Cell

(p)ppGpp Controls Bacterial Persistence by Stochastic Induction of Toxin-Antitoxin Activity

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SUMMARY

Persistence refers to the phenomenon in which isogenic populations of antibiotic-sensitive bacteria produce rare cells that transiently become multidrug tolerant. Whether slow growth in a rare subset of cells underlies the persistence phenotype has not be examined in wild-type bacteria. Here, we show that an exponentially growing population of wild-type *Escherichia coli* cells produces rare cells that stochastically switch into slow growth, that the slow-growing cells are multidrug tolerant, and that they are able to resuscitate. The persistence phenotype depends hierarchically on the signaling nucleotide (p)ppGpp, Lon protease, inorganic polyphosphate, and toxin-antitoxins. We show that the level of (p)ppGpp varies stochastically in a population of exponentially growing cells and that the high (p)ppGpp level in rare cells induces slow growth and persistence. (p)ppGpp triggers slow growth by activating toxin-antitoxin loci through a regulatory cascade depending on inorganic polyphosphate and Lon protease.

INTRODUCTION

Bacteria display remarkably high degrees of individuality or phenotypic heterogeneity in populations of genetically identical cells (Dubnau and Losick, 2006; Eldar and Elowitz, 2010; Lidstrom and Konopka, 2010). This cell-to-cell variation increases the probability that at least one offspring will survive under a given situation. In other words, one phenotype may be better adapted to a given environment, whereas other phenotypes may be predisposed to confer a higher fitness under certain other environmental conditions. An example of this so-called "bet-hedging" strategy is the bacterial persistence phenomenon (Losick and Desplan, 2008; Veening et al., 2008). Bacterial persistence was initially discovered by Joseph Bigger, who explored how bacteria were killed by penicillin (Bigger, 1944). Typically, when a growing culture of genetically identical bacteria was exposed to a bactericidal antibiotic, the bulk of the population was rapidly killed. After a few hours of treatment, the killing rate decreased dramatically, revealing the existence of rare cells, called persister cells, which were less sensitive to the antibiotic (Bigger, 1944). Descendants of persister cells were as sensitive to the antibiotic as their ancestors, showing that bacterial persistence is a noninherited, epigenetic trait (Bigger, 1944; Keren et al., 2004a).

Since the original discovery, bacterial persistence has been observed in almost all bacteria investigated, including the major pathogens *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, and *Salmonella enterica*. Unsurprisingly, bacterial persistence has been implicated in many recurrent and chronic infections (Allison et al., 2011; Lafleur et al., 2010; Lewis, 2007; Mulcahy et al., 2010). The understanding of the mechanisms underlying bacterial persistence is a prerequisite for the development of new drugs that can reduce persistence. However, the low frequency of persister cells (typically 10^{-4} to 10^{-6} of the bacterial population) has delayed the analysis of the phenomenon, and perhaps because numerous genes have been associated with the process, the molecular mechanisms leading to bacterial persistence remain unknown.

That several bactericidal antibiotics were active only against growing cells originally suggested that persisters might constitute a subpopulation of slow growing cells (Levin and Rozen, 2006). Work in *Escherichia coli* on *hi*gh *p*ersister mutants (*hip*) indicated that persisters constitute a pre-existing subpopulation of cells that is formed stochastically (Balaban et al., 2004). The resulting phenotypic variability revealed bimodality of the growth rate where the slow or nongrowing cells became tolerant to the lethal action of the antibiotics (Balaban et al., 2004). Most of the *hip* mutations mapped to the *hipA* locus (Moyed and Bertrand, 1983). HipA is a "toxin" encoded by the type II *hipBA* toxin-antitoxin (TA) locus (Korch et al., 2003). Since then, numerous research articles have highlighted that persistence of the model organism *E. coli* depends on TA loci (Dörr et al., 2010; Keren et al., 2004b; Maisonneuve et al., 2011; Shah et al., 2006; Vázquez-Laslop et al., 2006). Generally, prokaryotic TA loci code for two components, a stable toxin that inhibits cell growth and a labile antitoxin that counteracts toxin activity. Interestingly, toxin overproduction not only very efficiently inhibits cell growth but also induces a nongrowing state from which the cells can be rapidly resuscitated by the induction of cognate antitoxin genes (Christensen-Dalsgaard and Gerdes, 2006; Christensen-Dalsgaard et al., 2010; Pedersen et al.,

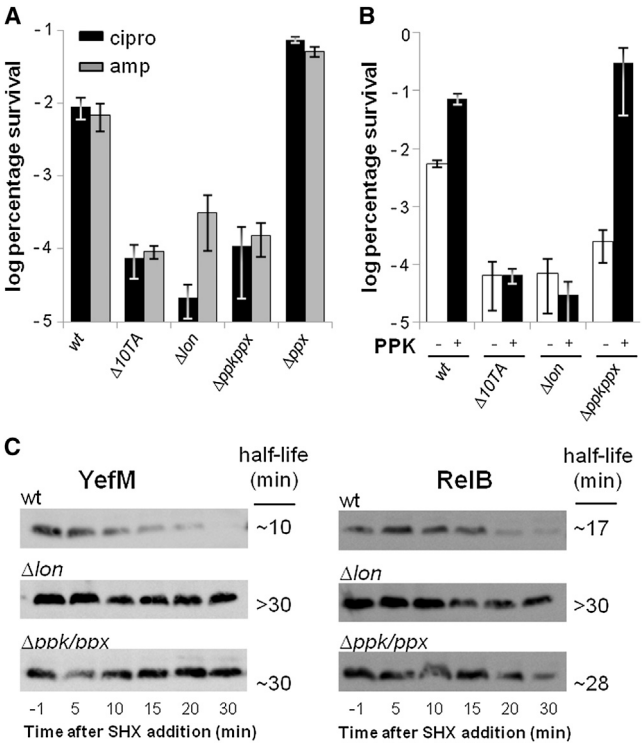

Figure 1. Bacterial Persistence Depends on PolyP·Lon-Mediated Degradation of Antitoxins

(A) Exponentially growing cells of MG1655 (WT) and isogenic deletion strains (*Δ10TA*, *Δlon Δ*(*ppk ppx*), and *Δppx*) were exposed to 1 μg/ml ciprofloxacin (black bars) or 100 μg/ml ampicillin (gray bars). Percentage of survival after 5 hr of antibiotic treatment was compared to that of the WT strain (log scale).

(B) Exponentially growing cells of MG1655 (WT), *Δ10TA Δlon*, and *Δ*(*ppk ppx*) overexpressing PPK (by plasmid pCA24N::*ppk*) were exposed to 1 μg/ml of ciprofloxacin. Percentage of survival after 5 hr was compared to that of a control strain carrying the pCA24N vector plasmid (white bars). The bars show averages of at least three independent experiments; error bars indicate SD.

(C) YefM and RelB were expressed from pEJM10 (pMG25::*yefM*) and pCA24N::*relB*, respectively in WT, *Δlon*, and *Δ*(*ppk ppx*) strains. Strains were grown in LB medium at 37°C, and at an OD_{600} of 0.3, 1 mM IPTG was added. After 30 min induction, amino acid starvation was induced by the addition of SHX (1 mg/ml), and samples for western blots were removed at the indicated times. See also Figure S1 and Table S1.

2002). We previously reported that progressive deletion of the ten type II TA loci encoding messenger RNA (mRNA) endonucleases (mRNases) of *E. coli* was associated with a cumulative reduction of persistence (Maisonneuve et al., 2011). Consistent with the observation that Lon degrades all known type II antitoxins of *E. coli*, we found that the deletion of *lon* dramatically reduced persistence. Based on these observations, we proposed a model explaining the RNase-based persistence phenomenon: the RNases encoded by TA loci are activated stochastically by Lon-mediated degradation of the antitoxins in a small subpopulation of growing cells (Maisonneuve et al., 2011). This view was also supported by the observation that Lon overproduction dramatically increased the persistence level of wild-type (WT) cells, but not of cells lacking the ten mRNases. Here, we show that WT *E. coli* cells stochastically switch into slow growth, that the slow-growing cells exhibit antibiotic tolerance, and that they are able to switch back to rapid growth. We also unravel the regulatory mechanism behind the persistence phenomenon. We present evidence that (p)ppGpp triggers persistence by activation of toxin-antitoxin loci through a regulatory cascade involving inorganic polyphosphate (PolyP) and Lon. Remarkably, the stochastic induction of slow growth is caused by fluctuations of the level of (p)ppGpp in single cells.

RESULTS

Bacterial Persistence Is Correlated with the Cellular Level of Inorganic Polyphosphate

We previously described that type II TA loci and Lon protease were both required for persistence of *E. coli* (Maisonneuve et al., 2011). Indeed, strains lacking ten TA loci (*Δ10TA*) or Lon exhibited highly reduced levels of persistence toward ciprofloxin (105- and 280-fold, respectively; Figure 1A and Table S1 available online). Because Lon is responsible for degradation of SulA, a cell-division inhibitor activated in response to DNA damage (i.e., ciprofloxacin treatment), we first evaluated the involvement of SulA in the observed phenotype by deleting *sulA* of the *Δlon* strain. The *Δlon ΔsulA* strain still showed a massive 65-fold reduction of persistence toward ciprofloxacin, indicating that, indeed, Lon is required for persistence (Figure S1A and Table S1). Altogether, the contributions of Lon and the ten TA loci in persistence were very similar (Table S1), supporting our model that persistence depends on Lon-mediated degradation of antitoxins (Maisonneuve et al., 2011).

That Lon protease controls bacterial persistence raised the possibility that Lon is stochastically turned ON at a low frequency (Maisonneuve et al., 2011). A straightforward explanation for such switching could be that the number of Lon molecules fluctuated in single cells. However, we did not observe significant fluorescence variation of cells carrying a functional Lon-GFP translational fusion inserted at the native *lon* gene serving as the sole form of active Lon (see Supplemental Information and Figures S1B and S1C).

The only known activator of Lon is inorganic polyphosphate (PolyP), a linear polymer of many hundreds orthophosphate residue (Kuroda et al., 2001). PolyP acts as a signaling molecule that binds to and directs Lon to degrade idling ribosomal proteins during amino acid starvation. In *E. coli*, PolyP is synthesized by polyphosphate kinase (PPK) and degraded by exopolyphosphatase (PPX) (Akiyama et al., 1992, 1993). To test the role of PolyP in bacterial persistence, we measured the persistence level of a *Δ*(*ppk ppx*) strain that has a very low level of PolyP (Crooke et al., 1994; Kuroda et al., 1997). Interestingly, exponentially growing cells of the *Δ*(*ppk ppx*) strain exhibited a dramatic 45- to 80-fold reduction of persistence with two bactericidal antibiotics (ciprofloxacin and ampicillin) that kill bacteria by two entirely different mechanisms (Figure 1A and Table S1). A similar effect was observed with the single *ppk* deletion mutant (Figure S1A). Cells of the *Δ*(*ppk ppx*) strain also exhibited a significant reduction in persistence in stationary phase or when grown in a model biofilm (Figures S1D and S1E).

Conversely, if PolyP programs Lon to degrade antitoxins, then the persistence level should increase in cells with an increased

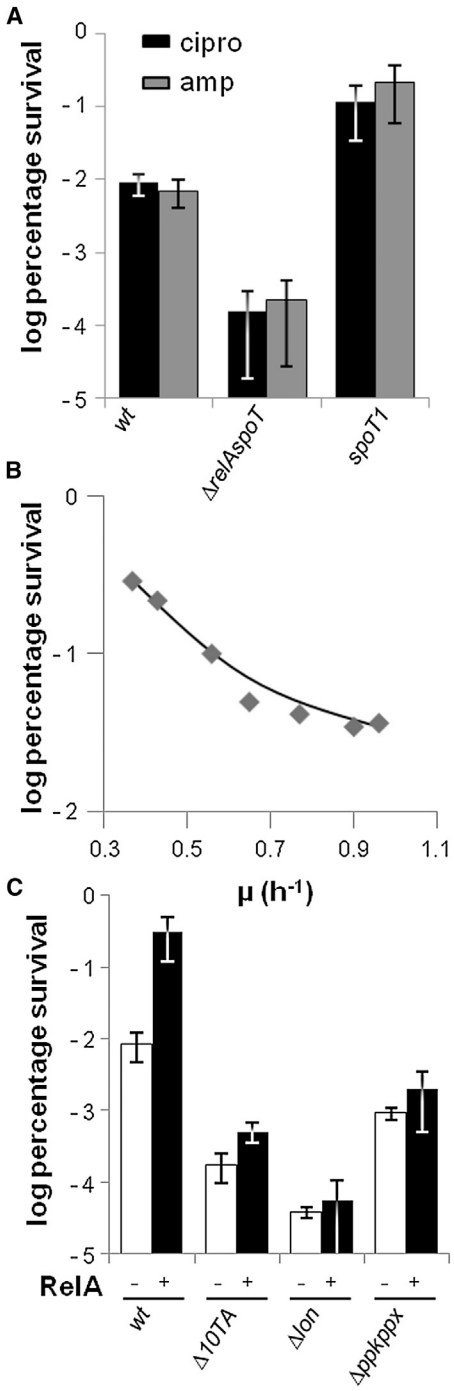

Figure 2. Bacterial Persistence Is Correlated with the (p)ppGpp Level

(A) Exponentially growing cells of MG1655 (WT), *Δ(relA spoT)*, or *spoT1* strains were exposed to 1 μg/ml ciprofloxacin (black bars) or 100 μg/ml ampicillin (gray bars). The bars show cell survival after 5 hr of antibiotic treatment (as percentage in log scale). See also Figure S1 and Table S1.

(B) Persistence toward ciprofloxacin as a function of growth rate. Different steady-state growth rates in minimal medium were obtained by varying the αMG/glucose ratio as described in Figures S2A and S2B.

(C) Exponentially growing cells of MG1655 (WT), *Δ10TA*, Δ*lon*, and *Δ(ppk ppx)* strains overexpressing RelA′ from plasmid pALS13 were exposed to 1 μg/ml of ciprofloxacin (RelA′ has constitutive (p)ppGpp synthase I activity). Percentage of survival after 5 hr was compared to that of control strains carrying the pALS14 plasmid vector expressing an inactive truncated form of RelA (RelA″) (white bars). The bars show averages of at least three independent experiments; error bars indicate SD.

level of PolyP. Indeed, a strain that accumulated PolyP because it carries a deletion of *ppx* (Zhao et al., 2008) exhibited a dramatically increased persistence level with both antibiotics used (Figure 1A). Thus, the persistence level correlated positively with the cellular level of PolyP.

We used controlled overexpression of PPK from a plasmid to increase the cellular level of PolyP. To minimize artifacts due to PolyP toxicity, we used a level of inducer that did not significantly change the cell growth rate. Mild overproduction of PPK in the WT strain resulted in a 13-fold increase in persistence (Figure 1B). Overproduction of PPK in the Δ(*ppk ppx*) strain produced a similar or even a higher level of persistence. These results showed that increasing the PolyP level induced a high level of persistence. Strikingly, however, increasing the PPK level in the *Δlon* or *Δ10TA* strains did not significantly increase persistence (Figure 1B). These results show that PolyP-induced persistence depends on both Lon and TA loci. Therefore, we tested the hypothesis that PolyP programs Lon to degrade antitoxins.

PolyP Programs Lon to Activate Toxin-Antitoxins

We measured the in vivo rate of degradation of an N-terminally his-tagged YefM antitoxin (hereafter called YefM) in exponentially growing cells. After 60 min of induction, serine hydroxamate (SHX) was added, and the approximate rate of YefM degradation was determined (Figure 1C). SHX is a serine analog that causes amino acid starvation by inhibiting aminoacylation of seryl-transfer RNA (tRNA) (Tosa and Pizer, 1971). SHX therefore triggers the stringent response and promotes PolyP accumulation (Kuroda et al., 1997). In the WT strain, YefM had a half-life ($t_{1/2}$) of ~11' (Figure 1C). YefM was stabilized in a *Δlon* strain ($t_{1/2}$ > 30'), which was consistent with previous observations (Maisonneuve et al., 2011). Finally, YefM was stabilized in the *Δ(ppk ppx)* strain ($t_{1/2}$ > to 30'), showing that PolyP was also required for degradation of YefM (Figure 1C). A similar pattern was obtained with the degradation of RelB antitoxin, showing that the requirement for both Lon and PolyP was general (Figure 1C).

To support these in vivo analyses, we tested whether PolyP stimulated Lon-mediated degradation of YefM in vitro. Indeed, we found that PolyP was required for degradation of YefM by Lon in vitro (Figure S1F). These results strongly support the notion that, in *E. coli*, PolyP programs Lon to degrade antitoxins.

(p)ppGpp Is Required for Persistence

The PolyP concentration is controlled by (p)ppGpp that competitively inhibits PPX (Kuroda et al., 1997). Consequently, we measured the level of persistence of exponentially growing cells of a Δ(*relA spoT*) strain. Indeed, this so-called (p)ppGpp^0 strain showed a massive decrease in persistence after ciprofloxacin treatment (60-fold) and also showed a significant decrease after ampicillin treatment (30-fold) (Figure 2A and Table S1). Similar effects were obtained in stationary phase cultures and in a biofilm model (Figures S1D and S1E).

If indeed (p)ppGpp positively controls persistence, then cells with a high (p)ppGpp level should exhibit increased levels of persistence. RelA and SpoT are (p)ppGpp synthetases, whereas SpoT is also a (p)ppGpp hydrolase (Gentry and Cashel, 1996). SpoT hydrolase activity is attenuated by the *spoT1* allele, while leaving the synthase activity unaffected. Thus, cells carrying the *spoT1* allele exhibited an elevated steady-state level of (p)ppGpp (~10-fold compared to WT) (Laffler and Gallant, 1974). Strikingly, the *spoT1* strain had 12- and 30-fold increases in persistence after treatment with ciprofloxacin and ampicillin, respectively (Figure 2A).

The (p)ppGpp level varies inversely with the growth rate in *E. coli* (Ryals et al., 1982). Given the above results, we hypothesized that persistence should be correlated with the growth rate. To challenge this inference, we measured the persistence level as a function of growth rate. The growth rate was controlled by varying the concentration of α-methylglucoside (αMG), a nonmetabolizable glucose analog. As expected, the ratio between glucose and αMG determined the growth rate (Figures S2A and S2B) (Hansen et al., 1975). Indeed, the persistence level varied inversely with growth rate (Figure 2B). Altogether, our results convincingly show that the level of persistence is positively correlated with the (p)ppGpp level.

Increased (p)ppGpp Levels Induce Persistence by Activating TA Loci via PolyP and Lon

To further investigate the involvement of (p)ppGpp in persister cell formation, we used controlled overexpression of a constitutively active form of RelA (i.e., RelA′) from a plasmid to artificially increase the level of (p)ppGpp (Svitil et al., 1993). To minimize artifacts due to (p)ppGpp toxicity, we used the leakiness of the promoter to express RelA′; this level of expression did not significantly change the growth rate of the culture. The resulting mild overproduction of RelA′ in the WT strain resulted in a 35-fold increase in the level of persistence (Figure 2C).

In a similar way, the (p)ppGpp level was artificially elevated in the *Δ10TA*, *Δlon*, and *Δ(ppk ppx)* strains, and their persistence levels were measured. Interestingly, only modest increases were observed (3-, 1.5-, and 2-fold, respectively) (Figure 2C). These results showed that the (p)ppGpp-induced increase in persistence depended on PolyP, Lon, and TAs.

Moreover, WT *E. coli* cells from carbon-starved biofilms (a condition known to induce (p)ppGpp synthesis) exhibited a strong, 50-fold increase of persistence toward ciprofloxacin (Figure S2C). Most importantly, this increase was not seen with the isogenic *Δ(relA spoT)*, *Δ(ppk ppx)*, *Δlon*, or *Δ10TA* strains (Figure S2C). Finally, as also shown by other groups, we observed that production of persisters was strongly dependent of the growth stage (Figures S2D and S2E) (Keren et al., 2004a).

Together, these results supported that (p)ppGpp increased persistence via PolyP-mediated activation of Lon to specifically degrade antitoxins.

Stochastic Activation of Toxin-Antitoxin Transcription Is Correlated with Persistence

Next, we investigated whether transcription of TA loci in single cells correlated with cessation of growth and, hence, persistence. For that purpose, we constructed transcriptional fusions between two TA operons (*yefM yoeB* and *relBE*) and a gene (*gfp*) encoding GFP (Figure 3A) and determined fluorescence levels of individual cells during steady-state growth. As seen from Figures 3B and S3A, only very few cells exhibited a threshold fluorescence level higher than that of the majority of the population. Statistical analysis of more than 150,000 cells revealed that 2.67×10^{-4} (SD $\pm$ 7.29×10^{-5}) of the population had elevated TA transcriptional levels (Figure S3B). These results showed that TA transcription was stochastically switched ON at a low frequency in the population.

As ectopic induction of toxins is known to induce reversible growth inhibition (i.e., dormancy), we next followed how TA expression affected the growth rates of the individual cells. For that purpose, cells from an exponential culture were introduced in a microfluidic device and subjected to growth in rich medium injected over a period of 80 min. Strikingly, high TA expression was correlated with an almost complete cessation of cell growth (Figure 3C and Movie S1).

We next directly observed the behavior of the cells having activated TA operon transcription toward a high dose of ampicillin antibiotic using our microfluidic device. Interestingly, cells with the highest levels of TA transcription did not lyse, even after 60 min of a high dose of ampicillin (500 μg/ml) (Figure 3D and Movie S2). A statistical analysis of this scenario revealed that 16 cells of 1,542 were fluorescent, and 15 (94%) of these did not lyse after 120 min of a high dose of drug treatment (Movie S3).

We then tested whether nongrowing cells could resume growth after antibiotic treatment has ended. To do this, ampicillin-treated cells were observed after the removal of ampicillin by the injection of fresh medium. Remarkably, after a latency period of 1.5 hr, the cell shown in Figure 3D (Movie S2) began to elongate and finally divided to produce a microcolony. These results showed that TA locus transcription was stochastically switched ON at a low frequency and thereby triggered a slow-growing, drug-tolerant state from which the cells could regrow.

Stochastic Variation of the (p)ppGpp Levels in Single Cells

To directly test the possibility that persistence is controlled by stochastic variation of (p)ppGpp levels, we aimed to develop a single-cell fluorescent reporter protein fusion as a proxy of (p)ppGpp. Testing of a number of (p)ppGpp-activated promoters fused transcriptionally to GFP was not successful (data not shown). We then constructed an *rpoS-mcherry* translational fusion because of the dual effect of (p)ppGpp on RpoS expression level: (1) (p)ppGpp stimulates *rpoS* transcription and (2) (p)ppGpp inhibits proteolytic degradation of RpoS (Battesti et al., 2011). Thus, (p)ppGpp simultaneously induces *rpoS* transcription and accumulation of RpoS (Figure 4A). We engineered an *E. coli* strain in which *rpoS-mcherry* translation fusion served as the sole form of active RpoS. Analysis of exponentially growing cells harboring this fusion revealed that a few cells had a fluorescence level higher than that of the bulk of the population (Figure 4B). As judged by statistical analysis of more than 150,000 cells, these rare cells occurred at a frequency of 4.86×10^{-4} (SD $\pm$ 6.46×10^{-5}) (Figure S4A).

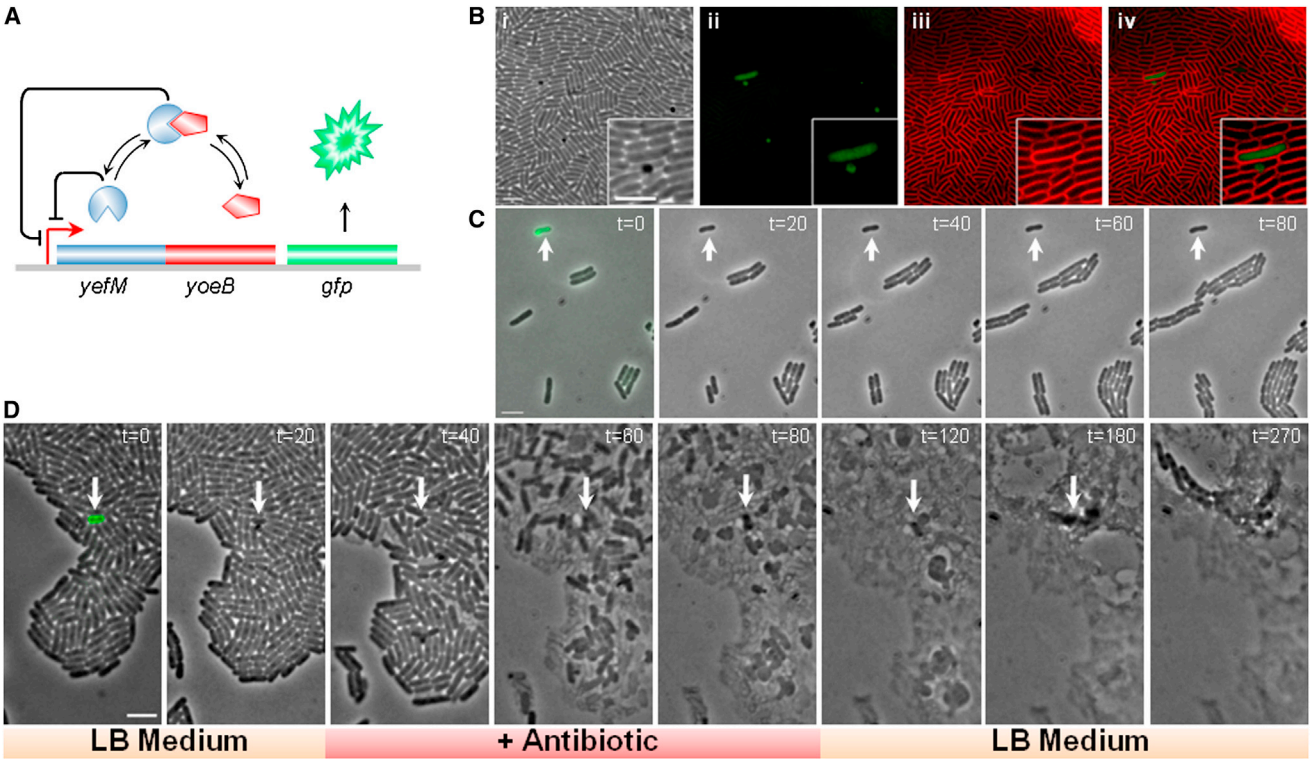

Figure 3. Toxin-Antitoxin Gene Transcription in Single Cells Is Correlated with Persistence

(A) Genetic setup of the fluorescent reporter used to monitor TA transcription using *yefM yoeB* as the model.

(B) Microscopic snapshot of MG1655 *yefM yoeB:: gfp* cells taken from an exponentially growing flask culture. (i) phase contrast; (ii) *yefM yoeB::gfp* fluorescence; (iii) membrane stained with the fluorescent FM-959 dye; (iv) merged view of (ii) and (iii).

(C and D) Exponentially growing cells of MG1655 *yefM yoeB:: gfp* analyzed by microfluidics (see Experimental Procedures).

(C) Time-lapse microscopy images showing a growth-arrested cell (arrow) revealing a high level of TA transcription (from Movie S1). The first image is the overlay of phase-contrast and fluorescence image showing the initial level of *yefM yoeB* expression (separate phase-contrast and fluorescence images are shown in Figure S3C). The following time points show phase-contrast images.

(D) Resuscitation of a growth-arrested cell with an initial high level of TA transcription. Time-lapse microscopy images showing persistence of a cell with a high level of *yefM yoeB* transcription (from Movie S2). The first image is the overlay of a phase-contrast and fluorescence image showing the initial level of *yefM yoeB::gfp* expression (separate phase-contrast and fluorescence images are shown in Figure S3D). The antibiotic content (ampicillin; 0.5 mg/ml) of the growth medium in the microfluidic device is indicated at the bottom of (D). The arrow indicates the bacterium having the highest level of *yefM yoeB* transcription (scale bar, 4 μm) (t = time in min).

See also Movie S3.

To obtain independent support that the above fluctuations in *rpoS-mCherry* expression indeed reflected variations in the level of (p)ppGpp, we also employed a promoter that was negatively regulated by (p)ppGpp. For that purpose, we used an *rrnB* P1*:: gfp*^{unstable} construction that expressed an unstable GFP variant (Shah et al., 2006; Sternberg et al., 1999) (Figure 4A). *rrnB* P1 is very active during exponential cell growth but is strongly inhibited by (p)ppGpp (Barker et al., 2001; Gourse et al., 1986) (Figure 4A). We confirmed that, in exponentially growing cells harboring *rrnB* P1*::gfp*^{unstable}, most of the bacteria were bright, whereas a few remained dim (Figure 4C). Finally, the combination of the two fluorescent reporters revealed a high coincidence of cells exhibiting a high RpoS-mCherry level and a low level of GFP (Figure 4D). This result strongly suggested that the RpoS-mCherry translational fusion reliably reported the intracellular level of (p)ppGpp. We conclude that the (p)ppGpp levels varied stochastically in single cells in an exponentially growing *E. coli* culture.

Stochastic Variation of (p)ppGpp Levels Triggers Slow Growth and Persistence

We tested the hypothesis that stochastic variation of the (p)ppGpp levels in single cells induced the persistent, drug-tolerant state from which the cells could be resuscitated. Cells of an exponentially growing culture harboring *rpoS-mcherry* were introduced in the microfluidic device and subjected to growth in rich medium over a period of 80 min. Strikingly, cells with a high level of RpoS-mCherry neither grew nor divided (Figure 5A and Movie S4). Next, we managed to directly observe the response of cells with high RpoS-mCherry toward a high dose of ampicillin. Remarkably, the cell that had the highest level of mCherry (indicating high (p)ppGpp) did not lyse, even after 60 min of ampicillin treatment (Figure 5B and Movie S5). A statistical analysis of this strain revealed that 18 cells of 1,868 were fluorescent, and 16 (88%) of these did not lyse after 120 min of a high dose of drug treatment (Movie S6).

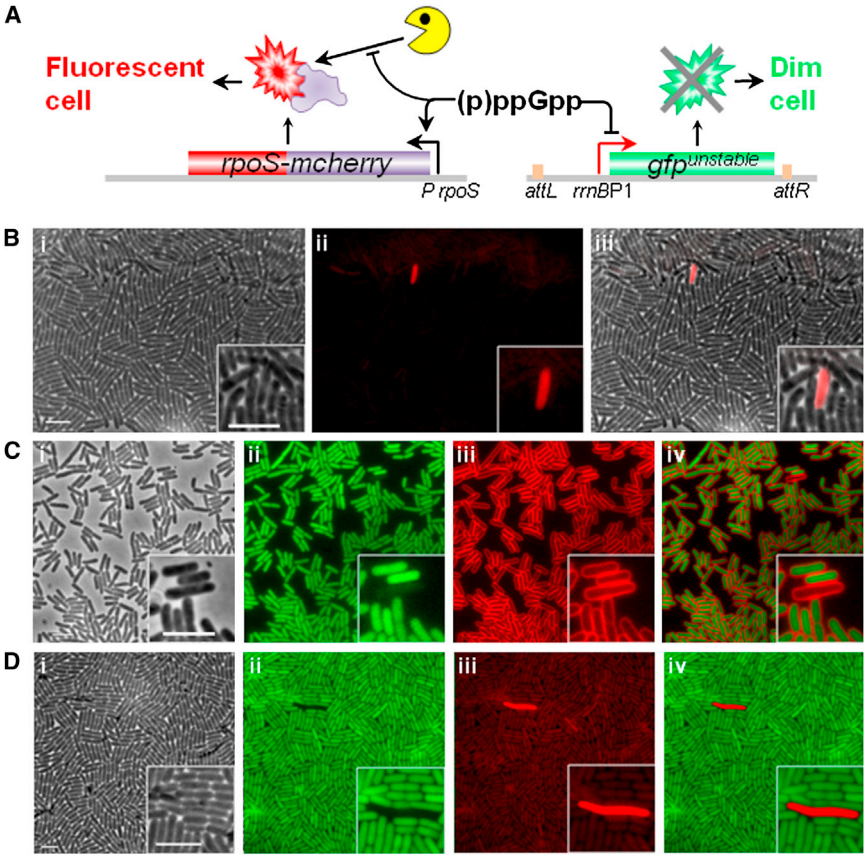

Figure 4. The (p)ppGpp Level Varies Stochastically in Single Cells

(A) Genetic setup and regulation of the fluorescent reporters used as a proxy of the level of (p)ppGpp in single cells (see Results for details).

(B) Snapshot of exponentially growing cells of MG1655 carrying an *rpoS::mcherry* translational fusion (i) phase contrast, (ii) RpoS-mCherry fluorescence, and (iii) overlay of (i) and (ii).

(C) Snapshot of exponentially growing cells of MG1655-ASV carrying an *rrnB* P1::$gfp^{unstable}$ transcriptional fusion. (i) Phase contrast, (ii) *rrnB* P1::$gfp^{unstable}$ expressed fluorescence, (iii) membrane stained with FM-959, and (iv) overlay of (ii) and (iii).

(D) Microscopic analysis of an exponentially growing cells harboring both transcriptional *rrnB*P1::$gfp^{unstable}$ and translational *rpoS::mcherry* fusions. (i) Phase contrast, (ii) *rrnB* P1::$gfp^{unstable}$, (iii) *rpoS::mcherry*, and (iv) overlay of (ii) and (iii). Scale bar, 4 μm.

We then tested whether this nongrowing cell could resume growth after removal of Ampicillin. After a latency period of 1 hr, the cell started to elongate and finally gave rise to progeny cells (Figure 5B and Movie S5). Together, these results showed that the (p)ppGpp levels stochastically fluctuated in the population, thus triggering cells to enter a reversible slow-growing and persistent state. See Extended Results for more information.

DISCUSSION

Our work shows that exponentially growing WT *E. coli* cells become persistent by stochastic switching into a slow-growing state from which they can regrow. Previously, a persistence mechanism involving stochastic switching into dormancy was inferred from the analysis of an *E. coli* strain carrying a mutation in the *hipA* toxin gene that increased persistence ~100-fold (Black et al., 1991; Moyed and Bertrand, 1983), thereby facilitating the microscopic analysis of the persistence phenotype at the single-cell level (Balaban et al., 2004; Gefen et al., 2008; Rotem et al., 2010). Thus, the general inference that persistence is caused by a small fraction of cells that grow slowly has hereby gained experimental support. Slow growth readily explains bacterial multidrug tolerance because the cellular targets corrupted by lethal antibiotics are much less vulnerable in slow-growing than in fast-growing cells (Lewis, 2010; Tuomanen et al., 1986).

It was previously suggested that bacterial persistence is a "bet-hedging" strategy by which a bacterial culture prepares itself for future but unknown insults. Our observations show that persistence emerges from stochastic events that render bacterial cells transiently multidrug tolerant. Mathematical modeling suggested that stochastic switching compared to sensing mechanisms yields a superior survival rate when the environment changes infrequently (Kussell and Leibler, 2005). As such, persistence may be a trait that has been selected for during evolution. We believe that the mechanism that we have unraveled here is consistent with a model stating that persistence is an evolved trait.

Regulatory Cascade Underlying Bacterial Persistence

Previously, we established that TA loci and Lon were required to maintain a WT level of persistence in *E. coli* (Maisonneuve et al., 2011). Here, we extended these ensemble level measurements of persistence to include PolyP (Figure 1) and (p)ppGpp (Figure 2). Overproduction of PolyP or (p)ppGpp led to dramatic increases in persistence of exponentially growing cells and cells growing in a biofilm. Persistence depended hierarchically on (p)ppGpp, PolyP, Lon, and toxin-antitoxins and allowed us to generate a model that we tested at the single-cell level (Figure 6). It should be noted that (p)ppGpp has multiple direct targets (Kanjee et al., 2012), and we do not exclude that (p)ppGpp may be involved in persister cell formation by mechanisms that do not involve the PolyP, Lon, and TA-dependent regulatory pathway.

The finding that high (p)ppGpp levels can mediate drug tolerance has been noted before. Thus, controlled induction of the *relA* gene led to inhibition of phospholipid and peptidoglycan synthesis and a simultaneous dramatic increase in penicillin tolerance (Rodionov and Ishiguro, 1995). Korch et al. (2003) observed that the high persistence level induced by a *hipA*

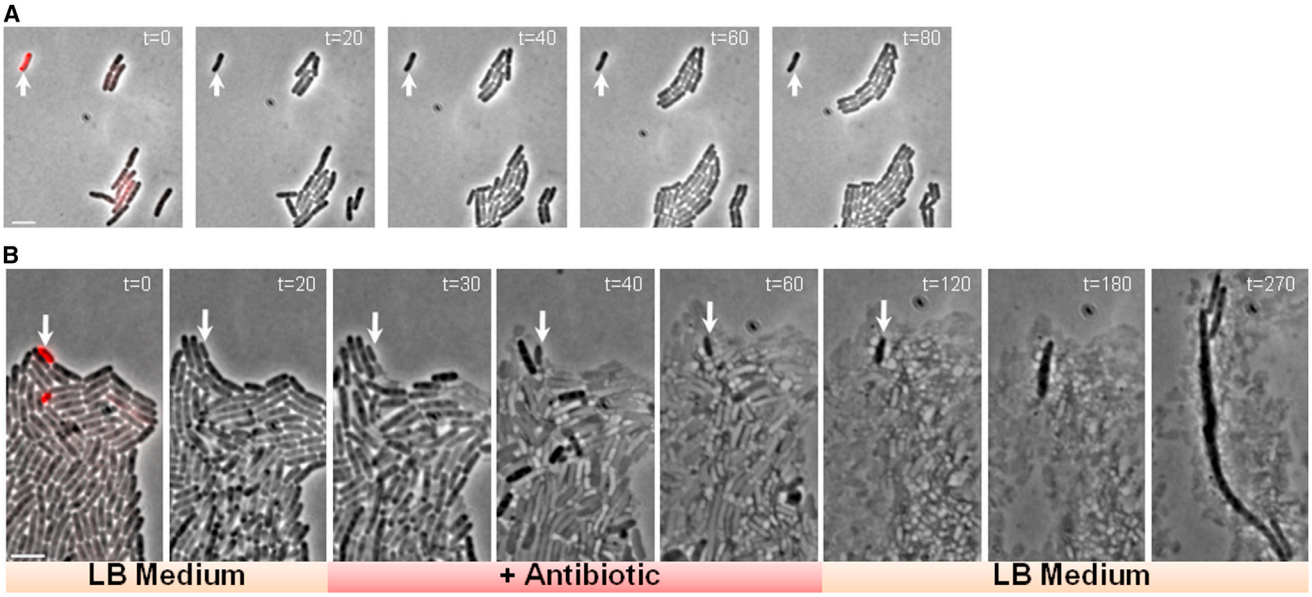

Figure 5. (p)ppGpp Triggers a Nongrowing and Persistence State

Exponentially growing cells of MG1655 *rpoS::mcherry* were introduced into a microfluidic device and were subjected to growth medium.

(A) Time-lapse images showing cells carrying *rpoS::mcherry*; the arrow indicates a growth-arrested cell with a high level of RpoS-mCherry (from Movie S4). The first image was generated by overlay of phase-contrast and fluorescence images (separate phase-contrast and fluorescence images are shown in Figure S4B).

(B) Time-lapse images showing persistence of cells of MG1655 *rpoS::mcherry* expressing a high level of RpoS-mCherry (from Movie S5). The first image is the overlay of phase-contrast and fluorescence images showing the initial level of RpoS-mCherry (separate phase contrast and fluorescence images are shown in Figure S4C). Cells were grown upon given growth medium at the indicated time. The arrow indicates a cell with a high level of RpoS-mCherry (scale bar, 4 μm.) (t = time in min).

See also Movie S6.

allele (*hipA7*) depended on both *relA* and *spoT* and suggested that HipA7 increased persistence by stimulation of (p)ppGpp synthesis. Both of these observations are consistent with our findings (Figure 2C) but did not offer an explanation of the underlying molecular mechanism of how persistence was generated.

Interestingly, ectopic induction of WT *hipA* increased persistence, and the period of growth arrest increased with increased levels of HipA (Rotem et al., 2010). Moreover, the level of HipA had to exceed a threshold before persistence became dominant. Close to the threshold value, HipA induced a condition at which persisters and growing cells coexisted (Rotem et al., 2010).

Bacterial Persistence Caused by Phenotypic Switching

The model presented in Figure 6 predicts that TA transcription and (p)ppGpp levels should be high in single cells at a frequency comparable to that of persister cells (i.e., ~1/10,000 cells) and, importantly, correlate with slow growth and drug tolerance of the single cells. Indeed, transcriptional fusions between two representative TA loci and *gfp* revealed that the ON rate of TA locus transcription was similar to that of persister cell frequency (Figure 1A and S3B). Moreover, TA locus "ON" cells grew slowly or not at all (Figure 3C), and these cells were drug tolerant and able to resume growth after removal of the drug (Figure 3D). As with TA operon transcription, the ON rate of the *rpoS::mcherry* fusion [(p)ppGpp reporter] ($\sim 5 \times 10^{-4}$) was comparable to that of the persistence frequency ($\sim 1 \times 10^{-4}$) and correlated with slow growth and drug tolerance of single cells (Figures 5A and 5B). This result strongly supports that the (p)ppGpp level controls persistence by peaking at a low frequency in single cells.

The level of persisters is strongly dependent on the growth phase (Figures S2D and S2E), being very low in early exponential phase and peaking at the midexponential growth phase (Figures S2D and S2E). It is thus possible that the very few persisters detected in early exponential phase were formed in the stationary phase, which is consistent with the observation that keeping the cells in early exponential phase leads to a gradual dilution of the persisters (Keren et al., 2004a). Therefore, we tested whether this was also the case under our experimental conditions. We found that keeping the cells in midexponential phase did not lead to dilution of the persistence level, showing that, under these conditions, persister cells indeed form de novo (Figure S2F).

The molecular basis of the apparent stochastic variation of the (p)ppGpp level in single cells can only be speculated upon. However, a parsimonious and therefore attractive model is that rare cells in the population encounter starvation ("microstarvation") that triggers (p)ppGpp synthesis and thus persistence. This suggestion is consistent with the strong growth-phase dependency of the persistence level discussed above (Figures S2D and S2E).

Interestingly, single-molecule tracking experiments showed that RelA is on the ribosome under nonstarved conditions but

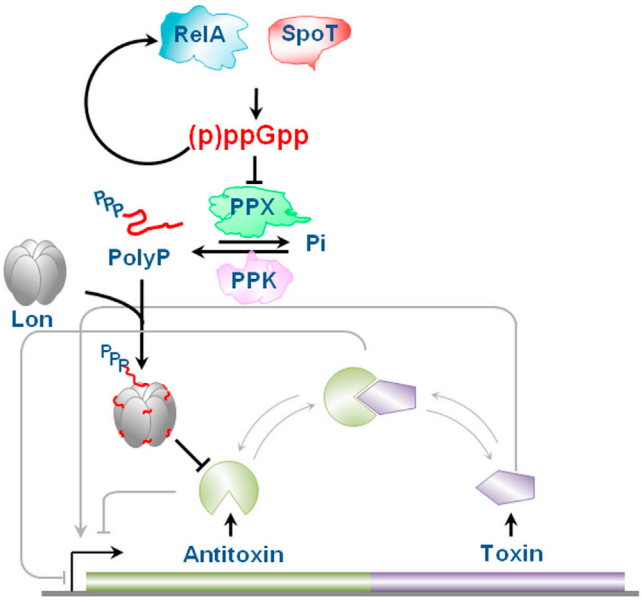

Figure 6. Molecular Model Explaining Bacterial Persistence

RelA or SpoT synthesize (p)ppGpp that inhibits exopolyphosphatase (PPX), the cellular enzyme that degrades PolyP. PolyP is constitutively synthesized by polyphosphate kinase (PPK). PolyP combines with and stimulates Lon to degrade the 11 antitoxins of *E. coli* K-12, thereby activating the toxins that inhibit translation, and inhibits cell growth. The arrow from the toxin to the TA promoter (indicated by an arrow pointing right) indicates that excess toxin ([toxin] > [antitoxin]) derepresses the TA promoter that, in turn, may lead to rapid quenching of toxin activity as a prerequisite for resuscitation of persister cells (Cataudella et al., 2012) (see the Discussion for further details).

stays off the ribosome for an extended period of time after activation by amino acid starvation (English et al., 2011). Thus, a molecular mechanism may keep RelA active by preventing it from reuniting with the ribosome once it has come off and thus may contribute to an increased level of (p)ppGpp in single cells. Remarkably, RelA is positively stimulated by (p)ppGpp, thereby invoking a positive feedback loop in the synthesis of (p)ppGpp (Shyp et al., 2012). Such feedback enables fast amplification of a small input signal and thus could generate the molecular basis of the heterogeneity of the (p)ppGpp levels in single cells (Figure 6). According to our model, such fast amplification of (p)ppGpp would in turn amplify both intrinsic (i.e., decrease transcription and translation rates) and extrinsic noise (fluctuation of PolyP resulting in Lon activity) that may contribute to activation of toxins and thereby cessation of growth and persistence.

Interestingly, Rel_{Mtb} ((p)ppGpp synthetase I) is required for the long-term survival and persistence of *M. tuberculosis* in mice (Dahl et al., 2003; Primm et al., 2000). Moreover, a positive feedback loop generates bistable induction of Rel, the main protein responsible for stringent response in *M. smegmatis*, thus offering a possible explanation for the heterogeneity of growth rates and the high persistence levels seen with mycobacterial populations (Ghosh et al., 2011; Sureka et al., 2008).

Recently, Amato et al. (2013) proposed a model explaining how the "(p)ppGpp network" may lead to fluoroquinolone persistence. Their model was based on observations obtained during diauxie—that is, during growth on a mixture of two different carbon sources (often two sugars). In the transition phase between the utilization of the two carbon sources, the cells encountered carbon starvation stress and therefore elicited the stringent response (i.e., (p)ppGpp synthesis) (Harshman and Yamazaki, 1971).Therefore, the cells transiently entered slow growth and, hence, became tolerant to ciprofloxacin (Amato et al., 2013).

Even if we assume that both models seeking to explain persistence were valid (i.e., microstarvation and glucose starvation during diauxie), the regulatory cascade suggested by Amato et al. (2013) was conceptually different from our model, which was based on the observation that (p)ppGpp stochastically switched ON in unstressed cells. In contrast, Amato et al. (2013) suggested that diauxie-induced persistence depended not only on (p)ppGpp but also on the transcription factor DksA and nucleoid-associated proteins that contribute to DNA supercoiling.

We believe that the two models are not mutually exclusive because they emerged from observations obtained in two entirely different environments (unstressed and stressed cells). Finally, we find it attractive to propose that stochastic activation of chromosomally encoded toxin-antitoxins triggered by (p)ppGpp may also actively contribute to the stress-induced, (p)ppGpp-dependent persistence observed by Amato et al. (2013) during diauxie.

Persistence, Virulence, and the Stringent Response

In general, virulence of bacterial pathogens depends on (p)ppGpp (Dalebroux et al., 2010). During pathogenesis, bacteria often form biofilms that increase their survival during harsh conditions as in a host organism, for example. Deep within biofilms, bacteria have limited access to nutrients and therefore increase their levels of (p)ppGpp. Consistently, multidrug tolerance of *P. aeruginosa* and *E. coli* grown in biofilms depended on (p)ppGpp (Bernier et al., 2013; Nguyen et al., 2011). We observed here that amino acid starvation failed to induce persistence in biofilms of *E. coli* unable to synthesize (p)ppGpp—lacking PolyP, Lon, or ten TA loci (Figure S2C)—supporting the conjecture that our model is valid also in more realistic settings.

Persistence Outgrowth

To generate viable offspring, cells in the persistent state must be able to resume growth. Therefore, cognate antitoxins must be replenished in excess of toxin when the signal that generates the high (p)ppGpp levels vanishes. Interestingly, in all cases tested, transcription of TA operons is regulated by a mechanism called conditional cooperativity (Afif et al., 2001; Garcia-Pino et al., 2010; Overgaard et al., 2008; Winther and Gerdes, 2012). Conditional cooperativity refers to the ability of the toxin to function both as corepressor and derepressor of its cognate TA operon. When [antitoxin] > [toxin], the TA complex binds tightly to the TA promoter and strongly represses the operon. However, when [antitoxin] < [toxin], the toxin destabilizes the promoter-bound TA complex and thereby induces strong transcription of the operon (Overgaard et al., 2008; Winther and Gerdes, 2012). In rapidly growing, nonstressed cells, [antitoxin] > [toxin], TA operons are repressed and toxin activity is quenched by the

excess antitoxin. However, during nutritional stress or in persister cells, the high (p)ppGpp level destabilizes the antitoxin due to rapid Lon•PolyP-mediated degradation and thereby derepresses TA operon transcription that, in turn, leads to increased synthesis rates of both toxin and antitoxin. Obviously, for a toxin to be active in a persister cell, the antitoxin degradation rate must be higher than its synthesis rate. Mathematical modeling argued that conditional cooperativity functions to rapidly quench toxin activity when the signal (i.e., high levels of (p)ppGpp) that triggers antitoxin degradation disappears (Cataudella et al., 2012). Other molecular events required for resuscitation of persister cells can only be speculated upon. However, our model raises the possibility that the activity of the Lon•PolyP complex may be negatively regulated. Alternatively, the Lon•PolyP complex is sufficiently unstable as to allow for its rapid inactivation when the (p)ppGpp level drops to the basal level.

This work shows that WT bacteria become persistent by temporarily switching into slow growth, a switch that allows the bacteria to evade eradication by multiple antibiotics. We also reveal the regulatory network behind the switching phenotype and establish that (p)ppGpp is at the top of the regulatory cascade that results in the triggering of toxin activity and stasis. We believe that these results for several reasons are important. Are similar or related mechanisms operational in pathogenic bacteria? If so, can we exploit the knowledge to combat persistent infections? We suggest that inhibitors of (p)ppGpp-synthesizing enzymes could have a future as useful drugs, and the search for such potential antibiotics has already begun (Wexselblatt et al., 2010, 2012).

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids

Bacterial strains and plasmids are described in the Supplemental Information and are listed in Table S2. DNA oligonucleotides are listed in Table S3.

Persistence Assay

Persistence was measured as described previously (Maisonneuve et al., 2011) and is described in detail in the Supplemental Information.

PPK Overexpression and Persistence

WT and an isogenic *E. coli* strain harboring pAC24N::*ppk* were grown in LB medium but with the addition of 10 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 2.5 hr. At this level of induction, the growth rate was identical to that of the same culture without IPTG (data not shown). Aliquots of the culture were subjected to antibiotic (ciprofloxacin), and persistence was determined. The value was then compared to that of control strains carrying the empty vector pCA24N treated in a similar way.

RelA Overexpression and Persistence

WT and derivative *E. coli* strain harboring pALS13 (P_{lac}::*relA′*) were grown in rich medium for 2.5 hr without inducer. Then, aliquots of cells were subjected to antibiotic (ciprofloxacin), and persister cell formation was determined as described in the Supplemental Information. The value was then compared to that of control strains carrying the pALS14 plasmid vector expressing an inactive truncated form of RelA (RelA″) treated in a similar way.

Microscopy

For phase contrast and fluorescence microscopy, cells were grown in LB to midexponential phase at 37°C, centrifuged, resuspended in M9 medium containing 10× diluted LB medium, and mounted on prewarmed microscope slides covered with a thin film of 1.2% agarose (in a 10× diluted LB in M9 medium). Cell membrane and DNA were visualized by staining with FM-595 and DAPI, respectively. Images were acquired with a Cool-Snap HQ CCD camera (Roper Scientific) attached to a Nikon Eclipse Ti-U microscope. The images were acquired and analyzed with Metamorph 6. Final image preparation was performed in ImageJ.

Microfluidic Assay and Device

Cells were grown in LB to midexponential phase at 37°C, centrifuged, resuspended in a 10× diluted LB in M9 medium, and mounted on prewarmed agarose-based microfluidic device (for more information, see Supplemental Information). The microfluidic device was assembled in a heating chamber (37°C) attached to the microscope. Medium (M9 medium supplemented with a 10-fold diluted LB with or without 500 μg/ml of ampicillin) perfusion was accomplished by gravity at a constant rate of ~5–10 μl/s. During medium transition, medium was injected manually by supplying two times the volume of the chamber with a 2 ml syringe to minimize the time required for the drug (i.e., ampicillin) to reach the cells. For details, see the Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, four figures, three tables, and six movies and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.07.048.

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