Fate of Genetically Engineered 2,4-D-Degrading Microorganisms in Natural Soils and Waters

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To analyze the effects of host versus plasmid on survival of 2,4-D-degrading bacteria in environmental samples, strains *Pseudomonas cepacia*/pJP4, *Alcaligenes* JMP228/pJP4, *P. cepacia*/p712, and *Alcaligenes* JMP228/p712 were separately inoculated into samples of field soil, paddy soil, lake water, and river water, and then the changes of their populations were measured. The strains used contained a 2,4-D degradative plasmid, either pJP4 conferring fast-growing property to the host or p712 conferring slow-growing property, and were resistant to antibiotics such that the inoculated strains could be enumerated against the indigenous microbial populations. In sterile environmental samples, these strains were stably maintained at the levels used for inoculation, except in sterile paddy soil where *Alcaligenes* JMP228 strains died rapidly. In natural soil samples the four strains declined steadily with time, but in natural water samples their populations fell rapidly at the early phase and then remained almost constant. When the environmental samples were treated with 2,4-D, *P. cepacia*/pJP4 and *P. cepacia*/p712 maintained significant numbers, while *Alcaligenes* JMP228/pJP4 and *Alcaligenes* JMP228/p712 declined significantly in most of the samples. The results indicated that the survivability of genetically modified microorganisms could vary depending on the environments and that their abundance in the environments under 2,4-D selection was markedly influenced by the nature of the 2,4-D degradative plasmid as well as the type of the host strain.

Key words: Fate, genetically engineered microorganism, 2,4-D degradative plasmid

Much attention has recently been paid to the investigation of survivability and fate of genetically-engineered microorganisms in the environment. These organisms may be introduced into soils and waters as a result of accidental release or planned inoculation. The population dynamics and activities of these organisms in natural environments are crucial factors for removing of pollutants, enhancing in crop production, or assessing of environmental risks (18, 19). However, little information is available on their fate in the natural environments primarily due to the absence of appropriate techniques for enumerations in the natural environment.

Genetically modified laboratory microorganisms may encounter a number of problems when introduced into the natural environments. The major factors affecting their fate are substrate availability for growth, biotic interactions such as predation and competition, and abiotic environmental parameters such as pH and moisture (10~

12, 16, 19). Hence, their behavior could be differently affected by the nature of the environment where they were introduced. In addition to the survival of the host organisms, the stable maintenance of engineered genes is required for their successful applications in the environment.

Plasmids are the most frequently used vectors in genetic engineering because of the ease of manipulation and the possible gene copy effects on levels of gene expression. Several studies have shown that plasmid-bearing hosts get a survival advantage in conditions when plasmid-encoded function is required, but they are negatively affected under nonselective conditions due to the additional metabolic load exerted by the presence of plasmids (2, 21). The absence of selective pressure, hence, frequently resulted in loss of plasmid from host bacteria (2, 20). In addition to selective pressure, host type and environmental factors such as nutrient and phosphate were shown to affect the stability of plasmids (1, 3, 6).

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In a previous study on the competitiveness of 2,4-D-degrading bacteria (5, 8), it was shown that hosts *Alcaligenes* JMP228 and *P. cepacia* were good competitors in 2,4-D mineral medium under laboratory conditions and that one 2,4-D degradative plasmid pJP4 conferred fast-growing property to its host, while another plasmid p712 conferred slow-growing property. In this study, therefore, we used various environmental samples to investigate the effects of host versus plasmid on survival of the inoculated 2,4-D-degrading bacteria. Two different host bacteria, *Alcaligenes* JMP228 and *P. cepacia*, were genetically modified to carry a 2,4-D degradative plasmid, either pJP4 or p712, and inoculated into microcosms of field soil, paddy soil, lake water, and river water. Then their behaviors and plasmid stability were assessed under sterile and nonsterile conditions with and without 2,4-D selection.

Materials and Methods

Bacteria and environmental samples

Strain *Pseudomonas cepacia* is resistant to antibiotics kanamycin (75 µg/ml) and bacitracin (50 µg/ml) through Tn5 introduction. *Alcaligenes* JMP228 is resistant to rifampicin (100 µg/ml) through spontaneous mutation. A 2,4-D degradative plasmid, either pJP4 or p712, was introduced into these two strains through filter mating using *Alcaligenes* JMP134/pJP4 and *Pseudomonas pickettii*/p712 as a respective donor (5).

The field and paddy soils used in this study were obtained from the Long-term Agricultural Experimentation Station of Seoul National University in Suwon. The lake and river waters were obtained from a agricultural reservoir in Ochun, Kyunggido, and the Han river in Cheongju, Chungcheongbukdo, respectively.

Media and culture conditions

All strains were maintained on MMO mineral medium (17) containing 2,4-D at a concentration of 500 ppm (µg/ml). Strains to be inoculated into soils and waters were cultured to the late log phase at 30°C in peptone-tryptone-yeast extract-glucose (PTYG) medium (7). The numbers of the inoculated strains were determined by plating appropriate dilutions of soil or water suspensions onto PTYG agar containing the antibiotics. The most probable numbers for 2,4-D degraders (4) were determined by inoculating the serial dilutions of soil or water suspensions into 2,4-D mineral medium.

Inoculation of bacteria

2,4-D-degrading bacteria, *P. cepacia*/pJP4, *Alcaligenes* JMP228/pJP4, *P. cepacia*/p712, and *Alcaligenes* JMP228/p712, were grown at 30°C, harvested by centrifugation at 10,000×g for 10 min at 4°C, washed twice with an equal volume of 15 mM sodium phosphate buffer, kept on ice, and enumerated using a counting chamber.

Field and paddy soils were sifted through a 2 mm pore-size sieve, adjusted to a water content 20% and 35% (wt/wt), respectively. A 250 g (dry weight) portion was placed in the sterile glass beaker and mixed with the inoculum at the concentration of ca. 1.0×10^7 cells/g soil. For microcosms in which sterile soil was used, the soil was sterilized by autoclaving for 1 hr before inoculation of the strain. For microcosms under 2,4-D selection, the soil was treated every 10 days with 2,4-D dissolved in 0.1 M NaH_2PO_4 buffer (pH 7.0) at a concentration of 100 ppm and thoroughly mixed. All the experiments were carried out in two replicates.

The water microcosms were also prepared in the similar ways to those for soil microcosms. 250 ml portions of water samples were placed in 1 liter Erlenmeyer flasks and then mixed with the inoculum. For long-term starvation-survival studies (13, 14), the soil microcosms and the water microcosms were incubated at room temperature for 120 days and 90 days, respectively, and then samples were regularly taken for analysis of total viable counts, MPN determinations, and viable counts for the inoculated strains.

Results and Discussion

Survival and plasmid stability in field soil

After introduction of the microorganisms into natural field soil samples at the densities of about 2.0×10^7 cells/g soil, the populations of *P. cepacia*/pJP4 and *P. cepacia*/p712 declined more significantly than those of *Alcaligenes* JMP228/pJP4 and *Alcaligenes* JMP228/p712 (Fig. 1 and 2). The populations of the formers fell to ca. 5.5×10^3 cells/g soil at the end of the experiment period, while those of the latters were present at relatively high levels, ca. 4.3×10^5 cells/g soil, at the end of the incubation period. Although these cell numbers are lower than total viable counts, ca. 1.0×10^7 cells/g soil, measured on PTYG plates at the end of the experiment, the result suggests that the introduced strains can still survive in this field soil for quite a long time without 2,4-D selection.

The behavior of these strains was different under 2,4-D selection pressure. The added strains could take advantage of their 2,4-D degradative plasmids to utilize 2,4-D as a carbon source for growth and maintenance. The strains containing the plasmid pJP4 were stably maintained at the inoculated levels throughout the experiment, while those containing the plasmid p712 were slightly declined to ca. 5.8×10^5 cells/g soil at the end of

the incubation period (Fig. 1 and 2). Since the 2,4-D degradative plasmid pJP4 confers a fast-growing property to the host bacteria and the plasmid p712 confers a slow-growing property (5, 8), the result indicates that the nature of the degradative plasmid appears to play an important role in determining the abundance of bacterial strains in the environment under selection.

In sterile soil, the number of the introduced bacteria were initially increased upon starvation-survival and then maintained at high levels throughout the ex-
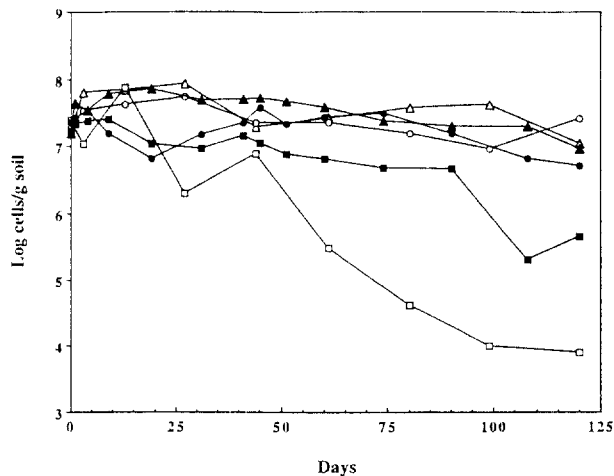
Fig. 1. Changes in numbers of 2,4-D-degrading bacteria after their introduction into field soil. Symbols: △ and ▲, sterile samples; □ and ■, natural samples; ○ and ●, 2,4-D-treated natural samples. Open symbols, strain *P. cepacia*/pJP4; solid symbols, strain *Alcaligenes* JMP228/pJP4. Each point represents an average value of replicate microcosm flasks.
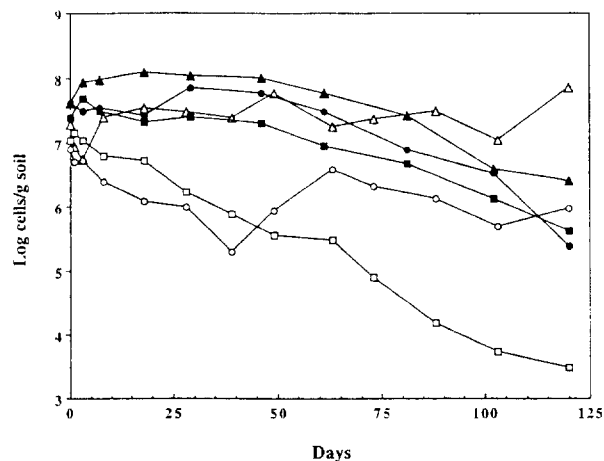

Fig. 2. Changes in numbers of 2,4-D-degrading bacteria after their introduction into field soil. Symbols: △ and ▲, sterile samples; □ and ■, natural samples; ○ and ●, 2,4-D-treated natural samples. Open symbols, strain *P. cepacia*/p712; solid symbols, strain *Alcaligenes* JMP228/p712. Each point represents an average value of replicate microcosm flasks.

periment (Fig. 1 and 2). The 2,4-D-degrading microbial populations measured by MPN procedure were close to the viable counts on antibiotics agar medium (Fig. 3), suggesting that most of the inoculated 2,4-D-degrading bacteria have stably carried the genetic information of the 2,4-D degradative plasmid in the sterile field soil.

## Survival and plasmid stability in paddy soil

In natural paddy soil, the populations of all of the inoculated bacteria were declined steadily and $1.1 \times 10^2 \sim 3.0 \times 10^2$ cells per g soil were detected at the end of the incubation period (Fig. 4 and 5).

When the paddy soil was regularly treated with 2,4-D, the populations of *P. cepacia*/pJP4 and *P. cepacia*/p712 were maintained at high levels throughout the experiment, with the former being more abundant than the latter (Fig. 4 and 5). On the other hand, under the same 2,4-D selection condition, the populations of *Alcaligenes* JMP228/pJP4 and *Alcaligenes* JMP228/p712 fell rapidly to ca. 1.0×10^5 cells/g soil at day 4, persisted at these levels by day 47, and thereafter died quickly, with no cells being detected from day 80. The abundance of *Alcaligenes* JMP228 strains also declined rapidly in the sterile paddy soil, whereas the populations of *P. cepacia* strains grew and persisted at high levels. The *Alcaligenes* JMP228 strains probably owe their short persistence in part to their sensitivity to abiotic stresses such as pH. The initial pH value of sterile paddy soil, 4.5, was relatively low compared to that of the natural soil, 5.1, and also the degradation of 2,4-D releases chlorine ions (9), thus gradually lowering the pH of 2,4-D treated soil. Competition
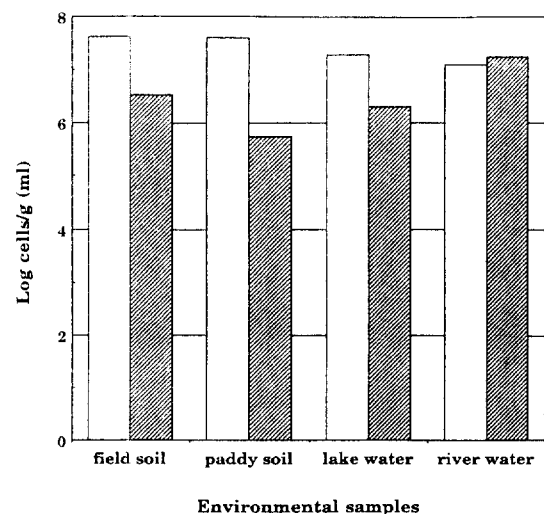

Fig. 3. Survival of *P. cepalcia*/pJP4 and plasmid maintenance after introduction into sterile environmental samples. Symbols: viable counts on antibiotics agar plates (□); MPN determinants in 2,4-D mineral medium (▨). The data represent the mean value of two replicate experiments.

for carbon source with indigenous microbial populations appears to be another factor affecting the abundance of *Alcaligenes* JPM228 strains in paddy soil under 2,4-D selection. This is apparent from the observation that their populations decreased with time until no cells were detected at day 100 in 2,4-D treated soil, while the numbers of 2,4-D degraders measured by MPN procedure were maintained at high levels throughout the experiment in this paddy soil (Fig. 6).
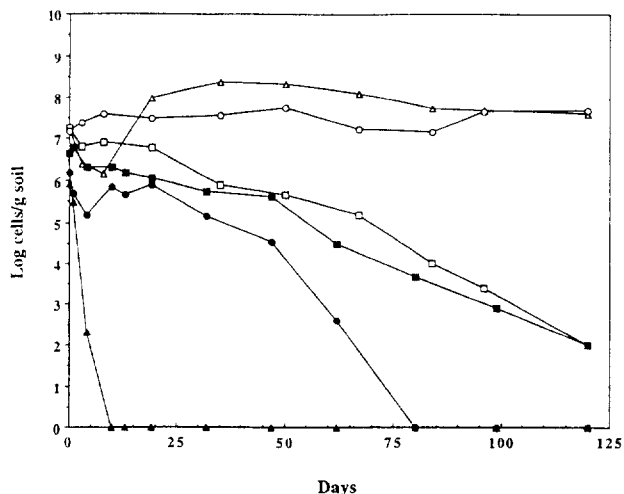
Fig. 4. Changes in numbers of 2,4-D-degrading bacteria after their introduction into paddy siol. Symbols: △ and ▲, sterile samples; □ and ■, natural samples; ○ and ●, 2,4-D-treated natural samples. Open symbols, strain *P. cepacia*/pJP4; solid symbols, strain *Alcaligenes* JMP228/pJP4. Each point represents an average value of replicate microcosm flasks.
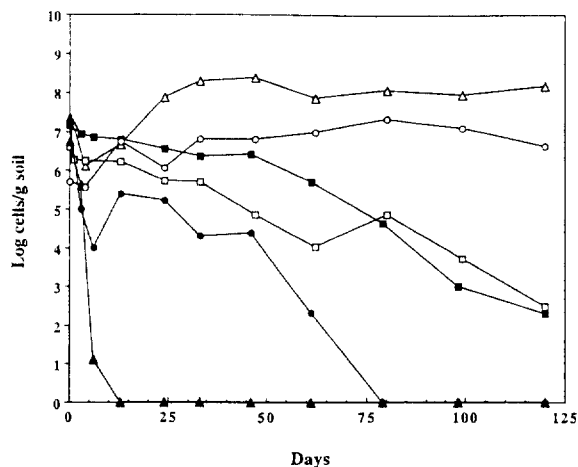

Fig. 5. Changes in numbers of 2,4-D-degrading bacteria after their introduction into paddy siol. Symbols: △ and ▲, sterile samples; □ and ■, natural samples; ○ and ●, 2,4-D-treated natural samples. Open symbols, strain *P. cepacia*/p712; solid symbols, strain *Alcaligenes* JMP228/p712. Each point represents an average value of replicate microcosm flasks.

In sterile paddy soil, the 2,4-D MPN counts for *Alcaligenes* JMP228 strains were about 2.0×10^2 cells/g soil at the end of the incubation period. The result was unexpected because no viable cells were detected on PTYG agar plates at the end of the experiment. It seemed that the stressed *Alcaligenes* JMP228 strains became no longer viable on the PTYG agar medium, but some of them could still grow in a more favorable medium like a buffered 2,4-D liquid medium. This observation would be a good demonstration that laboratory-selected microorganisms could persist in stressful environments, although they might not be viable any more on certain laboratory media (15). The MPN counts for *P. cepacia*/pJP4 and *P. cepacia*/p712 were 5.6×10^5 and 7.8×10^5 cells/g soil, respectively, at the end of the incubation period in the sterile soil. These values were relatively lower than their viable counts, 4.0×10^7 and 1.4×10^8 cells/g soil, respectively, on antibiotics agar plates (Fig. 3, 4, and 5), indicating that some of the plasmids were not stably maintained in these strains inoculated into the paddy soil.

Survival and plasmid stability in lake water

In natural lake water, the populations of the inoculated strains fell initially, but their numbers subsequently remained at a level of either about 3.4×10^3 cells/ml for *Alcaligenes* JMP228 strains or about 1.0×10^2 cells/ml for *P. cepacia* strains (Fig. 7 and 8). This phenomenon typically represented line D among four responses of bacteria to the lack of substrates in the environment (12).

Under 2,4-D selection, all of the inoculated populations sharply declined at the initial phase of the experiment, but their numbers subsequently recovered, with the populations of *P. cepacia* strains being maintained at higher
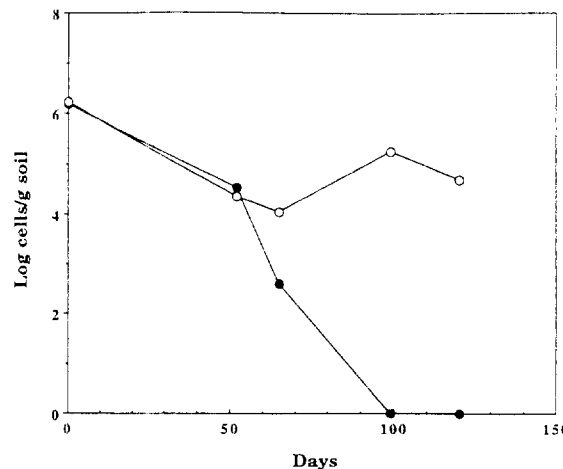
Fig. 6. Changes in numbers of the inoculated strain *Alcaligenes* JMP228/pJP4 (●) and total 2,4-D degraders (○) in 2,4-D-treated paddy soil. Each point represents an average value of replicate microcosm flasks.

levels than those of *Alcaligenes* JMP228 strains. Since these strains remained stably in sterile lake water, they appeared not to be destroyed by abiotic stresses. Instead their initial reduction in nonsterile lake water appeared to be due to protozoa predation and competition for carbon source with indigenous microbial populations.

In sterile lake water, the MPN values for the 2,4-D-degrading microbial populations were close to the viable counts on antibiotics agar medium measured at the end of the incubation period (Fig. 3), suggesting that the 2,4-D degradative plasmids were stably maintained in the

strains inoculated into the sterile lake water.

## Survival and plasmid stability in river water

In natural river water, the abundance of the inoculated strains also declined initially and thereafter remained stably as in lake water (Fig. 9 and 10). When the water samples were regularly treated with 2,4-D, only the population of *P. cepacia*/pJP4 was maintained at a higher level than that in natural water samples, while the other three populations presented at lower levels than those in
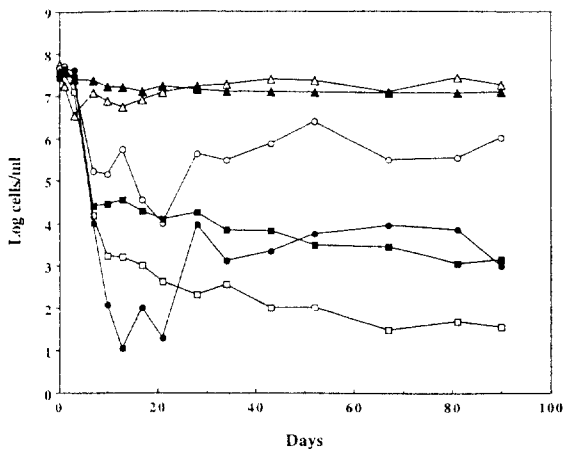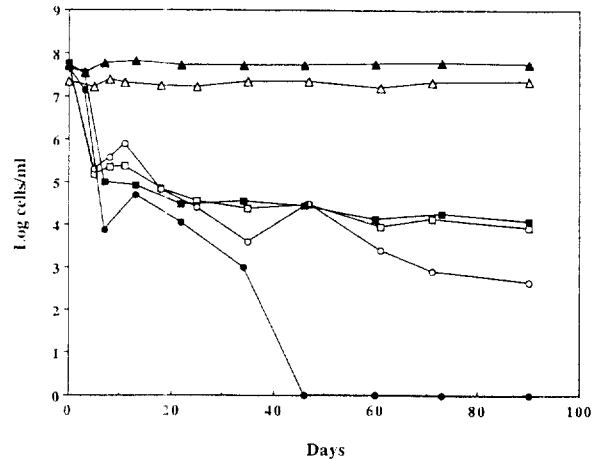

Fig. 7. Changes in numbers of 2,4-D-degrading bacteria after their introduction into lake water. Symbols: △ and ▲, sterile samples; ☐ and ■, natural samples; ○ and ●, 2,4-D-treated natural samples. Open symbols, strain *P. cepacia*/pJP4; solid symbols, strain *Alcaligenes* JMP228/pJP4. Each point represents an average value of replicate microcosm flasks.
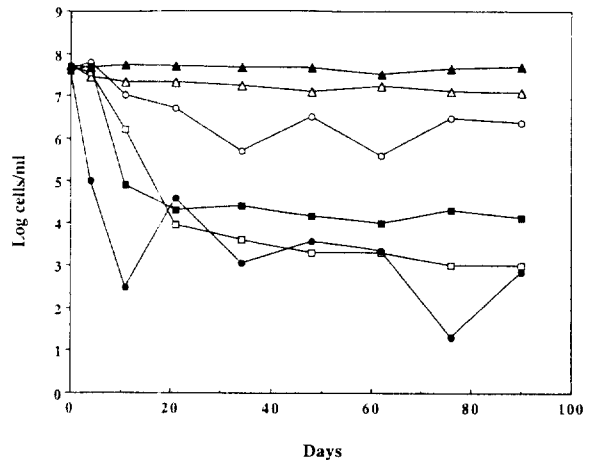
Fig. 9. Changes in numbers of 2,4-D-degrading bacteria after their introduction into river water. Symbols: △ and ▲, sterile samples; ☐ and ■, natural samples; ○ and ●, 2,4-D-treated natural samples. Open symbols, strain *P. cepacia*/pJP4; solid symbols, strain *Alcaligenes* JMP228/pJP4. Each point represents an average value of replicate microcosm flasks.
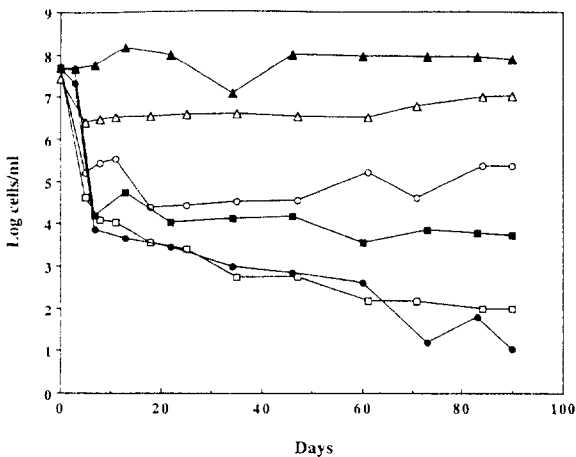
Fig. 7. Changes in numbers of 2,4-D-degrading bacteria after their introduction into lake water. Symbols: △ and ▲, sterile samples; ☐ and ■, natural samples; ○ and ●, 2,4-D-treated natural samples. Open symbols, strain *P. cepacia*/p712; solid symbols, strain *Alcaligenes* JMP228/p712. Each point represents an average value of replicate microcosm flasks.

Fig. 10. Changes in numbers of 2,4-D-degrading bacteria after their introduction into river water. Symbols: △ and ▲, sterile samples; ☐ and ■, natural samples; ○ and ●, 2,4-D-treated natural samples. Open symbols, strain *P. cepacia*/p712; solid symbols, strain *Alcaligenes* JMP228/p712. Each point represents an average value of replicate microcosm flasks.

natural water not treated with 2,4-D. Since these strains were stably maintained in sterile water, their populations in natural river water appeared not to be significantly affected by abiotic stresses. The most imortant factor involved in the abundance of the introduced 2,4-D-degrading bacteria seemed to be competition for carbon source with indigenous microbial populations. This is apparent from the observation that the host bacteria containing the 2,4-D degradative plasmid pJP4 of fast-growing property better persisted than those containing the plasmid p712 of slow-growing property in the river water sample under 2,4-D selection. Strain *Alcaligenes* JMP 228/p712, especially, disappeared quickly at the initial phase of the experiment and no cells were detected from day 46 under 2,4-D selection (Fig. 10), while the MPN value for total 2,4-D-degrading microbial population was 4.9×10^4 cells/ml at the end of the incubation period, indicating that other indigenous 2,4-D degraders outcompeted this strain under 2,4-D selection. In streile river water, the 2,4-D degradative plasmids appeared to be stably maintained in the inoculated strains throughout the experiment (Fig. 3).

These results suggested that the survival of 2,4-D-degrading bacteria in the environment under 2,4-D selection was markedly influenced by both the type of the host strain and the nature of the plasmid involved in 2,4-D degradation. Overall, strain *P. cepacia* better survived than strain *Alcaligenes* JMP228 under 2,4-D selection and the 2,4-D degradative plasmid pJP4 conferred better competitiveness to its host than the plasmid p712 in the environmental samples.

Acknowledgement

References

1. **Godwin, D. and J.H. Slater,** 1979. The influence of the growth environment on the stability of a drug resistance plasmid in *Escherichia coli* K12. *J. Gen. Microbiol.* **111,** 201-210.

2. **Gowland, P.C. and J.H. Slater,** 1984. Transfer and stability of drug resistance plasmids in *Escherichia coli* K12. *Microb. Ecol.* **10,** 1-13.

3. **Helling, R.B., T. Kinney, and J. Adams,** 1981. The maintenance of plasmid-containing organisms in populations of *Escherichia coli. J. Gen. Microbiol.* **123,** 129-141.

4. **Holben, W.E., B.M. Schroeter, V.G.M. Calabrese, R.H. Olsen, J.K. Kukor, V.O. Biederbeck, A.E. Smith, and J. M. Tiedje,** 1992. Gene probe analysis of soil microbial po-

pulations selected by amendment with 2,4-dichlorophenoxyacetic acid. *Appl. Environ. Microbiol.* **58,** 3941-3948.

5. **Hong, S.M., Y.J. Ahn, Y.K. Park, K.H. Min, C.K. Kim, and J.O. Ka,** 1995. Effects of genetically different 2,4-D-degradative plasmids on degradation phenotype and competitiveness of soil microorganisms. *Jour. Microbiol.* **33,** 208-214.

6. **Jones, S.A. and J. Melling,** 1984. Persistence of pBR322-related plasmids in *Escherichia coli* grown in chemostat cultures. *FEMS Microbiol. Lett.* **22,** 239-243.

7. **Ka, J.O. and J.M. Tiedje,** 1994. Integration and excision of a 2,4-D degradative plasmid in *Alcaligenes paradoxus* and evidence of its natural intergeneric transfer. *J. Bacteriol.* **176,** 5284-5289.

8. **Ka, J.O., W.E. Holben, and J.M. Tiedje,** 1994. Analysis of competition in soil among 2,4-dichlorophenoxyacetic acid-degrading bacteria. *Appl. Environ. Microbiol.* **60,** 1121-1128.

9. **Kelly, M.P., K.B. Hallberg, and O.H. Tuovinen,** 1989. Biological degradation of 2,4-dichlorophenoxyacetic acid: chloride mass balance in stirred tank reactors. *Appl. Environ. Microbiol.* **55,** 2717-2719.

10. **Kilbane, J.J., D.K. Chatterjee, and A.M. Chakrabarty,** 1993. Detoxification of 2,4,5-trichlorophenoxyacetic acid from contaminated soil by *Pseudomonas cepacia. Appl. Environ. Microbiol.* **45,** 1697-1700.

11. **Liang, L.N., J.L. Sinclair, L.M. Mallow, and M. Alexander,** 1982. Fate in model ecosystems of microbial species of potential use in genetic engineering. *Appl. Environ. Microbiol.* **44,** 707-714.

12. **Morita, R.Y.,** 1992. Survival and recovery of microorganisms from environmental samples, p. 435-453. *In* M.A. Levin, R.J. Seidler, and M. Rogul (ed.), Microbial ecology: principles, methods, and applications. McGraw-Hill, NY.

13. **Moyer, C.L. and R.Y. Morita,** 1989. Effect of growth rate and starvation-survival on the viability and stability of a psychrophilic marine bacterium. *Appl. Environ. Microbiol.* **55,** 1122-1127.

14. **Moyer, C.L. and R.Y. Morita,** 1989. Effect of growth rate and starvation-survival on cellular DNA, RNA and protein of a psychrophilic marine bacterium. *Appl. Environ. Microbiol.* **55,** 2710-2716.

15. **Roszak, D.B., D.J. Grimes, and R.R. Colwell,** 1984. Viable but nonrecoverable stage of *Salmonella enteritidis* in aquatic systems. *Can. J. Microbiol.* **30,** 334-338.

16. **Scheuerman, P.R., J.P. Schmidt, and M. Alexander,** 1988. Factors affecting the survival and growth of bacteria introduced into lake water. *Arch. Microbiol.* **150,** 320-325.

17. **Stanier, R.Y., N.J. Palleroni, and M. Doudoroff,** 1966. The aerobic pseudomonads: a taxonomic study. *J. Gen. Microbiol.* **43,** 159-271.

18. **Sussman, M., C.H. Collins, F.A. Skinner, and D.E. Stewart-Tull,** 1988. The release of genetically engineered

microorganisms. Academic Press, London.

19. **Tiedje, J.M., R.K. Colwell, Y.L. Grossman, R.E. Hodson, R.E. Lenski, R.N. Mack, and P.J. Regal,** 1989. The planned introduction of genetically engineered organisms: ecological considerations and recommendations. *Ecology* **70**, 298-315.

20. **Trevors, J.T., T. Barkay, and A.W. Bourquin,** 1987. Gene transfer among bacteria in soil and aquatic environments: a review. *Can. J. Microbiol.* **33**, 191-198.

21. **Zund, P. and G. Lebek,** 1980. Generation time-prolonging R plasmids: correlation between increases in the generation time of *Escherichia coli* caused by R plasmids and their molecular size. *Plasmid* **3**, 65-69.