Identification and characterization of genes responsible for high persistence in Salmonella enterica serovar Typhimurium

Alec DeMars Houlihan Victoren

Iowa State University

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Identification and characterization of genes responsible for high persistence in *Salmonella enterica* serovar Typhimurium

by

Alec DeMars Houlihan Victorsen

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

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Major: Molecular, Cellular and Developmental Biology

Program of Study Committee:
Gregory J. Phillips, Major Professor
Stephen H. Howell
Kristen Johansen

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## IDENTIFICATION AND CHARACTERIZATION OF GENES RESPONSIBLE FOR HIGH PERSISTENCE IN *SALMONELLA ENTERICA* SEROVAR TYPIMURIUM
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GENERAL INTRODUCTION

Introduction

Persistence is the ability of a subpopulation of cells to survive exposure to antibiotics, and other stresses that reduce the viability of a bacterial culture. Persistent cells have apparently entered into a distinct physiological state allowing them to survive environmental stresses. Despite increased survivability, persisters are genetically identical to the rest of the population, as cultures grown from surviving cells remain as susceptible to antimicrobial stress as the original culture (5). Although persistence has been observed for many decades, the phenomenon remains poorly understood, in part due to the relatively small number of persistent cells within a bacterial culture. Persisters typically only comprise ~1×10^{-5} of the total population (62).

Understanding the mechanisms of persistence is vital, as it could potentially reduce the efficacy of antibiotics and disinfectant treatments. Furthermore, it may also contribute to the antibiotic tolerance of microbial biofilms. It is well established that antibiotics kill planktonic cells more effectively than those within a biofilm in part due to the metabolically inactive state of cells within the biofilm (2, 80). Furthermore, the ability of persisters to survive exposure to antibiotics may contribute to the development of true resistance since bacteria remaining viable for extended periods may mutate or acquire resistance via horizontal gene transfer. The identification of genes responsible for persistence represents an important strategy to understand the genetic basis for the phenotype, as well as to potentially identify new targets for drug treatments to increase the efficacy of antibiotics.
Objective of the study

The objective of this study was to identify and characterize genes responsible for increased persistence in the food borne pathogen *Salmonella enterica* serovar Typhimurium.

Thesis organization

This thesis contains two manuscripts that include the masters degree candidate, Alec Victorsen as first author. Work by a previous student, including the isolation of high persistent strains and basic characterization of high persistence mutants, are also included and appropriately noted. Both manuscripts are preceded by a literature review and conclude with a general conclusion.
LITERATURE REVIEW

Persistence

Joseph Bigger first described persistence in 1944 when he showed that cultures of *Staphylococcus aureus* consistently failed to be sterilized by penicillin. Bigger hypothesized that the incomplete effectiveness of penicillin was due to a small number of cells within the population that have entered into an altered physiological state rendering them insensitive to the antibiotic. Persistent cells were not truly antibiotic resistant however, since cultures grown from surviving cells remained as susceptible to penicillin as the original culture. Bigger hypothesized that surviving cells were dormant, non-dividing cells, a physiological state that he referred to as persistence (5).

Since the first report by Bigger, persistence has been observed in multiple bacterial species, including *Escherichia coli*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Salmonella enterica* and *Streptococcus pyogenes*. In addition, persistence has been observed in response to a variety of classes of antibiotics and other lethal stresses (5, 91).

Persistence also explains the biphasic killing response typically observed when bacterial cultures are treated with antibiotics. As shown in Figure 1, immediately following addition of an antibiotic, cell viability is reduced by several orders of magnitude. However, after the initial killing phase, cell viability is not substantially reduced further due to the ability of persistent cells to survive antibiotic exposure. Also shown in Figure 1 is the behavior of resistant mutants, which continue to grow in the presence of the antibiotic. Although not shown in Figure 1, removal of the antibiotic results in resumption of growth of the surviving persistent cells.
Persistence in *Escherichia coli*

Despite the prevalence of persistence in the prokaryotic world, relatively little is known on the molecular mechanisms of phenomenon. This limitation results in part, from the relative scarcity of persistent cells. In *Escherichia coli*, for example, persistent cells typically comprise only between $10^{-5}$ or $10^{-6}$ of the total number of cells (62). In order to better understand the genetic basis of persistence, Moyed and Bertrand isolated *E. coli* K-12 mutants that showed in increase in the level of persistence (62). To accomplish this, Moyed and Bertrand treated cultures with a chemical mutagen and subsequently exposed the cells to ampicillin for multiple rounds to enrich the culture for high persistence mutants.

The resulting mutants were shown to exhibit ~100-fold increase in the level of persistence in response to β-lactam antibiotics. In addition, the mutants also were shown to have increased tolerance to other stresses, such as elevated temperature, and starvation of thymine and diaminopimelic acid (62, 76, 94). Subsequent mapping of the gene responsible for these phenotypes led to the identification of a two-gene operon named *hipAB* (high persistence). Specifically, mutations in *hipA* lead to elevated persistence of *E. coli*. 

Figure 1. Typical response of a bacterial culture to an antibiotic exposure. See text for details. Figure from (54).
Although the exact mechanism of how hipAB contributes to persistence remains unclear, subsequent work has led to a testable model. The hipAB locus has been identified as a member of the toxin-antitoxin (TA) family of genes (7, 48). TA modules were originally discovered as plasmid-encoded genes that ensure faithful distribution of plasmids to daughter cells, also known as post-segregational killing (67). TA modules function by expressing two interactive polypeptides. When in complex, the antitoxin prevents the toxin protein from inhibiting an essential cellular process, such as replication or translation. When a plasmid-less cell segregates in a population, the more stable toxin protein kills the cell because the less stable antitoxin is no longer being synthesized.

Subsequent to their discovery on plasmids, TA modules were found encoded on the chromosome of several bacterial species (41). For example, the mazEF locus encoded by the E. coli chromosome, was originally referred to as chpAB for chromosomal homologue of plasmid-encoded genes (59).

Studies of hipAB reveal that hipA encodes a toxin, and hipB is an antitoxin that interacts directly with HipA. Furthermore, in addition to controlling activity of the HipA toxin, the toxin/antitoxin complex also regulates transcription of hipAB (6). Black et al. observed that HipB is capable of forming dimers that bind several sites within the hipAB operator. HipB contains a helix-turn-helix motif allowing it to bind four operators with the sequence TATCCN₈GGATA.

In addition, the observation that hipA cannot be expressed in cells without hipB also being expressed is consistent with the theory that the hipAB locus as a TA module (6, 7). Additional studies showed that when HipA was synthesized at higher levels relative to HipB, the levels of persistence increased (48). Collectively, these results suggest a model for how
hipAB functions in persistence. When the balance of HipA becomes elevated with respect to HipB, the toxin inhibits cell growth by interaction with an as yet unknown cellular target. The signal causing lowering of levels of HipB in relation to HipA has also not been identified.

Characterization of the hipA7 mutant has provided insights into how genetic alteration of the toxin gene leads to high persistence. The hipA7 allele contains two point mutations resulting in the amino acid substitutions G22S and D291A. The first mutation renders hipA7 non-toxic, while the second mutation is necessary for increased persistence. The high persistence caused by these mutations represents a gain of function, as hipAB null mutants do not demonstrate high persistence (47). However, hipA7 can be complemented by the wild-type hipA, indicating the gain of function is recessive (7, 63). It has been proposed that the hipA7 gene product has lower affinity for HipB. However, the significance of the G22S change is not clear.

Biochemical studies indicated that HipA is a kinase capable of autophosphorylation, which is required for persistence (20). Persister cells created by HipA are formed after the inoculation of cells from mid-log or stationary phase into fresh media (3, 29, 44). Furthermore, hipA7 mutants show an extended lag phase when introduction into fresh media (3).

Other TA Modules

Much like HipA, the toxins of other TA modules form complexes with their cognate antitoxins. As summarized in Figure 2, the resulting complexes typically regulate the transcription of the TA module (7, 68). The toxic effect of the toxin gene product is typically induced through proteolytic cleavage of the antitoxin, freeing the toxin from the TA-
complexes and allowing it to bind its target (88, 89). Concomitantly, with less antitoxin present, transcription of the operon results in synthesis of more antitoxin (70).

Again like hipAB, most TA modules are structured as two-gene operons, with the second open reading frame encoding the toxin. However, higBA and ygiUT are exceptions to this generalization; the toxin in these TA modules seems to be encoded by the first open reading frame (79, 86). The open reading frames of TA modules also frequently overlap suggesting translational coupling of the toxin and antitoxin (69, 79).

In the hok/sok module, which encodes a plasmid stabilization system, a small non-coding RNA acts as an antitoxin (34). This tripartite TA module includes a transmembrane toxin encoded by hok, whose translation is dependent on the product of mok (31). The third gene, sok, encodes an antisense RNA that blocks translation of mok, therefore preventing translation of hok (85). Similar to protein antitoxins, siRNA antitoxins are more prone to degradation and require constant transcription in order to control expression and activity of the toxin (33). Although multiple TA modules that use small RNAs rather than a protein have been found, these still represent a small fraction of known TA modules.

The targets of the TA toxins are typically essential for cell growth, including translation, transcription and DNA replication. For example, mazEF, relBE, and higBA are all inhibitors of translation. MazF halts translation by acting as a riboendonuclease, cleaving mRNA (18, 97). RelE and HigB function in a similar manner to MazF, but rather than cleave free mRNA, the toxins digest mRNA within the ribosome (17, 19, 39, 71). The TA modules ygiUT and parDE appear to act by inhibiting DNA replication. YgiU is specifically tolerant towards fluoroquinolones, which interrupt DNA replication, suggesting YgiU targets DNA
gyrase or topoisomerase IV, whereas ParE has been observed to bind directly to DNA gyrase (42, 79).

Some TA modules act as part of the stringent response in that they are activated by amino-acid starvation resulting in preferential stalling of translation of the antitoxin. Furthermore, mazEF transcription is directly regulated by ppGpp, a cell signaling molecule present during amino acid starvation (1). Although direct regulation has not been observed, the production of persisters by hipA is also dependent on ppGpp (47).

There are many theories that serve to explain why bacteria maintain multiple TA modules within their genomes. Similar to their use on plasmids, they may be used to stabilize large portions of chromosomal DNA, such as super-integrons, integrons located within the chromosome that typically encode hundreds of genes, preventing them from being deleted (74, 84). Another possibility is that chromosomal TA modules serve as “anti-addiction” systems whereby a chromosomal TA module could allow bacteria to easily lose plasmids that are stabilized by a homologous TA module (75).

However, the theory most relevant to persistence is that TA modules allow bacteria to adapt their growth to changing environments. In fact, there seems to be a correlation between the number of TA modules within a bacterial genome and the diversity of environments in which those bacteria encounter. For example obligate intracellular pathogens such as Chlamydia trachomatis are devoid of TA modules, while bacteria such as Vibrio cholerae, which inhabit both fresh and salt water environments, contain approximately 13 TA modules (69). Whatever the function of TA modules, there must be a powerful selection to maintain a potentially lethal gene in the bacterial chromosome.
As a way to understand the function of TA modules, Tsilibaris et al. deleted all known TA modules in *E. coli* K-12 (87). However, these cells did not show any phenotypic differences in comparison to wild-type cells. This suggests that either the TA modules do not significantly contribute to survival under the laboratory conditions tested, or that there are unidentified TA modules that function in a similar manner to those that were deleted. In fact, following the report by Tsilibaris et al. several additional TA modules have been found in *E. coli*, including *yhaV/prlF, yeeUV, yafW/ykfI*, and *yfjZ/ypjF* (10, 77). The later three *yeeUV, yafW/ykfI*, and *yfjZ/ypjF* represent a new class of TA modules that were identified by searching for small open reading frames (10).

In order to identify new TA modules, the RASTA-bacteria machine learning program, was created (78). This program uses homology as well as searching for short tandem, overlapping open reading frames to identify potential TA modules. Although this program represents a potentially useful way to identify new TA modules, putative genes will still need to be confirmed experimentally.

**Figure 2. Autoregulation of the TA module *relBE*.** The RelBE complex can bind to the promoter, negatively regulating translation. Degradation of RelB results in the increase in free RelE toxin that can globally inhibit translation (70).
**Toxins and persistence**

To better understand how TA modules may contribute to persistence, Keren *et al.* used microarrays to investigate the changes in transcription in a *hipA7* mutant. They observed that many genes were significantly upregulated shortly after an antibiotic challenge. Interestingly, some of the genes represented other TA proteins, such as *mazEF* and *relBE* (44). *relBE* is a TA module whose product cleaves mRNA within ribosomes resulting in a cessation of translation (71). Keren *et al.* also observed that cells overexpressing RelE showed an increase in persistence in response to fluoroquinolones (44). These results suggested that additional TA modules may also contribute to persistence. As summarized in Figure 3, Lewis proposed a model where toxin proteins, which target fundamental cellular processes, such as DNA or RNA synthesis, serve to protect essential targets from corruption by antibiotics (53). This inhibition is reversible; by increasing the levels of antitoxin protein, cells can reemerge from their persistent state at which point the antibiotic killing may resume (48, 70).

Since persistent bacteria represent such a small number of cells within the culture, they are difficult to isolate in sufficient number to characterize. In an attempt to isolate persister cells, Shah *et al.* used florescence-activated cell sorting to separate cells based on differential expression of green fluorescent protein (GFP). After separation of populations of dormant and non-dormant cells, they used microarrays to identify differentially regulated genes in persistent cells. Interestingly, the highest upregulated gene found was *ygiU*, part of a two-gene operon resembling the TA module *higBA*. Shah *et al.* was unable to knockout the second gene in the operon, *ygiT*, suggesting it encodes the antitoxin of the module (79). It was proposed that the target of the toxin may be DNA gyrase or topoisomerase IV, which are
the affected by fluoroquinolones, as overexpression of $ygiU$ increased persistence against ofloxacin and cefotaxime. Although $ygiUT$ may be a TA module, $ygiU$ has been reported to be a global regulator that regulates the two-component regulatory system $qseBC$ that is necessary for biofilm formation (35).

![Figure 3. Proposed mode of action of toxin/antitoxins on antibiotic activity.](image)

Whereas an antibiotic (circle) will bind to its target causing cell death by corrupting the target, in persisters the antibiotic still binds the antibiotic, yet does not result in a corrupted target (53).

**Other mechanisms of persistence**

*hipQ*. A second locus resulting in high persistence was found by Wolfson *et al.* that was increased persistence in *E. coli* in response to quinolones and as $\beta$-lactam antibiotics (94). The gene responsible for this trait was designated *hipQ* and maps to approximately 0.7 min downstream from the *leu* operon. However, the gene remains unidentified and uncharacterized.

*Global regulators*. Strains of *E. coli* with altered levels of persistence have also been found that result from mutations in genes other than TA modules. Li and Zhang observed
that when \textit{phoU} was interrupted by a transposon the level of persistence drastically decreased (56). The authors proposed that in addition to being a negative regulator of the phosphate regulon, including the phosphate-induced two-component system \textit{phoBR}, PhoU is actually a global negative regulator. Although \textit{phoU} can apparently alter the levels of persistence, the relevant genes regulated by PhoU have not been discovered.

\textbf{Persistence and metabolism.} A study by Spoering \textit{et al.} described additional two genes, \textit{glpD} and \textit{plsB} that can alter persistence in \textit{E. coli} (83). GlpD converts glycerol-3-phosphate (G3P) into dihydroxyacetone phosphate under aerobic conditions and may participate in formation of persisters during stationary phase. Over expression of GlpD increases the intercellular levels of G3P, which in turn negatively regulates the levels of the \textit{glp} regulon via the repressor \textit{glpR}. Although deletions in \textit{glpD} and \textit{glpR} could increase the levels of persistence independently, the increase was greatest when the G3P concentration increased from the loss of \textit{glpD}, in addition to a loss of \textit{glpR} suppressing the \textit{glp} regulon.

PlsB uses G3P in the biosynthesis of phospholipids. The authors argue that the G3P concentration can determine the number of persisters that are formed. Interrupting these processes regulated by G3P would induce a state dormancy either as a result of a lack of phospholipids, or by the repression of \textit{glpR}.

Expression of proteins that are toxic when ectopically overexpressed has also been observed to affect persistence when expressed at sub-lethal levels. This further supports the theory that the inhibition a cellular process is the cause persistence (90).

\textbf{Classes of high persistent mutants}

Careful examination of growth kinetics of persistent cells using single-cell microscopy revealed high persistent mutants can be organized into two classes. Type-I
persisters, including *hipA* mutants, must reach stationary phase before being inoculated into fresh media for persisters to develop (3, 43).

Type-II persisters, on the other hand, including *hipQ* mutants, form persister cells during exponential phase rather than during stationary phase. This class of persisters, rather than stay completely dormant, continue to divide at a much slower rate than the remaining cells in the population (3).

**Persistence and biofilms**

Most bacteria, including *E. coli* and *S. enterica* are able to form biofilms, a solid exopolysaccharide matrix created by a bacterial population to adhere to a solid surface (37). This matrix is able to protect the biofilm from the host’s immune cells, as well as some antimicrobial agents (51, 92). While protracted diffusion through the matrix protects against aminoglycosides, fluoroquinolones antibiotics are still able to freely diffuse (2, 80).

The protective nature of the matrix decreases the efficacy of antibiotics to treat persistent biofilm infections. Persisters surviving antibiotic exposure, which would normally be killed by the immune system, are protected within the biofilm. It has been proposed that repopulation of biofilm infections from persister cells leads to chronic infection, but may also allow the cells to acquire true resistance further hindering treatment.

For these reasons, understanding the molecular mechanisms of persistence is relevant and may yield new potential targets for the treatment of biofilms that may synergistically create better treatments of biofilm infections (54).

**Salmonella persistence**

Salmonella is an important pathogen of humans and animals. It is the causative agent of a common form of food-borne illness and causes significant economic impact each year in
the United States. Approximately 45% of *S. enterica* serovar Typhimurium isolates also now demonstrate multi-drug resistance with outbreaks of these strains occurring frequently (12-16).

While Salmonella is able to survive antimicrobial exposure, the mechanisms of persistence in this pathogen are also not known. Although Salmonella encodes multiple TA modules (Table 1), it does not contain homologues of *hipAB* or *mazEF*. It does however, contain homologues of *phoU* and *glpD* (69). However, the contribution of any of these genes to persistence in *Salmonella* remains unknown.

<table>
<thead>
<tr>
<th>Homologous TA module</th>
<th>Salmonella genes</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>relBE</em></td>
<td>STM1551/STM1550</td>
</tr>
<tr>
<td><em>relBE</em></td>
<td>STM3517/STM3516</td>
</tr>
<tr>
<td><em>relBE</em></td>
<td>STM4449/STM4450</td>
</tr>
<tr>
<td><em>parDE</em></td>
<td>STM2955.S/STM2954.2N</td>
</tr>
<tr>
<td><em>higBA</em></td>
<td>STM3907/STM3906</td>
</tr>
<tr>
<td><em>higBA</em></td>
<td>STM4033/STM4032.2N</td>
</tr>
<tr>
<td><em>vapBC</em></td>
<td>STM3034/STM3033</td>
</tr>
<tr>
<td><em>phdl/doc</em></td>
<td>yhhV/STM3558</td>
</tr>
</tbody>
</table>

Table 1. *Salmonella* Typhimurium genes homologous to known TA modules (69).
A METHOD FOR MAPPING TRANSPOSON INSERTIONS BY RECOMBINEERING

A paper to be submitted to the journal of Biotechniques

Alec Victorsen and Gregory J. Phillips

ABSTRACT

Transposable elements have been utilized for many years as genetic tools to study a wide variety of cellular processes in bacteria. Several molecular techniques have been developed to determine the site of transposon insertion within the bacterial chromosome. Transposons that contain a conditional origin of replication, so called plasposons, have recently become popular as they provide a convenient and effective method to clone and map transposon insertion sites. Plasposons, however, cannot be used to map the insertion sites of transposons that do not contain an origin of replication, such as those created with derivatives of Tn10. We have developed a way to convert a transposon into a plasposon by using homologous recombination to introduce a conditional origin of replication from the plasmid R6K. Using this technique we have mapped insertions sites of Tn10 and Tn10d in several strains of Salmonella enterica serovar Typhimurium.

INTRODUCTION

Transposons have proven to be an invaluable tool to study gene function in bacteria (45). The transposon Tn10, initially identified in 1975, as well as Tn5 have been modified numerous times to create a large variety of useful genetic elements (46, 93). For example, a common derivative is the mini-Tn10 derivative, Tn10d, which lacks the transposase gene, stabilizing the element in the chromosome (93). The precise point of insertion of Tn10d, as well as other Tn10 and Tn5 derivatives, is now routinely determined by DNA sequencing.
Several methods, including direct sequencing of genomic DNA and arbitrary PCR are commonly used (52). However, these methods can be tedious, and require the optimization of reaction conditions. Alternatively, the Tn element identified via southern blot, can be cloned into a plasmid vector directly from the chromosome and the site of insertion determined by DNA sequencing.

In order to make cloning the site of Tn insertion more convenient, new elements that incorporate a conditional origin of replication (ori) within the Tn element have been developed. These elements, referred to as plasposons, are converted to self-replicating plasmids by isolating genomic DNA, digesting the DNA with appropriate restriction enzymes and religating the products (26, 73). The ligation products are then transformed into a strain of *E. coli* that supports replication of the plasmid origin.

Plasposons typically utilize the R6Kγ ori and used in combination with *E. coli* hosts that express the trans-acting replication protein π. The *pir* gene, encoding π, has been incorporated into the chromosome of multiple *E. coli* strains, including strains that are commercially available. The R6Kγ ori does not initiate replication when introduced to cells that do not express π; rather, in these cases, it represents an inert sequence of DNA. However, upon transformation into a *pir*+ host, it supports replication of extra-chromosomal elements. Multiple plasmids have been developed that contain R6Kγ-ori along with selectable markers such as kanamycin resistance (24, 50).

Recombineering is a novel tool utilizing the mechanism of homologous recombination of bacteriophage λ to alter plasmid and chromosomal DNA sequences in bacteria (24, 96). Several systems have been developed in which the products of the λ
recombination system, including the genes: *exo*, *beta*, and *gam*, are expressed from temperature sensitive plasmids (25).

When cells expressing the Red proteins are transformed with linear DNA containing as little as 45-bp of homology to the host’s DNA, the Red proteins promote efficient integration of the DNA at the region of homology. Since such a sort homologous sequence is required for recombination, PCR can be used to generate the linear DNA for transformation. The primers used to create the linear DNA are hybrid primers, containing as few as 45-bp of sequence homologous to the target, as well as sequences that anneal to the sequence being inserted. Typically the template for these PCR reactions includes an antibiotic resistance marker to directly select for recombinants.

We take advantage of the known DNA sequences of Tn10 and Tn10d to introduce an R6Kγ ori and kanamycin resistance (Kan^R^) cassette into the tetAR genes carried by the Tn elements. This strategy has allowed us to map the site of insertion of several *S. enterica* mutants that were previously generated with Tn10 or Tn10d. This method should also be a useful strategy to map the insertion sites in collections of mutants generated by other Tn elements.

**MATERIALS AND METHODS**

**Bacterial strains and genetic manipulation.** TT169, TT173, TT215 and TT744 were obtained from the Salmonella Genetic Stock Center (SGSC). New Tn10d mutant insertions were generated by transforming *Salmonella enterica* LT2 derived strains with pNK972, a temperature sensitive plasmid expressing the Tn10 transposase (93). P22 phage lysates were prepared from AS30 containing Tn10d and used to transduce the Tn element into the pNK972 transformants to create random insertions. Transductants were grown at
42°C overnight and screened for their inability to grow on minimal media by patching individual colonies onto minimal M9 glucose plates. The *pir*<sup>+</sup> host for recovery of R6Kγ ori plasmids was EC100D (Epicentre, Madison WI).

**Media.** All strains were grown on LB agar plates or in LB broth unless otherwise noted (60). Antibiotics were used at the following concentrations: kanamycin (30 µg/ml), tetracycline (20 µg/ml), ampicillin (100 µg/ml). Minimal M9 media was supplemented with amino acids, phenylalanine and threonine, at a concentration of 0.3 mM.

**Primers.** The PCR primers used for recombineering are shown in Table 1.1. In general, they contained ~45 bases homologous to the *tetAR* genes of Tn10 along with regions that anneal to the template (underlined sequence). In addition to these two regions, the pRL27 primers contain additional bases creating *Bam*HI or *Eco*RI restriction sites.

**PCR templates.** Templates for PCR included pRL27 (50) or pPL1 (Figure 1.1) and included a Kan<sup>R</sup> cassette and the R6Kγ ori. The pPL1 plasmid is a derivative of pRL27 and was constructed by PCR amplification of pRL27 using the *Nhe*I-pRL27 primers (Table 1.1). The 2.77-kbp product was digested with *Nhe*I and religated. After recovering the circular plasmid from EC100D, the plasmid was further modified by replacing the ~350-bp *Pfl*MI and *Sma*I segment from within the Kan<sup>R</sup> gene with a DNA fragment synthesized without recognition sites for the restriction enzymes *Bsa*WI or *Bsr*FI (Integrated DNA Technologies, Coralville IA).

In order to maximize the amount of PCR product that was recovered, an initial PCR reaction was gel purified to remove the template. This PCR fragment was in turn used as a template for a second, scaled up round of PCR. The products were pooled and purified using the Qiagen PCR purification kit (Qiagen, Valencia CA).
Recombineering. Strains were recombineered using the Quick and Easy *E. coli* Gene Deletion Kit Genome Engineering by Red®/ET® Recombination (Gene Bridges, Dresden Germany). Approximately 1000 ng of PCR product was used per reaction. Electrocompetent cells were prepared by diluting overnight cultures 1:50 and growing to an $\text{OD}_{600}$ of 0.6. 1.4 ml of cells were washed three times with 1 ml of MOPS solution (1 mM MOPS in 20% glycerol) and resuspended in 20 µl MOPS solution. Recombinants were selected for resistance to Kan$^R$ and screened for their loss of resistance to tetracycline (Tet$^S$) (Figure 1.2).

Genomic plasmids. Genomic DNA was isolated using the MasterPure Complete DNA and RNA Isolation kit (Epicentre, Madison WI) from four Kan$^R$, Tet$^S$ recombinants. When digesting with *BamHI*, *EcoRI*, 4 µg of DNA was digested in 20 µL reactions. The digestions were incubated at 37°C overnight. *EcoRI* reactions were heat inactivated at 65°C for 20 minutes. The *BamHI* reaction was purified using the Qiagen Qiaex II kit because it could not be heat inactivated. Each digestion was then directly used in a 50 µl ligation reaction incubated at 14°C overnight. The ligations were incubated at 65°C for 10 minutes and either used to transform 100 µL chemically competent EC100D, or purified and electroporated into electrocompetent EC100D. The entire culture was plated on LB plates with the appropriate antibiotics.

When genomic DNA was digested with *HaeII*, *BsrFI* and *BsaWI* this same method was used. However due to the fact that these enzymes cut more frequently, they yield more fragments. In order to remove many of the fragments shorter than 1-kbp, the digestions were purified with the NucleoSpin Extract II kit (Macherey-Nagel, Bethlehem, PA) using a 1:10 dilution of NT buffer.
Sequencing data. The Tn10R primer was used to sequence plasmids generated by digestion with BamHI and HaeII. The Tn elements contain restriction sites for both these enzymes between the Tn10L priming site and tetA, therefore Tn10L could not be used (Figure 1.2). For this same reason, Tn10L primer was used when sequencing, plasmids generated by EcoRI digestion. It is also noted that when reporting the site of insertion, 9-bp duplications are generated during the transposon reaction (38).

RESULTS

Mapping of Tn10 from the SGSC collection. In order to confirm the veracity of this method, we mapped the locations of Tn10 in two auxotrophs obtained from SGSC. The Tn10 elements within TT173 and TT744 had been previously genetically mapped to cysC and argA respectively (Table 1.3). However, the exact site of insertion was not known. The tetAR genes within the Tn elements were replaced with a DNA fragment containing KanR and R6Kγ ori generated from pRL27. One of the limitations to Tn insertion site mapping using the strategy described here is that digestion with 6-bp cutting restriction enzyme often yields large plasmids that transform relatively inefficiently. To reduce the size of the plasmids, we incorporated sequences encoding restriction enzymes recognition sites for BamHI or EcoRI into the PCR primers (Table 1.1). After digestion the genomic DNA with either of these two enzymes, plasmids were recovered from EC100D and sequenced using the Tn10L primer (Table 1.4). Using this approach, the Tn10 insertions in TT173 and TT744 were found to be in cysN and argA respectively. Due to the higher resolution of this new mapping technique, we found the Tn10 in TT173 to be in cysN, which is immediately upstream from cysC that was the insertion site that was originally reported.
Mapping of new Tn10d mutants. We also generated new Tn insertions mutants of S. Typhimurium using Tn10d as described in Materials and Methods. The mutagenized cells were screened to identify auxotrophs. Several auxotrophs that failed to grow on minimal media were identified, and AV22 and AV23 were selected for mapping. Also, AV24 was selected because of its poor growth on minimal medium.

After recombineering with the PCR products generated from pRL27 as described, plasmids were isolated following digestion of with EcoRI or BamHI. Sequencing the EcoRI-generated plasmids with the Tn10d-L primer and the BamHI with the Tn10d-R primer showed the insertions in the auxotrophs AV22 and AV23 were in pheA, and the promoter region of thr respectively (Table 1.4). The mutant AV24 was a result of an insertion in yegS, identified as a lipid kinase (65). This proved to be an interesting mutation due to its phenotypic growth displayed on minimal plates. Although yegS is not an essential gene, it is clearly required for normal growth.

To confirm the identity of the genes disrupted in AV23 and AV22, the mutants were grown on minimal media and minimal media supplemented with the phenylalanine or threonine. Each strain grew on the appropriately supplemented plates as predicted.

An improved PCR template for mapping Tn insertions. Despite engineering restriction sites (EcoRI and BamHI) into the PCR products, several of the plasmids we isolated were still relatively large, (greater than 5-kbp), and low number of transformants were occasionally obtained. By further reducing the size of the resulting plasmids, we predicted that we would continue to improve the efficiency of this mapping technique.

Consequently, we used restriction enzymes that recognize 5-bp recognition sites, which cut genomic DNA more frequently. To implement this strategy, we first identified 5-
bp cutting enzymes that do not cut within Tn10, and that also consistently generate compatible 5’ or 3’-prime overhangs. Although BsaWI (WCCGGW) and BsrFI (RCCGGY) fulfill these requirements, they are found within the kan gene of pRL27.

In order to use these enzymes for mapping, we constructed a new plasmid pPL1 (Figure 1.1) that resulted in a PCR product that was devoid of BsaWI and BsrFI restriction sites. Use of this plasmid as PCR template was used to map the location of Tn10 in the auxotrophs TT169 and TT215. As shown in Table 1.5, Tn10 was inserted into serA, and lysA respectively; the same locations as reported by the SGSC.

A third enzyme HaeII (RGCGCY) was also used in these studies to map and confirm the insertions of Tn10d in AV25, AV26, and AV27, the recombineered strains that had been mapped with BamHI and EcoRI. Utilizing this enzyme showed no difference in the location of the Tn elements, yet it proved to be more efficient in yielding self-ligated products. However, HaeII cannot be used to map the insertion site of Tn10, since there are multiple sites within the element.

During the mapping of the Tn site insertions, we frequently obtained multiple insertions into the plasmids that did not represent contiguous regions of the chromosome. While these insertions did not interfere with identification of the Tn insertion site (Table 1.5), they did enlarge the plasmid sizes. To improve the efficiency of recovering plasmids without multiple inserts, we routinely purified the chromosomal digestions to remove small digestion products prior to ligation.

DISCUSSION

We have taken advantage of recombineering technology to map the site of transposon insertions in bacterial genomes. In addition to the strategy of incorporating a selectable KanR
marker and the R6Kγ ori into Tn10 and Tn10d elements, we also constructed a new plasmid to serve as a PCR template for recombineering. Our attempts at mapping Tn insertion sites by recombineering included adding unique restriction sites, (BamHI or EcoRI) within the PCR product in order to reduce the size of the recovered plasmids. While this strategy was used to map insertions, the resulting plasmids remained relatively large, making their recovery inefficient. Additionally, while DNA sequencing the plasmids revealed the junction between the Tn element and the chromosomal DNA, non-contiguous inserts joined at restriction sites were common in the plasmids, further increasing plasmid size. In addition to the insertion of additional DNA fragments during the ligation reaction, illegitimate recombination within the plasmids was another likely source of sequence variability within the plasmids. This phenomenon has been observed previously (26). Since we propagated the plasmids in strains where copy numbers would be high, overproduction of gene products could be detrimental to cell growth. This metabolic burden could be relieved by recombination resulting in deletions of portions of the plasmid.

Use of a modified plasmid for PCR that is devoid of restriction enzyme sites that recognize 5-bp recognition sites further improved the mapping strategy. Restriction enzymes HaeII, BsaWI and BsrFI sites, which cut the Salmonella chromosome on average of every 1024 bases, but do not cut within Tn10 (with the exception of HaeII) or Tn10d yielded smaller plasmids that were more efficiently recovered by transformation. As a consequence, use of these enzymes has proven to be the most efficient in the creation of plasmids from genomic DNA.

We have used the recombineering method to successfully map the locations of eight Salmonella strains containing Tn10 or Tn10d. With properly designed PCR primers, this
method can be used to map other Tn elements in any bacterial species in which recombineering is feasible.

TABLES AND FIGURES

**Figure 1.1. pPL1 plasmid.** Shown are the locations of the sense (S) and antisense (AS) priming sites for recombineering PCR products, origin of transfer (oriT), R6Kγ origin of replication (R6K ori), and kan (KanR).
Figure 1.2. Recombination of PCR products into Tn10 and Tn10d. Tn10 (top) and Tn10d (bottom) elements showing the locations of EcoRI, BamHI, HaeII, BsaWI, and BsrFI restriction sites. pRL27 PCR fragment (middle) contains regions (grey) homologous to Tn10 and Tn10d. Sequencing primer sites are shown in white.

**Table 1.1. PCR and sequencing primers used in this study.** The underlined sequences represent regions homologous to pRL27 or pPL1. The sequences in bold were added to introduce restriction sites within the primer.
Table 1.2. Bacterial strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Previously Reported Gene</th>
<th>Mapped gene</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT173</td>
<td>cysC</td>
<td>cysN</td>
<td>3074124</td>
</tr>
<tr>
<td>TT744</td>
<td>argA</td>
<td>argA</td>
<td>3143900</td>
</tr>
<tr>
<td>TT215</td>
<td>lysA</td>
<td>lysA</td>
<td>3173089</td>
</tr>
<tr>
<td>TT169</td>
<td>serA</td>
<td>serA</td>
<td>3223934</td>
</tr>
<tr>
<td>AV24</td>
<td>-</td>
<td>yegS</td>
<td>2234798</td>
</tr>
<tr>
<td>AV23</td>
<td>-</td>
<td>pheA</td>
<td>2808511</td>
</tr>
<tr>
<td>AV22</td>
<td>-</td>
<td>thrL promoter</td>
<td>187</td>
</tr>
</tbody>
</table>

Table 1.3. Tn10 and Tn10d mutants mapped in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AV5</td>
<td>Tn10L</td>
<td>AATTGATTTTTACCAAAATCATTAGGGGATTCATCAGGGCGAGCGTGAGCAGGGCATTACTATTGACGTCGCGTACCGCTATTTCTC CACGGAAAGACGAAAATTTATTAT</td>
</tr>
<tr>
<td>AV4</td>
<td>Tn10L</td>
<td>AATTGATTTTTACCAAAATCATTAGGGGATTCATCAGGGCGCTGTATCCTTTTGTGGAAGAGAAAATAGGCGAAATGGCCTGCGTAGC GGTACATCCGGATTACCGCAGCTC</td>
</tr>
<tr>
<td>AV27</td>
<td>Tn10d-R</td>
<td>TAACTTAATGATTTTTACCAAAATCATTAGGGGATTCATCAG TGCACCGCATGAAATCACGTTTAATCTGGATGGCGAACCACTCAGCGGGCA AGAATTTCATATTGAGGT</td>
</tr>
<tr>
<td>AV25</td>
<td>Tn10d-L</td>
<td>TAACTTAATGATTTTTACCAAAATCATTAGGGGATTCATCAG CACGGAGTCTTCAATGATAAGCTGGAACAGACGGGTAATGTAGTGTGCGTC GAGATGGTGGGCTTTACC</td>
</tr>
<tr>
<td>AV26</td>
<td>Tn10d-L</td>
<td>TAACTTAATGATTTTTACCAAAATCATTAGGGGATTCATCAG TGTTGTGTACTCTGTCATTTTTATTTGTCTGTCTTGTATTCCTATATTGGT TAAAGTATCTGCCCGCCT</td>
</tr>
</tbody>
</table>

Table 1.4. Sequence data from plasmids generated by digestion with BamHI and EcoRI. The underlined sequences represent Tn10 or Tn10d.
Table 1.5. Sequence data from plasmids generated by digestion with HaeII, BsaWI, and BsrFI. Underlined sequences represent portions of the Tn10d transposon. The GGCGCC of AV24, shown in bold, represents a HaeII site, after which a sequence representing another region of the genome occurred.
IDENTIFICATION AND CHARACTERIZATION OF GENES RESPONSIBLE FOR HIGH PERSISTENCE IN SalmoNella Enterica seroVar TyPimurium

A paper to be submitted to the Journal of Bacteriology

Alec Victorsen, Andrew Slattery* and Gregory J. Phillips

ABSTRACT

The ability of bacteria to survive lethal challenges of antibiotics, and other environmental stresses, is known as persistence. To better understand the genetic basis of persistence, we have isolated new mutants of Salmonella enterica serovar Typhimurium that show an increased level of persistence to antibiotic exposure. Two loci were mapped and characterized. One first includes two previously uncharacterized genes (STM4528 and STM4529) that were renamed shpAB for Salmonella high persistence. The second locus was mapped to metG, which encodes a methionyl-tRNA synthetase. Genetic analysis of the shpAB locus using deletion mutants, gene expression studies, as well as characterization of promoter activity and by lacZ gene fusions reveal that these genes appear to encode a toxin/antitoxin (TA) module. Similar to studies in Escherichia coli K-12, changes in the expression or activity of TA modules components can also lead to high persistence in S. enterica.

BACKGROUND

The phenomenon of antimicrobial persistence was originally characterized in Staphlococcus by J. Bigger (5). Cultures treated with penicillin were rarely sterilized, apparently the result of a relatively small subpopulation of cells that survived antibiotic

* Andrew Slattery was responsible for the isolation, initial characterization, and mapping the location of the transposon insertions by arbitrary PCR in the shp-mutants.
exposure. These cells were distinct from antibiotic resistant mutants and were referred to as persisters. They appeared to represent cells that had entered a state of dormancy, altruistically forgoing division in order to increase the survival of the population to lethal conditions. While persistence has been observed for decades, relatively little is known about the molecular mechanisms of this trait (53, 54).

Persistence has been documented in both Gram-positive bacteria such as *Staphylococcus pyogenes*, *Streptococcus pneumoniae* and *Staphylococcus aureus*, as well as Gram-negative species including *E. coli*, *Salmonella enterica*, *Pseudomonas aeruginosa* and *Haemophilus influenza* (5, 91). In all cases, persisters are genetically identical to non-persister cells (3). Cultures grown from enriched populations of persistent cells remain as susceptible to antibiotics as cells in the original culture (5). The paucity of persister cells within a bacterial culture has hindered elucidating the mechanisms of persistence on a genetic and molecular level.

The first successful attempt to understand persistence required the isolation of *E. coli* K-12 mutants that showed elevated levels of antibiotic tolerance. By enriching for mutants that survived exposure to ampicillin, Moyed and Bertrand identified the *hipAB* high persistence locus. Specific mutations in *hipA* resulted in a 100-1000-fold increase in survival to exposure to antibiotics (62, 94). Furthermore, these mutants showed an enhanced survival to other lethal conditions, such as elevated temperature, thymine and diaminopimelic acid starvation (76). More recent studies have carefully examined the *hipA* mutants using novel techniques to monitor the growth of single cells. These studies have revealed that *hipA* mutants have an extended lag phase when inoculated into fresh media and that persister cells formed by *hipA* arise upon exit from stationary phase (29).
Characterization of *hipA* revealed that it encodes the toxic component of a toxin-antitoxin (TA) module. Typically, multiple TA modules are found on the chromosome of bacteria, as well as on plasmids where their function is to ensure stable inheritance of extrachromosomal elements (32, 72). The toxic effects of HipA can be inhibited through direct interaction with the antitoxin HipB, which also serves as a negative regulator or the *hipAB* promoter (6, 7). The exact target of the HipA toxin remains unknown.

Since the discovery of *hipAB*, it has also been shown that other TA modules such as *mazEF*, *relBE* and *ygiUT* can increase persistence when the levels of toxin relative to antitoxin are altered as a result of expressing the toxin on an inducible plasmid (44, 79, 90). These toxins are known to inhibit essential processes such as translation and transcription (17-19, 39, 42, 71, 79, 97). Therefore, it has been proposed that persistence is a result of the regulated inhibition of macromolecular synthesis (44, 79). By this model, inhibition of essential cellular processes such as protein synthesis or DNA replication enable a cell to induce a state of dormancy, protecting them from conditions that would typically kill actively growing cells such as antibiotics. Even expression of non-TA proteins, which are toxic when overexpressed, can also induce persistence when expressed at sub-lethal levels(90).

Continued characterization of persistence in *E. coli* has revealed that genes involved in other cellular processes can also lead to elevated persistence. For example, mutations in *glpD* and *plsB* can increase the level of persistence. PIsB appears to achieve this increase by slowing phospholipid synthesis causing cell division to stall thus achieving a state of dormancy. While *glpD* seems to function indirectly by increasing the intracellular levels of glycerol-3-phosphate resulting in a decrease in expression of genes in the *glp* regulon (83).
Another gene that is involved in persistence is the transcriptional repressor encoded by \textit{phoU}. \textit{E. coli} strains containing a transposon insertion interrupting \textit{phoU} show a decrease in persistence (56). It has been proposed that in addition to being a negative regulator of the Pho regulon, PhoU is a global negative regulator of gene expression. However, the genes regulated by PhoU that cause high persistence are still unknown.

Understanding the cellular mechanisms leading to persistence is important as any process that reduces the effectiveness of antibiotics or antimicrobials could potentially have consequences to human and animal health. For example, persistence is thought to contribute to the antibiotic tolerance of biofilms (55). It is known that cells within a biofilm are more tolerant of antibiotics than those growing planktonically. Understanding the process whereby cells enter a state of persistence could lead to new treatments increasing the effectiveness of antimicrobials. Furthermore, persistence could potentially contribute to resistance by allowing surviving cells to gain resistance by mutation or horizontal gene transfer (82).

A vast majority of research on persistence has been conducted using \textit{E. coli} K-12. In order to better understand persistence in other human pathogens, we chose to characterize \textit{Salmonella enterica} serovar Typhimurium. Approximately 45% of \textit{S. enterica} serovar Typhimurium isolates demonstrate multi-drug resistance, with outbreaks of these strains occurring frequently (12-16). Despite the similarity between \textit{Salmonella enterica} and \textit{E. coli}, inspection of the \textit{Salmonella enterica} (strain LT2) genomic DNA sequence reveals no homologues of \textit{hipAB} even though our studies indicate that is capable of entering into a state of persistence. Therefore, to identify the mechanisms of persistence in this pathogen, we have isolated several mutants that show elevated levels of persistence. This report describes
the mapping and preliminary characterization of two loci responsible for high persistence, 
metG and shpAB.

MATERIALS AND METHODS

**Media.** All bacteria were grown on LB agar plates or in LB broth unless otherwise noted (60). LBEDO broth is LB broth containing 1 M D-Glucose and 1x E Salts and was used for bacteriophage P22 transduction experiments (57). Antibiotics were purchased from Sigma Chemical Co., St. Louis, Mo. and were used at the following concentrations: kanamycin (50 µg/ml), tetracycline (20 µg/ml), ampicillin (100 µg/ml), ofloxacin (5 µg/ml), piperacillin (200 µg/ml), or chloramphenicol (20 µg/ml). EGTA was used at a concentration of 10mM. The penicillinase stock was made at 2500 U/ml in 50mM Tris-HCl pH 7.5, 50mM NaCl, 1mM EDTA, 1mM dithiothreitol (DTT), in 20% glycerol. 100 µl of penicillinase stock were diluted in 4.9 ml LB to create the working solution.

**PCR primers.** Primers used in this study are found in Table 2.1.

**Bacterial strains and plasmids.** Bacterial strains are listed in Table 2.2. DH5α was used for all cloning with the exception of cloning shpA, which required EPI400 (Epicentre, Madison WI) to be used. TransforMax™ EC100D™ pir+ electrocompetent E. coli cells (Epicentre, Madison WI) was used for selection of transformants with R6K origin of replication (ori).

**Isolation of high persistence mutants.** Salmonella enterica serovar Typhimurium strain LT2 was mutagenized with N-methyl-N-nitro-N-nitrosoguanidine (MNNG). Cells were grown to a concentration of $10^8$ CFU/ml and exposed 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 persistence mutants were screened by inoculating 40 ml of LB in a 250 ml baffled flask with 40 µl of a saturated culture resuspended in sterile saline. The flask was incubated with shaking for 1 hour at 37°C before ampicillin was added and grown for another 3 hours. The cells were centrifuged and resuspended in 40 ml of fresh LB. This enrichment was repeated a second time. After centrifugation, the cells were plated on LB plates containing ampicillin and incubated at 37°C for 24 hours. The plates were then sprayed with penicillinase working solution delivered by a martini mister and incubated again at 37°C for an additional 24 hours.

Colonies were scored individually for increased persistence to ampicillin using the high persistence assay, described below. Spots that showed confluent growth were scored as having high persistence to ampicillin.

**High persistence assays.** Strains were inoculated in 100 µl or 200 µl of LB broth with appropriate antibiotics in 96-well microtiter plates, covered with parafilm and grown overnight with shaking at 37°C. Each well was diluted 1:1000 into fresh media, covered with parafilm, and incubated for 1hr before spotting 5-10 µl from each well onto 100 mm square LB plates containing ampicillin. After growth for approximately 24 hours at 37°C, the plates were sprayed with approximately 150 µl of penicillinase working solution and again cultured overnight at 37°C. The next morning, spots were inspected; high persistent mutants typically showed confluent growth, while control strains yielded only a few colonies (Figure 2.1).

To better quantify persistence, assays were performed by diluting overnight cultures were diluted to $1 \times 10^{-3}$, $1 \times 10^{-5}$ and $1 \times 10^{-6}$ in LB. 100 µl of the $10^{-3}$ and $10^{-5}$ dilutions were plated on LB with ampicillin while the $10^{-6}$ dilution was plated on LB. Cultures were plated
in triplicate and grown 24 hours at 37°C before being sprayed with 150µl of penicillinase working solution and again incubated overnight at 37°C. The number of colonies on each plate was counted and the level of persistence was calculated.

**MIC determination.** Using sterile cotton swabs, overnight cultures were spread evenly onto 150 x 15 mm Mueller-Hinton II plates (Becton Dickinson and Co., Franklin Lakes NJ). Five different antibiotic strips were transferred to each plate (as directed by the manufacturer), and 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.

**Assay for persistence to other antibiotics.** 20 µl from a 5 ml overnight culture was transferred to a 125 ml baffled flask containing 20 ml of LB. After incubation for one hour, 0.5 ml was transferred to test tubes containing 0.5 ml of LB media with ampicillin, ofloxacin, chloramphenicol, kanamycin, or piperacillin with 2x antibiotic concentrations. At the same time, 1:10 serial dilutions of the flask contents were diluted and plated on LB or plated on LB with ampicillin. The tubes were incubated for 4.5 hours at 37°C, 1:10 serial dilutions were plated on LB. Cultures containing ofloxacin and kanamycin were collected by centrifugation and resuspended in fresh LB before diluting to minimize transfer of antibiotics. All plates were incubated overnight at 37°C. Ampicillin plates were sprayed with penicillinase after the first incubation and incubated again for 24 hours. All plates were incubated at room temperature for an additional 5 days to allow cells with delayed growth to form colonies. At least 5 antibiotic tests were done on each strain using multiple plates.

**Growth rates of high persistent mutants.** 20 µl of overnight cultures were transferred to 20 ml of LB contained in a 125 ml baffled flask and incubated at 37°C with
shaking. Every 30 minutes an OD_{600} reading was recorded using a Bio-Rad Smartspec 3000 spectrophotometer.

**Effect of growth phase on persistence.** 20 µl from a 5 ml overnight culture was transferred to a 125 ml baffled flask containing 20 ml of LB. At various times 20 µl samples were removed from the cultures and 1:10 serial dilutions were plated on LB plates to assay the CFU/ml and on ampicillin plates. At the same time, 500 µl were removed and added to 5ml cultures of LB with ampicillin or ofloxacin. The 5ml cultures were incubated at 37°C for 4.5 hrs. and diluted in several 1:10 serial dilutions before being plated on LB plates. The ofloxacin culture was washed once with LB before dilution to minimize transfer of the antibiotic.

All plates were incubated for 24 hours after plating. Ampicillin plates were sprayed with penicillinase after the initial incubation and again incubated for 24 hours at 37°C. All plates were allowed to incubate for an additional 5 days at room temperature before colonies were counted. Each mutant was assayed at least three times.

**Assaying delayed growth.** To measure the delay in the onset of growth, a protocol from Balaban et al. was used (3). Overnight cultures were diluted and plated on LB plates. Colonies were counted at several times during growth at 37°C. These values were compared to the number of colonies observable after 80 hours.

**Isolation of Tn10d markers linked to high persistence.** Strains containing Tn10d transposons carried by an F-factor were used as donors for generalized P22 transduction into high persistent strains containing the temperature sensitive plasmid pNK972 expressing the Tn10 transposase (93). Tetracycline resistant transductants were selected at 42°C. The cells were pooled and resuspended in 1.5 M NaCl and used as donors for bacteriophage P22
generalized transduction into LT2. Individual tetracycline resistant transductants were then screened for high persistence by the spot persistence assay described above.

**Mapping Tn10d insertions by arbitrary PCR.** Arbitrary PCR was used to map the locations of Tn10d elements in AS19, AS20, AS23 and AS24. Two rounds of PCR were run using primers listed in Table 2.1. The first round of arbitrary PCR used the primers ARB3 or ARB1 and Tn10L, 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 sequencing and Synthesis Facility, Ames IA.

**Recombineering.** Antibiotic resistance markers were inserted into the chromosome of *S. Typhimurium* by recombineering using the Quick and Easy *E. coli* Gene Deletion Kit (Gene Bridges, Dresden Germany) as per the instructions provided by the supplier with the following modifications. 1 µg of PCR product was used per recombination reaction. *S. Typhimurium* was transformed with pRED-ET were grown to mid-exponential phase, washed three times with 1 mM MOPS in 20% glycerol and resuspended to 20 µl in MOPS solution prior to electroporation (28, 64). PCR products for recombineering were generated using pKD3, pKD4 (24), or pRL27 (50) as templates. Each plasmid contains an antibiotic cassette, either chloramphenicol (pKD3) or kanamycin (pKD4 and pRL27), and an R6K ori. Primers were synthesized with ~45-48-bp of homology to the targeted genomic sequence along with regions that would allow for the amplification of the template sequence (Integrated DNA Technologies, Coralville IA). In order to maximize the amount of PCR product that was recovered, an initial PCR reaction was gel purified to remove the plasmid template. The resulting PCR fragment was used as a template for a second round of PCR
using multiple reactions. The products were pooled and purified using the Qiagen PCR purification kit (Qiagen, Valencia CA).

**P22 Transduction and linkage analysis.** 2 ml of overnight cultures were combined with 8 ml P22 HT broth (~1×10^{11} PFU/ml in LBEDO broth) and incubated at 37°C overnight. Several drops of chloroform were added, vortexed and aerated for several minutes. Cultures were transferred to a 15 ml Falcon tube without disturbing the chloroform and centrifuged at 2.5×g for 10 min. The supernatant was transferred to several microcentrifuge tubes and centrifuged at 14,000 rpm for 30 min at 4°C. The supernatant was removed and the pellets were combined in 100µl 1.5M NaCl to which several drops of chloroform were added. Overnight cultures of the recipients were grown at 37°C. 100 µl of overnight recipient culture was combined with 10 µl of donor lysate (~1×10^{11} PFU/ml), which translates to a multiplicity of infection of ~10. The cultures were incubated for 15 min at 37°C. 900 µl of LB + EGTA was added to each transduction and incubated for 20 min at 37°C to prevent further infection. 200 µl were plated on LB with EGTA and appropriate antibiotics and incubated overnight at 37°C. Colonies were then scored for the rate of cotransduction either by patching on LB agar plates with antibiotics for the cotransduction between antibiotic markers or using the high persistence assay for the cotransduction of shp.

**Mapping Tn10d insertions by cloning.** A new technique was developed to more efficiently map the location of Tn10d insertions (Victorsen and Phillips, manuscript in preparation). Briefly, genomic DNA was isolated from strains showing linkage between Tn10d and high persistence using the MasterPure Complete DNA and RNA Isolation kit (Epicentre, Madison WI). 4µg of DNA was digested using 1 µl of one of the following restriction enzymes: EcoRI, ScaI, MluI, BsrGI, or BamHI in 20 µl reactions. Digestions were
incubated overnight at 37°C. After heat inactivation, 50 µl ligation reactions were prepared using the entire digestion solution and incubated at 14°C overnight. The entire ligation reaction was used to transform chemically competent pir \( E. coli \). Half the cells were plated on LB agar plates with the appropriate antibiotics and grown overnight at 37°C. Plasmid DNA was isolated from recombinant DNA, and sequenced.

**Cloning and complementation.** Genes under the control of their native promoter were amplified by PCR using 5’-phosphorylated primers and Phusion polymerase (Finnzymes, Espoo Finland). The products were gel purified and ligated into pSMART-LCKan (Lucigen, Middleton WI). The quantitative high persistence assay was used for the complementation tests using kanamycin in all plates, and without plating dilutions in triplicate. The assay was repeated at least three times.

Genes under the control of the araBAD promoter were amplified from genomic DNA using rTaq (Takara, Otsu Japan) and cloned using the pBAD TOPO TA expression kit (Invitrogen, Carlsbad CA). Plasmids expressing shpA or shpB individually were digested with NcoI to remove the leader peptide sequence present on the pBAD TOPO plasmid before being religated. The ampicillin cassette was interrupted by inserting a spectinomycin cassette into the unique ScaI site found within bla. The quantitative high persistence assay was used for the complementation experiments. Plates were supplemented with spectinomycin to maintain selection for the plasmids. The assays were repeated at least three times.

**shpAB regulation.** The shpAB-promoter region was cloned by PCR from genomic DNA using shpAB-prom-NheI primers (Table 2.1) into the pCR2.1 vector (Invitrogen, Carlsbad CA). The resulting product included the region upstream of shpAB and contained
the putative promoter and ribosome binding site for the genes. The resulting plasmid was digested with NheI, and the insert was gel purified and ligated into the unique XbaI site of pLacZ2 (Table 2.2) (40). This plasmid was then transformed into the strains, LT2, AV53, AS24 and AS26 and the resulting transformants were assayed for β-galactosidase activity.

The β-galactosidase activities of cells grown to mid-log phase or from overnight cultures in LB at 37°C with appropriate antibiotics were measured. 100 µl of mid-log culture or 100 µl of a 1:10 dilution of overnight culture were combined with 900 µl Z buffer (60 mM Na$_2$HPO$_4$, 40 mM NaH$_2$PO$_4$, 10 mM KCl, 1mM MgSO$_4$·7H$_2$O, 50 mM β-mercaptoethanol), one drop of 0.1% SDS and two drops of chloroform. Reactions were vortexed for 10 sec and equilibrated to 28 ºC. 200 µl of 13.3 mM o-nitrophenyl-β-D-galactopyranoside was added. Once a yellow color was observable 500 µl 1 M Na$_2$CO$_3$ was added. The OD was measured at 420, 550 and 600 nm, and the Miller units were calculated (61). The assays were repeated three times.

RESULTS

**Isolation of high persistent mutants.** Although Salmonella does not contain obvious homologues to hipAB, we reasoned that, like the *E. coli* hip mutants, mutants showing elevated levels of persistence could be isolated in *Salmonella*. These mutants should give new insights into high persistence in this food borne pathogen. Since the *hip* mutants of in *E. coli* were the result of specific base pair changes, we mutagenized *S. Typhimurium* with the alkylating agent MNNG and screened for mutants displaying high persistence (47). Since it has been observed that persisters are formed during late exponential to stationary phase in *E. coli*, we allowed the cells to recover in sterile saline for 18 hours prior to screening for high persistent mutants (3, 49). Following enrichment of
mutagenized bacteria, several mutants were isolated that showed an increase in persistence to ampicillin. A majority of these mutants showed a significant decrease in growth rate, as measured by observing colony size and growth rate measurements, and were not characterized further. Those that showed growth commensurate with LT2 were tested for an increase in persistence to a second antibiotic, ofloxacin. Six *Salmonella* high persistent mutants (*shp*) were isolated in this manner.

**Growth rates.** Although the *shp* mutants showed growth rates comparable to wild type LT2 during exponential growth phase, the mutants showed an extended lag phase (Figure 2.3). This extended lag phase was evident by measuring the time it took for cells to form visible colonies after plating without antibiotics selection. As shown in Figure 2.4, a significant number of the *shp* mutants remained dormant after plating.

**Persistence to other antibiotics.** To ensure that the new mutants imparted persistence to multiple lethal treatments, their response to ampicillin, ofloxacin, kanamycin, chloramphenicol, or piperacillin was tested. The number of persisters in mid-log growth phase has been shown to remain relatively constant, therefore, cells were grown to mid-lag phase prior to exposure (43). As shown in Figure 2.5, AS13, AS17 and AS18 showed between 2 to 3 orders of magnitude increase in persistence to all antibiotics, showing a general tolerance to multiple antibiotics.

In *E. coli*, the number persistent cells appear to be dependent on the growth phase of the culture (3, 49). In order to determine if persistent cells in *Salmonella* were also only generated during a specific growth phase, we determined the number of persister cells during different periods of cell growth (Figure 2.6). Similar to *E. coli*, the number of persistent *Salmonella* did not change between lag and early log phase. However, the number of
persistent cells increased dramatically when cells reached mid-exponential phase. The increase was observed in response to both ampicillin and ofloxacin, and a similar result was obtained when the experiment was repeated using ampicillin plates, which is a more sensitive assay of persistence (Figure 2.7).

**Role of relA in persistence.** RelA is essential for (p)ppGpp synthesis, a molecule that signals amino acid starvation in bacteria (9). Previous reports have indicated that the high persistence of *hipAB* *E. coli* mutants requires a functional *relA* gene to impart the high persistence phenotype. Using a *relA* mutant, Korch *et al.* showed that the persistence of the *hipA7* mutant decreased by 3 orders of magnitude (47). Also, other TA modules like *mazEF* are regulated by (p)ppGpp (1). To determine if the LT2 *hip* mutants have a similar requirement for functional *relA*, we constructed a knockout mutation and measured persistence in each *shp* mutant. No significant change in persistence was observed (Figure 2.8), indicating that persistence in LT2 can be achieved in the absence of (p)ppGpp synthesis.

**Selectable markers linked to *shp*.** In order to identify the locus responsible for high persistence, we attempted to isolate random insertions of the Tn10d transposon linked to high persisters in the mutants. The resulting strains: AS19 and AS20 were isolated with Tn10d linked to the *shp* mutation in AS13, AS26 and AV1 each contained transposons linked to the *shp* mutation in AS17, and AS23 and AS24 each had a transposon linked to the *shp* mutation in AS18 (Table 2.3).

Two strategies were used to map the location of these Tn10d insertions. The transposons in the AS13 and AS18 derived strains were mapped by sequencing arbitrary PCR products. The transposons in the AS17 derived strains were mapped using an alternative technique developed for this study. These transposons were retrofitted with an
R6K conditional origin of replication to essentially create a “plasposon” (26). To accomplish this, the Tn10ds were recombineered with a PCR product amplified from a region of pKD4 that included the R6Kγ origin of replication and kanamycin resistance. Total genomic DNA was isolated from the kanamycin resistant, tetracycline sensitive recombinants and digested with multiple enzymes, as described in Materials and Methods. After selecting for transformants in a pir⁺-E. coli strain, plasmid DNA was isolated and sequenced. DNA sequencing revealed that the Tn10d in both AS26 and AV1 had inserted within hsdM, only 1 base away from the insertion site of AS24 (Table 2.3).

Cotransduction by P22 is limited to 44,000 bp regions and correlates to the distance between the two phenotypes (95). Once the location of the transposon was known, the distance to the shp mutation was approximated by a formula proposed by Wu. The frequency of co-transduction between the transposons and the shp mutations are shown in Table 2.3.

**Linkage analyses of Tn10d insertions.** In order to identify selectable markers with higher linkage to the persistence genes, we used recombineering to insert either kanamycin or chloramphenicol resistance at various locations surrounding the Tn10d elements. We initially chose insertion sites based on the linkages of the Tn10d transposons. However, after repeated rounds of inserting new markers in this fashion, the rate of cotransduction remained very low. Therefore, we systematically inserted markers roughly every 5-kbp within the region surrounding our Tn10d markers that could be transduced by P22. Based on the Wu formula, the linkage of markers less than 3-kbp away from the shp allele should be at least 80%, well above the levels we had seen in our initial attempts to isolate linked markers (95).
Using this approach, we found that the high persistence mutation of AS17 and AS18 were very close to the insertion near *mrr*. The high persistence in AS13 mapped close to an insertion near *metG*. The cotransduction between the high persistence mutations and the recombineered markers are shown in Table 2.3.

**Mapping the high persistence locus of AS13.** Based on the linkage data, it appeared the mutation of AS13 was very close to *metG*, as insertion of *kan* immediately downstream of the *metG* coding region was 96.4% linked with high persistence. To determine if the *shp* mutation lay upstream or downstream from the *kan* site, a 5.3-kbp region downstream of *metG* was deleted. The metG-KD4.S and 2161-KD4.AS primers (Table 2.1) were used to generate a PCR product that was introduced to AS13 by recombineering (Figure 2.9). Although this region contains several uncharacterized genes, the level of persistence did not decrease in the AS13 derivative mutant, suggesting that the mutation lay upstream from the insertion site, likely in *metG*, encoding methionyl-tRNA synthetase.

To determine if the mutation was in *metG*, we cloned the entire coding region from wild type LT2 onto the low copy number plasmid pSMART-LCKan and attempted to complement the high persistence phenotype. As shown in Figure 2.10, persistence levels returned to those found in wild type LT2. We also cloned *metG* from AS20 and found that it also complemented the *shp* mutation.

DNA sequence analysis of the gene from AS20 confirmed that there was a mutation in *metG*. The *metG418* allele from AS13 contained a four base pair deletion at position 1877 within *metG*. The deletion of TGAT results in a frame shift mutation, resulting in a premature stop codon that truncates the last 47 amino acids of the gene product.
Mapping the high persistence locus of AS17 and AS18. To identify the location of the high persistence mutation with respect to *mrr*, we used the mrr-KD4.S and 4535-KD4.AS primers to generate a PCR product to delete a region between *mrr* and STM4535 (Figure 2.9). The resulting deletion mutant lost high persistence, restoring levels to that of LT2 and indicating that the *shp* mutation was located within the region between *mrr* and STM4535, as shown in Figure 2.11.

To further identify the *shp* alleles, we deleted the predicted genes identified as STM4528 and STM4529, the first putative operon within this region. Deleting these putative genes also restored the level of persistence to that of the wild type control strain (Figure 2.11). STM4528 and STM4529 were cloned from both AS17 and AS18 and sequenced. Both mutants contained identical C to T transition mutations in the second gene, STM4529, of the putative operon. As shown in Figure 2.13, the result is a nonsense mutation resulting in a truncation of 4 amino acids of the predicted gene product.

Complementation of high persistence. To perform complementation tests, STM4528 and STM4529 from both wild type LT2 and AS17 were cloned into the low copy number plasmid pSMART-LCKan and introduced to AS17 by electroporation. The AS24 transformants showed a decrease in the level of persistence, suggesting that the *shp* mutation in these strains is recessive. Likewise, when AS24 was transformed with the plasmid expressing STM4528 and STM4529 from the *shp* mutant, the level of persistence was also reduced (Figure 2.12).

Since STM4528 and STM4529 were clearly responsible for the high persistence phenotype in *S*. Typhimurium, the ORFs were renamed *shpA* and *shpB* and the mutant allele of *shpB* referred to as *shpB1*. Comparison of *shpA* and *shpB* with the genomes of all other
completely sequenced prokaryotic genomes, including other species of *Salmonella*, using BLAST revealed no significant matches. However, given the relatively small size of the *shpA* and *shpB* coding regions predicted to code for polypeptides of 95 and 100 amino acids respectively, and the tandem arrangement of the genes, we considered that these genes may encode a TA module, which show similar structural characteristics. To test this, we cloned *shpA* and *shpB* together, as well as individually, into pBAD-TOPO in order to regulate transcription of the genes by arabinose. Each plasmid was also modified by insertion of a spectinomycin (*spc*) resistance gene to disrupt *bla*, which encodes ampicillin resistance, so that persistence could be assayed using ampicillin.

While *S. Typhimurium* transformants expressing *shpA* and *shpB* gave rise to colonies indistinguishable from the pBAD-TOPO-*spc* control plasmid, transformants expressing *shpA* alone were small with reduced viability in the presence of arabinose (Figure 2.16). Similar results were also seen with *E. coli* transformants, although the lethal effects of *shpA* expression were even more pronounced (data not shown).

Using these plasmids, we observed that *shpB1* was complemented by *shpB*+ or *shpAB*+, even without the addition of arabinose (Figure 2.14). Furthermore, no decrease in persistence was observed in the mutant strain when transformed with the plasmid expressing *shpAB1*, while only a slight decrease was seen when transformed with the mutant *shpB1* alone. Conversely, we were able to induce a higher level of persistence by expressing the mutant *shpAB1* in the ∆*shpAB* strain AV53 (Figure 2.14). This increase in persistence was not achieved through expression of the *shpB1* allele alone, however.

**Expression of shpAB.** Another characteristic of TA modules is that they are frequently autoregulated (6, 36, 58, 77). To determine if this is also true of *shpAB*, we
constructed a gene fusion where expression of lacZ was placed under control of the putative
shpAB promoter. The β-galactosidase activity was measured in wild type LT2, AV53(ΔshpAB) and AS24 (shpB1) transformed with a plasmid expressing the lacZ construct. As shown in Table 2.4, the results indicated that shpAB is autoregulated, as the expression of lacZ increased in the absence of a wild type copy of shpB. This result also indicates that the shpB1 mutant is defective in autoregulation as β-galactosidase expression was even higher than in the shpAB deletion mutant AV53.

**Predicted TA modules in Salmonella.** Given the broad distribution of TA modules in bacteria, we sought to identify other genes that potentially encode toxin/antitoxin genes. We used the RASTA-bacteria program to identify additional putative TA modules encoded by the Salmonella Typhimurium LT2 genome (78). Genes with a score above 70%, their predicted associated genes, and shpAB are listed in Table 2.6. Interestingly, shpAB scored 51.6 and 27 respectively on the scale, below the cut off typically used to identify TA modules with a high confidence.

**DISCUSSION**

We have successfully isolated several mutant strains of Salmonella Typhimurium strain LT2 that show an increased level of persistence when exposed to multiple antibiotics. While most of these mutants grew slower than the wild type Salmonella strain LT2, several appeared to grow at a comparable rate and were chosen for further characterization. Upon closer examination, these mutants in general showed a prolonged lag phase before beginning growth comparable to LT2 (Figure 2.3). Furthermore, they did not exhibit higher antibiotic resistance, rather they were able to better tolerate antibiotic exposure (Figure 2.2 and Figure 2.5). We designated these alleles as shp for Salmonella high persistence.
Characterization of the *shp* mutants revealed that the persister cells are generated during mid to late log phase (Figure 2.6 and Figure 2.7). This is similar to what has been observed in the *hipA7* high persistent *E. coli* strain (3, 43). Unlike the *hipA7* strain however, the generation of persister cells was independent of (p)ppGpp synthesis (Figure 2.8). (p)ppGpp functions as a signaling molecule reporting amino acid starvation (9). Due to this difference between the *hipA7* mutation and our *shp* mutants, it seemed likely that the *shp* mutants were unrelated to *hipA*.

We further observed that a significant fraction of the *shp* mutants remained dormant for an extended period after plating on LB agar plates (Figure 2.4). Compared to wild type, the mutants took longer to form the same number of countable colonies. Since antibiotics are most effective against actively growing bacteria, these results suggest an explanation for high persistence. A subpopulation the *shp* mutant cells appear to delay their growth, even under favorable conditions, and in doing so are able to avoid the lethal effects of antibiotics. Unlike slow growing mutants however, where all of the cells grow at identical rates, the *shp* mutants represent a heterogeneous population of bacteria where only a subpopulation of cells are tolerant of antibiotics.

In order to better understand the mechanisms the *shp* mutants employ to delay growth and increase persistence, we used a combination of genetic and molecular approaches to identify the responsible genes. We first created a random pool of Tn10d mini-transposon insertions carrying a tetracycline resistance marker (TetR) in the *shp* mutants. Using generalized transducing bacteriophage P22, we isolated insertions that were linked to the *shp* mutations. Bacteriophage P22 was then grown on the pooled, randomized TetR mutants and used to infect wild type LT2. The TetR transductants were screened for high persistence
using the spot high persistence assay described in the Materials and Methods. Three mutant strains, AS13, AS17 and AS18 were found to cotransduce high persistence with Tet\(^R\). We believe that the remaining strains may have been the result of multiple mutations, since linked Tet\(^R\) markers were not found.

Two methods were used to determine the location of the linked Tn10d elements. The Tn10d in AS13 was mapped by arbitrary PCR as described by Das et al.\(^{(23)}\). A DNA fragment was amplified from genomic DNA using the primers Tn10L and ARB3. This product was in turn used as a template for a second round of PCR using the primers IS10R and ARB4. This amplified a smaller fragment, within the original PCR fragment, which was sequenced in order to determine the location of the Tn10d.

A second strategy was also developed to improve the efficiency of mapping the Tn10d elements (Victorsen and Phillips, manuscript in preparation). This method took advantage of recombineering to convert the Tn10d into a plasposon. When a conditional origin of replication was inserted within the transposable elements, plasmids could be created through the digestion and ligation of genomic DNA. The plasmids that contained the recombineered ori, when transformed into pir\(^+\) strains, are able to replicate allowing the location of the Tn10d to be determined by sequencing plasmid DNA.

Since the linkage between Tet\(^R\) and high persistence was relatively low, \(< 20\%\) (Table 2.3), we used recombineering to target additional selective markers in the vicinity of the Tn10d insertions. As described in Materials and Methods, we used recombineering to insert kanamycin resistance Kan\(^R\) markers throughout the genome surrounding the transposon insertion sites. This yielded several Kan\(^R\) markers that had a linkage of \(>90\%\) (Table 2.3).
Unfortunately, we were not able to accurately predict persistence genes based upon the Salmonella genomic sequence in order to guide our mapping efforts. For example, Tn10d linkage data initially suggested that the shp mutation in AS13 was near a group of genes encoding the two-component regulatory system baeRS. Associated with baeRS, are genes encoding a multidrug transporter (4). To determine the involvement of these loci in high persistence we used recombineering to separately delete baeRS and STM2126/yegNOB. Assays of persistence in these strains showed there to be no change in the level of persistence. Although these genes could have feasibly caused high persistence, upon repeating the linkage experiments it was clear that these were not close to the shp mutation as we had originally thought.

The lack of accuracy in mapping high persistence to the Tn10d markers may be due to the relative inefficiency of growing P22 on the mutations that show subtle changes in growth kinetics. However, continual use of recombineering to introduce selectable markers eventually proved successful, and the high persistence mutation in AS13 was mapped to a location near metG. A KanR marker was inserted just downstream of metG, which encodes methionyl tRNA-synthetase, and was found to show high linkage to persistence (96.4%). Downstream of this insertion were several ORFs of unknown function. To assess their contribution to persistence we deleted a region containing 6 ORFs. This deletion mutant however, showed no significant difference in persistence from wild type LT2 (data not shown).

Since metG is an essential gene, and could not be deleted, we attempted to complement the high persistence of AS20. To accomplish this, metG was cloned by PCR from wild type LT2 onto a low copy number plasmid. When AS20 was transformed with the
metG-expressing plasmid, persistence returned to a level comparable to wild LT2 (Figure 2.10). We also cloned metG418 from AS20, and upon repeating the assay, also observed lower levels of persistence. Although this result was somewhat unexpected, we attribute it to expressing of the mutant metG418 from a multiple-copy plasmid. This was also observed with hipA7 in E. coli when it was expressed on a multicopy plasmid (63).

In order to identify the mutation in metG418, we sequenced the mutant allele from AS20 and found that it contained a 4-bp deletion that resulted in a frame shift leading to a premature stop codon. This mutation results in a truncation of the last 47 residues in the C-terminal domain (CTD) of MetG.

Prior studies of MetG from E. coli have shown the CTD is necessary for dimerization of the protein, as well as additional binding to tRNA (11, 21). These two activities enable MetG to demonstrate anti-cooperative binding (8, 27). The first binding site has a higher affinity for tRNA, likely due to its interaction with one of the CTDs. At high concentrations of free tRNA, the second subunit binds a second tRNA aiding in the disassociation of the tRNA bound to first subunit, resulting in an increased $k_{cat}$ under these circumstances. It has also been proposed that the affinity of the CTD for tRNA may also allow it to regulate translation by binding to a tRNA-like structure in within the 5’ end of its own mRNA (22).

While the manner in which MetG can increase the level of persistence remains unclear, these functions of the CTD provide some insight as to the possible ways in which MetG could affect persistence. Loss of the ability to dimerize, and likely lowering of the affinity of MetG for tRNA$^{\text{Met}}$, may starve the cell of Met-tRNA stalling protein synthesis. Another likely possibility that would result in a similar situation is if the loss of the CTD
affects protein stability. However, the loss of the CTD may be affecting persistence indirectly either through translational regulation or by inducing a cell stress response.

Using recombineering we also created several additional deletions mutations to identify the alleles responsible for persistence in AS17 and AS18. The insertion near mrr was 91.7% and 96.4% linked respectively with high persistence. Therefore we deleted two small ORFs of unknown function, STM4528 and STM4529. Deletion of both genes lowered persistence to the levels seen in LT2 (Figure 2.11). To further characterize these genes, they were cloned from both wild type LT2 and AS18 and complementation tests performed. Expression of either the wild type or mutant alleles complemented the high persistence of AS17 and AS18 (Figure 2.12).

Sequencing the two ORFs from wild type and both mutants revealed the second gene in the putative two gene operon contained a nonsense mutation that would result in truncation of the last four residues from the gene product. The identical mutation was also found in AS18, and is consistent with their similar behavior in experiments to characterize high persistence (Figure 2.3, Figure 2.4, Figure 2.5 and Figure 2.8). Since these two ORFs were associated with high persistence they were named shpAB (Salmonella high persistence), and the mutant allele was referred to as shpB1.

To further understand how each ORF contributes to persistence, shpAB from wild type LT2 and AS24 was cloned, both together and individually, under the control of the araBAD promoter from E. coli (66). This E. coli promoter is functional and tightly regulated in Salmonella. Even without arabinose, expression of shpB1 complemented the shpAB1 mutants, as did expression from the mutant shpAB1 allele (Figure 2.14) indicating some level of transcription still occurs in the absence of arabinose.
To clarify the role of shpAB in persistence, we also performed complementation tests in AV53, an LT2 mutant that was deleted for shpAB. When shpAB1 were expressed together in AV53, high persistence was observed (Figure 2.14). However, expression of shpB1 alone did not induce persistence suggesting that shpA is actually causing persistence.

If this were the case, expression of shpA alone should increase persistence. Expression of shpA in LT2 was able to increase persistence, but not to the levels seen in the original mutants (Figure 2.15). This result may be due to the toxicity caused by expression of shpA.

When constructing plasmids for complementation tests, we observed that E. coli transformants expressing shpA alone grew poorly and were inviable upon addition of arabinose (data not shown). Transformants expressing shpB, shpB1, shpAB or shpAB1 however, grew normally. Similar results were also seen for the Salmonella transformants (Figure 2.16).

These results strongly suggest that shpAB represents a new TA module where the product of shpB is an antitoxin that prevents the lethal effects of ShpA through direct interaction. Typically, TA modules encode relatively small proteins, similar in size to the predicted gene products of shpA and shpB, and are found in a tandem orientation in the chromosome (30). Although most TA modules have a structure where the toxin is encoded by the downstream open reading frame, shpAB is similar in structure to higBA where the orientation is reversed (19). Furthermore, it appears that the target of ShpA is conserved in both Salmonella and E. coli, as ShpA is toxic in both species (Figure 2.16, data not shown).

Another feature of TA modules is that they are autoregulated (6, 36, 58, 77). To test if this also holds true for shpAB, we constructed a reporter gene fusion where the putative
promoter of \textit{shpAB} and a region of \textit{shpA} encoding the first few amino acids were fused to \textit{lacZ}. The \(\beta\)-galactosidase assays demonstrate that \textit{shpAB} expression was elevated in the absence of \textit{shpAB} (AV53), as well in the \textit{shpB1} mutant (Table 2.4).

While not tested directly, these results are consistent with the behavior of other TA modules where the toxin, ShpA, forms a complex with ShpB \textit{in vivo}. This complex serves both to inactivate the growth inhibition of the toxin, as well as function as a repressor of transcription of the TA module.

The \textit{shpAB} locus is located in a region that has been dubbed the immigration control region for its abundance of restriction-modification systems (81). This region is highly variable between bacteria. Neither \textit{shpA} nor \textit{shpB} share significant homology to any characterized genes including other characterized TA modules. Using the web-based program RASTA-bacteria, neither \textit{shpA} nor \textit{shpB} was predicted to belong to a TA module (Figure 2.6) (78). This program searches both for homology to known TA modules, as well as for several motifs of TA modules such as short overlapping ORFs. The program predicted 27 genes in \textit{S. Typhimurium} as having a high probability of encoding a TA module. While fifteen of the genes had previously been predicted by homology alone (Table 2.5), the remaining TA modules remain uncharacterized (69). Interestingly, both \textit{shpA} and \textit{shpB} gave relatively low probability scores and would not be considered as belonging to a family of TA modules. This result also suggests that additional, as yet identified TA modules are encoded by the \textit{S. Typhimurium} chromosome.

Discovery of \textit{shpAB} in \textit{Salmonella} represents the second example of a TA module that is involved in persistence, and the first example in \textit{Salmonella}. We propose a similar model for the function of \textit{shpAB} persistence in \textit{S. Typhimurium}. Through an unknown
cellular trigger, the balance of toxin to antitoxin changes, leading to increased expression and activity of the toxin protein. By targeting an essential cellular process, the toxin is able to halt growth, leading to the formation of persistent cells. By this model, the shpB1 mutation elevates the toxin levels and activity even in the absence of environmental stress, leading to an elevated number of persisters.

Despite these similarities, there are differences in the systems. For example, while hipAB is dependent on (p)ppGpp synthesis, shpAB is not. The mutation in hipAB was within the toxin gene hipA, the mutation in shpAB was the result of a mutation in the antitoxin shpB.

Future studies will be required to better understand how shpAB contributes to persistence in Salmonella, including identifying the target of ShpA. Our results also suggest that there are additional TA modules to be discovered in the Salmonella genome. The fact that shpAB is unrelated to hipAB yet can also cause persistence further supports the idea that there is redundancy in the function of TA modules. Insights from continued study of persistence in Salmonella will likely be applicable to all bacteria.
### TABLES AND FIGURES

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**Table 2.1. Primers used in this study.** The underlined sequences in the recombineering primers represent the portion of the primers that will anneal to pKD3 and pKD4.
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Table 2.2. Strains and plasmids used in this study.
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Plasmids

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<td>Genebridges</td>
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<td>Cloning vector</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pBAD-TOPO</td>
<td>Expression vector</td>
<td>Invitrogen</td>
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<td>Lucigen</td>
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<td>pAV1-mlu</td>
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<th>Parent strain or plasmid</th>
<th>Source</th>
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<td>pLacZ2</td>
<td>Promoterless <em>lacZ</em> cloning vector</td>
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<td>pSMART</td>
<td>this paper</td>
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<tr>
<td>pLT2 MetG</td>
<td><em>metG</em> from LT2</td>
<td>pSMART</td>
<td>this paper</td>
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<tr>
<td>pLT2 shpAB</td>
<td><em>shpAB</em> from LT2 with native promoter</td>
<td>pSMART</td>
<td>this paper</td>
</tr>
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<td><em>shpAB</em> from AS24 with native promoter</td>
<td>pSMART</td>
<td>this paper</td>
</tr>
<tr>
<td>pAS26 shpAB</td>
<td><em>shpAB</em> from AS26 with native promoter</td>
<td>pSMART</td>
<td>this paper</td>
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<td>pBAD-TOPO</td>
<td>this paper</td>
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<td>pBAD AS24 shpAB-1</td>
<td><em>shpAB</em> from AS24</td>
<td>pBAD-TOPO</td>
<td>this paper</td>
</tr>
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<td>pBAD LT2 shpB-1</td>
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<td>pBAD-TOPO</td>
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<td>this paper</td>
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<td><em>Nhe</em>I fragment from pSHP prom.</td>
<td>pLacZ2</td>
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Table 2.2. Continued.
Figure 2.1. Screening for high persistence. Small aliquots of cultures were tested for high persistence using the spot assay described in Materials and Methods. Controls in the top row (left to right, in duplicate): LT2, AS26, AV38. Transductants that have lost high persistence are circled.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Ampicillin (µg/ml)</th>
<th>Piperacillin (µg/ml)</th>
<th>Amoxicillin (µg/ml)</th>
<th>Chloramphenicol (µg/ml)</th>
<th>Ofloxacin (µg/ml)</th>
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<td>1.5 - 2</td>
<td>1.5 - 2</td>
<td>3 - 4</td>
<td>0.125-0.19</td>
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<td>3</td>
<td>3 - 4</td>
<td>0.125-0.19</td>
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<td>AS18</td>
<td>1</td>
<td>1.5 - 2</td>
<td>2</td>
<td>4</td>
<td>0.125-0.19</td>
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Figure 2.2. Minimum inhibitory concentration determination. Top: A Mueller-Hinton II plate spread with wild type LT2 and antibiotic test strips were applied as described in Materials and Methods. MIC values were determined as the point where bacterial growth inhibition intersected with each strip, as shown by the arrows. A: chloramphenicol; B: ampicillin; C: amoxicillin; D: ofloxacin; E: piperacillin. Bottom: MIC values of LT2 and high persisting mutants determined using antibiotic strip method.
Figure 2.3. Growth curves and the growth rate constants of *Salmonella Typhimurium* strains. LT2 (diamonds), *shp* mutants: AS13 (squares), AS17 (triangles), and AS18 (circles). Growth was measured as described in Materials and Methods. The OD<sub>600</sub> was measured at various times during the growth of each strain.

<table>
<thead>
<tr>
<th>Strain</th>
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<td>1.98 35</td>
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<td>AS13</td>
<td>1.86 37</td>
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<td>AS17</td>
<td>2.11 33</td>
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<tr>
<td>AS18</td>
<td>1.85 37</td>
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</table>

Figure 2.4. Delay in growth of LT2 and *shp* high persistent strains. Overnight cultures of LT2 (diamonds), AS13 (squares), AS17 (triangles) and AS18 (circles) were diluted and plated on LB and incubated at 37°C. The observable colonies were counted at various times and compared to the total number observable after 80 hrs.
Figure 2.5. Change in the level of persistence of *Salmonella* Typhimurium strains in response to antibiotics. Ampicillin, ofloxacin, kanamycin, chloramphenicol, piperacillin and ampicillin in agar plates were tested as shown. Persistence was measured according to Materials and Methods.

Figure 2.6. The effect of growth phase on persistence. As described in Materials and Methods, aliquots of cultures during different phases of cell growth were plated on LB plates (squares) after 4.5 hrs. of incubation with ampicillin (circles) or ofloxacin (triangles) in order to measure the number of persisters.
Figure 2.7. The effect of growth phase on persistence. Aliquots of growing cells were taken at different times and plated on LB plates to measure total CFU/ml (squares). Aliquots were also plated on LB + ampicillin plates to assay the number of persisters (circles) as described in Materials and Methods.

Figure 2.8. The effect of relA deletions on the level of persistence in LT2 and shp mutant strains.
**Table 2.3. Linkage analysis of Tn10d strains and recombineered strains.** Antibiotic markers were inserted in close proximity to the predicted location of the shp mutations initially either by random insertion of Tn10d or via recombineering. The frequency at which those antibiotic markers were cotransduced with the high persistence phenotype by bacteriophage P22 transduction as described in Materials and Methods is shown. Strains are organized based on the shp mutant strain from which they were derived. The insertion sites in the genomic site at which the Tn10d or recombineered DNA inserted at.
Figure 2.9. Summary of the chromosomal location of shp alleles and recombineering strategies. Top: Map of AS13 genome in the vicinity of metG. Bottom: Map of AS17 and AS18 genomes in the vicinity of shpAB. A KanR marker was used to replace the chromosomal regions by recombineering, as shown in the middle. The region between yehR and STM2161 from AS13 was deleted (top) and STM4528 and STM4534 were deleted from AS17 and AS18 (bottom). Length of DNA is not to scale.

Figure 2.10. Complementation of metG418. The levels of persistence LT2 (wild type) and AS20 (metG418) along with a plasmid control as described in Materials and Methods.
Figure 2.11. Persistence levels of deletion mutants. Regions of chromosome between shpA and STM4535 or of shpAB were deleted in LT2, and the shpB1 mutants AS17 and AS18. Deletions were made by recombineering as described in Materials and Methods.

Figure 2.12. Complementation of shpAB1. The levels of persistence in LT2 and AS24 when expressing shpAB or shpAB1 under their native promoter or a plasmid control (pSMART-LCKan) were measured as described in Materials and Methods and shown on the abscissa.
Figure 2.13. The shpB1 mutation. The C to T transition mutation results in a nonsense mutation resulting in a truncation of the C-terminal QNKK residues of ShpB. Top: chromosomal region of AS24, middle: shpAB, bottom: DNA sequence following the mutated thymine showing the truncated residues.

Figure 2.14. Persistence levels of mutants transformed with plasmids expressing shpAB alleles under araBAD regulation. AS24 (shpB1) AV53 (ΔshpAB) were transformed pBADtopo-derivative plasmids expressing shpAB, shpAB1, shpB or shpB1 as shown. Persistence was measured in the absence of arabinose according to Materials and Methods.
Figure 2.15. Persistence levels in strains expressing \textit{shpA} under \textit{araBAD} control. LT2 (wild type) and AS24 (\textit{shpB1}) were transformed with plasmids expressing \textit{shpA} (grey) or a control plasmid (white). Persistence was measured as described in Materials and Methods.

Figure 2.16. Effects of expression of \textit{shpAB} alleles on growth of \textit{S. Typhimurium}. Strain AV53 (\textit{ΔshpAB}) was transformed with pBADtopo-derivative plasmids expressing \textit{shpAB}\textsuperscript{+} (top panel), \textit{shpAB1} (second panel), \textit{shpB}\textsuperscript{+} (third panel) and \textit{shpA}\textsuperscript{+} (bottom panel) either in the presence of glucose or arabinose, as indicated.
Table 2.4. **Promoter activity of shpAB as measured by β-galactosidase activity.** The β-galactosidase activity was measured from cells in stationary or in mid-log phase. Assays were performed on LT2, AV53 or AS24 transformed with a plasmid expressing lacZ under the control of the shpAB promoter.

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Table 2.5. **Salmonella genes homologous to other TA modules** (69).
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Table 2.6. Prediction of TA modules in *Salmonella Typhimurium* by the RASTA-bacteria program. Genes predicted by the RASTA-bacteria prediction program with a score above 70, signifying a high likelihood of being a TA module gene, as well as the scores of *shpAB* (STM4528/29).
GENERAL CONCLUSION

Persistence enables bacteria to better survive lethal changes, including exposure to antibiotics. Since only a relatively small subpopulation of cells within a bacterial culture enter into a state of persistence, identifying the molecular mechanisms of persistence has proven difficult. For this reason, we have pursued a genetic approach to isolate mutants that exhibit increased levels of persistence in order to study this phenotype.

We chose to characterize persistence in the pathogen *Salmonella enterica* serovar Typhimurium. In addition to being a leading cause of food borne illnesses, new strains resistant to multiple antibiotics have recently appeared. Since persistence has important implications for use of antibiotics and antiseptics, and may contribute to antibiotic resistance by delaying the lethal effects of antimicrobials, determining the molecular mechanisms of persistence has important implications for human and animal health. To this end, we have isolated several new mutants of *Salmonella* Typhimurium that demonstrate higher levels of persistence in comparison to wild type. Similar high persistence mutants of non-pathogenic strains of *E. coli* K-12 have been isolated previously. However, the hipAB locus responsible for this phenotype in *E. coli* is not found in *Salmonella* Typhimurium.

Since *E. coli* hipAB mutants result from gain of function mutations, we chose to use a chemical mutagenesis to isolate new *Salmonella* mutants, rather than rely on more conventional transposon mutagenesis. While this approach ensured that a wide variety of mutant alleles were generated, mapping the mutations is a greater challenge.

We attempted to identify the genes responsible for the phenotype in six mutants by first isolating transposon insertion mutants genetically linked to high persistence. We successfully isolated Tn10d linked to high persistence in three of the *Salmonella* mutants.
The remaining mutants may represent strains that carry multiple mutations, and were not further characterized.

To further identify the locations of the transposon insertions, we used the technique of recombineering to insert selectable antibiotic resistance markers to create markers that had higher linkages to high persistence, and to generate deletion mutants. Recombineering also allowed us to develop a novel technique to map transposons by retrofitting them with a conditional R6K origin of replication converting them into a “plasposons”. This allowed the locations to be easily mapped by directly sequencing plasmids generated from restriction enzyme digestions of *Salmonella* genomic DNA. This strategy should be useful to map the location of transposable elements generated from previous studies in both *Salmonella* and *E. coli*.

After identifying the general region of the chromosome to within 2 min. of the genetic map or 80-kbp, recombineering was used to insert additional selectable markers to increase the linkage with the high persistence genes. After taking this approach it became evident that the linkage relationships between antibiotic resistance and high persistence did not accurately reflect the physical distance between the genes, requiring us to saturate the 2 min. region with markers. This was likely due to either changes in efficiency of growth or infection of bacteriophage P22, or with the variability of the high persistent phenotype.

Nonetheless, the strategy of introducing additional selectable markers into the *Salmonella* chromosome closely linked to the genes responsible for high persistence did eventually yield markers close to the *shp* mutants. Utilizing recombineering, we generated deletion mutants to specifically identify the genes responsible for the high persistence phenotype. Unfortunately, given the unique nature of this phenotype, we were seldom able
to predict which genes in the vicinity of a linked maker could give rise to high persistence. For example, \textit{baeRS} encodes a two-component regulatory system related to antimicrobial stress responses. It is plausible that this locus could yield high persistence by regulation of other genes, as has been observed with \textit{phoU}. Although initial linkage data suggested that a \textit{shp} mutation was within these genes, deletion of \textit{baeRS} showed no effect on persistence and reevaluation of the linkage confirmed the error in the initial linkages. Likewise, several open reading frames of unknown function in the vicinity of \textit{metG} could have yielded high persistence, yet their deletion also did not alter persistence in the mutants, leading us to identify the mutation in \textit{metG418}.

Despite these challenges, genes responsible for high persistence for the three mutants were identified. First, the mutant AS13 achieved high persistence by alteration of \textit{metG}. The product of \textit{metG} is methionyl-tRNA synthetase, an essential bacterial gene. Although this gene could not be deleted, complementation tests showed that expression of wild type \textit{metG} reduced persistence to wild type levels. Sequencing the \textit{metG418} allele revealed the mutation created a truncated protein lacking a portion of the C-terminal domain (CTD). The CTD is known to be responsible for dimerization of the enzyme, additional binding to tRNA, as well as possible translational regulation of the metG mRNA (11, 21, 22).

How this mutation results in high persistence is unknown. It is possible that the loss of the CTD domain destabilizes the protein. Alternatively, the loss of dimerization, which lowers the $K_m$ of tRNA binding at low concentration of enzyme, likely results in reduced levels of Met-tRNA$^{\text{Met}}$ that may slow initiation or elongation of protein synthesis (21). The effects of the mutant MetG must be subtle however, since growth of the \textit{metG418} mutant in exponential phase is not significantly different from wild type.
Consequently, we propose that this alteration has an indirect effect on persistence. For example, it is plausible that changes in protein synthetic capacity could trigger expression of a stress response, resulting in elevated persistence. This hypothesis could be tested using proteomics or transcriptional profiling to identify other genes whose expression is upregulated in the mutant. Furthermore, determining the levels of both Met-tRNA and fMet-tRNA would show whether persistence was caused by initiation or elongation of protein synthesis or by some regulatory function of MetG.

The other two high persistent mutants contained the same mutation. The shpAB locus appears to represent a new toxin/antitoxin (TA) module in salmonella. Interestingly, mutations in hipAB, also encoding a TA module, result in increased persistence in *E. coli* K-12. The TA module identified in this study was named shpAB, for *Salmonella* high persistence and represent the first example of TA genes participating in persistence outside of *E. coli* K-12. While shpAB is not found in *E. coli*, hipAB is also not found in *Salmonella*. This is likely reflective of the diverse origins of these genes. Furthermore, this supports the utility of studying persistence in a variety of bacteria other than *E. coli*.

Both of these *Salmonella* high persistence mutants were the result of a nonsense mutation in shpB1, resulting in the deletion of the terminal 4 amino acids from the predicted gene product. Through complementation tests, we found the ShpB protein to be the antitoxin and ShpA to be the toxin in this new TA module. In addition, through the use of a lacZ gene fusion we confirmed that shpAB is autoregulated, similar to other TA systems.

From these results we propose a model where high persistence results from an imbalance of the ratio of toxin to antitoxin. The shpB1 mutation could reduce the affinity of the antitoxin for the toxin, or result in an unstable gene product. In either event, we predict
that the levels of free toxin will be elevated in the high persistent mutants resulting in a delay of growth for a subpopulation of mutant cells. Under normal conditions, we propose that a defined ratio of toxin to antitoxin protein limits the number of cells that enter into a state of persistence. Upon encountering an environmental stress, including antibiotic exposure, the levels of the antitoxin protein are reduced, resulting in a growth inhibition due to excess toxin. The imbalance of toxin to antitoxin appears to occur stochastically in a bacterial culture, as persistent cells are found in all bacterial populations. The \textit{shpAB} mutants have apparently altered the balance of toxin to antitoxin resulting in formation of higher numbers of persistent cells.

Future studies will be required to better understand how \textit{shpAB} contributes to persistence in \textit{Salmonella}, including identifying the target of ShpA. This could be achieved through a molecular strategy such as looking for mutations that reduce toxicity of ShpA. Alternatively, a biochemical approach could be used identify the pathway of macromolecular synthesis that is disrupted by toxin expression. Cellular processes such as transcription or translation are typically the targets of toxin proteins. Biochemical approaches will also be required to test the hypothesis that ShpA and ShpB interact \textit{in vivo} and serve to regulate their own synthesis.

Our results also suggest that there are additional TA modules that remain to be identified in the \textit{Salmonella} genome. A combination of bioinformatics and genetics could be used to delete all known as well as newly discovered TA modules before again testing for persistence. While this represents a considerable effort, the fact that multiple TA modules exist throughout the genome suggests redundancy in their function. In any case, their function must be important for bacteria to maintain toxic proteins with the ability to limit
growth. The potential role of TA modules in disease should also be tested using animal models. These insights from continued study of persistence in *Salmonella* will likely be applicable to all bacteria.
REFERENCES


