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Isolation of Highly Persistent Mutants of *Salmonella enterica* Serovar Typhimurium Reveals a New Toxin-Antitoxin Module

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Bacterial persistence is characterized by the ability of a subpopulation within bacterial cultures to survive exposure to antibiotics and other lethal treatments. The surviving persisters are not the result of genetic changes but represent epigenetic variants that are in a physiological state where growth is inhibited. Since characterization of persisters has been performed mainly in *Escherichia coli* K-12, we sought to identify mechanisms of persistence in the pathogen *Salmonella enterica* serovar Typhimurium. Isolation of new highly persistent mutants revealed that the *shpAB* locus (*Salmonella* high *p*ersistence) imparted a 3- to 4-order-of-magnitude increase in survival after ampicillin exposure throughout its growth phase and protected the population against exposure to multiple antibiotics. Genetic characterization revealed that *shpAB* is a newly discovered toxin-antitoxin (TA) module. The high-persistence phenotype was attributed to a nonsense mutation in the 3′ end of the *shpB* gene encoding an antitoxin protein. Characteristic of other TA modules, *shpAB* is autoregulated, and high persistence depends on the Lon protease.

The phenomenon of bacterial persistence has been observed for decades, following the observation by Joseph Bigger that a small subpopulation of growing *Staphylococcus aureus* cells consistently survived treatment with penicillin. These "persisters," as coined by Bigger, represented cells that did not inherit antibiotic resistance, as drug sensitivity was regained by the progeny of the surviving bacteria (1). Subsequent studies have shown that most, if not all, bacteria can enter into a physiological state that renders them tolerant of antimicrobial drugs, as well as other lethal stresses (2–7). Even though persisters are not drug resistant, their occurrence has been implicated in the failure of antibiotic therapy, as well as contributes to the inherent drug tolerance of biofilms (2, 6, 8, 9).

Persisters represent a small subpopulation within a bacterial culture that is phenotypically distinct (7, 10). In *Escherichia coli*, for example, persistent cells typically comprise only between 10^{-5} and 10^{-6} of the total population (11). The relatively low frequency of persistent cells has hampered the identification of the mechanisms responsible for antibiotic tolerance.

An initial attempt to understand the genetic basis for persistence was initiated by Moyed and Bertrand, who isolated highly persistent mutants of *E. coli* where a significantly greater proportion of the population (~1,000-fold) had entered into a drug-tolerant state compared to cultures of wild-type bacteria (11). To isolate *hip* (high-persistence) mutants, Moyed and Bertrand treated cultures with a chemical mutagen and exposed the cells to lethal doses of penicillin to enrich for survivors. The resulting mutants showed a significant increase in persistence in response to β-lactam antibiotics, in addition to showing increased tolerance to other stresses, such as elevated temperature and deprivation of thymine and diaminopimelic acid (11). The locus responsible for this phenotype led to the identification of a two-gene operon named *hipBA* (11, 12). *hipA7* is a gain-of-function mutation, as deletion of *hipBA* did not change the levels of persistence (11, 13).

More recent characterization of the *hipA7* mutant used microfluidics to monitor the growth of individual cells. From this study, *hipA7* mutants were classified as type I persisters, signifying that while persistent cells arise during stationary phase, a state of dormancy was not achieved until 1.5 h after transfer to fresh medium (10, 14). Apparently, signals triggering persistence are generated in stationary phase, but differentiation into persisters requires additional time for signal processing. Similar characterization of another highly persistent mutant, *hipQ* (15), revealed it to be a type II persister, which was continuously generated during the exponential growth phase (10). Despite its distinction from *hipA*, the function of *hipQ* remains unknown.

Genetic analysis of *hipBA* revealed it to encode a toxin-antitoxin (TA) module (13). Although TA modules were originally discovered as plasmid-borne genes responsible for plasmid partitioning by postsegregational killing (16, 17), they are now also known to reside throughout the chromosome of most bacteria (18–22). Consistent with the structure and function of most type II TA modules, *hipBA* form an operon of two tightly linked genes (12, 23). The product of *hipA* is a toxin, while that of *hipB* is an antitoxin that interacts directly with HipA (24, 25). Also in common with most other TA module family members are the findings that HipA cannot be expressed in cells without HipB (12, 13) and that *hipBA* autoregulate their own transcription (24). Biochemical studies indicated that HipA is a kinase that phosphorylates the translation factor EF-Tu, suggesting a mechanism whereby *hipBA* induce a state of dormancy in *E. coli* by interfering with translation (25).

In addition to *hipBA*, other evidence has emerged that TA modules are important contributors to persistence (26, 27). While initial studies to delete multiple TA modules from *E. coli* revealed no obvious phenotypes (28), a more extensive analysis recently revealed that persistence decreased when a sufficient number of genes was deleted (29). The functional redundancy of the multiple TA modules encoded throughout the bacterial chromosome ap-

Received 1 August 2012 **Accepted** 25 November 2012
Published ahead of print 30 November 2012
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doi:10.1128/JB.01397-12

parently masks the contribution of individual genes to persistence. Another indirect link between TA modules and persistence comes from microarray analysis, which showed that genes encoding TA components, including *mazEF* and *relBE* (7), were among the genes whose expression was upregulated following an antibiotic challenge.

Other genetic approaches have included screening for insertion mutations that decrease persistence, as well as identifying genes whose products increase persistence when overexpressed. The results of these studies consistently showed that, in addition to TA modules, multiple cellular processes can contribute to persistence and that functional redundancy is prevalent. For example, the Keio collection of *E. coli* deletion mutants was screened in an attempt to find genes required for persistence (30). While no insertion mutations completely abolished persistence, 150 mutations affecting a variety of cellular functions, including chaperones and global transcriptional regulators, were found to decrease persistence (30).

Transposon mutant libraries have also been screened to identify mutations that cause either decreased or increased persistence in *E. coli* (31–33) and *Pseudomonas aeruginosa* (34). In general, these attempts revealed genes that likely only indirectly affect persistence. More recently, a high-density library of transposon insertion mutants was screened to identify new high persisters (31). Consistent with other studies, several different genes with diverse functions answered the selection for antibiotic tolerance (31).

In addition to toxins, including HipA (26, 27, 35–39), elevated expression of multiple gene products that inhibit *E. coli* growth can also elevate persistence (36). For example, an expression library screen yielded high persisters resulting from overexpression of GlpD with *sn*-glycerol-3-phosphate dehydrogenase activity. Deletion of *glpD* also decreased persistence in stationary phase (33).

In general, these studies reveal that multiple metabolic pathways can contribute to persistence in bacteria (3). Despite the challenge to identify specific mechanisms of persistence, the phenomenon requires additional study in efforts to improve the effectiveness of antibiotics (40). Since genetic and physiological studies of persistence have been performed primarily in *E. coli* K-12 strains, we sought to understand persistence in a bacterial pathogen. *Salmonella enterica* serovar Typhimurium is an important pathogen of humans and animals. It is the causative agent of a common form of food-borne illness and causes significant health and economic impacts each year in the United States (41). *Salmonella* strains with multidrug resistance are also emerging, with outbreaks of these strains occurring at a higher frequency (42).

Inspection of the *S.* Typhimurium strain LT2 genome reveals no obvious orthologues to *hipBA* (43). However, the pathogen shares many other genes, including a host of TA family members, implicated in *E. coli* persistence. Therefore, to better understand the mechanisms of persistence in a bacterial pathogen, we isolated mutants that show elevated levels of persistence. This report describes the identification and characterization of a mutant that results from a single base pair change in a newly discovered locus that we term *shpAB*, for *Salmonella h*igh *p*ersistence.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains are listed in Table 1. *E. coli* DH5α (NEB5α; New England BioLabs, Ipswich, MA) was used as a host for cloning experiments, and *E. coli* EPI400 (Epicentre, Madison, WI) was used to clone *shpA*, whose expression is toxic to bacteria.

Bacterial growth media and chemicals. All bacteria were grown on LB agar plates or in LB broth unless otherwise noted (48). LBEDO broth (49) was used for bacteriophage P22 transduction experiments. Mueller-Hinton II plates (Becton, Dickinson and Co., Franklin Lakes, NJ) were used for MIC measurements using AB Biodisk (Solna, Sweden) strips. Antibiotics and penicillinase (Pen) were purchased from Sigma Chemical Co., St. Louis, MO, and were used at the following concentrations: kanamycin (Kan) at 50 μg/ml, tetracycline (Tet) at 20 μg/ml, ampicillin (Amp) at 100 μg/ml, ofloxacin (Ofx) at 5 μg/ml, piperacillin (Pip) at 200 μg/ml, and chloramphenicol (Cam) at 20 μg/ml. The Pen stock was made at 2,500 U/ml in 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol (DTT) in 20% glycerol. One hundred microliters of Pen stock was diluted in 4.9 ml LB to prepare a working solution. Restriction enzymes, T4 DNA ligase, and thermostable polymerases were obtained from New England BioLabs, Ipswich, MA.

PCR primers. The sequences of the PCR primers used in this study are shown in Table 2. All primers were obtained from Integrated DNA Technologies (Coralville, IA).

Isolation of high-persistence mutants. *Salmonella enterica* serovar Typhimurium strain LT2 was mutagenized with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) as described previously (48). This included growing cells to a concentration of 10^8 CFU/ml, followed by exposure to 50 μg/ml of MNNG for 10 min. Cells were then washed twice with 8.5% NaCl and resuspended to a concentration of 10^9 CFU/ml and incubated overnight.

High persisters were isolated as described by Moyed and Bertrand (11), with modifications. A saturated overnight culture of mutagenized cells was pelleted and resuspended in sterile saline. Forty microliters of cells was used to inoculate 40 ml of LB in a 250-ml baffled flask. The flask was incubated with shaking for 1 h at 37°C before addition of Amp, followed by continued growth for an additional 3 h. The cells were then centrifuged and resuspended in 40 ml of fresh LB, and the enrichment was repeated a second time. After the second round of Amp treatment, cells were washed with saline, plated on LB plates containing Amp, and incubated at 37°C. After 24 h, the plates were sprayed with the Pen working solution delivered by a martini mister and incubated again at 37°C for an additional 24 h.

Mutants were scored for increased tolerance to Amp by transferring individual colonies, along with unmutagenized control colonies, into 100 μl of LB broth with Amp in 96-well microtiter plates. The plates were covered with Parafilm and incubated overnight with shaking at 37°C. The contents of each well were diluted 1:1,000 with fresh LB before spotting 5 to 10 μl onto 100-mm-square LB-plus-Amp plates. After overnight growth at 37°C, the plates were sprayed with Pen and again cultured overnight at 37°C. The next morning, the plates were inspected, and highly persistent mutants were identified as having confluent growth, while controls yielded only a few colonies (Fig. 1A).

Assay for persistence. To quantify persistence using Amp, overnight cultures of high persisters and controls were diluted 10^{-3}, 10^{-5}, and 10^{-6} in LB. One hundred microliters of the 10^{-3} and 10^{-5} dilutions was plated on LB with Amp, while the 10^{-6} dilution was plated on LB. Cultures were plated in triplicate and grown overnight at 37°C before being sprayed with Pen, followed by overnight incubation. The number of colonies on each plate was counted, and the level of persistence was calculated as the percentage of surviving cells.

To measure tolerance to antibiotics in broth culture, 20 μl from 5-ml overnight cultures was transferred to 125-ml baffled flasks containing 20 ml of LB. After 1 h incubation at 37°C with shaking, 0.5 ml was transferred to culture tubes containing 0.5 ml of LB medium containing Ofx, Cam, Kan, or Pip. Serial dilutions were also plated on LB. The tubes were incubated for 4.5 h at 37°C, after which cells were collected by centrifugation and resuspended in fresh LB to minimize transfer of antibiotics. Serial dilutions were plated onto LB plates to measure viability. The plates were

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Strains		
E. coli		
NEB5α	*fhuA2* Δ(*argF-lacZ*)*U169 phoA glnV44* ϕ80 Δ(*lacZ*)M15 *gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	New England Biolabs
LMG194	F$^-$ Δ(*lacIPOZY*)*X74 galE galK thi rpsL* Δ*phoA ara714*	Invitrogen
EPI400	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ(*ara leu*)*7697 galU galK* λ$^-$ *rpsL* (Str^r) *nupG trfA tonA pcnB4 dhfr*	Epicentre
BW25113	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ(*ara leu*)*7697 galU galK* λ$^-$ *rpsL* (Str^r) *nupG trfA tonA pcnB4 dhfr*	Coli Genetics Stock Center
JW0429-1	F$^-$ Δ(*araD-araB*)*567* Δ*lacZ4787*(::*rrnB3*), Δ*lon-725*::*kan* λ$^-$ *rph-1* Δ(*rhaD-rhaB*)*568 hsdR514*	Coli Genetics Stock Center
S. enterica		
LT2	Wild type	Salmonella Genetics Stock Center
AS17	LT2 *shpB1*	This study
AS26	LT2 *hsdM*::Tn*10d shpB1*	This study
AV1	LT2 *hsdM*::Tn*10d shpB1*	This study
AV7	LT2 Δ*relA*::*kan*	This study
AV9	AS17 Δ*relA*::*kan*	This study
AV47	LT2 Δ(STM4528-STM4534)::*kan*	This study
AV53	LT2 Δ(STM4528-STM4529)::*kan*	This study
Plasmids		
pKD46	*repA*(Ts) Amp^r (expression of λ Red genes)	44
pNK972	ColE1 *ori* Tet^r Amp^r (Tn*10d* mutagenesis)	45
pBAD-TOPO	ColE1 *ori* Amp^r (P_{araBAD}-regulated gene expression)	Invitrogen
pTrcHisA	ColE1 *ori* Amp^r (P_{trc} gene-regulated expression, ColE1 *ori* Amp^r)	Invitrogen
pJPC12	pSC101 *ori* Cam^r (pSC101-derivative cloning vector)	46
pKRP13	ColE1 *ori* Spc^r Amp^r (source of Spc^r cassette)	47
pSMART-LCKan	ColE1 (pBR322) Kan^r (cloning vector)	Lucigen
pSMART-*shpAB*	pSMART-LCKan *shpAB*$^+$	This study
pSMART-*shpAB1*	pSMART-LCKan *shpAB1*	This study
pBADshpAB	pBAD-TOPO *shpAB*$^+$	This study
pBADshpAB1	pBAD-TOPO *shpAB1*	This study
pBADshpB	pBAD-TOPO *shpB*$^+$	This study
pBADshpB1	pBAD-TOPO *shpB1*	This study
pBADshpA	pBAD-TOPO *shpA*	This study
pJPC-Trc-shpB	pJPC12 (P_{trc}-*shpB*)	This study

sealed with Parafilm and incubated at 37°C for up to 5 days to allow cells with delayed growth to form colonies. Each assay was performed in triplicate, and the results were averaged.

Time course experiments were also performed to determine the effect of growth phase on persistence. For this, cultures were prepared as just described using Amp and Ofx. At regular intervals, samples were removed from the cultures and serial dilutions were plated on LB plates to assay the numbers of CFU/ml. When assaying for Amp persistence, dilutions were plated onto LB-Amp plates, as described above.

To measure the levels of persistence of *E. coli* K-12 strains, the plasmid pSMART-*shpAB1* (Table 1) expressing *shpAB1* was transformed into BW25113 and the *lon* derivative JW0429-1. Tolerance to Amp was measured as described above.

MIC determination. Overnight cultures were spread evenly onto Mueller-Hinton II plates (150 by 15 mm; Becton, Dickinson and Co., Franklin Lakes, NJ) with sterile cotton swabs. Antibiotic test strips representing 5 different antibiotics (see Table 3) were transferred to each plate per the manufacturer's instruction, and the plates were incubated overnight at 37°C. MIC values were determined by recording the antibiotic concentration on the strip that intersected with the point of visible inhibition of bacterial growth.

Growth rates measurements. Twenty microliters of overnight cultures was transferred to 20 ml of LB contained in a 125-ml baffled flask and incubated at 37°C with shaking. The optical density at 600 nm (OD_{600}) reading was recorded every 30 min over the course of several hours.

To measure the delay in the onset of growth, overnight cultures were diluted and plated on LB plates and sealed with Parafilm. Colonies were counted at regular intervals following growth at 37°C. These values were compared to the number of colonies observable after 80 h.

Isolation and mapping of Tn*10d* markers linked to high persistence. Tn*10d*(Tc) libraries were prepared in strain LT2 as described previously (45, 49). Tet-resistant (Tet^r) colonies were pooled and resuspended in 1.5 M NaCl for preparation of bacteriophage P22 lysates (49). Generalized transduction was used to transfer Tn*10d* insertions into the highly persistent mutant AS17 (Table 1) (49). Individual Tet^r transductants were then scored for high persistence as described above.

Arbitrary PCR (50, 51) was used to map the locations of Tn*10d* elements in AS17 (Table 1). Two rounds of PCR were run using primers listed in Table 2. The first round of arbitrary PCR used the primers ARB3 or ARB1 and Tn10U (52), followed by a second round of PCR that used the primers ARB4 and IS10R. PCR products were gel purified and sequenced by the Iowa State University DNA Facility.

Recombineering. *S.* Typhimurium was prepared for electroporation as described previously by washing mid-exponential-phase cells with 1 mM MOPS (morpholinepropanesulfonic acid) in 20% glycerol (53). Antibiotic resistance markers were generated by using pKD3 (Cam) or pKD4 (Kan) (44) as the PCR templates. Primers with ~45 to 50 bases of homology to the targeted genome sequence were synthesized (Table 2). The PCR products were gel eluted using a Qiagen MinElute purification kit (Qiagen, Valencia CA). For recombineering, 1 μg of PCR product was used for electroporation into *S.* Typhimurium transformed with pKD46 (44).

TABLE 2 DNA primers used in this study

Primer name	Sequence (5′-3′)[a]
Tn10U	ACCAACCATTTGTTAAATCAGTTTTTGTTGTGA
IS10R	CAAGATGTGTATCCACCTTAACTTAATGATTTT
ARB1	GGCCAGGCCTGCAGATGATG
ARB2	GGCCACGCGTCGACTAGTACNNNNNNNNNNACGC
ARB3	GGCCACGCGTCGACTAGTACNNNNNNNNNNACGCC
ARB4	GGCCACCCGTCGACTAGTAC
STrelA-KD4.S	ATG GTC GCG GTA AGA AGT GCA CAT ATT AAT AAA GCT GGT GAA TTT GAT GTG TAG GCT GGA GCT GCT TC
STrelA-KD4.AS	ACC GAG CAC CCG GCC CAG CAC CTG CAG GTT GTA GAT CTC GAT GGT CAT ATG AAT ATC CTC CTT AG
STM4518-KD4.S	AGG CGA TAG AAA CAG CGG CTG CGG ATG TAC TGA ATT TCA TGT AAT TGT GTA GGC TGG AGC TGC TTC G
STM4518-KD4.AS	AGA ACG CGT CTC TTC CTG TCT GTG TTT TCA CCG GCA GCG TTT TTG CAT ATG AAT ATC CTC CTT AG
STmrr-KD4.S	ATT ACT TTG AGT AGT GGT AAC GCT GGA ACC TGA TTG CGG GTA ATT TGT GTA GGC TGG AGC TGC TTC G
STmrr-KD4.AS	TTC TGG GCG AGA GGA AAC ACA GGT GCC CAC AAC GTG GTC CAT CTC CAT ATG AAT ATC CTC CTT AG
STrelA-KD4.S	ATGGTCGCGGTAAGAAGTGCACATATTAATAAAGCTGGTGAATTTGATGT GTA GGC TGG AGC TGC TTC G
STrelA-KD4.AS	ACCGAGCACCCGGCCCAGCACCTGCAGGTTGTAGATCTCGATGGTCAT ATG AAT ATC CTC CTT AG
STM4535-KD4.S	TTC CCT CTG TTA ACC CGC ATC TTT TTT CGG CGC GCC ACT TTT TCT TGT GTA GGC TGG AGC TGC TTC G
STM4535-KD4.AS	TCT AAA ACA CAT TCC ACT GTG ATA AAA GAG AGC AAG CGG CGT GCC CAT ATG AAT ATC CTC CTT AG
STM4529-KD4.AS	CAT TAA GCG CTT ATC TGG CCT GGC GGG AAT TTG TAG GCC GGA TAA CAT ATG AAT ATC CTC CTT AG
STM4528.S	GGC GTA TCC TCA CGT TTA CTC AAG
STM4529.AS	GGG AAT TTG TAG GCC GGA TAA G
STM4528-ara.S	ACC ATG GAG TTT GAA TGG GAT GCG AAC
STM4528-ara.AS	GAT GCG TTG CCG CGT TTA TGT T
STM4529-ara.S	ACC ATG GGA AGC ATG GTT AAA CAT AAA CGC GGC
PshpAB-NheI.S	GCT AGC AAA GTT GAA GGT CTG GTG CTG GTG
PshpAB-NheI.AS	GCT AGC CTT GTT CGC ATC CCA TTC AAA CTC

[a] The underlined sequences represent the portion of the primers that anneal to pKD3 and pKD4 for recombineering.

When appropriate, drug resistance cassettes were removed by site-specific recombination using pCP20 (44).

Recombinant plasmids. *shpAB* and *shpAB1* were cloned under the control of their native promoters and amplified by PCR using 5′-phosphorylated primers STM4528.S and STM4529.AS (Table 2). Amplification products were gel purified and ligated into pSMART-LCKan (Lucigen, Middleton, WI). The *shp* genes were also placed under the control of the *araBAD* promoter using primers STM4528-ara.S and STM4529.AS (*shpAB* or *shpAB1*), STM4528-ara.S and STM4528-ara.AS (*shpB* or *shpB1*), and STM4529-ara.S and STM4529.AS (*shpA*) (Table 2) and cloned into pBAD-TOPO (Invitrogen, Carlsbad, CA). Each sense primer included sequences to generate an NcoI restriction site. After cloning, plasmids were digested with NcoI to remove the leader sequence found on plasmid pBAD-TOPO before religating. The Amp^r of the resulting plasmid was converted to spectinomycin (Spc) resistance by inserting a blunt-ended (SmaI) Spc^r cassette from pKD13 (47) into the unique ScaI site within *bla*. All plasmid inserts were confirmed by DNA sequencing and were purified from *E. coli* NEB5α before introduction into *Salmonella* by electroporation. Gene expression was induced with L-arabinose at a final concentration of 0.01%.

The *shpB* gene was also expressed from the *tac* promoter by cloning an NcoI-PmeI fragment from pBADshpB into pTrcHisA (Invitrogen). A DNA fragment representing *lacI*-P_{trc}-*shpB* was then inserted into the pSC101-derived cloning vector pJPC12 (46), generating pJPC-Trc-shpB (Table 1). Gene expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM.

The *shpAB* promoter region was cloned by PCR from LT2 genomic DNA using primers PshpAB-NheI.S and PshpAB-NheI.AS (Table 2). The resulting PCR product included the region upstream of *shpAB* and contained the putative promoter and ribosome binding site for the targeted genes. The PCR product was digested with NheI, gel purified, and ligated into the unique XbaI site of the *lacZ* fusion vector pLacZ2 (54), generating a translational gene fusion.

β-Galactosidase assays. The pLacZ2-derivative plasmid expressing the Φ(*shpA′-lacZ*)Hyb gene fusion was introduced into strains LT2, AV53 (Δ*shpAB*), and AS17 (*shpB1*). The transformants were assayed for β-galactosidase activity (48) on cultures grown to mid-logarithmic phase or from saturated overnight cultures in LB at 37°C.

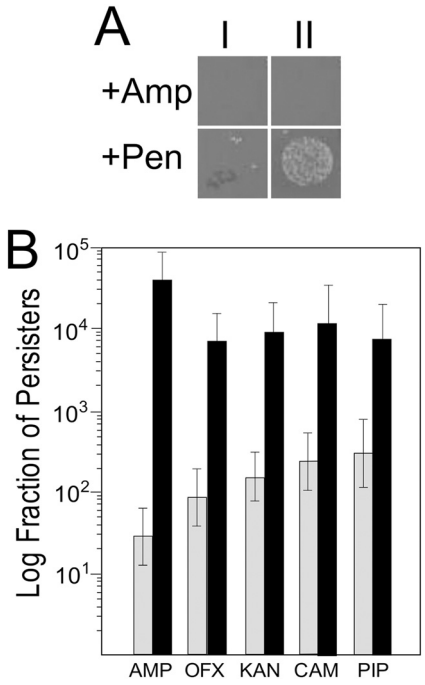

FIG 1 Phenotypes of higher persisters. (A) Wild-type stain LT2 (I) and highly persistent mutant AS17 (II) were spotted onto LB-Amp plates and incubated for 24 h (+Amp). Plates were sprayed with penicillinase and incubated for another 24 h (+Pen). (B) The levels of persistence of strains LT2 (wild type, gray bars) and AS17 (*shpB1*, black bars) were measured following exposure to the antibiotics Amp, Ofx, Kan, Cam, and Pip.

RESULTS

Isolation of highly persistent mutants. Although *S.* Typhimurium does not contain an orthologue to *hipBA*, we nonetheless predicted that high-persistence mutants could be isolated in this serovar. Since the *hipBA* mutants found in *E. coli* K-12 were the result of specific base pair changes and not a complete-loss-of-function mutation (13), we mutagenized *S.* Typhimurium strain LT2 with the alkylating agent MNNG and screened for mutants

TABLE 3 Measurement of MICs

Strain	MIC (μg/ml)				
	Ampicillin	Piperacillin	Amoxicillin	Chloramphenicol	Ofloxacin
LT2	1	1.5–2	1.5–2	3–4	0.125–0.20
AS17	1	1.5–2	3	3–4	0.125–0.20

that survived prolonged exposure to Amp (see Materials and Methods). We identified several dozen mutants that showed increased survival after Amp exposure while remaining antibiotic sensitive. Basic characterization of the mutants revealed that the majority had obvious growth defects, as revealed by formation of small colonies compared to the size of the colonies of wild-type LT2 (data not shown). These mutants were not characterized further. Here we describe the characterization of an *shp* (*Salmonella h*ighly *p*ersistent) mutant with elevated tolerance to Amp (Fig. 1A) and with a growth rate similar to that of wild type.

***shp* mutant characterization.** To determine if the *shp* mutant showed high tolerance to multiple antibiotics, we measured the mutant's ability to survive exposure to Amp, Ofx, Kan, Cam, and Pip. As shown in Fig. 1B, the mutant showed a greater than 2-order-of-magnitude increase in tolerance to Amp and a 20- to 30-fold increase in tolerance to Cam, Kan, and Pip. In contrast, the MICs of multiple antibiotics were not altered (Table 3).

To more accurately measure the growth of the *shp* mutant, we compared changes in the OD_{600} compared with that of wild-type strain LT2 over the course of several hours. As shown in Fig. 2A, the *shp* mutants showed growth rates comparable to the growth rate of wild-type LT2 during exponential growth phase (each with a mass doubling time of 34 min at 37°C) but exhibited a modest extension in lag phase. In plating the *shp* mutant, we also consistently observed that colony size was heterogeneous, especially after prolonged incubation. To better understand the basis for this, we measured the time that it took for cells to form visible colonies after plating in the absence of antibiotic selection. As shown in Fig. 2B, a significant number of the *shp* cells did not immediately initiate growth after plating; i.e., they remained dormant. An equivalent number of colonies eventually appeared after continued incubation.

A previous report indicated that the high persistence of *hipBA E. coli* mutants requires a functional *relA* gene. Korch et al. showed that the persistence of the *hipA7* mutant decreased by 3 orders of magnitude in the absence of *relA* (13). To determine if the highly persistent mutant had a similar requirement for *relA*, we constructed *relA* deletion mutants in wild-type and *shp* backgrounds (Table 1) and measured tolerance to Amp. In contrast to *E. coli*, no significant change in persistence was observed (Fig. 2C), indicating that *shp*-mediated high persistence was achieved in the absence of (p)ppGpp synthesis by RelA.

In *E. coli*, the fraction of the culture that is in a persistent state is dependent on the growth phase (55). For comparison, we determined the fraction of persisters in cultures of *S.* Typhimurium during different periods of cell growth. As shown in Fig. 3, the number of cells persistent in the presence of Amp tracked with the number of cells in the culture, reaching their maximum in stationary phase, indicating density dependence; i.e., quorum-sensing regulation is not involved in high persistence. Both wild type and the *shp* mutant followed the same trends, with the latter strain showing elevated persistence throughout each of the growth phases (Fig. 3). A similar profile was observed when tolerance to ofloxacin was measured (data not shown).

Mapping the gene responsible for the Shp phenotype. To identify the gene(s) responsible for high persistence in *S.* Typhimurium, we isolated Tn*10d* insertions linked to the *shp* locus. As described in Materials and Methods, we first constructed a random Tn*10d* library in LT2 and then transduced the Shp mutant AS17 to Tet^r. Approximately 100 Tet^r transductants were then tested for loss of tolerance to Amp. This screen, an example of which is shown in Fig. 4A, yielded two transductants with transposons linked to the *shp* mutation in AS17 (Table 1).

Bacteriophage P22 was then used to transduce wild-type LT2 to Tet^r and high persistence, yielding AS26 and AV1 (Table 1). Characterization of the transductants revealed that they had the

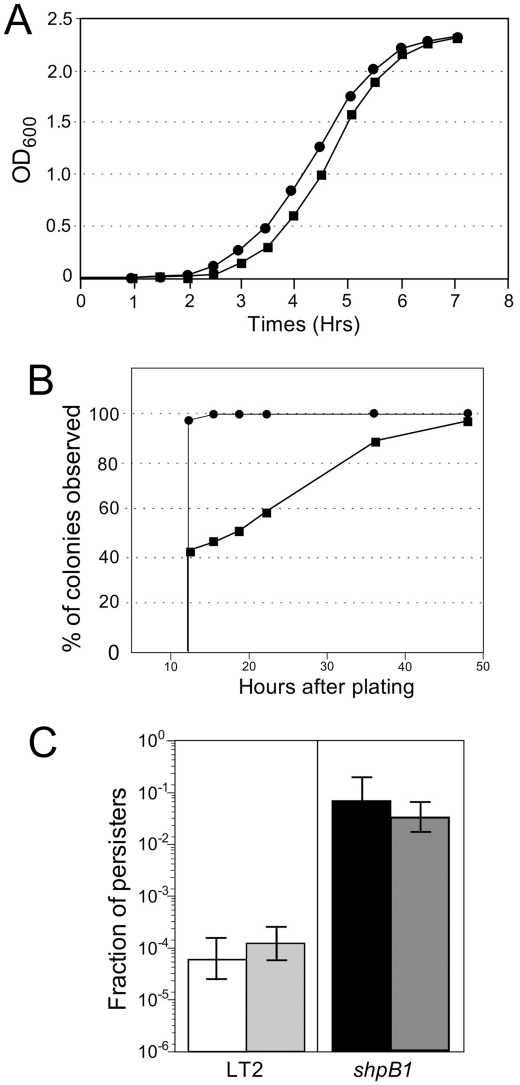

FIG 2 Characterization of the high persister. (A) Growth curves of strains LT2 (circles) and AS17 (squares). (B) Appearance of colonies on agar plates. Overnight cultures of LT2 (circles) and AS17 (squares) were diluted, plated onto LB plates, and incubated overnight at 37°C. (C) RelA is not required for formation of high persisters. A comparison between LT2 (white bar) and AV7 (LT2 *relA*::*kan*, light gray bar) on the left with the high persister AS17 (black bar) and its *relA*::*kan* derivative AV9 (dark gray bar) on the right is shown.

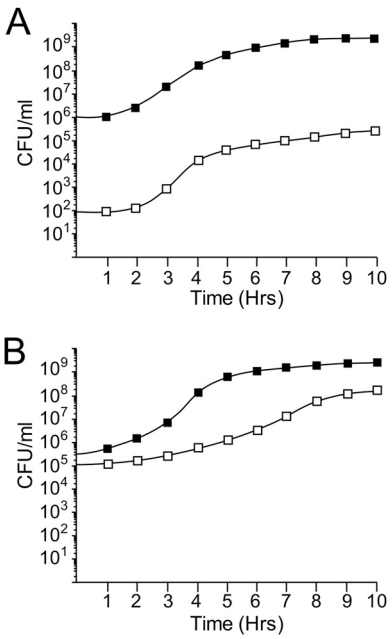

FIG 3 Levels of persistence correlate with growth phase. Strains LT2 (wild type) (A) and AS17 (high persister) (B) were inoculated into fresh LB from overnight cultures, and the numbers of CFU (closed squares) and tolerance to Amp (open squares) were measured at 1-h intervals, as described in Materials and Methods.

same growth characteristics and levels of persistence as AS17, indicating that high persistence could be attributed to the single mutation in *shpB* (data not shown). Arbitrary PCR was then used to map the location of these Tn*10d* insertions. DNA sequencing revealed that Tn*10d* inserted at different positions within *hsdM* (Fig. 4B), indicating that *shp* was in the vicinity of the *hsdSMR* locus.

To identify the *shp* locus, we used bacteriophage λ-mediated homologous recombination (recombineering) to introduce Kan^r markers at different locations in the vicinity of *hsdM* (Fig. 4B). This effort resulted in isolation of three different Kan^r insertions linked to *shp*, with an insertion in *mrr* being most tightly linked (92% cotransduction; Fig. 4B). Inspection of the genomic DNA sequence of LT2 (43) in the vicinity of *mrr* revealed multiple open reading frames of unknown function that were likely candidates for the *shp* locus. To find which of these open reading frames was responsible for generating high persisters, we constructed a derivative of AS17 by replacing the open reading frames distal to *mrr* (STM4528 to STM4536) with a *kan* cassette (Fig. 4B). After deletion of the *kan* cassette by site-specific recombination (44), persistence was measured and found to be significantly reduced (Fig. 4C).

Within the STM4528 to STM4536 deletion, we were intrigued that two of the predicted open reading frames, STM4528 and STM4529, were unusually small and appeared to compose a two-gene operon (Fig. 4B). Indeed, deletion of STM4528 and STM4529 also abolished high persistence in the AS17-derivative mutant (Fig. 4C). Since the open reading frames STM4528 and STM4529 were previously uncharacterized and are responsible for high persistence, they were named *shpA* and *shpB*, respectively.

To understand the basis for how the mutation in *shpB* imparts high persistence, we performed complementation tests by cloning *shpAB*$^+$ onto pBR322-derivative, medium-copy-number plasmids (see Materials and Methods). This plasmid, along with an empty-vector control, was introduced into strain LT2 and the high persister AS17, and persistence was measured. As shown in Fig. 4D, expression of *shpAB*$^+$ complemented the mutation in AS17 and restored persistence to the level observed in wild-type LT2.

Roles of *shpA* and *shpB* in conferring high persistence. The *shpAB* locus was also cloned, and comparison of the DNA sequences with the sequence of the wild-type allele revealed a single transition mutation that changed a cytosine to a thiamine near the 3′ end of *shpB*, yielding the *shpB1* allele. The predicted result of this change is a truncation of the last four amino acids of the ShpB gene product (Fig. 4B).

To understand how this mutation results in high persistence, we constructed additional plasmids by placing *shpAB*$^+$, *shpAB1*, *shpB*$^+$, and *shpB1* under the control of the *araBAD* promoter. Each plasmid, along with an empty-vector control, was introduced into AS17 (*shpB1*) and the Δ*shpAB* mutant AV53, and persistence was measured. As shown in Fig. 5A, expression of *shpAB1* in the Δ*shpAB* mutant imparted high persistence, even in the absence of L-arabinose. In the *shpAB1* background, expression of *shpAB*$^+$ or *shpB*$^+$ alone complemented the mutation, while expression of *shpB1* partially reduced persistence. In contrast, persistence remained high when *shpAB1* was expressed in the *shpAB1* background. These results reveal that while the *shpB1* mutation can be fully complemented with *shpB*$^+$, it is only partially complemented by *shpB1*, consistent with a defect in its gene product. Furthermore, we observed that *shpA* is required for high persistence, since ectopic expression of *shpAB1* is capable of conferring high persistence.

Despite repeated attempts, we were unable to clone *shpA*$^+$ on a plasmid without *shpB*, except where *shpA*$^+$ was under the control of the *araBAD* promoter. Transformants grown with L-arabinose were inviable, indicating that ShpA is a toxic gene product to both *E. coli* and *Salmonella* (data not shown). Expression of *shpA*$^+$ alone elevated persistence in LT2 (Fig. 5B).

***shpAB* comprises a toxin-antitoxin module.** The result that ectopic expression of *shpA* is lethal unless *shpB* is also expressed and the genetic configuration in which the two small genes are tightly linked in an operon highly resemble the findings observed for most type II toxin-antitoxin (TA) systems. TA modules have also been linked to persistence (3, 29). To more directly test if *shpAB* comprises a previously undiscovered TA module in *Salmonella*, we expressed *shpA* and *shpB* separately from different compatible plasmids under the control of the *araBAD* and *trc* promoters, respectively, in *E. coli*. As shown in Fig. 6A, expression of *shpB* significantly improved the growth of transformants expressing *shpA*, while expression of *shpB* alone did not significantly affect growth. The relatively poor growth of the transformants expressing *shpA* from the *araBAD* promoter (Fig. 6A) was achieved even in the absence of L-arabinose, indicating that even low levels of the toxin are sufficient to inhibit growth. Likewise, expression of *shpB* from the *trc* promoter in the absence of IPTG was sufficient to negate the effects of ShpA expression.

Another characteristic of many TA modules is that toxin expression is either bacteriostatic or lethal and growth inhibition can be relieved by expression of the cognate antitoxin (56). To determine if this is the case for ShpAB, we quantified the effect of *shpA*

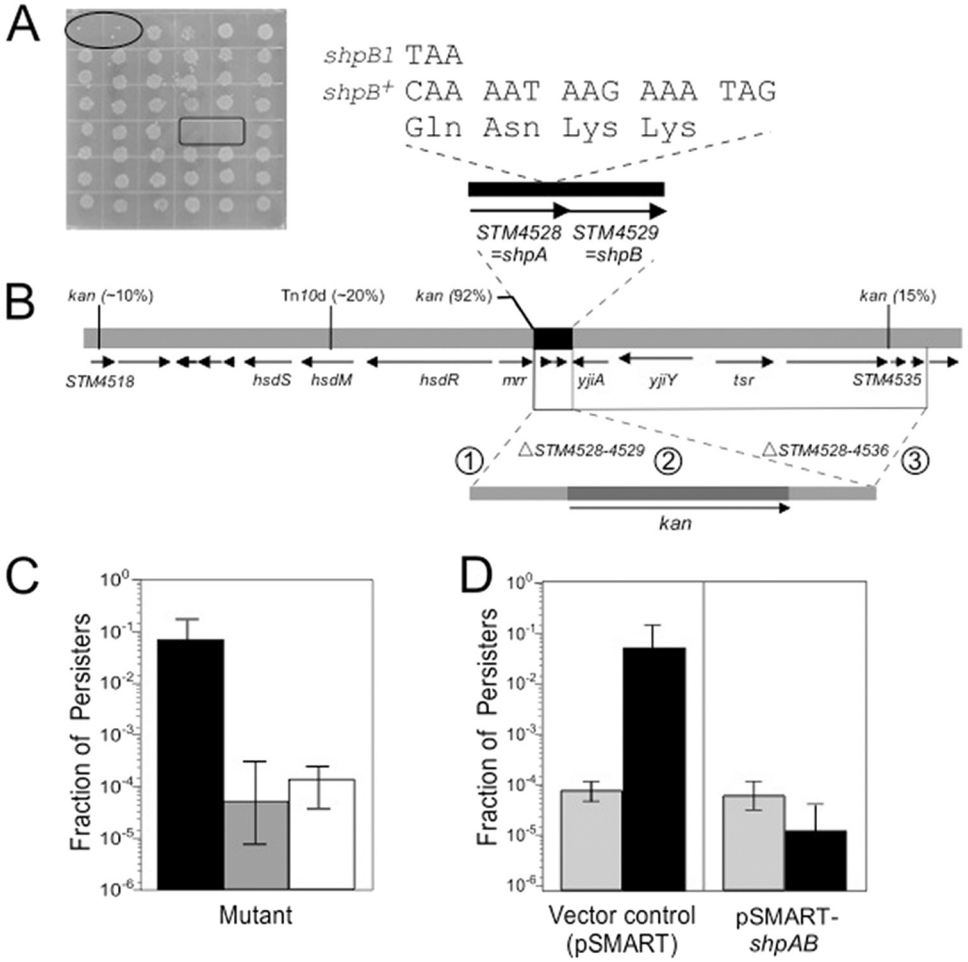

FIG 4 Mapping and characterization of the locus responsible for high persistence in *Salmonella*. (A) Screen used to identify wild-type transductants, two of which are shown within the rectangle. Wild-type controls are shown within the oval. (B) Identification of wild-type and mutant *shpAB* on the *Salmonella* Typhimurium chromosome. The linear map in the middle shows the positions of *shpAB* and flanking genes, as well as the sites of insertion of Tn*10d* and *kan* markers. The transduction linkages to *shpAB* are given in parentheses. *shpAB* (STM4528 and STM4529) genes are shown in black. The locations of the *kan* insertion/deletion mutations are shown above the linear chromosome map. Deletion of STM4528 to STM4536 (AV47) corresponds to recombination events 1 and 3 (circled numbers), while deletion of STM4528 and STM4529 (AV53) corresponds to events 1 and 2. Nucleotide sequences and predicted amino acid sequences of the $shpB^+$ and *shpB1* alleles are shown above the linear map. (C) Deletion of *shpAB* results in loss of high persistence. Persistence of AS17 (*shpB1*, black bar) along with mutants with deletions of STM4528 to STM4536 (gray bar) and STM4528 and STM4529 (*shpAB*, white bar) is shown. (D) Complementation of the Shp phenotype. A ColE1-derivative plasmid (pSMART-LCKan) expressing $shpAB^+$ (pSMART-*shpAB*), as well as the empty vector, was transformed into LT2 (gray bars) or AS17 (*shpB1*, black bars).

expression on cell viability both with and without *shpB*. While prolonged elevated expression of *shpA* dramatically reduced viability, growth inhibition could be overcome by elevated expression of *shpB* (Fig. 6B).

Type II TA modules are also known to be autoregulated, where the toxin-antitoxin complex functions as a transcriptional repressor (18). To determine if *shpAB* is autoregulated, we constructed a *lacZ* gene fusion so that the activity of the *shpAB* promoter could be measured by β-galactosidase activity (see Materials and Methods). The fusion was constructed on a ColE1-derivative plasmid and expressed in wild-type, Δ*shpAB*, and *shpAB1* backgrounds. While β-galactosidase activity in LT2 was 330 (±50) Miller units, it was significantly elevated in the two *shpB* mutants: 1,550 (±150) Miller units in AV53 (Δ*shpAB*) and 3,100 (±450) Miller units in AS17 (*shpB1*).

There are numerous examples of cases where the antitoxin protein is a substrate of the Lon protease (29, 57–60). If ShpB is also degraded by Lon, we predicted that high persistence will be dependent upon a functional Lon protease. To test this, we expressed *shpAB1* from a medium-copy-number plasmid with and without functional Lon. In *E. coli*, similar to LT2, ectopic expression of *shpAB1* elevated the fraction of persisters in the culture (2×10^{-1}). However, in the absence of *lon*, the fraction of persisters was significantly decreased (4×10^{-6}).

With the recent characterization of several different TA modules, gene products have been classified into different families depending on the target of the toxin. One of the most common families of toxins includes mRNA nucleases, which exert their bacteriostatic effects by degrading mRNA transcripts, either by themselves or in association with the ribosome (20, 61). Recently, a comprehensive analysis of amino acid sequence alignments has revealed sequence signatures of RelE-like RNases and ribbon-he-

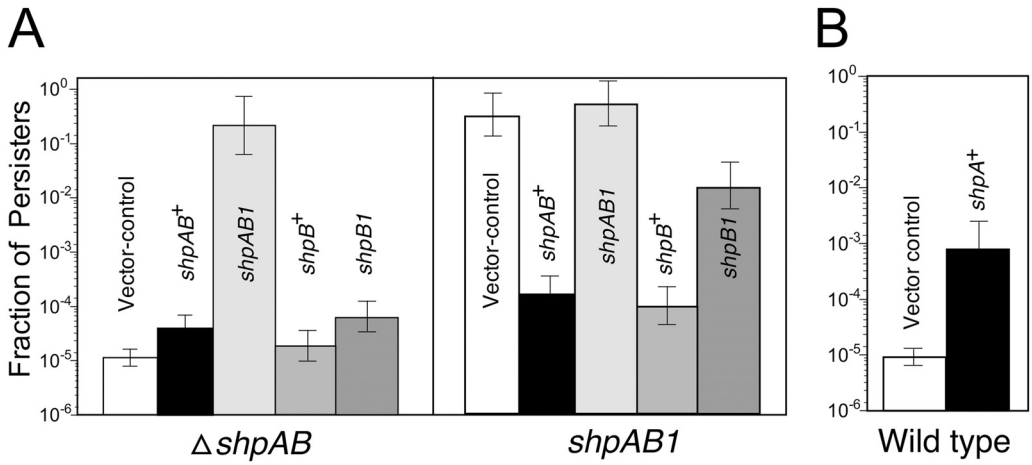

FIG 5 Effect of expression of *shpAB* on persistence. (A) Persistence of AV53 (Δ*shpAB*) or AS17 (*shpB1*) transformed with a plasmid expressing *shpAB*$^+$, *shpAB1*, *shpB*$^+$, or *shpB1*, as indicated; (B) persistence of LT2 (*shpAB*$^+$) transformed with either an empty vector (vector control) or a plasmid expressing *shpA*$^+$.

lix-helix (RHH) DNA binding motifs common to many antitoxins (22). Comparison of the ShpA amino acid sequence with the sequences of other RelE-like members strongly suggests that, indeed, ShpA is another member of this RNase family (Fig. 7A). Also, analysis of the ShpB sequence revealed it to have an amino acid sequence signature of an RHH domain, consistent with the role of ShpB as an antitoxin (Fig. 7B) (22).

DISCUSSION

The results of this study are consistent with *shpAB* representing a newly discovered TA module. Despite the diversity of type II TA modules, in general, the systems include genes that encode a toxin protein along with its cognate antitoxin polypeptide. In complex, the antitoxin prevents the toxin from inhibiting one of several essential cellular processes, including translation, transcription, DNA replication, or cell division (18–20). TA modules were first recognized as determinants of plasmid stability (16, 62, 63) but have since been implicated in many other cellular processes (19, 64–66), as they are found to be encoded throughout chromosomes of free-living bacteria (67). Most antitoxins also have DNA binding activity that enables TA complexes to repress their own transcription (20). Disruption of the balance of the TA complex by cell division or by the activity of cellular proteases such as Lon destabilizes the antitoxin, leading to bacteriostasis or cell death (3, 20, 68).

Although multiple mechanisms can contribute to persistence (3), the role of TA modules is becoming well established (2, 69). The first locus responsible for high persistence, *hipBA*, also encodes a TA module, and ectopic expression of multiple toxin genes increases persistence (26, 27, 35–39). Deletion of the type I *tisAB* TA system decreased persistence under SOS-inducing conditions (26). Similarly, deletion of the *mqsRA* TA module reduces persistence, as well as affects biofilm formation and motility (27, 70). However, deletion of other TA modules, including *hipBA*, does not alter persistence levels (13, 29). This is explained by the high degree of redundancy of TA modules; genes representing different TA families are encoded throughout the genomes of most bacteria (64). Recently, this has been confirmed, since deletion of 10 TA

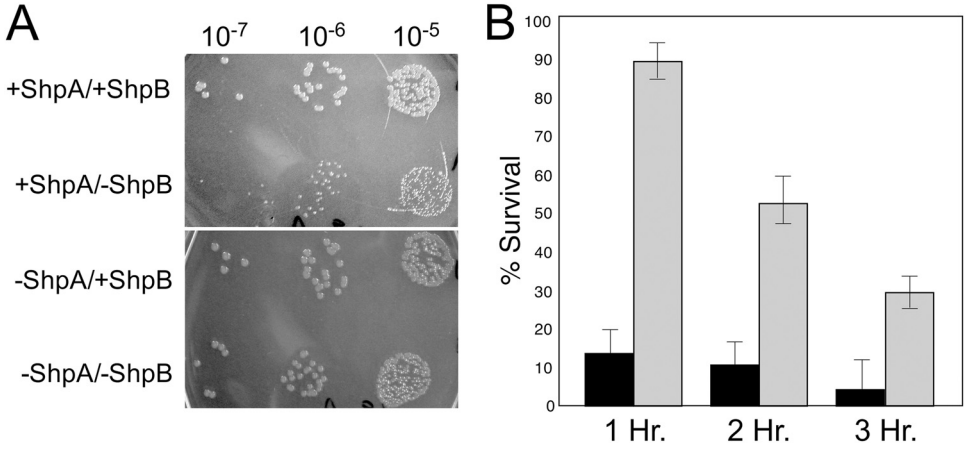

FIG 6 The detrimental effect of *shpA* expression on cell growth is overcome by expression of *shpB*. Plasmids expressing the *shpA* gene product from the *araBAD* promoter (pBAD) and *shpB* from the *trc* promoter (pTrc) were transformed into *E. coli*, as described in Materials and Methods. (A) Colonies expressing either *shpA* or *shpB* alone or in combination formed. Dilutions of transformants were grown in the absence of inducers (L-arabinose for pBAD and IPTG for pTrc). (B) Survival of cells following induction of *shpA* (black bars) by induction with L-arabinose, followed by addition of IPTG of *shpB* (gray bars), for the times indicated.

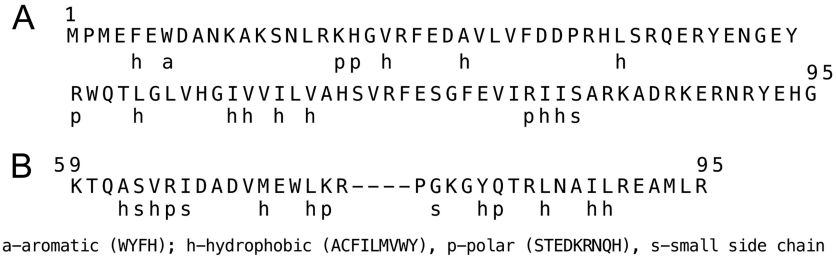

FIG 7 ShpA and ShpB homology comparisons. (A) The amino acid sequence of ShpA (residues 1 to 95) is compared to the consensus amino acid properties of RelE-like RNases, as shown below the sequence. (B) The amino acid sequence (residues 59 to 95) representing the HRR region of ShpB antitoxin is shown above the consensus amino acid properties for HRR DNA binding proteins (22).

modules predicted to encode toxins with mRNA nuclease activity decreased the level of persistence naturally occurring in *E. coli* (29).

Collectively, these studies suggest a model for bacterial persistence where increased toxin activity within a subpopulation of cells leads to inhibition of a cellular process necessary for growth, resulting in a state of dormancy that protects against the lethal effects of antibiotics (2, 3, 7, 66, 71). Since antitoxins can be degraded stochastically under normal growth conditions (72), as well as in stressed cells by increased proteolytic activity (73), persisters are continually present in bacterial populations.

We propose the following mechanism for *shpAB*-mediated persistence. The *shpB1* mutation causes a truncation of four carboxy-terminal amino acids, two of which are lysine residues (Fig. 4). Consistent with other antitoxins, the carboxy terminus is typically unstructured and can serve as a substrate for Lon protease or is important for binding the toxin (57, 74). Many DNA binding proteins also have unstructured amino- or carboxy- terminal tails rich in lysine and arginine residues that assist the folded domain of the protein in contacting DNA (75–77). We propose that the product of *shpB1*, with its altered carboxy terminus, has reduced affinity for ShpA and, consequently, is not able to fully subdue toxin activity. In addition, elevated expression of *shpA* as a result of lost repressor activity likely contributes to increased toxin activity in the mutants. Together, the increased ShpA toxin expression and activity induce a state of dormancy in a larger subpopulation of cells, likely by inhibiting translation.

Although *shpAB* represents another example of a mutation in a TA module that causes high persistence, it is distinct from the *E. coli hipA7* mutation. The *hipA7* allele encodes an altered protein that is nontoxic to the cell (13). Since ectopic expression of the *hipA7* product in a Δ*hipBA* background confers high-level persistence, the antitoxin HipB must play no part in persistence (37). Korch et al. also showed that *relA* and *spoT* are necessary for high persistence in the *hipA7* mutant, leading them to propose that high persistence results from an increased rate of (p)ppGpp synthesis (13). In contrast, *shpAB1*-mediated persistence was independent of *relA*, indicating that *shp* is not acting through the (p)ppGpp signaling. Characterization of the *shpAB1* mutant also revealed that the persister cells were generated throughout exponential growth (Fig. 3), while *hipA7* high persisters occurred primarily at high cell densities (13).

Recently, another example of a mutation in a TA module yielding high persistence was reported (58). CcdAB, well established as a determinant of plasmid stability for the F factor (63), was also linked to persistence since a mutant CcdB toxin can cause release of wild-type toxin when expressed in diploid (58). The *shpB1* mutant reported here represents the only example of a mutation in an antitoxin gene linked to high persistence.

One of the first reports identifying the STM4528 and STM4529 (*shpAB*) open reading frames was made by Sibley and Raleigh, who characterized the immigration control region (ICR) of *E. coli* and related bacteria (78). The ICR was originally defined as a 14-kb region near the origin of the *E. coli* K-12 genetic map encoding three distinct restriction-modification systems (79). Comparative genomics of this chromosomal region of several *E. coli* strains and *S.* Typhimurium LT2 revealed significant variability in genetic content. Genes within this region appear to be part of mobile cassettes that potentially integrate by site-specific recombination (78). Interestingly, *shpAB* is found in this highly variable region, but only in *S.* Typhimurium (78). TA modules similar to *shpAB* have also been found in bacteriophages and plasmids, suggesting that they may be widely distributed through horizontal gene transfer (22).

ACKNOWLEDGMENTS

This work was supported by the USDA (2003-35201-13889).

We thank Tom Hill and Jim Slauch for helpful suggestions and bacterial strains.

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