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Dissemination of Catabolic Plasmids Among Desiccation-Tolerant Bacteria in Soil Microcosms

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Abstract

The dissemination of catabolic plasmids was compared to bioaugmentation by strain inoculation in microcosm experiments. When *Rhodococcus erythropolis* strain T902, bearing a plasmid with trichloroethene and isopropylbenzene degradation pathways, was used as the inoculum, no transconjugant was isolated but the strain remained in the soil. This plasmid had a narrow host range. *Pseudomonas putida* strain C8S3 was used as the inoculum in a second approach. It bore a broad host range conjugative plasmid harboring a natural transposon, RP4::Tn4371, responsible for biphenyl and 4-chlorobiphenyl degradation pathways. The inoculating population slowly decreased from its original level (10^6 colony-forming units [CFU]/g of dry soil) to approx 3×10^2 CFU/g of dry soil after 3 wk. Transconjugant populations degrading biphenyl appeared in constant humidity soil (up to 2×10^3 CFU/g) and desiccating soil (up to 10^4 CFU/g). The feasibility of plasmid dissemination as a bioaugmentation technique was demonstrated in desiccating soils. The ecologic significance of desiccation in bioaugmentation was demonstrated: it upset the microbial ecology and the development of transconjugants.

Index Entries: Bioaugmentation; drought tolerance; conjugation; plasmid dissemination; microcosm; isopropylbenzene.

Introduction

Bioaugmentation, the addition of microorganisms to enhance a specific activity, has been used in several areas such as agriculture *(1)* and

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wastewater treatment *(2)*. However, it is not considered an efficient technique for the remediation of soils polluted with hydrocarbons. In many cases, the advantages of increasing the biocatalyst activity do not offset the advantages of niche fitness demonstrated by indigenous microorganisms *(3,4)*. Therefore, bioaugmentation should be considered only when the intrinsic catabolic activity is not present in the soil. The bioaugmentation product should combine the advantages of good catalytic activity and niche fitness.

A way to fulfill these two criteria is the dissemination of catabolic plasmids to indigenous bacterial strains adapted to the environment. Then, the inoculating microorganism could be a plasmid-delivering strain chosen according to technologic and ecologic selective criteria:

1. A degrading activity toward the more recalcitrant hydrocarbons, borne on plasmids or not.
2. A high productivity in bioreactors preferentially with inexpensive growth media.
3. A good tolerance to desiccation in order to prepare a starter culture in a desiccated form.
4. A good shelf-life with full maintenance of metabolic activity on rehydration *(5)*.
5. Adaptation to soil physicochemical conditions, to survive long enough to transmit its plasmid or to achieve pollution removal itself.
6. Compatibility with autochthonous bacterial strains, which could be measured by the ability of the inoculating strain to disseminate its catabolic plasmid to the indigenous flora.

For microorganisms to meet these criteria, drought-tolerant bacteria were selected from desiccated polluted soils *(6)*. Desiccation tolerance is relevant for the production of a dry bioaugmentation product, but it also has ecologic significance in soils submitted to daily or seasonal variations of hydration *(7)*.

The introduction of new catabolic genes borne on plasmids into the "technologic strains" was achieved by means of natural conjugation in order to improve their catabolic activity *(8)*. Many catabolic genes are located on plasmids that are self-transmissible and have a broad host range *(9)*. The newly obtained strains could be used in microcosm experiments either as bioaugmentation strains with good catalytic activity able to remove the pollution or as plasmid delivery systems. They should exhibit a high catalytic activity and be adapted to the soil biotope.

This article compares the catabolic gene dissemination strategy and bioaugmentation by strain inoculation. Some studies have dealt with catabolic plasmid dissemination *(10–14)*, but none have addressed the ecologic importance of desiccation or used the concept of technologic strains as tools to produce and deliver the plasmid to the autochthonous microflora.

In the present study, two strains were used as model systems. In the first experiment, a *Rhodococcus erythropolis* strain was used as the starter

strain and as the plasmid delivery system. These Gram-positive nocardioform *Actinomycete* eubacteria should survive and develop in polluted soils. The *R. erythropolis* strain carries a conjugative plasmid responsible for mercury resistance, trichloroethene (TCE) and isopropylbenzene (IPB) degradation that could disseminate. It has a narrow host range, but in vivo experiments have shown that unexpected transfers could occur in field conditions *(15,16)*. This strain is desiccation tolerant and exhibits good technologic properties. The fate of this strain, the dissemination of the plasmid, and the appearance of IPB-degrading autochthonous microflora were monitored in IPB-treated and untreated soil microcosms.

In the second approach, a *Pseudomonas putida* strain was used as the inoculum. It carried a broad host range plasmid responsible for biphenyl (BP) and 4-chlorobiphenyl pathways. This strain did not exhibit technologic properties but was adapted to the soil biotope since it was first isolated from the soil in which it served as the inoculum. Its survival in BP-treated and untreated soil microcosms and the dissemination of the catabolic plasmid were monitored. The BP concentration was measured.

When desiccation stress was imposed on the ecosystems, the appearance of drought-tolerant strains expressing the plasmid was monitored. The behavior of desiccating systems was compared to constant water activity systems and the ecologic significance of desiccation was evaluated.

Materials and Methods

Bacterial Strains and Plasmids

In the first microcosm experiment, *R. erythopolis* strain T902.1 was used as the inoculum. It was first isolated from a desiccated polluted soil *(6)*. It was mated with another *R. erythropolis* strain harboring pBD2, a conjugative megaplasmid responsible for mercury resistance and TCE and IPB degradation, in order to broaden its metabolic activity *(8)*. This plasmid has a narrow host range *(17)* as measured in vitro. A rifampicin mutant of this strain was used as the inoculum in the microcosm experiments.

The second microcosm experiment was conducted with an IncP, broad host range conjugative plasmid harboring a natural transposon RP4::Tn4371 responsible for BP and 4-chlorobiphenyl pathways *(18)*. It also confers resistance to ampicillin, tetracycline, and kanamycin. This plasmid was originally hosted in *Escherichia coli* strain CM844, but it was transferred by conjugation to *P. putida* strain C8S3 prior to soil inoculation.

Transfer of Plasmids to Soil Isolates

The *E. coli* strain CM844 harboring the RP4::Tn4371 plasmid is a polyauxothroph and is not adapted to soil conditions. To inoculate the soil with a strain adapted to it and harboring the plasmid, a filter mating was done between *E. coli* strain CM844 and a soil microbial extraction obtained by adding 5 g of soil to 45 mL of $MgSO_4$ solution (10 m*M*) with 0.1% of

Tween-80 and shaking for 1 h at 30°C. The transconjugant was selected on minimal medium 284 *(5)* with tetracycline (50 mg/L) and glucose (2 g/L) as the sole source of carbon. It did not express the biphenyl (BP) degradation capability. This property enabled good counterselection of the donor. A rifampicin mutant of this strain, C8S3, was used as donor throughout this study. C8S3 was identified with BIOLOG Fingerprinting (BIOLOG, Hayward, CA) as *P. putida*.

Soil Microcosms

Two types of soil were used in the first microcosm experiment: The first was a silt soil collected from woods with a pH (H_2O) of 3.9, a very low organic matter content, and a field capacity moisture content of 51% on dry soil. The second was a clay soil collected from a field plot with a pH (H_2O) of 7.0, an organic matter content of about 2%, and a field capacity moisture content of 50% on dry soil. Only clay soil was used in the second experiment. Soil samples were moistened to 75% of their water-holding capacities and preincubated at 20 ± 2°C for 3 wk prior to the start of the experiment. The soil microcosms were prepared by adding 100 g of soil to 250-mL tightly closed glass jars for the IPB-amended soil and to opened jars for the BP-amended soils.

Four different experimental conditions were set up: the first set of microcosms was inoculated with the plasmid donor strain (10^6 colony-forming units [CFU]/g of dry soil) and amended with 1000 ppm of either IPB or BP, the second was not inoculated but amended with the pollutant, the third was inoculated but not amended, and the fourth was untreated and served as the control.

Strains used as inoculant were grown overnight at 30°C in selective 869 based [21] rich media (869 + 5 μ*M* $HgCl_2$ + 150 mg/L of ripampicin for T902.1 and 869 + 50 mg/L of tetracycline + 150 mg/L of ripampicin for C8S3), centrifuged at 10,000*g*, washed in $MgSO_4$ solution (10 m*M*), and resuspended in $MgSO_4$ solution (10 m*M*). A Bürker's chamber count was made to determine the required volume of this suspension to reach 10^6 CFU/g of dry soil in the microcosms.

The total heterotrophic count of the soil was determined by plating 0.1 mL of serial 10-fold dilutions of soil in $MgSO_4$ solution (10 m*M*) onto 869 rich medium containing cycloheximide (150 mg/L). Strain T902.1, the inoculating strain in the first experiment, was enumerated on 284 minimal medium containing cycloheximide (150 mg/L) and rifampicin (150 mg/L) with IPB as the sole source of carbon. Transconjugants with strain T902.1 were sought on minimal selective medium with cycloheximide (150 mg/L), $HgCl_2$ (5 μ*M*), and IPB as the sole source of carbon. No counterselection of strain T902.1 could be made. Strain C8S3, the inoculating strain in the second experiment, was enumerated on 869 rich medium with cycloheximide (150 mg/L), tetracycline (50 mg/L), and rifampicin (150 mg/L). Transconjugants with strain C8S3 were sought by plating the dilutions onto 284 minimal medium with cycloheximide (150 mg/L), tetracycline (50 mg/L),

and BP as the sole source of carbon. The donor strain could not grow on this medium.

Owing to IPB volatility, IPB-amended microcosms were incubated in closed jars. No variation in water content could be made. Therefore, desiccation-tolerant transconjugants were sought by plating the dilution of freeze-dried microcosm soil samples onto the minimal selective medium. Because BPs are not volatile, microcosm jars could be left open and two experimental conditions could be set up. One set of microcosms was kept at 75% of the water-holding capacity by daily weighed additions of water. The other set of microcosms was submitted to cycles of desiccation and rehydration in order to impose a water depletion stress to the biotope. The desiccation-tolerant transconjugants were sought by plating dilutions from the desiccating microcosms onto the 284 selective medium.

BP Analysis of Soil Samples

Concentrations of BPs in the soil were determined as follows. Five-gram samples were collected in triplicate from the microcosms and were extracted with 45 mL of cyclohexane by mixing the soil and the solvent in a blender for 1 min. The supernatant was collected and filtered, and the absorbance at 268 nm was measured and compared to a standard curve.

Identification of Strains

A few strains exhibiting transconjugant properties were identified to the species level with BIOLOG fingerprinting. The procedure was realized according to the manufacturer's instructions (BIOLOG).

Plasmid DNA Isolation

Individual colonies of transconjugants were isolated by streaking them onto selective agar. They were cultured in 869 selective broth. DNA isolation was performed as described previously *(8)*, and the plasmids were resolved on a 0.8% agarose gel essentially as described.

Drought-Tolerance Measurements

The strains were cultivated overnight in 869 broth, centrifuged, washed in $MgSO_4$ solution (10 m*M*), and resuspended in the same volume of solution. Then they were freeze-dried as described previously *(19)*. Soil samples were freeze-dried under the same conditions.

Results and Discussion

Bioaugmentation in IPB-Amended Soil Microcosms

The first microcosm experiment was conducted with *R. erythropolis* strain T902.1 used as the inoculum. This strain harbored a plasmid responsible for IPB and TCE degradation as well as mercury resistance. In vitro experiments have shown that this linear plasmid has a narrow host range

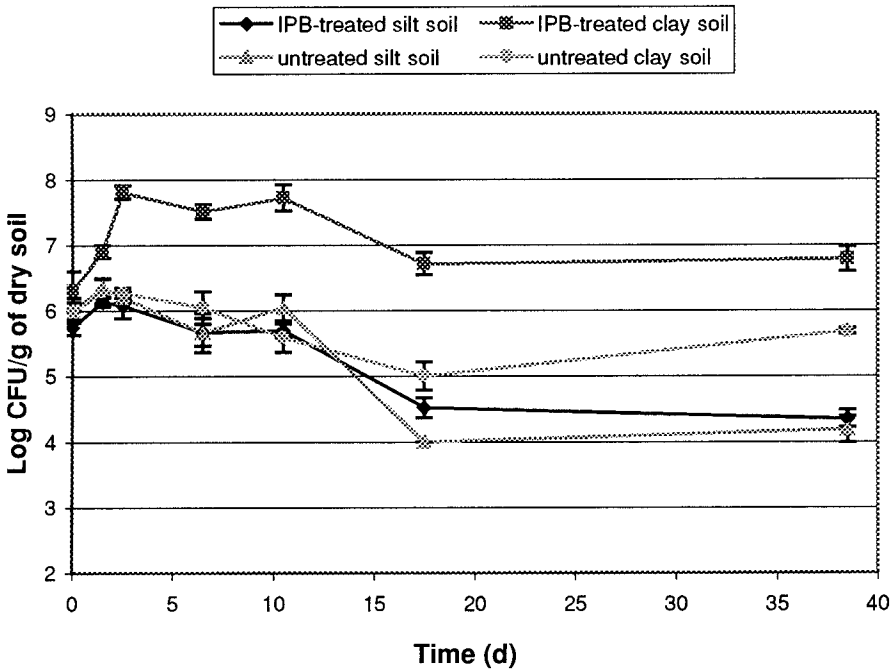

Fig. 1. Fate of strain T902.1 in treated and untreated silt soil and clay soil.

(17). However, in vivo observations may differ from what occurs in vitro *(15,16)*. This strain is drought tolerant and adapted to the technologic production and conditioning constraints. It could either deliver the plasmid and disappear or develop and do the remediation itself.

Two types of soil were used : a forest acid silt soil and an agricultural clay soil. The fate of the starter culture was monitored in both soils (Fig. 1). The behavior is different according to the nature of the soil.

In IPB-amended clay soil, strain T902.1 increased 30-fold after only 2 d. Selective pressure owing to the presence of IPB gave an advantage to strain T902.1. In nonamended soil, T902.1 maintained its count to its original level (10^6 CFU/g of dry soil) during the time of the experiment (40 d). Strain T902.1 was well adapted to these soil conditions. By maintaining its population in the soil, the starter culture guaranteed the presence of IPB degradation activity in the soil. This presence reinforced the standardization of the bioaugmentation treatment whether or not plasmid dissemination occurred and independently of the nature of the indigenous microflora. No transconjugants could be isolated from either of the two soils. This was probably owing to the narrow host range nature of the pBD2 plasmid. Although no plasmid dissemination occurred, the strain bioaugmentation approach was successful because the inoculated strain developed in the clay soil.

In the acid silt soil, the T902.1 population declined slowly from its original level (10^6 CFU/g of dry soil) to 10^4 CFU/g in amended or

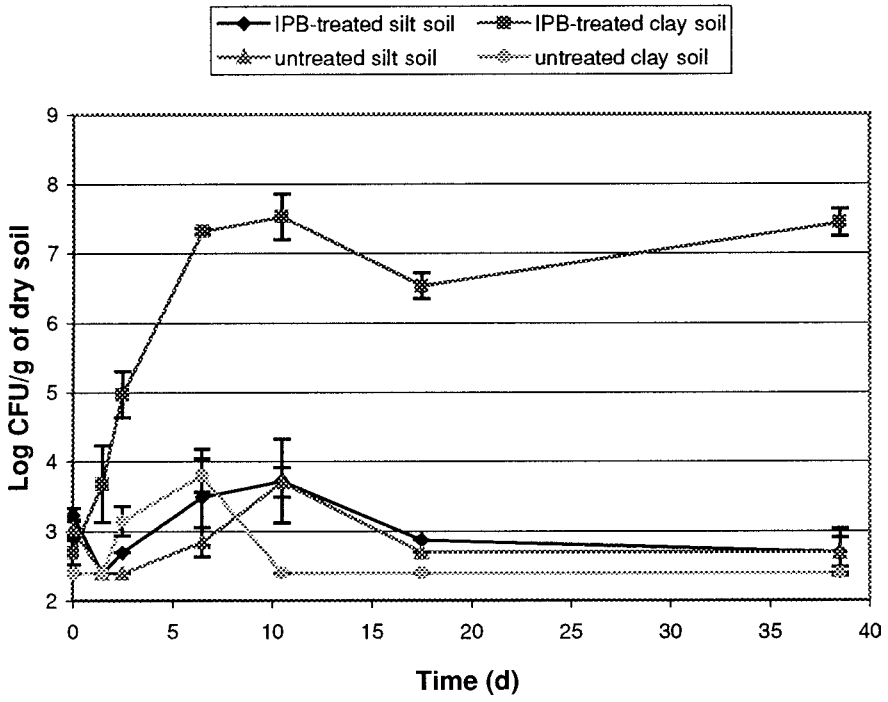

Fig. 2. Evolution of indigenous IPB-degrading populations in silt soil and clay soil.

nonamended soil. The starter maintained the presence of the catabolic activity in the soil.

The autochthonous population of the uninoculated clay soil adapted rapidly to IPB pollution and reached 2×10^7 CFU/g of dry soil (Fig. 2). IPB was not recalcitrant and intrinsic degrading activity was present and could develop. Two different strains were isolated among this population: a *Pseudomonas pseudoalcaligenes* strain and an *R. erythropolis* strain. They both exhibited IPB 2,3-dioxygenase activity. Gram-negative *Pseudomonas* strains are known to decompose a large variety of aromatic compounds *(20)*. A high homology was found between IPB dioxygenase from *Rhodococci* and dioxygenase of other aromatic compounds found in *Pseudomonads (21)*, which explained how they had similar activities in IPB-amended soils.

Desiccation of microcosm samples was achieved in order to select drought-tolerant IPB-degrading microorganisms (Fig. 3) and to monitor the ecologic significance of desiccation in soils. The sensitivity to desiccation of the IPB-degrading population increased as this population appeared and developed. Before IPB treatment, the desiccation-tolerant population was of the same order of magnitude as the overall population. After 7 d, only 0.5% of the global population was still desiccation tolerant. The microorganisms responsible for IPB degradation were more drought sensitive than the original average population of the soil. IPB amendment disturbed the soil ecology, resulting in a change in the drought tolerance of the

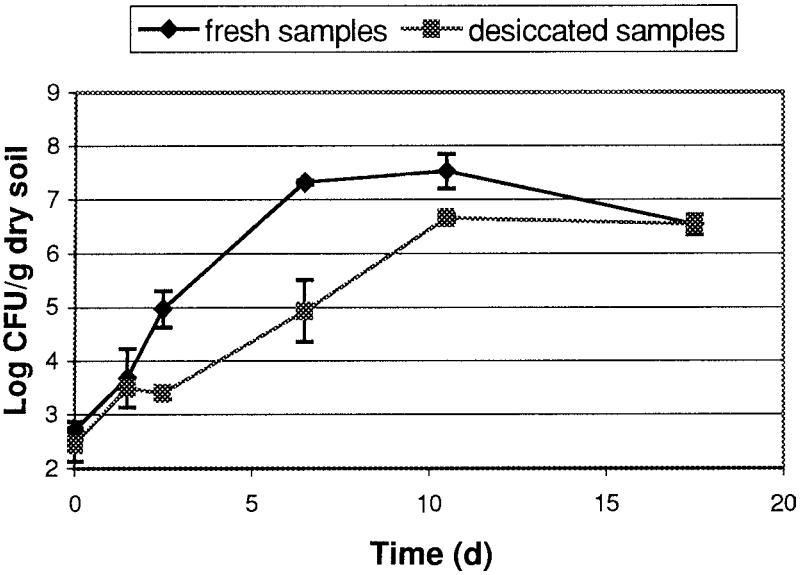

Fig. 3. Evolution of indigenous IPB-degrading population in clay soil in fresh samples and after desiccation.

autochthonous microflora. Because the microcosm itself was not submitted to desiccation stress, it did not exert a selective pressure on the IPB-degrading population. In the second experimental setup, some microcosms were submitted to desiccation in order to measure the ecologic significance of desiccation as a selective agent.

Plasmid Dissemination in BP-Treated Soil Microcosms

In the second approach, a broad host range IncP plasmid harboring a natural catabolic transposon (RP4::Tn4371) was used to study plasmid dissemination as an alternative bioaugmentation approach. This plasmid was previously transferred in Gram-positive *Arthrobacter* bacteria *(16)*, a microorganism usually found in soils *(22)*. The plasmid was originally hosted in *E. coli* strain CM844. This strain was polyauxothrophic and not adapted to the soil. To inoculate the microcosms with an adapted strain, a filter mating was achieved between *E. coli* strain CM844 and an extract of the soil to be treated. Because of its polyauxothrophic character, the donor strain was easily counterselected on selective minimal medium. A transconjugant was isolated on medium 284+tetracycline (50 mg/L) with glucose as the sole source of carbon. This transconjugant was also resistant to kanamycin and ampicillin but did not express the BP degradation property. This enabled easy counterselection of the inoculum in microcosm isolates. The presence of the plasmid in the strain was confirmed by extraction and isolation by agarose gel electrophoresis. The transconjugant was identified by BIOLOG fingerprinting as *P. putida*. A rifampicin mutant of this strain was used to inoculate the microcosms. The clay soil was used in this experiment.

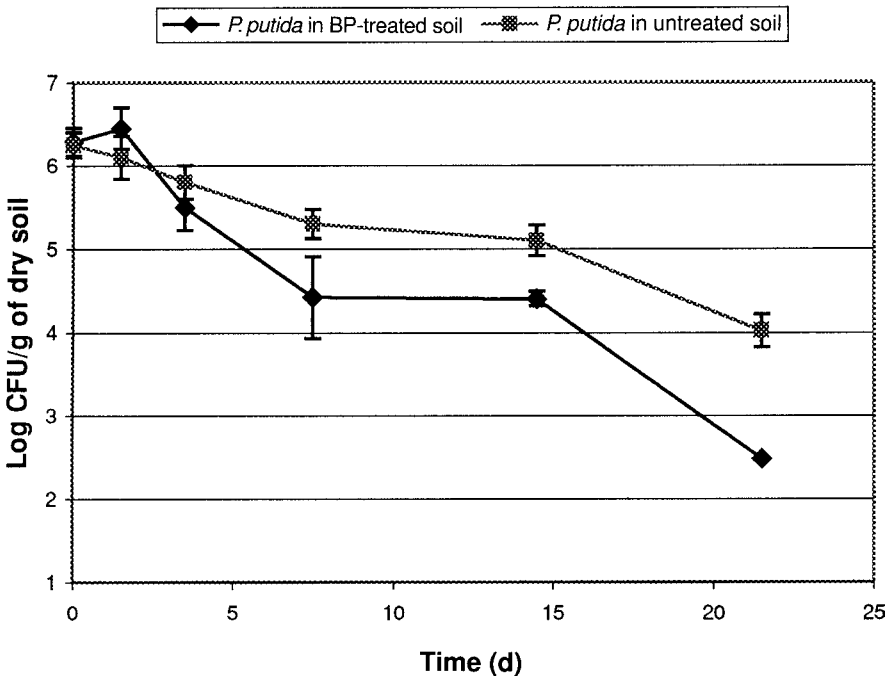

Fig. 4. Fate of the inoculum (*P. putida*) in BP-treated and untreated microcosms under constant-humidity conditions.

To measure the influence of desiccation on microbial ecology in polluted soils, desiccating soil microcosms were set up in parallel with constant humidity systems.

The fate of *P. putida* was monitored in constant-humidity soil (Fig. 4). Its population slowly declined from its original level (2×10^6 CFU/g) to 10^4 CFU/g in untreated soil and to 3×10^2 CFU/g in BP-treated soil after 21 d. This inoculating strain could not maintain its population in the soil. Furthermore, the decline was faster in the BP-amended soil than in the untreated control, which means that it was sensitive to the presence of BP. A BP-degrading population appeared after 12 d in the constant-humidity soil (Fig. 5). Individual colonies of this population were collected and further analyzed. They exhibited resistance to tetracycline, kanamycin, and ampicillin, which is a good indication that these strains are transconjugants. They were identified by BIOLOG fingerprinting as *Pseudomonas fluorescens*, *Acinetobacter johnsonii*, and *Sphingobacterium* sp. RP4 is an IncP plasmid. These plasmids are well expressed in γ-proteobacteria *(23)*, to which the isolated strains belonged.

In the inoculated soil in which transconjugants were found, a decrease of 20% in the BP concentration was measured after 30 d as compared with the uninoculated soil in which BP concentration remained unchanged after the same time. The lack of any addition of nutrients could have limited the biomass development and blocked the BP degradation in this experiment.

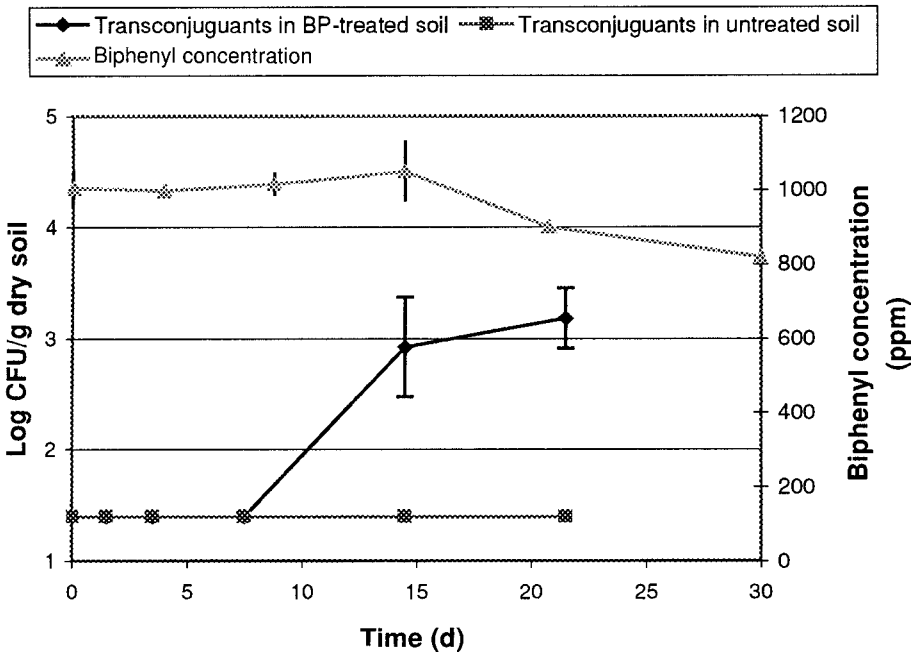

Fig. 5. Fate of transconjugants in BP-treated and untreated soil under constant-humidity conditions and BP concentrations.

In parallel, microcosms were submitted to desiccation. The *P. putida* population was monitored in BP-treated and nontreated desiccating microcosms (Fig. 6). Soil water content was measured daily.

The overall behavior of the inoculum was the same as in constant-humidity soil. Its population declined with time and the rate of mortality was higher in BP-treated soil than in untreated soil. The rate of disappearance of the starter culture over the duration of the experiment was higher in the desiccating microcosm than in the constant-humidity microcosm. However, as the soil began to desiccate, the population of *P. putida* rose 10-fold. This could be owing to the decrease in competition under these conditions. When the soil was rehydrated, the competition became more important again and the decline in the inoculum accelerated. This reflected the upset effect of desiccation on the microbial ecology of the soil.

Transconjugants in the inoculated BP-treated soil appeared after 5 d, but desiccation of the soil slowed the rate of growth of this population (Fig. 7). It increased again when the soil was rehydrated. The second cycle of desiccation made this population disappear under the limit of detection and it reappeared on rehydration. Desiccation upset the development of transconjugants. Transconjugants developed faster in desiccating soil than in constant-humidity soil, also likely owing to decreased competition.

In both soil conditions (desiccating or constant humidity), no BP-degrading population was observed in noninoculated soil. As opposed to IPB-dioxygenase activity, no intrinsic BP-degrading activity was present in

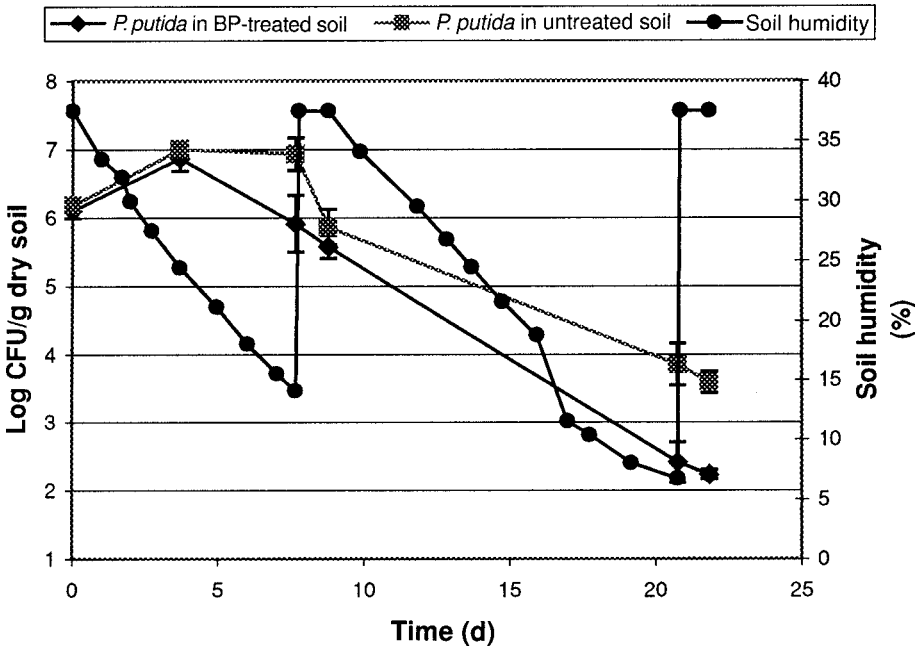

Fig. 6. Fate of the inoculum in desiccating BP-treated and untreated soil microcosms and soil humidity.

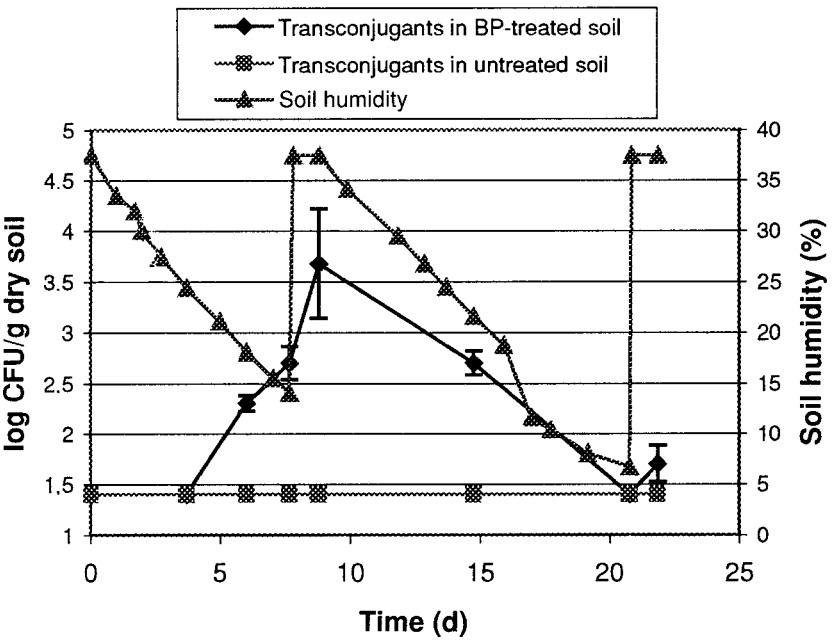

Fig. 7. Fate of transconjugants in desiccating BP-treated and untreated soil microcosms.

Table 1
Drought Tolerance of Transconjugants

Strain	Origin	Survival to desiccation (%)	Gram stain
Escherichia coli CM844	Plasmid collection	0.005	–
Pseudomonas putida C8S3	Inoculum	<0.002	–
Pseudomonas fluorescens	Inoculated BP-treated microcosm under constant humidity	0.0005	–
Sphingobacterium sp.	Inoculated BP-treated microcosm under constant humidity	ND[a]	–
Acinetobacter johnsonii	Inoculated BP-treated microcosm under constant humidity	0.4	–
Alcaligenes denitrificans	Desiccating inoculated BP-treated microcosm	1	–
ND[a]	Desiccating inoculated BP-treated microcosm	3	–

[a]ND, not determined.

the soil. With this pollution, inoculation was necessary. The plasmid dissemination strategy yielded catalytically active, adapted indigenous microorganisms responsible for partial removal of pollution.

The transconjugants from the desiccating microcosms were isolated and further analyzed. They all exhibited resistance to tetracycline, kanamycin, and ampicillin. They were identified with the BIOLOG system as *Alcaligenes denitrificans*. Another transconjugant strain could not be identified. The drought tolerance of these strains was determined to compare with the tolerance of the strains isolated from a constant-humidity microcosm (Table 1). It was 10- to 1000-fold higher than the drought tolerance of the transconjugants isolated from the constant-humidity microcosm. The nature and phenotype of the transconjugants were fundamentally different owing to the desiccation of the soil.

Although the strains from the desiccating soil were more resistant to desiccation than the strains from constant-humidity soil, their survival was still low (about 1%). All isolated transconjugants were Gram-negative strains expressing the RP4 plasmid. Although Gram-positive strains could express this plasmid *(16)* and are more resistant to desiccation *(19)*, no desiccation-tolerant Gram-positive transconjugants could be selected under these conditions.

Conclusion

When an *R. erythropolis* strain was used as the inoculum, it maintained its population in the polluted soil but could not disseminate its plasmid to

indigenous soil microorganisms. The pollution removal activity could be guaranteed by the presence of the inoculating strain in the soil.

When a broad host range plasmid was introduced in the soil by means of a soil isolate, transconjugants appeared rapidly and BP concentration decreased as compared to the uninoculated control. However, some limiting factor blocked the pollution removal process before it was completed. The addition of nutrient could solve this problem. When water availability decreased, the ecology of the system was upset. The transconjugant nature was different and their development was compromised, limiting the biodegradation of the pollutant.

The dissemination of plasmids from technologic strains as remediation strategy should be used with broad host range plasmids. The desiccation tolerance of the inoculum could be an important factor when the treated soil is submitted to hydration variations. This property could guarantee the maintenance of the catalytic activity in the soil when desiccation-sensitive inocula or transconjugants could disappear, jeopardizing the pollution removal procedure.

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