ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 2007, p. 2092–2099 Vol. 51, No. 6
0066-4804/07/$08.00+0 doi:10.1128/AAC.00052-07

PhoU Is a Persistence Switch Involved in Persister Formation and Tolerance to Multiple Antibiotics and Stresses in *Escherichia coli*▽†

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Received 14 January 2007/Returned for modification 2 March 2007/Accepted 30 March 2007

When a bactericidal antibiotic is added to a growing bacterial culture, the great majority of the bacterial population is killed but a small number of metabolically quiescent bacteria called persisters survive antibiotic treatment. The mechanism of this bacterial persistence is poorly understood. In *Escherichia coli*, we identified a new persistence gene, *phoU*, whose inactivation leads to a generalized higher susceptibility than that of the parent strain to a diverse range of antibiotics, including ampicillin, norfloxacin, and gentamicin, and stresses, such as starvation, acid pH, heat, peroxide, weak acids, and energy inhibitors, especially in stationary phase. The PhoU mutant phenotype could be complemented by a functional *phoU* gene. Mutation in PhoU leads to a metabolically hyperactive status of the cell, as shown by an increased expression of energy production genes, flagella, and chemotaxis genes and a defect in persister formation. PhoU, whose expression is regulated by environmental changes like nutrient availability and age of culture, is a global negative regulator beyond its role in phosphate metabolism and facilitates persister formation by the suppression of many important cellular metabolic processes. A new model of persister formation based on PhoU as a persister switch is proposed. PhoU may be an ideal drug target for designing new drugs that kill persister bacteria for more effective control of bacterial infections.

The phenomenon of bacterial persisters was first described by Joseph Bigger in 1944 when he found that penicillin could not completely sterilize staphylococcal cultures in vitro (3). The small number of persister bacteria not killed by the antibiotic was still susceptible to the same antibiotic when subcultured in fresh medium. The nonsusceptibility to antibiotics in persisters is phenotypic and distinct from stable genetic resistance. The persister bacteria are due to preexisting metabolically quiescent bacteria that are not susceptible to antibiotics (1). In log phase cultures, there are only a very small number of persister bacteria, presumably due to carryover from the inoculum, but the number of persisters increases as the cultures enter stationary phase (1, 3). The persister phenomenon is presumably a protective strategy bacteria deployed to survive under adverse conditions, such as starvation, stress, and antibiotic exposure. The persister bacteria present in biofilms (14, 20) and also during the natural infection process in the host with or without antibiotic treatment (15) pose a formidable challenge for effective control of a diverse range of bacterial infections (14, 15, 26).

Despite the discovery of the persister phenomenon over 60 years ago (3), the mechanism behind bacterial persistence has been elusive as the persisters represent a small fraction of the bacterial population and are constantly changing. The first molecular study of bacterial persistence was carried out by Moyed and Bertrand in 1983 when a gene in *Escherichia coli* called *hipA*, whose mutation caused about a 100- to 1,000-fold increase in penicillin-tolerant persister bacteria, was identified (16). *hipA* forms an operon with *hipB* as a toxin-antitoxin (TA) module where HipA as a toxin is tightly regulated by the repressor HipB, which forms a complex with HipA (4). A mutant *hipA7* containing two mutations (G22S and D291A) (12) is involved in persistence to different antibiotics and to stress conditions (8, 18), although how *hipA7* mediates persister formation is unclear. Most recently, HipA has been shown to be a serine kinase (6). The significance of HipAB in bacterial persistence in some gram-negative bacteria that have HipA homologs (8, 12) cannot explain the universal persister phenomenon in other gram-negative bacteria, especially gram-positive bacteria that do not have HipA homologs. Based on the microarray analysis of *E. coli* persisters not killed by ampicillin (10), Lewis and colleagues proposed a persister model where persister formation is dependent on various TA modules, such as *HipBA* and *RelBE*, which can inhibit peptidoglycan, RNA and DNA synthesis, and protein synthesis (4, 17), leading to multidrug tolerance (10). The overexpression of toxins such as HipA (8, 13, 21), RelE (10), and MazF (13, 21) could increase persister formation. However, a recent study showed that the overexpression of unrelated toxic proteins, such as heat shock protein DnaJ and protein PmrC, also caused higher persister formation (21). This finding challenges the significance of TA modules as a specific and universal mechanism for persister formation. In this study, we report the identification of a new persister gene, *phoU*, whose inactivation causes pan-susceptibility to various antibiotics and stresses, and propose a new model of persister formation based on PhoU as a persister switch.

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† Supplemental material for this article may be found at http://aac.asm.org/.

▽ Published ahead of print on 9 April 2007.

MATERIALS AND METHODS

Culture media, antibiotics, and chemicals. Luria-Bertani (LB) broth or agar was used as the growth medium for most experiments. MOPS (morpholinepropanesulfonic acid) minimal medium or M9 minimal medium was used a nutrient-deficient medium. Glucose was added as a carbon source to a final concentration of 0.4%. Saline (0.9% NaCl) was used in the starvation experiment. The antibiotics ampicillin, norfloxacin, gentamicin, trimethoprim, and kanamycin and stress agents hydrogen peroxide, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), salicylic acid, pyrazinoic acid, and pyrazinamide (PZA) were obtained by Sigma Chemical Co., and their stocks were dissolved in appropriate solvents and used at appropriate concentrations as indicated below.

Bacterial strains, construction of mutant library and library screen, DNA manipulations, inverse PCR, and DNA sequencing. *E. coli* K-12 W3110 is F$^-$ *mcrAmcrB* IN(*rrnD-rrnE)1* lambda$^-$. Bacteriophage λ NK1316, containing Tn*10* kan cI857 *P*am80 *nin*5 *b*522 att$^-$ (11), was used for the construction of the *E. coli* transposon mutant library. Wild-type *E. coli* K-12 strain W3110 was subjected to mini-Tn*10* (kanamycin) transposon mutagenesis using a method described previously (11). The mutant library consisting of 11,748 clones was grown in LB medium containing 50 μg/ml kanamycin in 384-well plates overnight. The library in 384-well plates was replica transferred to fresh LB medium in 384-well plates, which were incubated at 37°C for 5 h to log phase when ampicillin was added to 100 μg/ml. The plates were further incubated for 24 h when the library was replica transferred to LB plates to score for clones that failed to grow after ampicillin exposure.

Inverse PCR was used to localize the mini-Tn*10* insertions in mutant *E. coli*. Two oligonucleotide primers at the end of IS*903* of the mini-Tn*10* derivative 103 (11) were synthesized (primer I, 5′-TTA CAC TGA TGA ATG TTC CG-3′, and primer II, 5′-GTC AGC CTG AAT ACG CGT-3′). Chromosomal DNA of mutant strains was isolated and digested by the restriction enzyme HaeII or AvaII, and DNA restriction fragments were then circularized using T4 DNA ligase (Invitrogen). The PCR cycling parameters were 1 min at 96°C, followed by 30 cycles, each consisting of 10 s at 96°C, 30 s at 55°C, and 2 min at 65°C. PCR products were subjected to DNA sequencing with primer I as the sequencing primer. The DNA sequences of the PCR products were subjected to a homology search in the NCBI database using the BLAST algorithm.

The primers used for the construction of the plasmid containing a functional *phoU* gene are F(5′CGCATATGTTATGTACCTGGGCGAATTG3′) and R (5′CCGGATCCTCATTATTTGTCGCTATCTTTCC3′). The purified PCR product was cloned using a pCR8/GW/TOPO TA cloning kit (Invitrogen, Carlsbad, Calif.) according to the manufacturer's protocol. The plasmid construct containing the *phoU* gene and a vector control were used to transform the PhoU mutant by electroporation. The deletion mutants of *phoR*, *phoB*, *phoU*, *hipA*, and *hipAB* were constructed as described previously by Datsenko and Wanner (7).

Susceptibility of PhoU mutant to various antibiotics. MICs and minimum bactericidal concentrations (MBCs) of ampicillin, gentamicin, trimethoprim, and norfloxacin were determined by using serial twofold dilutions of the antibiotics in LB broth. The initial cell densities were 10^6 to 10^7 bacteria/ml of log phase cultures, and the samples were incubated for 16 h at 37°C. The MIC was recorded as the minimum drug concentration that prevented visible growth, and the MBC was recorded as the drug concentration that reduced CFU by 100-fold over the seeded inoculum.

The susceptibilities of the log phase and stationary-phase PhoU mutant and wild type W3110 cultures to various antibiotics, including ampicillin (100 μg/ml), norfloxacin (3 μg/ml), gentamicin (20 μg/ml), trimethoprim (16 μg/ml), and PZA (2 mg/ml), were evaluated in a drug exposure experiment in MOPS minimal medium (pH 5.0). The antibiotic exposure was carried out over a period of several hours to 10 days at 37°C without shaking. Aliquots of bacterial cultures exposed to antibiotics were taken at different time points and washed in saline before plating for viable bacteria (CFU) on LB plates.

The sensitivity of bacterial strains to antibiotics or stresses was also assessed by the Kirby-Bauer method (2) using paper discs. *E. coli* bacteria were grown to log phase (10^8 bacteria) in LB broth. An inoculum from this culture was spread across the surfaces of LB plates to provide confluent growth. Nitrocellulose discs (7 mm in diameter) soaked with appropriate antibiotics or stress agents (100 mM H_2O_2) were placed on the agar surface. After incubation at 37°C for 48 h, the diameter of the zone of growth inhibition was measured and scored according to the size of the zone of inhibition, which is directly proportional to the sensitivity of the organism to the antibiotic. The results obtained were reproducible.

Susceptibility of PhoU mutant to various stresses. Overnight cultures of the PhoU mutant and the wild-type strain W3110 grown in LB broth at 37°C were incubated with acid, pH 4, at 37°C and 58°C, respectively, and incubated for various times, and the number of CFU per milliliter was determined by plating serial dilutions of cells on LB plates. For carbon starvation, cultures were grown overnight in M9 minimal medium with 0.4% glucose and then washed twice with saline. The cultures were diluted 1:100 in saline and incubated without shaking at 37°C at different time points. The susceptibilities of the PhoU mutant and the wild-type strain W3110 to weak acids were tested by incorporating salicylate (80 μg/ml) and pyrazinoic acid (230 μg/ml) into LB agar with acid at pH 5.0 in an MIC experiment wherein the growth inhibition was assessed by visible growth after incubating the LB plates at 37°C overnight.

DNA microarray analysis and qRT-PCR. The Affymetrix *E. coli* Genome 2.0 array was used in DNA microarray analysis of the PhoU mutant with the wild-type strain W3110 as a control. The PhoU mutant and the wild-type strain were grown in MOPS minimal medium overnight, and the RNA was isolated using a MasterPure RNA purification kit and reverse transcribed for making probes for array hybridization. The array was performed according to the manufacturer's instructions at the Johns Hopkins Malaria Research Institute Gene Array Core Facility. Triplicate samples of the PhoU mutant and the wild-type strain W3110 were used for each individual array (six arrays total), and the array data were analyzed using SAM (significance analysis of microarrays) software. For quantitative real-time PCR (qRT-PCR), the SuperScript III Platinum SYBR green one-step qRT-PCR kit was used. For qRT-PCR, the *phoU* primers were TATT GGCGACGTGGCGGAC and ATGAATGACGCGACAAGACG; the *phoE* primers were TCAACTGACTGGTTATGGTCG and TGTTGAAATACTGGT TTGCGC; and the *fliA* primers were ACTTGACGATCTGCTACAGG and TAGCGGTTTACAACGAGCTG.

TABLE 1. MIC and MBC determination for W3110 and JHU-313[a]

Antibiotic	MIC/MBC (μg/ml) for indicated strain			
	W3110	JHU-313	JHU-313 containing pPhoU	JHU-313 containing pVector
Ampicillin	3.1/12.5	1.5/6.25	3.1/12.5	1.5/6.25
Gentamicin	2.5/5	1.25/2.5	2.5/5	1.25/2.5
Trimethoprim	2/8	0.25/1	2/4	0.5/1
Norfloxacin	0.5/1	0.125/0.5	0.5/1	0.125/0.5

[a] MICs and MBCs of the above antibiotics (μg/ml) were determined by using serial twofold antibiotic dilutions in LB broth.

RESULTS

Identification of a new persister gene, *phoU*. To better understand the mechanism of persisters and to identify new genes involved in persister formation, in this study, we performed a genetic screen by identifying potential mutants with defective persistence in *E. coli* using mini-Tn*10* transposon mutagenesis. The screen identified several mutants that failed to grow on LB plates after ampicillin exposure. One mutant, JHU-313, which consistently gave the phenotype associated with inability to grow after ampicillin exposure, was further characterized. Sequence analysis revealed that this mutant harbored a transposon insertion in the C terminus-encoding region at bp 654 of the *phoU* gene, which encodes a negative regulator for phosphate metabolism (23). A homology search revealed that PhoU is widely present in numerous bacterial species.

PhoU mutant is more susceptible to various antibiotics. Since persisters are known to be tolerant or nonsusceptible to different antibiotics (14, 15, 26), we tested the susceptibilities of the PhoU mutant and the wild-type strain W3110 as a control to a variety of antibiotics, including ampicillin, norfloxacin, gentamicin, tetracycline, and trimethoprim. Interestingly, the PhoU mutant was found to be more susceptible than W3110 to all the antibiotics tested in both MIC and MBC experiments (Table 1). The PhoU mutant was generally 2- to 10-fold more susceptible than the wild-type strain to various

TABLE 2. Sensitivity of the *E. coli* PhoU mutant (JHU-313) and the complemented strain to antibiotics and peroxide as measured by zone of inhibition[a]

Antibiotic	Concentration (μg/ml)	Inhibition zone size (mm) of indicated strain			
		W3110	JHU-313	JHU-313 containing pPhoU	JHU-313 containing pVector
Ampicillin	100	35	40	40	45
	25	28	35	36	40
	6	25	30	32	34
	1.5	21	25	25	27
Gentamicin	10	25	27	28	31
	2	22	26	24	30
	0.5	20	22	22	29
	0.1	14	20	17	22
Tetracycline	50	23	32	32	34
	25	23	32	32	34
	12.5	22	26	30	34
Trimethoprim	2	30	42	36	44
	1	26	38	33	40
	0.5	24	31	32	36
Norfloxacin	4	32	37	36	44
	2	30	34	35	40
	0.5	27	30	31	35
	0.1	24	28	27	29
Hydrogen peroxide (30%)		30	37	39	46

[a] The sensitivity of bacterial strains to antibiotics or peroxide was determined by Kirby-Bauer's method.

antibiotics (Table 1). The higher susceptibility of the PhoU mutant than the wild-type strain to various antibiotics was also shown by a larger inhibition zone in the paper disc assay (Table 2). The transformation of the PhoU mutant with the functional *phoU* gene conferred increased resistance to the antibiotics compared to the wild-type level in the MIC/MBC experiments (Table 1) and also in the paper disc zone inhibition assay (Table 2).

In a killing curve experiment, the PhoU mutant was initially killed as much as wild-type strain W3110 during the first hour but showed increased susceptibility to ampicillin such that by 3 h the PhoU mutant was killed about 100-fold more than W3110 (Fig. 1A). Complementation of the PhoU mutant with the functional *phoU* gene restored the level of antibiotic susceptibility to that of the wild-type strain, whereas the PhoU mutant transformed with vector control remained susceptible to ampicillin (Fig. 1B). In log phase cultures, the PhoU mutant was only slightly more susceptible than the wild-type strain W3110 to ampicillin (100 μg/ml) exposure (Table 3). A more dramatic difference between the PhoU mutant and the wild-type strain in susceptibility to ampicillin was seen for stationary-phase cultures (Table 3). It is well known that stationary-phase cultures are not susceptible to penicillin (9). Surprisingly, the stationary-phase PhoU mutant was completely sterilized by ampicillin after 3 days, but the wild-type strain showed the typical high tolerance to ampicillin, with only a slight drop in viable counts at 10^8 CFU/ml (Table 3). It is of interest to note the huge drop in CFU resulting from ampicillin exposure, from 10^7 CFU/ml at day 1 to zero at day 3 (Table 3). A similar trend of susceptibility to ampicillin of the PhoU mutant compared with that of the wild-type strain was also seen for norfloxacin for both log phase cultures and stationary-phase cultures (Table 3). For log phase cultures, no viable bacteria were found in the PhoU mutant, whereas the wild type had 5×10^4 CFU/ml left after 3 days of exposure to norfloxacin (3 μg/ml) (Table 3). The stationary-phase PhoU mutant was more susceptible than the wild-type strain to norfloxacin such that no viable bacteria were left in the PhoU mutant after 5 days of exposure, while the wild-type strain had about 4×10^6 CFU/ml remaining (Table 3).

Persister specificity. To determine whether the persisters, after ampicillin treatment, are susceptible to other antibiotics, we subjected the persister bacteria of wild-type strain W3110 and the PhoU mutant from log phase cultures preexposed to ampicillin (100 μg/ml) for 1.5 h to gentamicin (20 μg/ml), trimethoprim (16 μg/ml), norfloxacin (5 μg/ml), and also ampicillin again in LB broth and incubated for 20 h, at which time the CFU count was determined. Both W3110 and the PhoU mutant had a 3-log decrease in CFU count, with comparable numbers of persisters after the initial 1.5-h ampicillin treat-

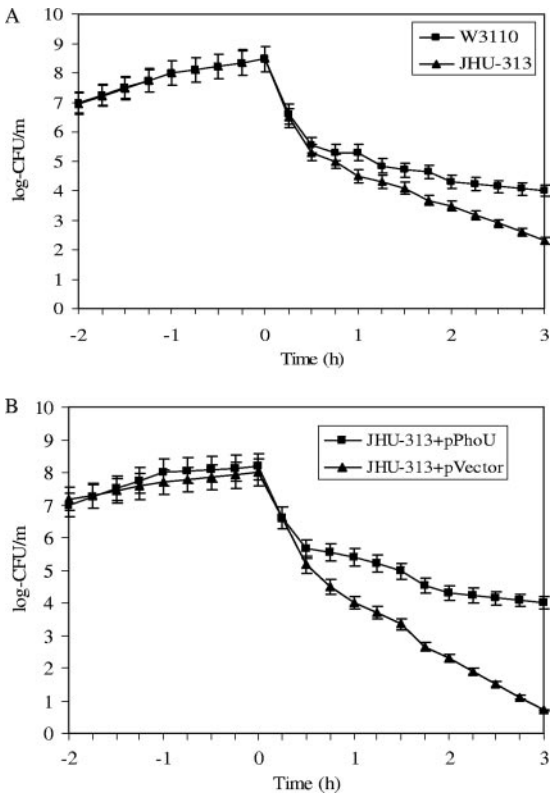

FIG. 1. Killing curve of the PhoU mutant and the wild-type strain W3110 upon ampicillin treatment (A) and the effect of complementation with the functional *phoU* gene (B). Log phase cultures of the *E. coli* PhoU mutant and wild-type W3110 and the PhoU mutant transformed with the *phoU* gene and the vector control were exposed to ampicillin 100 μg/ml for various times up to 3 h. The viability of the bacterial cultures was determined by CFU counts on LB plates. Error bars indicate standard deviations.

TABLE 3. Survival of the PhoU mutant and wild type *E. coli* strain W3110 with antibiotic exposure over time[a]

Antibiotic	Time point	No. of bacteria (CFU/ml)					
		W3110		PhoU mutant		Δ*phoU*	
		Log phase	Stationary phase	Log phase	Stationary phase	Log phase	Stationary phase
Ap	Start	4×10^8	7×10^9	3×10^8	4×10^9	4×10^8	7×10^9
	5 h	3×10^3	5×10^9	20	2×10^8	6×10^2	6×10^8
	1 day	10	3×10^9	0–1	1×10^7	0–1	1×10^8
	3 days	0	1×10^8	0	0	0	0
	5 days	0	3×10^7	0	0	0	0
	1 wk	0	5×10^6	0	0	0	0
	10 days	0	2×10^5	0	0	0	0
Norf	5 h	1×10^8	5×10^9	2×10^7	3×10^8	5×10^7	6×10^8
	1 day	4×10^6	4×10^9	4×10^5	4.5×10^7	1×10^6	2×10^8
	3 days	5×10^4	1×10^8	0	2×10^5	0	3×10^5
	5 days	7×10^2	4×10^6	0	0	0	0
	1 wk	0	1.5×10^4	0	0	0	0
	10 days	0	2×10^2	0	0	0	0

[a] The susceptibilities of log phase and stationary-phase cultures of the PhoU insertion mutant (JHU-313 transposon mutant), the PhoU deletion mutant, and the wild type to ampicillin (Ap) (100 μg/ml) and norfloxacin (Norf) (3 μg/ml) were determined. CFU values were determined at different times of exposure to antibiotics for both log phase and stationary-phase cultures of the above strains. See Materials and Methods for Δ*phoU* deletion construction.

ment (Table 4). Interestingly, the persisters in the wild-type W3110 that were not killed by the 1.5-h ampicillin treatment remained tolerant to ampicillin and were also nonsusceptible to other antibiotics after a 20-h exposure to these antibiotics, indicating that the persisters are multidrug tolerant, a finding consistent with the previous observation (25). Surprisingly, unlike what was found for the wild-type strain, the surviving bacteria that were not killed by the short ampicillin exposure (1.5 h) in the PhoU mutant continued to be killed by the bactericidal antibiotics ampicillin, norfloxacin, and gentamicin but not by the bacteriostatic antibiotic trimethoprim (Table 4).

PhoU mutant is more susceptible to a variety of stresses, including starvation, heat, oxidative stress, acid pH, weak acids, and energy inhibitors. To determine the effect of starvation on the survival of the PhoU mutant, we subjected the PhoU mutant and the wild-type strain W3110 grown in M9 minimal medium to starvation in saline for various times and assessed the abilities of the strains to survive starvation. During the first 3 days of starvation, there was no apparent difference between the PhoU mutant and the wild-type strain (Fig. 2A).

TABLE 4. Persister specificity[a]

Time of antibiotic exposure	Antibiotic	No. of bacteria (CFU/ml)	
		W3110	JHU-313
0 h		5×10^7	1.4×10^7
1.5 h	Ampicillin	7×10^4	4×10^4
20 h	Ampicillin	7×10^5	0
	Gentamicin	5×10^5	2×10^3
	Trimethoprim	6×10^5	6×10^5
	Norfloxacin	7×10^5	0

[a] Cells were grown in LB to the exponential phase, at which point they were exposed to ampicillin 100 μg/ml. Values for 0 h are starting CFU values. After exposure to ampicillin for 1.5 h, cells were washed by centrifugation and resuspended in LB containing gentamicin 20 μg/ml, trimethoprim 16 μg/ml, norfloxacin 5 μg/ml, and again ampicillin 100 μg/ml and incubated for 20 h. CFU was determined at the start, after 1.5 h ampicillin treatment and at 20 h after second antibiotic exposure.

However, a more pronounced susceptibility to starvation was seen in the PhoU mutant after 1 week of starvation. No surviving bacteria were detected in the PhoU mutant after 3 weeks of starvation, whereas the wild type had 3×10^4 viable bacteria (Fig. 2A). This result indicates that the PhoU mutant was more sensitive to starvation than the wild-type strain was.

We next determined the sensitivity of the PhoU mutant to a variety of other stress conditions. The PhoU mutant was much more sensitive to heat treatment, as demonstrated by the fact that there were no survivors after 2 h of exposure at 58°C for both log phase and stationary-phase cultures, whereas the wild-type strain W3110 had 3×10^3 and 1.7×10^4 surviving bacteria for the log phase and stationary-phase cultures, respectively. We also tested the PhoU mutant for its ability to grow at 42°C. While wild-type strain W3110 grew normally at 42°C on LB plates, the PhoU mutant grew very poorly (not shown).

The PhoU mutant was also more sensitive than the wild-type W3110 to hydrogen peroxide, and the complementation of the PhoU mutant with the functional *phoU* gene restored peroxide resistance (Table 2). Under anaerobic conditions, the PhoU mutant was more susceptible than the wild-type strain, with about 100-fold fewer viable bacteria after 3 days of incubation. In an acid, pH 4.0, exposure experiment, the PhoU mutant was more sensitive to acid at pH 4.0 than was the wild type for the stationary-phase bacteria, such that after 7 days of exposure, no viable bacteria were recovered from the PhoU mutant, whereas the wild type had about 10^8 CFU/ml (Fig. 2B). The defect in survival in acid at pH 4.0 for the PhoU mutant was restored by complementation with the functional *phoU* gene, whereas the PhoU mutant transformed with the vector control remained as susceptible as the mutant itself (Fig. 2B). The PhoU mutant was also more susceptible than the wild-type strain W3110 to the energy inhibitors *N*,*N*′-dicyclohexylcarbodiimide (5 mM) (an F1F0 ATPase inhibitor) and CCCP (1 mM) (a proton carrier that dissipates proton motive force), such that there was an approximately 1,000-fold drop in CFU count in the PhoU mutant over that of the wild-type strain

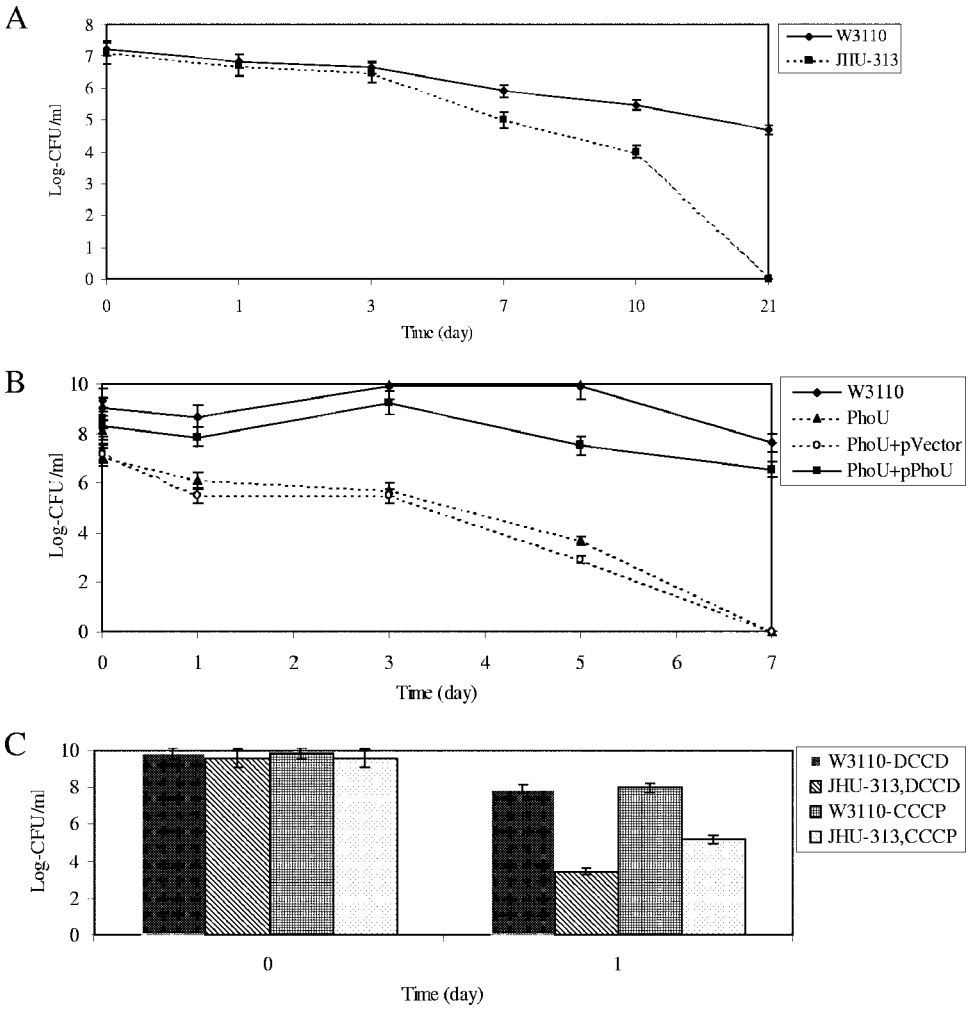

FIG. 2. Susceptibilities of the PhoU mutant JHU-313 and *E. coli* wild-type strain W3110 to stresses and energy inhibitors. More experimental details are described in Materials and Methods. (A) Susceptibilities to starvation in saline. (B) Susceptibilities to acid at pH 4.0 in LB. (C) Susceptibilities to energy inhibitors, 1 mM CCCP and 5 mM DCCD in MOPS minimal medium at pH 5.0. Error bars indicate standard deviations.

W3110 after 1 day of exposure in MOPS minimal medium at pH 5.0 (Fig. 2C). In addition, the PhoU mutant was more sensitive to the weak acids salicylic acid (80 μg/ml) and pyrazinoic acid (230 μg/ml) at pH 5.0, as shown by lack of growth at one-third the MIC compared with that of the wild-type strain W3110, which was resistant under such conditions (not shown).

Since weak acid susceptibility in *Mycobacterium tuberculosis* is correlated with susceptibility to the frontline tuberculosis drug PZA (a weak acid pyrazinoic acid amide) (29), a persister drug that depletes membrane energy, kills nonreplicating persister tubercle bacilli, and shortens the tuberculosis therapy (27, 28), we determined the susceptibility of the PhoU mutant to PZA. Interestingly, the stationary-phase PhoU mutant was more susceptible to PZA than was the wild-type strain W3110 (2 mg/ml at pH 5.0 in MOPS minimal medium) (Fig. 3). The PhoU mutant and wild-type strain had similar beginning CFU (10^8/ml) counts, and there was little difference in CFU counts between the two strains on the first day of incubation with PZA (Fig. 3). However, upon extended incubation, the stationary-phase PhoU mutant was much more susceptible to PZA by day 3 and was completely sterilized at day 6, whereas the stationary-phase wild-type strain W3110 had 6.7×10^6 CFU/ml remaining (Fig. 3). The log phase PhoU mutant was less susceptible than the stationary-phase PhoU mutant to PZA but was more susceptible to PZA than the log phase wild-type strain W3110 was, such that by day 10, the log phase PhoU mutant was completely killed, whereas the log phase wild-type W3110 had about 10^6 CFU/ml left (Fig. 3). These findings are surprising considering that normal-growing *E. coli* is highly resistant to PZA with a MIC of >2 mg/ml at pH 5.0 in the MIC experiment (22).

PhoU is a global negative regulator beyond its role in phosphate metabolism. PhoU is known to be a negative regulator of the Pho regulon, which consists of about 40 genes involved in phosphate metabolism (23). Mutation in PhoU leads to the activation of the two-component system sensor PhoR, which in turn activates the transcription factor PhoB to turn on the Pho regulon genes (7). However, the exact function of PhoU is not well understood (23). To understand how the loss of PhoU might be involved in the defect in persister formation as shown

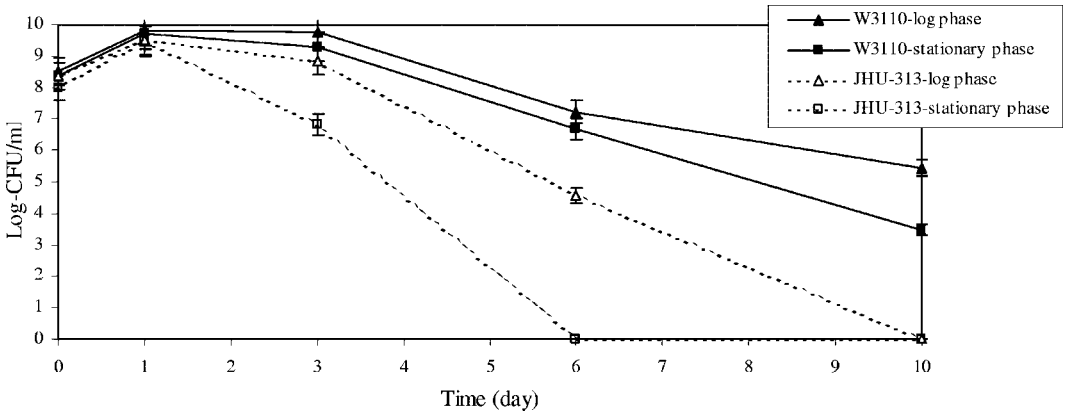

FIG. 3. Susceptibilities of the log phase and stationary-phase PhoU mutant and the wild-type strain W3110 to pyrazinamide (2 mg/ml) exposure in MOPS minimal medium at pH 5.0. Error bars indicate standard deviations.

by increased susceptibility to various antibiotics and stresses described above, we performed a microarray analysis comparing the PhoU mutant and the wild-type strain W3110. A total of about 350 genes were upregulated twofold and above (see the supplemental material), and many of these genes with known functions are listed in Table 5. As expected, genes involved in phosphate metabolism (*phoE*, *phoB*, *phoR*, *pstS*, *pstC*, *pstA*, *pstB*, *phnC*, *phnD*, *psiF*, *ugpB*, etc.) were induced in the PhoU mutant due to the inactivation of the PhoU as a negative regulator for the Pho regulon. Surprisingly, genes involved in energy production (*sdhBD*, *nuo* operon genes, *atpB*, *acnB*, *mdh*, *ugpC*, *ugpE*, *cyoAB*, etc.), some membrane transporters of various nutrients (*proV*, *X*, *artJ*, *fiu*, *proW*, *kptP*, *livJ*, *hisJ*, *copA*, *aroP*, *yhgL*, *yaeC*, *yebM*, *yicE*, *gltI*, *livG*, *oppA*, *metN*, *I*, *T*, *trxB*, *secG*, *fhuE*, *cusF*, *B*, and *X*), transcription factors (*arcA*, *pdhR*, *flhD*, *betI*, *osmE*, *fecI*, *soxS*, and *sspA* [stringent starvation protein A, a global regulator that associates with RNA polymerase]), and regulatory RNA (small antisense RNA SgrS) and, in particular, genes involved in flagella synthesis (over 40 flagella genes) and chemotaxis were upregulated at much higher levels in the PhoU mutant than in the wild type (Table 5). These findings suggest that PhoU is a global negative regulator beyond its role as a negative regulator of the Pho regulon in phosphate metabolism; it is a negative regulator for a more general cellular metabolism, whose inactivation leads to a metabolically hyperactive status of the cell and a defect in persister formation.

TABLE 5. Genes in the PhoU mutant JHU-313 that are upregulated twofold and above in DNA microarray analysis relative to wild-type strain W3110[a]

Description	Genes
Pho regulon genes	*phoE*, *-A*, *-U*, *-B*, *-R*, *-H*
	pstS, *-B*, *-A*, *-C*
	phnC, *-D*
	psiF, *ugpB*
Flagellar genes	*fliA*, *-C*, *-D*, *-E*, *-F*, *-G*, *-H*, *- I*, *-J*, *-K*, *-L*, *-M*, *-N*, *-O*, *-P*, *-Q*, *-R*, *-S*, *-T*, *-Z*
	flgA, *-B*, *-C*, *-E*, *-F*, *-G*, *-H*, *-D*, *-I*, *-K*, *-J*, *-L*, *-M*, *-N*
	motA, *-B*
	flhB, *-C*, *-A*, *-D*, *-E*
Chemotaxis genes	*cheA*, *-B*, *-W*, *-Y*, *-R*
	aer, *trg*, *tar*, *tsr*
Two-component systems and TA modules	*arcA*, *cheA*, *cheZ*, *yoeB*
Regulators, repressor, and small RNA	*sgrS*, *betI*, *spoT*, *malT*, *stpA*, *glnK*, *yefM*, *ycfQ*, *crl*, *rtT*, *isrB*, *rpsU*, *iscR*, *iscU*, *iclR*, *trpR*, *sspA*
Transporter systems	*proV*, *X*, *artJ*, *fiu*, *proW*, *kptP*, *livJ*, *hisJ*, *copA*, *aroP*, *yhgL*, *yaeC*, *yebM*, *yicE*, *gltI*, *livG*, *oppA*, *metN*, *I*, *T*, *trxB*, *secG*, *fhuE*
	cusF, *-B*, *-X*
Metabolic enzymes	*purK*, *-E*, *-M*, *-D*, *-N*, *-C*, *-F*, *-L*, *-H*, *-B*
	carA, *-B*
	nuoA, *-B*, *-C*, *-E*, *-F*, *-G*, *-M*
	argG, *-A*, *-D*, *-C*
	cyoA, *-B*
	sdhA, *-D*, *-C*, *-B*
	pyrB, *-I*, *-D*, *-C*, *-L*
	sucA, *-B*, *-C*
	aceB, *-E*
	nrdF, *-I*, *-H*, *-E*
	sodA, *-B*
	yeaA, *-F*
	aroL, *ilvC*, *acnB*, *aceA*, *folE*, *ent*, *yojH*, *fadA*

[a] Selected genes from a list of 350 genes upregulated twofold and above are grouped according to function and are listed here. See the supplemental material for complete array data.

Persister formation is independent of the PhoR-PhoB two-component system. To determine whether the persister formation is dependent on other genes in the Pho regulon besides *phoU*, we constructed deletion mutants for PhoB and PhoR and for PhoU as a positive control. As expected, the PhoU deletion mutant had the same persister deficiency phenotype (Table 6) as that of the PhoU transposon insertion mutant JHU-313 as described above (Table 3), although the PhoU deletion mutant grew more poorly and was not stable as reported previously (19). However, the deletion of *phoB* or *phoR* alone or the deletion of both *phoB* and *phoR* did not affect persister formation in *E. coli*; as expected, the *phoU* deletion mutant behaved like the *phoU* transposon insertion mutant in having a defect in persister formation (Table 6). This result suggests that persister formation is not dependent on the PhoR-PhoB two-component system and supports our array data above indicating that PhoU has the additional function of persister formation independent of its role in regulation of phosphate metabolism. We also evaluated the role of HipA in persister formation and found that the deletion of *hipA* had no effect on persister formation (Table 6), which is consistent with previous observations (4, 12).

PhoU expression is regulated by nutrient availability and age of the culture. To determine how PhoU, which is involved

TABLE 6. Survival of the deletion mutants Δ*phoB*, Δ*phoR*, and Δ*phoBR* with antibiotic exposure

Time of exposure to Ap	No. of bacteria (CFU/ml)											
	W3110		Δ*phoB*		Δ*phoR*		Δ*phoBR*		Δ*phoU*		Δ*hipA*	
	Log phase	Stationary phase	Log phase	Stationary phase	Log phase	Stationary phase	Log phase	Stationary phase	Log phase	Stationary phase	Log phase	Stationary phase
Start CFU	3.7×10^8	8×10^9	1×10^8	6×10^9	1×10^8	7×10^9	1×10^8	5×10^9	1×10^8	1×10^9	2×10^8	7×10^9
5 h	9×10^3	6×10^9	6×10^3	4×10^9	1×10^3	4×10^9	7×10^3	3×10^9	1×10^2	2×10^8	1×10^4	6×10^9
24 h	1×10^2	2×10^9	1×10^2	1.8×10^9	1×10^2	1.5×10^9	2×10^3	1.7×10^9	0	7×10^6	1×10^3	1.8×10^9
72 h	0	2×10^8	0	1×10^8	0	1×10^8	0	2×10^8	0	0	0	1×10^8

The susceptibilities of log phase and stationary phase cultures of the deletion mutants Δ*phoB*, Δ*phoR*, Δ*phoBR*, Δ*phoU*, and Δ*hipA* and the wild type to ampicillin (Ap) (100 μg/ml) were determined. CFU values were determined at different times of exposure.

in persister formation, is regulated in response to changes in nutrient availability and during different growth phases, we monitored the expression of PhoU protein under such conditions by Western blot analysis. It was found that PhoU was either not expressed or hardly expressed during nutrient sufficiency in rich LB medium (Fig. 4). However, PhoU was highly expressed in a nutrient-limiting condition in MOPS minimal medium (Fig. 4). In addition, as the culture grew to stationary phase in LB medium, there was a slight increase in PhoU expression compared with that of the log phase culture in LB medium, presumably due to nutrient limitation in stationary phase (Fig. 4). Exposure to ampicillin also increased the expression of PhoU (data not shown). These findings suggest that nutrient availability, stationary phase, and antibiotic exposure could induce PhoU expression, which in turn facilitates persister formation. The above data are also consistent with the previous observation that expression of the *pstSCAB-phoU* operon is influenced by nutrient availability and the age of bacteria (19).

DISCUSSION

In this study, we have identified a new persister gene, *phoU*, involved in persister formation, whose inactivation leads to increased susceptibility to multiple antibiotics and stresses. The various phenotypes of the PhoU mutant are generally more obvious than those of the wild-type strain W3110 in stationary-phase or starved cultures than in log phase cultures (Table 3). This is presumably because persister formation in stationary phase is defective in the PhoU mutant such that the stationary-phase culture without persisters will remain susceptible to antibiotics and stresses, whereas the wild-type strain can develop persisters normally and become tolerant to antibiotics and stresses. It should be emphasized that "persisters" are relative and should be defined by highly specific conditions, such as the type of antibiotics, the length of antibiotic or stress exposure, the culture medium, and the growth phase. For example, during short ampicillin exposure (1.5 h) when log phase PhoU mutant bacteria are killed to the same extent as wild-type bacteria and are not completely killed, there can be a specific "persister" frequency (Fig. 2C; Table 4), but with longer ampicillin exposure for 1 day (Table 3) or norfloxacin exposure for 3 days (Table 3) or stress exposure, such as starvation for 3 weeks (Fig. 2A), when no viable bacteria are left, as found in the PhoU mutant, persister frequency has no meaning.

FIG. 4. Expression of PhoU in *E. coli* wild-type strain W3110 in response to nutrient availability by Western blot analysis. Bacterial extracts were loaded onto a 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. After electrophoresis and electrotransfer, the nitrocellulose membrane was stained with polyclonal antiserum against *E. coli* PhoU peptides to monitor PhoU expression. Lane 1, 27-kDa molecular mass marker; lane 2, W3110 grown overnight in MOPS minimal medium with 2 mM K2HPO4; lane 3, log phase growth of W3110 grown in rich LB medium; lane 4, stationary-phase growth of W3110 grown in LB medium.

PhoU was originally identified as a specific negative regulator for the Pho regulon (19). However, our findings that the PhoU mutant has such a diverse phenotype as being highly susceptible to various antibiotics (ampicillin, norfloxacin, gentamicin, tetracycline, and trimethoprim) and stress conditions (starvation, heat, peroxide, acid pH, weak acids, and energy inhibitors), along with our array data that show higher metabolic activity, as demonstrated by increased expression of flagella synthesis genes and energy production genes (Table 5), strongly suggest that the function of PhoU is beyond its role in phosphate metabolism and that it serves as a global negative regulator that shuts down cellular metabolism to facilitate persister formation. The very striking induction of numerous flagella and chemotaxis genes, along with increased expression of energy production enzymes in the PhoU mutant, suggest that loss of the negative regulator PhoU makes the cells hyperactive and makes them try to "escape" or seek nutrients. The highly metabolically active status of the cells provides an explanation for why the PhoU mutant is more susceptible to various antibiotics and stresses. PhoU as a negative regulator causes the PhoU mutant to lose the ability to suppress the metabolic processes necessary for persister formation so that no persisters can be produced, causing the cells without PhoU to become more susceptible to antibiotics and stresses. Our finding of increased expression of energy production and flagella and chemotaxis genes in the PhoU mutant is also consistent with the previous observation that *E. coli* persisters had decreased expression of energy production genes and flagella genes (13). This study provides the first evidence that PhoU is a master regulator involved in persister formation and whose inactivation leads to the loss of persisters as the underlying mechanism for the increased sensitivity to antibiotics and stresses.

It has previously been shown that the *pstSCAB-phoU* operon expression manifests the interesting property of "phase variation"

as demonstrated by switching on and off in response to diverse environmental changes, such as the type of medium (rich medium versus minimal medium) and carbon source and the age of bacteria (19), which are associated with conditions that facilitate persister formation. Based on our findings on the role of PhoU in persister formation and the effect of nutrient availability on PhoU expression (Fig. 4) and the "phase variation" property of the *pstSCAB-phoU* operon (19), we propose a new persister model with PhoU as a master switch whose expression correlates with persister formation as follows. When bacteria are growing in the presence of sufficient nutrients (including phosphate) in rich medium such as LB medium, PhoU, as a negative regulator for cellular metabolism, is repressed or not expressed in the majority of the bacterial population (Fig. 4), which makes the bacteria susceptible to antibiotics and stresses. However, a small number of bacteria express low amounts of PhoU because of incomplete repression of the *pstSCAB-phoU* operon due to "phase variation," presumably caused by competing transcription activators and repressors in the promoter region of this operon (24), thus causing low level oscillatory or rhythmic transcription of the *pstSCAB-phoU* operon in response to changes in fluctuating environments, which allows persister formation in a small number of bacteria even during log phase growth. However, as bacteria enter stationary phase or encounter nutrient starvation, including phosphate starvation, PhoU is induced and expressed to a higher level (Fig. 4), which allows more persisters to form. The function of PhoU is to serve as a negative global regulator, which suppresses the overall cellular metabolic activity of the bacteria by affecting the genes or proteins involved in energy production, membrane transporters, etc., to facilitate persister formation, although the exact mechanism by which PhoU suppresses the cellular metabolic activity remains to be determined.

Our persister model based on PhoU, which needs to be confirmed by further studies, seems to best explain the pleiotropic phenotype of persisters that exhibit tolerance to various antibiotics and stresses and also the stochastic nature of persister generation in response to fluctuating environmental changes. Since PhoU is widely present in many gram-negative and gram-positive bacteria, PhoU is likely to be involved in persistence in other bacteria. It is of interest to note that *M. tuberculosis*, which is notorious for its persistence (15, 26), has two PhoU homologs, PhoY2 and PhoY1, in its genome (5). Persister bacteria pose enormous public health problems (14, 15, 26). Our finding that PhoU is a persister switch has implications for the design of new drugs that target persister bacteria and may find application for improved treatment of many persistent bacterial infections.

ACKNOWLEDGMENTS

Y.Z. was supported by NIH grants AI44063 and AI49485.

We thank Diane Griffin for support and encouragement; A. Jedlicka and A. Scott at the Gene Array Core Facility, Johns Hopkins Malaria Research Institute, for help with the microarray experiment; and Yanqin Yang for help with the array data analysis.

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