Survival and Impact of Genetically Engineered *Pseudomonas putida* Harboring Mercury Resistance Gene in Soil Microcosms

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The survival of genetically engineered and wild-type *Pseudomonas putida* PpY101, that contained a recombinant plasmid pSR134 conferring mercury resistance, were monitored in andosol and sand microcosms. The survival of genetically engineered and wild-type *P. putida* was not significantly different in andosol. The population change of the two strains was dissimilar in andosol and sand. The survival of genetically engineered and wild-type *P. putida* strains was affected by the water content of andosol, and increased with the increment of the water content. The impact of the addition of genetically engineered and wild-type *P. putida* strains on indigenous bacteria and fungi was examined. Inoculation of both strains had no apparent effect on the density of indigenous microorganisms.

Genetically engineered microorganisms (GEMs) are being developed for various commercial applications, including those in agriculture, industry, and pollution control.\(^1\)–\(^3\) The risk related to the release of GEMs depends on their fate in the environment. Therefore the survival or growth of each GEMs in soil microcosms must be measured and the risks of environmental damage should be assessed before its release.

Many studies have dealt with soil environmental factors such as water content\(^4\)–\(^7\) and bacterial habitat,\(^8\)–\(^10\) that affect nonrecombinant bacterial survival. Although more recent studies have been done to determine the fate of GEMs in the soil environment,\(^11\)–\(^20\) the factors that affect their survival, persistence, and growth in the soil environment are poorly understood. Although *Pseudomonas putida* and other pseudomonads are important elements of any indigenous microflora, their persistence and the effects of their introduction into the environment have not been fully examined.

For the study described in this paper, we constructed genetically engineered *P. putida* strains with a specific marker, such as mercury resistance.\(^21\) This specific marker is useful in studying the fate and impact of GEMs in the environment because of its high sensitivity for detection and convenience. The investigation was aimed at assessing the persistence of genetically engineered and wild-type *P. putida* strains in soil microcosms, to evaluate the factors that affect the survival of the both strains, and to determine the potential impact of genetically engineered and wild-type *P. putida* strains on indigenous bacteria and fungi in the soil environment.

**Materials and Methods**

*Bacterial strains. P. putida* PpY101, pSR134 and PpY101 were used in this study. Plasmid pSR134 (18.6 kb) was constructed by inserting two EcoRI DNA fragments, H (4.9 kb) and I (4.2 kb), encoding the mercury resistance gene from *N. r. plasmid*\(^22\) into a broad host range vector, pSUP104 (9.5 kb), encoding the tetracycline resistance gene. The recombinant plasmid pSR134 was maintained stably in the wild-type *P. putida* PpY101 for 80 generations. The wild-type *P. putida* PpY101 was nalidixic acid resistant and harbored no plasmids. The presence of the recombinant plasmid pSR134 in *P. putida* PpY101 was determined by the growth on Luria-Bertani (LB) agar\(^23\) (prepared with 5 g instead of 10 g of NaCl per liter) containing 20 μg of HgCl₂ ml⁻¹, and confirmed by plasmid isolation and restriction analysis.

Genetically engineered and wild-type *P. putida* strains were cultured in LB broth with and without the addition of 20 μg of HgCl₂ ml⁻¹ at 30°C, respectively. The two strains were cultivated until the late logarithmic phase, and harvested by centrifugation at 8000 x g for 10 min at 4°C. The cells were washed three times with ice-cold sterile distilled water and suspended in distilled water to obtain a population of 10⁶ CFU ml⁻¹, before use in survival experiments.

**Microcosms and inoculation.** Survival studies were done using soil microcosms. Andosol from a vegetable field (Tsukuba, Ibaraki) and sand from the Kinugawa River (Mitakaidou, Ibaraki) were used. The water contents of andosol and sand were 33 and 15% (w/w), respectively. The fresh moist soil samples of andosol and sand were passed through a 5-mm sieve without air-drying, and sprayed with sterile distilled water containing genetically engineered or wild-type *P. putida* strains. The soil samples were then mixed well, to secure homogenous inoculation (about 10⁶ CFU g⁻¹ dry weight). Portions (330 g dry weight of andosol, 600 g dry weight of sand) of inoculated and noninoculated soil were put in 500-ml glass jars with lids, called soil microcosms.

To study the effects of the water content of the andosol on genetically engineered and wild-type *P. putida*, the soil was air-dried and passed through a 2-mm sieve. Sterile distilled water was added to andosol, to obtain water contents of 14, 23, and 33%. The soil samples were sprayed with the inoculum after moistening, the inoculated soil (about 10⁶ CFU g⁻¹ dry weight) was then mixed well, so that after inoculation the water contents of the soil samples were 15, 25, and 34%, respectively. Each 350-g portion (dry weight) of the inoculated and noninoculated soil was put in 500-ml glass jars. The microcosms were incubated at 25°C, and all microcosm experiments were duplicated.

**Sampling of microcosms.** Samples were withdrawn from microcosms at fixed intervals. After the soil in the microcosms was mixed, samples (1 g) were removed randomly, and placed in test tubes containing 9 ml of sterile distilled water (i.e., a 10⁻¹ dilution of soil) and then blended with a Vortex at maximum speed for 3 min. Further dilutions were prepared from this 10⁻¹ dilution.

Moisture loss was measured by weight at each sampling period, and sterile distilled water was added to the soil microcosms to maintain the soil moisture at the initial level. The water content of the microcosm soil was kept constant throughout the experiment.

**Enumeration of bacteria.** Soil dilutions were made in 10-fold increments in test tubes containing 9 ml of sterile distilled water. Dilutions were plated onto appropriate media. For counting the genetically engineered *P. putida*, 0.1-ml samples were placed in a test tube containing 2 ml of 0.7% LB top agar, with 40 μg of HgCl₂ and 5 mg of cycloheximide to suppress the
growth of fungi. The soft agar was then poured onto LB agar containing 250 μg of nalidixic acid ml⁻¹. The wild-type strain was enumerated on LB agar containing 250 μg of nalidixic acid ml⁻¹ without HgCl₂. The densities of the genetically engineered and wild-type P. putida strains were estimated from the colony numbers on plates after two days of incubation at 30°C.

Total bacteria were enumerated on 1:10 LB agar. The quantity of fungi was measured on Czapek Dox agar (0.2% NaNO₃, 0.1% K₂HPO₄, 0.05% MgSO₄·7H₂O, 0.05% KCl, 0.001% FeSO₄·7H₂O, 3% sucrose, 1.5% agar, and pH 5.2) containing 100 μg of streptomycin and 100 μg of chloramphenicol ml⁻¹. Densities of total bacteria and fungi were estimated from plates incubated at 25°C for 7 days.

Soil for measurement of water content was removed at the time of sampling, and results were expressed as CFU g⁻¹ of dry weight.

Results
Effects of soil source
The population changes of genetically engineered and wild-type P. putida strains in andosol and sand microcosms are shown in Fig. 1. In andosol, the detection limit of genetically engineered and wild-type P. putida strains were 10² and 10⁴ CFU g⁻¹, respectively. In sand, the detection limit of the both strains was 10⁴ CFU g⁻¹. The densities of genetically engineered and wild-type P. putida strains decreased moderately in andosol during the experiment (Fig. 1A). No significant difference in survival was observed between genetically engineered and wild-type P. putida strains. In sand, however, the densities of the two strains decreased rapidly for 7 days, then very moderately from 7 to 46 days (Fig. 1B).

Effects of water content
The effects of the water content of andosol on the survival of genetically engineered and wild-type P. putida strains were examined. The population changes of both strains in andosol at 15, 25, and 34% are shown in Fig. 2. With 15% water content the population of both genetically engineered and wild-type P. putida strains decreased rapidly to about 1% of their initial numbers, within 2 h of inoculation. The survival of the two strains increased with the increment of water content.

Effects on total bacteria
The samples of sand and andosol contained initially about 10⁷ and 10⁸ CFU g⁻¹ of total indigenous bacteria, respectively. Figure 3 shows the density changes of the total indigenous bacteria in andosol and sand microcosms after inoculation of 10⁷ CFU g⁻¹ genetically engineered or wild-type P. putida strains. For one month the density of total indigenous bacteria was unchanged by the inoculation of genetically engineered or wild-type strains in sand and andosol. Figure 4 shows the population change of total indigenous bacteria in andosol with various levels of water content. There was no significant difference in the density of the total indigenous bacteria in microcosms inoculated with genetically engineered or wild-type P. putida strains and the noninoculated microcosms.

Effects on fungi
The density changes of the fungi in soil microcosms after inoculation of genetically engineered or wild-type P. putida strains are shown in Figs. 5 and 6. The samples of sand and andosol contained initially about 5 x 10⁴ and

![Fig. 1. Survival of Genetically Engineered (●) and Wild-type P. putida Strains (○) in Andosol with 33% (A) and Sand with 15% (B) Water Content, Respectively. Dotted lines indicate minimum levels of detection.](image)

![Fig. 2. Survival of Genetically Engineered (●) and Wild-type P. putida Strains (○) in Andosol with 15% (A), 25% (B), and 34% (C) Water Content, Respectively. Dotted lines indicate minimum levels of detection.](image)

![Fig. 3. Density of Total Indigenous Bacteria in Andosol (A) and Sand (B) Microcosms Inoculated with the Genetically Engineered Strain (○), the Wild-type Strain (●), and in Noninoculated Microcosms (●).](image)
10^6 CFU g^{-1} of fungi, respectively. No difference was observed in the fungal density between the inoculated and noninoculated microcosms.

**Discussion**

We investigated the survival of genetically engineered and wild-type *P. putida* strains in soil microcosms. Yeung *et al.*\(^{17}\) reported no significant differences between the fate of wild-type and genetically engineered strains of *P. putida* and *Pseudomonas aeruginosa* harboring recombinant plasmids in freshly moistened soil. Morel *et al.*\(^{12}\) noted the similar fate in the corn rhizosphere of wild-types and genetically modified strains of *Escherichia coli* and *P. putida* harboring recombinant plasmids. Devanas *et al.*\(^{13}\) reported that the survival in soil of several strains of *E. coli* containing various size of plasmids depended on the host strain rather than on the type or size of the plasmid. Wang *et al.*\(^{11}\) reported that the survival of wild-type and recombinant *Streptomyces* released into soil was not influenced by the presence of plasmids. Orvos *et al.*\(^{14}\) observed that a population of a genetically engineered strain of *Erwinia carotovora*, including an antibiotic resistance gene in its chromosome, declined slightly faster than did the wild-type. We did not observe significant differences in survival between genetically engineered and wild-type *P. putida* strains in soil. From these studies, the survival of wild-types and GEMs was similar in the soil environment. Therefore, it seems probably that the survival of GEMs can be predicted from the results of survival tests of parent strains.

The factors affecting survival of genetically engineered and wild-type *P. putida* in soil microcosms were investigated. Trevors *et al.*\(^{19}\) observed that the survival of genetically engineered *Pseudomonas fluorescens* was different between loam soil and loamy sand. They noted that adverse biological factors such as competition, antagonism, predation, and niche exclusion as well as abiotic factors likely caused the differential survival in the two soils. Ramos *et al.*\(^{15}\) noted that the survival of genetically engineered *P. putida* strain harboring a recombinant TOL plasmid depended on soil type. However, they found no direct correlation between survival of the strain and soil type, or between organic content, C/N ratio or calcium content of the soil. Our study also indicates that the survival of genetically engineered and wild-type *P. putida* strains was different in andosol and sand microcosms (Fig. 2). This suggests the importance of determining the fate of GEMs in site soil before release.

We found no reports on the effects of water content on the survival of GEMs, while there were some reports about nonrecombinant strains. Regarding *Rhizobium leguminosarum* Tn5 mutants introduced into soil, it was observed that differences in water content before inoculation did not influence the population of *R. leguminosarum*.\(^5\) Postoma and van Veen\(^7\) also noted that nonrecombinant rhizobial numbers in sterile soil were not affected or decreased only slightly when water content increased. In contrast, Dupler and Baker\(^7\) reported the significantly greater survival of nonrecombinant *P. putida* at higher water content levels. We observed that the survival of wild-type and genetically engineered *P. putida* was affected by the water content of andosol (Fig. 2). It would appear that the characteristics of the parent strain were the important factors with respect
to the effect of water content on the survival of GEMs.

The reasons for the rapid decrease of genetically engineered and wild-type *P. putida* were discussed as follows. As the correlation between water content and protozoan predation, Vargas and Hattori suggested that the low soil water content might be a factor preventing the dispersion of protozoan cells among soil aggregates. It is also known that such predators as protozoa are more active with higher soil water content. Therefore it seems probable that the decrease of genetically engineered and wild-type *P. putida* strains in low soil water content is not caused by protozoan predation but physiological characteristics of the parent strains. Senoo et al. indicated the survival of the indigenous *Sphingomonas paucimobilis* was better than that of the inoculated *S. paucimobilis*. They suggested that the indigenous *S. paucimobilis* were located in micro-pores in the soil. The bacteria living in "the outer-part" of a soil crumb are more directly exposed to influences of environmental conditions than the bacteria in "the inner-part". It would appear that the introduced *P. putida* strains could not inhabit the inner-part of soil, which might be one of the important factors for the rapid decrease in low soil water content. On the other hand, Zechman and Casida noted that rapid death of nonrecombinant *P. aeruginosa* began at approximately 1 week of incubation, when natural soil containing *P. aeruginosa* cells was allowed to dry slowly. However the cells moderately decreased when soil was not dried. It may be necessary to determine the fate of GEMs under the conditions of wet-dry cycles in soil microcosms, before intentional release.

It is very important to evaluate the impact on indigenous microbial communities before the introduction of GEMs. In our study the densities of total indigenous bacteria and fungi were not changed by the inoculation of genetically engineered or wild-type *P. putida* strains in sand and andosol (Figs. 3 and 5). Neither were differences observed in the bacterial and fungal densities between the inoculated and noninoculated microcosms at various levels of water content of andosol (Figs. 4 and 6). Further, neither strain affected the indigenous bacterial density in aquatic microcosms. From that it appears that neither genetically engineered nor wild-type *P. putida* strains affect the population of indigenous microorganisms in the water and soil environment. Orvos et al. also noted that the effects of genetically engineered and wild-type *E. carotovora* strains on indigenous soil bacteria were not statistically significant. Genetically engineered *Pseudomonas* sp. did not measurably influence ecosystem parameters such as photosynthesis, densities of selected heterotrophic bacteria, thymidine incorporation rate, and oxygen microgradients in aquatic sediment microcosms. Jones et al. reported that five GEMs and their wild-type strains in soil showed no consistent and significant effect on the kinetics of nitrogen transformation. However, recombinant *Streptomyces lividans* significantly affected the short-term rate of soil organic carbon turnover. Armstrong et al. reported that the total epiphytic population increased approximately fourfold after 2 days, while genetically engineered *Pseudomonas cepacia* decreased to 2–30% of their initial numbers. Scanferlato et al. also reported that the total bacterial density was significantly increased by the introduction of genetically engineered *E. carotovora* strains for 16 days at a rate of 10⁴ CFU ml⁻¹ in an aquatic microcosm. As mentioned above, there were different effects of GEMs on the ecosystem parameters. Therefore, it would be necessary to evaluate the impact on the environment for each GEM prior to intentional release. In addition, much more research is required to assess the impact of GEMs on ecosystem parameters, and so to reflect essential characteristics of the natural environment.

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References