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Survival in Soil of Different Toluene-Degrading *Pseudomonas* Strains after Solvent Shock

MARÍA-JOSÉ HUERTAS, ESTRELLA DUQUE, SILVIA MARQUÉS, AND JUAN L. RAMOS*

Department of Plant Biochemistry, Consejo Superior de Investigaciones Científicas, Estación Experimental del Zaidín, E-18008 Granada, Spain

Received 2 June 1997/Accepted 16 October 1997

We assayed the tolerance to solvents of three toluene-degrading *Pseudomonas putida* strains and *Pseudomonas mendocina* KR1 in liquid and soil systems. *P. putida* DOT-T1 tolerated concentrations of heptane, propylbenzene, octanol, and toluene of at least 10% (vol/vol), while *P. putida* F1 and EEZ15 grew well in the presence of 1% (vol/vol) propylbenzene or 10% (vol/vol) heptane, but not in the presence of similar concentrations of octanol or toluene. *P. mendocina* KR1 grew only in the presence of heptane. All three *P. putida* strains were able to become established in a fluvisol soil from the Granada, Spain, area, whereas *P. mendocina* KR1 did not survive in this soil. The tolerance to organic solvents of all three *P. putida* strains was therefore assayed in soil. The addition to soil of 10% (vol/wt) heptane or 10% (vol/wt) propylbenzene did not affect the survival of the three *P. putida* strains. However, the addition of 10% (vol/wt) toluene led to an immediate decrease of several log units in the number of CFU per gram of soil for all of the strains, although *P. putida* F1 and DOT-T1 subsequently recovered. This recovery was influenced by the humidity of the soil and the incubation temperature. *P. putida* DOT-T1 recovered from the shock faster than *P. putida* F1; this allowed the former strain to become established at higher densities in polluted sites into which both strains had been introduced.

Toluene is widely used as an organic solvent, and its worldwide production is estimated to be more than 80,000 metric tons per year (1). Toluene and other solvents, such as xylenes, benzene, and ethylbenzene, are ubiquitous pollutants (2, 8), and several environmental protection agencies have declared the removal of these pollutants to be a high priority. In many environments indigenous microorganisms are able to remove aromatic hydrocarbons. This is to be expected, as many catabolic pathways for the metabolism of these compounds have been described, particularly in strains belonging to the genus *Pseudomonas*. Gibson et al. (7) reported that *Pseudomonas putida* F1 used a toluene dioxygenase pathway that yielded the corresponding *cis*-glycol, which was subsequently converted into 3-methylcatechol. Worsey and Williams (29) found that the TOL plasmid of *P. putida* mt-2 metabolized toluene via oxidation to benzoate. More recently, three cresol-yielding pathways have been described for the metabolism of this compound. Depending on the position at which the hydroxyl group is incorporated, the pathways are known as the *o*-, *m*-, and *p*-cresol pathways for toluene metabolism (14, 17, 24, 28, 30).

Duetz et al. (5) showed with chemostat experiments that in competition assays performed with pairs of *Pseudomonas* strains that used different toluene degradation pathways, the strain that became dominant was the strain with the higher affinity for toluene. In these experiments *Pseudomonas mendocina* KR1, which metabolized toluene via the *p*-cresol pathway, was the winning strain in competition with *P. putida* mt-2 (bearing the TOL plasmid), *P. putida* F1, or *Pseudomonas cepacia* G4, a strain which metabolized toluene via *o*-cresol. However, in heterogeneous habitats, such as sewage treatment plants, underground waters, and soils, other factors in addition to affinity for a compound are important. These factors include the ability to use multiple substrates simultaneously, the ability to adhere to surfaces and colonize the corresponding niche, and the ability to respond to physicochemical alterations (6, 12, 16, 21). In addition, aromatic hydrocarbons are extremely toxic for microorganisms, because they dissolve in the cell membrane (25). Therefore, a critical issue in the degradation of aromatic hydrocarbons in polluted sites is tolerance to these compounds.

Recently, a number of *Pseudomonas* strains have been shown to be tolerant to organic solvents such as toluene, xylenes, styrene, and others, when the solvents are supplied at supersaturating concentrations in liquid medium (3, 10, 15, 18, 27). Solvent tolerance in bacteria belonging to the genus *Pseudomonas* involves (i) an increase in cell membrane rigidity as a result of increases in both the level of the *trans* isomers of unsaturated fatty acids and the level of cardiolipin, a component of phospholipid head groups (18, 26), and (ii) removal of solvents from membranes via efflux pumps (11, 17).

We report here the solvent tolerance of different toluene-degrading *Pseudomonas* strains and the survival of these organisms in soil after solvent shock. Our results show that the viability of *P. putida* DOT-T1 and F1 was not lost upon heptane shock and propylbenzene shock; however, the viability of these strains was severely affected by toluene shock, although both strains recovered and colonized polluted niches. This recovery was influenced by the soil humidity and the incubation temperature.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in this study are shown in Table 1. Strains were grown on Luria-Bertani (LB) medium or M9 minimal medium supplemented with toluene in the gas phase or 0.5% (wt/vol) glucose in the liquid phase as the C source. Spontaneous rifampin-resistant mutants of all of the *Pseudomonas* strains were isolated. The rifampin resistance marker was introduced into the strains because no rifampin-resistant bacteria were recovered from the fluvisol soil used in this study (see below). When required, antibiotics were added to the culture medium as follows: ampicillin, 50 μg/ml; chloramphenicol, 30 μg/ml; kanamycin, 25 μg/ml; rifampin, 20 μg/ml; and streptomycin, 50 μg/ml.

* Corresponding author. Mailing address: Estación Experimental del Zaidín, Apdo. 419, 18008 Granada, Spain. Phone: 34-58-121011. Fax: 34-58-129600. E-mail: jlramos@eez.csic.es.

TABLE 1. Catabolic pathways and tolerance to organic solvents of the strains used in this study

Strain	Catabolic pathway	Marker(s)[a]	Reference(s)
P. putida DOT-T1	Toluene dioxygenase	Rif^r, Cm^r	13, 17
P. putida DOT-T1-Km	Toluene dioxygenase	Cm^r, Km^r Mini-Tn*5*-Km derivative of DOT-T1	13
P. mendocina KR1	*p*-Cresol	Rif^r	28
P. putida F1	Toluene dioxygenase	Rif^r, Cm^r	6
P. putida F1-Te	Toluene dioxygenase	Te^r, mini-Tn*5*-Te derivative of F1	This study
P. putida EEZ15(pWWO-EB62)	Benzyl alcohol	Ap^r, Km^r	19

[a] Ap^r, Cm^r, Km^r, Rif^r, and Te^r, resistance to ampicillin, chloramphenicol, rifampin, kanamycin, and telurate, respectively.

Mutagenesis of *Pseudomonas* strains by mini-Tn*5* transposons. Triparental matings involving *P. putida* F1 as the recipient, *Escherichia coli* CC118λ*pir* harboring the suicide vector pJMSB4 as the mini-Tn*5* transposon donor strain (23), and *E. coli* HB101(pRK600) as the helper strain were performed as described by de Lorenzo and Timmis (4). Transconjugants of *P. putida* F1 were selected on minimal medium plates containing toluene in the gas phase as the sole C source and supplemented with telurate (20 μg/ml), the selection marker in the pJMSB4 mini-transposon. *P. putida* DOT-T1 was labelled with mini-Tn*5*Km basically as described above except that the donor strain was *E. coli* CC118λpir(pUT-Km) (4) and the selection medium contained kanamycin instead of telurate.

Soil microcosm assays. For soil assays we used a fluvisol soil (6.4% [wt/wt] organic matter, 3.5% [wt/wt] $CaCO_3$). The soil was sieved through a 4-mm mesh, and the humidity was usually adjusted to 30% of the field water-carrying capacity (16). Pots containing 90 g of soil were incubated at room temperature (17 to 20°C) or at the temperature indicated below. Bacteria were uniformly distributed in the soil. The number of CFU per gram of soil was determined as described by Ramos et al. (16) in minimal medium containing toluene as the sole C source and the appropriate antibiotics.

RESULTS

Solvent tolerance of several toluene-degrading strains in liquid medium. Although many aromatic hydrocarbon-degrading strains have been isolated (8), they are usually solvent sensitive, and degradation of aromatic hydrocarbons occurs only when these compounds are supplied at low concentrations (8, 25). However, a number of toluene-resistant *Pseudomonas* strains, some of which were unable to use this aromatic compound as a sole C source, were recently isolated (3, 10, 17, 27). The results described above and other results from the four laboratories that isolated these strains suggested that metabolism of monocyclic aromatic hydrocarbons and tolerance to organic solvents are unrelated characteristics (3, 10, 16, 18, 25, 27). To further explore this possibility, we tested the solvent tolerance of four gram-negative toluene degraders, *P. putida* DOT-T1, EEZ15(pWW0-EB62), and F1 and *P. mendocina* KR1, which metabolized toluene by using the catabolic pathways shown in Table 1. The degrees of tolerance to organic solvents with $\log P_{o/w}$ values between 4.5 and 2 (heptane [$\log P_{o/w} = 4.5$], propylbenzene [$\log P_{o/w} = 3.5$], toluene [$\log P_{o/w} = 2.5$], and benzene [$\log P_{o/w} = 2.0$] [$\log P_{o/w}$ values are from references 10 and 25) and to different amounts of solvents (0 to 10%, vol/vol) were tested in LB liquid medium cultures. In these assays the cultures were incubated under aerobic conditions at 30°C with moderate shaking (100 strokes per min). All four strains grew in LB medium supplemented with 0, 0.1, 1, and 10% (vol/vol) heptane (data not shown), and none of the strains grew in the presence of benzene ($\log P_{o/w} = 2.0$) supplied at concentrations of 0.2 to 10% (vol/vol) (data not shown). The growth of the four strains when propylbenzene was added to the liquid phase was also assayed. *P. putida* DOT-T1 tolerated concentrations of this aromatic compound greater than 10% (vol/vol), whereas *P. putida* F1 and EEZ15-Km tolerated 1% (vol/vol) propylbenzene and *P. mendocina* KR1 tolerated only 0.1% (vol/vol) propylbenzene (data not shown). *P. putida* DOT-T1 was also shown to be tolerant to toluene concentrations greater than 10% (vol/vol), in agreement with previous findings (16). *P. putida* F1 and EEZ15-Km (pWW0) and *P. mendocina* KR1 tolerated up to 0.1% (vol/vol) toluene in the culture medium. These results corroborate the finding that solvent tolerance is a strain characteristic rather than a property associated with the metabolism of a compound by a microorganism.

Survival of toluene degraders in soils after organic solvent shock. To study the response of the *Pseudomonas* strains described above to solvent shocks in a different context, we determined survival in a fluvisol soil from the Granada, Spain, area before and after solvent shocks. This fluvisol soil is relatively rich in organic matter, and previous studies have shown that certain *Pseudomonas* strains are able to become established in it at a level of 10^5 to 10^6 CFU per g of soil (16, 22).

The four strains described above were introduced into unsterile soil at a density of approximately 10^6 CFU per g of soil, and the number of cells of each strain was followed with time in each of the microcosms. In short-term assays (30 days) the number of CFU of each *P. putida* strain introduced per g of soil remained relatively constant with time. In contrast, *P. mendocina* KR1 was no able to become established in this soil, and this strain could not be recovered after 30 days (data not shown). For this reason in subsequent studies we used only the three *P. putida* strains.

To soil microcosms into which the *P. putida* strains had been introduced at a density of about 10^6 CFU/g we added 0.1 to 10% (vol/wt) heptane or 0.1 to 10% (vol/wt) propylbenzene. After heptane or propylbenzene was added, the three strains survived well. The number of CFU per gram of soil remained relatively constant (range, 10^7 to 10^6 CFU/g of soil) throughout the assay (Fig. 1).

To soil microcosms into which we introduced the different *P. putida* strains toluene was also added to an initial concentration of 0, 0.1, 1, or 10% (vol/wt). Then the number of cells of each strain was followed. The addition of 0.1% (vol/wt) toluene had no significant effect on the survival of any of the strains (Fig. 2). However, the strains were extremely sensitive to toluene shock at a toluene concentration of 1% (vol/wt) or higher. Immediately after the addition of 1% (vol/wt) toluene the number of *P. putida* EEZ15(pWW0-EB62) cells decreased by more than 6 orders of magnitude, and after 24 h no bacteria were recovered from the soils (data not shown). For *P. putida* F1 and DOT-T1 the initial decrease was also large, and the number of organisms decreased to 10^2 to 10^4 CFU/g of soil. Then the number of organisms increased to about 10^5 to 10^6 CFU/g of soil (Fig. 1 and 2). When 10% (vol/wt) toluene was added, the number of CFU per gram of soil for all three introduced strains was below our detection limit. Since our detection limit was about 100 CFU/g of soil, we attempted to determine whether viable cells remained in the preparations. One gram of soil was suspended in 10 ml of LB medium

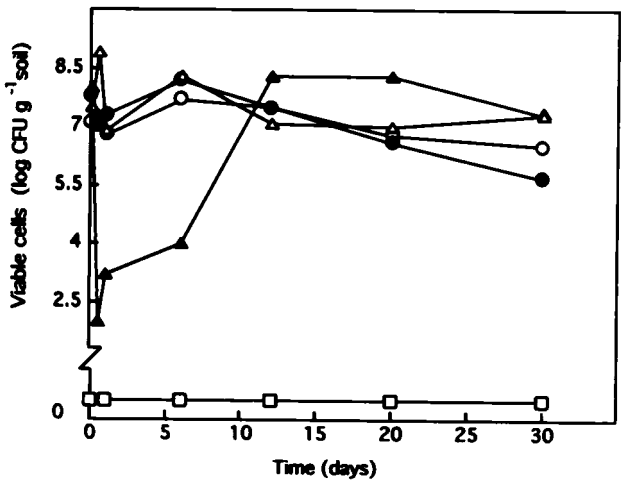

FIG. 1. Survival in soil of *P. putida* F1 after solvent shock. About 10^7 CFU of *P. putida* F1 per g of soil was introduced into independent pots to which 10% (vol/wt) heptane (●), 10% (vol/wt) propylbenzene (△), 1% (vol/wt) toluene (▲), 1% (vol/wt) benzene (□), or nothing (○) was added. At different times the number of CFU per g of soil was determined.

containing the antibiotics to which each strain was resistant, and after growth for 20 h at 30°C serial dilutions of the cultures were spread onto minimal medium containing toluene as a C source and the appropriate antibiotics. Recovery of cells was taken as evidence that viable cells were present, whereas the absence of recovery was considered to indicate complete disappearance of cells. *P. putida* EEZ15(pWW0-EB62) disappeared completely from soils after a 10% (vol/wt) toluene shock, whereas a few *P. putida* DOT-T1 and F1 cells remained viable, but the cell density was very low. The soils were incubated further, and the numbers of CFU of *P. putida* DOT-T1 and F1 per g of soil were followed with time. Both strains were able to multiply in the soils, and they reached densities of about 10^5 to 10^6 CFU per g of soil 30 days after the addition of toluene (Fig. 2).

We previously found that the survival of certain *P. putida* strains in soils is influenced by the humidity of the soil and the

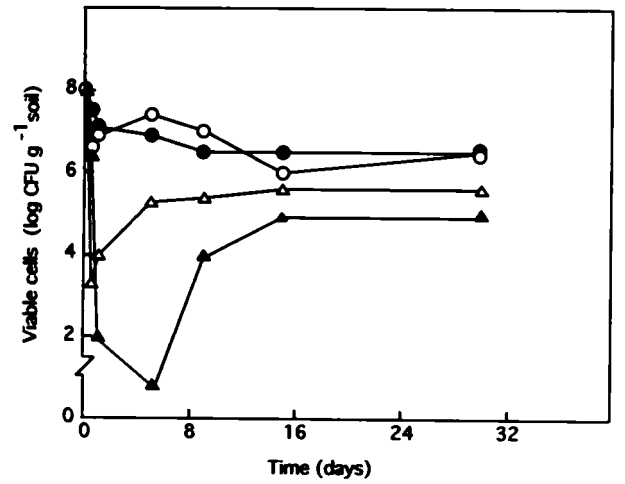

FIG. 2. Survival in soil of *P. putida* DOT-T1 after toluene shock. About 10^8 CFU of *P. putida* DOT-T1 per g of soil was introduced into independent pots, which were supplemented or not supplemented with toluene. Symbols: ○, no addition; ●, 0.1% (vol/wt) toluene; △, 1% (vol/wt) toluene; ▲, 10% (vol/wt) toluene. At different times the number of CFU per g soil was determined.

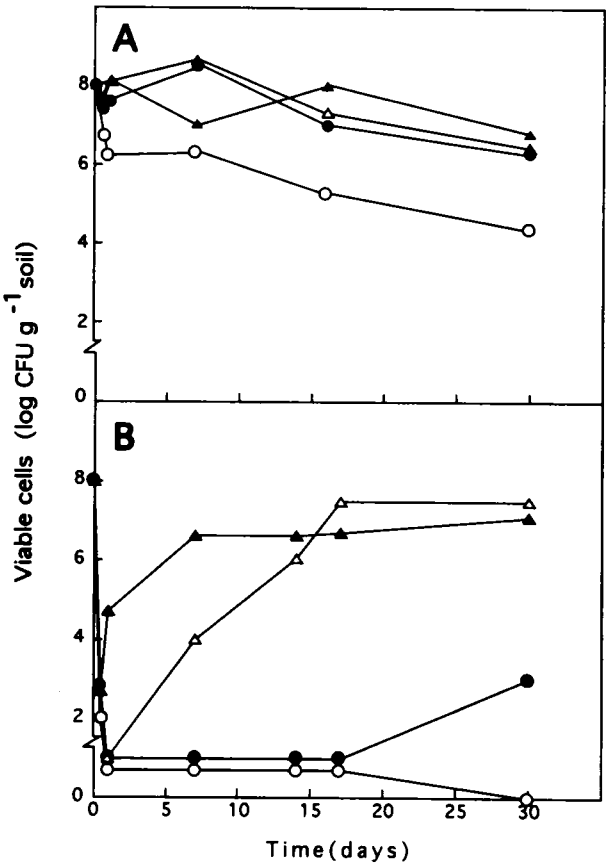

FIG. 3. Survival of *P. putida* DOT-T1 in a fluvisol soil maintained at different humidities in the absence of toluene (A) or after a 1% (vol/wt) toluene shock (B). The humidity of the soil was adjusted to 5% (○), 15% (●), 30% (△), or 45% (▲) of the field carrying capacity before bacteria were introduced. Then 10^8 CFU per g of soil was added, and 1% (vol/wt) toluene was added to one set of pots (B). At different times the number of CFU per g of soil was determined.

incubation temperature (16). We assayed how these two factors influence the multiplication in soils of *P. putida* DOT-T1 and F1 cells after toluene shock. As a control, cells were incubated under the same conditions, but no toluene was added. In the absence of toluene, survival of these two strains was high when the water content of the soil was 15 to 45% of the field carrying capacity, but survival decreased (by about 2 log units) when the water content was lower (Fig. 3A). After the addition of toluene, regardless of the soil water content, the number of viable cells decreased by 5 to 6 log units. No *P. putida* DOT-T1 or F1 cells were recovered from soil with a humidity below 15% of the field carrying capacity, whereas cells were recovered from soils with humidities in the range from 15 to 45% of the field carrying capacity (Fig. 3B). The lag time required for recovery was influenced by the soil humidity; the higher the humidity, the faster the recovery (Fig. 3B). At a humidity of 45%, cells were present at a relatively high density 24 h after the shock, whereas at a humidity of 30%, cells were found 1 week after the shock and at a humidity of 15% cells were found at a density greater than 10^2 CFU per g of soil 15 days after the toluene shock. When the soil humidity was about 5% of the field carrying capacity, we never found more than 10^2 CFU per g of soil, and eventually cells could not be recovered (Fig. 3B).

At a humidity of 30% of the field carrying capacity, temperature also affected recovery. Recovery was best at 25 to 30°C, and higher or lower incubation temperatures decreased the recovery rate. At 4 and 37°C no cells were recovered (Table 2).

TABLE 2. Effect of incubation temperature on recovery of *P. putida* DOT-T1 and F1 after toluene shock in soil[a]

Incubation temp (°C)	Density (CFU/g of soil)	
	P. putida DOT-T1	*P. putida* F1
4	$<10^2$	$<10^2$
18	10^4	10^3
25	4×10^5	2×10^3
30	2×10^5	5×10^3
37	$<10^2$	$<10^2$

[a] *P. putida* DOT-T1 and F1 were introduced at a density of 10^6 CFU per g of fluvisol soil which contained water at a level of 30% of the field carrying capacity. Then 10% (vol/wt) toluene was added, and samples were incubated for 8 days at different temperatures. Serial dilutions were then plated onto selective culture medium, and the numbers of CFU per g of soil were determined.

In these studies the loss of viability by *P. putida* DOT-T1 and F1 in soils upon toluene shock may have been influenced not only by the addition of the solvent, but also by the fact that cells might not have had enough time to become established in the soil. We therefore tested whether introduction of *P. putida* DOT-T1 or F1 into soils before the toluene shock had a beneficial effect on tolerance. Incubation of *P. putida* DOT-T1 for 2 or 14 days before the toluene shock had no beneficial effect on tolerance to this compound; immediately after the addition of the aromatic hydrocarbon the number of CFU per g of soil decreased by between 4 and 5 orders of magnitude (data not shown). Similar results were obtained when the assays were done with *P. putida* F1 (data not shown).

Given that the levels of toluene tolerance of *P. putida* F1 and EEZ15(pWW0-EB62) in soil were greater than the levels of tolerance in liquid medium, we also tested whether *P. putida* DOT-T1 and F1 could tolerate benzene shocks. After the addition of 1% (vol/wt) benzene to the soil, the number of CFU decreased to below our detection limits, and cells could not be recovered even after enrichment as described above (data not shown).

Establishment in soil of different strains able to degrade toluene after toluene shock. To determine how the two toluene-tolerant strains behaved when they were introduced simultaneously into soil in the absence and in the presence of toluene, the strains were labelled with different resistance markers so that they could be easily distinguished from each other and from indigenous soil microbiota. We generated kanamycin-resistant and telurate-resistant derivatives of *P. putida* DOT-T1 and F1, respectively. When these labelled strains were introduced independently into soils, they behaved like the unmarked wild-type strains described above (data not shown). The two labelled strains were introduced into soil microcosms at a ratio of 1:1 and at a cell density of either 10^8 or 10^6 CFU per g of soil with and without 10% (vol/wt) toluene.

In nonpolluted soils with a humidity of 30% of the field carrying capacity, no apparent competition between the strains was observed (Fig. 4A). Regardless of the initial cell load, both strains tended to become established at a level of approximately 10^6 to 10^7 CFU per g of soil (Fig. 4A).

The addition of 10% (vol/wt) toluene to soils inoculated with 10^8 CFU of the two strains per g of soil led to an immediate decrease of 5 to 6 log units in the number of CFU per g of soil. Twenty-four hours later we detected between 10^2 and 10^3 CFU of each strain per g of soil (Fig. 4B). *P. putida* DOT-T1 recovered faster than strain F1; *P. putida* DOT-T1 reached a density of about 10^5 CFU per g of soil 3 days after the shock, whereas the density of *P. putida* F1 remained about 10^2 CFU per g of soil and reached 10^5 CFU per g of soil after 30 days. After 30 days the density of *P. putida* DOT-T1 was about 10^7 CFU per g of soil (Fig. 4B).

DISCUSSION

Toluene and related aromatic hydrocarbons are highly toxic for living organisms because the preferential partitioning of these compounds in cell membranes disrupts the membrane structure, which leads to cell death (25). Toluene-degrading microbes are not immune to this general toxic effect and are sensitive to toluene shocks. This is particularly relevant for *P. putida* EEZ15 (a *P. putida* mt-2 derivative), *P. putida* F1, and *P. mendocina* KR1, which in liquid culture medium were not able to grow when toluene was present at a concentration of 0.2% (vol/vol). In contrast to these sensitive strains is *P. putida* DOT-T1, one of the two strains described so far that are able to tolerate high concentrations of toluene in liquid medium (10, 17).

Our results showed that the three *P. putida* strains studied were able to become established in nonpolluted soil at levels of approximately 10^5 to 10^6 CFU per g of soil, whereas *P. mendocina* KR1 was not able to become established in this soil. Also, *P. mendocina* KR1 was the winning strain in competition assays in C-limited chemostats (5). The inability of *P. mendocina* KR1 to become established in soil makes this strain the least competitive. This finding suggests that the results of com-

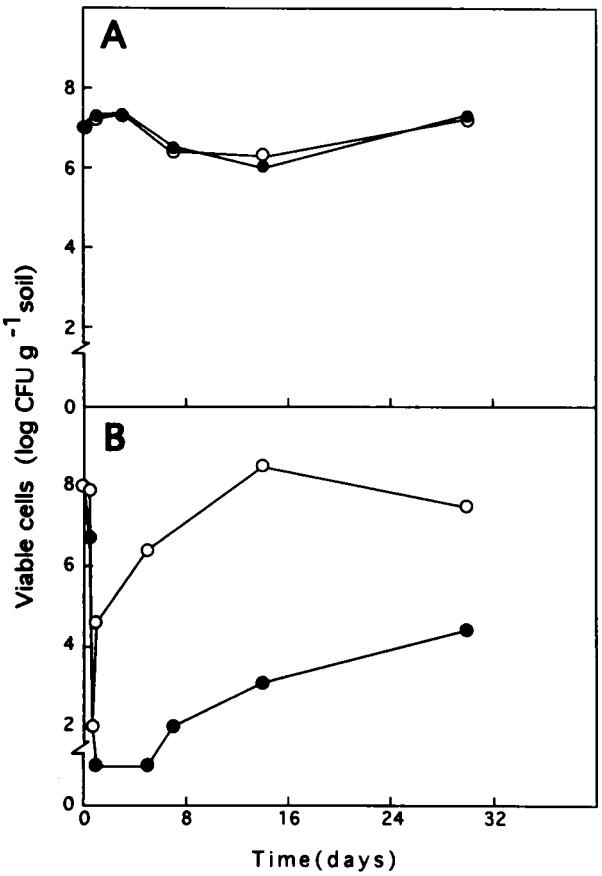

FIG. 4. Survival of *P. putida* DOT-T1 and F1 in a fluvisol soil without toluene (A) and after a 10% (vol/wt) toluene shock (B). A telurate-resistant derivative of *P. putida* F1 (●) and a kanamycin-resistant derivative of *P. putida* DOT-T1 (○) were introduced into the same soil at a density of about 10^8 CFU per g of soil. The number of CFU per g of soil was determined at different times.

petition assays performed in the laboratory cannot always be extrapolated to a complex environment, such as soil.

Compared with liquid cultures, *P. putida* EEZ15(pWW0-EB62) and F1 tolerated greater amounts of organic solvents in soil. Both of these strains tolerated shocks consisting of 10% (vol/wt) propylbenzene in soil, whereas this concentration prevented cell growth in liquid medium. Furthermore, *P. putida* F1 tolerated shocks consisting of 1 and 10% (vol/wt) toluene, in contrast to the results observed in liquid cultures. This might reflect the limited access of the solvent to the cells in soil either because of sequestration of part of the toluene by soil particles or because of the absence of homogeneous mixing of toluene in soil, in contrast to the situation in agitated liquid culture medium.

P. putida DOT-T1 has been shown previously to tolerate high concentrations of toluene in liquid medium (17), and in agreement with this observation is our finding that this strain tolerates shocks of propylbenzene and toluene in liquid medium and in soil. However, the number of CFU of *P. putida* F1 and DOT-T1 per g of soil after shocks consisting of 1% (vol/wt) (or more) toluene deserves attention. We found that after the addition of toluene, the number of CFU of both *P. putida* F1 and DOT-T1 per g of soil decreased by about 5 log units and then increased. This suggested that most cells were not able to tolerate the initial solvent shock and that those cells able to respond multiplied and colonized the niche. The recovery of the more tolerant strain, *P. putida* DOT-T1, was faster than the recovery of *P. putida* F1. In assays performed with both strains in the same soil, *P. putida* DOT-T1 colonized the site more rapidly than *P. putida* F1 (Fig. 4B).

The rate of recovery of both strains after toluene shock was influenced by the soil humidity and the incubation temperature. Both strains recovered best at a humidity between 30 and 45% of the soil carrying capacity; lower water levels resulted in slower recovery or no recovery. Humidities below 5% of the field carrying capacity in the soil reduced the survival of *P. putida* strains, whereas the survival was optimal at a humidity of 30% of the field carrying capacity (16). The faster recovery at the higher humidity was unexpected, as the presence of water probably facilitated the movement of unbound toluene. The faster recovery probably reflected the fact that under these conditions the metabolic activity of the cells was higher, which helped to remove toluene from the cell membrane, to increase cell membrane rigidity, or to metabolize toluene (9, 11, 18, 27). The activity of the *Pseudomonas* strains was optimal in the temperature range from 25 to 30°C, which should favor metabolic activities and therefore solvent exclusion. In general, our results show that *Pseudomonas* strains are more resistant to solvents in soils than in liquid culture medium; this may explain why these microbes are able to deal with these pollutants in biofilms and soils (12, 20). However, the level of tolerance in soil is related to the level of tolerance in liquid; the higher the tolerance in liquid, the faster the recovery of the strain in soil after toluene shock. Therefore, in sites heavily polluted by aromatic hydrocarbons, solvent-tolerant strains would be expected to become established first, to colonize the site, and to become predominant in the removal of these compounds.

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