* SUB37, pWWO (78×10^6) TOL plasmid from *P. putida* mt-2, and pLC plasmid from *Lactobacillus casei*.

for CAM plasmid.

Although the standard curve did not provide a precise molecular size, it allows to determine reproducible, relative and approximate size. The TOL plasmid of *P. cepacia* SUB37 was similar to pWWO ($78 \times 10^6 = 117$ kb) plasmid of standard curve, as shown by their slower migration during electrophoresis. On the basis of five bands, the molecular size of TOL plasmid of *P. cepacia* SUB37 was estimated 79.3×10^6 (119 kb) by a linear extrapolation.

Duggleby *et al.* (1977) have shown that in at least some of the soil *Pseudomonas* the TOL function is plasmid borne. It is striking that in a number of independently isolated strains, the TOL plasmids must be very closely related. This group could also be distinguished from the other TOL strains studies here by genetic and physiological criteria.

Molecular weight of TOL plasmid from *P. cepacia* SUB37 also distinguished from the other TOL plasmids from *P. putida* mt-2, mt-16, mt-17, mt-18 and mt-21 (Duggleby *et al.*, 1977). These results indicate strongly that some plasmids may be very widly distributed in nature.

Conjugation of TOL plasmid

In addition to the production of cured, nonrevertible strains, the ability of the wild type to transfer the genes back into the cured strains by conjugation is a major criterion, which has been applied to characterize a set of genes as being plasmid carried.

The majority of matings were carried out using streptomycin selection against the donor, but in addition auxotrophic mutants of several of the isolates were made; this was essential for *P. putida* TN1126, TN1032, TN1307 and *P. aeruginosa* PAO2003 and PAO303, which were very resistant to streptomycin.

The result of the intraspecies mating is given in Table 2. *P. capacia* SUB37 was able to transfer their putative TOL plasmids back into their cured strain at a frequency comparable to *P. putida* strains and *P. aeruginosa* strains as interspecies mating, and other strains as intergeneric mating. *P. cepacia* SUB37 was also able to transfer its plasmid at a lower, but detectable, frequency into different species of *P. putida, P. aeruginosa* and *P. fluorescens* strains, but transfer of TOL plasmids from *P. cepacia* SUB37 into *Rhodopseudomonas sphaeroides* and *Flavobacterium devorans* strains could be detected at the lower frequencies. No measurable transfer from *P. cepacia* SUB37 into *Flavobacterium multivorum* and *Acinetobacter calcoaceticus* was observed.

It is interesting that, whereas *P. cepacia* SUB37 can readily transfer TOL plasmid into the cured strain, the ability to transfer into other species of the same genus is limited to those strains which very closely resemble *P. cepacia* SUB37.

Generally, degradation of catechol is made by two enzyme systems. Those are meta-pathway producing 2-hydroxymuconic semialdehyde according to catechol-2, 3-oxygenase, and ortho-cleavage pathway according to catechol-1, 2-oxygenase.

We can clearify the fact that enzyme system participating meta-pathway be all in plasmid in case of strain belonging to *Pseudomonas* and enzyme system participating ortho-pathway be in chromosome. Initial velocities of catechol-1, 2-oxygenase, catechol-2, 3-oxygenase were measured and observed to be roughly linear until about 15 min after the reactions were started. From these preliminary experimental results, it scheduled that the enzyme activities were assayed at 3 min after starting the enzyme reaction. The results of enzyme activity assay are shown in Table 3. When *P. cepacia* SUB37 was grown on *m*-toluate the key enzyme catechol-2, 3-oxygenase of the meta-pathway but not the key enzyme catechol-1, 2-oxygenase activity of *P. cepacia* SUB37 containing TOL plasmid was higher compared with those of in absence of TOL plasmid.

Kunz and Chapman (1981) indicated that, in strains carrying the plasmid, benzoate is metabolized via the meta-pathway since benzoate serves as the inducer of catechol-2, 3-oxygenase and the meta-pathway whereas induction of the ortho-pathway requires the initial converstion of benzoate to catechol whith must then first be converted by basal levles of catechol-1, 2-oxygenase to cis, cis-muconic acid its product inducer.

This communication shows that the pathways by which these hydrocarbons are degraded by *P. cepacia* SUB37 are similar to those described *P. putida* mt-2 and other *Pseudomonas* (Kunz and Chapman, 1981; Williams and Murray, 1974; Williams and Worsey, 1976; Worwey and Williams, 1975) and, as shown for *P. putida* mt-2 provides

Table 2. *Conjugal transfer of TOL plasmid to various strains*

Donor	Recipient	Selective marker	Mating time(h)	Frequency of* transfer ($\times 10^7$)
P. cepacia SUB37	*P. cepacia* SUB37-1	TOL$^+$,Sm^r	1	310
			2	690
(TOL$^+$,Sm^s)			3	850
	P. putida TN 1126	met$^+$,trp$^+$,TOL$^+$,Sm^r	1	0.4
			2	1.7
			3	1.9
	P. putida TN 1032	trp$^+$,leu$^+$,TOL$^+$,Sm^r	1	0.9
			2	3.2
			3	4.4
	P. putida TN 1307	trp$^+$,leu$^+$,TOL$^+$,Sm^r	1	3.0
			2	6.6
			3	7.9
	P. aeruginosa PAO2003	arg$^+$,TOL$^+$,Sm^r	1	–
			2	1.3
			3	2.1
	P. aeruginosa PAO303	arg$^+$,TOL$^+$,Sm^r	1	1.1
			2	2.3
			3	4.3
	P. fluorescens	TOL$^+$,Sm^r	1	0.5
			2	1.3
			3	3.8
	R. sphaeroides	TOL$^+$,Sm^r	1	–
			2	–
			3	0.06
	F. devorans	TOL$^+$,Sm^r	1	–
			2	0.01
			3	0.03
	F. multivorum	TOL$^+$,Sm^r	1	–
			2	–
			3	–
	A. calcoaceticus	TOL$^+$,Sm^r	1	–
			2	–
			3	–

*Frequency of transfer = $\frac{\text{Number of conjugants}}{\text{Number of donors}}$

evidence that the genes coding for the catabolism of these compounds are also plasmid encoded.

The activity of *P. putida* TN1307/conjugants among exconjugants were higher than those of *P. cepacia* SUB37 containing TOL plasmid as donor cell.

This result suggests that *P. putida* could be better host strain for TOL plasmid than *P. cepacia* in their gene expression.

Determination of incompatible group of TOL plasmid

One of the major criteria in the classification

Table 3. *Specific activities of catechol-1,2-oxygenase and catechol-2,3-oxygenase of various Pseudomonas strains and their growth on m-toluate minimal medium*

Strains	Sp. activity of catechol 1,2-oxygenase (μmole/min/mg protein) $\times 10^{-2}$	Sp. activity of catechol 2,3-oxygenase (μmole/min/mg protein) $\times 10^{-2}$	Growth at *m*-toluate* minimal medium (A 550 nm)
P. cepacia SU37 (TOL$^+$)	84.3	276.2	2.14
P. cepacia SU25 (TOL$^-$)	0.1	0.3	0.39
P. putida TN 1307 (TOL$^-$)	0.3	0.3	0.86
P. aeruginosa PA0303 (TOL$^-$)	0.1	0.2	0.35
P. putida TN1307/conjugant (TOL$^+$)	89.4	355.4	2.46
P. aeruginosa PA0303/conjugant (TOL$^+$)	66.6	213.3	2.24

*Growth was carried out overnight at 30 °C

Table 4. *Determination of incompatibility group by conjugation*

Donor	Recipient	Plasmid (Inc group)	Selective marker	Mating time(h)	Frequency of transfer($\times 10^{-6}$)	Growth on selective medium after 3 generations
P. cepacia	*P. aeruginosa*	R18(Km^r)		1	1.05	+
SUB37	PAO8	(IncP-1)	Km^r, Rif^r, TOL$^+$	2	1.42	+
(TOL$^+$,Rif^r)				3	2.23	+
	P. aeruginosa	pMG1(Sm^r)		1	2.55	+
	PAO303	(IncP-2)	Sm^r, Rif^r, TOL$^+$	2	2.87	+
				3	3.4	+
	P. putida	RSF1010(Sm^r)		1	0.008	−
	TN1126	(IncP-4)	Sm^r, Rif^r, TOL$^+$	2	0.0086	−
				3	0.009	−
	P. aeruginosa	Rms 148(Sm^r)		1	0.79	+
	PAO303	(IncP-7)	Sm^r, Rif^r, TOL$^+$	2	1.46	+
				3	1.7	+

*Frequency of transfer = $\frac{\text{Number of conjugants}}{\text{Number of donors}}$

of *Pseudomonas* plasmids has been their incompatibility characteristics. Since many plasmids of *Pseudomonas* are atransmissible to other bacteria where their incompatibility characteristics can be studied in relation to other bacteria plasmids. Thus a new classification system of plasmids has devised on the basis of their interaction.

On the basis of the result that TOL plasmid from *P. cepacia* SUB37 can be transferred to *P. aeruginosa* and *P. putida*, the incompatible group of TOL plasmid was examined into several groups of incompatibility. TOL plasmid from *P. cepacia* SUB37 was transferred to *P. aeruginosa* PAO8-1 (R18; IncP-1), *P. aeruginosa* PAO303-2 (pMG1; IncP-2), *P. aeruginosa* PAO303-7 (Rms148; IncP-7) using *m*-toluate and antibiotics selection, respectively, at frequencies of approximately 10^{-6} transconjugants per donor cell. However, TOL plasmid from *P. cepacia* SUB37 was also transferred to *P. putida* TN1126-4 (RSF1010; IncP-4) to determine if TOL plasmid residence in *P. putida* TN1126-4 limited entry of TOL plasmid. This TOL

plasmid transferred to *P. putida* TN1126-4 at a low frequency of approximately 10^{-9} transconjugants per donor cell. Their transconjugants from each conjugation was purified by single colony isolation and growth through approximately 3 generations on the respective selection medium. Tests for growth on *m*-toluate of two single clonies from each of the purified transconjugants indicated that all of the TOL transconjugants retained with R18 pMG1, and Rms148 but none of the TOL transconjugants tested retained with RSF1010.

To verify that the presence of TOL was not interfering with expression of streptomycin resistance, strains were obtained from which TOL had segregated spontaneously. All segregants remained unable to utilize *m*-toluate in presence of antibiotics, respectively. These results suggest that TOL plasmid from *P. cepacia* SUB37 belongs to the same incompatibility group as RSF1010 and the oringinal TOL plasmid, that is the P4 incompatibility group. Dunn *et al.* (1980) isolated catabolic plasmids conding for the degradation of naphthalene in *P. putida* both plasmids are transmissible belong to the P9 incompatibility group with TOL plasmid.

However, our result is distinguished from that of the above result by Dunn *et al.* (1980). Therefore, TOL plasmid from *P. cepacia* SUB37 could be derivatives of that from *P. putida*.

The detailed determination of the relationship between the different TOL plasmids may also provide useful information on their evolutionary origin.

적 요

Toluate 분해 플라스미드를 *Pseudomonase cepacia* SUB37 에서 분리하여 분자량을 한천 겔 전기영동으로 측정한 결과 79.3×10⁶(119 kb)로 확인되었다. 이 TOL플라스미드는 *Pseudomonas* 의 다른 균주와 다른 속의 균주에 전달되었다. *m*-toluate 분해에서 가장 중요한 역할을 하는 catechol-2,3-oxygenase 활성을 *P. cepacia* SUB37 과 transconjugant 의 조효소액으로부터 측정한 결과, *P. putida* mt-2 에서와 같이, meta pathway 를 거쳐 *m*-toluate 를 분해하는 유전자들이 plasmid 에 암호화됨을 알 수 있었다. *P. cepacia* SUB37 유래의 새로운 TOL plasmid 는 IncP-4 불화합성군에 속하였고, 이것은 아마도 *P. putida* 의 IncP-9 그룹의 TOL 플라스미드의 유도체로 사료된다.

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