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Toxin/Antitoxin System Paradigms: Toxins Bound to Antitoxins Are Not Likely Activated by Preferential Antitoxin Degradation

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Periodically, a scientific field should examine its early premises. For ubiquitous toxin/antitoxin (TA) systems, several initial paradigms require adjustment based on accumulated data. For example, it is now clear that under physiological conditions, there is little evidence that toxins of TA systems cause cell death and little evidence that TA systems cause persistence. Instead, TA systems are utilized to reduce metabolism during stress, inhibit phages, stabilize genetic elements, and influence biofilm formation (bacterial cells attached via an extracellular matrix). In this essay, it is argued that toxins bound to antitoxins are not likely to become activated by preferential antitoxin degradation but instead, de novo toxin synthesis in the absence of stoichiometric amounts of antitoxin activates toxins.

1. Review

1.1. TA Systems

TA systems are found in most bacteria and archaeal strains[1] and usually consist of two components, a protein toxin that disrupts some key cellular process, functioning like an antibiotic, and an RNA or protein antitoxin that masks the toxin activity. Since the genes for TA systems are malleable, for example, antitoxins can be converted into toxins and antitoxins can be made to inhibit different toxins,[2] they can be readily adapted for various applications by the cell, once they are acquired through horizontal gene transfer.[3] TA systems are often autoregulated,[4] and they may also exist in cascades, as is the case for endonuclease toxin MqsR of the MqsR/MqsA TA system, which activates membrane-damaging toxin GhoT by selectively degrading antitoxin GhoS.[5] Note that for consistency, in this essay, we list the toxin followed by the antitoxin for TA systems (e.g., toxin/antitoxin) regardless of the order of the genes which encode them.

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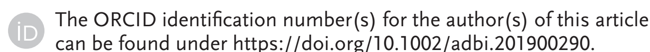
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1.2. TA System Nomenclature Update

Though becoming unwieldy, TA systems are classified into eight groups based on the how the antitoxin masks toxin activity. In type I systems (the first example is Hok/Sok[6]), the antitoxin is an anti-sense RNA that inhibits toxin mRNA translation. In type II systems (the first example and also the first TA system described is CcdB/CcdA[7]), the antitoxin protein binds and inhibits the toxin. In type III systems (the first example is ToxN/ToxI[8]), the antitoxin RNA binds the toxin to inhibit it. In type IV systems (the first example is CbtA/CbeA[9]), the antitoxin protein prevents binding of the toxin with its target. In type V systems (the first example is GhoT/GhoS[10] and notably the first antitoxin that acts as an enzyme), the antitoxin is an RNase that degrades specifically the toxin mRNA. In type VI systems (the first example is SocB/SocA[11]), the antitoxin protein stimulates degradation of the toxin. In type VII systems (the first example is Hha/TomB,[12] the second system in which the antitoxin is an enzyme), the antitoxin inactivates the toxin by oxidizing a cysteine residue. Here, we propose the type VIII classification system (the first example is SdsR/RyeA[13]) in which for the first time the toxin is a small RNA, and the antitoxin masks its activity by anti-sense binding.

1.3. TA Systems Do Not Cause Cell Death

After the initial discovery of TA systems as plasmid stabilization systems (the type II CcdB/CcdA system),[7] the TA field was plagued by the idea that cell death is associated with toxin activation. For example, in the second report of a TA system (Hok/Sok) "post-segregational killing" was claimed for the TA system Hok/Soks[14] but there is no evidence of it.[15] Furthermore, the claim of programmed cell death by MazF[16,17] is not warranted.[18–21] Similarly, the claim of phage inhibition by altruistic host death[22,23] is not warranted.[15] Therefore, there is no evidence linking TA systems with cell death under physiological conditions.

1.4. TA Systems Are Weakly Related to Persistence

Persistence is a stress-tolerant state in which a small subpopulation of cells (usually less than 1%) become dormant to

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weather the stress without undergoing mutation; upon removal of the stress and in the presence of nutrients, the persister cells resuscitate and resume growth as wild-type cells.[24] The best evidence that TA systems are related to persistence is that deleting some genes which encode toxins reduces persistence; for example, deleting *mqsR*,[25,26] *tisB*,[27] and *yafQ*[28] reduce persistence. However, the changes in persistence is on the order of only tenfold for the *mqsR* deletion (and the result was not complemented[25]) and for the *tisB* deletion, and changes of this magnitude are not likely to be relevant for persistence since cell populations are reduced by 100 000 to 1 000 000-fold in these type of experiments.[24] Furthermore, the *yafQ* deletion had an effect as high as 2400-fold in biofilms, but there was no effect in planktonic cultures.[28] Hence, for the few instances where inactivating toxins has a reduction in persistence phenotype, the evidence is not compelling.

Since non-physiological production of toxic proteins not related to TA systems increases persistence in a manner similar to overproducing toxins of TA systems,[29] overproduction of toxins[25,30] is also not strong evidence of their relationship to persistence. Similarly, evidence based on the overproduction of a non-toxic toxin such as HipA7 is not convincing for establishing a link between persistence and TA systems.[31] Critically, several reports have shown that inactivation of 10 TA systems (but not including HipA) has no effect on the persister level of *Escherichia coli*[32–34] and deletion of 12 TA systems in *Salmonella enterica* has no effect on persistence.[35] Notably, these studies are imperfect in proving persistence is not related to TA systems since *E. coli* has at least about 40 TA systems[24] which are interconnected (e.g., toxin MqsR activates toxin GhoT as part of a cascade[5]) so only a small percentage of the total number of TA systems were deleted.

If TA systems are related to persistence, there should also be evidence they impact resuscitation from the persister state. However, there is little robust data regarding resuscitation from persisters due to inactivation of a TA system toxin. For example, it has been claimed incorrectly that the peptidyl-tRNA hydrolase Pth counteracts toxin TacT in *Salmonella typhimurium* during resuscitation, for example, by this passage[36]: "In *Salmonella typhimurium* persisters, tRNAs acetylated by the TacT toxin are deacetylated by the peptidyl-tRNA hydrolase Pth, reversing the effect of the toxin and allowing cells to resume growth," but there are no data showing Pth plays a role in persister resuscitation in the cited work.[37]

Similarly, it has been claimed that deactivation of HokB toxin in *E. coli* controls persister waking; however, instead of single-cell observations, delays in resuscitation were estimated from growth data,[38] which are not direct proof of HokB inactivation in recovering persister cells. Critically, the persister resuscitation work related with HokB is based on non-physiological levels of toxin from overproduction studies.[38,39] In addition, there is little convincing proof that HokB is related to persistence in wild-type cells since deleting *hokB* has no effect on persistence.[40] Furthermore, HokB was identified as related to persistence based on work with the GTPase Obg; however, reduction in persistence based on reducing Obg levels occurs without HokB,[40] and production of Obg causes a likely inconsequential increase in the induction of *hokB* (1.7-fold). Therefore, to date, the bulk of the data suggest TA systems are not the primary means of forming or resuscitating persister cells.

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1.5. Bona Fide Roles of TA Systems

To date, there appear to be four bona fide roles of TAs in bacterial cell physiology: i) growth diminution during stress, ii) phage inhibition, iii) genetic element maintenance, and iv) biofilm formation. In this section, we touch on some of the key works that establish these roles for TA systems.

For the role of TA systems in growth diminution, this was proposed in 2000[41] and demonstrated in 2001 with the RelE/RelB TA system for nutritional stress.[42] Another clear example of growth diminution during stress is that the MqsR/MqsA TA system, which was first identified as relevant in biofilms[43,44] and later shown to reduce growth during bile acid stress in the GI tract through toxin MqsR[45] and through derepression of the general stress response by degradation of antitoxin MqsA.[46] Since most bacteria experience nutrient stress[47] and other forms of stress, TA systems are likely the means by which they exquisitely reduce their growth rates.

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For the role of TA systems in phage inhibition, the first example of a TA system inhibiting phage was that of the type I system Hok/Sok, which inhibited T4 phage[22]; 8 years later, the type II system MazF/MazE was found to inhibit phage P1,[48] 13 years later the type III system ToxN/ToxI was found to inhibit phage φA2 and φM1,[8] and 18 years later, the type IV AbiEii/AbiEi was found to inhibit the 936 phage family.[49] Also, antitoxins have been found on phage that inhibit host toxins.[50] Hence, phage inhibition has been shown to be an important role of TA systems.

For the role of TA systems in genetic element maintenance, this role was established by the discovery of TA systems (CcdB/CcdA) as a means to maintain the mini-F plasmid[7] in 1983. Since this initial report, there have been many examples of TA systems stabilizing various plasmids as well as other genetic elements such as the integrative and conjugative element SXT in *Vibrio cholera* via the MosT/MosA TA system.[51] In a related manner, TA systems are also present in cryptic prophages such as the RalR/RalA TA system in the *E. coli* rac cryptic prophage[52] and the RelE/RelB TA system in the *E. coli* Qin cryptic prophage.[53] Hence, TA systems have a clear role in the maintenance of genetic elements.

For the role of TA systems in biofilm formation, two TA systems were first identified as relevant in *E. coli* biofilms in 2004 through a whole-transcriptome study[43]: Hha/TomB[12,54] and MqsR/MqsA.[55] Toxin Hha decreases biofilm formation by reducing fimbriae production by repressing the transcription of genes that encode tRNAs for rare codons.[54] MqsA inhibits biofilm formation by binding to the palindrome present in the promoter of *csgD*, which encodes the master regulator of curli and cellulose,[56] and by binding to the palindrome present in the *rpoS* promoter which encodes the stress sigma factor[46]; repression of *rpoS* by MqsA serves to reduce c-di-GMP,[46] a well-known positive regulator of biofilm formation. Toxin MqsR increases biofilm formation through quorum-sensing signal autoinducer 2, which enhances biofilm formation[44] as a chemotaxis signal[57]; *E. coli* chemotaxis toward AI-2, and the positive role of AI-2 on biofilm formation was re-discovered a decade later.[58] Critically, many groups have reported phenotypes related to the MqsR/MqsA TA system in *E. coli*, including its impact on heat shock,[59] on biofilm formation,[60] on nitrogen starvation through its impact on RpoS,[61] on nitric oxide regulation,[62] and on persistence upon deletion of *mqsR*.[26,63] Therefore, MqsR/MqsA is important for cell physiology in *E. coli*. Also, other labs have demonstrated the importance of the MqsR/MqsA TA system in non-*E. coli* systems. These include *Xylella fastidiosa,* where MqsR/MqsA plays a role in copper stress,[64] is secreted via vesicles,[65] and impacts biofilm formation.[66] Also, MqsR/MqsA impacts biofilm formation in *Pseudomonas fluorescens*[67] and impacts persistence and biofilm formation in *Pseudomonas putida*.[68] In addition, deletion of five TA systems was found to alter biofilm formation by affecting fimbriae production through TabA.[69] Therefore, TA systems have a clear role in biofilm formation.

1.6. Antitoxins Are Unlikely to Be Degraded When Bound to Toxins

One of the main paradigms in the toxin/antitoxin (TA) field is that the type II toxins that are inactivated by binding to antitoxins are reactivated by preferential degradation of the antitoxin, which frees the toxin to inhibit cell growth. For example, a recent TA system review indicates, "Under conditions of stress the antitoxins are *selectively* degraded. This leaves the toxins to exert their toxic effects, which leads to growth arrest and dormancy."[4] Hence, it is common to assume a protease degrading antitoxin bound to toxin is the means to activate toxins.[70–72] We suggest that the current model of reactivation of toxins in this manner is unlikely and unsupported in that to the best of our knowledge, there are no reports showing the degradation of antitoxins bound to toxins in vitro or in vivo. Hence, it seems this paradigm has been established, like cell killing, without experimental evidence.

Aside from the lack of experimental data showing antitoxins bound to toxins are degraded, there are several reasons why this is unlikely. First, not all antitoxins are unstructured so there is no clear mechanism by which a protease like Lon would recognize the antitoxin as a substrate since Lon preferentially degrades unstructured proteins.[73] For example, MqsA is structured even before binding toxin MqsR,[55] so it is not clear why it should be degraded. Also, antitoxins bound to toxins are not likely to be degraded preferentially over toxins, since in most cases, both proteins are structured, and it seems unlikely that proteases like Lon can distinguish bound structured antitoxin from bound structured toxin. Furthermore, since intracellular conditions are unlikely altered much during stress, it is not clear how Lon protease would be stimulated to degrade bound antitoxins of type II TA systems instead of toxins.

The tight interactions between toxin and antitoxin also preclude dissociation of an active toxin as well preclude free toxin (assuming antitoxins are produced in excess). For example, the binding constant (K_d) for RelB and RelE is 0.33 nM.[70] For PezT/PezA, the binding constant is on the order of a 65 femtomolar, which makes the bound antitoxin PezA resistant to proteolysis.[74]

The problem is that in many experiments, unbound antitoxins are studied, and their lability is then attributed to bound antitoxin. For example, antitoxin MqsA was shown to be degraded by Lon protease during oxidative stress but these experiments were based on overproduction of MqsA from a multi-copy plasmid where MqsA is likely not bound to toxin MqsR.[46] Similarly, antitoxin HipB is degraded readily by Lon protease both in vivo and in vitro in the absence of toxin HipA; however, HipA is not degraded significantly in the presence of HipA, even with a disordered 16 aa C-terminus region that remains disordered upon HipA binding. Also, antitoxin RelB is degraded readily by Lon in vitro in the absence of toxin RelA but the degradation is reduced in its presence.[70] Therefore, proteases like Lon can degrade antitoxins and play a role in their removal during stress, but there is little evidence that they degrade antitoxins bound to toxins.

1.7. Toxins Are Likely Activated by De Novo Synthesis

Instead of activation of bound toxins, we propose that toxins are activated by their de novo synthesis in a background of insufficient antitoxin for rapid binding and inactivation. Hence, for toxins to inhibit growth, toxins must be synthesized de novo and in excess

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of antitoxins. For type II TA systems in which toxins and antitoxins are co-transcribed, it appears there are factors during stress that inactivate the antitoxin mRNA portion while leaving the toxin portion of mRNA free for translation. A post-transcriptional approach like this has been seen for activation of toxin GhoT where endonuclease toxin MqsR was shown to degrade preferentially antitoxin GhoS mRNA in vivo and in vitro since the GhoT toxin portion of the mRNA lacks 5’GCU MqsR cleavage sites whereas the antitoxin GhoS transcript has three 5’GCU sites.[5] Addition of two artificial MqsR 5’GCU sites to the GhoT mRNA led to its rapid degradation.[5] Hence, there is at least one TA system where de novo antitoxin production is prevented under oxidative stress and the resulting de novo toxin production leads to toxin-related phenotypes, specifically membrane damage by GhoT.

In summary, there are at least eight types of TA systems, and TA systems have at least four bona fide roles in cell physiology, which do not include a role in the phenotypic switch to produce persister cells as well as do not include a role in cell death. In addition, it is likely that de novo synthesis of toxins, in the absence of stoichiometric amounts of antitoxin, is required for toxin activation.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

T.K.W. conceived and wrote the manuscript. S.S. helped write the manuscript.

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