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Survival of *Pseudomonas putida* KT2440 in soil and in the rhizosphere of plants under greenhouse and environmental conditions

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Abstract

Pseudomonas putida KT2440 is a root colonizer of potential interest for the rhizoremediation of pollutants and the biological control of pests. The short- and long-term survival of this strain, as well as the possible effects of its introduction on different populations of indigenous soil bacteria, were tested in soil under greenhouse and field conditions. The greenhouse studies showed that inoculated *P. putida* KT2440 was able to establish itself after 3 d in nonvegetated soils at a density of $8 \pm 2 \times 10^3$ CFU g^{-1} soil. The introduction of this strain had no significant effect on the number of several soil bacteria including those that were resistant to tetracycline; those that utilized *p*-hydroxyphenylacetic acid as the sole C-source, and total fluorescent pseudomonads. In four independent field assays in nonplanted soils, the numbers of *P. putida* KT2440 decreased during 50 d from an initial density of 1×10^6 CFU g^{-1} soil to approximately $2 \pm 1 \times 10^2$ CFU g^{-1} soil. Thereafter, the number of cells was below detection limits (i.e. $< 10^2$ CFU g^{-1} soil), although they were still present because they could be recovered using selective enrichment from the soil for up to 200 d after the beginning of the experiment. This suggested that *P. putida* was maintained at a low cell density long after inoculation. In contrast, when *P. putida* KT2440 was introduced in the soil as a coating of corn (*Zea mays*) or broad bean (*Vicia faba*) seeds, the bacteria established at high cell densities in the rhizosphere (10^4–10^5 CFU g^{-1} soil in corn; 10^6–10^7 CFU g^{-1} soil in broad beans) during the growth of the crops over 12 to 16 weeks. The numbers of *P. putida* in the bulk soil after 2 weeks were 1 to 2 orders of magnitude below those in the rhizosphere. During the field assays, the population of *p*-hydroxyphenylacetic acid users was also monitored in the rhizosphere and the bulk soil. No significant seasonal variations were found.

Keywords: Bacterial survival; Field test; *lux* genes; *Pseudomonas*; Root colonization

1. Introduction

Bacteria of the genus *Pseudomonas* are ubiquitous and have been isolated from many habitats, including different soil types, fresh and marine waters, plant leaves, plant roots, animal skin and animal tissues (Galli et al., 1992). The ability of pseudomonads to colonize a wide variety of habitats resides not only in their capacity to adhere to solid particles (Duque et al., 1993) and their motility, but also in their metabolic versatility, which allows them to use many natural and xenobiotic compounds as C-, N-, S- and P-sources

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(Leisinger, 1983; Chaudlhry and Chapalamadugu, 1991; Dawson and Chang, 1992; Murooka and Imanaka, 1994; Ramos et al., 1994).

Pseudomonas putida mt-2 is a soil isolate which utilises toluene, xylenes and alkylbenzoates as sole C-sources because it possesses the TOL plasmid, pWW0 (Nakazawa and Yokota, 1973; Williams and Murray, 1974). The restriction-deficient derivative of this strain, *P. putida* KT2440 (Franklin et al., 1981), has been the subject of many studies aimed at elucidating specific aspects of the catabolic pathways encoded by the TOL plasmid and different aspects of its physiology, biochemistry and genetics (Ramos et al., 1991, 1994, 1997). This strain can use a variety of compounds as C- and N-sources, it can stably maintain and express heterologous genes, and is able to colonize efficiently different soils (Ramos et al., 1991, 1994; Duque et al., 1992, 1993; Nusslein et al., 1992). These properties make this strain of interest for the development of recombinant strains that express heterologous segments of catabolic pathways that enable them to degrade recalcitrant pollutants, such as alkyl-, sulfur- and chlorosubstituted aromatics (Ramos et al., 1991). It has been hypothesized that recombinant derivatives of this strain, and others similar to it, can be used as biopesticides and in phytoremediation (Compeau et al., 1988; Ramos et al., 1991, 1994; Haggblom, 1992; Ronchel et al., 1995; Thompson et al., 1995). The successful use of the derivatives of *P. putida* mt-2 for specific environmental applications requires that these strains survive and colonize the selected niches under field conditions and that they express the target genes in situ (De Weger et al., 1987; Drahos et al., 1988; Dwyer et al., 1988; Delgado et al., 1992; Simons et al., 1996; Benizri et al., 1997).

We have determined the short- and long-term survival of derivatives of *P. putida* mt-2 in soil under greenhouse and field conditions, as well as the possible effects of the introduction of the derivatives of this strain on natural communities of soil bacteria. The results show that derivatives of *P. putida* mt-2 survive best in the rhizosphere.

2. Materials and methods

2.1. Bacterial strains, culture media and growth conditions

Derivatives of *P. putida* mt-2 were marked with antibiotic resistance or *lux* genes to facilitate their tracking in soil (Franklin et al., 1981; Prosser et al., 1996). *P. putida* KT2440 is an *hsdR* mutant deficient in DNA restriction enzymes (Franklin et al., 1981). *P. putida* KT2442 is a spontaneous rifampicin-resistant derivative of *P. putida* KT2440 (Franklin et al., 1981). *P. putida* S1B1 is a kanamycin-resistant Lux^+ derivative of *P. putida* KT2442, which was obtained after random mutagenesis with a mini-Tn*5*-*luxAB* transposon (Ramos, unpublished). Growth rates of *P. putida* strains KT2440, KT2442 and S1B1 were almost identical in LB medium (doubling time was 42 ± 4 min [$n = 6$]), in minimal medium M8 with proline as the sole C- and N-source (doubling time was 108 ± 7 min [$n = 4$]), and in minimal medium M9 with glucose or benzoate as C-sources (doubling time in both cases was 90 ± 8 min [$n = 5$]). All strains were motile and produced halos of similar size in semisolid LB agar (0.3% w/v) plates, a property essential for root colonization (Duque et al., 1992; Reniero et al., unpublished).

P. putida EEZ201, *P. chlororaphis* EEZ204, and *P. fluorescens* EEZ206 were isolated from agricultural soil on King's agar plates. *P. putida* EEZ201 was resistant to 30 μg ml^{-1} of rifampicin and chloramphenicol; *P. chlororaphis* EEZ204 and *P. fluorescens* EEZ205 were resistant to 30 μg ml^{-1} of nalidixic acid and chloramphenicol (Molina, L., unpublished).

Bacteria were routinely grown on modified M9 minimal medium supplemented with 0.5% (w/v) glucose, 10 mM *p*-hydroxyphenylacetic acid or 10 mM benzoic acid (Maniatis et al., 1982). When proline (10 mM) was used as the sole C- and N-source, ammonium was omitted from the M9 minimal medium. Solid medium was made by supplementation of liquid medium with 1.5% (w/v) agar.

The selective solid growth medium for *P. putida* KT2442 and EEZ201 was M9 minimal medium with 10 mM benzoate as the sole C-source and 30 μg rifampicin ml^{-1}. No indigenous soil bacteria were able to grow on this medium when 10 g of soil was suspended in 90 ml of phosphate buffer (pH 7.0), and 0.2 ml of the suspension was spread on the selective medium. For the growth of *P. putida* S1B1, the medium also contained 50 μg kanamycin ml^{-1}. *P. chlororaphis* EEZ204 and *P. fluorescens* EEZ205 were selected on M9 minimal medium plates containing 10 mM benzoate as the sole C-source and 30 μg of nalidixic acid ml^{-1}. To enumerate indigenous soil bacteria tolerant to tetracycline, solid LB medium (Maniatis et al., 1982) supplemented with 15 μg of tetracycline ml^{-1} was used.

2.2. Measurements of light emission

Luciferase activity of intact cells of *P. putida* S1B1 was determined as described by Prosser et al. (1996). To 1 ml of a cell suspension of *P. putida* S1B1 with a turbidity of 0.1 at 660 nm, 0.4 ml of 0.01% (v/v) *n*-decylaldehyde was added, and light emission was determined for 1 min with a LKB 1250 luminometer.

2.3. Seed coating and soil inoculation

In all greenhouse and field assays, a natural soil whose content was 6% (w/w) $CaCO_3$ and 0.5% (w/w) organic matter was used. For these assays cells were grown on M9 minimal medium containing 10 mM benzoic acid, harvested by centrifugation when the turbidity of the culture at 660 nm was between 1 and 1.5, washed twice in 50 mM phosphate buffer (pH 7.2) and 100 mM NaCl, and resuspended in the same buffer to about 1×10^8 CFU ml^{-1}. When bacteria were introduced to nonplanted soils, pots (10 cm dia) with 1 kg of soil were used. The bacterial inoculum was added to the pots and mixed thoroughly to a density of $2 \pm 1 \times 10^6$ CFU g^{-1} soil. In planted soils in the greenhouse, 40 cm diameter pots each containing 40 kg of soil were used. The water content of the soil was adjusted to 30–50% of the field capacity. The pots were kept in a greenhouse at 18 to 22°C with natural light–dark cycles. One hundred seeds of corn (*Zea mays*) or broad bean (*Vicia faba*) were soaked in 200 ml of the bacterial cell suspension containing about $5 \pm 1 \times 10^8$ CFU ml^{-1} for 30 min with gentle shaking at room temperature. The seeds were washed with water and sown at a depth of 5 cm in pots in the greenhouse. The number of bacteria per seed was determined within 2 h of planting and estimated to be about $2 \pm 1 \times 10^6$ CFU for corn and $8 \pm 2 \times 10^7$ CFU for broad bean.

2.4. Design of the environmental field release

P. putida KT2442 was used in field releases. Growth of the cells and coating of seeds was as above. Seeds coated with this strain were planted at a depth of 5 cm in parallel rows in a fenced 200 m^2 site located within the 2000 m^2 experimental area at the Estación Experimental del Zaidín of the Consejo Superior de Investigaciones Científicas, Granada, Spain. To minimize edge effects, seeds were separated from each other by 1 m. The entire release area was completely surrounded by a 2 m wide buffer zone where no cells of *P. putida* KT2442 were introduced.

There were four field releases, two in the winter (1996 and 1997), in which broad bean plants were used, and two in the summer (1997 and 1998), in which corn was the test plant.

2.5. Monitoring bacteria in the soil and in the rhizosphere

After germination, individual plants in duplicate were sampled after the appearance of the first true leaf, i.e., 11 d (corn) and 21 d (broad bean) after sowing. Whole plants were gently removed from the soil, and bacteria in the soil attached to the roots (the rhizosphere), and in the rest of the soil (bulk soil) were counted in triplicate. Ten grams of soil were placed in a 250 ml Erlenmeyer flask with 90 ml of M9 minimal medium without a C-source and shaken for 30 min on a Heidolph bench shaker at 250 strokes min^{-1}. Soil suspensions were then serially diluted (10-fold) in the above medium, and 0.1 ml aliquots were spread on selective media.

The strains tested were counted on selective minimal media (see above). In addition, the following microbial soil populations were usually examined: (i) Cells that exhibited resistance to tetracycline. The number of tetracycline-resistant bacteria in this soil were counted on LB agar medium containing 15 μg tetracycline ml^{-1}. (ii) Bacteria that were able to use *p*-hydroxyphenylacetic acid as the sole C-source. This population was stable throughout the year, and it represented the most abundant indigenous bacterial population in this soil capable of using of aromatic compounds and (iii) total fluorescent pseudomonads which were counted on King's agar medium (Smibert and Krieg, 1994).

Data were tested for statistical significance using the analysis of variance package included in Microsoft Excel 98.

2.6. Recombinant DNA techniques and phylogenetic reconstruction

The DNA encoding the 16S rRNA of different *Pseudomonas* strains was amplified by using the following oligonucleotides: 5′-AGAGTTTGAT(C or T (A or C)TGGCTCAG-3′ and 5′-CA(G or T)AAAGGAGGTGATCC-3′. DNA was sequenced on both strands by the dideoxy sequencing termination method, with specific 20-mer oligonucleotides to prime synthesis.

New 16S rRNA sequences were compared with the set of available 16S RNA primary structures (Maidak et al., 1999). Phylogenetic trees were constructed based on representative sequences of members of the gamma subclass of proteobacteria (Maidak et al., 1999).

3. Results and discussion

3.1. Short-term survival of P. putida S1B1 in soil, and its effect on indigenous bacterial populations

Derivatives of *P. putida* mt-2 can establish in the short-term in planted and unplanted soils (Duque et al., 1992, 1993; Ramos et al., 1994). However, the possible effects of these microorganisms on indigenous microorganisms have been overlooked. The influence, under controlled conditions in a greenhouse, of introducing $2 \pm 1 \times 10^5$ CFU of Lux^+ *P. putida* S1B1 g^{-1} soil on different populations of microorganisms in an

agricultural soil was tested. Fig. 1 shows the number of viable cells over time of several indigenous populations in the soil in the absence and in the presence of *P. putida* S1B1. The number of viable cells of the introduced S1B1 strain decreased from about $2 \pm 1 \times 10^5$ CFU g^{-1} soil to about $8 \pm 2 \times 10^3$ CFU g^{-1} soil in 30 d. Throughout the assay, the numbers of the indigenous populations examined remained relatively constant, regardless of the presence of *P. putida* S1B1; i.e. the number of tetracycline resistant bacteria and the number of bacteria that used *p*-hydroxyphenylacetic acid as the sole C-source was in the order of 8×10^3 and 8×10^5 CFU g^{-1} soil, respectively. The number of total fluorescent pseudomonads was around 10^5 CFU g^{-1} soil.

To study in detail the effect on a specific population, three colonies that differed in morphology and colour were selected from the King's agar plates. The strains were characterized taxonomically and identified by fatty acid analysis (Microbial Identification Systems, Inc.) as belonging to the genus *Pseudomonas*. The sequence of the gene encoding the 16S rRNA was determined and deposited in GenBank under accession number AF099802. These strains were identified as *P. putida* EEZ201, *P. fluorescens* EEZ205, and *P. chlororaphis* EEZ204. The three strains were able to use benzoic acid as the sole C-source and were Nal^R Cm^R Km^S. The pattern of antibiotic resistance can be used to distinguish these strains from the Rif^R Km^R *P. putida* S1B1 on selective medium. The numbers of Ben^+ (able to use benzoic acid as a C-source), Nal^R, Cm^R bacteria in the soil of the experimental area used in these assays were around 10^3 CFU g^{-1} soil. About $8 \pm 2 \times 10^5$ CFU g^{-1} soil of *P. putida* S1B1 and either *P. putida* EEZ201, *P. chlororaphis* EEZ204, or *P. fluorescens* EEZ205 were introduced in the soil, which was kept under greenhouse conditions. As a control, soils were also inoculated with only one of these microorganisms in the absence of *P. putida* S1B1. (Note that the number of Ben^+ Nal^R Cm^R cells introduced in the soil exceeded by three orders of magnitude the number of indigenous bacteria that exhibited this phenotype, and the number of indigenous microbes with this phenotype was considered of minor significance for counting bacteria.) The colonization of the soil by the introduced bacterial strains in each pot was followed on selective plates. *P. putida* S1B1, *P. chlororaphis* and *P. fluorescens* tended to establish in the soil at about $6 \pm 4 \times 10^4$ CFU g^{-1} soil when the incubation temperature was in the range of 18 to 22°C (Fig. 2). Similar results were obtained for *P. putida* EEZ201 (not shown). The difference in CFU g^{-1} soil for each strain (inoculated individually or in all possible permutations of pairs) was not significant, and therefore, it can be concluded that the pattern of establishment of each strain in the soil was independent of co-inoculation with the other strain. None of the four strains were able to establish in soils stored at 37°C. This is in agreement with previous observations regarding the behavior of *P. putida* in soils with regard to temperature (Ramos et al., 1991; Delgado et al., 1992).

In another series of assays, a soil high in organic matter (10% w/w) used for growing ornamental plants was inoculated with *P. putida* S1B1, *P. fluorescens*

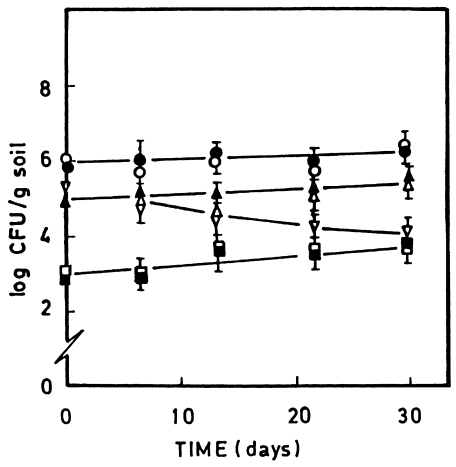

Fig. 1. Effect of the introduction of *P. putida* S1B1 on indigenous soil bacteria under greenhouse conditions. Six pots with 1 kg of soil each were used. Three pots were kept as control (open symbols); to the other pots, $2 \pm 1 \times 10^5$ CFU of *P. putida* g^{-1} soil (closed symbols) were added. At the indicated times, the number of bacteria that used *p*-hydroxyphenylacetic acid as a sole C-source (△), bacteria that were resistant to tetracycline (□), and fluorescent pseudomonads (○) were counted. *P. putida* S1B1 was also enumerated (▽) in the second series of pots. Error bars represent 1 standard deviation.

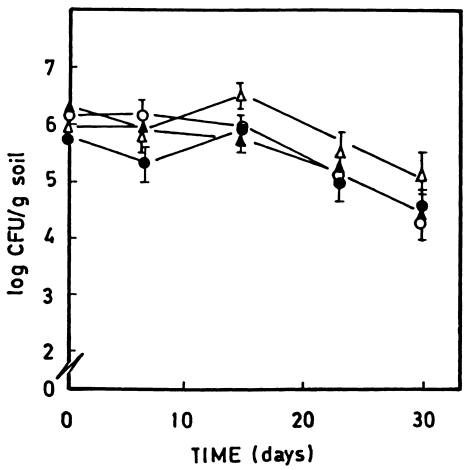

Fig. 2. Co-inoculation of *P. putida* S1B1 and *P. chlororaphis* EEZ204 in soil. Three pots were used: one received $2 \pm 1 \times 10^5$ CFU g^{-1} soil of *P. chlororaphis* (△), another received the same number of *P. putida* (○) and a third received *P. chlororaphis* (▲) and *P. putida* (●). At the indicated times, the number of viable cells were counted on selective medium as described in Materials and Methods. The assay was repeated three times. Error bars represent 1 standard deviation.

EEZ205 and *P. chlororaphis* EEZ204. These strains were introduced individually or in all possible permutations of pairs. Cell numbers were determined thereafter on selective media. The numbers of each strain established within 72 h of inoculation were about $3 \pm 1 \times 10^7$ CFU g^{-1} soil, and remained relatively constant during 30 d (not shown). These results indicated that the pattern of establishment of each strain was independent of co-inoculation with the other strains.

3.2. Long-term survival of P. putida S1B1 in unplanted soils under environmental conditions

This assay was carried out in pots maintained outdoors for about 200 d from February to August 1997. Uninoculated control pots and pots to which $2 \pm 1 \times 10^6$ CFU of *P. putida* S1B1 g^{-1} soil was added were used. The population of microorganisms that used *p*-hydroxyphenylacetic acid as the sole C-source, the numbers of microorganisms that formed colonies on King's agar (fluorescent *Pseudomonas*), and the numbers of *P. putida* S1B1 were determined. The numbers of indigenous microorganisms that degraded *p*-hydroxyphenylacetic acid ($8 \pm 2 \times 10^5$ CFU g^{-1} soil) and the numbers of fluorescent pseudomonads remained relatively constant with time ($9 \pm 1 \times 10^4$ CFU g^{-1} soil). In contrast, the numbers of *P. putida* S1B1 decreased to about 1×10^2 CFU g^{-1} soil in the first 10 d and remained at this value during the subsequent 40 d. Thereafter, their numbers were always below detection limits (i.e., less than 10^2 CFU g^{-1} soil). In cases in which S1B1 was below detection limits, 1 g of soil was suspended in 3 ml of LB medium supplemented with 30 μg rifampicin ml^{-1} and 50 μg kanamycin ml^{-1}. After 24 h of incubation at 30°C, bacteria were spread on the same medium. In all cases, luminiscent colonies were present, indicating that although the numbers of *P. putida* S1B1 were low in the soil, cells were still present and could be recovered after selective enrichment.

Our observation of the lack of effect on indigenous soil microorganisms when *P. putida* was introduced, is in contrast with the observations of Doyle et al. (1991) and Short et al. (1991). They showed that the introduction of *P. putida* PPO301 (pRO103) in a soil with 2,4-dichlorophenoxyacetate lead to the accumulation of 2,4-dichlorophenol and had a significant ecological effect on soil microbes.

3.3. Long-term survival of derivatives of P. putida mt-2 in the rhizosphere and surrounding bulk soil.

In the winter assays, colonization of broad been plant roots by *P. putida* KT2442 was determined. Throughout the two winter assays (November 1996 to February 1997 and November 1997 to February 1998), the numbers of *P. putida* KT2442 were about $4 \pm 2 \times 10^6$ CFU g^{-1} rhizosphere soil. In the rhizosphere of the same plants, the numbers of indigenous bacteria able to use *p*-hydroxyphenylacetic acid as the sole C-source were in the range of 1×10^7 to 1×10^8 CFU g^{-1} rhizosphere soil (Fig. 3). The population of microorganisms that used *p*-hydroxyphenylacetic acid as the sole C-source was also in the range of 1×10^7 to 1×10^8 CFU g^{-1} rhizosphere soil of uninoculated plants (Fig. 3). In bulk soil the numbers of indigenous bacteria and *P. putida* S1B1, when this strain was used, were 1 to 2 orders of magnitude less than in the rhizosphere (not shown).

Similar field trials were performed in the summer (May to August 1997 and 1998), with corn plants. In the rhizosphere soil of plants whose seeds had been coated with *P. putida* KT2442, the cell density of this strain varied between 5×10^3 and 6×10^5 CFU g^{-1} soil; and the density of the indigenous community that was able to use *p*-hydroxyphenylacetic acid as the sole C-source was approximately 10^7 CFU g^{-1} soil. As with broad bean, the level of establishment of the population of KT2442 and the indigenous community that used *p*-hydroxyphenylacetic acid were both about 1 order of magnitude lower in the bulk soil (Fig. 4).

These results showed that the density of the population of *P. putida* KT2442 in soil was less in the summer than in the winter. The establishment of *P. putida* KT2442 at higher numbers in the rhizosphere of the broad bean plants than in corn, could be due to biotic factors. On one hand, the number of CFU that coated broad bean seeds was higher than the number of CFU that coated corn seeds. In addition, the exudates of

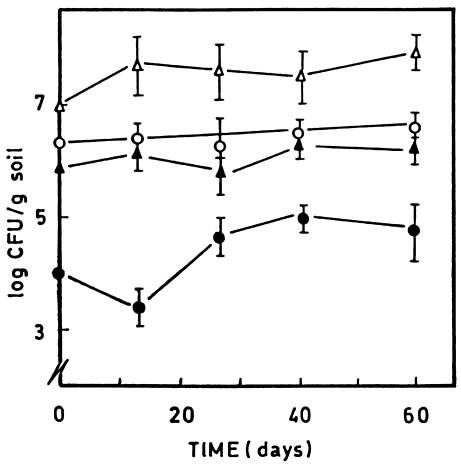

Fig. 3. Establishment of *P. putida* (○) and the indigenous bacterial population that used *p*-hydroxyphenylacetic acid (△) in the rhizosphere (open symbols) and bulk soil (closed symbols) of *V. faba* plants in a field release in the winter of 1997. Samples were taken at the indicated times, and the values given are the average of duplicate counts of three samples. Day 0 corresponds to the appearance of the first true leaf (see Materials and Methods). Error bars represent 1 standard deviation.

broad bean plants allowed for the slightly better growth of this strain (Vílchez and Ramos, unpubl.). These two biotic factors may, in themselves, explain the better colonization of the rhizosphere of broad bean plants. However, abiotic factors, such as temperature, could also influence the establishment in rhizosphere, as survival of this strain at relatively high temperatures (between 24 and 45°C in the summer during the day) is worse than at versus milder temperatures as those registered in the winter (between 3 and 18°C during the day).

To monitor the possible effects of the release of *P. putida* KT2440 on natural bacterial populations, microorganisms that used *p*-hydroxyphenylacetic acid as a sole C-source, a bacterial community that was relatively stable in the soil throughout the year, were enumerated. The introduction to soil of *P. putida* KT2442 had no significant effect on the survival of this natural community, neither in greenhouse assays nor in field releases. These results indicated that *P. putida* KT2442 was a good root colonizer, although it did not out-compete indigenous microbes.

Survival of derivatives of *P. putida* mt-2 was better in the rhizosphere than in bulk soil (Figs. 3 and 4). This has also been observed with different strains of *P. syringae*, several fluorescent pseudomonads (Goldstein et al., 1985; De Weger et al., 1987; Compeau et al., 1988; Drahos et al., 1988; Dwyer et al., 1988; Lindow and Panapolous, 1988; Laville et al., 1992, 1998; Thompson et al., 1995; Simons et al., 1996; Benizri et al., 1997; Nautiyal, 1997), rhizobacteria (Geels et al., 1986; Beringer and Barth, 1988), and enterobacteria (Rattray et al., 1995).

The potential use of derivatives of *P. putida* mt-2 to augment or initiate in situ degradation of pollutants and as a biological control agent depends not only on the successful survival of the microorganisms, but also on the stability and expression of the genetic information carried by the strain (Ramos et al., 1991; Duque et al., 1992, 1993). Although this was not specifically addressed in our study, it has been reported that the catabolic pathways of the TOL plasmid of this strain are expressed in bacteria introduced in soil because mineralization of ^{14}C-labeled substrates was observed (Duque et al., 1993). In our study, all S1B1 cells maintained their luminescent character after selective recovery on benzoate as a C-source, suggesting that the genetic information borne by *Pseudomonas* S1B1 was stably maintained in soils.

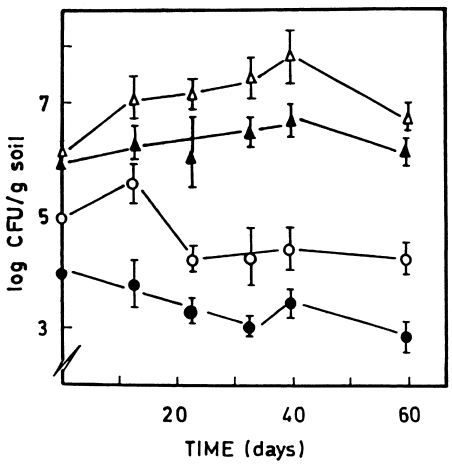

Fig. 4. Establishment of *P. putida* and the indigenous population that used *p*-hydroxyphenylacetic acid in the rhizosphere and bulk soil of *Z. mays* plants in a field assay in the summer of 1997. Conditions and symbols are as in the legend for Fig. 3.

Acknowledgements

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References

Benizri, E., Schoeny, A., Picard, C., Courtade, A., Gukert, A., 1997. External and internal root colonization of maize by two *Pseudomonas* strains: enumeration by enzymelinked immunoabsorbent assay. Current Microbiology 34, 297–302.

Beringer, J.E., Barth, M.J., 1988. The survival and persistence of genetically-engineered microorganisms. In: Sussman, M., Collins, C.H., Skinner, F.A., Stewart-Tull, D.E. (Eds.), The Release of Genetically-Engineered Microorganisms. Academic Press, London, pp. 19–46.

Chaudlhry, G.S., Chapalamadugu, S., 1991. Biodegradation of halogenated organic compounds. Microbiological Reviews 55, 45–48.

Compeau, G., Al-Achi, B.J., Platsouka, E., Levy, S.B., 1988. Survival of rifampicin-resistant mutants of *Pseudomonas fluorescens* and *Pseudomonas putida* in soil systems. Applied and Environmental Microbiology 84, 2432–2438.

Dawson, T.D., Chang, F.H., 1992. Screening test of the biodegradative capability of a new strain of *Pseudomonas gladioli* (BSU 45124) on some xenobiotic organics. Bulletin of Environmental Contamination and Toxicology 49, 10–17.

Delgado, A., Duque, E., Ramos, J.L., 1992. Behavior in agricultural soils of a recombinant *Pseudomonas* bacterium that simultaneously degrades alkyl and haloaromatics. Microbial Releases 1, 23–28.

De Weger, L.A., van der Vlugt, C.I., Wijfjes, A.H., Bakker, P.A., Schippers, B., Lugtenberg, B., 1987. Flagella of a plant-growth-stimulating *Pseudomonas fluorescens* strain are required for colonization of potato roots. Journal of Bacteriology 169, 2769–2773.

Doyle, J.D., Short, K.A., Stotzky, G., King, R.J., Seidler, R.J., Olsen, R.H., 1991. Ecologically significant effects of *Pseudomonas putida* PPO301(pRO103), genetically engineered to degrade 2,4,-dichlorophenoxyacetate, on microbial populations and processes in soil. Canadian Journal of Microbiology 37, 682–691.

Drahos, D.J., Barry, G.F., Hemming, B.C., Brandt, E.J., Skipper, H.D., Kline, E.L., Kluepfel, D.A., Hughes, T.A., Gooden, D.T., 1988. Prerelease testing procedures: US field test of a *lacZY*-engineered soil bacterium. In: Sussman, M., Collins, C.H., Skinner, F.A., Stewart-Tull, D.E. (Eds.), The Release of Genetically-

Engineered Microorganisms. Academic Press, London, pp. 181–191.

Duque, E., Ramos-González, M.I., Delgado, A., Contreras, A., Molin, S., Ramos, J.L., 1992. Genetically engineered *Pseudomonas* strains for mineralization of aromatics: survival, performance, gene transfer, and biological containment. In: Galli, E., Silver, S., Witholt, B. (Eds.), *Pseudomonas*: Molecular Biology and Biotechnology. American Society for Microbiology, Washington, pp. 429–437.

Duque, E., Marqués, S., Ramos, J.L., 1993. Mineralization of *p*-methyl-^{14}C-benzoate in soils by *Pseudomonas putida* (pWW0). Microbial Releases 2, 175–177.

Dwyer, D.F., Rojo, F., Timmis, K.N., 1988. Fate and behaviour in an activated sludge microcosm of a genetically-engineered microorganism designed to degrade substituted aromatic compounds. In: Sussman, M., Collins, C.H., Skinner, F.A., Stewart-Tull, D.E. (Eds.), The Release of Genetically-Engineered Microorganisms. Academic Press, London, pp. 77–88.

Franklin, F.C.H., Bagdasarian, M., Bagdasarian, M.M., Timmis, K.N., 1981. Molecular and functional analysis of the TOL plasmid pWW0 from *Pseudomonas putida* and cloning of genes for the entire regulated aromatic ring *meta*-cleavage pathway. Proceedings of the National Academy of Sciences USA 78, 7458–7462.

Galli, E., Silver, S., Wiltholt, B. (Eds.), 1992. *Pseudomonas*: Molecular Biology and Biotechnology. American Society for Microbiology, Washington.

Geels, F.P., Lamers, J.G., Hoekstra, O., Schippers, B., 1986. Potato plant response to seed tuber bacterization in the field in various rotations. Netherland Journal of Plant Pathology 92, 257–272.

Goldstein, M.I., Mallory, M., Alexander, M., 1985. Reasons for possible failure of inoculation to enhance biodegradation. Applied and Environmental Microbiology 50, 977–983.

Haggblom, M.M., 1992. Microbial breakdown of halogenated aromatic pesticides and related compounds. FEMS Microbiology Reviews 9, 29–71.

Laville, J., Blumer, C., von Schroetter, Ch., Gaia, V., Défago, G., Keel, Ch., Haas, D., 1998. Characterization of the *hcnABC* gene cluster encoding hydrogen cyanide synthase and anaerobic regulation by ANR in the strictly aerobic biocontrol agent *Pseudomonas fluorescens* CHA0. Journal of Bacteriology 180, 3187–3196.

Laville, J., Voisard, C., Keel, C., Maurhofer, M., Défago, G., Haas, D., 1992. Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root rot of tobacco. Proceedings of the National Academy of Science USA 89, 1562–1566.

Leisinger, T., 1983. Microorganisms and xenobiotic compounds. Experientia 39, 1183–1191.

Lindow, S.E., Panapolous, N.J., 1988. Field test of recombinant *Ice*$^-$ *Pseudomonas syringae* for biological frost control in potato. In: Sussman, M., Colling, C.H., Skinner, F.A., Stewart-Tull, D.E. (Eds.), The Release of Genetically-Engineered Microorganisms. Academic Press, London, pp. 121–138.

Maidak, B.L., Cole, J.R., Parker, Ch.T., Garrity, G.M., Larssen, N., Li, T.G., Lilbuurn, M.J., McCaughey, G.J., Olsen, R., Overbeek, R., Pramanik, S., Schmidt, T.M., Tiedje, J.M., Woese, C.R., 1999. A new version of the RDP (Ribosomal Data Project). Nucleic Acids Research 27, 171–173.

Maniatis, T., Fritsch, E.F., Sambrook, J., 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Murooka, Y., Imanaka, T., 1994. Recombinant microbes for industrial and agricultural applications. Marcel Dekker, New York, pp. 723–837.

Nakazawa, T., Yokota, T., 1973. Benzoate metabolism in *Pseudomonas putida* (arvilla) mt-2: demonstration of two benzoate pathways. Journal of Bacteriology 115, 262–267.

Nautiyal, C.S., 1997. A method for selection and characterization of rhizosphere-competent bacteria of chickpea. Current Microbiology 34, 12–17.

Nusslein, K., Maris, D., Timmis, K.N., Dwyer, D.F., 1992. Expression and transfer of engineered catabolic pathways harbored by *Pseudomonas* spp. introduced into activated sludge microcosms. Applied and Environmental Microbiology 58, 3380–3386.

Prosser, J.I., Killham, K., Glover, L.A., Rattray, E.A., 1996. Luminescencebased systems for detection of bacteria in the environment. Critical Reviews in Biotechnology 16, 157–183.

Ramos, J.L., Díaz, E., Dowling, D., de Lorenzo, V., Molin, S., O'Gara, F., Ramos, C., Timmis, K.N., 1994. The behavior of bacteria designed for biodegradation. Bio/Technology 12, 1349–1356.

Ramos, J.L., Duque, E., Ramos-González, M.I., 1991. Survival in agricultural soils of an herbicide-resistant *Pseudomonas putida* bearing a recombinant TOL plasmid. Applied and Environmental Microbiology 57, 260–261.

Ramos, J.L., Marqués, S., Timmis, K.N., 1997. Transcriptional control of the *Pseudomonas* TOL plasmid catabolic operons is achieved through an interplay of host factors and plasmid-encoded regulators. Annual Reviews of Microbiology 51, 341–373.

Rattray, E.A., Prosser, J.L., Glover, L.A., Killham, K., 1995. Characterization of rhizosphere colonization by luminescent *Enterobacter cloacae* at the population and single-cell levels. Applied and Environmental Microbiology 61, 2950–2957.

Ronchel, M.C., Ramos, C., Jensen, L.B., Molin, S., Ramos, J.L., 1995. Construction and behavior of biologically contained bacteria for environmental applications in bioremediation. Applied and Environmental Microbiology 61, 2990–2994.

Short, K.A., Doyle, J.D., King, R.J., Seidler, R.J., Stotzky, G., Olsen, R.H., 1991. Effect of 2,4-dichlorophenol, a metabolite of a genetically engineered bacterium, and 2,4-dichlorophenoxyacetate on some microorganism-mediated ecological processes in soil. Applied and Environmental Microbiology 57, 412–418.

Simons, M., van der Bij, A.J., Brand, I., de Weger, L.A., Wijffelman, C.A., Lugtenberg, B.J., 1996. Gnotobiotic system for studying rhizosphere colonization by plant growth-promoting *Pseudomonas* bacteria. Molecular Plant/Microbes Interactions 9, 600–607.

Smibert, R.M., Krieg, N.R., 1994. Phenotypic characterization. In: Gerhardt, P., Murray, R.G.F., Woods, W.A., Krieg, N.R. (Eds.), Methods for general molecular bacteriology. ASM, Washington, pp. 611–651.

Thompson, I.P., Ellis, R.J., Bailey, M.J., 1995. Autoecology of a genetically modified fluorescent pseudomonad on sugar beet. FEMS Microbiology Ecology 17, 1–14.

Williams, P.A., Murray, K., 1974. Metabolism of benzoate and the methylbenzoates by *Pseudomonas putida* (arvilla) mt2: evidence for the existence of a TOL plasmid. Journal of Bacteriology 120, 416–423.