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Isolation of Salicylate-Degrading Plasmid from *Pseudomonas putida*

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*Pseudomonas putida*로부터 Salicylate 분해 Plasmid의 분리

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Abstract: The large plasmid (about 180 megadaltons) was isolated from the aquatic strain of *Pseudomonas* which was found to degrade salicylate. It was found that the plasmid could be isolated under gentle conditions in comparison with other methods. The yield of covalently closed circular DNA was enhanced by heat treatment at 55°C after denaturing the chromosomal DNA with alkaline sodium dodecyl sulfate (pH 12.45), and the plasmid DNA was selectively concentrated by utilizing 10% polyethylene glycol as final concentration. It was also found that the cured strains with mitomycin C did not show any growth on the medium containing salicylate, therefore, it was concluded that the plasmid might play an important role on the salicylate degradation.

Key Words: plasmid, *Pseudomonas putida*, salicylate, curing

The genetic basis of the biodegradation of aromatic hydrocarbons such as naphthalene, toluene, xylene, benzoate, and salicylate has been studied in a number of laboratories. Recent work has indicated that in certain stains of *Pseudomonas*, the genes for catabolism of these less common substrates are carried on transmissible plasmids (Chakrabarty, 1972; Dunn and Gunsalus, 1973; Williams and Murray, 1974; Williams and Worsey, 1976). We have also isolated a strain of *P. putida* which has a versatile catabolic capacity, and the degradative pathway seems to be plasmid-borne (Hong *et al.*, 1986).

In order to determine whether a given bacterium harbors plasmids, it is necessary to isolate plasmid DNA. Various extraction methods (Currier and Nester, 1976; Hansen and Olsen, 1978) have been used for the physical analysis of the large plasmids involved in the degradation of aromatic compounds by *Pseudomonas*.

Isolation procedures of large plasmids have been developed by Humphreys *et al.* (1975), Palchaudhuri and Chackrabarty (1976), and Currier and Nester (1976). Many plasmids in genus *Pseudomonas* are often quite difficult to isolate, due to their large size and their instability.

There appear to be two problems in isolation procedures of large plasmid. With the methods that depend on the selective precipitation of chromosomal complexes by high-gravity centrifugation (Clewell and Helinski, 1969) or high-salt precipitation (Guerry *et al.*, 1973), lage plasmids do not remain in the supernatant. Other methods employ a shearing step to break chromosomal DNA, which may act to help release plasmid. However, shearing appears also to break much of

the large plasmid DNA (Currier and Nester, 1976). Under these above mentioned conditions, it is necessary that an increase of input cells to provide sufficient plasmid yield. Increasing the input, however, required a further elaboration: excess linear DNA is removed before concentration of plasmid DNA (Humphreys *et al.*, 1975; Palchaudhuri and Chakrabarty, 1976).

To enhance the yield and stability of large plasmid, the procedures of plasmid isolation were modified: the disruption of cells and the denaturation of chromosomal DNA were carefully carried out and heat treatment of high salt-precipitated chromosome-membrane complex was examined.

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacterial strains and plasmids used are listed in Table 1.

Media and culture conditions

Pseudomonas putida B3 strain was grown in minimal medium described by Hong *et al.* (1986). Other cells were grown in L broth (containing Tryptone 10g, Yeast extract 5g, NaCl 5g per liter of distilled water, pH 7.0). Cell cultures inoculated from single colonies were grown to approximately 2×10^8 cells per ml in broth medium. All cultures were incubated with shaking at 30°C. All strains were maintained on appropriate minimal media containing carbon sources at concentration of 5mM.

Buffers and reagents

TE buffer contained 0.05 M Tris (hydroxymethyl) aminomethane (Tris) and 0.02M disodium ethylenediaminetetraacetate (Na_2EDTA) (pH 8.0). TES buffer consisted of 0.01 M Tris, 0.01M NaCl, and 1mM Na_2EDTA. Storage buffer contained 10mM 2-mercaptoethanol in TES buffer. Tris-borate running buffer contained 89 mM Tris, 2.5 mM Na_2EDTA, and 89 mM boric acid.

Lysing solution was 6.9% sodium dodecyl sulfate (SDS) (w/v) in TE buffer. The solution was adjusted to pH 12.45 with freshly prepared 3 N NaOH. Polyethylene glycol (PEG) solution was 42% (w/v) in 0.01 M sodium phosphate buffer (pH 7.0).

Cell lysis and plasmid isolation

All bacterial cells except *P. putida* B3 were lysed with lysozyme and SDS. Plasmid isolation was performed by the method of Franklin and Williams (1980).

In order to isolate the plasmid of *P. putida* B3, following procedures were used. Cells were cultured in salicylate-minimal medium. The cells were harvested at 10,000 rpm for 10 min, and washed with 100 mM Tris-HCl (pH 8.0). The washed pellet was resuspended by mixing in a vortex mixer in 5ml of 25% sucrose -0.05M Tris-HCl (pH 8.0) per gram of cells. At ambient temperature, 2 volumes of lysing buffer was added and the mixture was stirred with a magnetic stirrer at 100 rpm for 3 min before incubation at 37°C for 1 hr. The pH was then lowered to 8.5 by adding 2M Tris-HCl buffer (pH 7.0) and stirring the mixture at 100 rpm for 10 min. The addition of SDS (20%, w/v, in TE) to 4% final concentration was carried out under gentle stirring. The lysate was adjusted to 1M NaCl by addition of 5 M NaCl, and stirring the mixture at 100 rpm for 30 min. Then the mixture was chilled in an ice-water bath and refrigerated for 6 hr or overnight as conve-

Table 1. *Bacterial strains and plasmids*

Bacterial strains	Plasmids	Molecular size (Mdal)	Origin
Pseudomonas aeruginosa			
PAO303	Rms148	95	Y. N. Lee[a]
Pseudomonas putida			
mt-2	TOL	78	ATCC 23973
PpG1	CAM	170	ATCC 17453
PpG7	NAH	56	ATCC 17485
B3	SAL	?	Hong *et al.* (1986)
Agrobacterium tumefaciens			
C-58	Ti	120	R. Dickey

Abbreviations: Mdal: mega-dalton, TOL: toluene, CAM: camphor, NAH: naphthalene, SAL: salicylate, Ti: tumor-inducing

a: *P. aeruginosa* PAO 303 was a kind gift from Y. N. Lee, Korea University.

nience.

After thawing the mixture at 55°C for 10 min, centrifugation was carried out at 12,000 rpm (4°C, 1hr). The supernatants were collected into chilled tubes and 0.313 volume of PEG solution was added, giving a concentration of 10%. Mixing was done by stirring. After the tubes were chilled at 4°C for 2 hr, centrifugation for 5 min at 2,500 rpm (4°C) yielded pellets: these were resuspended in appropriate volumes of cold storage buffer and kept in refrigerator.

Agarose gel electrophoresis

Samples of 10 to 20 μl of crude plasmid DNA preparations were subjected to electrophoresis by the method of Meyers *et al.* (1976) in a horizontal gel apparatus at 100 V for 3 hr in 0.7% agarose (Type II, Sigma Chemical Company) using Tris-borate buffer. A dye solution consisting of bromophenol blue (0.25%), xylene cyanol (0.25%), and Ficoll type 400 (15%) in water was added at 5 μl per sample to DNA samples prior to electrophoresis. The gel was stained for 30 min in 0.4 μg of ethidium bromide per ml of water and photographed with a Polaroid MP-3 Land Camera equipped with a Kodak 22A Wratten (red) filter using Polaroid 667 film. The gels were illuminated during photography with a transilluminator C-62 (Ultraviolet Products). To avoid overheating, the gel-holding base was placed on an ice-water bath.

Curing with mitomycin C

Tubes of L broth containing various concentrations of mitomycin C were inoculated with suitable dilutions of early stationary phase cells to give concentration of 10^4 to 10^5 cells per ml. These tubes were incubated on a shaker at 30°C for 2 days. Samples from tubes that showed some growth were transferred in the same series of tubes of L broth containing mitomycin C. Such transfer was repeated. Samples from tubes that showed growth were then diluted and spread on L agar plate. After growth at 30°C for 24hr, individual colonies were transferred to L agar plates and salicylate-minimal plates to give the portion of salicylate-negative (Sal^-) derivatives.

Chemicals

SDS, Tris, Na_2EDTA, PEG 6,000, Ficoll type 400, and ethidium bromide were purchased from Sigma Chemical Co., St. Louis, U.S.A.. Phenol, chloroform and ethanol were obtained from E. Merck., Darmstadt, Germany. Sodium salicylate and others were the products of Wako Pure Chemical Industries, Ltd., Tokyo, Japan.

RESULTS AND DISCUSSION

A procedure for plasmid isolation

Our aim was to detect the large plasmid in *P. putida* B3 (PpB3). We, therefore, required a method suitable for screening for plasmid of large molecular weight.

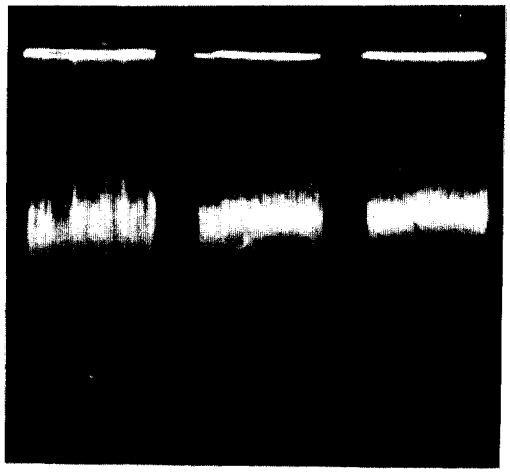
Meyers *et al.* (1976) have described a method for rapid detection and identification of plasmids based on agraose gel electrophoresis of DNA crude extracts. However, neither the SDS-salt precipitation method (Hansen and Olsen, 1978) used for preparation of this crude extracts, nor the akaline extraction method (Birnboim and Doly, 1979) allowed efficient isolation of large plasmid DNA from PpB3.

In preliminary experiments, covalently closed circular (CCC) DNA of the large plasmid from PpB3 was found to migrate through agarose gel during electrophoresis. We, therefore, modified the procedure proposed by Casse *et al.* (1979) for isolating CCC-DNA in an attempt to make it more suitable for handling a large plasmid.

Alkaline cell lysis and denaturation of DNA: To obtain a supercoiled DNA of plasmid of molecular weight higher than 100 megadaltons we had to limit DNA shearing, because the plasmid DNA larger than 100 megadaltons was also sheared when we applied shearing force such as vortex mixing in order to reduce the large bulk of chromosomal DNA (Currier and Nester, 1976; Casse *et al.*, 1979). In the Currier and Nester method (1976), lysis at pH 8.0 gives rise to a very viscous lysate. This lysate has to be sheared by passage through a syringe or in a mixer to decrease its viscosity and allows the NaOH solution to have access to the DNA thus providing alkaline denaturation. This shearing which is a very critical step, could be responsible for an important

variation in the recovery of large CCC-DNA molecules (Currier and Nester, 1976). If lysis is carried out in an alkaline buffer, that is, at the pH used for denaturation (arround 12.3), the viscosity of the lysate is low and shearing is not required (Kado and Liu, 1981). In addition, lysis in highly alkaline buffer provides a protein denaturation effect which may serve to reduce the enzymatic degradation of plasmid DNA. Alkaline denaturation was achieved by adding a constant volume of an alkaline lysing buffer (pH 12.45 and 6.9% SDS) to a small volume corresponding to a constant weight of bacterial suspension. Adjustment of the lysing solution to pH 12.45 has to be carried out carefully with electrodes resistant to Tris and alkaline pH and calibrate just before the experiment with a freshly prepared alkaline reference buffer (equal volume of dibasic and tribasic phosphate buffer, of which the pKa is 12.3). Incubation for 1 hr at 37°C was required to achieve good lysis and denaturation.

Removal of denatured DNA: After renaturation of the lysate, denatured DNA was precipitated by adjusting the lysate to 4% SDS and 1M NaCl, the lysate was chilled in an ice-water bath and refrigerated (4°C) for 6 hr. Centrifugation at 12,000 rpm (4°C) caused the salt-precipitated chromosome-membrane complex to form a large white pellet (Hansen and Olsen, 1976). Figure 1 shows the yield of plasmid after the NaCl precipitation. As expected, the upper phase of the salt-precipitated lysate yielded plasmid DNA (lane 2,3). However, plasmid DNA was still remained in the salt-precipitated chromosome-membrane complex protion (lane 1).

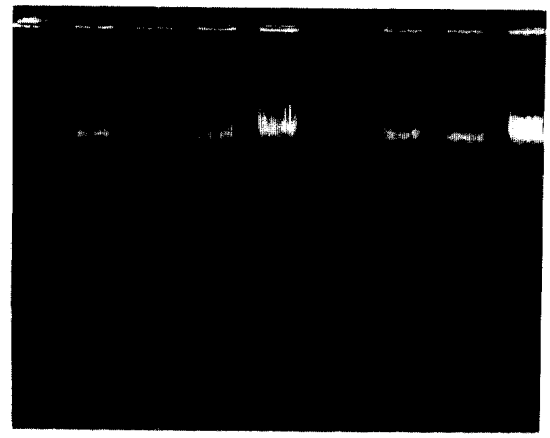


1 2 3

Fig. 1. *Yield of plasmid in various phases after NaCl precipitation.*
1; salt-precipitated chromosome-membrane portion, supernatant after centrifugation
2,3; upper phase of the precipitate

Effect of heat treatment: To enhance the yield of plasmid DNA, the plasmid DNA remained in the salt-precipitated chromosome-membrane complex was recovered by heat treatment. Kado and Liu (1981) have reported that the heat treatment at 55°C usually eliminated most of the chromosomal DNA in *E. coli*, at 65°C in *Xanthomoas*, and at 95°C in *Agrobacterium*. We also examined the effect of heat treatment at various temperature. As shown in Figure 2, the 55°C treatment for 15 min was effective for the recovery of plasmid DNA from the salt-precipitated chromosome-membrane complex.

Concentration of DNA: To concentrate the plasmid DNA, several methods were examined. Ethanol precipitation with magnesium phosphate (Currier and Nester, 1976) gave rise to large pellets which took a long time to dissolve in the storage buffer. Preparation with 0.3 M sodium acetate and 2 volumes of ethanol (Meyers *et al.*, 1976) produced a large pellet of chromosomal



1 2 3 4 5 6 7 8 9

Fig. 2. *Effect of heat treatment.*
1; 95°C 1 min 2,3; 65°C, 10 min
4,5; 55°C, 15 min 6; untreated
7,8; 37°C, 20 min 9; 55°C, 10 min

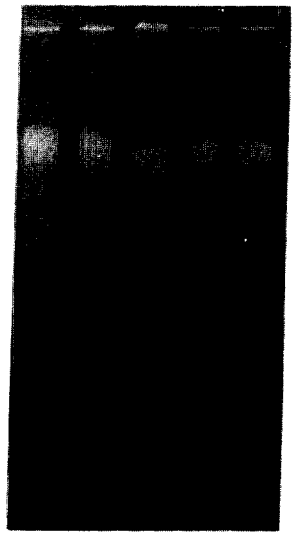


Fig. 3. *Effect of 2-mercaptoethanol for DNA storage*
1; 2-ME added (after 1 week)
2; 2-ME added (after 2 weeks)
3; not added (after 1 week)
4; not added (after 3 days)
5; not added (after 1 day)

DNA. Precipitation of plasmid DNA with PEG solution (42%, w/v) for 6 hr to overnight (Hansen and Olseon, 1978) produced pellets that were contaminated with large amount of protein. So as to decrease the protein concentration, refrigeration for 2 hr provided better result. The plasmid DNA prepared in this procedure was stable at 4°C when 2-mercaptoethanol was added to TES buffer in 10mM, the plasmid DNA was stable for 4 or more weeks (Figure 3). While the plasmid DNA of PpB3 detected by the methods of Casse *et al.* (1979) and Kado and Liu (1981) was unstable and degraded after 1 day. It was assumed that phenol extraction step of the methods was ineffective for the isolation of plasmid DNA from PpB3.

Estimation of molecular size

Figure 4 showed the standard curve that we constructed from agarose gel electrophoresis data for 5 plasmids whose range of molecular size varied from 56 megadaltons for NAH plasmid to 170 megadaltons for CAM plasmid (see Table 1). Although the standard curve did not provide a precise molecular size, it did allow to determine reproducible, relative and approximate size.

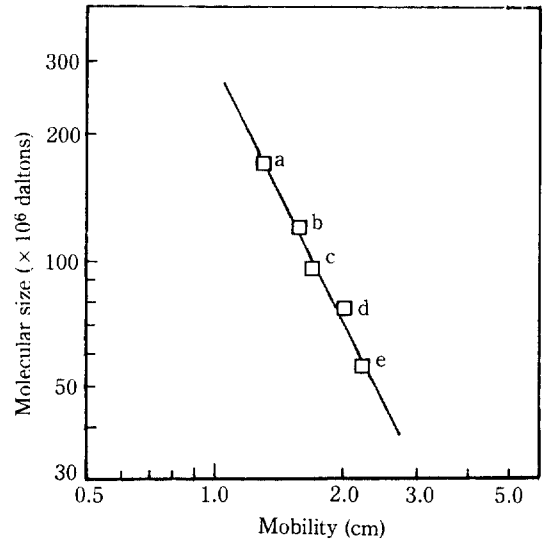


Fig. 4. *Standard curve for molecular size.*
a; CAM b; Ti c; pRms148 d; TOL e; NAH
(See Table 1 for the molecular size)

We take the standard curve to estimate the molecular size of the plasmid from PpB3 ("SAL" plasmid) whose mobility data was normalized to the standard curve. The SAL plasmid was larger than the largest plasmid of the standard curve, as shown by their slower migration during electrophoresis (Figure 5). On the basis of six bands, the molecular size of SAL plasmid was estimated 180 megadaltons by a linear extrapolation. In order to estimate the accurate size, the plasmid purifica-

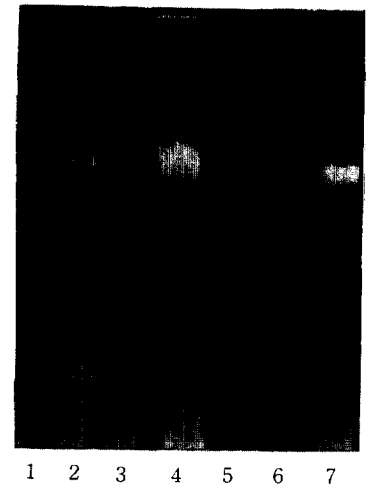


Fig. 5. *Gel electrophoresis of various plasmids.*
1; pRms148 2; Ti 3; CAM 4; SAL 5; TOL
6; Ti 7; NAH

Table 2. *Curing frequency of PpB3 with mitomycin C.*

Experiment	Concentration of mitomycin ($\mu g/ml$)				
	0	5	10	15	20
1	0	0	0	0	1.8%
2	0	1.6	–	2.7	3.2
3	0	7.0	–	–	11.5

tion and restriction map should be carried out.

Curing experiment

The loss of the ability of salicylate-positive (Sal^+) cells to grow on salicylate when treated with different concentration of mitomycin C is seen in Table 2. The Sal^+ character was not lost spontaneously. However, when Sal^+ cells were treated repeatedly with different concentrations of mitomycin C and after 1 to 2 days-growth, and the resultant cells were tested for the presence of the Sal^+ character, the frequency of the occurrence of Sal^- segregants was found to be greatly increased.

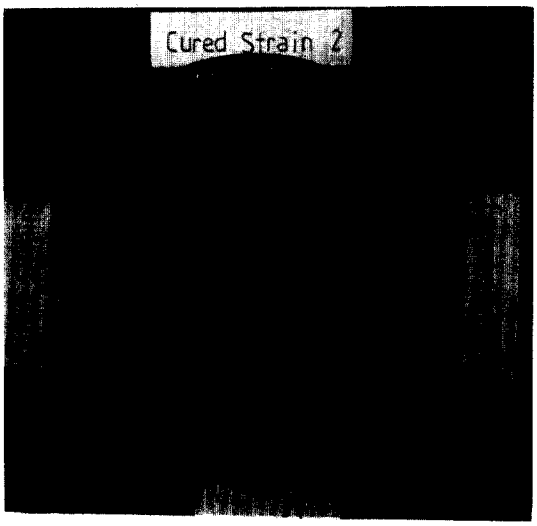


Fig. 6. *Growth in salicylate mininal plate.*

Fig. 7. *Detection of plasmid from Sal^- derivatives.*
1; PpB3 parent 2; cured strain 1
3; cured strain 2 4; cured strain 3

When Sal^- derivatives were grown in salicylate minimal plate, none of them showed growth (Figure 6). These derivatives never reverted to the Sal^+ character (less than 10^{-10} per cell division). On the other hand, plasmid was not detected in those Sal^- derivatives by the method described above (Figure 7). These results indicate that the Sal^+ character is encoded on plasmid.

Although the molecular size of SAL plasmid was reported as about 50 megadaltons (Johnston and Gunsalus, 1977; Lehrbach *et al.* 1983), the plasmid described in this presentation is about 180 megadaltons in its molecular size and represents salicylate-degrading capacity. Therefore, this one is a new type of salicylate-degrading plasmid.

적 요

자연계에서 분리한 *Pseudomonas*에서 크기가 매우 큰 plasmid(약 180 megadaltons)를 분리하였다. 이 plasmid는 기존의 plasmid 검출방법보다 더 온화한 조건에서 분리되었다. Alkaline sodium dodecyl sulfate(pH 12.45)를 사용하여 chromosomal DNA를 변성시킨 뒤, 55℃로 열처리 하여 covalently closed circular DNA의 손실을 최대한으로 방지하였고, polyethylene glycol을 최종농도 10%로 첨가하여 plasmid DNA를 선택적으로 농축하였다. 한편, 이 plasmid는 mitomycin C를 처리하여 얻은 cured strains에서는 나타나지 않아 salicylate를 분해하는 새로운 plasmid로 확인되었다.

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