Hypothesis: type I toxin–antitoxin genes enter the persistence field—a feedback mechanism explaining membrane homoeostasis

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Bacteria form persisters, cells that are tolerant to multiple antibiotics and other types of environmental stress. Persister formation can be induced either stochastically in single cells of a growing bacterial ensemble, or by environmental stresses, such as nutrient starvation, in a subpopulation of cells. In many cases, the molecular mechanisms underlying persistence are still unknown. However, there is growing evidence that, in enterobacteria, both stochastically and environmentally induced persistence are controlled by the second messenger (p)pGpp. For example, the ‘alarmone’ (p)pGpp activates Lon, which, in turn, activates type II toxin–antitoxin (TA) modules to thereby induce persistence. Recently, it has been shown that a type I TA module, hokB/sokB, also can induce persistence. In this case, the underlying mechanism depends on the universally conserved GTPase Obg and, surprisingly, also (p)pGpp. In the presence of (p)pGpp, Obg stimulates hokB transcription and induces persistence. HokB toxin expression is under both negative and positive control: SokB antisense RNA inhibits hokB mRNA translation, while (p)pGpp and Obg together stimulate hokB transcription. HokB is a small toxic membrane protein that, when produced in modest amounts, leads to membrane depolarization, cell stasis and persistence. By contrast, overexpression of HokB disrupts the membrane potential and kills the cell. These observations raise the question of how expression of HokB is regulated. Here, I propose a homoeostatic control mechanism that couples HokB expression to the membrane-bound RNase E that degrades and inactivates SokB antisense RNA.

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1. Introduction

Persisters arise stochastically in bacterial populations and are tolerant to multiple antibiotics, which has led to growing interest in understanding the phenomenon, given the current global problems with antibiotic resistance [1,2]. However, the molecular mechanisms underlying this phenotype are poorly understood and it has been suggested that several redundant mechanisms may contribute to the phenomenon [3]. As slow growth is associated with reduced killing by antibiotics and other environmental insults, by inference, slow growth was initially suggested to be central to the drug-tolerance phenotype [4,5]. My group discovered that type II toxin–antitoxin (TA) genes code for ‘toxins’ that efficiently and reversibly inhibit cell growth, raising the possibility that these gene modules could be involved in persistence [6]. Moreover, persister cell fractions have increased levels of TA mRNAs, further supporting this notion [7–9]. Indeed, solid evidence now supports that both type I and II TAs are crucial to the persistence of enterobacteria.

Prokaryotic genomes, plasmids and bacteriophages encode a bewildering number of TA genes [10,11]. The known TA gene families can be divided...
into six different types, depending on how the antitoxins inhibit cognate toxins [12,13]. Here I focus on type I and II TAs that, remarkably, can mediate persistence by different mechanisms that are both controlled by (p)ppGpp [14,15]. In type I TAs, the antitoxins are antisense RNAs inhibiting toxin mRNA translation [16], whereas in type II TAs, the antitoxins are proteins sequestering cognate toxin activity by direct protein::protein contact [12,17]. Type I and II TA modules have several features in common: both types were discovered by their ability to increase plasmid maintenance in growing bacterial populations by a mechanism called post-segregational killing (PSK) [18–21], and both are found on plasmids, phages and chromosomes, often in multiple copy numbers [22,23]. Based on toxin sequence similarities, both type I and II TA modules have been further divided into evolutionarily independent families. For example, the type II module families relBE and mazEF encode RNases that are unrelated and, therefore, have evolved independently. The following briefly describes the genetic set-up, cellular targets and regulatory properties of paradigm type I and II TA modules, beginning with the almost ubiquitous type II TA modules.

The ccdAB locus of *Escherichia coli* plasmid F was discovered due to its ability to increase the maintenance of F-derived replicons and was proposed to couple cell division with plasmid segregation [18], but later analyses indicated that CcdB toxin, a lethal DNA gyrase inhibitor, was activated post-segregationally in plasmid-free cells, thereby inhibiting the frequency of appearance of these cells in a population of growing cells [19]. Soon thereafter, the type II *penIK* (also called *parD* or *kis/kid*) locus of plasmid R1/R100 was also discovered due to its ability to increase plasmid stability [24]. A few years later the first two chromosome-encoded type II TA loci, homologues of *penIK* (also called *mazEF*), were identified [23]. We discovered the relBE module of *E. coli* (figure 1a) and showed that relE encodes an efficient inhibitor of global cellular translation [28,31] that cleaves mRNA codons at the ribosomal A-site [25,26]. Over the years, we have developed relBE of *E. coli* into a paradigm model system, not least because of its simplicity in single cells of exponentially growing cells and that the rare cells with a high level of (p)ppGpp can survive penicillin treatment (figure 2a). We could then show that, in single cells, (p)ppGpp induces type II TA activity via a cascade that involves (p)ppGpp-mediated accumulation of polyphosphate, activation of Lon, degradation of antitoxins and activation of toxins that inhibit translation [14]. This model (figure 2b) then raises the question of how induction of mRNAse activity induces persistence.

TAs and (p)ppGpp also contribute to persistence during stressful conditions, such as during stationary phase and amino acid starvation [14], in a process that has been called ‘responsive diversification’ [44]. Moreover, type II TA modules are also vital for the generation of non-replicating persisters of *Salmonella* within macrophages [45] and for survival within fibroblasts [46], thus adding to the general validity of the proposal that type II TAs are major players in persistence in enterobacteria.

It has been proposed that inhibition of translation *per se* is sufficient to induce persistence [47]. However, we showed recently that this may not always be the case. The first ‘persister’ gene that was discovered, *hipA* of *E. coli* K-12, was identified by a screening for mutants that exhibited a reduced killing kinetics by penicillin [48]. Many years later, we and others showed that *hipA* encodes a kinase that inhibits translation by inactivation of GltX, the tRNA*Glu*-charging enzyme of *E. coli* K-12 [43,49]. Remarkably, persistence induced by ectopic overexpression of HipA depended not only on RelA-mediated (p)ppGpp synthesis but also on Lon, polyphosphate and the TA modules encoding RNases, thereby further substantiating the model in figure 2b [50]. Interestingly, overexpression of HipA in the absence of 10 type II toxins was able to halt growth but not induce persistence, suggesting that growth inhibition and thus translation inhibition are not alone sufficient for persister formation. In addition, analysis of single cells that had high levels of (p)ppGpp showed that inhibition of growth in the absence of TAs was insufficient to induce persistence. These observations raise the possibility that type II TA-encoded RNases induce persistence by an as yet unknown mechanism.

### 2. Control of persistence by type II toxin-antitoxin modules

How type II TA modules induce persistence has been reviewed recently [2,39,40] and will be described only briefly in the following. *Escherichia coli* K-12 encodes up to 17 different type II TA modules (figure 1b) many of which encode RNases that inhibit translation by cleavage of mRNA, tRNA or rRNA [33]. We showed that progressive deletion of 10 canonical TA modules (7 *relBE*, 2 *mazEF* and 1 *hicAB*) gradually reduced the persistence levels of *E. coli* K-12 (figure 1c). The cumulative effect on persistence yielded, for the first time, a common phenotype to type II TA modules [35]. The persistence phenotype mediated by TA modules depended strongly on Lon protease, consistent with the observation that Lon degrades all 10 type II antitoxins of *E. coli* K-12 [35]. This observation led to the important and straightforward question of how Lon is activated to degrade type II antitoxins.

The only known activator of Lon is polyphosphate [41]: during amino acid starvation, Lon is activated by polyphosphate to degrade idling ribosomal proteins that are used for *de novo* protein synthesis and adaption to starvation. Moreover, the increase in polyphosphate during amino acid starvation is caused by (p)ppGpp that competitively inhibits exopolyphosphatase, the enzyme that degrades polyphosphate [42]. Inspired by these observations, we were able to show that (p)ppGpp is the ‘master’ regulator of persistence in *E. coli*, during both exponential growth and stressful conditions [14]. We found that (p)ppGpp varies stochastically in single cells of exponentially growing cells and that the rare cells with a high level of (p)ppGpp can survive penicillin treatment (figure 2a). We could then show that, in single cells, (p)ppGpp induces type II TA activity via a cascade that involves (p)ppGpp-mediated accumulation of polyphosphate, activation of Lon, degradation of antitoxins and activation of toxins that inhibit translation [14]. This model (figure 2b) then raises the question of how induction of RNAse activity induces persistence.

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Figure 1. (a) Genetic organization and regulatory properties of relBE, a paradigm type II toxin antitoxin module of E. coli K-12. RelE toxin inhibits translation by cleavage of mRNA at the ribosomal A-site [25–27]. RelB antitoxin sequesters RelE activity by direct protein–protein interaction. RelB is also a transcriptional repressor that, via its ribbon–helix–helix motif, binds to operators in the relBE promoter region [28,29]. Binding of the RelBE complex to the operators is highly cooperative and represses the relBE promoter very efficiently [30]. Excess RelE ([RelE] > [RelB]) disrupts the RelBE–operator complex by a mechanism that we called ‘conditional cooperativity’ [30]. Lon protease degrades RelB antitoxin [31]. (b) Confirmed type I (red) and type II (blue) TA modules encoded by the chromosome of E. coli K-12. Data were from the EcoCyc database [32] and from Yamaguchi & Inouye [33]. All of the TA modules shown here have confirmed activity except the hokA, hokC, hokD and hokE loci that are inactivated by mutation [6]. The four ldr genes have a genetic organization very similar to that of the hok/sok modules and also encode small toxic membrane proteins [16,34]. (c) Type II TA modules are required for the persistence of E. coli K-12. Effects of multiple TA gene deletions on persistence. Survival rates in persister assays using ciprofloxacin (1 \( \mu \)g ml\(^{-1}\)) or ampicillin (100 \( \mu \)g ml\(^{-1}\)). Numbers on the x-axis refer to the number of TA loci that were deleted in the strains tested. Adapted from Maisonneuve et al. [35].
3. Persistence by type I toxin-antitoxin modules

The first type I TA module discovered was hok/sok of plasmid R1 that increases plasmid maintenance by PSK [20,21]. Over the years, hok/sok became a paradigm system in the study of how an mRNA whose translation is inhibited by a cis-acting antisense RNA can be activated in the absence of the antisense [16,51]. The hok/sok locus codes for three genes, hok, sok and mok (figure 3a). The Hok toxin is a small membrane protein whose ectopic production kills bacteria by damaging the inner cell membrane by dissipating the proton motive force (PMF) [22,53]. The damaged membrane leads to efflux of small molecules, in particular, ATP, and thereby confers rapid cell death. A similar mechanism leads to Hok-dependent death of newborn, plasmid-free cells [21,54]. The sok gene encodes a small, unstable and cis-acting antisense RNA, called Sok-RNA, which inhibits translation of the mok reading frame (figure 3a). Because hok translation depends on mok translation, Sok-RNA also inhibits translation of hok [55]. The translational coupling between mok and hok is indirect, that is, the ribosome translating mok continues to the end of the mok reading frame, thereby exposing the hok translation-initiation region and allowing a different ribosome to be loaded there [55].

4. Complex regulation of the hok/sok module of plasmid R1

Cis-acting antisense RNAs are fully complementary to their target RNAs and usually form highly stable duplexes with their targets, thereby inactivating the target RNA [56]. The duplexes are then rapidly cleaved by RNase III, leading to decay of the RNAs. Initially, the antisense and target RNAs interact transiently, generating a so-called kissing complex, as has been shown for paradigm antisense RNA I/II of plasmid ColEI/pMB1 and the CopA/CopT RNA of plasmid R1 [57,58]. After the initial interaction, the two fully complementary RNAs refold into very stable, complete duplexes that are stably cleaved by RNase III [59]. We showed that hok and Sok-RNA also form a duplex that is cleaved by RNase III [60,61]. This clear observation posed a conundrum: how can an mRNA whose translation is irreversibly inhibited by a cis-acting antisense RNA become activated in plasmid-free cells? The answer to this question was unravelled in a long series of papers in which we focused on the hok–sok interaction. The first clue to the mechanism came from the observation that hok mRNA exists in two forms: an inactive full-length, primary transcript of 400 nucleotides (nt) and a 3’-end processed, translationally active mRNA of 360 nt [54,62]. Using both genetic, phylogenetic and biochemical approaches, we identified several regulatory elements in hok mRNA: we showed that the 5’-end of full-length hok mRNA base-pairs with the very 3’-end and that the resulting, highly folded mRNA is inactive with respect to both Sok-RNA and ribosome binding. The inert full-length hok mRNA is more stable than Sok-RNA but is slowly activated by 3’-end processing [63]. On the basis of nucleotide covariation analysis and computer simulations of RNA folding pathways during transcription, we proposed a model that fully explains all conserved elements in hok mRNA and, crucially, allowed us to understand how
**Figure 3.** (a) Genetic organization of hok/sok of plasmid R1, a type TA module. sokT denotes the Sok-RNA target in hok mRNA (indicated by a small grey box). The mok reading frame (black filled box) is required for hok to be translated but the mok gene product itself has no known function. The hok reading frame is out of frame with that of mok and terminates just downstream of m. FL denotes the full-length, inactive hok mRNA; TR denotes the 3′-truncated, active hok mRNA that can either bind the antisense RNA or be translated. Sok-RNA is the 65 nt long antisense. The 5′-element base-pairs with three different sequence elements within hok mRNA: in the metastable hairpin, with the SD–AUG elements base-pair to form the stable, inactive mRNA. The full-length hok mRNA functions as a reservoir from which the active truncated version of hok mRNA can be generated by 3′-end processing. (b) hok mRNA folding pathway: (i) the nascent transcript contains the metastable hairpin that prevents Sok-RNA and ribosome binding during transcription; (ii) the full-length hok mRNA in which the fbi–tac elements base-pair to form the stable, inactive mRNA. The full-length hok mRNA functions as a reservoir from which the active truncated version of hok mRNA can be generated by 3′-end processing. tac denotes the translational activator sequence that sequentially base-pairs with three different sequence elements within hok mRNA: in the metastable hairpin, with the upstream complementary box (ucb) and with a fold-back inhibition (fbi) element. The ucb element is complementary to both tac and SD of mok; see text for a further explanation; (iii) truncated, active hok mRNA is generated by 3′ processing of the full-length mRNA. Removal of 40 nt at the 3′ end of FL hok mRNA disrupts the fbi–tac interaction and triggers refolding into the truncated, active version of the mRNA that can either bind the antisense or be translated. Everted bases indicated with dashes symbolize the U-turn loop structure present in the refolded hok mRNA, which functions as the initial recognition element for antisense RNA binding and thereby increases the recognition reaction approximately 10-fold [52]. SD, Shine and Dalgarno elements. (iv) plasmid-carrying cell, Sok-RNA present; (v) plasmid-free cell, Sok-RNA absent. Sok-RNA present on the nascent transcript in an inactive configuration that binds neither to ribosomes nor to Sok-RNA. In this way, the metastable hairpin prevents ‘fortuitous’ translation or inactivation by the antisense during hok mRNA transcription (figure 3b(i)). The tac-element in the metastable hairpin is complementary to the fbi-element present in the very 3′-end of the full-length hok mRNA (figure 3b(ii)). After the fbi element has been synthesized, hok mRNA refolds into the FL form of the initial metastable hairpin is to keep the nascent
that is stabilized by the \( \text{fbi:tae} \) base-pairing (figure 3b(ii)). The FL mRNA is slowly processed by 3' exonucleases (ribonuclease II and PNPase) to a 40 nt shorter version called truncated (TR) \( \text{hok} \) mRNA (figure 3b(iii)). This exonucleolytic processing disrupts the \( \text{fbi:tae} \) interaction and triggers a dramatic refolding of the mRNA 5'-end, thus generating the active, truncated mRNA (figure 3b(iii)). The truncated mRNA has two fates: it can either bind Sok-RNA (figure 3b(iv)) or, if Sok-RNA is absent, such as in plasmid-free cells, it can be translated and lead to Hok synthesis and cell death (figure 3b(v)). Thus, the paradoxical activation of Hok translation in plasmid-free cells is explained by the properties of the two different \( \text{hok} \) mRNA species.

5. Hypothesis: RNase E controls SokB-RNA level

The chromosome of \( E. \ coli \) K-12 codes for at least 18 different type I TA modules (figure 1b), 5 of which are \( \text{hok}/\text{sok} \) homologous loci [66]. Obviously, these chromosomal \( \text{hok}/\text{sok} \) loci do not function to increase plasmid maintenance, and their possible biological function was not readily uncovered [16]. However, recently Jan Michels’s group discovered that the universally conserved GTPase Obg can induced persistence in \( E. \ coli \) K-12 by a mechanism that depends on (p)ppGpp and the \( \text{hokB}/\text{sokB} \) locus [15]. Thus, reduced or increased levels of Obg reduced or increased the persistence level, respectively. Transcriptomic analysis revealed that Obg, in the presence of (p)ppGpp, stimulates \( \text{hokB} \) transcription [15]. Jan Michels’s group also elegantly showed that modest levels of HokB induced a dramatic decrease in the membrane potential and simultaneously induced cell stasis and persistence but not cell killing [15]. Cell growth could be restored by the expression of proteorhodopsin that provides an active proton pump able to increase the membrane potential. Thus, whether HokB induces cell stasis (and persistence) or cell killing appears to depend on its level of expression. A working model explaining how HokB expression is controlled is shown in figure 4.

Induction of persistence by HokB was surprising, given the clear killing phenotype seen both by ectopic overexpression of R1 Hok [21,22] and by activation of R1 Hok translation in plasmid-free cells [54]. It should be noted that all the regulatory features present in the plasmid-encoded \( \text{hok}/\text{sok} \) locus are also present in the \( \text{hokB}/\text{sokB} \) locus and that ectopic expression of chromosomal HokB induces rapid cell killing [66]. The observations by Jan Michels’s group raise the question of how HokB expression is regulated. One possibility is that a homoeostatic control mechanism shuts down HokB expression such that unintended and irreversible membrane damage and cell killing is avoided. How might such a homoeostatic regulatory mechanism function? We showed previously that RNase E inactivates Sok-RNA by rapidly cleaving the RNA at its single-stranded 5’-end. Here, I propose that RNase E is a crucial factor in determining the level of SokB-RNA and thus the rate of \( \text{hokB} \) mRNA translation. There is now solid evidence that RNase E is anchored to the inner cytoplasmic membrane via a membrane targeting sequence (MTS) and that membrane association is correlated with the activity of the enzyme [68–71]. Thus, to be fully active, RNase E must be associated with the membrane [70], raising the possibility that membrane association plays a regulatory role. We propose that

![Figure 4](http://rstb.royalsocietypublishing.org/ Downloaded from on December 31, 2017)

**Figure 4.** Model explaining how the universally conserved Obg GTPase and (p)ppGpp control persistence by inducing transcription of the \( \text{hokB} \) gene of \( E. \ coli \) K-12. Grey connectors symbolize experimentally verified connections, whereas a blue connector symbolize hypothetical connections (\( \downarrow \) indicate stimulation and \( \downarrow \) indicate inhibition). The regulatory cascade in which (p)ppGpp and Obg induce \( \text{hokB} \) transcription is shown in the top of the figure and is according to Verstraeten et al. [67]. The \( \text{hokB}/\text{sokB} \) locus produces \( \text{hokB} \) mRNA and SokB antisense RNA that inhibits translation of the truncated form of \( \text{hokB} \) mRNA. During steady-state cell growth, HokB toxin is not produced due to an excess of SokB-RNA. However, during stress, Obg and (p)ppGpp stimulate transcription of \( \text{hok} \), thereby leading to accumulation of \( \text{hokB} \) mRNA, which, in turn, overrides the inhibition by SokB-RNA, thus leading to HokB synthesis and to depolarization of the inner cell membrane. Membrane depolarization has two effects: it leads to increased persistence [67] and hypothetically leads to detachment of RNase E from the membrane that, in turn, leads to reduced degradation of SokB-RNA. Accumulation of SokB-RNA inhibits translation of truncated \( \text{hokB} \) mRNA, thus establishing a negative, homoeostatic control loop that prevents irreversible membrane damage and cell killing.

the interaction between RNase E and the inner cell membrane may provide a homoeostatic control mechanism that protects the membrane from irreversible damage by HokB and perhaps other small toxic membrane proteins. The model is outlined in figure 4. During rapid cell growth, when energy and growth substrates are plentiful, the membrane potential
is high and RNase E is associated with the inner membrane and degrades SokB-RNA at a rate sufficient to prevent translation of hokB mRNA. Even though RNase E degradation of SokB-RNA is relatively rapid [66], the steady-state rate of SokB-RNA synthesis is sufficient to prevent translation of hokB mRNA that would be toxic. However, when a cell encounters stressful conditions, increased levels of (p)ppGpp, together with Obg, stimulate hokB transcription. The increased hokB mRNA level overrides inhibition by SokB-RNA, thus leading to HokB production and reduction of the membrane potential [15]. This change in membrane potential is ‘sensed’ by RNase E, leading to its detachment from the membrane and enzymatic activity. The reduced RNase E activity, in turn, will cause an accumulation of SokB-RNA, inhibit translation of hokB mRNA and prevent further HokB synthesis, thus limiting the potential membrane damage, allowing only for cell stasis rather than cell killing. Thus, the proposed control mechanism will allow for depolarization of the membrane by HokB without leading to cell killing. A simpler version of this model proposes that HokB, by direct interaction with RNase E at the cell membrane, reduces degradation of SokB-RNA by RNase E without the release of the RNase from the membrane.

Other regulatory mechanisms may contribute to membrane integrity after HokB expression. One such mechanism could involve the phage shock proteins (Psps) that are induced by damage to the membrane that decrease the PMF, which helps mitigate the damage [72]. Moreover, similar to TA genes, the psp genes are induced in cell fractions enriched for persisters [8] and have been implicated in the survival of non-replicating persisters of Mycobacterium tuberculosis [73]. It should now be tested if cell stasis by HokB induces the phage shock genes in E. coli.

The model proposed in figure 4 is readily tested. For example: (i) amino acid changes in the MTS of RNase E that reduce or abrogate membrane binding should increase the stability and therefore the level of SokB-RNA and thereby prevent HokB-mediated persistence; (ii) ectopic expression of HokB at a level that induces persistence but not cell killing should also stabilize SokB-RNA; (iii) by contrast, ectopic expression of proteorhodopsin that can restore the membrane potential of HokB-induced persisters should lead to reduced stability of SokB-RNA; and (iv) energy poisons causing membrane depolarization, such as sodium azide, should increase SokB-RNA levels.

The above considerations do not finally explain why the chromosomal hok/sok homologues, similar to the plasmid-encoded modules, are equipped with such complicated regulation of the hok encoding mRNAs. One possibility is that the slowly processed, inactive full-length hokB mRNA functions as a reservoir that does not require de novo RNA synthesis to become translationally active at the onset of sudden starvation because slow processing of the already-present full-length mRNA leads to formation of the truncated, active HokB mRNA while SokB-RNA is simultaneously depleted by RNase E (figure 4). Similar considerations apply to type II TA loci in which the TA complexes may function as reservoirs from which the toxins can be activated without the requirement for de novo protein synthesis [31].

Competing interests. I have no competing interests.

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References

15. Verstraeten N et al. 2015 Obg and membrane depolarization are part of a microbial bet-hedging strategy that leads to antibiotic tolerance. Mol. Cell 59, 9 – 21. (doi:10.1016/j.molcel.2015.05.011)


