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Properties of biparental clones formed by spheroplast fusion of *Pseudomonas putida*

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원형질체 융합에 의한 *Pseudomonas putida*의 biparental clones의 형성과 성질

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ABSTRACT: Biparental clones and recombinant clones were obtained by spheroplast fusion of *Pseudomonas putida* KU218R-3 and *P. putida* KU428. Formation of the fusion product was the most effective when the *Pseudomonas* spheroplast mixture were treated with 40% plyethyleneglycol(PEG) 6000 for 10min at room temperature. The fusants which selected by indirect method were obtained at an average frequency of 10.8%. Most of the fusants were biparental clones(10.4%) and the recombinant clones were produced in low yield(0.42%). Fusants, at the frequency of 4% were obtained without PEG 6000, which shows that fusion is not strictly dependant on PEG. The stability of fusants were examined. Most of the biparental clones were segregated to parental form and late recombinants were formed on further propagation of biparental clone but the recombinant clones were very stable.

KEY WORDS□*Pseudomonas putida*, spheroplast fusion, biparental clone.

Polyethyleneglycol mediated protoplast fusion has been recognized as a potential tool for the research in genetics and the genetic manipulation of organisms. The first successful fusion of Gram-positive bacterial protoplast with PEG, was achieved with polyauxotrotrophic strains of *Bacillus subtilis* by Schaeffer *et al,* (1976) and of *B. megaterium* by Fodor and Alföldi (1976). After these successes bacterial protoplast fusion was most frequently studied using species of *Bacillus* (Hadlaczky *et al,* 1976: Fodor *et al,* 1978; Fodor and Alföldi, 1979; Gabor and Hotchkiss, 1979; Sanchez-Rivas, 1982) and of *Streptomyces* (Hopwood and wright, 1979; Baltz and Matsushima, 1981). The mechanism of fusion was recently studied by the use of electron microscope (Frehel *et al,* 1979). Gumpert (1980) has recently proposed a model for membrane alteration during fusion at the molecular level. The genetic properties of the fusants were studied. Among the products of PEG-treated *B. subtilis* protoplasts, three types of clone that are phenotypically recombinant clones (Schaeffer *et al,* 1976), complementing diploid clone (Sanchez-Rivas, 1982) and biparental clone (Hotchikiss and Gabor, 1980) have been described. However, little is known about fusion of Gram-negative bacteria except for *E. coli* (Tsenin *et al,* 1978) and *Providence alkarifacience* (Coetzee *et al,* 1979). In this study, *Pseudomonas putida,* Gram-negative bacteria, was used to examine the possibility of the fusion in *Pseudomonas* and the properties of the fusants.

MATERIALS AND METHODS

Bacterial strains and media

Pseudomonas putida KU428 and *P. putida* KU218R-3 used in fusion experiments. The stra-

ins were isolated and identified in this laboratory (Kim and Lee, 1984; Oh and Lee, 1986). *P. putida* KU428 is wild type which resistant to kanamycin, streptomycin, and tetracyclin. *P. putida* KU218-3 is rifampicin resistance mutant of *P. putida* KU218. Mutation was induced by UV according to Miller's method (1972) to use in this experiment. Bacteria were cultured in nutrient broth and spheroplasts were regenerated on a rich regeneration media (RRM). For selection of fusants nutrient agar media containing antibiotics were used.

Spheroplast formation, regeneration and fusion

The method used for the production and regeneration of spheroplasts were those described previously by Lee and Lee (1986). Spheroplast fusions were performed according to Fodor and Alföldi, (1976). Equal amount of spheroplast suspensions (5m*l* each) were mixed and centrifuged at 7,000 r.p.m. for 10min at room temperature. The pellet was suspended in 1m*l* spheroplasting buffer (10mM Tris-HCl buffer pH 8.0 supplemented with 0.5 M sucrose and 20mM $MgCl_2$) and this suspension was added to 9m*l* of 40% (w/v) PEG 6000 in isotonic buffer and shaken. After 3min of standing at room temperature PEG-treated spheroplast mixtures were serially diluted and plated on RRM. In case of using $CaCl_2$ as fusogenic agent, the spheroplast pellet was suspended in 2m*l* of spheroplasting buffer. Then 0.1m*l* of 0.02M KH_2PO_4 and 1.0M $CaCl_2$ were added to the spheroplast suspension. After 3min of gentle shaking, the spheroplasts were serially diluted and plated on RRM.

Analysis of fusants

Selection and classification of fusants were carried out as described by Hotchkiss and Gabor (1980). After 48hr of incubation on RRM plate at 30°C, well seperated regenerated colonies from 10^{-6}, 10^{-5} and occasionally 10^{-4} dilutions were picked at random with sterile toothpicks and replicated to selective media containing antibiotics such as kanamycin, rifampicin, and both. Regenerated colonies were classified as parents growing on one of the parental media (Nutrient agar containing kanamycin only or rifampicin only.), biparental clone growing on both parental media but not on media containing both kanamycin and rifampicin, and recombinant clones growing on media containing both kanamycin and rifampicin. Biparental clones (BP) and recombinant clones were determined as fusants.

Subcloning of fusion products

Colonies classified as BP and recombinant clones were picked with sterile toothpicks from different media, inoculated seperately into nutrient broth, and cultured overnight at 30°C with shaking. One loop of this culture was streaked out on nutrient agar plate. After 24-48hr at 30°C, well isolated single colonies were transferred to selective media containing antibiotics with toothpicks. The colonies were again analysed and reclassified as parental, recombinant, or BP.

Electron microscopy

PEG-treated spheroplasts were prefixed for 3hr at 4°C with 1% (w/v) glutaraldehyde in 0.2M cacodylate buffer (pH6.2) containing 0.5M sucrose and 20mM $MgCl_2$. After centrifugation, the pellet was washed several times and post fixed with 1% (w/v) osmium tetraoxide (OsO_4). Dehydration was performed with acetone (30%-100%) and embeded with Epon. Sections cut with a diamond knife on LKB 2088 ultratome, stained with uranylacetate and lead citrate and observed in EM.

RESULTS AND DISCUSSION

Effect of PEG treatment on spheroplast fusion

The effects of PEG and $CaCl_2$ on the spheroplast fusion of *Pseudomonas putida* KU218 R-3 and *P. putida* KU428 were examined. The formation frequency of fusant was showed 1.4×10^{-1} and 1.0×10^{-1} after treatment of PEG 6000 and $CaCl_2$ respectively (Table 1). These yields were two fold high as simillar to that of untreated spheroplast mixtures. However, the frsants were formed well with a frequency of 6.5% in absence of PEG. We wanted to know whether PEG is effective to *Pseudomonas* spheroplast fusion or not. Therefore, the effects of PEG were observed under different experimental conditions to find op-

Table 1. *Effect of PEG 6000 and $CaCl_2$ on Pseudomonas spheroplast fusion.*

Fusion	Addition to spheroplast mixture	Frequency of fusant formation(%)
P. putida KU218R-3 × *P. putida* KU428	[a] none	6.5
	[b] PEG 6000	14.0
	[c] $CaCl_2$	10.7
	[d] PEG + $CaCl_2$	8.5

a; Spheroplast buffer (0.9 m*l*) was added to spheroplast mixture of *P. putida* KU218R-3 and *P. putida* KU428 (0.1 m*l*) in place of PEG solution.
b; Spheroplast mixture was treated with 40% (w/v) PEG 6000 for 3 min at room temperature.
c; 0.05 volume of 0.01M KH_2PO_4 and 1M $CaCl_2$ were added to the mixture.
d; PEG 6000 (40%, w/v) was added immediately after $CaCl_2$ treatment.
e; $\frac{\text{Number of (BP clone + recombinant clone)}}{\text{Number of colonies tested}} \times 100(\%)$

timal conditions for *P. putida* spheroplast fusion. The conditions tested were the following: (a)effect of different concentrations of PEG 6000 (Fig.1); (b)effect of the duration of treatment with 40% (w/v) PEG 6000 (Fig.2); (c)effect of temperature on 40% (w/v) PEG 6000 treatment. (a)The effect of different concentrations of PEG 6000 ranging from 10 to 60% (w/v) on spheroplast fusion and regeneration was examined. As shown in Fig.1, The fusion frequency was highest at the concentration of 40% PEG 6000 whilst the regeneration frequency decreased with increasing the concentration of PEG 6000. (b)To determine optimal treatment time of PEG 6000, spheroplast mixtures were treated with 40% (w/v) PEG 6000 for various times ranging from 1 to 30min at room temperature. The fusion frequency increased from 4% to 21% with increasing treatment time until 10min but the yield did not increased after 10 min (Fig.2). On the other hand the regeneration frequency fell drastically as the PEG treatment time increased. (c)Spheroplasts were treated with 40% PEG 6000 for 3min at various temperatures (0°C, room temp., 30°C). The fusion frequency was highest when PEG treatment performed at

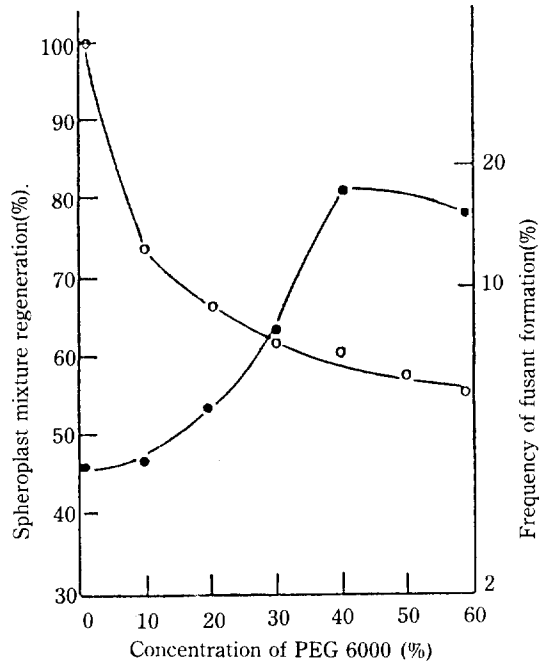

Fig. 1. *Effect of concentration of PEG 6000 on regeneration and fusion of spheroplast mixture of Pseudomonas putida KU218R-3 and P. putida KU428.* PEG 6000 was used in concentration from 10% (w/v) to 60% (w/v); treatment was for 3min at room temperature ○, spheroplast mixture regeneration; ●, frequency of fusant formation.

room temperature (Table 2). Studied optimal conditions for spheroplast fusion of *Pseudomonas putida* were similar to the effective conditions of *Bacillus* and of *Micrococcus* (Rastogi *et al,* 1983). However, the efficiency of PEG to *Pseudomonas* spheroplast fusion was different markedly with those of the other researches (Kuruno *et al,* 1983; Ochi *et al,* 1979). In kuruno's research, fusant was not produced in the absence of PEG. Also, Frehel *et al,* (1979) have detected fused pairs go on to a more complete cellular fusion during subsequent incubation and only after PEG treatment. On the other hand, treatment of PEG in spheroplast mixtures of *Pseudomonas putida* could improve the yield of fusants as much as 2 to 5 folds (Fig.1, 2; Table 1,2,6). In the studies by *Bacillus* of Hotchkiss and Gabor (1980) and Fodor *et al* (1983) fusants were observed at a frequency of 3.3% and 2% respectively in the absence of PEG. The fusants of the experiment in previous and that of this

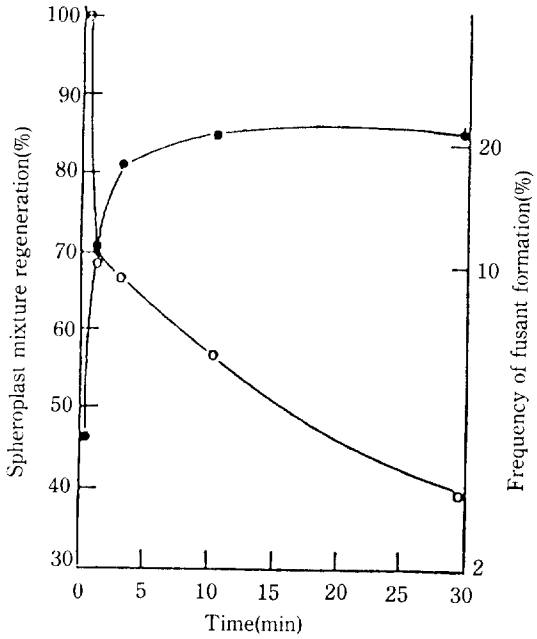

Fig. 2. *Effect of duration of PEG treatment on regeneration and fusion of spheroplast mixture of Pseudomonas putida KU218R-3 and P. putida KU428.* The mixture was treated with 40%(w/v) PEG 6000 for various time intervals ranging from 1 to 30 min at room temperature.○, spheroplast mixture regeneration; ●, frequency of fusant formation.

experiment were BP clones.

Hotchkiss and Gabor (1980) described BP clones as follows. Some, if not all, of the biparentals are noncomplementing diploid(Ncd) carring two chromosomes but show preciesely the phenotype of one (either one) of the parental strains. Segregation analysis and DNA transformation experiments showed that Ncd's carry the two parental genotype, and nucleoids of non complementing diploids from protoplast fusion of *B. subtilis* were isolated. However, only one of the two parental chromosomes is expressed, the other being phenotypically 'silent'. (Hotchkiss and Gabor, 1980; Bohin *et al,* 1982; Guillen *et al,* 1982). On the other hand Foder *et al,* (1983) have different interpretation of BP. According to the data by Foder *et al,* (1983), BP phenotype was not necessarily a genetic category in *B. megaterium.* Specially, Hotchkiss and Gabor (1980) supposed that biparental clones found in absence of PEG were transient

Table 2. *Effect of temperature of PEG treatment on the spheroplast fusion.*

fusion	Temp. (°C)	Frequency of fusant formation(%)
P. putida KU218R-3	0	11.7
×	Room temp.	13.5
P. putida KU428	30	4.5

Spheroplast mixture of *P. putida* KU218R-3 and *P. putida* KU428 was treated with PEG6000 (40%) for 3 min at various temperature ranging from 0°C to 30°C.

BPs did not distinguish between actual fusion and an early, short-lived association of protoplasts. The BPs formed in no PEG were segregated more completely to parental form and did not form late recombinant on subsequent culturing.

BP clones formed by spheroplast mixtures of *Pseudomonas putida* were slightly different from previous BPs. In *Pseudomonas putida,* the efficiency of BP production was low in case of no PEG, but the stability and late recombinant formation frequency were similar to BPs formed in PEG (unpublished data). Therefore, the properties of BP clone are unknown accurately. Further experiment must be made to find out the nature of biparental clones.

Analysis of fusion products and their properties

As shown in methods, regenerated colonies were analysed and classified as parents, biparental clones (BP), and recombinant clones. In optimal conditions the fusants which selected by indirect method were obtained at an average frequency of 10.8% (Table 3). Most of the fusion products were BPs, and BPs occured at a proportion of 10.4%. On the other hand the recombinant clones produced in low yield (0.42%). However, the recombinant clones were more stable than BP clones. As indicated Table 4, most of the BPs were segregated to parental form on further propagation, but all tested recombinant clones were stable. Small parts of BP subclones were persisted BP nature. A certain proportions of BP give rise to additional recombinants (late recombinants) as they continue to grow.

Table 3. *Analysis of primary products from fusion of Pseudomonas putida KU218 R-3 and P. putida KU428.*

Expt	Colonies growing on NA containing				fusant		Frequency of fusant formation(%)
	None	Km	Rif	Both Km and Rif	BP	rec	
1	299	68	244	0	13	0	4.3
2	146	43	122	0	19	0	13.0
3	388	92	361	0	65	0	16.7
4	399	79	359	3	36	3	9.8
5	378	111	332	5	60	5	17.1
6	297	143	159	0	5	0	1.7
Total	1907	536	1577	8	198	8	10.8

PEG treated spheroplast mixtures were serially diluted and plated on RRM. After 48 hr of incubation on RRM plate at 30°C, well seperated regenerated colonies from 10^{-6} and 10^{-5} were picked at random with sterile toothpicks and replicated to selective media. The colonies were classified as parents growing on one of the parental media (NA containing rifampicin or kanamycin) biparental clones growing on both parental media but not on NA containing both kanamycin and rifampicin, and recombinant clones growing not only on both parental media but also on NA containing both antibiotics. Biparental clones and recombinant clones determined as fusants.

Check for possible involvement of genetic mechanisms other than cell fusion

The possibilities of transformation and spontaneous mutation as a cause of fusant trait were investigated. To exclude possibility of transformation, deoxyribonuclease I (final concentration; 5μg/m*l*) was added to suspending buffers and media after spheroplast formation and the influence of DNase on the fusion frequency was examined. Also, the possibility that one parent acquired spontaneously other parental phenotype was examined in order to know whether spontaneous antibiotic mutant of parents was contained to fusants or not. As shown in table 5 and 6, the pre-

Table 4. *Segregation of biparental clones and recombinants on further propagation.*

Selection medium	BP colonies selected		Recombimants selected
	from Rif	from Km	from Rif + Km
Rif	190	0	25
Km	0	200	25
Sm	0	200	25
Tc	14	200	25
Rif + Km	0	0	25
Rif + SM	0	0	25
Rif + Tc	10	0	25

Colonies classified as BP and recombinant clone were picked with sterile toothpicks from different media, inoculated seperately into nutrient broth, and cultured overnight at 30°C with shaking. One loop of this culture was streaked out on nutrient agar plate. After 24-48 hr at 30°C, well isolated single colonies were transferred to selective media containing antibiotics with toothpicks. The colonies were again analysed and reclassified as parental, recombinant or BP.

Table 5. *Spontaneous artibiotics resistant mutant derived from Pseudomonas putida.*

Strain	Mutant phenotype	Mutant formation frequency
P. putida KU218R-3	Km^r	$>1\times10^{-9}$
P. putida KU428	Rif^r	$>1\times10^{-9}$

Table 6. *Effect of DNase I on PEG-induced Spheroplast fusion between P. putida* KU428 and *P. putida* KU218 R-3

Exp't No.	Addition to fusion mixture		Frequency of fusant formation
	PEG	DNase	
I	-	-	2.0×10^{-2}
II	+	-	7.0×10^{-2}
III	-	+	3.0×10^{-2}
IV	+	+	6.5×10^{-2}

Spheroplast mixture was treated with 40% (w/v) PEG6000 for 3 min at room temperature. In case of exp't 1, spheroplast buffer was used in plase of PEG sol. DNase I (5μg/m*l*) was added to the reaction mixture for PEG treatment and the medium for regeneration.

Fig. 3. *Electron microscopic observation of fusion between P. putida KU218 R-3 and P. putida KU428 spheroplasts after PEG 6000 treatment.*
A; Contacting stage of *P. putida* spheroplasts.
B,C; Combining stage of *P. putida* spheroplasts.
; multiple combining could be observed.
D; Fusing stage of *P. Putida* spheroplasts.

sence of DNase had no significant effects on the fusion frequency and the possibility of appearance of spontaneous antibiotics resistant mutant was less than 1.0×10^{-9}. These data showed that the fusants were the result of fusion in mixed spheroplasts.

Ultrastructural observation

Fig.3 shows the ultrastructure of *P. putida* spheroplasts after PEG treatment. The spheroplasts contain wall remnants. Also, a clumping of the spheroplasts could be observed. Fig.3 (a) shows two spheroplasts contacting each other. Spheroplasts fusing could be observed between not only two spheroplasts but also more than two spheroplasts (Fig.3;b,c). Fig.3 (d) shows two spheroplasts which seem to be fused to each other. The process of fusion of *Pseudomonas putida* agree with other electron microscopic studies of *Bacillus subtillis* (Frehel *et al,* 1979), *Streptomyces* (Gumpert, 1980), *Mycobacteria* (Rastogl *et al,* 1983), and *Coryneform* bacteria (Park *et al,* 1985).

적 요

P. putida KU218R-3와 *P. putida* KU428의 원형질체 혼합액에 PEG 6000을 처리하여 biparental형과 recombinant형의 융합체를 얻었다. *Pseudomonas*의 원형질체가 융합하는 형태는 TEM으로 관찰하였고, 원형질 융합체의 선별은 항생제 내성을 유전적 지표로 하여 간접적인 방법으로 선별하였다. *Pseudomonas*의 원형질체 융합에는 40%(w/v) PEG 6000을 상온에서 10분간 처리하는 것이 가장 효과적이었으나 PEG를 처리하지 않은 실험구에서도 융합체가 4%의 빈도로 생성되므로 PEG의 효과는 절대적인 것이 아니었다. 생성된 융합체의 대부분은 biparental clone이었고 (10.4%), recombinant clone의 생성빈도는 대우 낮았다(0.42%). 또한 biparental clone의 대부분은 further propagation 중에 모균주의 형태로 분리되었고, 이 과정에서 late recombinant를 생성하였다. biparental clone에 반해 recombinant clone은 여러 세대 후에도 안정하였다.

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