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Biodegradation of phenoxyacetic acid in soil by *Pseudomonas putida* PP0301(pR0103), a constitutive degrader of 2,4-dichlorophenoxyacetate

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Abstract

The efficacy of using genetically engineered microbes (GEMs) to degrade recalcitrant environmental toxicants was demonstrated by the application of *Pseudomonas putida* PP0301(pR0103) to an Oregon agricultural soil amended with 500 μg/g of a model xenobiotic, phenoxyacetic acid (PAA). *P. putida* PP0301(pR0103) is a constitutive degrader of 2,4-dichlorophenoxyacetate (2,4-D) and is also active on the non-inducing substrate, PAA. PAA is the parental compound of 2,4-dichlorophenoxyacetic acid (2,4-D) and whilst the indigenous soil microbiota degraded 500 μg/g 2,4-D to less than 10 μg/g, PAA degradation was insignificant during a 40-day period. No significant degradation of PAA occurred in soil inoculated with the parental strain *P. putida* PP0301 or the inducible 2,4-D degrader *P. putida* PP0301(pR0101). Moreover, co-amendment of soil with 2,4-D and PAA induced the microbiota to degrade 2,4-D; PAA was not degraded. *P. putida* PP0301-(pR0103) mineralized 500-μg/g PAA to trace levels within 13 days and relieved phytotoxicity of PAA to *Raphanus sativus* (radish) seeds with 100% germination in the presence of the GEM and 7% germination in its absence. In unamended soil, survival of the plasmid-free parental strain *P. putida* PP0301 was similar to the survival of the GEM strain *P. putida* PP0301(pR0103). However, in PAA amended soil, survival of the parent strain was over 10 000-fold lower (< 3 colony forming units per gram of soil) than survival of the GEM strain after 39 days.

Keywords: biodegradation, soil, PAA, 2,4-D, GEM, pseudomonad

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Introduction

Bacterial degradation of phenoxyacetic acid (PAA) has been described in liquid culture studies with mutants of *Alcaligenes eutrophus* JMP134(pJP4) (Don & Pemberton 1981; Pemberton *et al.* 1979) and recently in the genetically altered *P. putida* PP0300(pR0103) strain (Harker *et al.* 1989). Plasmid pR0103 carries most of the genes for degradation of 2,4-dichlorophenoxyacetic acid (2,4D) and 3-chlorobenzoate (Don *et al.* 1985) and is deregulated for TFD degradation due to a deletion of a negative regulatory gene, *tfd*R (Kaphammer *et al.* 1990). The *tfd*R gene negatively regulates expression of the *tfd*A gene product, tfd monoxygenase (Don *et al.* 1985; Streber *et al.* 1987; Harker *et al.* 1989). The deregulated *tfd* monooxygenase recognizes the non-inducing substrate PAA and converts PAA to phenol. *P. putida* strains PP0300(pR0103) and PP0301(pR0103) express a phenol hydroxylase and use PAA as a sole source of carbon (Harker *et al.* 1989; King *et al.* 1991).

The use of *P. putida* PP0301(pR0103) to degrade 2,4-D in different soil types has previously been demonstrated (Short *et al.* 1990, 1991; Doyle *et al.* 1991). In the present study, the ability of *P. putida* PP0301(pR0103) to degrade PAA was tested in soil. PAA is not directly used as an agricultural herbicide. Therefore, the purpose of this research was to demonstrate the potential use of GEMs to remediate chemically contaminated soil, and to provide

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further information on the environmental fate and activity of GEMs.

P. putida PP0301, the inducible 2,4-D degrader *P. putida* PP0301(pR0101), and the constitutive 2,4-D degrader *P. putida* PP0301(pR0103) were added to a PAA-amended agricultural soil with a natural bacterial population capable of degrading 2,4-D (Short *et al.* 1990). PAA was recalcitrant to native 2,4-D degraders and to the inducible 2,4-D degrader *P. putida* PP0301(pR0101). In addition, although PAA has been reported to be non-herbicidal (Cole & Loughman, 1982), we present evidence that PAA is phytotoxic and toxic to bacteria.

Materials and methods

Bacterial cultures

P. putida strains PP0301, PP0301(pR0101) and PP0301-(pR0103) are resistant to nalidixic acid (nal) (500 μg/ml). *P. putida* PP0301 was derived from *P. putida* PP0300 (ATCC17514) by sequential transfer of naturally resistant subclones onto increasing concentrations of nal-supplemented medium (unpublished data). Plasmids pR0101 and pR0103 were derived from plasmid pJP4 (Don & Pemberton 1981) as described previously (Harker *et al.* 1989), and in addition to carrying the genes for degradation of 2,4-D, they encode the degradation of 3-chlorobenzoate, resistance to mercury (Hg) and tetracycline (tet) (located on transposon Tn*1712*) (Altenucher *et al.* 1983). Plasmid pR0103 was derived from plasmid pR0101 (Harker *et al.* 1989) by a serendipitous deletion of the *trd*R regulatory gene (Kaphammer *et al.* 1990). In minimal phosphate-buffered medium (Stanier *et al.* 1966), PP0301(pR0103) used PAA as a sole source of carbon (King *et al.* 1991).

Bacterial culturing and preparation of soil

To initiate each experiment, frozen (−80°C) stock cultures were plated on the appropriate selective medium. *P. putida* PP0301 was plated onto nutrient medium (Olsen & Shipley 1973) supplemented with 500-μg/ml nal (Sigma Chemical Co., St Louis, Miss.). *P. putida* strains PP0301-(pR0101) and PP0301(pR0103) were plated onto brain-heart infusion medium (Difco Chemical Co., St Louis) supplemented with 25-μg/ml $HgCl_2$ (Aldrich Chemical Co., Milwaukee, Wisc.). Cultures were incubated overnight at 30°C. The next day, several isolated colonies were used to inoculate 125 ml of nutrient broth in a 250-ml Erlenmeyer flask. Cultures were shaken overnight at 200 rpm *g* at 30°C. Cells were pelleted by centrifugation at 8000 *g* for 10 min and washed twice in 200 ml of 50-mM K_2HPO_4/NaH_2PO_4 buffer (pH 6.8). The final cell pellet was resuspended in 30 ml of buffer prior to topical application of suspended cells to the soil surface.

Table 1 Chemical characteristics of the soil

Variable	Measured value
pH	6.1
Total organic carbon (%)	4.41
Total N (%)	0.19
P (μg/g)	21
K (μg/g)	390
NO_3^- (μg/g)	41.9
NH_4 (μg/g)	10.9
Cation exchange capacity (mequiv/kg)	198
Ca (mequiv/kg)	132
Mg (mequiv/kg)	19.5

Soil characteristics, preparation and incubation

The soil used in this study was collected from a farm in the foothills of the Oregon Coastal Mountain Range in the Willamette Valley, Oregon, USA. The soil is classified as a Veneta series, characterized by a 48-cm surface layer of Veneta silt loam and silty clay loam. The chemical characteristics, as determined by the Oregon State University Soil Testing Laboratory, are shown in Table 1. Prior to inoculation or addition of liquid, the soil was first passed through a 2-mm-mesh-size seive. The first soil group—uninoculated soil—was wetted with buffer and amended with PAA or PAA and 2,4-D, mixed and brought to −33-kPa water tension (Orchard & Cook 1983). The second soil group—unamended and inoculated soil—was inoculated with washed suspensions of *P. putida* strains PP0301, PP0301(pR0101) or PP0301(pR0103) and adjusted to −33-kPa water tension (Orchard & Cook 1983). A third soil group—amended and inoculated soil—was amended with PAA, inoculated with *P. putida* strains PP0301, PP0301(pR0101) or PP0301(pR0103), mixed, and adjusted to −33-kPa water tension. PAA was added at 500 μg/g of soil (free acid, 99% pure, Sigma Chem. Co.) as a dry powder or as a 1% solution in water (pH 7.0). 2,4-D was added at 500 μg/g (Aldrich Chem. Co) as a dry powder. These concentrations of chemical were chosen based on similar or higher dose rates of 2,4-D in other studies (Ou *et al.* 1978; Sattar & Paasivirta 1980; Stott *et al.* 1983). All soils were placed in Petri dishes and incubated at room temperature in a humidified chamber.

Seed germination

Soil was first amended with 500-μg/g PAA and either uninoculated are inoculated by mixing with a washed cell

suspension of *P. putida* strains PP0301, PP0301(pR0101) or PP0301(pR0103). The soil was then placed within Petri dishes and seeded with *Raphanus sativus* (radish), cultivar Cherry Belle Short Top. The dishes were covered to minimize evaporation and incubated at room temperature in a lighted humidified chamber. For these experiments, five seeds were placed within each dish and there were 3 dishes per condition. The dishes were monitored daily for seed germination and appearance of dicotyledonous leaves. In the absence of PAA, 100% (15 out of 15) of the seeds germinated.

Soil sampling

Bacterial cell numbers were determined by vortexing, in triplicate, 1.0 g of soil in a final volume of 10 ml of H_2O, serially diluted to a 10^8 dilution and spread-plated, in duplicate for each dilution, onto nutrient medium supplemented with (500-μg/ml) nal. For determination of low numbers of bacteria, 1 g of soil was mixed with 2 ml of H_2O and plated directly or serially diluted prior to plating. Preservation of plasmid-encoded mercury resistance was assessed by replica plating nal-resistant colonies onto brain–heart infusion medium supplemented with 25-μg/ml $HgCl_2$. There was no evidence for loss of plasmid in either chemically unamended or PAA-amended soil.

Extraction of PAA, Phenol and 2,4-D

A 2.0-g soil subsample was placed in a 30-ml screw-top test tube with a Teflon-lined cap. The initial alkaline extraction of soil was performed as described previously (Purkayastha 1974; Jensen 1978; Short *et al.* 1990). Subsequent extraction was conducted as described by Smith *et al.* (1984) except that diethyl ether was substituted for chloroform.

Analysis of PAA, phenol and 2,4-D

Ether soil extract samples were analysed for PAA and phenol content with an HP-1090 high-performance liquid chromatograph (HPLC) (Hewlett-Packard Co., Avondale, Penn.) coupled to a diode-array UV detector. Samples were separated by an isocratic mobile phase (35% H_2O, 65% CH_3OH, 0.1% CH_3COOH) on a C18 reverse-phase micro-bore and guard column at a flow rate of 1.0 ml/min. Sample absorbance was monitored at 254, 272 and 280 nm. External PAA, phenol and 2,4-D reference standards (NBS certified, Research Triangle, N.C.) were incorporated to bracket samples and analysed in triplicate every tenth sample. The limit of detection for PAA, phenol and 2,4-D using HPLC was 0.1 μg. Extraction efficiency for soil used in two experimental runs was 60% and 66% for PAA and 2,4-D, respectively. All values are reported without adjustment for extraction efficiency. Unless otherwise stipulated, chemicals used in extraction and analysis of PAA and 2,4-D were of Analytical Reagent (AR) grade (Aldrich Chemical Co.). The extraction efficiency of PAA added to sterile soil was initially 60% and did not change over a 3-week period.

Statistical analysis

Data for bacterial survival and chemical extraction of PAA were analysed for statistical significance by comparison of mean daily values using the Tukey–Kramer method (SAS Institute Inc. Cary, N.C.). P values ≤ 0.05 were considered significant. Seed germination data was analysed for statistical significance by the Fisher's exact test (Steel & Torrie 1980). Plotted values are the means ± SEM.

Results

Degradation of PAA occurred only in the presence of the GEM strain, *P. putida* PP0301(pR0103). After 28 days, there was no significant degradation of PAA in soil inoculated with either the parental strain *P. putida* PP0301 or the regulated 2,4-D degrader strain *P. putida* PP0301-(pR0101) (Fig. 1). The indigenous soil microbiota had not degraded any PAA after 40 days' incubation (data not shown). The co-amendment of 500 μg of 2,4-D per g of soil and 150 μg of PAA per g of soil did not result in metabolism of PAA. After 40 days' incubation, no 2,4-D

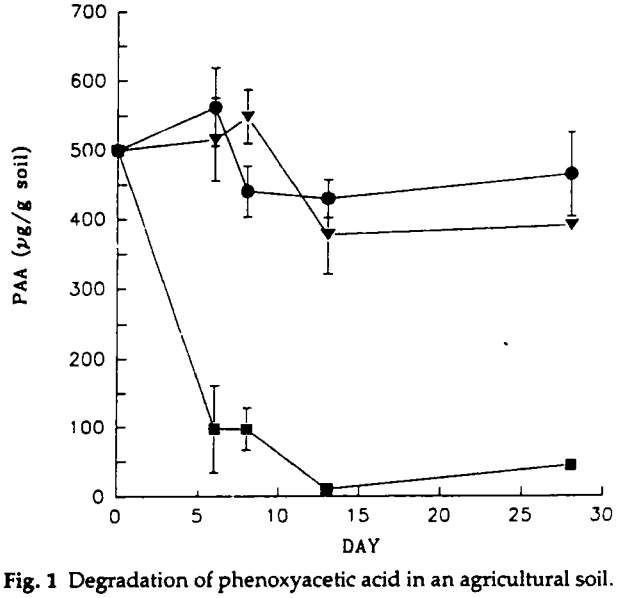

Fig. 1 Degradation of phenoxyacetic acid in an agricultural soil. Soil was inoculated with *P. putida* PP0301 (●), *P. putida* PP0301-(pR0101) (▼), or *P. putida* PP0301(pR0103) (■) at approximately 10^8 CFU/g of soil. Plotted values are means ± SEM. Some standard error bars fall within the area of the symbol.

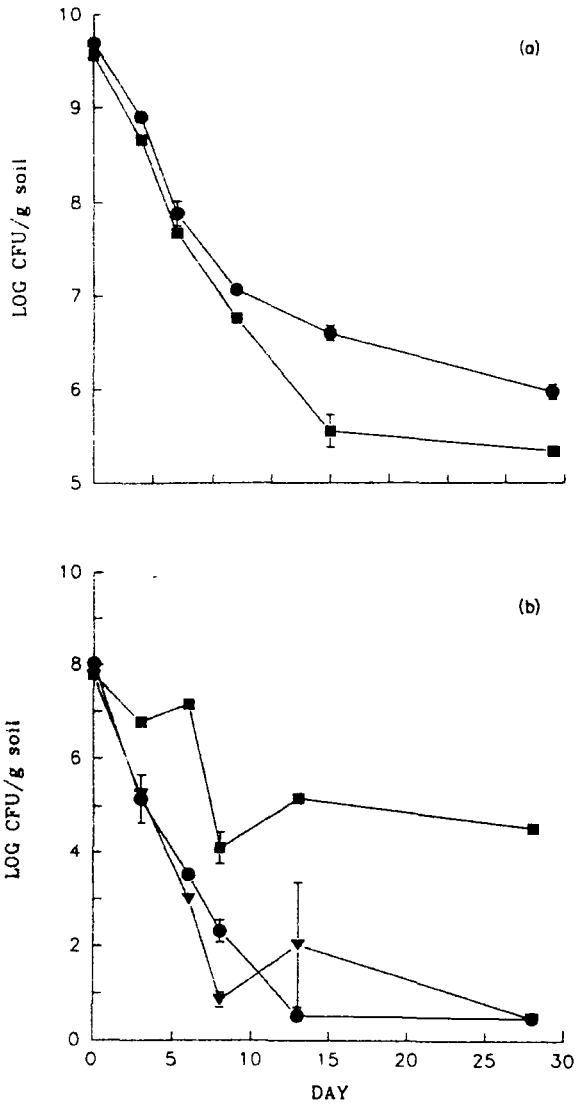

Fig. 2 Numbers of *P. putida* PP0301 (●), *P. putida* PP0301(pR0101) (▼), and *P. putida* PP0301(pR0103) (■) in (a) unamended agricultural soil and (b) the presence of 500-μg/g PAA. Plotted values are means ± SEM. Some standard error bars fall within the area of the symbol.

was detected whilst PAA was detected at the initial concentration (data not shown).

A comparison of survival of *P. putida* PP0301, *P. putida* PP0301(pR0101) and *P. putida* PP0301(pR0103) in the presence and absence of PAA demonstrates that PAA is bacteriotoxic (Fig. 2). In unamended soil, survival of the parental strain *P. putida* PP0301 and the GEM strain *P. putida* PP0301(pR0103) were similar (Fig. 2a). When inoculated at approximately 10^9 colony forming units (CFU)/g soil, *P. putida* PP0301 had declined to approximately 10^6 CFU/g after 39 days in unamended soil. In PAA-amended soil, an initial inoculum density of *P. putida* PP0301 at approximately 10^8 CFU/g had declined to fewer than 3 CFU/g after 13 days (Fig. 2b). Survival of the inducible strain *P. putida* PP0301(pR0101) was similar to that of the parental strain *P. putida* PP0301 in unamended (data not shown) and PAA-amended soil. In contrast, the survival of the GEM strain *P. putida* PP0301(pR0103) was very similar in both unamended and PAA-amended soil, and strikingly different from the survival of the parental strain in PAA amended soil.

In a second study, the affect of PAA mineralization by *P. putida* PP0301(pR0103) was demonstrated biologically. Seeds of *R. sativus* were planted in native soil amended with 500-μg/g PAA in the presence and in the absence of cells of the constitutive PAA degrader. In the presence of *P. putida* PP0301(pR0103), 100% of the seeds (15 of 15) germinated. In uninoculated soil, only 7% of the seeds germinated.

Discussion

The results of these studies suggested that the PAA degrader *P. putida* PP0301(pR0103) had a selective advantage releative to either of the non-degraders of PAA (*P. putida* PP0301 and *P. putida* PP0301(pR0101)) in soil amended with PAA. *P. putida* PP0301(pR0103) expresses a chromosomally encoded phenol hydroxylase (Harker *et al.* 1989), which enables this GEM to mineralize PAA. In fact, 500-μg/g PAA was mineralized to less than 10 μg/g within 13 days (Fig. 1) without an accumulation of greater than 10-μg/g phenol. However, although *P. putida* PP0301-(pR0103) can use PAA as a source of carbon, there was no apparent increased survival of *P. putida* PP0301(pR0103) in the presence of PAA, relative to unamended soil. Thus these data suggest that all strains of *P. putida* PP0301 experience toxicity to PAA and that the increased fitness of *P. putida* PP0301(pR0103), relative to *P. putida* strains PP0301 and *P. putida* PP0301(pR0101), was not due to some inherent resistance of PP0301(pR0103) to PAA. Rather, the increased fitness of *P. putida* PP0301(pR0103) in PAA-amended soil, relative to *P. putida* strains PP0301 and PP0301(pR0101), was due to its ability to degrade and thereby reduce the toxicity of PAA. In previous studies with the same soil type there was no significant difference in survival of *P. putida* PP0301 in unamended versus 2,4-D amended soil (Short *et al.* 1990). Newman (1947) evaluated the depressive effects of various phenoxy-herbicides on soil bacteria and fungi, and concluded that phenoyxacetate-type herbicides are inhibitory and taken up by cells only when in the undissociated form. The apparent higher toxicity of PAA, than 2,4-D, could therefore be explained if PAA passes through the cell membrane more easily than 2,4-D.

When induced by 2,4-D, *P. putida* PP0301(pR0101) and

Alcaligenes eutrophus JMP134(pJP4) express a 2,4-D monooxygenase active on the non-inducing substrate PAA (Harker *et al.* 1989). If 2,4-D-degrading bacteria native to the Oregon farm soil express a similar 2,4-D monooxygenase, then we propose that the persistance of PAA in the agricultural soil, with a native microbiota capable of degrading 2,4-D (Short *et al.* 1990) indicates that PAA did not induce the expression of 2,4-D monooxygenase by the indigenous soil microbiota. Moreover, these results suggested that native 2,4-D degraders are induced in the presence of 2,4-D and corroborated evidence that the usual delay in 2,4-D degradation (Audus 1960; Altom & Stritzke 1973), is the time necessary for enzyme induction (Audus 1960; Loos 1975). However, the fact that co-amendment of soil with PAA and 2,4-D did not result in the degradation of 100-μg/g PAA after 40 days, although 500-μg/g 2,4-D was degraded after 19 days, is supportive of an alternate possibility, that the enzyme systems of the indigenous 2,4-D degraders differ from plasmid pJP4 encoded enzymes in their substrate specificity.

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