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# Spheroplast fusion of *Pseudomonas* spp. using plasmid as selective marker

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# 선별표지로 plasmid를 이용한 *Pseudomonas* spp.의 원형질체 융합

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**ABSTRACT: Antibiotic resistance plasmids (RP4, pMG1, R679 and R91-5) were used as primary selective markers to detect the fusants in *Pseudomonas* spp. By using plasmid marker, clones containing both plasmids of parental strains were obtained as fusants by direct selection. The spheroplast fusion was occured not only between strains of interspecies, but also between strains of intergenus such as *Pseudomonas* and *E. coli*. The frequencies of fusant formation were variable from $2.8\times10^{-1}$ to $6.0\times10.^{-4}$ In addition, chromosomal recombinants were formed among the clones with both parental plasmids in frequencies of 0.44-1.3%. The fusant formation frequency between interspecies or intraspecies was not different markedly, but stability of plasmids in fusants correlated with the phylogenic smilarity of the parental strains.**

**KEY WORDS** □ ***Pseudomonas*, spheroplast fusion, R plasmid**

Fusion of protoplasts results in genetic recombination between two cell types, by which DNA transfer is biderectional, and cytoplasm as well as whole genome of a donor is transferred into a recipient(Fodor *et al.*, 1980). As far as we are informed, most of the attempts at fusing bacterial protoplasts have been reported in Gram-positive bacteria such as *Bacilus subtilis* (Schaeffer *et al.*, 1976), *B. megaterium* (Fodor and Alföldi, 1976) and species of *Streptomyces* (Hopwood *et al.*, 1977; Baltz, 1978; Hopwood and Wright, 1979; 1981). However, fusion of spheroplasts between Gram negative bacteria have been reported only in some bacteria such as *E. Coli* (Tsenin *et al.*, 1978), *Providence alkalifacience* (Coetzee *et al.*, 1979), and *Pseudomonas putida* (Lee *et al.*, 1987).

After transformation of *Streptomyces* protoplasts by plasmid DNA was reported at first (Bibb *et al.*, 1978), Chang and Cohen(1979) showed that protoplasts of *B. subtilis* in the presence of PEG could be transformed by plasmids prepared from *Staphylococcus aureus*. And plasmids were transferred among Bacilli by protoplast fusion and regeneration (Dancer, 1980). Also, Götz *et al.*, (1981) suggested that plasmids were available as selective marker to detect the fusants in the protoplast fusion of *Staphylococcus aureus*.

In the previous report about the spheroplast fusion in *P. putida*, fusants were selected indirectly. The method of indirect selection to detect fusant was rather complex. Also, most of the fusants selected by the indirect selection were biparental clones. Therefore, we want to devise a rather simple selection method to detect the fusant.

In this paper, we report the usefulness of plasmids as selective marker in various fusion experiments between intra, interspecies, and intergenus.

## MATERIALS AND METHODS

### Bacterial strains and media

The strains and plasmids used in fusion are listed in Table 1. The strains having plasmid used in this study were conjugants mostly. RP4 and pMG1 were transferred to various strains of *Pseudomonas* by broth mating method (Puhler and Timmis, 1984). The transferred plasmids in transconjugants were isolated and confirmed by electrophoresis.

The strains were cultivated in nutrient broth (NB) and spheroplasts were regenerated in rich regeneration medium(RRM). To investigate antibiotic resistance and auxotropic characteristics of the used strains and fusants, nutrient agar(NA) and M9 minimal medium(Maniatis *et al.*, 1982) each containing antibiotics and amino acids were used respectively.

### Spheroplast preparation, regeneration, and fusion

The method used for the preparation and regeneration of *Pseudomonas* spheroplasts was those previously described by Lee and Lee(1986). Also, spheroplast fusion of *Pseudomonas* spp. were performed according to Lee *et al.*, (1987).

### Detection of fusant

Plasmid coded antibiotic resistance character was used as a selective marker for detection of the fusant. In indirect selection, after the process of fusion experiment, each regenerated colony on RRM was transferred to selective media which only parental strains could grow and other one which only fusants could grow with sterile toothpick. The clones expressed both parental resistance characters were determined as fusants having both plasmids. Fusion frequency was given as the ratio of the number of fusants to total tested colonies.

In direct selection, appropriate dilutions ($10^{-2}$-$10^{-4}$) of PEG treated spheroplast mixtures were spreaded on none selective RRM and on

**Table 1.** *Bacterial strains and plasmids.*

| Strain/Plasmid | Relevant characteristics | Reference or source |
|---|---|---|
| *Pseudomonas putida* | | |
| KU188 | $sal^+$ | Kim and Lee (1984) |
| KU212 | $sal^+$ | " |
| KU218 | $sal^+$ | " |
| KU218 R-5 | $sal^+$, $rif^r$ | Mutant of KU 218 (this expt.) |
| KU220 | $sal^+$ | Kim and Lee (1984) |
| KU428 | $sal^+$, $Km^r$, $str^r$, $trc^r$ | Oh and Lee (1986) |
| *Pseudomonas aeruginosa* | | |
| KU141 | $sal^+$, $Ap^r$ | Kim and Lee (1984) |
| PAO5/R91-5 | $trp^-$, $rif^r$, fonl/$Cb^r$, $Tra^+$ | Carrigan *et al.* (1978) |
| PAO8/R18 | $met^-$, $ilv^-$, $str^r$/$Km^r$, $Cb^r$, $Tra^+$, Inc P1 | Issac and Holloway (1968) |
| PAO303/pMG1 | $arg^-$/$Sm^r$, $Su^r$, $Tra^+$, Inc P2 | Hansen and Olsen (1978) |
| PAO1670/R679 | $pur^-$, $leu^-$, $rif^r$/$Sm^r$, $Tra^-$ | Stanisich (1974) |
| *Escherichia coli* | | |
| C600/RP4 | $leu^-$, $thr^-$/$Ap^r$, $Tc^r$, $Km^r$, $Tra^+$, Inc P1 | Depicker *et al.* (1977) |

Most of the strains having plasmid used in this study were conjugants. RP4 and pMG1 (R. plasmids) were transferred to various strains of *Pseudomonas*, especially *P. putida* by broth mating method. The transconjugants were not showed in Table 1. All plasmids in used strains were not segregated during the preparation and regeneration of spheroplasts.

selective RRM containing antibiotics respectively. Colonies growing on selective RRM were determined as fusants, and fusion frequency was estimated by comparing colony counts from selective RRM with those from none selective RRM.

**Stability of plasmids in fusant**

Colonies having both parental plasmids were picked into 5m*l* of nutrient broth and incubated with shaking at 30 °C until $OD_{600}$ of 0.4-0.8 were reached. These cultures were diluted and spreaded on NA to obtain single colonies. Each colony was replicated to selective media with toothpick and examined as to whether both plasmids in fusants after several generations were stable or not.

**Isolation of plasmids**

Plasmid DNAs in transconjugants and fusants were isolated in accordance with the procedure of Hansen and Olsen (1978). Plasmids were routinely checked by agarose gel electrophoresis.

## RESULTS AND DISCUSSION

**Usefulness of plasmids as selective marker**

Spheroplasts of *Pseudomonas aeruginosa* PAO5 (R91-5) and *P. aeruginosa* PAO303(pMGl) were fused under various experimental conditions. Double antibiotic resistant(which coded in each parental plasmids) clones were detected as fusants (Table 2).

Formation frequency of the fusant in indirect selection method was 5-10 times higher than in direct method. However, when plasmid marker was used as selective marker for fusant, direct selection might be profitable because many fused clones could be obtained practically and experimental process was rather simple.

**Plasmid transfer in intra-, interspecies and intergenus by spheroplast fusion.**

By using the fusion technique, plasmid was transferred between strains of the interspecies as well as strains of intraspecies. Besides, intergenus spheroplast fusion between *Pseudomonas* spp. and *E. coli* using plasmid coded resistance to antibiotics was attempted (Table 3).

In the interspecies fusions of *Staphylococcus,*

**Table 2.** *Transfer of plasmid by fusion of P. aeruginosa PAO5 (R91-5) and P. aeruginosa PAO303 (pMG1).*

| Expt. | Fusogenic agent | Addition to fusion mixture | Fusion frequency direct[b] | Fusion frequency indirect |
|---|---|---|---|---|
| 1 | None[c] | | $1.9 \times 10^{-2}$ | $1.4 \times 10^{-1}$ |
| 2 | PEG6000 | | $3.0 \times 10^{-2}$ | $1.4 \times 10^{-1}$ |
| 3 | PEG6000 | DNase I | $4.1 \times 10^{-2}$ | $2.0 \times 10^{-1}$ |
| 4 | $CaCl_2$ | | $1.5 \times 10^{-2}$ | $1.9 \times 10^{-1}$ |
| 5 | PEG6000 and $CaCl_2$[d] | | $1.6 \times 10^{-2}$ | $1.5 \times 10^{-1}$ |

Antibiotic resistance character coded by plasmid was used selective marker for detection of fusants. R91-5 and pMG1 have resistance to ampicillin and streptomycin respectively. The experimental procedure of direct and indirect selection method was performed as described in the method

the frequencies of plasmid transfer were reduced by about one order of magnitude in compansion with the intraspecies fusions(Götz *et al.*, 1981). However, in present experiments, there were no marked differences in the frequencies of transfer in intra- and interspecies fusions, although the lower frequency in intergenus was showed. These results suggested that transfer of cytoplasm by contacting of spheroplasts is available not only in intra- and interspecies but also in intergenus.

**Chromosomal recombinants among the clones having both plasmids of parents**

Among the clones having two plasmids of parents, various phenotypic recombinants of chromosomal gene were obtained in frequencies of 0.44 to 1.3%(Table 4). When the chromosomal gene was used as a selective marker to detect a fusant, obtaining recombinant by direct selection was practically impossible. Also, only a few recombinants were obtained by indirect selection (Lee *et al.*, 1987).

These results showed that the antibiotic resistance plasmids could be used as indicators of fusion and the use of plasmid genes as primary selective markers was reliable in selecting chromosomal recombinants among fused clones having both plasmids of each parent.

**Characteristics of fusants**

When spheroplasts of *P. aeruginosa* PAO5

**Table 3.** *Plasmids as markers in spheroplast fusion in intra, interspecies and intergenus.*

| Fusion | Regenerated cells on RRM (Colony forming unit/ml) | Regenerated cells on RRM containing antibiotics(c.f.u./ml) | | | frequency of fusant formation |
|---|---|---|---|---|---|
| | | Km + Sm | Sm + Tc | Sm + Ap | |
| 1. *P. aeruginosa* PAO303(pMG1) *P. aeruginosa* PAO5(R91-5) | $2.4 \times 10^9$ | | | $8.0 \times 10^7$ | $3.3 \times 10^{-2}$ |
| 2. *P. aeruginosa* PAO303(pMG1) *P. aeruginosa* KU141(RP4) | $4.2 \times 10^8$ | $1.2 \times 10^7$ | | | $2.8 \times 10^{-1}$ |
| 3. *P. aeruginosa* PAO303(R679) *P. aeruginosa* KU141(RP4) | $2.8 \times 10^8$ | | $2.1 \times 10^6$ | | $7.5 \times 10^{-3}$ |
| 4. *P. aeruginosa* PAO303(pMG1) *P. putida* KU218(RP4) | $1.6 \times 10^8$ | $2.1 \times 10^7$ | | | $1.3 \times 10^{-1}$ |
| 5. *P. aeruginosa* KU141(RP4) *P. putida* KU218R-5(pMG1) | $5.0 \times 10^6$ | $3.0 \times 10^3$ | | | $6.0 \times 10^{-4}$ |
| 6. *P. aeruginosa* PAO1670(R679) *P. putida* KU218(RP4) | $1.2 \times 10^8$ | | $1.2 \times 10^6$ | | $1.0 \times 10^{-2}$ |
| 7. *P. aeruginosa* PAO303(pMG1) *E. coli* C600(RP4) | $6.8 \times 10^8$ | $1.1 \times 10^6$ | | | $1.6 \times 10^{-3}$ |
| 8. *P. putida* KU218R-5(pMG1) *E. coli* C600(RP4) | $3.2 \times 10^8$ | $2.7 \times 10^6$ | | | $8.4 \times 10^{-3}$ |

Frequency of fusant formation = $\frac{\text{Regenerated cells on RRM containing antibiotics}}{\text{Regenerated cells on RRM}}$

**Table 4.** *Distribution of chromosomal recombinants among colonies obtained both plasmids by fusion.*

| Fusion | Chromosomal marker | Recombinants phenotype | Frequency of recombinants formation |
|---|---|---|---|
| I. *P. aeruginosa* PAO5(R91-5) | $trp^-$ | | |
| | | $trp^+, arg^+$ | $7.5 \times 10^{-3}$ |
| *P. aeruginosa* PAO303(pMG1) | $arg^-$ | | |
| II. *P. aeruginosa* PAO303(pMG1) | $arg^-$ | $arg^+, leu^+$ | $1.3 \times 10^{-2}$ |
| *E. coli* C600 (RP4) | $leu^-, thr^-$ | $arg^+, thr^+$ | $4.4 \times 10^{-3}$ |

(R91-5) and *P. aeruginosa* PAO303(pMG1) were fused, chromosomal phenotype of fused clones having both plasmids of parental strains was examined. Among them 98% was arginine auxotroph that was the phenotype of *P. aeruginosa* PAO303 (Table 5). This suggested that two plasmids coexisted within *P. aeruginosa* PAO303 in most cases. These unidirectional transfer of the plasmid to one of the parental strains says that both parents do not contribute equivalently to plasmid transfer by fusion. The reasons why these phenomenon happened were not discovered. Further experiments are required to find out the reasons.

**Stability of plasmids in fusants after propagation**

The stability of plasmids in fusants after propagation was examined as shown in method. As can be seen from expt. 1,2 in Table 6, stability of plasmids in fusants correlated with the phylogenic similarity of the parental strains. Plasmid R679 and RP4 in fusants between intraspecies fusion were maintained in frequency of 100%(Expt. 1 in Table 6). On the other hand, the stability of R679 and RP4 in fusants between interspecies fusion was 100% and 32% respectively(Expt. 2). Besides, in intergenus fusion (Expt. 3) plasmid pMG1 was

**Table 5. Chromosomal phenotype of fusants having both plasmids.**

| Fusion | Chromosomal marker | Primary selection marker | Tested colonies[a] (No.) | Chromosomal phenotype of fusant[b] | | | |
|---|---|---|---|---|---|---|---|
| | | | | recombinant | *P. aeruginosa* PAO5 | *P. aeruginosa* PAO303 | *E. coli* C600 |
| I. *P. aeruginosa* PAO5(R91-5) | trp⁻ | | | | | | |
| | | Ap$^r$, Sm$^r$ | 400 | 3 | 5 | 392 | |
| *P. aeruginosa* PAO303(pMG1) | arg⁻ | | | | | | |
| II. *P. aeruginosa* PAO303(pMG1) | arg⁻ | | | | | | |
| | | Sm$^r$, Km$^r$ | 250 | 4 | | 245 | 1 |
| *E. coli* C600 (RP4) | leu⁻, thr⁻ | | | | | | |

[a]; Colonies regenerated on RRM containing both antibiotics (Ap + Sm or Sm + Km) were determined as fusants having both plasmids.

[b]; Fusants having both plasmids were replicated on the minimal media containing amino acid which was needed by each parental strain and the minimal media. Only recombinant could grow on the minimal media.

very stable but RP4 was lost at high frequency. What was the cause of the wide difference of plasmid stability in fusants. The stability of plasmids shown in Table 6 and chromosomal phenotype of fused clones in Table 5 may correspond. In the case the strain used in fusion was a original host of the plasmid, the plasmid in fusants was found stable.

**Table 6.** *Segregation of the clones obtained both plasmids by cell fusion.*

| Expt. | Fusion | Total tested clone | Plasmid selection marker Km | Sm | Tc | Km+Sm | Sm+Tc |
|---|---|---|---|---|---|---|---|
| 1. | *P. aeruginosa* PAO1670/R679 *P. aeruginosa* KU141/RP4 | 39 | | 39 | 39 | | 39 |
| 2. | *P. aeruginosa* PAO1670/R679 *P. putida* KU218/RP4 | 140 | | 140 | 45 | | 45 |
| 3. | *P. aeruginosa* PAO303/pMG1 *E. coli* C600/RP4 | 70 | 5 | 65 | | 0 | |

pMG1: Sm$^r$, RP4: Km$^r$, Tc$^r$, R679: Sm$^r$

**Isolation of plasmids in fusants**

As the results of electrophoresis of plasmids obtained from fusants, only pMG1 was isolated from fusants having pMG1 and RP4, whereas only RP4 was identified from fusants having R679 and RP4 (Fig. 1). The former was understood by the stability of plasmids in fusants (Table 6). How to explain the latter results such as only RP4 was

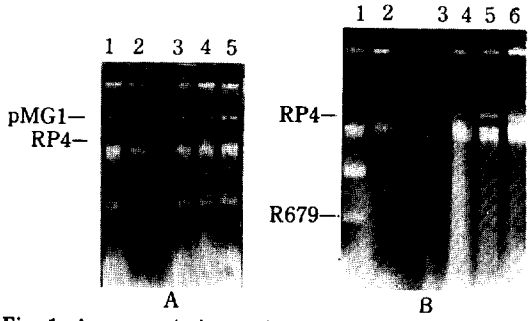


**Fig. 1.** *Agrose gel electrophoresis of plasmids obtained from fusants.*

A. Fusion between *P. aeruginosa* PAO303 / pMG1 and *E. coli* C600/RP4, lane 1; pMG1, lane 2; RP4, lane 3,4,5; plasmid in fusants.

B. Fusion between *P. aeruginosa* PAO1670 / R679 and *P. aeruginosa* KU141/RP4, lane 1; R679 lane 2; RP4, lane 3,4,5,6; plasmid in fusants.

identified, although both R679 and RP4 were very stable in fusants (Table 6, Fig. 1). As a result, R679 was supposed to be integrated into chromosome.

**Possibility of involving of gene transfer mechanisms other than fusion**

Presence of deoxyribonuclease(DNase I) at a concentration of 5 $\mu$g/m*l* in spheroplast mixtures at the stage of PEG treatment and in soft agar overlay had no significant effect on plasmid transfer (Table 2). The finding shows that plasmid transfer between strains of *Pseudomonas* was not the result of PEG-induced transformation due to partial lysis of spheroplasts and release of DNA. As we can see in Table 7, the frequency of plasmid transfer by fusion was temperature-independent whereas the conjugation process was temperature dependent. Therefore, the conjugation also was not involved in the emergence of double antibiotic resistant products in the present experiment.

**Table 7.** *Effect of temperature on the transfer of plasmids by fusion and conjugation.*

| Temp. (°C) | Fusion<br>*P. aeruginosa* KU141(RP4)<br>*P. putida* KU218R-5(pMG1) | Conjugation*<br>Donor; *P. aeruginosa* KU141(RP4)<br>Recipient; *P. putida* KU218R-5 (pMG1) |
|---|---|---|
| 0 | $1.26 \times 10^{-1}$ | $1.0 \times 10^{-7}$ |
| Room temp. | $1.70 \times 10^{-1}$ | $3.4 \times 10^{-6}$ |
| 30 | $8.50 \times 10^{-2}$ | $1.3 \times 10^{-5}$ |

* Overnight culture of recipient strain was diluted with equal volume of nutrient broth (2 m*l*).
And mixture of donor strain (0.2 m*l*) and diluted recipient strain (1.8 m*l*) were incubated at 30 °C for 5-6 hrs without shaking. 0.1 m*l* of mixture was spreaded on selective media and incubated overnight at 30 °C.

$$\text{Frequency} = \frac{\text{colony no. on selective medium} \times 20}{2 \times 10^8 \text{ (donor cell no. in 5m}l\text{NB overnight culture)}}$$

## 적 요

항생제에 대한 내성을 갖는 R plasmid(PR4, pMG1, R679, R91-5)를 *Pseudomonas* spp.의 원형질체 융합의 일차적인 선별표지로 사용하여 양쪽 모균주의 plasmid를 함께 가진 융합체를 얻을 수 있었다. 이 때 형성된 융합체는 직접적 선별방법에 의해 판별되었으며 두개의 plasmid를 갖는 융합체들 중에는 chromosomal recombinant가 0.44-1.3%의 빈도로 존재하였다. 동종간, 이종간 *Pseudomonas*의 원형질체 융합 뿐만 아니라 이속간인 *E. coli*와의 융합을 plasmid를 선별표지로 하여 시도하였다. 이때 *Pseudomonas*속의 동종간과 이종간의 융합율은 큰 차이가 없었으나, 융합체 내에서의 plasmid는 모균주들의 유연관계가 가까울수록 안정하였다.

## REFERENCES

1. Baltz, R.H. 1978. Genetic recombination in *Streptomyces fradiae* by protoplast fusion and cell regeneration. *J. Gen. Microbiol.* **107,** 93-102.
2. Bibb, M.J., J.M. Ward, and D.A. Hopwood. 1978. Transformation of plasmid DNA into *Streptomyces* at high frequency. *Nature,* **274,** 398-400.
3. Carrigan, J.M., Z.H. Helman, and V. Krishnapillai, 1978. Transfer-Deficient Mutants of the Narrow-Host-Range Plasmid R91-5, *J. Bacteriol.* **135,** 911-919.
4. Chang, S., and S.N. Cohen. 1979. High frequency transformation of *Bacillus subtilits* protoplasts by plasmid DNA. *Mol. Gen. Genet.* **168,** 111-115.
5. Choi, D.S., and Y.N. Lee, 1986, Characterization of MCPA degrading plasmid isolated from *Pseudomonas* sp., Master thesis, Korea university.
6. Coetzee, J.N., F.A. Sirgel, and Lecatsas. 1978. Genetic recombination in fused spheroplasts of *Providence alkalifaciens. J. Gen. Microbiol.,* **3250**313-322.
7. Dancer, B.N. 1980. Transfer of **plasmids** among *Bacilli. J. Gen. Microbiol.* **2210**263-266.
8. Depicker, A., M. Van Montagu, and J. Schell. 1977. Physical map of RP4. p. 678. In A.I. Bukhari. J.A. Shapiw, and S.L. Adhya (ed), DNA Insertion elements, plasmids. and epi-

somes. Cold Spring Harbor Laboratory, Cold Spring Harbo, N.Y.

9. Fodor, K., and L. Alföldi. 1976. Fusion of protoplasts of *Bacillus megaterium. Proc. Natl. Acad. USA.* **830**2147-2150.
10. Fodor, K., K. Rostas, and L. Alföldi, 1980. Bacterial protoplasts and their possible use in bacterial genetics. Advances in Protoplast research.
    by protoplast fusion in *Stapylococci. J. Bacteriol.* **145,** 74-81.
11. Götz, F., S. Ahrne, and M. Lindberg, 1981. Plasmid transfer and genetic recombination by protoplast fusion in *Stapylococci. J. Bacteriol.* **145,** 74-81.
12. Hansen, J.B., and R.H. Olsen, 1978. Isolation of large plasmids and characterization of the P2 incompatibility group plasmids pMG1 and pMG5. *J. Bacteriol.* **135,** 227-238.
13. Hopwood, D.A., H.M. Wright, M.J. Bibb, and S.N. Cohen. 1977. Genetic recombination through protoplast fusion of *Streptomyces. Nature, London* **268,** 171-174.
14. Hopwood, D.A., and H.M. Wright, 1981. Protoplast Fusion in *Streptomyces:* Fusions involving ultraviolet-irradiated protoplasts. *J. Gen. Microbiol.* **126,** 21-27.
15. Issac, J.H., and B.W. Holloway. 1968. Control of pyrimidine biosynthesis in *Pseudomonas aeruginosa. J. Bacteriol.* **96,** 1732-1741.
16. Kim, J.K., and Y.N. Lee. 1984. Isolation and identification of *Pseudomonas* utilizing hydrocarbon. *Kor. J. Microbiol.* **22,** 29-34.
17. Kurono, M., Y. Hirachi, Y. Kato, Y. Toda, N. Takenmasa, and S. Kotani, 1983. Intergenus cell fusion between L-form cells of *Pseudomonas aeruginosa* and *Escherichia coli. Biken. J.* **26,** 103-111.
18. Lee, J.S., and Y.N. Lee, 1986. The spheroplast formation and regeneration of *Pseudomonas* spp. *Kor. Jour. Microbiol.* **24,** 24-31.
19. Lee, J.S., Y.W. Lee, and Y.N. Lee, 1987. Properties of biparental clones formed by spheroplast fusion of *Pseudomonas putida. Kor. Jour. Microbiol.* **25,** 198-204.
20. Maniatis, T., E.F. Fristsch, and J. Sambrook, 1982. Molecular cloning a laboratory manual, 68-69. Cold Spring Harbor.
21. Oh, K.H., and Y.N. Lee, 1986. Isolation and identification of *Pseudomonas* utilizing chlorinated aromatic hydrocarbons. Master thesis, Korea university.
22. Pemberton, J.M., B. Corney, and R.H. Don. 1979. Evolution and spread of pesticide degrading ability among soil microorganisms, p. 287-299. In K.N. Timmis and A. Puhler (ed.), Plasmids of medical, environmental and commercial importance. Elsevire/North-Holland Biomedical Press, Amsterdam.
23. Puhler, A., and K.N. Timmis. 1984. Advanced Molecular Genetics. p41-42.
24. Schaeffer, P., B. Cami, and R. Hotchkiss, 1976. Fusion of bacterial protoplasts. *Proc. Natl. Acad. Sci. USA.* **73,** 2151-2155.
25. Stanisich, V.A. 1974. The Properties and host range of malespecific bacteriophages of *Pseudomonas aeruginosa. J. Gen. Micro.* **84,** 332-342.
26. Tsenin, A.N., G.A. Karimova, and V.N. Rybchin. 1978. Recombinaciya prisliyanii protoplastov *Escherichia coli*K12. *Dokl. Akad. Nauk. SSSR* **243,** 1066-68.

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