Conditional Activation of Toxin-Antitoxin Systems: Postsegregational Killing and Beyond

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ABSTRACT

Toxin-antitoxin (TA) systems are small genetic modules formed by a stable toxin and an unstable antitoxin that are widely present in plasmids and in chromosomes of Bacteria and Archaea. Toxins can interfere with cell growth or viability, targeting a variety of key processes. Antitoxin inhibits expression of the toxin, interacts with it, and neutralizes its effect. In a plasmid context, toxins are kept silent by the continuous synthesis of the unstable antitoxins: in plasmid-free cells (segregants), toxins can be activated owing to the faster decay of the antitoxin, and this results in the elimination of these cells from the population (postsegregational killing [PSK]) and in an increase of plasmid-containing cells in a growing culture. Chromosomal TA systems can also be activated in particular circumstances, and the interference with cell growth and viability that ensues contributes in different ways to the physiology of the cell. In this article, we review the conditional activation of TAs in selected plasmidic and chromosomal TA pairs and the implications of this activation. On the whole, the analysis underscores TA interactions involved in PSK and points to the effective contribution of TA systems to the physiology of the cell.

INTRODUCTION

Toxin-antitoxin (TA) genes are small genetic modules coding for a toxin and an antitoxin. Toxins inhibit cell proliferation or viability, and antitoxins neutralize this inhibition. The toxin (always a protein) is the stable component and the antitoxin (a protein or a regulatory RNA) is less stable, and this differential stability plays an important role in the conditional activation of TAs. The term postsegregational killing (PSK) was introduced to define the toxin-dependent elimination of plasmid-free cells that occurs as a consequence of the loss of TA-containing plasmids at cell division. Conditional activation of the toxins in these cells requires a differential decay of the antitoxins compared with the toxins. This differential stability is due to the action of proteases or RNases on the antitoxin half-life. In plasmid-containing cells, the toxin is kept under control because the levels of the antitoxin are replenished by de novo synthesis. In plasmid-free cells, the toxins are activated as the consequence of the faster decay of the antitoxins, and this leads to the elimination of these cells from the population (PSK) and to an increase of the percentage of plasmid-containing cells (1, 2) (Fig. 1). Since the discovery of TA systems as auxiliary maintenance modules in plasmids (3), they have been found in phages and chromosomes of Bacteria and Archaea.
often in multiple copies (4). Conditional activation of TA pairs has also been detected in chromosomal systems in response to particular signals. Furthermore, some of the chromosomal TA systems have the potential to stabilize plasmids via PSK, implying that the differential stability of toxins and antitoxins plays a role in their activation. Conditional activation of TA systems has consequences beyond plasmid stabilization, such as in plasmid competition, phage-abortive infection, stress response, stabilization of particular genomic regions, biofilm formation, and bacterial persistence (5). Most recently, TA systems have been found tightly associated with other defense systems that can be found in Archaea and in Bacteria and that include the so-called CRISPR-Cas immunity system (6).

In TA systems so far described, the antitoxins are either small RNAs or labile proteins; thus, RNases or proteases can lead to degradation of the antitoxin component and, therefore, to the activation of the cognate toxin (7, 8). To date, TAs are classified in five main types depending on the nature and activities of their antitoxins. In type I and type III systems, the antitoxins are small RNAs that inhibit translation of the toxin (type I) or neutralize its activity (type III). In type II TAs, the antitoxins are proteins that neutralize their cognate toxins by direct interactions (9). A basic representation of these three TA types is shown in Fig. 2. Two additional TA-type systems, named IV and V, have been described. In these systems, the antitoxins are also proteins but they do not interact directly with the toxin. Instead, in type IV systems (10), the antitoxin interacts with its target, protecting it from the toxin activity, and, in type V systems (11), the antitoxins cleave and inactivate the mRNA of the toxin. Toxins can act on different targets, but in all cases they inhibit cell growth or affect cell viability (9). Expression of TAs is regulated at the transcriptional and/or posttranscriptional levels. Depending on the similitude of their amino acid sequences or on their structural homologies, toxins and antitoxins are grouped into different families and superfamilies (12).

So far, proper TA pairs have not been found in eukaryotic cells. However, it has been proposed that toxin/immunity systems found in lineal plasmids of unicellular fungi could mediate plasmid stabilization via the PSK mechanism (13). These systems code for toxins

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**FIGURE 1** TAs determine plasmid maintenance by PSK. A bacterial population contains cells with a plasmid that encodes a TA system. (A) In plasmid-containing cells, both the antitoxin and the toxin will be continuously expressed. The inhibitory activity of the toxin will keep neutralized its cognate antitoxin. (B) In plasmid-free cells, a specific depletion of the antitoxin levels by cellular RNases or proteases activates the more stable toxin. This activation induces cell death or arrests the growth of plasmid-free cells (PSK) and increases the number of plasmid-containing cells in the growing population (plasmid maintenance phenotype). doi:10.1128/microbiolspec.PLAS-0009-2013.f1
that, as microcines, are secreted to the growth medium and can inhibit the proliferation of competitors. Cells losing these plasmids and therefore the immunity determinants are susceptible to the action of these toxins and, as a consequence, can be eliminated from the cell population by them. This potential extends the signature of PSK beyond the bacterial and archaeal kingdoms. Even more, the bacterial toxin-antitoxin systems can be engineered to mimic in eukaryotic cell lines the selective elimination of particular cell lines as the result of the differential expression of the toxin and the antitoxin (14, 15).

Owing to experimental and bioinformatic searches, the number of TAs is continuously increasing. In parallel, the interest in the basic and applied implications of these systems has developed to the point of establishing TAs as a new and fertile field of microbiology. A recent compilation of the contribution of the different TAs to the field can be found in reference 16.

**Focus**

Because of the large expansion of the number of putative TA systems and the broad nature and mechanism of action of toxins, we will concentrate on some well-defined TA systems present in plasmids or in chromosomes rather than lavishly covering the PSK subject. In the plasmid context, we will concentrate in several systems found in *Escherichia coli*: *hok-sok* (type I) and *kis-kid* (type II) systems of plasmid R1, *ccd* (type II) of plasmid F, *parDE* (type II) of plasmid RK2, and *phd-doc* (type II) of bacteriophage P1; we will also review the conditional activation of the *toxIN* system found in

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**Figure 2** TA regulation and activation. TA systems are operons that codify a toxin (T) and an antitoxin (A). They share common features: (i) Expression of the operon is regulated at the transcriptional or posttranscriptional levels; (ii) the antitoxin binds and neutralizes the toxic activity of the toxin; and (iii) the antitoxin is unstable and the toxin is stable. The decay of the more unstable antitoxin leads to toxin activation. A, B, and C show the basic features of the regulation and activation of type I, II, and III TAs. (A) Type I TAs: the antitoxin is a small antisense RNA, and the toxin is a protein; processing of the toxin mRNA and cleavage of RNA-RNA hybrids regulate the activity of these systems. (B) Type II TAs: Both toxin and antitoxin are proteins; proteases targeting specifically the antitoxin regulate activation of the toxin. (C) Type III TAs: the antitoxin is an RNA that inactivates the toxin. Toxin activation can occur in response to bacteriophage infection leading to the elimination of these cells and thus preventing the spread of the infection. doi:10.1128/Microbiolspec.PLAS-0009-2013.f2
pECA1039 of the Gram-negative phytopathogen Pectobacterium atrosepticum, the prototype of type III TA systems. At the chromosomal level, we will focus on three type II TA systems: parDE of Vibrio cholerae and yefM-yoeB and pesAT of Streptococcus pneumoniae. We will also discuss the plasmid-chromosome cross-talk between TAs and the implications of PSK in these interactions. As a summary, the systems analyzed include the main three type TA systems found in plasmids, in bacteriophages, or in the bacterial chromosome and toxins that reach different targets; on the whole, they illustrate PSK modulated by RNA or protein decay. In addition to the reviews mentioned above, other excellent reviews covering different aspects of the TA field can be found in references 17, 18, 19, 20, 21, 22, 23, and 24.

PSK MEDIATED BY PLASMID-ENCODED TAS

PSK Mediated by a Fertility Factor: ccd TA of Plasmid F

The coupling cell division (ccd) system of the fertility factor F1 was the first TA plasmid maintenance system described (3). This system, which moderately stabilizes the plasmid (10-fold), codes for an antitoxin protein, CcdA, and a toxin protein, CcdB, that inhibits cell growth and/or cell viability. ccd-mediated stabilization was initially thought to be due to the inhibition of cell division occurring when the copy number of the plasmid was one per cell; this prevented the subsequent accumulation of plasmid-free cells (3). However, it was later found that these cells could indeed divide, giving rise to two cell types: plasmid-containing cells that continue dividing and plasmid-free cells in which its division was arrested after a few residual divisions (2). The result was an increase in the percentage of plasmid-containing cells in the population, i.e., a plasmid maintenance phenotype. These observations formulated the first definition of PSK: increased plasmid maintenance associated with the inhibition of proliferation of plasmid-free cells due to the conditional activation of the toxin in these cells. It was later found that this activation was due to the cleavage and inactivation of the antitoxin by the Lon protease (see below).

CcdB, the toxin of the system, targets and inhibits the dimeric subunit A of the DNA gyrase at a stage in which the DNA strands are cleaved and unsealed (25, 26); it stabilizes this cleavage complex and inhibits DNA gyrase activity (27). Broken DNA can be released from this complex probably owing to its interactions with transcription and/or replication machineries. As a result of this DNA damage, the SOS regulon is induced. SOS induction is not required for CcdB toxicity, but it increases genetic variation associated with DNA repair, mutagenesis, chromosomal rearrangements, and transposition events, thus speeding up genetic variation and adaptation (18).

CcdA antitoxin interacts with CcdB and neutralizes its toxic effect (28, 29). In addition, CcdA can remove the toxin from a CcdB-gyrase complex, thus “rejuvenating” the enzyme (30, 31). Adding to this control of toxin activity, CcdA represses transcription of the ccd operon (29), and this regulation is more efficient in the presence of the CcdB toxin. Interestingly, the efficient repressor is a CcdA2-CcdB2 heterotetramer. This repressor is formed in excess of the antitoxin and is disrupted when the toxin is in excess, a condition in which the main form observed is a CcdA2-CcdB4 heterohexamer (32). This implies that the relative dosage of toxin and antitoxin regulates in a reversible way the expression of the system. The molecular interactions regulating this switch involved two sites of TA interactions, one of high affinity and the other of low affinity (33). Transcriptional regulation and neutralization of the active toxin by the antitoxin are the first level at which PSK is regulated. An additional point of control is antitoxin stability. This stability is modulated by the action of Lon protease that degrades preferentially CcdA (34, 35). The preferential decay of CcdA is at the base of the activation of CcdB in plasmid-free cells and of the elimination of these cells (PSK). CcdB protects the CcdA antitoxin from the action of Lon and, in this way, also influences the efficiency of PSK. Presumably, CcdA is available for Lon-mediated degradation when it assembles from the CcdA-CcdB complex. Alternatively, CcdA antitoxin is cleaved far less efficiently by this protease when it is in complex with the CcdB toxin.

PSK Mediated by a TA Present in a Multiresistance Conjugative Plasmid: hok-sok of Plasmid R1

parB or hok-sok locus of the low-copy-number plasmid R1 was the first type I TA maintenance module described (1) and the prototype of these important TA systems. hok-sok increased by 2 orders of magnitude the stability of plasmid R1 without changing its copy number. It stabilized the plasmid by promoting the elimination of plasmid-free segregants and contributed, jointly with ccd, to establishing the PSK concept. This locus contains two genes hok and sok encoding, respectively, a killing protein (Hok, host killing) and an
unstable RNA antitoxin (Sok, suppressor of killing). Genes sok and hok are constitutively expressed; a strong promoter directs Sok-mRNA synthesis, while hok-mRNA synthesis is directed from a weak promoter. This ensures an increased dosage of the Sok over hok-mRNA. Sok, a small and unstable antisense RNA, inhibits the expression of Hok at the posttranscriptional level. The structure of the Hok toxin and the way in which it acts is not accurately known, but indirect evidence has suggested that it is a protein that disrupts the membrane potential. Cells killed by this toxin have a particular fragile aspect (ghost cells). The way in which this killing potential is maintained under control in plasmid-containing cells and activated in plasmid-free cells has been elegantly elucidated (36): full-length hok-mRNA is inactive but, owing to three processing steps and subsequent refolding, the mRNA precursor becomes an active mRNA. In plasmid-containing cells, this mRNA is silenced by interactions with antisense Sok-RNA, and this RNA-RNA complex is removed because of RNase III cleavage. In plasmid-free cells, decay of the unstable antisense Sok-RNA leaves the active form of hok-mRNA available for translation; and the accumulation of the Hok toxins eliminates these cells (PSK). The hok-sok model of PSK remains a paradigm in the field, but, as could be expected, different mechanisms to prevent irreversible inactivation of toxin mRNA by RNA-RNA interactions do occur (37, 38).

A Second PSK System in an Antibiotic Resistance Factor: The kis-kid TA of Plasmid R1

In addition to hok-sok, plasmid R1 contains a TA close to its basic replicon: kis-kid or parDE (39). This system, which was also found in plasmid R100 (40), encodes a toxin, Kid (Killing determinant), and an antitoxin, Kis (Killing suppressor). Both of them are proteins. The wild-type kis-kid system shows low activity as a maintenance system. It remained undetected until its discovery, by serendipity, following the isolation of a mutation in kis, the antitoxin gene, that increased by over 3 orders of magnitude the stability of the plasmid (39). This mutation inhibited cell growth in rich medium, an effect that was enhanced at high temperature. Both phenotypes, maintenance and cell growth inhibition, were abolished by mutations in kid, the gene adjacent to kis, pointing to Kid as the toxin and Kis as the antitoxin of a plasmid stabilization system (41). The great increase in plasmid stabilization observed in the original mutant was not correlated with a parallel increase in plasmid copy number; this pointed to PSK mediated by the Kid toxin as the explanation of this increased maintenance. Following, the identification of this mutated system, it was also found that the wild-type system had a poor but detectable plasmid stabilization activity, similar or slightly lower than the stabilization determined by the ccd system (39, 42). Kid, the toxin of this system, is a site-specific RNase that cleaves mRNA and inhibits protein synthesis, and, therefore, cell growth is thwarted (43, 44). Kis antitoxin interacts with the toxin and prevents the binding and cleavage of the RNA substrate and the subsequent growth inhibition (45, 46). Expression of the Kis antitoxin up to 30 min after expression of the Kid toxin can rescue the growth of cells arrested by this toxin (our unpublished results), implying an initial bacteriostatic and reversible effect of the toxin.

PSK activation determined by this system is regulated at the transcriptional level by the coordinated action of the toxin and the antitoxin (47, 48). Regulation is also modulated, at a posttranscriptional level by coupling translation of the toxin to the synthesis of the antitoxin and by the differential processing of the polycistronic kis-kid-mRNA (49); this posttranscriptional regulation can determine an increased dosage of the antitoxin compared with the toxin. The activation of the kis-kid system in response to inefficient replication (see “Coupling Plasmid Replication and TA Maintenance Modules” below) adds a new complexity to the regulation of this system. Another level of regulation is modulated by ClpAP protease which targets the Kis antitoxin but not the Kid toxin (50); this proteolysis also modulates the relative dosage of toxin and antitoxin and is at the base of the activation of the toxin. The antitoxin is protected from the protease action by interaction with the Kid toxin. Furthermore, this protection is more efficient when the toxin is in excess (50). The relative dosage of the toxin and antitoxin proteins modulates both transcription of this system and the antitoxin stability, thus influencing PSK in a complex way. Mutations that inactivate the Kid toxin or a deletion of the clpP gene that increased the stability of the Kis antitoxin decreased plasmid stability which is consistent with the role of Kid activity and of Kis proteolysis in PSK (50).

PSK by a TA Present in a Broad-Host-Range Plasmid: The parDE System of Plasmid RK2

parDE is the TA module of plasmid RK2/RP4, a broadhost-range replicon of Gram-negative bacteria (51, 52). ParD is the antitoxin of this system and ParE is the toxin. The parDE system increases 100-fold the maintenance of the RK2 replicon. PSK modulated by parDE
is effective in *E. coli* host and also in other Gram-negative bacteria, thus mimicking the broad-host-range character of the replicon. As CcdA, ParE interacts with the DNA gyrase and inhibits this enzyme at a stage in which DNA strands are cleaved (cleavable complex) (53). It has been proposed that broken DNA can be released from this complex owing to collisions with the replication or transcription machineries; as a consequence, the SOS regulon is induced. Owing to its antitopoisoerase activity, ParE inhibits DNA replication *in vitro*, induces cell filaments, and affects cell growth and viability. Toxin expression and activity are regulated at different levels. In contrast to *ccd* and *kis-kid* in which efficient repression requires the coordinated action of the toxin and antitoxin, repression of the ParDE operon is basically modulated by the ParD antitoxin, being negligible the contribution of the ParE toxin (54, 55, 56, 57). This suggests that in this system conditional cooperativity is not involved in transcriptional regulation of the PSK potential. In addition, ParD interacts with ParE and neutralizes the activity of this toxin (56, 58). Furthermore, ParD-ParE interactions can rescue the ParE toxin when in complex with DNA gyrase, thus rejuvenating a ParE-poisoned DNA gyrase (53). This is an additional safety mechanism that contributes to neutralize the effects of possible uncontrolled release of ParE in plasmid-containing cells. In *parDE*, the differential stability of toxin and antitoxin required for PSK is due to the action of the Lon protease on the ParD antitoxin (52). In plasmid-containing cells, the levels of the ParD required to neutralize ParE are restored by *de novo* synthesis. In plasmid-free cells, the continuous decay of the antitoxin leads to activation of ParE and to plasmid stabilization by PSK. The fact that both ParE and CcdB toxins act on the same target but that the plasmid maintenance mediated by *parDE* is 10-fold higher than *ccd*, suggests that this difference could be due to a different initial load of TA proteins in these cells.

**PSK by a TA Present in a Lysogenic Bacteriophage: The *phd-doc* System of Plasmid P1**

The bicistronic *phd-doc* TA system was first discovered in P1 bacteriophage as a maintenance system acting by a PSK mechanism during lysogeny (59); *phd* codes an antitoxin, and *doc* codes a toxin. Interactions between Phd antitoxin and Doc toxin neutralize the inhibitory potential of this toxin (Phd, Prevent host death; Doc, Death on curing). This system makes the cell addicted to the antitoxin and, by extension, to the system and to the plasmid. Later on, homologs of this system were found on the chromosomes of many bacteria (60). *doc* encodes a toxin, Doc, that prevents translation elongation by interacting with the ribosome, inhibiting cell growth/viability (61). This activity is at the base of PSK mediated by *phd-doc* requires, in *E. coli*, the chromosomal *mazEF* TA, thus underlining complexities at the base of the PSK phenotype and the interaction between plasmidic and chromosomal TA systems (62). As in other systems, *phd-doc* is regulated at the transcriptional level by the coordinated interaction of the toxin and the antitoxin, and the antitoxin pilots the specific interactions at the promoter-operator region (63, 64). The translation initiation signals of *doc* overlap with *phd*, suggesting that synthesis of the toxin is coupled to the synthesis of the antitoxin. The regulatory complex is formed when the antitoxin is in excess and, as in *ccd*, the *phd-doc* system is de-repressed in excess of the Doc toxin. This protein-dosage-dependent regulation (conditional cooperativity) is due to a low- to high-affinity switch in the interaction between Phd and Doc (65). Thus, conditional cooperativity plays an important role in activation of the system and therefore in PSK. The ATP-serine protease ClpXP targets and degrades selectively the Phd antitoxin (66). The relevance of this activity in plasmid maintenance and in PSK is indicated by the fact that *rexB* gene, which product prevents the degradation of Phd by ClpXP, inhibits host death on curing of P1 (63, 67).

**toxIN: PSK That Protects from Bacteriophage Infection**

The *toxIN* locus is encoded on plasmid pECA1039 of the Gram-negative phytopathogen *P. atrosepticum*. This system was discovered because of its ability to confer resistance to bacteriophage, i.e., abortive infection (Abi) system (68, 69, 70). *toxIN* is the prototype of the type III TA systems. *toxIN* encodes a protein (ToxN) which is a sequence-specific (AA/UA) endoribonuclease that cleaves the RNA substrate in the absence of ribosomes (71, 72). *toxi* encodes a small and noncoding RNA (ToxI) that interacts with ToxN and neutralizes the RNase activity of this protein (68, 71, 72). ToxIN precursor contains a tandem array of direct repeats that are probably processed by the ToxN RNase to generate 36-nucleotide pseudoknot repeats that bind ToxN, RNA-Protein interaction, a heterohexameric assembly of three pseudoknots and three ToxN dimers, inactivates the RNase potential of ToxN (71). Because
of the differential stabilities of ToxN and ToxI components (68), antitoxin levels must be maintained by the continued expression of the toxIN operon. Transcription of the system occurs from a constitutive promoter (68) and is regulated by transcriptional termination at a short inverted repeat that precedes toxN. This signal determines the relative levels of the ToxI-RNA precursor and the toxN mRNA. ToxI RNA is degraded by an unknown mechanism shortly after infection; this releases ToxN RNase that arrests growth of the infected cell, thus preventing replication and propagation of the phage in the bacterial population (68, 69). It remains to be tested if, in addition to its function as an Abi system, ToxIN can also stabilize plasmids via PSK.

**Lessons from the Comparative Analysis of PSK of Some of the Above TAs**

In an attempt to obtain a general view on the stability mediated by PSK, the stabilization mediated by each of four early plasmidic TA systems described above was analyzed comparatively: the hok-sok, kis-kid, and ccd systems of narrow-host-range plasmids R1 and F, and the parDE system of plasmid RK2 (42). Based on the extensive knowledge of the R1 plasmid replication control (73), an elegant approach was developed to determine the individual contributions of the above TA systems to the maintenance of this replicon: the four selected systems were independently cloned in a transcription-free region of a TA-free mini-R1 vector and introduced into an *E. coli* host containing a compatible plasmid-expressing *copA* gene under the control of an inducible promoter. *copA* codes a small antisense RNA and inhibits, at the posttranscriptional level, the synthesis of RepA, the replication initiation protein of plasmid R1. The CopA RNA arrests very efficiently the replication of this plasmid. As a consequence, following *copA* transcription, the plasmid recombinants are eliminated. In this way, the effects of each of the four TAs on the maintenance of the same replicon and on the growth of plasmid-free cells could be followed in a comparative way. Consistently, with the maintenance effects of the individual systems, hok-sok or parDE stabilized the plasmid vector 100-fold while stabilization mediated by *ccd* and *kis-kid* was close to 10-fold, i.e., an order of magnitude lower. Further analysis of the cell growth, number of cells (particles), and viable cells suggested that stabilization mediated by the four systems occurred at the postsegregation level. Maintenance mediated by *ccd*, hok-sok, or parDE systems was associated to a decrease in the number of viable cells compared with the total number of cells following induction of *copA* and the loss of the plasmid. This suggested that the systems eliminate plasmid-free cells. For the *kis-kid* system, the effect on plasmid-free cells was a gradual inhibition of cell proliferation and cell division. This was indicated by (i) the parallel increase of cell counts and viable cells occurring during the first generations after *copA* induction and (ii) by an equivalent decrease of these parameters and by an increase in cell size occurring at later times. Both situations, killing or cell growth arrest of plasmid-free cells, led an increase of the percentage of plasmid-containing cells in the bacterial population, i.e., to a plasmid maintenance phenotype.

The analysis underlines the basic hallmark of plasmid stabilization mediated by PSK: (i) it is a specific feature of TA maintenance systems present in plasmids, (ii) it is associated with the loss of the plasmids at cell division and with the activation of the toxin due to the differential decay of the antitoxin in these cells, and (iii) it is the consequence of the nonproliferation of plasmid-free cells that either remain viable or die and of the proliferation of plasmid-containing cells.

**Coupling Plasmid Replication and TA Maintenance Modules**

A singularity of the *kis-kid* system is that the toxin of the system is activated in plasmid-containing cells when plasmid replication is inefficient; this coupling seems to be modulated by a decrease in the antitoxin levels and, surprisingly, can contribute to rescue plasmid replication (74, 75). This rescuing is due to the cleavage by Kid RNase of the *copB-repA* polycistronic mRNA at a preferred sequence located in the intergenic *copB-repA* region; the cleavage reduces the CopB repressor levels and increases transcription of the *repA* gene from an internal promoter, thus enhancing plasmid replication (76). Because this coupling is triggered by a decrease in plasmid gene dosage, it could play a role in situations in which this dosage is reduced, such as in plasmid propagation in unfavorable hosts (73). Note that *kis-kid*-mediated replication rescue does not exclude stabilization via PSK and that the combined effects of PSK and replication rescue are not sufficient to eliminate completely plasmid-free segregants when the efficiency of replication is reduced. An interesting case of the coupling of a plasmid TA system to the plasmid replication functions, and also to the plasmid-partitioning system, occurs in the *oweC* system of plasmid pSM19035 (see reference 126). The coupling of the basic maintenance modules of this plasmid results in a complete stabilization of this genetic element (77).
On the Success of PSK: Plasmid Stabilization or Plasmid Competition?

TA systems are widely present in plasmids and phages, mobile genetic elements, and chromosomes. It was first proposed that the selection of TAs in plasmids could be due to their contribution to their vertical stable inheritance (stability hypothesis). This hypothesis predicts that the number of plasmid-containing cells in a population should increase in the presence of TAs. However, an evaluation of this prediction made in independent cultures with or without TAs encoded in plasmids indicated that the total number of plasmid-containing cells remained similar in both types of cultures. An alternative hypothesis, the competition hypothesis, proposed that these modules were selected in plasmids because they allow them to exclude TA-free plasmids (78, 79). This last hypothesis implied that TA systems have been selected during horizontal plasmid propagation rather than associated with the vertical propagation. The competition hypothesis was strongly supported by the fact that a plasmid containing the parDE TA system excluded an isogenic plasmid devoid of this maintenance module (Fig. 3) (78). This same observation has been reported independently for the TA system formed by a restriction-modification pair (80, 81). The authors concluded that PSK does not increase plasmid stability but acts to mediate the exclusion of competing plasmids; they further proposed that the competition hypothesis could also apply to the evolution of virulence determinants or antibiotic resistance.

Some Evolutionary Considerations on TA Interactions

PSK associated with TA interactions could play a role in the evolution of TA systems. Some of these systems present in bacterial chromosomes could have been delivered by plasmids and thus acquired by horizontal gene transfer. A signature of this common origin has been detected in the ccd system present in the chromosome of Erwinia chrysanthemi. Because of the close similitude to the ccd system of plasmid F, the antitoxin of the chromosomal system protects the cell against the PSK associated with the loss of this plasmid (82). These authors suggested that this antiaddiction context could select plasmid toxin variants that no longer recognize the chromosomal antitoxin. The plasmid-encoded antitoxin could then evolve to achieve effective neutralization of this toxin variant. Eventually, this should originate independent TA systems. In fact, an intermediate situation in which a plasmidic CcdA antitoxin can neutralize the CcdB toxin of a ccd chromosomal system, but in which the chromosomal CcdA antitoxin does not neutralize the plasmidic CcdB toxin, has been reported. In this case, the host is not protected from PSK associated with the loss of the plasmid (see “Plasmid-Chromosome Cross-talk: One- or Two-Way Communications?” below).

FIGURE 3 PSK in plasmid maintenance and in plasmid competition. The random distribution, at cell division, of two plasmid copies with or without a TA system, are shown, in (A) and (B), respectively. Filled circle, plasmid containing TA; open circle, plasmid without TA. Toxin is activated in cells that lose the TA plasmid, and this results in cell death or inhibition of cell proliferation (PSK). Elimination of plasmid-free cells (crossed cell) increases the proportion of plasmid-containing cells in the culture (maintenance phenotype). In B, only one of the two plasmids contains a TA system. Proliferation of cells containing a TA-free plasmid requires the presence of the TA plasmid. This gives a reproductive advantage to cells containing the TA plasmid (competition). doi:10.1128/MicrobiolSpectrum.PLAS-0009-2013.f3
An interesting study of the possible regions targeted by evolutionary process to avoid antiaddiction has been done in the *kis-kid* system of plasmid R1 and in the homologous *chpA* TA found in the *E. coli* chromosome. Functional studies indicated a residual activity of the ChpAI antitoxin on the Kid toxin that could only be detected at high gene dosage of the chromosomal system (83). Subsequent NMR analysis of Kis-Kid and ChpAI-Kid TA complexes clearly identified the regions involved in TA interactions, and native mass spectrometry defined the stoichiometry of these complexes (46). This functional and structural analysis indicated two different ways in which toxin neutralization occurs, one of them being efficient (self-TA interactions: hetero-hexamers in excess of the toxin) and the other being inefficient (crossed TA interactions: ChpAI-Kid heterotetramers). In self-Kis-Kid interactions, the C-terminal region of the antitoxin invaded the interprotomeric region of the dimeric toxin and disrupted the connection between the two protomers, inactivating one of the RNase cleavage sites and disrupting regions required for the binding of the RNA substrate. In the heterologous ChpAI-Kid interactions, the two C-terminal regions of the dimeric antitoxin contact in a lateral way the two active sites of the dimeric toxin, thus preventing the access of the RNA to these sites. The interactions of the toxin and the amino-terminal region of the antitoxin that are crucial to orient the interaction of the C-terminal region of the antitoxin with the toxin in the Kis-Kid complex are lost in the ChpAI-Kid complex. This suggests that subtle changes in these interactions allowed efficient self-TA interactions and inefficient cross-TA interactions between homologous systems.

A signature of the evolution of TA systems from a common origin is found in members of the *kis-kid* and *ccd* systems of plasmid R1 and F, respectively. CcdB and Kid, the toxins of these two systems, have clearly different activities (RNase and antitopoisomerase, respectively) and share poor similitude at the amino acid sequencing level. Surprisingly, their structures are similar, indicating that they share a common structural module (84). The fact that critical regions for activity in CcdB and Kid toxins are located in different regions of this common module opens the possibility that the two toxins evolved from an ancestor protein containing both activities. This common ancestor remains to be identified. A comparative analysis of the toxins and antitoxins of these systems and their interactions suggested that the toxins and antitoxins of the *ccd* and *kis-kid* systems derive from a common ancestor (85).

Furthermore, the analysis indicated that the activities of their toxins are clearly separated: there is no residual antitopoisomerase activity in Kid nor a residual RNase activity in CcdB. In addition, the analysis showed that the Kis antitoxin could neutralize the CcdB toxin, albeit very inefficiently. Furthermore, a distorted trait of CcdA-Kid interactions resulting in enhanced RNase activity of the Kid toxin was demonstrated. The complexes involved in self- and cross-interactions and their stoichiometry were clearly identified and defined by native mass spectrometry. This analysis also revealed distorted interactions between heterologous toxin-antitoxin pairs. On the whole, the analysis suggested that the two systems evolved from a common ancestor in a process in which the toxins reached different targets and the antitoxin coevolved with their toxins to neutralize them.

A variant of this situation occurs between ParE, the toxin of the *parDE* system of plasmid RK2, and RelE, the toxin of the *relBE* system of *E. coli*. ParE inactivates DNA gyrase as CcdB does, but its structure is similar to RelE (86) rather than to CcdB. The structures of the RelE and Kid toxins are also different, pointing to the evolution of *kis-kid/cdd* and *parDE/relBE* superfamilies from different TA ancestors. Note that Kid and RelE are both RNases, but that they cleave RNA in different contexts: RelE cleaves mRNA in a ribosomal (translation) context (87), and Kid is an RNase that cleaves RNA independently of ribosomes (43, 88).

### PSK Via Chromosomally Encoded TAs

**Are Mobile Genetic Elements Stabilized by Chromosomally Encoded TA Systems?**

The finding that copies of genes encoding toxin-antitoxin pairs were found in many of the genomic islands integrated within the chromosome of many bacteria showed that these mobile genetic elements (MGEs) could make use of the ways already “discovered" by plasmids as to not be eliminated from the cell population; thus, the islands would have benefited by the use of TA systems (89, 90). An immediate question derived from this idea is whether these TAs are involved in the maintenance of the islands through PSK: excision from the chromosome could represent a risk for the island if the host cell divides before it is integrated back to the chromosome, transferred to another recipient cell, or is replicated, increasing its copy number and thus enhancing the probability of stable inheritance. Would these possibilities (depicted as a simplified model in Fig. 4) condition the success of the mobile islands in
A) Conjugative transfer of ICE from a donor cell to a recipient cell after ICE replication

B) Conjugative transfer of ICE from a donor cell to a recipient cell without ICE replication
spreading between its bacterial hosts? Would the presence of TAs on those islands, which are transferred by conjugation (without replication), be needed? Or would the TAs be operative as PSK systems for those that are lost without the chance of integrating back? An elegant, albeit partial, answer to these questions was provided by the work of Wozniak and Waldor on the V. cholerae 100-kb integrative and conjugative element (ICE) termed SXT (91). The authors showed that expression of the mosAT operon (a TA encoded within SXT) was increased when the mobile element was excised from the chromosome, thus leading to its stabilization by, most likely, PSK.

Superintegrons, which contain plenty of recombination sequences, surprisingly are found to be remarkably stable on the bacterial chromosome. Bioinformatics analyses revealed that genes encoding TAs were found on large superintegrons but not on the small ones. The stabilization of superintegron and a 165-kb dispensable chromosome region of E. coli by TAs were demonstrated, indicating that chromosomal TAs could limit the extensive genetic loss (92, 93). However, deletion of the entire chromosomal TA operon was feasible (94), which thus gives a hint that the presence of TA systems alone might not directly lead to stabilization of the bacterial genome. Perhaps the TAs expression levels or other yet to be discovered factors could influence the stable inheritance of some of these elements. An interesting finding showed that chromosomal TAs might exhibit PSK as plasmid TAs: V. cholerae contains two chromosomes and, strikingly, all the 13 annotated TAs were found on chromosome II within a 126-kb superintegron (4). Among them are three functional ParDE homologs, where parDE1 (which is identical in both coding and promoter sequence with parDE3) and parDE2 are regulated by their cognate ParD antitoxins, respectively, being ParE toxins only neutralized by their cognate ParD antitoxins. Expression of ParE toxin homologs, which inactivate DNA gyrase, led to DNA damage and SOS response in V. cholerae, presumably as a consequence of blocking cell division (95). More interestingly, the three ParE homologs were shown to degrade chromosome I in V. cholerae cells that had lost chromosome II, which indicates that chromosome mis-segregation stimulates ParE-mediated killing of aneuploid daughter cells (95), in the way similar to PSK by plasmid-encoded TAs. Nonetheless, although chromosome II of V. cholerae contains several essential genes that qualify it as a bona fide chromosome (96), this chromosome replicates like the plasmid/phage P1 (97). It is thus tempting to speculate that chromosome II could be evolved from an MGE.

In the case of the Gram-positive pathogenic bacterium S. pneumoniae, bioinformatics studies of the sequenced strains showed that it contained one or two mobile islands in many of them. One of these islands, a ∼27-kb ICE termed Pneumococcal Pathogenicity Island 1 (PPI-1), was found to contain the pezAT TA (98, 99). Not all the S. pneumoniae strains contain pezAT: only 67% of 26 strains that had been analyzed encode pezT (98), which also coincide with our study on bioinformatics search of PezAT homologs found in 48 of the 48 annotated strains (65% harbor pezAT) (22). Strikingly, some of the strains carry two copies of pezAT on

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**FIGURE 4** Proposed model for a possible role of TA in stabilizing ICE by PSK during conjugative transfer. Under normal conditions, the TA genes (antitoxin gene is depicted as blue arrow; toxin gene is represented by red arrow) on the ICE (purple fragment) are expressed at a basal level within the chromosome. Toxin and antitoxin proteins (red and blue ovals, respectively) form tight complexes that are inert to the cell. During conjugative transfer, the ICE is excised from the chromosome and forms a circular mobilome. (A) The ICE replicates (one copy or more), and one copy of the ICE is transferred to the recipient cell through rolling circle (single-stranded DNA is transferred to the recipient cell and its complimentary DNA strand will be degraded gradually in the donor cell); another copy of the ICE remains in the donor cell. In the donor cell, the ICE is integrated back into the chromosome, while the transferred single-stranded DNA in the recipient cell will replicate to form an intact ICE, followed by integration into the chromosome. (B) The ICE is transferred to the recipient cell without replication in the donor cells. Since the donor cell has lost the TA-containing ICE, the remaining TA complexes will be triggered. The antitoxin proteins that are more susceptible to the degradation of the host proteases are degraded and not replenished owing to the loss of the TA-containing ICE, thus releasing the toxin activity that poisons the donor cell. On the other hand, the recipient cell, which has newly acquired a TA-containing ICE, will thus incorporate the ICE into the chromosome. This recipient cell is subject to the same fate as the donor cell if the ICE is lost. doi:10.1128/microbiolspec.PLAS-0009-2013.f4
different ICEs: in strain ATCC700669, one copy of pezAT was found on PPI-1, and another copy of pezAT was discovered on an 81-kb Tn5253-like ICE (W. T. Chan and M. Espinosa, unpublished data). The absence of pezAT in some of the capsulated strains hinted that pezAT is not essential for virulence. However, the disruption of pezT resulted in strains with impaired virulence in mouse models of infection, showing its influence in virulence, despite the fact that pezT mutant did not show different growth patterns in laboratory broth, serum, or blood (98). Not all the reported ICEs from low-G+C bacteria harbor a cognate TA, as would be expected if the theory that the very existence of the ICE might be endangered by excision and lack of reintegration into the host in the case of lack of replication (see above and Fig. 4). We could speculate that replication of a TA-free ICE would generate more than one copy of it, which would increase the chances of the ICE to integrate back when there is no TA-mediated PSK. Although not mentioned by the authors, this could be, perhaps, the case of the ICEA genetic element from Mycoplasma agalactiae, a member of the Mollicutes class of bacteria related to lactococci and streptococci. We performed a search for TAs associated with this island and could not find any hint of a possible TA within the ICEA (100).

Chromosomally Encoded TAs: Is There Something Else?

As stated above, TAs were discovered on plasmids where they seem to function as ways to achieve successful plasmid maintenance via PSK (1, 21, 39, 41). In addition, more subtle processes, such as coupling plasmid replication and maintenance, were later shown for the R1-encoded kin-kid (75). However, when TAs are located on the chromosome their roles can, at least, be qualified as debatable, since different functions have been ascribed to them from stress response (101), to persistence and antibiotic tolerance (77, 102), and from programmed cell death and altruistic response (24) to ICE maintenance (91). However, it might be that chromosomally encoded TAs should be considered to have a different function depending on their genomic context or to provide any selective advantage to its host (22).

As for the pneumococcal YefM-YoeB TA, we have data showing that it is involved in biofilm formation (Moreno-Córdoba I, Chan WT, Moscoso M, Garcia E, Nieto C, Espinosa M, unpublished data), a role for TAs that has been shown for E. coli (103). The presence of a BOX element (a predicted mobile element present in more than 100 copies within the S. pneumoniae genome) placed upstream of the yefM-yoeB pneumococcal operon, had provided to the system an extra promoter that was independent of regulation by the YefM-YoeB protein complex (104). Location of BOX elements adjacent to genes related to competence and virulence has allowed us to speculate that at least some of the BOX elements might be involved in coordinating the expression and modulation of competence and virulence genes (22). Such a modulation could depend on the copy number of some of the BOX subelements and its orientation, as suggested by reference 105. It is worth noting that, just as with the TAs, competence and virulence can be also viewed as global responses of S. pneumoniae to stress.

PLASMID-CHROMOSOME CROSS-TALK: ONE- OR TWO-WAY COMMUNICATIONS?

To try to define a clear borderline between extra- and intrachromosomal elements can lead to confusion, since it is not so clear which is which. For instance, many rhizobia have a heavy genetic load of megaplasmids (considered as minichromosomes by some). Although undisputedly genetic loads to the bacteria, rhizobia are slow growers; some of the megaplasmids cannot be removed without loss of viability (106). Thus, the essentiality of the DNA cannot be considered as the basis to distinguish between extra- and intrachromosomal information: some megaplasmids cannot be considered as “dispensable” (107). A clearer example is, perhaps, provided by V. cholerae (see above), which has its genome divided into two chromosomes, chromosome I replicating like the E. coli oriC, whereas chromosome II replicates like the plasmid/phage P1 (97). Furthermore, bacterial genes including all contained within the bacteria-shared extrachromosomal gene pool, the so-called MGEs, are naturally dynamic entities, so that plasmid-plasmid and plasmid-chromosome interactions lead to exchanges of the gene cassettes; and these exchanges increase the overall bacteria biodiversity. Plasmids replicating by the rolling circle mechanism generate single-stranded DNA intermediates, a kind of molecule that is highly recombinogenic. Thus, these kinds of plasmids can cointegrate and excise by RecA-independent (illegitimate) recombination mechanisms using as little as 6- to 14 bp sequence homology (108, 109). Other MGEs, such as ICEs, integrative and mobilizable elements (IMEs), or pathogenicity islands (PIs), can promote transfer of chromosomal material to recipient cells, and many of these islands carry their own TAs.

In conclusion, dialogs between bacterial genomes and their mobilome do occur and can be facilitated by the
horizontal transmission of MGEs and, as a result, novel adaptive functions will appear (110). Such new functions pertain to adaptation of bacteria to novel niches or to newly acquired external elements (be they independent like plasmids or integrated into the chromosome as “islands”). This would lead, in turn, to increase the modularity of the bacterial genomes so that the so-called variable genome is the one that would contribute to bacterial biodiversity and adaptation to changing environments (111). Under these circumstances, mutual dialogs based on control of plasmid genes by chromosome-encoded regulators, together with that of chromosomal functions regulated by plasmids, may certainly take place. Examples of these dialogs can be found (112), even though most of them have been uncovered in bacteria grown in laboratory conditions. Nevertheless, an impressive example was the finding that up to 4% of the genes encoded by the *E. coli* chromosome were influenced by the presence of plasmid F; and, furthermore, significant differences of the presence of F on different strains were found (113). These findings provided an even more exciting example that the plasmid-chromosome cross-talks not only take place, but also that they can vary between strains, perhaps as a result of differences in fitness of the strain-plasmid pair. Moreover, bioinformatic analyses performed with the cyanobacterium *Synechococcus* sp. PCC 7002 and six of its plasmids showed that there were a number of cross-talks taking place between the different plasmids as well as between plasmids and the chromosome of the bacterium (114).

For TAs, cross-talks have been reported to take place among chromosomally encoded operons (115). Measurements of the transcription levels of the *E. coli* of the *relBE* operon as a response to several ectopically expressed toxins (MazF, MqsR, YafQ, HicA, and HipA) showed that these toxins were able to activate the transcription of the former operon (115). Furthermore, ectopic expression of the VapC toxins from *Salmonella enterica* serovar Typhimurium LT2 chromosome and from *Shigella flexneri* 2a plasmid pMYSH6000 into *E. coli* led to inhibition of translation and, unexpectedly, to Lon-dependent *trans*-activation of the *E. coli* YoeB toxin, indicative of strong cross-interactions between different TAs from different bacteria and from different genomic origins as well (116). An even more exciting example was provided by the finding that the type II (proteic) TA operon *mqsR-mqsA* was able to control type V TA *ghoT-ghoS* by a complex mechanism involving recognition of the toxin MqsR of the unprotected mRNAs mostly at 5′-GCU sites (117). Under steady state (that is, unstressful conditions), MqsA antitoxin generates a stable and harmless complex with its cognate toxin MqsR. Similarly, and under the same growth conditions, GhoS antitoxin would prevent expression of *ghoT* by cleaving its transcript. However, when the cells are placed under stress, the intracellular Lon protease would degrade MqsA; this, in turn, would release free toxin MqsR, and further induce *mqsR* expression (118). The toxin MqsR will degrade all mRNAs not protected at 5′-GCU sites, like the 5′ end of *ghoST* mRNA. As a consequence, *ghoST* mRNA would be preferentially degraded in the *ghoS* coding region, while the *ghoT* coding region would remain uncleaved. This, in turn, would lead to increased levels of GhoT toxin and, ultimately, to disruption of the cell membrane. Then, this complex interaction between two very different TAs would lead to an increase in bacteria persistence (117). The above findings, in conjunction with those from Gerdes’ group on the reduction of persisters as a consequence of the progressive deletion of 10 ribonuclease-encoding TAs (whereas deletion of individual operons had no detectable effects [119]), added another level of intricacy to the already complex scenario of the TA roles in the bacterial lifestyle. We conclude that it is important to take into account the possible cross-talks between the various TAs that exist within any given bacterium. In addition, scarce information, if any, is available on the possibility of cross-talks between plasmid-encoded and chromosomally encoded TAs.

In another context, bioinformatics approaches indicated that homologs of TAs are present within the genome of a bacterium (4); furthermore, the abundance of TAs of the same family in a single cell and whether they can be cross-talking to each other was somehow an intriguing possibility. A comprehensive functional analysis study reported that 20 VapBC homologs (of 47 putative VapBC homologs) of *Mycobacterium tuberculosis* were functional (120). In addition, a subset of four VapB antitoxin homologs were found to be able only to antagonize their own VapC toxicity but not the rest of the three VapC toxin homologs, indicating that cross-talk (in terms of counteracting individual toxicity) among the coexisting VapBC homologs in *M. tuberculosis* is unlikely (120).

In the case of the *ccd* TA, the chromosomally encoded *ccd*O157 and the *ccd*F from F plasmid are coexisting in *E. coli* O157:H7 isolates in nature. Cross-interaction experiments showed that the chromosomal CcdB*O157 toxin could be neutralized by the plasmidic CcdA*F antitoxin, but the plasmidic CcdB*F toxin was not able to
be counteracted by the chromosomal CcdA_{O157} antitoxin (121). These findings would allow the coexistence or maintenance of plasmid F to be feasible despite the presence of the ccd_{O157} in the chromosome, because losing plasmid F would lead to PSK mediated by the plasmidic CcdF (121). On the other hand, the chromosomally encoded Erwinia chrysanthemi ccd_{Ech} could instead hinder the PSK exerted by the plasmidic CcdF (82). Because chromosomal CcdA_{Ech} antitoxin was able to inhibit the toxicity of plasmidic CcdB_{F} toxin, the chromosomal ccd_{Ech} was thus capable of protecting the cells from the invasion of MGEs that carry TA (ccd_{F} in this case) that mediate PSK, and, therefore, this chromosomal TA was termed antiaddiction module (82).

**FURTHER THOUGHTS**

It appears that TA homologs, or the same family of TAs that are encoded on plasmid, can also be found on chromosome, i.e., ccd (2, 121), albeit they may be more distantly related to each other (82). The function of plasmid-encoded TAs is usually associated with PSK and the chromosomally encoded TAs are suggested to play more complex roles in cell processes than PSK. These roles include general stress response, biofilm formation, persistors, stabilization of mobile elements in chromosome, development processes, virulence, or even essential for niches and survival (see above).

**Do Chromosomally Encoded TAs Act Also as PSK?**

The two chromosomally encoded ccd (ccd_{O157} and ccd_{Ech}) were shown to be unable to stabilize plasmid (82, 122), and thus the chromosomal TAs were believed to play roles other than PSK. Conversely, the chromosomally encoded relBE locus of the E. coli was able to stabilize plasmid R1, although plasmid stabilization was not observed in a Lon-deficient strain, indicating that activation of RelBE-mediated PSK required the host Lon protease (123). In addition, as mentioned above, the ParE toxins within the superintegron of chromosome II of V. cholerae led to the PSK of cells that was missing chromosome II (124). Together with the examples of TAs stabilizing MGEs (91, 93), allows us to envisage a scenario in which a selfish invasion of a MGE to a bacterial cell should be counteracted by the invaded organism by means of a defensive behavior (6) or by an adaptive response, in which adaptation could lead to the acquisition of new selective advantages that could result in out-competing cognate and competitors, as well.

**Do Plasmid-Encoded TAs Respond to Stress Like Chromosomally Encoded TAs?**

An elegant study has shown an additional role of plasmid-encoded ccd_{F} as a transmissible persistence factor, besides PSK (125). A CcdB mutant, which is not toxic but still able to bind to the CcdA antitoxin, was found to be able to compete with the wild-type CcdB toxin to bind to its cognate CcdA antitoxin, and also de-repress the ccd promoter. With low expression (induced by 0.001% of arabinose) of CcdB mutant, cell growth inhibition was evident, and under this condition, the cells were exposed to lethal dosage of different antibiotics. As a consequence, the induced cells showed more surviving colonies in comparison with the un-induced ones, signifying that ccd_{F} increased tolerance and promote persistence (125).

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