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Overexpression of Outer Membrane Protein OprT and Increase of Membrane Permeability in *phoU* Mutant of Toluene-Tolerant Bacterium *Pseudomonas putida* GM730

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Eight toluene-sensitive mutants were previously isolated from the toluene-tolerant bacterium *Pseudomonas putida* GM730. One of these mutants was TOS6, in which Tn5 had been inserted into *phoU*. Susceptibility to multiple antibiotics, as well as toluene sensitivity, was increased in the *phoU* mutant of *P. putida* GM730. We compared the outer membrane proteins from the *phoU* mutant and wild-type via two-dimensional gel electrophoresis. A 45 kDa protein was dramatically overexpressed as the result of *phoU* inactivation, and this protein was identified by peptide mass fingerprinting and microsequencing as a conserved hypothetical protein consisting of 414 amino acids. The protein, designated as OprT, harbors a signal sequence and extended β-sheets, both of which are features common to the bacterial porins. The rate of ethidium bromide accumulation in TOS6 was higher than in GM730, which indicates that the TOS6 membranes may be more permeable to ethidium bromide than are the membranes of GM730. We propose that the toluene sensitivity and increased antibiotic susceptibility observed in the *phoU* mutant may be attributable to increased membrane permeability.

***Keywords*:** PhoU, *P. putida*, membrane permeability, outer membrane protein OprT, toluene

Organic solvents with low logP_{ow} (logarithm of partition coefficient in n-octanol/water) are known to be toxic to microorganisms (Sikkema *et al.*, 1995). Toluene, with a logP_{ow} of 2.69, is extremely toxic to microorganisms. Nevertheless, *Pseudomonas putida* strains capable of growth in the presence of excess quantities of toluene have been successfully isolated, and the mechanisms relevant to toluene-tolerance have been previously investigated (Ramos *et al.*, 2002; Volkers *et al.*, 2006; Bernal *et al.*, 2007). From these efforts, a general picture of the biochemical mechanisms underlying organic solvent tolerance has emerged (Duque *et al.*, 2001; Ramos *et al.*, 2002; Rojas *et al.*, 2003; Segura *et al.*, 2005). Solvent-tolerant bacteria manifest intrinsically low permeability to toxic chemicals. In addition, they possess active efflux pumps with broad specificity, which can be utilized to drive out toxic solvents. A solvent-tolerance mechanism predicated on low permeability and active efflux pumps is essentially identical to the mechanism proposed for the broad antibiotic resistance that has been previously observed in certain bacteria (Nikaido, 1994). In fact, some genes that have been identified as solvent tolerance genes have been demonstrated to perform a crucial function in drug resistance, and vice versa (Rojas *et al.*, 2001; Guazzaroni *et al.*, 2005; Muller *et al.*, 2007). Bacterial transporters in the *P. putida* strain DOT-T1E, TtgABC and TtgGHI, were shown to be involved in antibiotics extrusion and solvent tolerance (Terán *et al.*, 2003). Bacteria may not need to discriminate between toxic organic solvents and antibiotics in order to eliminate them from the cell.

A toluene-tolerant bacterium, *P. putida* GM730, was isolated and the mechanisms underlying its solvent tolerance characteristics were evaluated via the isolation and characterization of the toluene-sensitive mutants generated via Tn5 insertion in our previous study (Kim *et al.*, 1998). We identified 7 different genes, the inactivation of which induced toluene sensitivity. They included genes for two different transporters, one of which had been previously determined to be a toluene efflux pump (Kieboom *et al.*, 1998), three genes for the pyruvate dehydrogenase complex, and a gene coding for a *phoU* orthologue. The PhoU protein has been generally considered to be a negative regulator of the *pho* regulon; however, its inactivation in *E. coli* has been shown to influence the expressions of more than 350 genes with diverse functions (Li and Zhang, 2007). On the basis of this observation, *phoU* has been implicated as a global regulator.

Previous results obtained from our mutational study showed that intact *phoU* was required for toluene tolerance in *P. putida* GM730, but its mode of action in solvent tolerance could not be determined from the known functions of *phoU*. In an effort to elucidate the biochemical mechanisms of the toluene-sensitive phenotype of the *phoU* mutant, we conducted comparative proteomic studies with the *phoU* mutant and wild-type variant. A 45 kDa protein identified as a putative porin was shown to be dramatically overexpressed by

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phoU inactivation. We also showed that membrane permeability, as measured by the ethidium bromide (EtBr) uptake rate (Ocaktan *et al*., 1997), was increased in the *phoU* mutant, and that the mutant was thus more susceptible to various antibiotics than was the wild-type strain.

Materials and Methods

Bacterial strains, media, and growth conditions

P. putida GM730, its toluene-sensitive mutant strains TOS4, TOS5, and TOS6, and *P. putida* ATCC 12633 were all previously described (Kim *et al.*, 1998). TOS4 and TOS5 were identified as toluene-sensitive mutants that are mutated in pyruvate dehydrogenase and dihydrolipoamide acetyltransferase, respectively. They were grown in Luria-Bertani (LB) medium at 30°C. LB medium supplemented with 10 mM $MgCl_2$ was utilized when these bacteria were cultivated in the presence of toluene (Kim *et al.*, 1998). For the alkaline phosphatase expression test, morpholinepropanesulfonic acid (MOPS) minimal medium supplemented with 0.1 mM or 2 mM sodium phosphate and 5-bromo-4-chloro-3-indolyl phosphate (40 μg/ml) was used.

Determination of minimal inhibitory concentrations (MICs)

The MICs of norfloxacin, tetracycline, ceftriaxone, chloramphenicol, rifampicin, streptomycin, and trimethoprim were determined using serial two-fold dilutions of the antibiotics in LB broth. The initial cell densities were measured as the OD_{600}=0.005 of log-phase cultures, and the samples were incubated for 24 h at 30°C. The MIC was recorded as the minimum drug concentration required for the prevention of visible growth.

Uptake of EtBr

Bacteria were grown to an OD_{600} of 0.5 and washed twice in phosphate-buffered saline. Washed cell-suspensions were incubated with 10 μM of EtBr dissolved in water. Fluorescence was integrated every 0.5 sec with excitation and emission wavelengths of 500 nm and 580 nm, respectively. The slit widths were 5 nm for excitation and 10 nm for emission. This procedure was repeated at least three times. Fluorescence was measured with a Perkin Elmer LS 50B fluorometer with computer-controlled data acquisition (Ocaktan *et al*., 1997).

Preparation of membrane proteins

Outer membrane was prepared by Sarkosyl method with some modifications (Filip *et al*., 1973). The cells were harvested from 200 ml cultures during the late exponential growth phase via centrifugation (6,000×g, 10 min, 4°C) and suspended in 10 ml of 20 mM Tris-HCl (pH 7.6). The cell suspensions were then sonicated using 30 sec bursts at 40 W on ice. Any intact cells were removed via 10 min of centrifugation at 1,000×g at 4°C. The supernatants were centrifuged for 30 min at 105,000×g at 4°C. After washing the pellets with phosphate-buffered saline, Sarkosyl was added to a final concentration of 1% for 30 min at 25°C. The Sarkosyl-insoluble fraction was then separated via high-speed centrifugation (105,000×g, 1 h). The pellets (outer membrane fraction) were resuspended in buffer containing 7 M urea, 2 M thiourea, 2% (w/v) 3-[(3-Cholamidopropyl)Dimethyl-Ammonio]-1-Propanesulfonate, 0.5% (v/v) IPG buffer (GE Healthcare, USA), and 20 mM DTT.

2-D gel electrophoresis (2-DE)

Isoelectric focusing was conducted using an IPGphor system (GE Healthcare). The protein samples were mixed with a rehydration buffer [8 M urea, 2% (w/v) CHAPS, 0.5% (v/v) IPG buffer, 20 mM DTT] and applied to 13 cm Immobiline Drystrips, pH 4-7L or 3-10NL (GE Healthcare) via in-gel rehydration. The proteins were focused for a total of 30,000 volt-hour. IPG strips were reduced, alkylated, and applied to 7.5~17.5% polyacrylamide gradient gels. Electrophoresis was conducted overnight and the gels were stained with silver nitrate or Coomassie Brilliant Blue R-250. Gels were obtained from two biological replicas.

Protein identification by mass spectrometry

Protein spots were identified using peptide mass fingerprinting and nanoelectrospray tandem mass spectrometry (MS/MS) as described previously (Lee *et al.*, 2004). For peptide mass fingerprinting, the protein spots were excised, carbamidomethylated, and digested with trypsin (Promega, sequencing grade). Peptide mass maps of the tryptic peptides were generated using a MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) mass spectrometer Voyager-DE STR (Applied Biosystems, USA). Monoisotopic peptide masses were analyzed using the MS-Fit public software tool (http://prospector.ucsf.edu). The NCBInr databases were used to search for all species and all p*I*s.

Nanoelectrospray MS/MS was conducted in order to generate peptide sequence information. The tryptic digestion supernatant was loaded onto a microcolumn of POROS R2 (Applied Biosystems) packed into GELoader tips (Eppendorf, Germany) as described previously (Lee *et al.*, 2004). MS/MS data were obtained using a QSTAR pulsar-i mass spectrometer (AB/MDS Sciex, Applied Biosystems, USA) equipped with a nanoelectrospray ion source (Proxeon, Denmark). The ionspray voltage was set to a potential of 1,000 V. Scan data of tryptic peptides were acquired over an m/z range of 400-1,600 Da in positive mode. MS/MS experiments were conducted over an m/z range of 80~2,000 Da with manually optimized collision energy settings for each peptide. The data were processed and interpreted with BioAnalyst software (Applied Biosystems). The resultant peptide sequences were searched against the NCBInr database, using the BLAST program.

Results

Constitutive expression of alkaline phosphatase in TOS6

TOS6 was previously identified as toluene-sensitive and *phoU* mutant strain (Kim *et al.*, 1998). It has been demonstrated that PhoU had negative regulatory function in alkaline phosphatase synthesis, showing the constitutive synthesis of alkaline phosphatase in *phoU* deletion mutant (Muda *et al.*, 1992). Therefore, in order to observe the expression of alkaline phosphatase, the TOS6 strain and the GM730 parental strain were grown on plates containing 5-bromo-4-

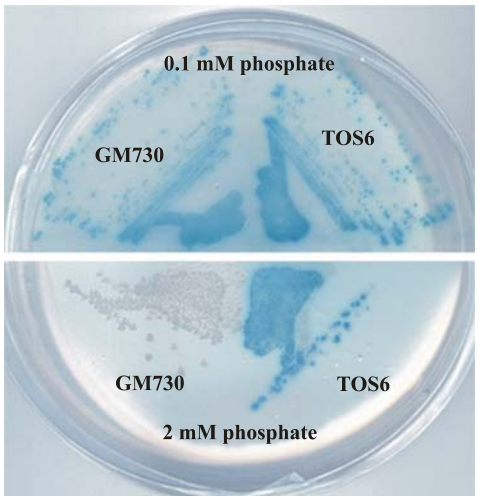

Fig. 1. Constitutive expression of alkaline phosphatase in TOS6. Wild type *P. putida* GM730 and *phoU* mutant TOS6 were streaked on the X-P (5-bromo-4-chloro-3-indolyl phosphate) plate with limiting (0.1 mM) and excess (2 mM) concentrations of phosphate. In GM730 the expression of alkaline phosphatase was controlled by available phosphate, but its expression was constitutive in the TOS6 cells.

chloro-3-indolyl phosphate (X-P) with limiting (0.1 mM) or excess (2 mM) concentrations of phosphate. As shown in Fig. 1, alkaline phosphatase in TOS6 was abundantly expressed regardless of the phosphate concentration, while its activity in *P. putida* GM730 was negatively controlled by phosphate concentration. Thus, in addition to its unexpected level of toluene sensitivity, the *phoU* mutant TOS6 cells constitutively expressed alkaline phosphatase.

The functions of *phoU* are broad, and include functions associated with virulence determination (Daigle *et al.*, 1995; Buckles *et al.*, 2006) and persister formation. Li and Zhang (2007) previously reported that the *phoU* mutant of *E. coli* was more susceptible to a variety of antibiotics. It was also shown that mutations resulting in the loss of solvent-tolerance often were accompanied by a concomitant loss of antibiotic resistance (White *et al.*, 1997). Considering these facts, we evaluated the antibiotic susceptibilities of TOS6 and GM730.

Multiple antibiotic susceptibility of *phoU* mutant

Pseudomonas species are known to be profoundly resistant to a variety of toxic chemicals (Hancock and Speert, 2000). In fact, *P. putida* GM730 and TOS6 were extremely resistant to bactericidal chemicals, including acriflavin, sulfadiazine, cloxacillin, novobiocin, deoxycholic acid, EtBr, sodium dodecyl sulphate, and tetraphenylphosphonium, with MICs of greater than 1,000 μg/ml (data not shown). Interestingly, however, TOS6 was more susceptible than GM730 to several antibiotics (Table 1). The MIC of rifampicin was decreased dramatically in TOS6, by approximately 60-fold. The MIC of norfloxacin was approximately 8-fold, and the MICs of tetracycline, ceftriaxone, and chloramphenicol were approximately two-fold lower in TOS6 than in GM730. The increased susceptibility to both antibiotics and toluene was suspected to be attributable to changes in the membrane permeability. Because we observed no significant differences in the fatty acid or lipopolysaccharide compositions of TOS6 and GM730 (unpublished data), we then attempted to detect any possible alterations of membrane proteins in the TOS6 strain.

Table 1. MIC values for GM730 and TOS6[a]

Strains \ Antibiotics[b]	Nf	Tc	Ct	Cm	Rf	Tm
GM730	3.1	12.5	100	100	62	100
TOS6	0.4	6.2	50	50	1.6	100

[a] MICs of the above antibiotics (μg/ml) were determined by using serial dilutions in LB broth.
[b] Abbreviations for antibiotics are: Nf, norfloxacin; Tc, tetracyclin; Ct, ceftriaxone; Cm, chloramphenicol; Rf, rifampicin; Tm, trimethoprim

Identification of outer membrane proteins differentially expressed in TOS6

Outer membrane proteins from the GM730 and TOS6 strains were separated via 2-DE. Spot 1, which evidenced an apparent molecular weight of 45 kDa, was differentially expressed between the two strains (Fig. 2A and B). This protein was not detected from GM730, but was one of the major proteins from TOS6. When an IPG strip with a pH gradient of 4-7L was used, the protein formed a train of spots, as is shown in the insert of Fig. 2B. The spot 1 protein was identified as an orthologue of the conserved hypothetical protein of *P. putida* W619 on the basis of peptide mass fingerprinting (PMF) and MS/MS data (Table 2). The peptide mass map and a representative of the MS/MS spectrum of this protein were provided in Fig. 2C and D, respectively. The conserved hypothetical protein (gi119856590) of *P. putida* W619 was composed of 414 amino acids. A protein highly homologous to it was found in the genomes of *P. entomophila* L48 and *P. fluorescens* PfO-1, as well as all four of the *P. putida* strains in the database. This protein, designated here as OprT, contains a signal sequence predicted by SignalP (http://www.cbs.dtu.dk/services/, signal peptide probability: 1.000) and extended β-sheets as predicted by Predictprotein (http://www.predictprotein.org/). These results may indicate that the identity of the spot 1 protein is an outer membrane porin induced by the inactivation of *phoU*.

Increased accumulation of EtBr in *phoU* mutant

Membrane permeability was assessed in GM730, three toluene-sensitive mutants (TOS4, 5, and 6), and the *P. putida* type strain ATCC 12633, by measuring the fluorescence of the intracellular ethidium-polynucleotide complex (Ocaktan *et al.*, 1997). TOS4 and TOS5 were identified previously as toluene-sensitive mutants that are defective in terms of pyruvate dehydrogenase and dihydrolipoamide acetyltransferase, respectively, and *P. putida* ATCC 12633 was identified as a toluene-sensitive wild-type strain. As is shown in Fig. 3, a distinct accumulation pattern was observed with the *phoU* mutant TOS6. In all strains, the accumulation of EtBr occurred in two discrete stages: a rapid increase in fluorescence intensity occurring within a minute after exposure, and a following slow accumulation period (Fig. 3). In the rapid

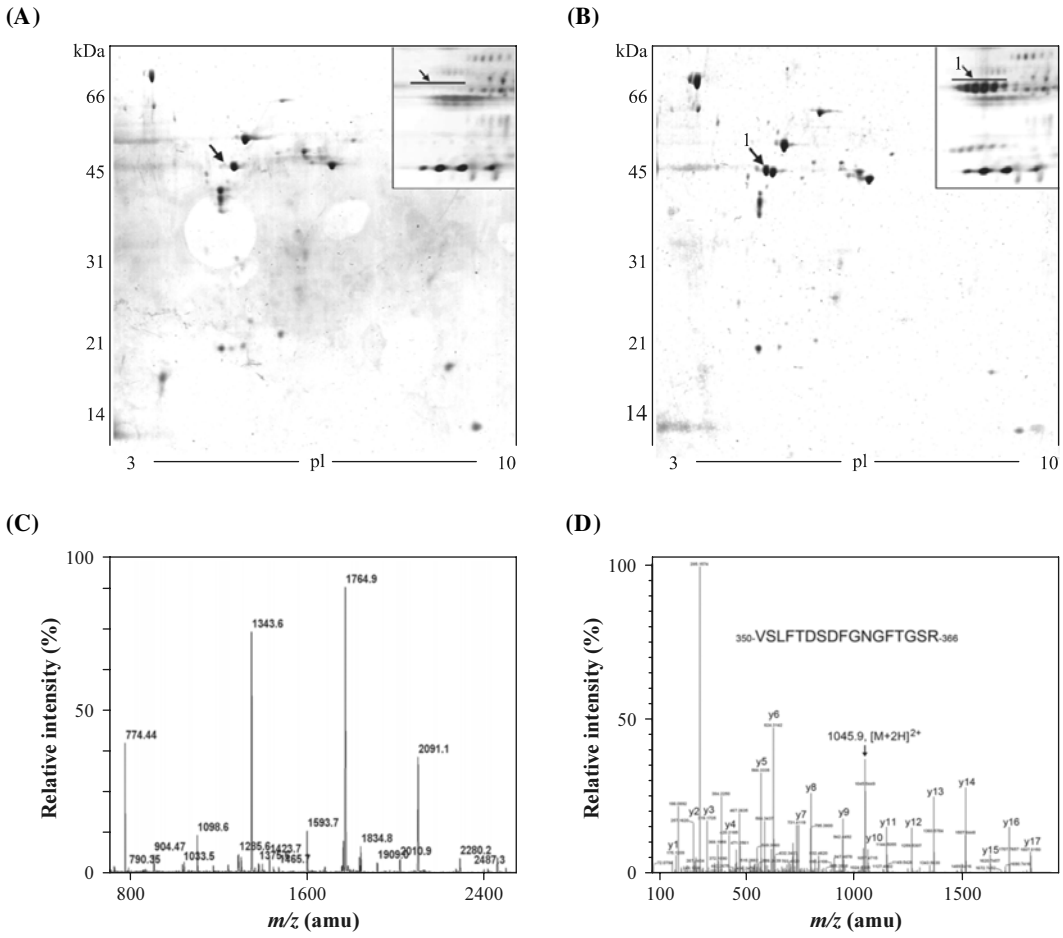

Fig. 2. Protein profiles of outer membrane and identification of differentially expressed protein in GM730 and TOS6. 2-DE of outer membrane proteins from *P. putida* GM730 (A) and TOS6 (B) using IPG strips with pH gradient 3-10NL. Gels were obtained from two biological replicas and representative images were shown. (C) Peptide mass fingerprinting of tryptic peptides from the spot 1. (D) Representative MS/MS spectrum of the doubly charged precursor ion (m/z 1045.5) of a peptide ^{350}VSLFTDSDFGNGFTGSR^{366} from the tryptic digest of spot 1. The inner boxes in (A) and (B) showed the cropped gel images when IPG strips with pH gradient 4-7L were used for 2-DE. Spot 1 was missing in (A), as indicated by arrows, but it formed a train of spots in the gel shown in the box of (B).

increase period, the fluorescence intensity of TOS6 was approximately 50% higher than that of GM730. The fluorescence intensity in TOS6 increased continuously for more than 10 min after the initial incremental stage. However, this second slower increment was not observed in GM730 or the other mutants, and the fluorescence intensities of these cells were flat after the initial stage. Interestingly, the toluene-sensitive wild type strain, *P. putida* ATCC 12633, evidenced a continual accumulation of EtBr. The eventual fluorescence intensity of TOS6 after 10 min of exposure was approximately 2.7 times higher than that of the parental strain. The higher rate of EtBr accumulation in the TOS6

Table 2. Protein identification of spot 1 shown in Fig. 2 by peptide mass fingerprinting (PMF) and tandem mass spectrometric (MS/MS) microsequencing

Protein	Theor Mr/pI[a]	Accession No. (NCBI)	PMF				MS/MS	
			MOWSE Score	Peptides No.[b]	Coverage (%)	Species	Peptide sequences	Species
Conserved hypothetical protein	45 kDa/4.8	119856590	6.63e+6	15	42	*P. putida* W619	NLHLIGGK	*P. putida* W619
							GAWSLDYNYR	*P. putida* W619
							GFYSEINPQVDAGIR	*P. putida* W619
							SDADVDTLQVDLEAK	*P. putida* W619
							LGGAEVFASAGQYTLK	*P. putida* W619
							VSLFTDSDFGNGFTGSR	*P. putida* GB-1

[a] Theoretical molecular weight (Mr) and isoelectic point (pI)
[b] Number of matched peptides

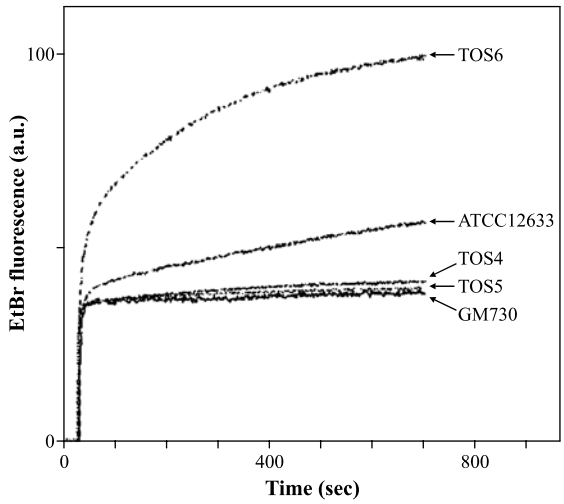

Fig. 3. Comparison of EtBr accumulation in TOS6 and GM730. EtBr fluorescence was measured in wild type (GM730) and three toluene-sensitive mutants (TOS 4, 5, and 6). *P. putida* type strain ATCC12633 was toluene-sensitive and included as a control. Three independent experiments were performed and a representative result was presented.

cells indicated that the membranes of the TOS6 strain might be more permeable to EtBr. It is currently not clear whether or not the increase of membrane permeability was due to the observed overexpression of OprT in TOS6. Future works, such as overexpression of OprT in a wild type strain, will be needed to resolve this interesting problem.

Discussion

We previously isolated 7 different genes, mutations of which resulted in a toluene-sensitive phenotype of the toluene-tolerant bacterium, *P. putida* GM730. One of those genes was *ttg*6, which was identified as an orthologue of *phoU*. In this study, we determined that the inactivation of the *phoU* of *P. putida* GM730 resulted in an increased sensitivity to several antibiotics, the overexpression of the 45 kDa outer membrane protein designated OprT, and a radical increase in permeability to EtBr. OprT harbored a signal sequence and its predicted secondary structure was predominantly that of the β-strand. All of these features are common to the bacterial porins. It has been demonstrated that the loss of function of OmpF, a common porin protein in *E. coli*, induced a reduction in susceptibility to antibiotics (Achouak *et al.*, 2001), thereby suggesting that the resistance to antibiotics and organic solvents may be associated with reduced membrane permeability as the consequence of porin changes in Gram-negative bacteria.

After our observation of the initial stage of mutant characterization, we were puzzled by the observation that TOS6 readily gave rise to toluene-tolerant revertants, which were never observed in the other 6 toluene-sensitive mutants. For example, when the mutant was inoculated into medium containing 20% toluene, cell growth was observed to occur after a long lag phase period, with the rate comparable to that of wild-type GM730 (Kim *et al.*, 1998). If the overproduction of membrane protein(s) resulting from the inactivation of *phoU* induces enhanced toluene permeability, as exemplified by the increased EtBr uptake, further mutations inducing the inactivation of such proteins in the *phoU* mutant would result in a toluene-tolerant phenotype. Such phenotypic revertants would still be *phoU* mutants, but would evidence reduced permeability. Further analyses of these revertants should facilitate our understanding of the mechanisms underlying toluene sensitivity and antibiotic susceptibility in *phoU* mutants.

It has been shown repeatedly in previous studies that growing bacterial cultures cannot be killed completely by antibiotic treatment, and a small fraction of cells normally persists (Kussell *et al.*, 2005). Recently, Li and Zhang (2007) proposed that *phoU* may be a "switch key" of persistent formation. Our data showed that the loss of *phoU* activity resulted in an increase of permeability to toxic chemicals, as demonstrated by the EtBr uptake measurements taken in this study. It may be that, in a natural population of wild-type cells, sporadic PhoU overexpression in a small subpopulation tightly represses the synthesis of membrane transporters, which enhances the survival of that subpopulation against exposure to antibiotics. In this regard, it is worth noting that the expression of *phoU* was increased in conditions that facilitate persistent formation, such as the stationary phase, nutrient-limiting conditions, and the presence of antibiotics (Steed and Wanner, 1993; Li and Zhang, 2007). Thus, the control of antibiotic permeability by *phoU* may constitute a general mechanism for persistent formation in bacteria.

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