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Molecular and Ecological Studies on 2,4-D Degrading Recombinant Microorganims.

Taesung Kim[1], Kyunghee Oh[1], Mi-Soon Kim[2] and Jong-Ok Ka[2]

[1]*Biological Resources Division, Biodiversity Research Department, NIER, Republic of Korea*
[2]*School of Agricultural Biotechnology, Seoul National University, Republic of Korea*

The recent developments in biotechnology have led to the construction of genetically modified microorganisms (GMMs) which can be used for a variety of commercial purposes including biocontrol and bioremediation. Some of these GMMs will be applied to the open environment and therefore are apt to escape from research laboratories. The release of these GMMs into the environment may have an impact on indigenous soil microorganisms. In an effort to assess environmental risk of genetically modified microorganisms (GMMs) to agro-ecosystems and environments, two case studies, as well as basic biology, have been performed.

The first case study was a model of environmental risk assessment on cleaning up polluted environments with genetically modified microorganisms. *Alcaligenes* sp. JMP228/pJP4, which has a 2,4-dichlorophenoxyacetic acid (2,4-D) degradative plasmid pJP4 was inoculated into natural soil with or without 2,4-D amendment. The applied 2,4-D was quickly degraded after a short lag period of 4 days in microcosms I and III, which were inoculated with 2,4-D-degrading bacteria, *Alcaligenes* sp. JMP228/pJP4 (Table 1) (Fig. 1.). In microcosms I and III inoculated with *Alcaligenes* sp. JMP228/pJP4, the initial populations of 2,4-D degraders were 8.4 x 10^6 cells/g soil and 8.7 x 10^6 cells/g soil, respectively, as measured by MPN counting. By day 14 after the 2,4-D amendment, the population densities had markedly increased to 3.7 x 10^7 and 4.2 x 10^7 cells/g soil, respectively, and these densities were stably maintained throughout the experiment (Fig. 1.). Transfer of pJP4 to indigenous soil bacteria was investigated with and without 2,4-D amendment. Plasmid pJP4 transfer was enhanced in the soils treated with 2,4-D, compared to the soils not amended with 2,4-D. Several different transconjugants were isolated from the soils treated with 2,4-D, while no indigenous transconjugant was obtained from the unamended soils (Fig. 2.). Inoculation of the soils with both the donor *Alcaligenes* sp. JMP228/pJP4 and a recipient *Burkholderia cepacia* DBO1 resulted in low species diversity of the transconjugants. Repetitive extragenic palindromic-PCR (REP-PCR) analysis of the transconjugants exhibited seven distinct genomic DNA fingerprints. Analysis of 16S rDNA sequences indicated that the transconjugants were related to members of the genera, *Burkholderia* and *Pandoraea*. Denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified 16S rRNA genes revealed that the inoculation of the donor caused clear changes in the bacterial community structure of the 2,4-D-amended soils (Fig. 3.). The new 16S rRNA gene bands in the DGGE profile corresponded with the

16S rRNA genes of 2,4-D-degrading transconjugants isolated from the soil. The results indicate that introduction of the 2,4-D degradative plasmid as *Alcaligenes* sp. JMP228/pJP4, has a substantial impact on the bacterial community structure in the 2,4-D-amended soil.

The second case study was a model of environmental risk assessment on genetically modified *E. coli* and introduced DNA in natural soil by unintentional release. *Escherichia coli* is generally used in gene cloning as a host for the expression of gene material from other organisms.

To investigate the fate of GM *E. coli* and engineered DNA in natural soil by unintentional release, *E. coli* HB101/pJP4, which is an enteric bacterium harboring plasmid pJP4, was used as a model. *E. coli* HB101/pJP4 was inoculated into natural soil and transfer of the plasmid pJP4 to indigenous soil bacteria was investigated with and without 2,4-D amendment.

The initial population density, 1.0 x 10^5 cells/g soil, of *E. coli* HB101/pJP4 inoculated into the soils declined quickly (Fig. 4.). About 3 weeks after the inoculation, its population became undetectable. When *E. coli* HB101/pJP4 was inoculated at a higher density 1.0 x 10^7 cells/g soil, its population also declined at a similar rate as 1.0 x 10^5 cells/g soil (Fig. 5.). The population of *E. coli* HB101/pJP4 declined to 1.0 x 10^2 cells/g soil in 3 weeks after the inoculation, and then this level was maintained throughout the experiment. The copy number of the *tfd*A gene of *E. coli* HB101/pJP4 also rapidly decreased to about 100 - 1000 per g of soil in three weeks (Fig. 6.).

The applied 2,4-D was not degraded throughout the experiment and no indigenous transconjugants were obtained from the 2,4-D amended and unamended soils. Moreover, DNA band patterns of DGGE profiles were not significantly changed throughout the experiments (Fig. 7.).

These results indicate that the inoculation of *E. coli* HB101/pJP4, which is very vulnerable in the soil environment, may not have a significant impact on gene transfer and microbial community structure in the soil used in this study.

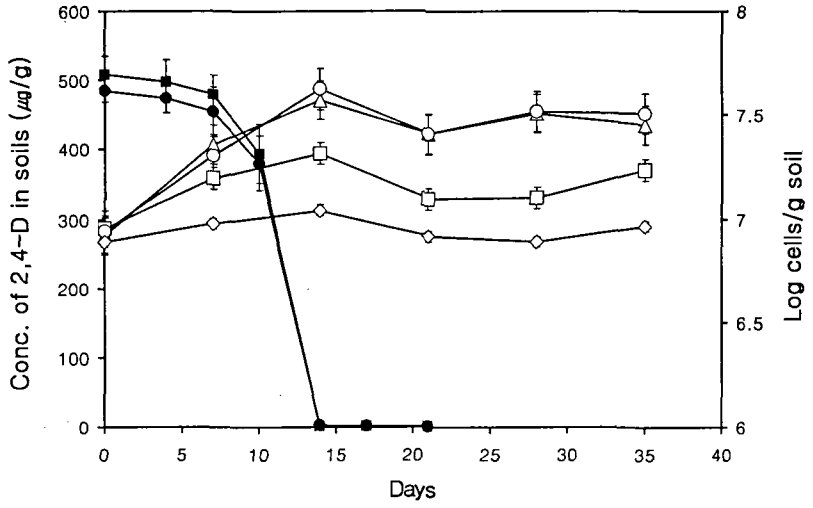
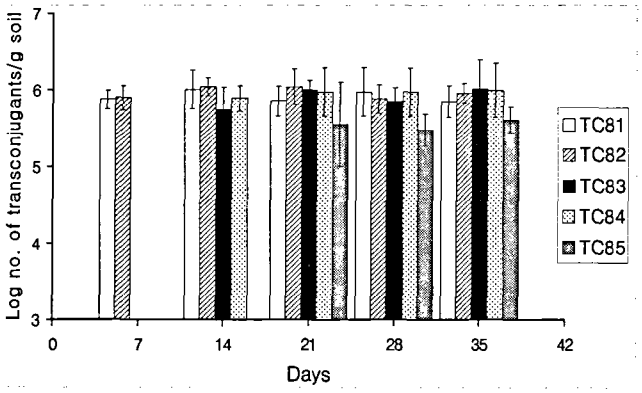


Fig. 1 Biodegradation of 2,4-D (solid symbol) and MPN counts of 2,4-D-degrading microorganisms (open symbol) in microcosm soils.
Symbols: ●, microcosm I; ■, microcosm III; △, microcosm I; □, microcosm II; ○, microcosm III; ◇, microcosm IV. Bars represent the standard deviations from duplicate microcosms.

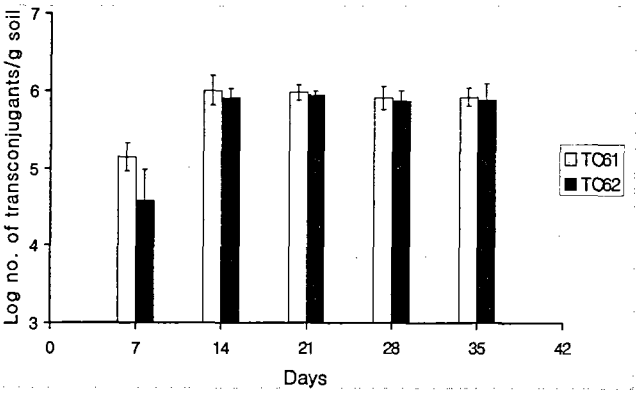
Table. 1 Microcosm design and treatment.

	Microcosm I	Microcosm II	Microcosm III	MicrocosmIV	Microcosm V
2,4-D (500ppm)	+	-	+	-	-
Alcaligenes sp. JMP228/pJP4	+	+	+	+	-
B.cepacia DBO1	+	+	-	-	-

(A)



(B)



(D)

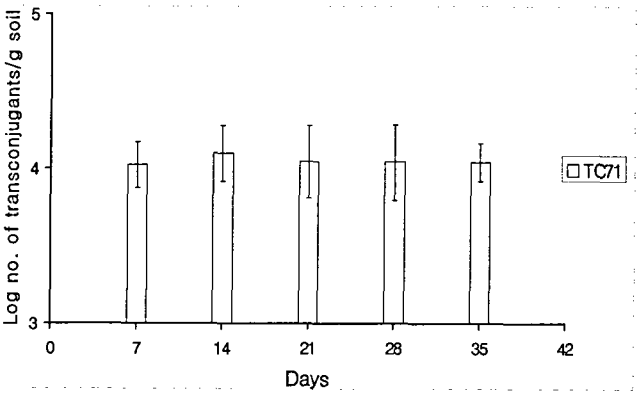


Fig. 2 Occurrence and distribution of the transconjugants in microcosm I (B), microcosm II (C), and microcosm III (A).

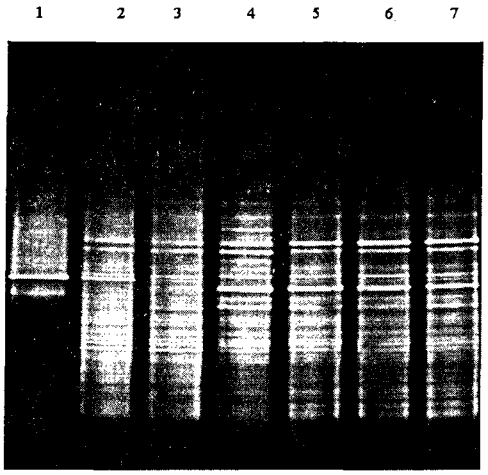


Fig. 3 DGGE analysis of 16S rDNA fragments obtained after PCR amplification with eubacterial primers 1070F and 1392R in microcosm Ⅲ. DGGE profiles for soil of Day 0 (lane 2), Day 7 (lane 3), Day 14 (lane 4), Day 21 (lane 5), Day 28 (lane 6), and Day 35 (lane 7) of microcosm Ⅲ. DGGE pattern from *Alcaligenes* sp. JMP228/pJP4 (lane 1) is shown.

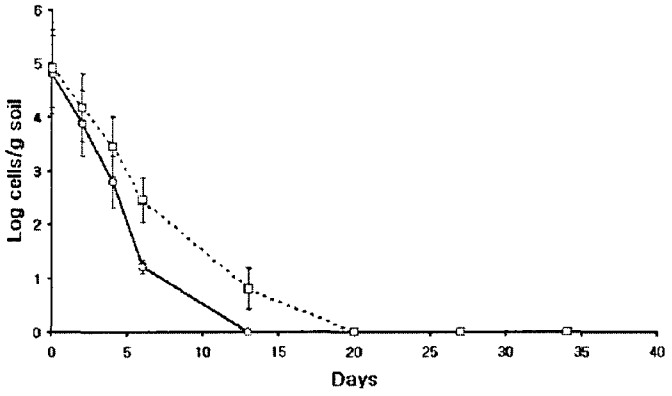


Fig. 4 Population dynamics of *E. coli* HB101/pJP4 inoculated into microcosm Ⅰ (○) and microcosm Ⅲ (□). Microcosm Ⅰ soils were treated with 500 ppm of 2,4-D.
*: **microcosm** Ⅰ was inoculated with the donor *E. coli* HB101/pJP4 at a density of 1.0 x 10^5 cells/g soil and amended with 2,4-D (500μg/mℓ); **microcosm** Ⅱ was inoculated with the donor at a density of 1.0 x 10^5 cells/g soil but not amended with 2,4-D; **microcosm** Ⅲ was inoculated with the donor at a density of 1.0 x 10^7 cells/g soil and amended with 2,4-D (500μg/mℓ); **microcosm** Ⅳ was inoculated with the donor at a density 1.0 x 10^7 cells/g soil but not amended with 2,4-D; **microcosm** Ⅴ was an uninoculated, unamended control.

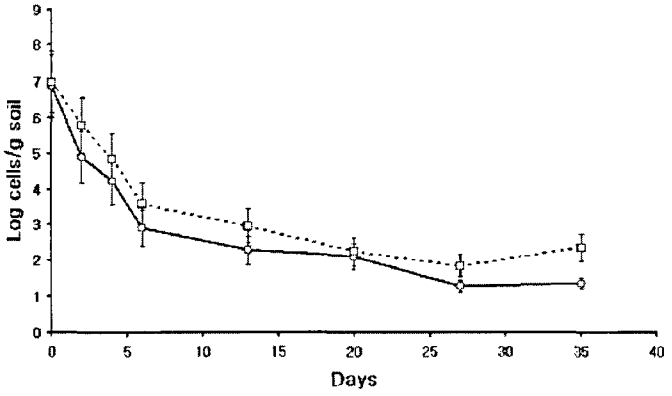


Fig. 5 Population dynamics of *E. coli* HB101/pJP4 inoculated into microcosm Ⅲ (○) and microcosm Ⅳ (□). Microcosm Ⅲ soils were treated with 500 ppm of 2,4-D.

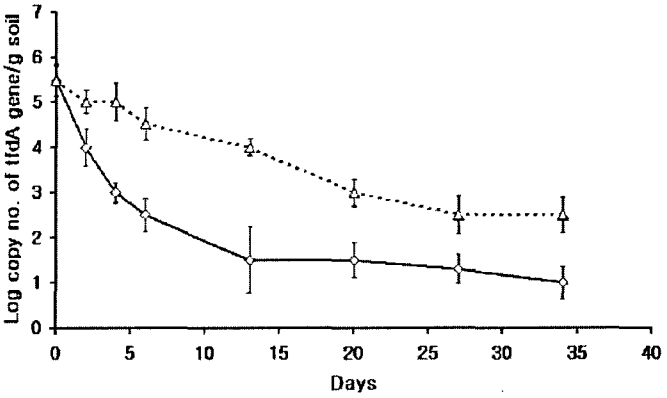


Fig. 6 The *tfd*A gene copies in microcosm soils treated with *E. coli* HB101/pJP4 and/or 2,4-D. Symbols: ◇, microcosm Ⅰ; △, microcosm Ⅱ. Bars represent the standard deviations from duplicate microcosms.

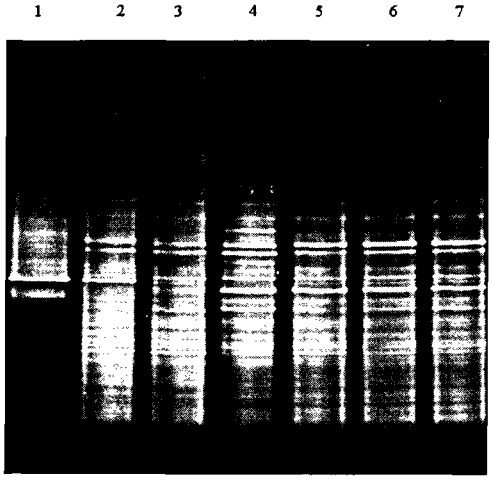


Fig. 7 DGGE analysis of 16S rDNA fragments obtained after PCR amplification with eubacterial primers 1070F and 1392R in microcosm Ⅲ. DGGE profiles for soil of Day 0 (lane 1), Day 2 (lane 2), Day 4 (lane 3), Day 6 (lane 4), Day 13 (lane 5), Day 20 (lane 6), Day 27 (lane 7), and Day 34 (lane 8) of microcosmⅢ.

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