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Growth and survival of *Pseudomonas cepacia* DBO1(pRO101) in soil amended with 2,4-dichlorophenoxyacetic acid

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Abstract

The 2,4-dichlorophenoxyacetic acid (2,4-D) degrading pseudomonad, *Pseudomonas cepacia* DBO1(pRO101), was inoculated at approximately 10^7 CFU/g into sterile and non-sterile soil amended with 0, 5 or 500 ppm 2,4-D and the survival of the strain was studied for a period of 44 days. In general, the strain survived best in sterile soil. When the sterile soil was amended with 2,4-D, the strain survived at a significantly higher level than in non-amended sterile soil. In non-sterile soil either non-amended or amended with 5 ppm 2,4-D the strain died out, whereas with 500 ppm 2,4-D the strain only declined one order of magnitude through the 44 days.

The influence of 0, 0.06, 12 and 600 ppm 2,4-D on short-term (48 h) survival of *P. cepacia* DBO1(pRO101) inoculated to a level of 6×10^4, 6×10^6 or 1×10^8 CFU/g soil was studied in non-sterile soil. Both inoculum level and 2,4-D concentration were found to have a positive influence on numbers of *P. cepacia* DBO1(pRO101). At 600 ppm 2,4-D growth was significant irrespective of the inoculation level, and at 12 ppm growth was stimulated at the two lowest inocula levels.

P. cepacia DBO1(pRO101) was able to survive for 15 months in sterile buffers kept at room temperature. During this starvation, cells shrunk to about one third the volume of exponentially growing cells.

Abbreviations: AODC – acridine orange direct count; CFU – colony forming units; PTYG-Agar – peptone, tryptone, yeast & glucose agar; TET – tetracycline; LB – Luria Bertani medium

Introduction

The success of introduced naturally occurring or genetically modified microorganisms in the in situ removal of chemicals from the environment depends on their survival and on the maintenance of their degradative genes in the natural ecosystem. The growing interest in the use of microorganisms in environmental bioremediation necessitates a detailed understanding of their behaviour in the environment. Despite the fact that the in situ efficacy of xenobiotic-degrading organisms clearly depends on their ability to compete and survive in the natural setting, most research has been conducted using laboratory culture media (for a review, see Chaudhry & Chapalamadugu 1991). The study of

fate and effects of xenobiotic-degrading microorganisms in natural environments can be performed using indirect methods as most probable number of degrading bacteria (Briglia et al. 1990) or modelling of the ability of the organisms to transform the compounds of interest (Focht & Brunner 1985). However, when studying introduced microorganisms, these approaches are hampered by the difficulty in distinguishing the introduced strains from the indigenous bacterial flora. Hence, in the absence of a marker to facilitate specific detection, the direct detection of introduced xenobiotic-degrading strains in natural soils has relied upon the use of very high inocula ($>10^8$ CFU/g soil) in order to enable strain identification from its colony morphology on unselective agar medium (Topp & Hanson 1990). However, Kilbane et al. (1983) have conducted studies using xenobiotic degrading strains carrying antibiotic markers that facilitate easy detection on selective agar-media; they found a growth of the introduced strain upon addition of high amounts of the test-compound (> 500 ppm). In a study of the influence of amendment of three different non-sterile soils with 1000 ppm *p*-ethylbenzoate on survival of a *p*-ethylbenzoate-degrading *P. putida*, survival decreased in one case, increased in a second case, and was unaffected in the third after 21 days (Ramos et al. 1991). More consistent findings were obtained by Short et al. (1990); in their study *P. putida* PPO301KS(pRO101) responded positively to amendment of non-sterile soil with 500 ppm 2,4-D.

In the present study we investigated the population dynamics of the 2,4-dichlorophenoxyacetic acid (2,4-D) degrading pseudomonad, *Pseudomonas cepacia* DBO1(pRO101), in a laboratory soil system amended with different concentrations of 2,4-D. We followed survival both in natural (unsterilized) soil and sterilized soil for 44 days, examined the influence of inoculation level on the short-term (48 h) growth and survival in natural soil, and finally examined long-term survival and culturability under starvation conditions in sterile buffers. Detection and enumeration of the strain in soil samples was facilitated by its tetracycline resistance and its characteristic colony morphology. Identification was confirmed by colony hybridization with a DNA-probe against the tfdA degrading gene on the plasmid.

Materials and methods

Bacterial strains

P. cepacia DBO1(pRO101) was obtained from R.H. Olsen, University of Michigan, MI, USA. The plasmid pRO101 is a Tn1721 derivative of pJP4, with the transposon inserted at a site that is not essential for the expression of the genes conferring the ability to degrade 2,4-D (Harker et al. 1989). The Tn1721 transposon confers tetracycline resistance. *Escherichia coli* K12(pKJS31) containing the tfdA-gene (Streber et al. 1987) was obtained from D. Dwyer, GBF, Braunschweig, Germany. The master and working cultures of both strains were stored at −80°C.

Soil system

The soil used was sandy loam from an area of an experimental farm in Roskilde, Denmark, which had not been sprayed with 2,4-D or other pesticides for at least 25 years. The soil characteristics were: pH–H_2O: 6.5, cation exchange capacity: 8.4 meq/100 g dry wt, organic matter: 2.4%, clay 10.9%, silt: 30.7%, fine sand: 31.9%, coarse sand: 24.1%. The soil was passed through a 4-mm sieve on the day of collection and thereafter stored refrigerated in the dark at its natural moisture content for a maximum of 90 days.

Three days prior to use, 50 g wet wt aliquots of soil were transferred to 300 ml Erlenmeyer flasks which were then sealed with a rubber stopper in order to prevent soil desiccation, and stored at 22°C in the dark. Sterile soil was obtained by autoclaving the flasks for 90 min at 120°C on two successive days. Sterilization was verified by spread plating on PTYG-agar (0.25 g/l Bacto-peptone, 0.25 g/l tryptone, 0.5 g/l yeast extract, 0.5 g/l glucose, 0.06 g/l $MgSO_4 \cdot 7H_2O$, 0.007 g/l $CaCl_2 \cdot 2H_2O$, 15 g/l Bacto-agar). After the addition of bacteria and 2,4-D (see below) the soil systems used in Experiment 1

(see below) were adjusted to a final moisture content of 17.5% of dry wt corresponding to a water potential, ψ, of -2.0 kPa, as determined by the filter-paper method (Fawcett & Collis-George 1967). Soil systems used in Experiment 2 were adjusted to a final moisture content of 20.4% of dry wt, corresponding to a ψ of -1.1 kPa.

The two experiments performed were: Experiment 1: Long-term survival of *P. cepacia* DBO1(pRO101) in soil. Experiment 2: Effect of inoculation level and 2,4-D concentration on short-term growth. In addition, the culturability and size of *P. cepacia* DBO1(pRO101) in sterile buffers was examined after 15 months.

Inoculation with P. cepacia *DBO1(pRO101)*

Experiment 1. Prior to use, 1 ml working culture was thawed, inoculated into 20 ml Luria Bertani (LB) medium with 30 µg tet/ml (Maniatis et al. 1982) and incubated for 18h at 30°C while rotating at 150 rpm on an orbital shaker. The late log phase culture was harvested, washed twice in phosphate buffer (0.015 M, pH 7.4) and adjusted to 5×10^8 cells/ml as estimated by microscopy counts (AODC) (Hobbie et al. 1977). One ml of this suspension, i.e. 5×10^8 cells, or sterile buffer in case of the controls, was transferred to and carefully mixed with each soil system using a sterile pipette.

Experiment 2. The soil systems were inoculated with *P. cepacia* DBO1(pRO101) to a level of 6×10^4, 6×10^6 or 1×10^8 colony forming units (CFU) per g prepared at the appropriate concentration as described for Experiment 1 above.

Amendment of soil with 2,4-D

Experiment 1. 2,4-D soil systems were prepared by adding either 25 µg or 2500 µg 2,4-D (Sigma Chem. Co., St. Louis, MO, USA) dissolved in 500 µl pure methanol to the soil systems so as to obtain a 2,4-D soil concentration of 5.87 and 587 mg/kg soil dry wt (corresponding to 5 ppm and 500 ppm on a wet wt basis), respectively. Control systems were prepared by adding 500 µl methanol to the soil. The methanol in all systems was allowed to evaporate for 15 min in a sterile hood before the 2,4-D was mixed with the soil using a sterile pipette.

Experiment 2. 0 µg, 0.03 µg, 60 µg, 2500 µg or 3000 µg 2,4-D dissolved in 0.1 M Na_2HPO_4 was added to the soil systems in order to obtain 2,4-D concentrations of 0, 0.06, 12, 500 and 600 ppm (wet wt), and the 2,4-D mixed with the soil using a sterile pipette.

Incubation, sampling and enumeration of bacteria

In order to prevent anaerobic conditions developing in the soil systems of Experiment 1, they were aerated for 15 min in a sterile hood at least once a week. One sample containing approximately 1 g of soil taken from three different places in each soil system was shaken with 9.5 ml phosphate buffer, diluted in buffer and plated in triplicate. Total culturable bacteria were enumerated after 5 days at 25°C on PTYG-agar supplemented with 25 µg/ml of natamycin (Delvocid®, Gist Brocades, Delft, Holland) to control fungal growth (J.C. Pedersen 1992). Enumeration of *P. cepacia* DBO1(pRO101) was facilitated by its resistance to tetracycline, a powerful suppressor of the indigenous bacterial flora in soils (Holben et al. 1989). Thus, *P. cepacia* DBO1(pRO101) was enumerated after 2 days at 30°C on the same agar additionally supplemented with 30 µg/ml tetracycline (PTYG-tet) (Sigma).

Verification of the detection of P. cepacia *DBO1(pRO101)*

The identity of 20 selected colonies was verified using the API 20NE system (API Systems, Montalieu-Vercieu, France). In addition, colony hybridization with a tfdA-probe was used to further verify the detection method. A 589 bp *Stu*I fragment from pKJS31, representing an internal fragment of the tfdA-gene of pRO101 (Streber et al. 1987), was separated on a 1% agarose gel, purified with GeneClean® according to the manufacturers recommen-

dations (BIO-101, LaJolla, CA, USA) and ^{32}P-labeled using a random primed method (Boehringer Mannheim, Mannheim, Germany). Colonies were transferred to nitrocellulose paper (Schleicher & Schuell BA 85), denatured, neutralised, baked and hybridized according to standard procedures (Sambrook et al. 1989).

Long-term culturability in sterile buffers

The long-term survival of *P. cepacia* DBO1 (pRO101) under starvation conditions was also examined. Screw-cap bottles (25 ml) containing 10 ml of either distilled water, Winogradsky salt solution (Pochon 1954), phosphate buffer (0.015M, pH 7.4) or 0.9% NaCl were inoculated as described above with 1×10^6 CFU/ml exponentially growing *P. cepacia* DBO1(pRO101) and stored at room temperature for 15 months. The strain was enumerated immediately after inoculation, and at the end of the 15 month period, by spreadplating and AODC. Acridine orange-stained cells were photographed and length (l) and width (w) were measured on randomly selected cells. Cell volume was calculated as $\pi/4 \cdot w^2 \cdot (l - w/3)$ (Bjørnsen 1986), and converted to biomass using a conversion factor of 0.32 pg/μm^3 (Schmidt & Paul 1982).

Calculations and statistics

Plate counts were converted to CFU/g soil (wet wt), log transformed and the mean and SEM calculated and plotted using Fig. P., version 5 (Biosoft, Cambridge, UK). The log transformed data were analyzed by ANOVA and Duncan's multiple range test using SAS/STAT version 6.04 (Statistical Analysis Systems, SAS Institute, Cary, NC, USA).

Results

Detection of P. cepacia *DBO1(pRO101) in soil*

The background level of tetracycline-resistant indigenous soil bacteria, approximately 5×10^2 CFU/g, impeded the enumeration of low populations of *P. cepacia* DBO1(pRO101) on PTYG-tet. The characteristic bulging morphology of *P. cepacia* DBO1 (pRO101) colonies facilitated differentiation of the introduced strain. That bulged colonies were indeed *P. cepacia* DBO1(pRO101) was verified by colony hybridization. The tfdA-probe hybridized to all bulged colonies (30/30) and to less than 3% of non-bulged colonies (3/120).

Loss of plasmid or the transposon-carried tetracycline marker might occur during growth of *P. cepacia* DBO1(pRO101). However, it was found that the number of CFU on PTYG-tet and PTYG after growth in sterile soil or soil amended with 500 ppm 2,4-D were not significantly different ($P > 0.05$), this indicates that neither the plasmid nor the transposon were lost. Further evidence for conservation of tetracycline resistance is that 280 randomly selected colonies from PTYG-agar were all able to grow on PTYG-tet.

Experiment 1: long-term survival in soil

The survival of *P. cepacia* DBO1(pRO101) was significantly influenced ($p < 0.0001$) by the indigenous flora as well as by 2,4-D (Figs. 1a and b). In sterilized non-amended soil (Fig. 1a), the population slowly declined from the inoculation level of 1×10^7 to 5×10^6 CFU/g soil on day 44, whereas in the 2,4-D amended soils there was a 5–10-fold increase ($p < 0.05$). Growth occurred earlier at 5 ppm 2,4-D than at 500 ppm, peaked in both systems on day 18, and declined slowly to 5×10^7 CFU/g soil by day 44. We found that mixing of a portion of natural soil with sterilized non-amended soil inoculated with *P. cepacia* DBO1(pRO101) resulted in a faster decline of the population than in the sterile soil (data not shown).

In non-sterilized soil (Fig. 1b), *P. cepacia* DBO1 (pRO101) population decreased and in each case was lower than in the equivalent sterilized soil system. Amendment with 500 ppm 2,4-D had a positive influence on the population, as compared with non-amended and 5 ppm 2,4-D soil systems. At the end of the experiment, day 44, the population in soil initially amended with 500 ppm 2,4-D was $1 \times$

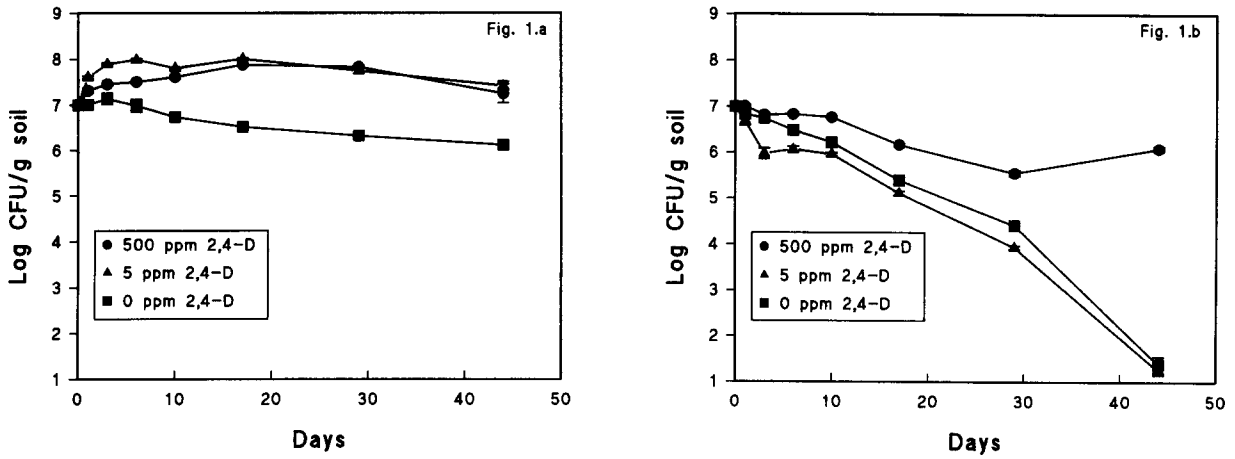

Fig. 1a and 1b. Survival of *P. cepacia* DBO1(pRO101) in sterilized (a) and non-sterilized (b) soil amended with different concentrations of 2,4-D. Each point represents the avarage of 3 samples from each of three soil systems. When larger than symbols, bars indicate SEM.

10^6 CFU/g soil, whereas it was less than 1×10^2 CFU/g soil in the non-amended and 5 ppm soils. Survival in the non-amended soil was better than that in the soil amended with 5 ppm 2,4-D ($p < 0.05$); however, in both cases a very low population level was reached. The numbers of total culturable bacteria remained constant throughout the experiment.

Experiment 2: inoculation level and 2,4-D concentration

Both inoculum level and 2,4-D concentration were found to have a positive independent influence on the recovery of *P. cepacia* DBO1(pRO101) 2 days after amendment with 2,4-D (Fig. 2). The effect of 2,4-D increased with increasing concentration of 2,4-D; the population decreased in all non-amended soils and in the soils amended with 0.06 ppm 2,4-D when the inoculum was 6×10^6 and 1×10^8 CFU/g soil and in soil amended with 12 ppm in the 1×10^8 CFU/g system, while it increased in the other cases. The highest population of *P. cepacia* DBO1(pRO101) (2×10^8 CFU/g soil) was reached in soils amended with 600 ppm 2,4-D and inoculated with 6×10^6 or 1×10^8 CFU/g soil.

Long-term survival in sterile buffers

P. cepacia DBO1(pRO101) was still viable after 15 months in the four different sterile buffers kept at room temperature, and numbers of cells and CFU per ml even increased slightly through the period. Culturability determined as the percentage of total cells (AODC) recovered by plating on LB or LB-tet (no difference) varied between 5% in phosphate buffer and 66% in distilled water. After 15 months the average cell volume (±SEM) in buffers was 0.367 ± 0.22 μm^3 while exponential phase cell volume was 1.05 ± 0.32 μm^3, corresponding to a biomass of 0.117 pg and 0.337 pg, respectively.

Discussion

Survival of P. cepacia *DBO1(pRO101)*

The presence of 2,4-D in the soil gave *P. cepacia* DBO1(pRO101) a competitive advantage over the indigenous flora. The low background of indigenous bacteria able to degrade 2,4-D (described in detail in the accompanying article, Jacobsen & Pedersen 1992) provided optimal conditions for the introduced strain to utilize 2,4-D as a carbon source. The cell yield in soil systems amended with 600 ppm in Experiment 2 was approximately 2×10^8 CFU/g soil, i.e. the same as can be predicted on

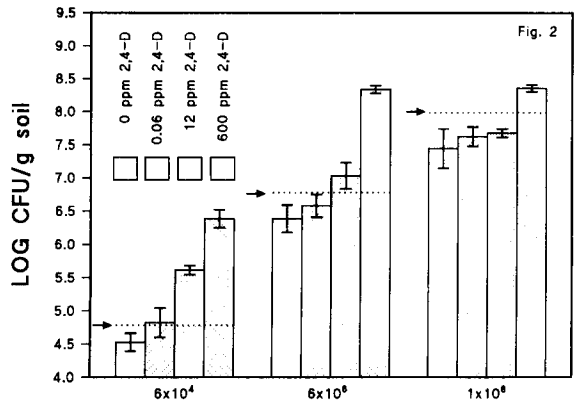

Fig. 2. CFU of *P. cepacia* DBO1(pRO101) 48 h after inoculation at levels of 6×10^4, 6×10^6 and 1×10^8 CFU/g soil into soils amended with 0, 0.06, 12, and 600 ppm 2,4-D. Bars indicate SEM, arrows indicate inoculation level.

the basis of the amount of carbohydrate in 600 μg 2,4-D and assuming that the formation of one cell requires 1 pg carbohydrate (Acea et al. 1988). That population growth at high 2,4-D concentrations was less in Experiment 1 compared to Experiment 2 is probably due to an effect of the methanol used as the 2,4-D solvent in Experiment 1. Methanol was therefore replaced with phosphate buffer in the subsequent experiments.

The numbers of *P. cepacia* DBO1(pRO101) were lower in non-sterilized soil than in steam-sterilized soil (Fig. 1). We found that mixing of a portion of natural soil into steam-sterilized soil inoculated with *P. cepacia* DBO1(pRO101) resulted in a decrease of the numbers of *P. cepacia* DBO1(pRO101) compared to steam-sterilized soil, hereby indicating that survival was negatively influenced by indigenous microflora. This finding is in accordance with the finding of Topp & Hanson (1990) that a pentachlorophenol degrading *Flavobacterium* sp. survived well in sterilized soil-slurries, but quickly died out after the addition of non-sterilized soil. Other studies indicate that survival of introduced xenobiotic-degrading organisms is affected by biotic factors (Acea et al. 1988; Wessendorf & Lingens 1989; Morel et al. 1989).

Another pre-requisite for long-term survival was demonstrated in the experiment with sterile buffers: *P. cepacia* DBO1(pRO101) was able to survive prolonged starvation, as other *Pseudomonas* spp. has shown to be in contrast to other gram negative strains (Byrd et al. 1991). During starvation the cells shrunk to approximately one third the volume of exponentially growing cells. This phenomena has been correlated with the ability to enter a dormant stage (Roszak & Colwell 1987). The average cell weight in the buffers was close to the average cell weight reported from subsurface aquifer sediments (Balkwill et al. 1988).

Plasmid-stability

We found that pRO101 was maintained in *P. cepacia* DBO1(pRO101) after growing more than 3 log-units in sterile soil both with and without 2,4-D. Stability of pRO101 in non-amended soil has also been reported by Short et al. (1990) in *P. putida* PPO301 under non-growing conditions. They studied the effect of 2,4-D on the survival in soil of different 2,4-D degrading strains of *P. putida* and found that spiking of soil with 500 ppm 2,4-D 50 days after introduction of the strain increased the population of culturable *P. putida* PPO301KS (pRO101). In contrast to Golovleva et al. (1988), who showed that the presence of the catabolic substrate in soil was necessary to maintain a keltane-degrading plasmid in *P. aeruginosa*, our study provides evidence for maintenance of plasmid pRO101, even under growing conditions in the absence of the catabolic substrate.

The inserted transposon could possibly transpose from the plasmid to the chromosome, thereby giving rise to false positive colonies if the plasmid was lost. However, colony hybridization showed that all tetracycline-resistant colonies with the characteristic bulged morphology of *P. cepacia* DBO1(pRO101) hybridized with the tfdA-probe, thus indicating that the majority of the detected colonies had maintained pRO101. In the present context Tn1721 seems therefore to be a valuable ecological marker, as does transposon Tn5 in other strains (Pillai & Pebber 1990; Fredrickson et al. 1989).

Conclusions

It can be concluded from the present study that *P. cepacia* DBO1(pRO101) is able to grow in soil amended with high amounts of 2,4-D. Furthermore, our study shows that the transposon Tn1721 seems to be a valuable ecological marker since both the transposon and the host-plasmid showed to be stable under environmental conditions.

As *P. cepacia* DBO1(pRO101) survived in soil for a much longer time than it takes to degrade 2,4-D and throughout 15 months of starvation in sterile buffers, the strain might well be able to establish itself as a permanent member of the soil bacterial community.

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