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Efflux Pumps Involved in Toluene Tolerance in *Pseudomonas putida* DOT-T1E

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The basic mechanisms underlying solvent tolerance in *Pseudomonas putida* DOT-T1E are efflux pumps that remove the solvent from bacterial cell membranes. The solvent-tolerant *P. putida* DOT-T1E grows in the presence of high concentrations (e.g., 1% [vol/vol]) of toluene and octanol. Growth of *P. putida* DOT-T1E cells in LB in the presence of toluene supplied via the gas phase has a clear effect on cell survival: the sudden addition of 0.3% (vol/vol) toluene to *P. putida* DOT-T1E pregrown with toluene in the gas phase resulted in survival of almost 100% of the initial cell number, whereas only 0.01% of cells pregrown in the absence of toluene tolerated exposure to this aromatic hydrocarbon. One class of toluene-sensitive octanol-tolerant mutant was isolated after Tn*5*-′phoA* mutagenesis of wild-type *P. putida* DOT-T1E cells. The mutant, called *P. putida* DOT-T1E-18, was extremely sensitive to 0.3% (vol/vol) toluene added when cells were pregrown in the absence of toluene, whereas pregrowth on toluene supplied via the gas phase resulted in survival of about 0.0001% of the initial number. Solvent exclusion was tested with 1,2,4-[^{14}C]trichlorobenzene. The levels of radiochemical accumulated in wild-type cells grown in the absence and in the presence of toluene were not significantly different. In contrast, the mutant was unable to remove 1,2,4-[^{14}C]trichlorobenzene from the cell membranes when grown on Luria-Bertani (LB) medium but was able to remove the aromatic compound when pregrown on LB medium with toluene supplied via the gas phase. The amount of ^{14}C-labeled substrate in whole cells increased in competition assays in which toluene and xylenes were the unlabeled competitors, whereas this was not the case when benzene was the competitor. This finding suggests that the exclusion system works specifically with certain aromatic substrates. The mutation in *P. putida* DOT-T1E-18 was cloned, and the knockedout gene was sequenced and found to be homologous to the drug exclusion gene *mexB*, which belongs to the efflux pump family of the resistant nodulator division type.

The sensitivity of microorganisms to toxic organic solvents is related to the logarithm of the partition coefficient of the solvent in a mixture of octanol and water (log P_{ow}). Aromatic hydrocarbons with a log P_{ow} of between 1.5 and 3.5 are extremely toxic to living organisms (47). These chemicals dissolve in the cytoplasmic membrane, disorganize it, and collapse the cell membrane potential; this, together with the induced loss of lipids and proteins, leads to irreversible damage resulting in the death of the cell (8, 47, 50).

Independent laboratories have isolated *Pseudomonas putida* strains tolerant to different aromatic hydrocarbons such as toluene, styrene, and *p*-xylene (6, 15, 42, 48). All four isolated strains were able to grow in liquid culture medium to which a high concentration (1% [vol/vol]) of these aromatic hydrocarbons was added. Tolerance to organic solvents in these *P. putida* strains is achieved by a series of biochemical mechanisms that actively remove the organic solvent from cell membranes (16, 43) and by physical barriers that help the cell to become (to a certain degree) impermeable to the solvent (13, 37, 43, 48). The physical barriers involve the ordered organization of the cell surface lipopolysaccharides (37) together with modified phospholipids (4, 37, 43, 49). Modifications in phospholipids upon exposure to an organic solvent involve both a short-term response, in which the level of the *trans* isomers of unsaturated phospholipids increases, and a long-term response consisting of a modification of the polar head groups of phospholipids (4, 43, 49) and an increase in the total amount of phospholipids per dry weight (49). For *P. putida* DOT-T1, it was suggested that an energy-dependent exclusion system (such as an efflux pump) is critical for tolerance to solvents (43). This conclusion was based on the following findings: (i) *P. putida* DOT-T1 treated with the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone accumulated higher levels of 1,2,4-[^{14}C]trichlorobenzene in cell membranes than did untreated cells, and (ii) *P. putida* DOT-T1 mutants which were sensitive to toluene, octanol, and other chemicals accumulated 5- to 20-fold-higher levels of 1,2,4-[^{14}C]trichlorobenzene in cell membranes than did the wild-type strain. Similar observations have been reported for *Pseudomonas* sp. strain S12 (16).

In this study, we report that *P. putida* DOT-T1 uses at least two efflux pumps for toluene exclusion, one that seems to be expressed constitutively and a second inducible one. A mini-Tn*5*′*phoA*-Km^r knocked out the constitutive efflux system of *P. putida* DOT-T1E. The mutant was shown to be hypersensitive to toluene but not to octanol. The Km^r marker of the mini-Tn*5* and the 3′ adjacent chromosomal DNA were cloned, and the wild-type gene was rescued by colony screening hybridization and sequenced. Sequence analysis showed that the knocked-out gene in the mutant was a homolog of the *mexB* gene, which belongs to the efflux pump family of the resistant nodulator division type (34–36, 38–41).

MATERIALS AND METHODS

Bacterial strains, culture media, and growth conditions. *P. putida* DOT-T1E is a rifampin-resistant derivative of the solvent-tolerant strain *P. putida* DOT-T1

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(42); *P. putida* DOT-T1E-18 is a mini-Tn*5*-*'phoA* mutant of *P. putida* DOT-T1E which shows sensitivity to toluene (see below). *P. putida* KT2440 is a natural toluene-sensitive strain (10). *Escherichia coli* CC118λ*pir* was the host of the suicide plasmid pUT-*'phoA*-Km, whose replication is dependent on the PIR protein (7). Plasmid pUT-*'phoA*-Km encodes Ap^r and Km^r; the latter marker together with *'phoA* is part of the mini-Tn*5* borne on this plasmid. *E. coli* HB101 (pRK600) was used as a helper strain in triparental matings; pRK600 encodes Cm^r and provides the *tra* functions for mobilization of the pUT plasmid. *E. coli* JM109 was used in cloning experiments.

Bacterial strains were routinely grown on liquid Luria-Bertani (LB) medium. When indicated, *P. putida* strains were grown on M9 minimal medium with 0.5% (wt/vol) glucose or 20 mM glycerol as the C source (1). For strains tolerant to high concentrations of toluene, aromatic hydrocarbons supplied as the sole C source were usually added at a concentration of 1% (vol/vol). For strains sensitive to toluene, aromatic hydrocarbons were supplied in the vapor phase. All flasks were sealed, incubated at 30°C, and shaken on an orbital platform operating at 150 to 200 strokes per min. Growth was usually determined as the most probable number of bacterial cells on LB solid medium supplemented with appropriate antibiotics.

Antibiotics were used at the following concentrations: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; and rifampin, 20 μg/ml.

Analysis of phospholipids. Phospholipids were extracted by the method of Bligh and Dyer (3). To measure fatty acids, phospholipids were saponified and esterified as described by Morrison and Smith (31). The fatty acids were identified by mass spectrometry after gas chromatographic separation.

Incorporation of 1,2,4-[^{14}C]trichlorobenzene into cell membranes. Exponentially growing cells were harvested by centrifugation, washed in LB, and suspended in 1.5 ml of LB to a cell density of about 150 to 200 μg of cell protein/ml. Then the cells were incubated for 10 min at 30°C and exposed to 2 μCi of 1,2,4-[^{14}C]trichlorobenzene, a compound that this strain is unable to metabolize (43). After 10 min, when the equilibrium level had been achieved, 250 μl of the cell suspension was filtered through a 0.45-μm-pore-size Millipore filter and washed with 2 ml of LB medium. The filters were dried, and the ^{14}C associated with the cell pellet (disintegrations/minute) was determined in a Packard Radiochemical detector.

SDS-polyacrylamide gels and Western blot analysis. Cell membrane proteins were prepared as described previously (32), and polypeptides were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gels as described by Laemmli (20). We used running gels of 8% (wt/vol) polyacrylamide and stacking gels of 5% (wt/vol) polyacrylamide. Polypeptides were transferred to a nitrocellulose membrane by electroblotting using a Bio-Rad electroblotter according to the manufacturer's instructions. Antigens were identified with the monoclonal antibody VIAP-1 against *E. coli* alkaline phosphatase (Caltag, Burlingame, Calif.) as recommended by Mutharia and Hancock (32).

Recombinant DNA techniques. Isolation of plasmid DNA, digestion of DNA with restriction enzymes, and agarose gel electrophoresis were done by standard methods (46). Competent *E. coli* cells were prepared as described by Sambrook et al. (46). *P. putida* total DNA was isolated as previously described (44). DNA was sequenced by the dideoxy sequencing termination method by using T7 phage DNA polymerase as well as universal or specific 20-mer fluorescently labeled oligonucleotides to prime synthesis.

Isolation of toluene-sensitive Tn*5*'*phoA* mutants of *P. putida* DOT-T1E. About 2,000 Tn*5* transconjugants of *P. putida* DOT-T1E were obtained after triparental mating of the latter strain with *E. coli* CC118λ*pir*(pUT-*'phoA*-Km) and *E. coli* HB101(pRK600). About 10% of the Km^r clones appeared as blue colonies in plates supplemented with 5-bromo-4-chloro-3-indolylphosphate (BCIP). Each Km^r blue transconjugant was tested for the ability to grow on LB medium supplemented with either 1% (vol/vol) toluene or 1% (vol/vol) octanol. One clone that failed to grow in the medium with 1% (vol/vol) toluene but did grow in the presence of the same amount of octanol was found and called *P. putida* DOT-T1E-18.

Cloning of the mutation in *P. putida* DOT-T1E-18 and analysis of the surrounding DNA sequence. To investigate where the mini-Tn*5*'*phoA*-Km^r was inserted in the mutant DOT-T1E-18, we digested chromosomal DNA from this mutant with *Sph*I. This enzyme cuts 5′ with respect to the Km^r gene within the *'phoA*-Km^r cassette (44). Ten micrograms of the digested total DNA was cloned into the *Sph*I site of the polylinker of pUC18 (3 μg of DNA). The ligation mixture was transformed in *E. coli* JM109 cells, and the resultant library was plated onto LB plates with kanamycin. Two Km^r colonies containing identical plasmids which carried an *Sph*I insert of about 2.3 kb were obtained. The resulting plasmid (pANA2) contained 1.7 kb of the mini-Tn*5* plus 551 bp of chromosomal DNA. The DNA was sequenced by using the M13 universal primer and a primer (5′-ACTTGTGTATAAGAGTCAG-3′) located at the end of the mini-Tn*5*; this made it possible to read outside of the Km^r gene and within the chromosomal insert.

Rescue of wild-type *P. putida* DOT-T1 genes from a gene bank. A *P. putida* DOT-T1 gene bank was established in *E. coli* JM109 by random cloning of *Bam*HI fragments in pUC18. Wild-type genes were rescued by colony screening hybridization with appropriate gene probes.

Computer analysis. Protein sequences were aligned with the Multiple Sequence Alignment program. DNA primary sequences were analyzed, and open reading frames were predicted with various programs included in the DNA Strider 1.1 package.

Nucleotide sequence accession number. The nucleotide sequence of the *P. putida* gene that encodes *ttgB* was submitted to the EMBL data bank under accession no. AF031417.

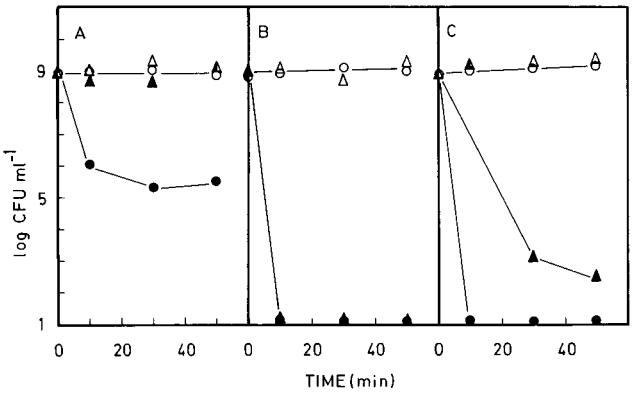

FIG. 1. Survival of *P. putida* DOT-T1E, its solvent-sensitive mutant strain DOT-T1E-18, and the naturally solvent-sensitive strain *P. putida* KT2440 upon toluene shock. Cells were grown in 30 ml of LB (circles) or LB with toluene in the gas phase (triangles) until the culture reached a turbidity of about 1 at 660 nm. These cultures contained about 10^9 CFU per ml. The cultures were divided in two halves; to one we added 0.3% (vol/vol) toluene (close symbols), and the other was kept as a control (open symbols). The number of viable cells was determined before toluene addition and 10, 30, and 50 min later. (A) *P. putida* DOT-T1E; (B) *P. putida* KT2440; (C) *P. putida* DOT-T1E-18.

RESULTS

Conditional survival of *P. putida* DOT-T1E to toluene shocks. Our previous studies showed that when *P. putida* DOT-T1E was introduced in soils, upon the addition of 1% (vol/wt) toluene the number of cells decreased 3 to 4 orders of magnitude; then bacteria surviving the solvent shock multiplied and colonized the niche (14). Here we studied the survival of *P. putida* DOT-T1E cells in liquid culture medium when cells had been pregrown on LB liquid medium without toluene or with toluene supplied via the gas phase. These cells were then challenged with 0.3% (vol/vol) toluene, and their short-term survival was determined (Fig. 1). Growth conditions were important in the response obtained; it was found that whereas almost 100% cells preexposed to low toluene concentrations survived the solvent shock (Fig. 1A), the number of CFU per milliliter of cells not preexposed to toluene decreased, so that after 30 min the number of cells was 0.01% of the initial number (Fig. 1A). After prolonged incubation, the cells that had survived the shock multiplied (not shown).

Exposure of *P. putida* DOT-T1E cells to toluene in the gas phase resulted in an increase in the rigidity of the cell membranes in comparison to nonexposed cells. This was evidenced by an increase in the amount of *trans* isomers of phospholipids, such that the *cis*/*trans* ratio of unsaturated phospholipids decreased from about 7.5 to 10 to about 2.5 to 1 in cells in different growth phases, in agreement with previous observations (43). Similar changes in rigidity of the cell membranes were also noted in toluene-sensitive *P. putida* KT2440 cells (not shown). To test whether the change in phospholipid composition explained why a proportion of the *P. putida* DOT-T1E cells pregrown with toluene in the gas phase were tolerant to the solvent shock, we tested the survival of *P. putida* KT2440 pregrown in the absence and in the presence of toluene in the gas phase to a toluene shock of 0.3% (vol/vol) (Fig. 1B). Cells

TABLE 1. Accumulation of 1,2,4-[^{14}C]trichlorobenzene in *P. putida* DOT-T1E cells grown under different culture conditions

Growth conditions[a]	cpm/mg of cell protein[b]
Prepn A	
Glycerol	48,800
Glycerol + toluene (g)	51,600
Glucose	86,400
Glucose + toluene (g)	107,600
Prepn B	
LB	54,400
LB + toluene (g)	67,600
LB + *m*-xylene (g)	92,800
LB + propylbenzene (g)	47,200

[a] *P. putida* DOT-T1E cells were grown (A) on M9 minimal medium with glucose or glycerol as the sole C source in the absence and in the presence of toluene supplied via the gas phase (g) (preparation A) or on LB medium in the absence and in the presence of different organic solvents supplied via the gas phase (preparation B).

[b] Accumulation of 1,2,4-[^{14}C]trichlorobenzene, assayed as described in Materials and Methods. The data are the averages of at least three independent determinations with standard deviations in the range of 10 to 20% of the given values.

of this strain were not able to withstand the solvent shock regardless of the pregrowth conditions (Fig. 1B).

These results were interpreted to mean that another mechanism(s), apart from a change in phospholipid composition, is responsible for toluene tolerance in *P. putida* DOT-T1E.

Extrusion of 1,2,4-[^{14}C]trichlorobenzene from *P. putida* DOT-T1E cell membranes. One mechanism that may be involved in toluene tolerance is exclusion of the aromatic hydrocarbon from the cell membranes (16, 43). We next investigated whether the culture conditions affect the ability of *P. putida* DOT-T1E to extrude aromatic compounds from the cell membranes. *P. putida* DOT-T1E cells were grown on rich LB medium and M9 minimal medium with glucose or glycerol as the sole C source, both in the absence and in the presence of toluene supplied via the gas phase. Cells were also grown on LB with and without toluene, *m*-xylene, and propylbenzene supplied via the gas phase. Exponentially growing *P. putida* DOT-T1E cells cells were harvested and exposed to 2 μCi of 1,2,4-[^{14}C]trichlorobenzene as described in Materials and Methods. The levels of radiochemical accumulated in the cell membranes (50,000 to 100,000 cpm/mg of cell protein) were similar regardless of the growth medium and the growth conditions (Table 1). These results were unexpected, because survival of *P. putida* DOT-T1E was notably influenced by preexposure of the strain to toluene, whereas the exclusion of the ^{14}C-labeled substrate was apparently not affected by such exposure.

To confirm that the exclusion system that removed 1,2,4-[^{14}C]trichlorobenzene from cell membranes also removed toluene, a fixed amount of the radiochemical was used together with increasing amounts of competing unlabeled toluene. We found that regardless of the growth conditions, the higher the amount of unlabeled compound, the higher the level of ^{14}C that accumulated in cell membranes (Fig. 2 shows results for cells grown on LB). This finding suggests that the exclusion system of *P. putida* DOT-T1E that extrudes 1,2,4-[^{14}C]trichlorobenzene also extrudes toluene.

P. putida DOT-T1E is tolerant to *m*-xylene but is sensitive to benzene (log P_{ow} = 2.0). Assays similar to that described above were done except that 2 mM *m*-xylene or benzene was used. The results showed that the exclusion system was not inhibited by benzene but was inhibited by *m*-xylene, although the level of inhibition was about half of that found with the same amount of toluene (not shown). These results suggest that the aromatic exclusion system does not work with benzene but that it does extrude *m*-xylene, toluene, and 1,2,4-trichlorobenzene.

***P. putida* DOT-T1E-18 mutant is specifically sensitive to toluene but not to other organic solvents.** To further elucidate the apparent paradox described above, we analyzed solvent sensitivity of the *P. putida* DOT-T1E-18 mutant. This mutant was generated by mutagenesis with mini-Tn*5′phoA*-Km^r and was selected as unable to grow on liquid LB medium supplemented with 1% (vol/vol) toluene as described in Materials and Methods, although it grew on LB medium with toluene supplied via the gas phase. The fatty acid composition of cell membranes was similar to that of the parental strain growing on LB and LB with toluene supplied via the gas phase.

Because alteration of cell surface proteins frequently induces pleiotropic effects, we tested growth characteristics of the wild-type *P. putida* DOT-T1E and its mutant DOT-T1E-18 in the presence of different concentrations of a chelating agent (EDTA), detergents (SDS and Triton X-100), aromatic organic acids, aromatic and nonaromatic organic solvents, and antibiotics (chloramphenicol, ampicillin, and tetracycline). We found that both the wild-type and mutant strains were able to grow in culture medium supplemented with up to 3 mM EDTA, 50 mg of SDS/liter, 3% (wt/vol) Triton X-100, and 12 g of *p*-hydroxybenzoate/liter. Both the wild-type and the mutant strain grew in LB liquid culture medium when one of the following organic solvents (1% [vol/vol]) was present: heptane (log P_{ow} = 4.5), propylbenzene (log P_{ow} = 3.5), *m*-xylene (log P_{ow} = 3.2), or octanol (log P_{ow} = 2.8). The wild-type strain tolerated up to 90 μg of chloramphenicol, 400 μg of ampicillin, and 15 μg of tetracycline per ml on solid LB medium, whereas these concentrations inhibited cell growth of the mutant strain. Double-diffusion assays revealed that the mutant strain toler-

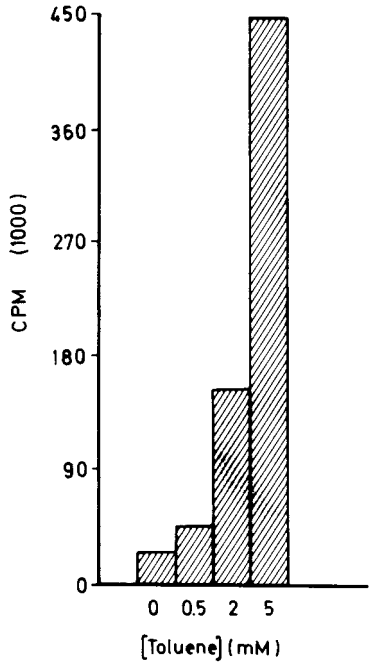

FIG. 2. Competition assay. *P. putida* DOT-T1E cells were grown on LB medium and treated as described in the footnote to Table 1 except that when 1,2,4-[^{14}C]trichlorobenzene was added, the indicated amount of toluene was also added. The accumulation of ^{14}C in the cell membranes was determined 10 min later.

ated up to about 20 μg of chloramphenicol and 50 μg of ampicillin per ml and did not tolerate tetracycline at concentrations as low as 2 μg/ml.

These results suggest that the toluene-sensitive mutant *P. putida* DOT-T1E-18 was specifically sensitive to toluene; in addition, it was more sensitive than the wild-type strain to certain antibiotics. Resistance to chloramphenicol, β-lactams, and tetracycline in certain *Pseudomonas* strains has been shown to be mediated via efflux pumps (17, 19, 21–23, 28, 34–36, 38–41), and this could account for the link between increased sensitivity to toluene and to antibiotics in this mutant strain.

Conditional sensitivity of *P. putida* DOT-T1E-18 to toluene. We tested the survival of *P. putida* DOT-T1E-18 cells grown in LB and LB with toluene in the gas phase upon sudden exposure to toluene (Fig. 1C). DOT-T1E-18 cells were hypersensitive to toluene shock when grown on LB, and after 10 min no viable cells were found. In contrast, when cells were pregrown with toluene in the gas phase, a significant proportion of the population (0.0001% of the cells) survived the solvent shock. These results suggest that toluene may induce at least one toluene exclusion mechanism that operates in conjunction with a constitutive system. If this were true, one would expect DOT-T1E-18 cells to be able to extrude toluene from the cell membranes when pregrown on toluene supplied via the gas phase. To test this hypothesis, we exposed DOT-T1E-18 cells grown in the absence and in the presence of toluene (gas phase) to 1,2,4-[^{14}C]trichlorobenzene and determined the accumulation of the radiochemical in the cell membranes. DOT-T1E-18 cells were able to extrude trichlorobenzene from the cell membranes when pregrown on toluene but not in the absence of this aromatic hydrocarbon (124,000 and 301,200 cpm/mg of cell protein, respectively, detected in the cell membrane; corresponding levels in the cell membrane of wild-type cells were 59,000 and 66,000).

In addition, we studied whether the inducible exclusion system in *P. putida* DOT-T1E-18 was also antagonized by cold toluene. To test this, we used an assay similar to that described above for the wild-type strain except that the mutant cells were pregrown on LB with toluene supplied via the gas phase. Then washed cells were exposed to 2 μCi of 1,2,4-[^{14}C]-trichlorobenzene in the absence and in the presence of 1 and 5 mM toluene. We found that the higher the toluene concentration in the culture medium, the higher the level of ^{14}C that accumulated in cell membranes: 70,000 ± 7,000, 137,000 ± 25,000, and 785,000 ± 150,000 for 0, 1, and 10 mM toluene, respectively. These results suggest that the inducible exclusion system also functions to pump out toluene.

Cloning of the mutation in *P. putida* DOT-T1E-18 and analysis of the surrounding DNA sequence. The gene disrupted by the mini-Tn*5′phoA*-Km^r was cloned as described in Materials and Methods, and the whole wild-type gene was rescued by colony screening hybridization from a library of the wild-type strain. The DNA sequence revealed that the knocked-out gene corresponded to an open reading frame that extended over 3,000 bp and encoded a putative polypeptide of about 110 kDa. Comparison of the amino acid sequence of the deduced polypeptide with sequences in several databases revealed that the *P. putida* DOT-T1E-18 polypeptide showed about 73, 64, and 60% identity with the MexB protein of *Pseudomonas aeruginosa*, the AcrB protein of *E. coli*, and the SrpB of *P. putida* S12, respectively (11, 18, 27, 40) (Fig. 3). MexB and AcrB are components of multidrug efflux pumps in *P. aeruginosa* and *E. coli* (27, 40), and SrpB in *P. putida* seems to be involved in antibiotic and solvent efflux (17, 18). Because the knocked-out gene in *P. putida* DOT-T1E seems to be specifically involved in the efflux of toluene and toluene tolerance in the wild-type *P. putida* DOT-T1E, we called the DNA sequence encoding the putative element of this efflux system *ttgB*, for toluene tolerance gene.

MexB, AcrB, and StgB are proteins of about 110 kDa in size (18, 27, 28, 36, 40). The cell membrane protein profiles of the wild-type DOT-T1E and the mutant DOT-T1E-18 strains were very similar, but Western blotting against an anti-PhoA monoclonal antibody showed that in the mutant strain the chimeric TtgB-′PhoA protein was about 130 kDa in size, as expected from the size of the insertion of *′phoA* in the *ttgB* gene.

DISCUSSION

Inoue and Horikoshi's report (15) of the isolation of a strain of *P. putida* tolerant to high toluene concentrations raised a series of questions regarding the molecular mechanisms that confer intrinsic solvent resistance to several strains of this species (6, 15, 42, 48). A series of factors that decrease the permeability of the outer membrane, such as an increase in unsaturated *trans* isomers of phospholipids and ordered organization of lipopolysaccharide, may contribute to resistance to toxic compounds (4, 13, 37, 43, 48). Our data suggest that the solvent-tolerant *P. putida* DOT-T1E strain grown in the presence of toluene had more rigid membranes that cells grown in the absence of toluene; however, organic solvents such as toluene and xylenes will reach half equilibration across the outer membrane of these cells in a few seconds, a very short period in comparison with the doubling time of the organism (34). This cannot in itself explain toluene tolerance in this microorganism. In agreement with this is our finding that although the membranes of solvent-sensitive *P. putida* strains are less permeable when cells are grown in the presence of toluene supplied via the gas phase, the cells are nonetheless unable to tolerate toluene shocks (Fig. 1B).

Studies by Isken and de Bont (16) and Ramos et al. (43) suggested that wild-type cells of *P. putida* S12 and DOT-T1E catalyze an efficient active system that expels toluene and related aromatic hydrocarbons and that this system is energy dependent. Our findings in this study identify at least two efflux pumps as the hitherto missing factor that makes a major contribution to toluene resistance in *P. putida* DOT-T1E. At least one aromatic hydrocarbon exclusion system is apparently expressed constitutively; this system was knocked out in mutant DOT-T1E-18, which became hypersensitive to toluene when grown on LB medium, as shown by the low level of ^{14}C-labeled aromatic that accumulated in the cell membranes of the wild-type strain grown on LB or minimal medium (see Results) and the high levels of ^{14}C in the cell membranes of the mutant strain grown on LB medium. An inducible aromatic hydrocarbon exclusion system also seems to operate in *P. putida* DOT-T1E. Growth of the wild-type strain in the presence of toluene did not increase the apparent level of exclusion of 1,2,4-[^{14}C]trichlorobenzene from cell membranes; however, the exclusion system functioned efficiently in mutant DOT-T1E-18 when cells were pregrown on LB with toluene in the gas phase. Under these conditions, the mutant strain was efficient in removing 1,2,4-[^{14}C]trichlorobenzene from the cell membranes. Operation of these efflux pumps in the toluene-tolerant *P. putida* DOT-T1E strain does not mean, however, that the outer membrane barrier (or some other unidentified factor[s] [2, 43]) is unimportant; the factor that slows influx through less permeable outer membranes is predicted to act synergistically with the active efflux pumps, and all factors are probably essential to intrinsic toluene resistance in *P. putida* DOT-T1E.

Competition experiments with 1,2,4-[^{14}C]trichlorobenzene and unlabeled aromatic hydrocarbons showed that toluene and

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TtgB    MSKFFIDRPIFAWVI  ALVIMLVGALSILKL  PINQYPSIAPPAIAI  AVTYPGASAQTVQDT  VVQVIEQQLNGIDNL  RYVSSESNSDGSMTI      90
MexB    MSKFFIDRPIFAWVI  ALVIMLAGGLSILSL  PVNQYPAIAPPAIAV  QVSYPGASAETVQDT  VVQVIEQQMNGIDNL  RYISSESNSDGSMTT      90
SrpB    MSRFFIDRPIFAWVL  AIVAMLAGALSLAKM  PISQYPNIAAPAVSI  QVSYPGASAQTVQDT  VVQVIEQQLSGLDGF  RYMSAESASDGSMTI      90

TtgB    TATFEQGTNPDTAQV  QVQNKLNLATPLLPQ  EVQQQGIRVTKAVKN  FLLVIGLVSEDGSMT  KDDLANYIVSNMQDP  ISRTAGVGDFQVFGA     180
MexB    TVTFEQGTDPDIAQV  QVQNKLQLATPLLPQ  EVQRQGIRVTKAVKN  FLMVVGVVSTDGSMT  KEDLSNYIVSNIQDP  LSRTKGVGDFQVFGS     180
SrpB    IVTFEQGTDPDIAQV  QVQNKLQLATPRLPE  EVQRQGLRVVKYQMN  FFLVMSLVDRSGKLD  NFDLGNLIASQLQDP  ISRIPGVGDFQLFGS     180

TtgB    QYAMRIWLDPAKLNK  FQLTPVDVKTAVAAQ  NVQVSSGQLGGLPAL  PGTQLNATIIGKTRL  QTAEQFESILLKVNK  DGSQVRLGDVAQVGL     270
MexB    QYSMRIWLDPAKLNS  YQLTPGDVSSAIQAQ  NVQISSGQLGGLPAV  KGQQLNATIIGKTRL  QTAEQFENILLKVNP  DGSQVRLKDVADVGL     270
SrpB    PYAMRIWLDPGKLNS  YQLTPTDVASAIREQ  NVQVSSGQLGGLPTR  SGVQLNATVLGKTRM  TTPSQFDEILVKVNP  DGSQVRVKDVGRAEL     270

TtgB    GGENYAVSAQFNGKP  ASGLAVKLATGANAL  DTAKALRETIKGLEP  FFPPGVKAVFPYDTT  PVVTESISGVIHTLI  EAVVLVFLVMYLFLQ     360
MexB    GGQDYSINAQFNGSP  ASGIAIKLATGANAL  DTAKAIRQTIANLEP  FMPQGMKVVYPYDTT  PVVSASIHEVVKTLG  EAILLVFLVMYLFLQ     360
SrpB    GADSFAISAQYKDSP  TASLALRLSTGGNLL  ETVDAVKKLMEQQKA  YLPDGVEVIYPYDTT  PVVEASIESVVHTIF  EAVVLVFLVMYLFLQ     360

TtgB    NFRATIITTMTVPVV  LLGTFGILAAAGFSI  NTLTMFAMVLAIGLL  VDDAIVVVENVERVM  SEEGLPPKEATKRSM  EQIQGALVGIALVLS     450
MexB    NFRATLIPTIAVPVV  LLGTFGVLAAFGFSI  NTLTMFGMVLAIGLL  VDDAIVVVENVERVM  AEEGLSPREAARKSM  GQIQGALVGIAMVLS     450
SrpB    SFRATLIPTLAVPVV  LLATFALLPYFGLNI  NVLTMYAMVLAIGLL  VDDAIVVVENVERLM  HDEGLSPLEATRKSM  DQISGALVGIGMVLS     450

TtgB    AVLLPMAFFGGSTGV  IYRQFSITIVSAMGL  SVLVALIFTPALCAT  MLKPLKKGEHHTAKG  GFFGWFNRNFDRSVN  GYERSVGTILRNKVP     540
MexB    AVFLPMAFFGGSTGV  IYRQFSITIVSAMAL  SVIVALILTPALCAT  MLKPIEKGDHGEHKG  GFFGWFNRMFLSTTH  GYERGVASILKHRAP     540
SrpB    AVFVPMAFFGGSAGI  IYQQFAITIVVCMGL  SILVALVFTPALCVT  ILKA-PEGNSHHERK  GFFGWFNRIFDRGTR  RFERGVGAMLKGRGR     539

TtgB    FLLAYALIVVGMIWL  FARIPTAFLPEEDQG  VLFAQVQTPAGSSAE  RTQVVVDQMREYLLK  DEADTVSSVFTVNGF  NFAGRGQSSGMAFIM     630
MexB    YLLIYVVIVAGMIWM  FTRIPTAFLPDEDQG  VLFAQVQTPPGSSAE  RTQVVVDSMREYLLE  KESSSVSSVFTVTGF  NFAGRGQSSGMAFIM     630
SrpB    YLLAFLLITGGTGYL  FTQIPKAFLPNEDQG  LMMIEVRTPANASAE  RTEGVLQEVRDYLAN  DEGALVEHFMTVNGF  NFAGRGQNSGLVLIT     629

TtgB    LKPWDER-SKENSVF  ALAQRAQQHFFTFRD  AMVFAFAPPAVFEVG  NATGLDVFFQDRGGV  GHAKLMEARNQFLAK  AAQSKIFSAVRPNGL     719
MexB    LKPWEERPGGENSVF  ELAKRAQMHFFSFKD  AMVFAFAPPSVLELG  NATGFDLFLQDQAGV  GHEVLLQARNKFLML  AAQNPALQRVRPNGM     720
SrpB    FKDWKERHGAGQDVF  SIAQRANQHFAKIKD  ASVMAFVPPAILEMG  NAMGFNLYLQDNLGL  GHEALMAARNQFLQL  ASQNPKLQAVRPNGK     719

TtgB    NDEPQYQLTIDDERA  SALGVTIADINNTLS  IALGASYVNDFIDRG  RVKKVYIQGEPSARM  SPEDLQKWYVRNGAG  EMVPFSSFAKGEWTY     809
MexB    SDEPQYKLEIDDEKA  SALGVSLADINSTVS  IAWGSSYVNDFIDRG  RVKRVYLQGRPDARM  NPDDLSKWYVRNDKG  EMVPFNAFATGKWEY     810
SrpB    DDEPQFQVNIDDEKA  RALQVSIASINETMS  AAWGSMYVNDFIDRG  RVKRVYVQGEDISRI  SPEDFDKWYVRNSLG  QMVPFSAFATGEWVN     809

TtgB    GSPKLSRYNGVEAME  ILGAPAPGYSTGEAM  AEVERIAGELPSGIG  FSWTGMSYEEKLSGS  QMPALFALSVLFVFL  CLAALYESWSIPIAV     899
MexB    GSPKLERYNGVPAME  ILGEPAPGLSSGDAM  AAVEEIVKQLPKGVG  YSWTGLSYEERLSGS  QAPALYALSLLVVFL  CLAALYESWSIPFSV     900
SrpB    GSPKLERYGGISSLN  ILGEPAPGYSTGDAM  IAIAEIMQQLPAGIG  LSYTGLSYEEIQTGD  QAPLLYALTVLIVFL  CLAALYESWSVPVSV     899

TtgB    VLVVPLGIIGALIAT  SLRGLSNDVYFLVGL  LTTIGLAAKNAILIV  EFAKELHE-QGRSLY  DAAIEACRMRLPPII  MTSLAFILGVVPLNI     988
MexB    MLVVPLGVIGALLAT  SMRGLSNDVFFQVGL  LTTIGLSAKNAILIV  EFAKELHE-QGKGIV  EAAIEACRMRLRPIV  MTSLAFILGVVPLAI     989
SrpB    IMVVPLGILGAVLAT  LWRDLTADVYFQVGL  MTTVGLSAKNAILIV  EFAKELYEKEGYPIV  KAAIEAAKLRLRPIL  MTSLAFTFGVLPMAI     989

TtgB    ASGAGAGSQHAIGTG  VIGGMISATVLAIFW  VPLFFLR---NVLAV  RQPRTGKRPHP----  -     1041
MexB    STGAGSGSQHAIGTG  VIGGMVTATVLAIFW  VPLFIV----AVSTL  FKDEASKQQASVEKG  Q     1046
SrpB    ASGAGAGSQHSIATG  VVGGMITATVLAVFF  VPLFYVVVVKLFEGL  MKRKPNAVKEVTHEV  -     1049
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FIG. 3. Alignment of the *P. putida* DOT-T1E TtgB sequence to the homologous *P. aeruginosa* MexB and *P. putida* S12 SrpB sequences (18, 38–41).

xylenes interfered with the removal of ^{14}C from the cell membranes of the wild-type strain grown on LB in the absence and in the presence of toluene and in the mutant strain when grown on LB with toluene in the gas phase. (Note that these cells did not remove the substrate when grown under noninducing conditions.) Our data also show that the *P. putida* DOT-T1E pumps were not able to remove benzene, a compound to which the wild-type strain was sensitive. These results suggest that the two efflux systems have a relatively broad substrate specificity with regard to aromatic hydrocarbons. The constitutive efflux pump may also be involved in the exclusion of chloramphenicol, ampicillin, and tetracycline, as suggested by the finding that the mutant strain DOT-T1E-18 was more sensitive than the wild-type strain to these antibiotics. However, given that the mutant strain still tolerated certain concentrations of these antibiotics, the efflux of these products may also involve other exclusion systems. This possibility is also suggested by a recent study by Li and Poole (24), which showed that the *P. aeruginosa* antibiotic efflux pumps are involved in solvent extrusion, and by the work of Isken et al. (17), which showed that preculture of *P. putida* S12 in the presence of antibiotics favors solvent extrusion in this strain. This broad substrate profile for efflux pumps has been observed with other exclusion systems; for example, the *Bacillus subtilis* Bmr transporter presumably pumps out ethidium bromide, chloramphenicol, and puromycin (33); the EmrB efflux system of *E. coli* confers resistance to carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone, nalidixic acid, and phenylmercury acetate (25); and the AcrE protein of *E. coli* apparently pumps out a wide variety of substrates (26).

Multidrug efflux transporters have been cloned from bacteria of the genus *Pseudomonas* and other organisms (5, 12, 27, 30, 51). The most frequently identified drug exclusion system is a family of export machineries (reviewed in references 28 and 36) such as AcrA-AcrB, AcrE-AcrF, MexA-MexB, MtrA-

MtrB, CzcB-CzcA, CnrB-CnrA, and NolF-NolGHI, in which the component listed second is a putative 12-transmembrane-segment efflux pump belonging to the resistant nodulator division family (45) and the component listed first belongs to the membrane fusion protein family, which is thought to connect the pump directly to an outer membrane channel (9). A third component of this system is an outer membrane protein that probably functions as a channel to pump out solute molecules directly into the medium, circumventing the outer membrane barrier. In *P. putida* DOT-T1E-18, we found that the knocked-out gene was a homolog of the *P. aeruginosa mexB* gene. We have shown in Western blot analyses that the mutant strain synthesized a fusion protein between the MexB homolog and the ′PhoA protein, which was exposed to the periplasmic space of the mutant strain as deduced from the blue color of the mutant strain in culture medium supplemented with BCIP. Despite its relatively high homology with *mexB*, the knocked-out gene was called *ttgB*, because we specifically showed that the main role of its gene product in *P. putida* DOT-T1E is to confer toluene tolerance.

Observations similar to those presented in this article have been reported by Kieboom et al. (18) regarding the solvent tolerant *P. putida* S12, in which a homolog of *mexB* called *srpB* was cloned and knocked out. In contrast to *P. putida* DOT-T1, the *P. putida* S12 mutant was sensitive to other solvents such as *p*-xylene and ethylbenzene in addition to toluene. This difference may be due to the existence in *P. putida* DOT-T1E of other pumps not present in *P. putida* S12.

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REFERENCES

1. **Abril, M. A., C. Michán, K. T. Timmis, and J. L. Ramos.** 1989. Regulator and enzyme specificities of the TOL plasmid-encoded upper pathway for degradation of aromatic hydrocarbons and expansion of the substrate range of the pathway. J. Bacteriol. **171:**6782–6790.
2. **Aono, R., and H. Kobayashi.** 1997. Cell surface properties of organic solvent-tolerant mutants of *Escherichia coli* K-12. Appl. Environ. Microbiol. **63:** 3637–3642.
3. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37:**911–917.
4. **Chen, Q., D. B. Janssen, and B. Witholt.** 1995. Growth on octane alters the membrane lipid fatty acids of *Pseudomonas oleovorans* due to the induction of *alkB* and synthesis of octanol. J. Bacteriol. **177:**6894–6901.
5. **Cohen, S. P., D. C. Hooper, J. S. Wolfson, K. S. Souza, L. M. McMurry, and S. B. Levy.** 1988. Endogenous active efflux of norfloxacin in susceptible *Escherichia coli*. Antimicrob. Agents Chemother. **32:**1187–1191.
6. **Cruden, D. L., J. H. Wolfram, R. D. Rogers, and D. T. Gibson.** 1992. Physiological properties of a *Pseudomonas* strain which grows with *p*-xylene in a two-phase (organic-aqueous) medium. Appl. Environ. Microbiol. **58:** 2723–2729.
7. **de Lorenzo, V., M. Herrero, U. Jakubzik, and K. N. Timmis.** 1990. Mini-Tn*5* transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J. Bacteriol. **172:**6568–6572.
8. **de Smet, M. J., J. Kingma, and B. Witholt.** 1978. The effect of toluene on the structure and permeability of the outer and cytoplasmic membranes of *Escherichia coli*. Biochim. Biophys. Acta **506:**64–80.
9. **Dinh, T., I. T. Paulsen, and M. H. Saier, Jr.** 1994. A family of extracytoplasmic proteins that allow transport of large molecules across the outer membranes of gram-negative bacteria. J. Bacteriol. **176:**3825–3831.
10. **Franklin, F. C. H., M. Bagdasarian, M. M. Bagdasarian, and K. N. Timmis.** 1981. Molecular and functional analysis of the TOL plasmid pWWO from Pseudomonas putida and cloning of genes for the entire regulated aromatic pathway. Proc. Natl. Acad. Sci. USA **78:**7458–7462.
11. **Gotoh, N., N. Itoh, H. Yamada, I. Nishino.** 1994. Evidence for the location of OprM in the *Pseudomonas aeruginosa* outer membrane. FEMS Microbiol. Lett. **122:**309–312.
12. **Grinius, L., G. Dreganiene, E. B. Goldberg, C. H. Liao, and S. J. Projan.** 1992. A staphylococcal multidrug resistance gene product is a member of a new protein family. Plasmid **27:**119–129.
13. **Heipieper, H.-J., R. Diefenbach, and H. Keweloh.** 1992. Conversion of *cis* unsaturated fatty acids to *trans*, a possible mechanism for the protection of phenol-degrading *Pseudomonas putida* P8 from substrate toxicity. Appl. Environ. Microbiol. **58:**1847–1852.
14. **Huertas, M. J., E. Duque, S. Marqués, and J. L. Ramos.** 1998. Survival in soil of different toluene-degrading *Pseudomonas* strains after solvent shock. Appl. Environ. Microbiol. **64:**38–42.
15. **Inoue, A., and K. Horikoshi.** 1989. A *Pseudomonas* thrives in high concentration of toluene. Nature (London) **338:**264–265.
16. **Isken, S., and J. A. M. de Bont.** 1996. Active efflux of toluene in a solvent-resistant bacterium. J. Bacteriol. **178:**6056–6058.
17. **Isken, S., P. A. M. C. Santos, and J. A. M. de Bont.** 1998. Effect of solvent adaptation on the antibiotic resistance in *Pseudomonas putida* S12. Appl. Microbiol. Biotechnol. **48:**642–647.
18. **Kieboom, J., J. J. Dennis, J. A. M. de Bont, and G. J. Zylstra.** 1998. Identification and molecular characterization of an efflux pump involved in *Pseudomonas putida* S12 solvent tolerance. J. Biol. Chem. **273:**85–91.
19. **Köhler, T., M. M. Hamzehpour, U. Henze, M. Gotoh, L. K. Curty, J. C. Péchère.** 1997. Characterization of MexE-MexF-OprN, a positively regulated multidrug efflux system of *Pseudomonas aeruginosa*. Mol. Microbiol. **23:**345–354.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
21. **Lei, Y., K. Sato, and T. Nakae.** 1991. Ofloxacin-resistant *Pseudomonas aeruginosa* mutants with elevated drug extrusion across the inner membrane. Biochem. Biophys. Res. Commun. **178:**1043–1048.
22. **Li, X.-Z., D. M. Livermore, and H. Nikaido.** 1994. Role of a efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: resistance to tetracycline, chloramphenicol, and urefluxacin. Antimicrob. Agents Chemother. **38:**1732–1741.
23. **Li, X.-Z., D. Ma, D. M. Livermore, and H. Nikaido.** 1994. Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: active efflux as a contributing factor to β-lactam resistance. Antimicrob. Agents Chemother. **38:**1742–1752.
24. **Li, X.-Z., L. Zhang, and K. Poole.** 1998. Role of the multidrug efflux systems of *Pseudomonas aeruginosa* in organic solvent tolerance. J. Bacteriol. **180:** 2987–2991.
25. **Lomovskaya, O., and K. Lewis.** 1992. *emr*, an *Escherichia coli* locus for multidrug resistance. Proc. Natl. Acad. Sci. USA **89:**8938–8942.
26. **Ma, D., D. N. Cook, M. Alberti, N. G. Pon, H. Nikaido, and J. E. Hearst.** 1993. Molecular cloning and characterization of *acrA* and *acrE* genes of *Escherichia coli*. J. Bacteriol. **175:**6299–6313.
27. **Ma, D., D. N. Cook, M. Alberti, N. G. Pon, H. Nikaido, and J. E. Hearst.** 1995. Genes *acrA* and *acrB* encode a stress-induced efflux system of *Escherichia coli*. Mol. Microbiol. **16:**45–55.
28. **Ma, D., D. N. Cook, J. E. Hearst, and H. Nikaido.** 1994. Efflux pumps and drug resistance in gram-negative bacteria. Trends Microbiol. **2:**489–493.
29. **McMurray, L., R. E. Petrucci, Jr., and S. B. Levy.** 1980. Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **77:**3974–3977.
30. **Morimyo, M., E. Hongo, H. Hama-Inaba, and I. Michida.** 1992. Cloning and characterization of the *mvrC* gene of *Escherichia coli* K-12 which confers resistance against methyl viologen toxicity. Nucleic Acids Res. **20:**3159–3165.
31. **Morrison, W. R., and L. M. Smith.** 1964. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride methanol. J. Lipid Res. **5:**600–608.
32. **Mutharia, L. M., and R. E. W. Hancock.** 1985. Monoclonal antibody for an outer membrane lipoprotein of the *Pseudomonas fluorescens* group of the family *Pseudomonadaceae*. Int. J. Syst. Bacteriol. **35:**530–532.
33. **Neyfakh, A. A., V. E. Bidnenko, and L. B. Chen.** 1991. Efflux mediated multidrug resistance in bacteria: similarities and dissimilarities with mammalian system. Proc. Natl. Acad. Sci. USA **88:**4781–785.
34. **Nikaido, H.** 1989. Outer membrane barrier as a mechanism of antimicrobial resistance. Antimicrob. Agents Chemother. **33:**1831–1836.
35. **Nikaido, H.** 1994. Prevention of drug access to bacteria targets: role of permeability barriers and active efflux. Science **264:**382–388.
36. **Nikaido, H.** 1996. Multidrug efflux pumps of gram-negative bacteria. J. Bacteriol. **178:**5853–5859.
37. **Pinkart, H. C., J. W. Wolfram, R. Rogers, and D. C. White.** 1996. Cell envelope changes in solvent-tolerant and solvent-sensitive *Pseudomonas putida* strains following exposure to *p*-xylene. Appl. Environ. Microbiol. **62:** 1129–1132.
38. **Poole, K.** 1994. Bacterial multidrug resistance—emphasis on efflux mechanisms in *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. **34:**453–456.
39. **Poole, K., D. E. Heinrichs, and S. Neshat.** 1993. Cloning and sequence analysis of an EnvCD homologue in *Pseudomonas aeruginosa*: regulation by iron and possible involvement in the secretion of the siderophore pyoverdine. Mol. Microbiol. **10:**529–544.
40. **Poole, K., K. Krebes, C. McNally, and S. Neshat.** 1993. Multiple antibiotic

resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. J. Bacteriol. **175:**7363–7372.
41. **Poole, K., K. Tetro, Q. X. Zhao, S. Neshat, D. E. Heinrichs, and N. Bianco.** 1996. Expression of the multidrug-resistance operon MexA-MexB-OprM in *Pseudomonas aeruginosa*: MexR encodes a regulator of operon expression. Antimicrob. Agents Chemother. **40:**2021–2028.
42. **Ramos, J. L., E. Duque, M. J. Huertas, and A. Haïdour.** 1995. Isolation and expansion of the catabolic potential of a *Pseudomonas putida* strain able to grow in the presence of high concentrations of aromatic hydrocarbons. J. Bacteriol. **177:**3911–3916.
43. **Ramos, J. L., E. Duque, J. J. Rodríguez-Herva, P. Godoy, A. Haïdour, F. Reyes, and A. Fernández-Barrero.** 1997. Mechanisms for solvent tolerance in bacteria. J. Biol. Chem. **272:**3887–3890.
44. **Rodríguez-Herva, J. J., M. I. Ramos-González, and J. L. Ramos.** 1996. The *Pseudomonas putida* peptidoglycan-associated outer membrane lipoprotein is involved in maintenance of the cell integrity of the cell envelope. J. Bacteriol. **178:**1699–1706.
45. **Saier, M. H., R. Tam, A. Reizer, and J. Reizer.** 1994. Two novel families of bacterial membrane proteins concerned with nodulation, cell division, and transport. Mol. Microbiol. **11:**841–847.
46. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
47. **Sikkema, J., J. A. M. de Bont, and B. Poolman.** 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiol. Rev. **59:**201–222.
48. **Weber, F. J., S. Isken, and J. A. M. de Bont.** 1994. *Cis*/*trans* isomerization of fatty acids as a defence mechanism of *Pseudomonas putida* strains to toxic concentrations of toluene. Microbiology **140:**2013–2017.
49. **White, D. G., J. D. Goldman, B. Demple, and S. B. Levy.** 1997. Role of the *acrAB* locus in organic solvent tolerance mediated by expression of *marA*, *soxS*, or *robA* in *Escherichia coli*. J. Bacteriol. **179:**6122–6126.
50. **Woldringh, C. L.** 1973. Effects of toluene and phenetyl alcohol on the ultrastructure of *Escherichia coli*. J. Bacteriol. **114:**1359–1361.
51. **Yerushalmi, H., M. Lebendiker, and S. Schuldiner.** 1995. EmrE, and *Escherichia coli* 12 kDa multidrug transporter, exchanges toxic cations and H^+ and is soluble in organic solvents. J. Biol. Chem. **270:**6856–6863.