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Tolerance to Sudden Organic Solvent Shocks by Soil Bacteria and Characterization of *Pseudomonas putida* Strains Isolated from Toluene Polluted Sites

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Upon a sudden addition of toluene to soil (10% vol/wt) a significant proportion (about 1%) of the soil indigenous bacteria survived, the survival ones recolonized the soil to reach a high cell density. Two bacterial strains called MTB5 and MTB6, which use toluene as the sole carbon and energy source, were isolated from the soil polluted with this large amount of toluene after 15 days incubation. The strains were taxonomically identified as *Pseudomonas putida* sensu stricto based on 16S rRNA sequences. Another strain (*P. putida* R1) isolated from a biofilm washed with toluene-polluted waters was also studied regarding toluene degradation and toluene tolerance. All three strains used the toluene dioxygenase pathway for toluene metabolism. Strain *P. putida* MTB6 was able to grow on liquid medium with 10% (vol/vol) toluene, whereas the other two strains did not grow at concentrations of toluene higher than 0.1% (vol/vol). All three strains grew in the presence of 1% (vol/vol) ethylbenzene. After exposure to aromatic hydrocarbons, all three strains isomerized the *cis*-fatty acid lipids C16:1ω9 and C18:1ω11 to their trans isomers. This change led to the rigidification of the cell membrane. All three strains exhibited efflux pumps for the removal of toluene from the cell membrane, but the efflux systems appeared to be more efficient in the MTB6 strain based on its higher tolerance to toluene and their increased capacity to remove toluene from the cell membranes. The solvent-tolerant MTB6 strain established best in soils polluted with toluene and mineralized this aromatic hydrocarbon in situ.

Introduction

Toluene is widely used as an organic solvent, and it is considered a ubiquitous pollutant (*1, 2*). Toluene is toxic for eukaryotic and prokaryotic organisms because it partitions in cell membranes, where it provokes pores and leads to the collapse of the cell membrane potential (*3*). For these reasons several environmental protection agencies have declared the removal of toluene to be of high priority. Several catabolic pathways for the metabolism of toluene have been described, particularly in strains belonging to the genus *Pseudomonas*. Gibson et al. (*4*) 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 (*5*) 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 (*6*−*9*). The *o*- and *m*-cresol pathways canalize the hydroxylated compounds to 3-methylcatechol, whereas the *p*-cresol pathway yields 3,4-dihydroxybenzoate as the distal product.

In heterogeneous habitats (i.e. sewage treatment plants, underground waters, and soils) biodegradation of a given pollutant depends on the bioavailability of the compound, the ability of the microorganism(s) to use multiple substrates simultaneously, their ability to adhere to surfaces and colonize the corresponding niche, and their ability to respond to physicochemical alterations (*10*−*13*). In the case of toxic chemicals (i.e., aromatic hydrocarbons), tolerance to the compound is a critical issue for the degradation of the pollutant.

Recently, a number of *Pseudomonas* strains have been shown to be tolerant to organic solvents such as toluene, xylenes, styrene, and others (*14*−*19*). The key elements in solvent tolerance in bacteria belonging to the genus *Pseudomonas* involve (i) an increase in cell membrane rigidity mainly as a result of an increase in the level of the trans isomers of unsaturated fatty acids (*18*−*21*) and (ii) the removal of solvents from membranes via efflux pumps (*16*−*24*). However, the response of indigenous soil bacteria to a sudden toluene shock has not yet been evaluated in detail. In this study we show that soil bacteria are able to survive toluene shocks. Two fast-growing bacteria from these soils were identified as belonging to the genus *Pseudomonas*; one of them tolerated toluene-shocks in liquid medium, whereas the other did not. This suggests that heterogeneity of the soil matrix may have a protective effect against solvent shock.

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 on M9 minimal medium supplemented with toluene in the gas phase or with glucose (0.5% [wt/vol]) in the liquid phase as the C- and energy source (*25, 26*). Spontaneous rifampicin-resistant mutants of all of the *Pseudomonas* strains were isolated after spreading 0.1 mL of an overnight culture with $6 \pm 2 \times 10^8$ colony forming units (CFU)/mL on an LB plate supplemented with 20 μg/mL rifampicin. The rate of spontaneous rifampicin resistant

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TABLE 1. Strains Used in This Study[a]

strains	characteristics	ref
P. putida DOT-T1E	Rif^R, Tol^R, Tol^+	(*25*)
P. putida KT2440	Tol^S, Tol^-	(*30*)
P. putida MTB5	Tol^S, Tol^+	this study
P. putida MTB6	Tol^R, Tol^+	this study
P. putida R1	Tol^S, Tol^+	(*11*)

[a] Rif^R stands for tolerance to rifampicin. Tol^R and Tol^S stand for tolerance and sensitivity to 1% (vol/vol) toluene in liquid medium, respectively. Tol^+ indicates the ability to use toluene as the sole C- and energy source.

mutants was $4 \pm 2 \times 10^{-7}$. The rifampicin resistance marker was introduced into the strains because no rifampicin-resistant bacteria were found in the soils used in this study (see below). When required, antibiotics were added to the culture medium as follows: ampicillin (Ap), 50 μg/mL; chloramphenicol (Cm), 30 μg/mL; kanamycin (Km), 25 μg/mL; rifampicin (Rif), 20 μg/mL; streptomycin (Sm), 50 μg/mL; and tetracycline (Tc), 20 μg/mL.

Soil Microcosm Assays. For soil assays we used three soils that had no known history of hydrocarbon exposure, and whose characteristics are as follows: Fluvisol soil-10 (3.2% [wt/wt] organic matter, pH 7.2); Cambisol soil-1 (0.6% [wt/wt] organic matter, pH 7.5); and agricultural soil from the garden of the Estación Experimental del Zaidín (1.8% [wt/wt]organic matter, pH 8.1). Each soil was sieved through a 4-mm mesh, and the humidity was usually adjusted to 50% of the field water-carrying capacity (12). 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 on selective medium as described (12).

Preparation of Cell-Free Extracts and Determination of Enzyme Activities. *Pseudomonas putida* strains were grown on minimal medium with glucose in the absence and in the presence of toluene in the gas phase until they reached the late logarithmic phase (culture turbidity 1.0−1.5). Cell-free extracts of *Pseudomonas putida* strains were prepared by disrupting cells at 120 MPa in a French press as described (*25*). Catechol 2,3-dioxygenase was determined by following the formation of 2-hydroxymuconic acid semialdehyde at 375 nm ($E = 33\,000$ mol^{-1} cm^{-1}) from catechol (*27*).

Polarographic Studies. Oxygen consumption by whole-cell suspensions was determined with a Clark-type oxygen electrode. *Pseudomonas putida* cells were grown on minimal medium with toluene in the gas phase, harvested by centrifugation, washed twice on minimal medium without a C-source, and suspended in this medium at a cell density of about 10^8 CFU/mL. Cells were transferred to the chamber of the oxygen electrode maintained at 30 °C. Oxygen consumption was recorded for 10 min to determine the endogenous respiration rate, then the substrate, dissolved in *N*,*N*-dimethylformamide, was added at a final concentration of 0.5 mM, and oxygen consumption was recorded for at least 5 min (*N*,*N*-dimethylformamide was not a substrate for the different strains used in this study to determine substrate-specific oxygen respiration rate.)

Analysis of Phospholipids. Phospholipids were extracted and processed as described before (*28, 29*). Fatty acids were identified by gas chromatographic-mass spectrometry (*18*).

Incorporation of 1,2,4-[^{14}C]-Trichlorobenzene into Cell Membranes. Cells growing exponentially in LB medium were harvested by centrifugation, washed in the same medium, and suspended in 2.5 mL of LB to a cell density of about 1 at 660 nm. Then the cells were incubated for 10 min at 30 °C and exposed to 2 μCi 1,2,4-[^{14}C]-trichlorobenzene. After 10 min, 250 μL of the cell suspension was filtered through a 0.45 μm Millipore filter and washed with 2 mL of LB medium. The filters were dried, and the ^{14}C associated with the cell pellet (disintegrations/min) was determined in a Packard Radiochemical detector.

Recombinant DNA Techniques. Isolation of plasmid DNA preparation of *P. putida* total DNA, DNA restriction, and southern blot analysis were done by standard methods (*26, 30*). The DNA encoding the 16S rRNA of different *Pseudomonas* strains was amplified by using the forward oligonucleotide 5′-AGAGTTTGAT(C or T)(A or C)TGGCTCAG-3′ [this primer hybridizes at the complement of positions 8−27 according to the *E. coli* 16S rRNA gene sequence numbering] and the reverse primer 5′-CA(G or T)AAAGGAGGTGATCC-3′ [this primer hybridizes at positions 1525−1541]. The intergenic 16S-23S DNA of indigenous soil bacteria was amplified by using the forward primer 5′-GGGCCCGCACAAGCGGTG-3′ and the reverse primer 5′-CTTTCCCTCACGGTAC-3′ (*31*). Amplification of the *todC1* gene encoding for one of the toluene dioxygenase subunits and the *todE* gene encoding catechol 2,3-dioxygenase of different *Pseudomonas* strains was done by PCR. For *todC1* the following oligonucleotides were used: (5′-CAATGAATCAGACCGACAC-3′ [this primer hybridizes at the complement of positions 2716−2734 according to the *P. putida* DOT-T1E *todC1* sequence numbering PPUY18275] and 5′-CAGCGTGTCGCCTTCAG-3′ [this primer hybridizes at positions 4069 to 4053 of the same sequence]). For *todE* the oligonucleotides 5′-GTCATGAG-CATTCAAAGATTGGGCT-3′ (this primer hybridizes at the complement of position 7146−7169 according to the *P. putida* DOT-T1E *todE* sequence numbering PPUY18275) and 5′-CAGGCGGGCGCCTGGAACTT-3′ (this primer hybridizes at positions 8003 to 8022 in the above sequence) were used. DNA was sequenced on both strands. Amplification of the *ttgB* gene encoding the efflux element of the RND TtgABC pump involved in toluene efflux was done by PCR using the primers 5′-AGCTGAACAAGTTCCAGCTGACCCC-3′ and 5′-GGCGGTGGACACTTCCGTCT-3′. These primers hybridize at the positions 1741−1765 and at the complement positions 1920−1940 of the *P. putida ttgB* gene. Amplification of the *ttgD* gene encoding the periplasmic fusion protein of the solvent efflux RND TtgDEF pump was done by PCR using the primers 5′-GATCGACGACCTGAGCGATGGCCGC-3′ and 5′-CGCTGCCAGGCGTCCTGACCCAAGGCA GTTT-3′ (these primers hybridize at positions 1 to 25 and at the complement positions 1009 to 1040 in the sequence Y19106) as described by Mosqueda and Ramos (*44*).

Phylogenetic Reconstruction. New sequences were added to an alignment of about 10 000 homologous bacterial 16S rRNA primary structures (*32*) by using the aligning tool of the ARB program package (*33*). Phylogenetic trees were constructed using subsets of data that included outgroup reference sequences as well as representative sequences of members of the gamma subclass of *Proteobacteria* (*32*). Topologies were evaluated by using the different approaches to elaborate a consensus tree (*34*).

Results and Discussion

Coexistence of Solvent-Tolerant and Solvent-Sensitive Strains in Soils. We simulated an accidental discharge of toluene onto the soil surface to analyze the immediate effect on the survival of soil bacteria. We added 0.1% (vol/wt), 1% (vol/wt), and 10% (vol/wt) toluene to an agricultural soil taken from the garden of the Estación Experimental del Zaidín and determined its effect on four different bacterial populations that maintained a relatively constant level throughout the year in this soil: (i) soil bacteria able to grow on solid LB medium (2 ± 10^5 CFU/g soil); (ii) soil bacteria able to use *p*-hydroxyphenylacetic acid (10 mM) as the sole C-source

TABLE 2. Tolerance to Organic Solvents of *Pseudomonas putida* Strains Isolated from Soil and Biofilm[a]

	solvent				
strain solvent	heptane (10% vol/vol)	propylbenzene (10% vol/vol)	ethylbenzene (10% vol/vol)	toluene (1% vol/vol)	toluene (10% vol/vol)
MTB5	$2 \pm 1 \times 10^9$	$6 \pm 2 \times 10^8$	$5 \pm 2 \times 10^8$	$<10^2$	$<10^2$
MTB6	$3 \pm 2 \times 10^9$	$8 \pm 4 \times 10^8$	$8 \pm 1 \times 10^8$	$8 \pm 4 \times 10^7$	$3 \pm 1 \times 10^6$
R1	$2 \pm 1 \times 10^9$	$3 \pm 1 \times 10^8$	$4 \pm 1 \times 10^8$	$<10^2$	$<10^2$
DOT-T1E	$3 \pm 1 \times 10^9$	$5 \pm 1 \times 10^8$	$6 \pm 2 \times 10^8$	$2 \pm 1 \times 10^8$	$7 \pm 1 \times 10^6$
KT2440	$2 \pm 1 \times 10^9$	$6 \pm 2 \times 10^8$	ND	$<10^2$	$<10^2$

[a] About 10^7 CFU/mL of bacterial cells growing exponentially on LB medium were challenged with the indicated solvent at a concentration of 1% (vol/vol), and 10% (vol/vol) as indicated. Twenty-four h later the number of CFU/mL was determined. The results are the average of three independent assays.

on M9 minimal medium ($5 \pm 2 \times 10^5$ CFU/g soil); (iii) soil bacteria tolerant to 50 μg/mL kanamycin in LB solid medium ($8 \pm 1 \times 10^4$ CFU/g soil); and (iv) soil bacteria able to use glucose (0.5 wt %/vol) as the sole C-source ($8 \pm 2 \times 10^5$ CFU/g soil). The results obtained were similar for all four populations. The addition of large amounts of toluene reduced the survival of indigenous soil bacteria, with a survival rate of about 1−10% of total CFU per g of soil after solvent shock. However, the number of CFU per g of soil subsequently increased with time to reach the initial numbers. These results were surprising because in a soil inoculated with the solvent-tolerant *P. putida* DOT-T1E strain, the survival rate of this microorganism after solvent shock was only about 0.001%, i.e., a few orders of magnitude below the rates obtained with indigenous bacteria. *P. putida* DOT-T1E grew and reached high cell densities thereafter (*35*).

To test whether this result with indigenous soil bacteria was due to a special characteristic of the bacteria in the soil, the same test was done with two different well-characterized soils: a fluvisol (soil-10) from the coast of Granada—relatively rich in organic matter—and a cambisol (soil-1) poor in organic matter from Sierra Nevada (Granada, Spain). The results were similar to those obtained with the soil taken from the gardens of the Estación Experimental del Zaidín. We concluded that solvent tolerance among indigenous bacteria could arise from the limited direct access of the solvent to bacteria attached to soil particles, from the partition of toluene in the soil's organic matter, and from the presence of solvent-tolerant microbes in the soil, or from the combination of these possibilities.

Nature of the Bacteria That Survived Solvent Shocks in Soil. To analyze in more detail the population of soil bacteria capable of tolerating solvents shocks, we decided to explore the nature of soil bacteria able to grow on LB plates before, 24 h and 15 days after the toluene shock. To this end serial dilutions of a soil suspension, either previous to the toluene treatment or after the addition of the chemical were spread on LB plates and the DNA encoding the 16S rRNA of 16−25 colonies with apparently distinct morphologies, color, etc. was sequenced. Although this limited number of sequences cannot be considered exhaustive, it can provide an initial view of the undergoing changes among the subpopulation of soil bacteria that formed colonies on LB medium. We first analyzed the whole 16S rDNA of 22 colonies of the soil bacteria before to toluene addition, among them we found bacteria belonging to the following species: *Bacillus subtilis* (*6*), *Bacillus thermoalcalophilus* (*1*), *Bacillus firmus* (*1*), *Bacillus megaterium* (*2*), *Bacillus purilus* (*1*), *Bacillus anthracis* (*1*), *Bacillus alcalophilus* (*1*), *Pseudomonas putida* (*3*), *Pseudomonas chlororaphis* (*1*), *Micrococcus sp.* (*1*), and four unidentified low G+C gram positive bacteria. We also determined the 16S rDNA of 25 colonies we had isolated on LB medium after the initial toluene shock. Among the bacteria that survived the initial toluene shock we found a representation of all the above genus. We tested whether the bacteria that tolerated the initial toluene shock survived the shock when growing on liquid LB medium. To this end bacteria were grown in LB medium until the turbidity of the culture was between 0.8 and 1.2 at 660 nm, then 1% (vol/vol) toluene was added, and the incubation continued for 24 h at 30 °C. No bacteria able to tolerate this shock were found.

Our previous results in soil (*35*) and those described above suggested that upon an initial drop in CFU per g of soil after toluene shock, bacterial growth occurred as the number of CFU per g of soil increased with time. We therefore proceeded to recover in LB medium bacteria from the soil exposed for 15 days to high toluene concentrations, and the 16S rDNA of 16 isolates was determined. We found maintenance of the diversity with bacteria of all the species of the genus *Bacillus* and *Pseudomonas* cited above. The results obtained suggest that the presence of a solid phase can influence tolerance to toluene. This seems to be due to the physicochemical conditions of the environment rather than resulting from modified physiological activities of the cell. This is in accordance with the conclusions raised by van Loosdrecht et al. (*13*) regarding the influence of solid matrixes on bacterial activities.

Isolation and Characterization of Two Toluene Degraders from the Soil Polluted with High Toluene Amounts. To concentrate on the study on a few of the isolates we checked which of the 16 isolates could grow on minimal medium with toluene as the sole C-source, as these microbes would be responsible of the progressive removal of this aromatic hydrocarbon in soil. Two out of 16 isolates grew on toluene. These isolates were called MTB5 and MTB6, and sequencing of the gene encoding the 16S rRNA identified the two strains as members of the phylogenetic genus *Pseudomonas*. The sequences of the genes encoding the 16S rRNA were deposited at GenBank under accession numbers AF131103 and AF131104. It was found that MTB5 and MTB6 affiliated with the pseudomonads in the same branch of "*P. fluorescens* intrageneric cluster" (*32*). The closest relatives of MTB5 and MTB6 were those sequences corresponding to *P. monteilii* lineage, *P. putida* F1, and *P. putida* R1; in all cases similarity values were above 99.3% (*32*).

To determine whether the two strains were similar in terms of tolerance to organic solvents, we tested their ability to thrive in liquid LB medium with different solvents at different concentrations. It was found that both strains tolerated similar concentrations of heptane and propylbenzene; however, MTB6 tolerated up to 1−10% (vol/vol) toluene and ethylbenzene, whereas MTB5 survived only with 0.1% (vol/vol) toluene and 1% (vol/vol) ethylbenzene (Table 1). This phenotypic character clearly distinguishes the two strains and shows that in terms of toluene tolerance MTB6 is similar to DOT-T1E (Table 2 and ref *36*), while MTB5 is similar to KT2440 (Table 2 and ref *27*). The results obtained in this study are in agreement with the findings of Huertas et al. (*36*), who showed that a given strain is more sensitive to

FIGURE 1. PCR amplification of *todC1* gene from *P. putida* MTB5, MTB6, R1, and F1. Conditions for amplification with *todC1* base primers are described in Materials and Methods. M, Lambda DNA digested with *Hind*III. A, *P. putida* DOT-T1E; B, *P. putida* MTB5; C, *P. putida* MTB6; and D, *P. putida* R1.

solvents in liquid medium than in soils. In this regard the behavior of *P. putida* MTB5 was similar to that described before for *P. putida* F1, which exhibited toluene sensitivity in liquid medium and toluene tolerance in soils (*35*).

Since *Pseudomonas putida* R1 strain is closely related to MTB5 and MTB6 and because this strain was isolated from a biofilm washed by wastewaters polluted with toluene (*11*), we tested this strain's tolerance to different solvents in LB medium. As shown in Table 2, the strain exhibited a level of tolerance similar to that of *P. putida* MTB5 and should thus be considered a sensitive strain.

Degradation of Toluene by *P. putida* MTB5, MTB6, and R1. Previous studies have shown that a number of toluene-tolerant strains do not use toluene as the sole C-source (*15*), which suggests that solvent tolerance and degradation of the solvent are independent phenotypic characters. This has been further supported by the fact that a mutant of strain DOT-T1E unable to use toluene was as tolerant as the wild-type to toluene (*38*). We have determined which of the five catabolic pathways described for aerobic toluene metabolism is used by each of the three isolates.

Cells of MTB5, MTB6, and R1 grown on minimal medium with toluene respired toluene (80−106 nmol O_2/min/mg dry weight) and ethylbenzene (60−80 nmol O_2/min/mg dry weight), but none of them consumed oxygen in response to *o*-, *m*-, and *p*-xylene or *o*-, *m*-, and *p*-cresol. These findings suggested that these strains use the toluene-dioxygenase pathway for toluene metabolism. This was further confirmed by the fact that by using oligonucleotide primers designed based on the *todC1* gene encoding one of the toluene dioxygenase subunits of *P. putida* strain DOT-T1E (see Materials and Methods), a specific 1.35 kbp PCR product was obtained (Figure 1). This PCR product was identical in size to that expected from the amplification of *todC1* of *P. putida* DOT-T1E and F1 (Figure 1). By using oligonucleotide primers based on the *todE* sequence encoding 3-methylcatechol 2,3-dioxygenase (see Materials and Methods) we amplified and sequenced a 875 bp DNA from each strain (not shown). The DNA sequences were found to be 99% identical to the *P. putida* DOT-T1E and F1 *todE* gene.

We also determined 3-methylcatechol 2,3-dioxygenase activity in cell-free extracts of the *P. putida* MTB5, MTB6, and R1 strains growing on glucose and glucose plus toluene. A high activity level (165−370 mU/μg protein) was found in all three strains when grown on toluene but not in its absence (less than 10 mU/μg protein). These results show that there is no correlation between tolerance to toluene and the nature of the pathway used by a strain to degrade it, as the toluene-tolerant MTB6 and the toluene-sensitive MTB5 and R1 strains used the same catabolic pathways.

TABLE 3. Relative Levels of *Cis/Trans* Isomers of the Unsaturated C16:1 and C18:1 Fatty Acids in Membranes of Different *Pseudomonas putida* Strains[a]

strain	toluene	C16:1*cis*	C16:1*trans*	C18:1*cis*	C18:1*trans*
MTB5	−	0.6	0.3	7.7	0.7
	+	2.2	10.8	7.3	7.7
MTB6	−	1.8	0.5	9.3	0.5
	+	6.4	25.2	11.6	8.2
R1	−	0.7	0.3	11.2	0.6
	+	18.2	10.9	16.3	3.0

[a] Bacterial cells were grown on LB medium in the absence (−) or in the presence of toluene (+) supplied via the gas phase. Cultures in the late exponential phase were harvested, and total fatty acids of phospholipids were determined. The value for the C16:1ω9 and C18:1ω11 isomers is given as a percentage of the total fatty acid content.

Physiological Response to Solvents by *P. putida* MTB5, MTB6, and R1. Two main factors have been shown to be involved in solvent tolerance in other *Pseudomonas* strains: (i) the ability to carry out *cis* to *trans* isomerization and (ii) the ability to pump out toluene (*18*−*20, 39*−*44*).

Cells of *P. putida* MTB5, MTB6, and R1 strains were grown on LB without and with toluene in the vapor phase, and the chemical composition of the fatty acid of phospholipids was determined (Table 3). All three strains responded to the solvent with the rigidification of their membranes by increasing the amount of *trans* isomers. This was achieved within minutes after exposure to toluene in the gas phase, which suggests that it represents an adaptive response. In *P. putida* DOT-T1E this reaction is mediated by a *cis*/*trans* isomerase. The *cti* gene that encodes the fatty acid isomerase of *P. putida* DOT-T1 was cloned (*40*) and was used as a probe against chromosomal DNA of the three strains used in this study. The results obtained revealed that all three strains contained the *cti* gene in a 2-kb *Bgl*II fragment, which is also the case for *P. putida* DOT-T1 (*40*).

Ramos et al. (*24*) reported that the TtgABC pump is involved in tolerance to toluene in *P. putida* DOT-T1E, and Mosqueda and Ramos (*44*) have identified a second toluene efflux pump, called TtgDEF, linked to the *tod* operons encoding the toluene dioxygenase pathway enzymes. Oligonucleotide primers based on the *ttgA* and *ttgD* genes (these genes encoded the efflux element of the RDN pumps (*20*)) genes were synthesized and used to amplify chromosomal DNA of the three strains under study. In all three cases amplification of DNA yielded DNA of the expected size. Partial DNA sequencing was carried out and the genes in these three strains were found to be almost 98% homologous at the DNA level along the sequence fragment (not shown).

On the basis of the above results, we tested the accumulation of 1,2,4-^{14}C-trichlorobenzene in cell membranes in the three strains grown on LB medium in the absence and in the presence of toluene supplied via the gas phase. Regardless of the growth conditions MTB5 and R1 accumulated larger amounts of the radiochemical than MTB6, particularly in cells not preexposed to toluene (Table 4). This may suggest that the MTB6 strain possesses a more efficient pump system to remove solvent from the membranes (Table 4). This was further confirmed when cells were treated with 200 μM CCCP previous to the addition of the radiochemical. Under these conditions the amount of the radiochemical accumulated in the cells was similar and much higher in the two strains. *P. putida* DOT-T1E strain lacking either the TtgABC or the TtgDEF pumps is toluene sensitive, we surmise that solvent tolerance relies primarily on the exclusion of the

TABLE 4. Solvent Efflux by Solvent-Sensitive and Solvent-Tolerant *P. putida* Strains[a]

strain	^{14}C in cell membranes /DO$_{660}$ −toluene	^{14}C in cell membranes /DO$_{660}$ +toluene
MTB5	$6 \pm 2 \times 10^5$	$4 \pm 1 \times 10^5$
MTB6	$1.5 \pm 0.5 \times 10^5$	$8 \pm 2 \times 10^4$
R1	$7 \pm 1 \times 10^5$	$6 \pm 1 \times 10^5$

[a] Cells were grown on LB medium and when the turbidity of the cultures was around 1 at 660 nm, 2 μCi of 1,2,4-[^{14}C]-trichlorobenzene was added, and accumulation of ^{14}C in the cell membranes assayed.

TABLE 5. Survival in Soil of *P. putida* MTB6, MTB5, and R1 after Toluene Shock[a]

strain	[toluene] % (vol/wt) 0	0.1%	1%	10%
P. putida MTB5	$4 \pm 1 \times 10^7$	$6 \pm 2 \times 10^7$	$5 \pm 1 \times 10^4$	$<10^2$
P. putida MTB6	$2 \pm 1 \times 10^7$	$7 \pm 2 \times 10^7$	$2 \pm 1 \times 10^6$	$9 \pm 3 \times 10^4$
P. putida R1	$2 \pm 1 \times 10^7$	$5 \pm 2 \times 10^7$	$4 \pm 2 \times 10^3$	$<10^2$

[a] About 10^7 CFU of each strain per g of soil was placed in separate pots which were supplemented with toluene at the indicated concentration. Fifteen days later samples were removed and bacteria counted on selective medium. Assays were run in triplicate.

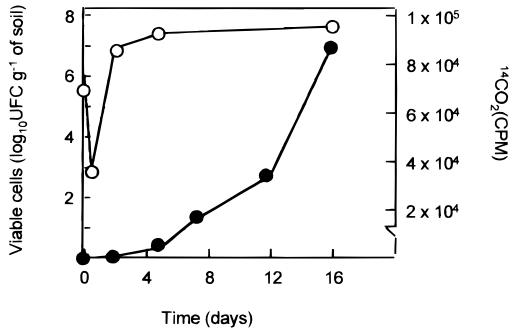

FIGURE 2. Evolution of $^{14}CO_2$ from ^{14}C-toluene in soils. Agricultural soil from the gardens of the Estación Experimental del Zaidín were inoculated with *P. putida* MTB6. $^{14}CO_2$ evolved (●) and viable cells (○) were determined at the indicated times. Values are the average of two independent duplicate assays, with standard deviations below 15% of the given values. Other conditions are described in Materials and Methods.

organic solvent from the cell membranes. Whether the difference in toluene tolerance between MTB5 and MTB6 lies in the differential rates of toluene removal or in the rate of expression of the different pumps under different growth conditions remains to be explored.

Tolerance to Toluene in Soils by *P. putida* MTB5, R1, and MTB6. Mineralization of ^{14}C-Toluene by MTB6. *P. putida* MTB5, MTB6, and R1 were introduced at about 10^7 CFU per g of soil in soil from the garden of Estación Experimental del Zaidín, and toluene was added to an initial concentration of 0.1, 1, and 10% (vol/wt) to determine the response of these strains to solvent shock. The addition of 0.1% (vol/wt) toluene had no significant effect on the survival of any of the strains (Table 5). However, MTB5 and R1 were more sensitive to toluene shocks of 1 and 10% (vol/wt) than was the toluene-tolerant MTB6 strain (Table 5). We also tested whether MTB6 was an efficient toluene-degrading strain in the soil. To this end, soil with 1% toluene (vol/wt) was supplemented with 20 μCi ^{14}C-toluene and was inoculated or not with MTB6 cells, and we observed that upon an initial drop in the number of viable MTB6 cells, growth took place and concomitant $^{14}CO_2$ evolution occurred (Figure 2). In the soil inoculated with MTB6 cells the initial rate of $^{14}CO_2$ evolution was 10-fold higher than in soils in which MTB6 had not been introduced (not shown).

These results therefore support the hypothesis that solvent-tolerant strains can be useful vehicles in bioremediation.

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