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Isolation and Characterization of Naturally Occuring Bacteria Carried TOL Plasmid

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TOL 플라스미드 세균의 분리와 특성

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Eighty two bacterial strains have been isolated from five different soil and sewage samples by selective enrichment culture on *m*-toluate minimal medium. Two of these were identified as *Pseudomonas cepacia,* one as *P. putida,* one as *Yersinia intermedia,* and one as *Flavobacterium odoratum. P. cepacia* SUB37 appeared to carry plasmid superficially similar to TOL plasmid previously described in *P. putida* mt-2 and other two plasmids from *Flavobacterium odoratum* and *Y. intermedia* larger than that of *P. putida* mt-2. *P. cepacia* SUB37 was sensitive to streptomycin but resistant to rifampicin. *P. cepacia* SUB37 carrying plasmid metabolizes the hydrocarbons to benzoate and toluates via the corresponding alcohols and aldehydes. By the curing experiment, it appears that *P. cepacia* SUB37 carries TOL plasmid encoding for the enzymes responsible for the catabolism of toluene and xylene via benzoate and the toluates and then by meta pathway in the process of degradation of aromatic hydrocarbons. *P. cepacia* SUB37 degraded *m*-toluate rapidly to be very low level when it was fully grown.

Pseudomonas sp. was one of the soil bacteria isolated by selective enrichment on *m*-toluate as sole carbon source (10, 14). A number of studies have revealed that genes coding for the enzymes for catabolism of several compounds in the genus *Pseudomonas* are plasmid encoded (4). One such plasmid, designated TOL as originally described for *Pseudomonas putida* mt-2 (13, 15, 16), codes for catabolic sequences which lead to the dissimilation of toluene and *m*-and *p*-xylene via initial reactions forming benzoate and *m*-and *p*-toluate, respectively, followed by meta-fission pathway. A number of *Pseudomonas* strains capable of utilizing toluene and *m*-and *p*-xylene have been isolated from soil, and in each of these, the genes encoding for the degradative enzymes have been shown to be plasmid encoded (14).

We have recently shown that pseudomonads and other strains capable of degrading toluene and *m*-xylene through benzoate, and *m*-toluate respectively and hence by the meta pathway, can readily be isolated from soil and sewage and that in each strain examine the genes coding for the catabolic enzymes appear to be present on a curable plasmid.

Materials and Methods

Bacterial strains

The bacterial strains and plasmids used in this experiment are listed in Table 1.

Key words: TOL plasmid, toluene, degradation
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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
SUB25		wild type	This work
P. cepacia			ATCC25416
Pseudomonas putida			
SUB48		wild type	This work
mt-2	pWWO (TOL)	Ben^+, $Mtol^+$, $Ptol^+$	ATCC23973
Flavobacterium odoratum			
SUB53	TOL	wild type (Tol^+)	This work
F. odoratum			ATCC4651
Yersinia intermedia SUB34	TOL	wild type (Tol^+)	This work
Y. intermedia			ATCC29909

Media

m-Toluate minimal medium (5) and M9 minimal medium (8), which were composed of 10 mM *m*-toluate instead of glucose as a carbon source, were used for the isolation of bacterial strains the utilization of *m*-toluates. Benzoate-yeast extract medium-containing 10 mM benzoate was used for the enzyme induction (2).

Isolation of bacterial strains for toluate utilization

For the isolation of bacterial strains utilizing *m*-toluate as sole carbon source, the enrichment broth was employed by means of *m*-toluate minimal broth. The strains were selected on the plates of M9 minimal medium containing 10 mM *m*-toluate and the growing strains were stored on the same medium at 4°C.

Identification of bacterial strains

Identification of bacterial strains for *m*-toluates utilization was carried out according to Bergey's Manual of Systematic Bacteriology (7) and API 20E kit (11).

Isolation of plasmid

Bacterial strains with plasmids were grown overnight in 10 m*l* of Luria Broth. Harvest cells were suspended with 0.4 m*l* of 25% sucrose-50 mM Tris-HCl (pH 8.0) and treated with 96.5 μl of lysozyme (5 mg/m*l*) in the same buffer for 5 min at 0°C, followed by incubation for another 5 min in presence of 50 μl of 6.25 mM EDTA. The cells were treated with 27.6 u*l* of 0.5% Brij-0.2% sodium deoxycholate-6.25 mM EDTA in 50 mM Tris-HCl (pH 8.0) for 10 min at 0°C, and centrifuged at 30,000g for 20 min at 2°C. Plasmid DNA was precipitated with 10% PEG in the presence of TES (50 mM Tris-HCl-5 mM EDTA-50 mM NaCl, pH 8.0) buffer. Then CsCl(5.8g) was added to the solution and centrifuged to remove insoluble PEG(9).

Curing experiment

Plasmid curing by mitomycin C was carried out according to method of Chakrabarty (3). Single colony from *m*-toluate agar plate was inoculated into culture tubes containing 5 m*l* of LB (Luria Broth). After overnight growth at 30°C, 50 μl of a 10^3 dilution of the culture (10^4 -10^5 cells/m*l*) was inoculated into 10 m*l* of LB contained various concentrations of mitomycin C (0-30 μg/m*l*), and incubated with shaking for 24 to 48 hrs until the cells grow well. Then appropriate dulutions were made and 0.1 m*l* of them was spread on nonselective LB plate and replicated to the *m*-toluate plate for scoring and selective individual clones for the TOL phenotype were obtained.

Results and Discussion

Isolation and identification of bacterial strains for toluate degradation

The eighty two isolates that utilized *m*-toluate as

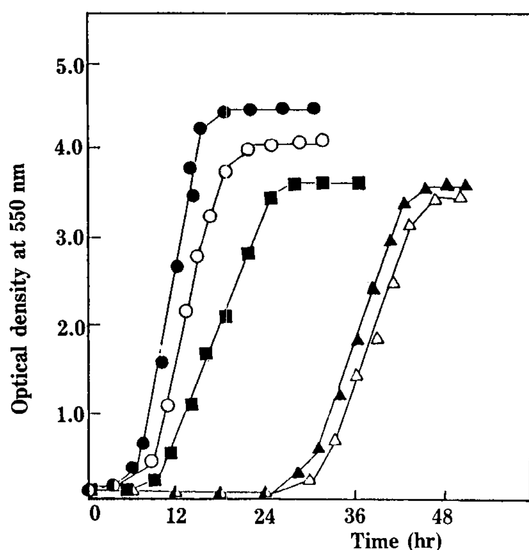

Fig. 1. Growth curve of the strains biodegrading *m*-toluate.

P. cepacia SUB25 (▲-▲), *Y. intermedia* SUB34 (■-■), *P. cepacia* SUB37 (●-●), *P. putida* SUB48 (△-△),and *F. odoratum* SUB53 (○-○).

sole carbon source were isolated from the sewage and soil samples around the several companies which is involved in hydrocarbon materials in Seoul area. Among them, five isolates growing well on *m*-toluate agar plates were selected and identified. The five isolates, SUB25, SUB34, SUB37, SUB48 and SUB53, were tested with morphological, physiological and biochemical characteristics. Some of the isolates were tentatively identified as the genus *Pseudomonas, Flavobacterium* and *Yersinia.* The isolates, SUB25 and SUB37, were identified as *Pseudomonas cepacia,* the isolate SUB34 as *Yersinia intermedia,* the isolate SUB48 as *P. putida,* and the isolate SUB53 as *Flavobacterium odoratum,* comparing with type cultures; *P. cepacia* ATCC25416, F. odoratum ATCC4651 and *Y. intermedia* ATCC29909.

The growth of toluate-degradation bacteria

The growth of five isolates on *m*-toluate minimal broth exhibited a typical sigmoid shape and the growth of the isolates were rapidly increased to approximately 4.0 of optical density at 550 nm, indicating that they utilize *m*-toluate as energy source (Fig. 1). Among them, *P. cepacia* SUB37 was shown to the best growth comparing with other strains, and the next was *F. odoratum* SUB53. The finally selected

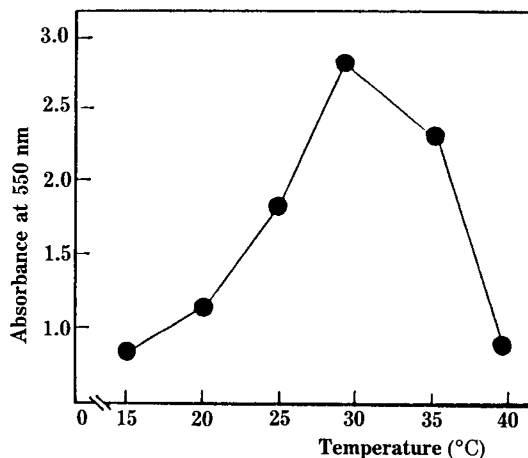

Fig. 2. Effect of temperature on the growth of *P. cepacia* SUB37.

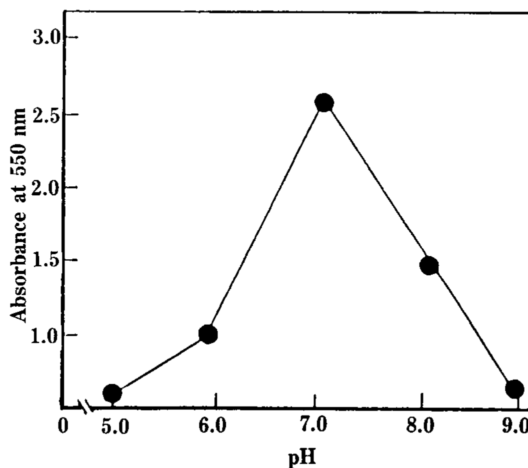

Fig. 3. Effect of pH on the growth of *P. cepacia* SUB37.

strain, *P. cepacia* SUB37, was tested the growth on the temperature, pH and *m*-toluate concentration in minimal medium. Fig. 2 and 3 are shown that *P. cepacia* SUB37 grew well at the optimum condition of 30°C and pH 7.0. The *m*-toluate concentration in minimal medium of growth of this strain was investigated to be optimum condition at 10 mM, but at higher concentration of *m*-toluate, it's growth was decreased gradually as shown in Fig. 4.

Plasmid isolation

It was reported that the most ability degrading hydrocarbon was existed on the genes of plasmid (4, 12). The presence of plasmid degrading *m*-toluate was applied to the agarose gel electrophoresis as shown

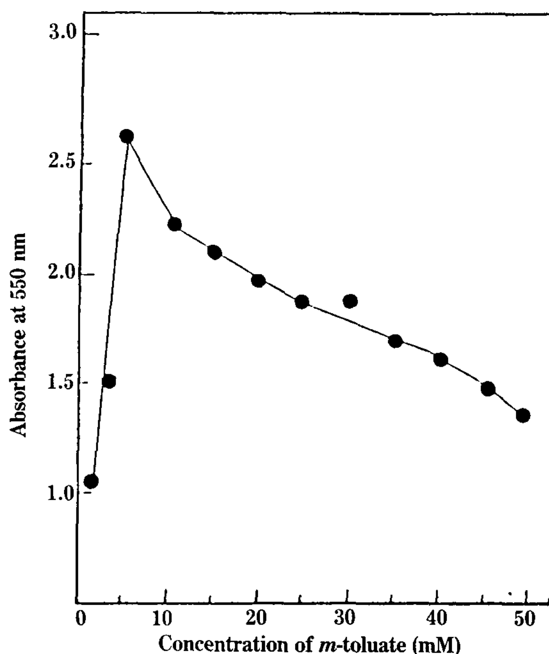

Fig. 4. Effect of *m*-toluate concentration on the growth of *P. cepacia* SUB37.

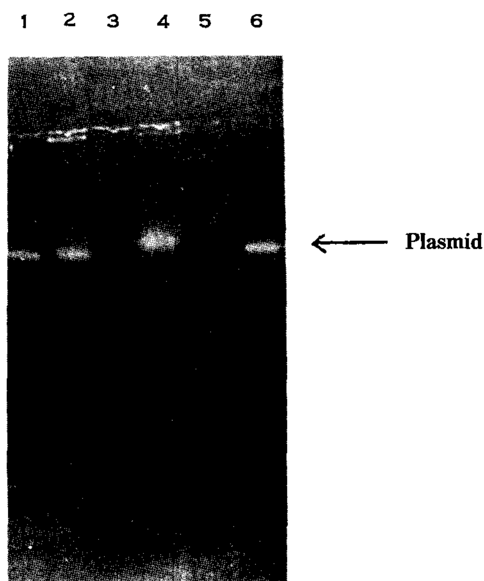

Fig. 5. Isolation of plasmid by agarose gel electrophoresis.
lane 1: *P. putida* mt-2, lane 2: *P. cepacia* SUB37, lane 3: *P. putida* SUB48, lane 4: *Y. intermedia* SUB34, lane 5: *P. cepacia* SUB25, lane 6: *F. odoratum* SUB53.

in Fig. 5. *Pseudomonas putida* mt-2 carrying TOL plasmid was used for standard culture comparing with other isolates. It was shown that *P. cepacia* SUB37, *Yersinia intermedia* SUB34, and *Flavobacterium odoratum* SUB53 were carried plasmids. Plasmid size of *P. cepacia* SUB37 was almost similar to TOL plasmid (117 kb) from *P. putida* mt-2, whereas plasmids of *F. odoratum* and *Y. intermedia* were larger than TOL plasmid from *P. putida* mt-2.

Hydrocarbon biodegradation

The growth characteristics of the isolates are shown in Table 2. All of them grow on *m*-toluate, xylene and catechol, and not grow on naphthalene, octane, camphor and 2,4-dichlorophenoxyacetic acid. In this respect their metabolism appeared to be identical to that of *P. putida* mt-2 which metabolizes the three hydrocarbons to benzoate and the toluates via the corresponding alcohols and aldehydes (14) although those alcohols and aldehydes did not used for the test of growth characteristics.

The superficial similarity between the isolates and *P. putida* mt-2 in their metabolism is argumented by the observation that all of them appeared to use the

Table 2. Utility of various hydrocarbons of selected strains.

Strains\hydrocarbons	TOL	XYL	NAH	OCT	CAT	SAL	BEN	CAM	2,4-D
1. *P. cepacia*									
SUB34	+	+	–	–	+	–	–	–	–
2. *Y. intermedia*									
SUB53	+	+	–	–	+	–	–	–	–
3. *F. odoratum*									
SUB37	+	+	–	–	+	–	–	–	–

Abbr.: TOL; toluene, XYL; xylene, NAH; naphtalene, CAT; catechol, SAL; salicylate, BEN; benzoate, CAM; camphor, 2,4-D; 2,4-dichlorophenoxyacetic acid.

Table 3. Curing frequency (%) of *P. cepacia* SUB37 with mitomycin C.

Exp.	Concentration of mitomycin C (μg/ml)				
	0	5	10	20	30
I*	0	0.2	0.6	1.3	2.2
II	0	0.8	1.7	2.4	4.1
III	0	1.5	2.8	3.9	6.1

*Experiment I, II and III was the serial inoculation from the original inoculation.

meta pathway for further metabolism of *m*-toluate. Cells grown on *m*-toluate showed the characteristics yellow color of 2-hydroxymuconic semialdehyde when incubated with catechol in the cleavage test.

Curing experiments

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 substrates. The main agent to cure catabolic plasmids in *Pseudomonas cepacia* SUB37 was used to treated with mitomycin C.

Experiment I was incubated with 5 μl of a 10^3 dilution of the culture at 1 m*l* of LB contained 5 μg/m*l*, 10 μg/m*l*, 20 μg/m*l* and 30 μg/m*l* of mitomycin C and then they were incubated until the cells were fully grown. In the case of 5 μg/m*l* of mitomycin C treatments, Experiment I to Experiment III were incubated with three times of inoculation of LB contained the same concentration of mitomycin C as described above.

From the results of Table 3, it appears that plasmids were effectively cured with the treatment of 30 μg/m*l* mitomycin C. Single colonies grown on the selective medium were appeared their phenotype. Since the ortho pathway is specific for benzoate, these cured cells loose the ability to metabolize *m*-and *p*-toluate. We, therefore, determined the proportion of the cured cells for all the isolates after growth on benzoate from a small inoculum. The frequency of curing produced after benzoate growth varies from 0.2 to 6.1% for different isolates. However, our experience with this mitomycin C leads us to conclude that it is not a proper general agent for plasmid curing. By this curing experiment, it appears that *P. cepacia* SUB37 carries plasmid coding for the enzymes responsible for the catabolism of toluene and xylene via benzoate and the toluates and then by meta pathway.

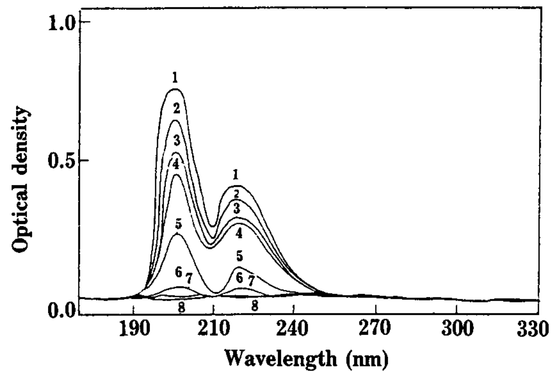

Fig. 6. Toluate biodegradativity by *P. cepacia* SUB37 on the spectra of ultraviolet scanning.
1; 0 hr, 2; after 3 hr,
3; after 6 hr, 4; after 9 hr,
5; after 12 hr, 6; after 15 hr,
7; after 18 hr, 8; after 24 hr incubation.

Toluate biodegradativity

In order to determine the degradation of *m*-toluate as a sole carbon source, *P. cepacia* SUB37 was grown on *m*-toluate minimal broth. The results of UV scanning on *m*-toluate degradation shown in Fig. 6 and it shows two peak at 205 nm and 228 nm. *m*-Toluate was degraded rapidly to be resulted very low level of residue with 15-18 culture times of *P. cepacia* SUB37, suggesting that *P. cepacia* SUB37 has completely degraded *m*-toluate when it was fully grown.

요 약

m-Toluate 최소배지에서 선택적 enrichment culture를 통하여 82개의 세균의 균주를 분리하였으며, 이들 중 두 균주는 *Pseudomonas cepacia*, 한 균주는 *P. putida*, 한 균주는 *Yersinia intermedia*, 그리고 한 균주는 *Flavobacterium odoratum*으로 동정되었다. *P. cepacia* SUB37은 *P. putida* mt-2의 TOL 플라스미드와 비슷한 크기의 플라스미드를 가지고 있었으며, *Flavobacterium odoratum*과 *Yersinia intermedia*는 이보다 더 큰 플라스미드를 갖고 있었다. *P. cepacia* SUB37은 streptomycin에 감수성을 나타내었으나, rifampicin에는 내성을 나타내었다. 플라스미드를 갖는 *P. cepacia* SUB37은 탄화수소를 해당 알콜과 알데히드를 거쳐서 benzoate와 toluate

로 분해하였다. 플라스미드 제거실험으로, *P. cepacia* SUB37의 플라스미드에는 탄화수소 분해과정 중 toluene과 xylene을 benzoate, toluate로 분해하는 효소와, 계속하여 meta pathway를 거치는 단계의 효소를 코딩하는 유전자들이 있음이 확인되었다. 또한 *P. cepacia* SUB37은 생장이 왕성하였을 때 *m*-toluate를 거의 분해하였다.

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