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Toxin–antitoxin systems as mediators of phage defence and the implications for abortive infection

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Bacteria have evolved a broad range of defence mechanisms to protect against infection by their viral parasites, bacteriophages (phages). Toxin–antitoxin (TA) systems are small loci found throughout bacteria and archaea that in some cases provide phage defence. The recent explosion in phage defence system discovery has identified multiple novel TA systems with antiphage activity. Due to inherent toxicity, TA systems are thought to mediate abortive infection (Abi), wherein the host cell dies in response to phage infection, removing the phage, and protecting clonal siblings. Recent studies, however, have uncovered molecular mechanisms by which TA systems are activated by phages, how they mediate toxicity, and how phages escape the defences. These new models reveal dazzling complexity in phage–host interactions and provide further evidence that TA systems do not in all cases inherently perform classic Abi, suggesting an evolved conceptual definition is required.

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Toxin–antitoxin systems as mediators of phage defence

Our understanding of bacteria–bacteriophage (phage) interactions is rapidly expanding. This is due to an unprecedented rise in the discovery of phage defence systems. Classic examples include restriction-modification systems [1], abortive infection (Abi) systems [2], and CRISPR-*cas* [3]. 'Guilt-by-association' approaches have now provided a much broader range of novel systems evolved by bacteria to protect from their viral parasites [4,5]. These can be clustered into genetic loci called 'defence islands' [6] that demonstrate widespread and conserved modes of regulation [7]. Amongst these discoveries are multiple examples of novel toxin–antitoxin (TA) systems involved in phage defence [8–10].

TA systems are canonically thought of as small loci encoding a toxin and an antagonistic antitoxin. They are sorted into families based on related structures [11], and types according to interactions between their components [12]. TA systems were first implicated in phage defence through demonstration that the type I TA system Hok/Sok inhibited T4 propagation [13]. Type II TA systems followed suit through MazEF inhibition of P1 [14], the type III system ToxIN was then shown to inhibit multiple phages [15], and the type IV AbiEi/AbiEii system similarly provided phage defence in *Lactococcus lactis* [16]. The AbiE system was first identified as an Abi system, and later shown to consist of the two AbiEi and AbiEii components acting as a TA system [17]. Phage defence is considered a primary evolutionary role for TA systems, though as many do not display clear antiphage activity, other functions should be considered [12].

Abi systems are themselves a grouping of disparate systems that have been identified as providing phage defence by enforcing cell death on the infected host [2,18,19]. In the classical Abi model, the phage will absorb and inject DNA as per a normal infection, but later infection stages are inhibited, resulting in fewer or zero phage progeny and host cell killing [19]. It follows that cell death removes the phage from the environment and protects the clonal bacterial population. Just as not all TA systems can be linked to phage defence, not all Abi systems are themselves TA systems. Furthermore, whilst recent articles have sometimes claimed TA-mediated phage defence occurs through an Abi mechanism, this has been considered a conflation between toxin activity and the outcome of an initiated phage infection [20]. For example, a toxin may provide phage defence by interfering with, or blocking production of, mature virions, but in itself does not kill the cell and may even be simply bacteriostatic when activated. Meanwhile, early infection phage products might be rapidly killing the cell through a range of processes (such as chromosomal degradation) that lead to cell death independently from TA system toxicity. The distinction as to the cause of cell death becomes more complex when

Table 1

Toxin–antitoxin systems involved in phage defence.

System	Demonstrated in		Activation mechanism	Toxicity mechanism	Escape strategies	Refs.
	Hosts	Phages				
AbiEi/AbieEii	*M. tuberculosis* *Serratia* sp.		Unknown	Prevents tRNA charging leading to cell death/ population arrest	None shown	[17,41,42]
CrlA	*P. aeruginosa*	PAP8 QDLS PAP-L5 PAOP5	Unknown TA module induced at stationary phase with [CrlA] > [CrlT]	CrlT cleaves mRNA	None shown	[40]
DarTG	*M. tuberculosis* *Thermus aquaticus* *E. coli*	RB69 T5 SECΦ18 Lust	Direct toxin activation/liberation	DarT specifically modifies thymidines on phage ssDNA via ADP-ribosylation. This blocks phage DNA replication elongation	Mutation of existing DarTG inhibitor (gp61.2) Mutation of SECΦ18 DNA polymerase	[9,35]
Gp29/Gp30 FaRel	*Mycobacteria* *Cellulomonas marina*	Phrann	Unknown	FaRel depletes cellular ATP and GTP pools and results in nucleoid condensation and rapid cessation of transcription	Phages can encode alarmone hydrolases	[30,37]
Hok/Sok	*E. coli* *Erwinia amylovora*	T4	Unknown Theorised global transcriptional shutoff	Hok caused membrane rupture and collapse of PMF	None shown	[13,52]
KKP	*P. aeruginosa* *Shewanella sp.* *E. Coli* *Vibrio tasmaniensis* *Salmonella enterica* sv. Typhi Str. CT18	T1 T4 T7	Unknown	Phosphoproteomics suggests targets: Cell division proteins, DNA replication proteins, transcription and translation proteins and stress-regulated genes	None shown	[32]
PfiAT	*P. aeruginosa*	Pf4 Prophage	Unknown	Unknown	None shown	[29]
PemIK	*S. aureus* *K. pneumoniae*	MS2 vB_Kpns-VAC7	Unknown Possible stress induction	Sequence-specific endoribonuclease recognising UAUU, with antitoxin physically preventing toxin activity leading to metabolic arrest	None shown	[38,39]
Retrons	*E. coli* (Ec48, Eco8, Ec67) *S. enterica* (Sen2) Se72		Phage protein inhibits antitoxin component of module, liberating active toxin (e.g. for Retron-Sen2: RT-Sen2 and msDNA-Sen2 comprise antitoxin, RcaT is the toxin) Prophage RT-Eco1 is an anti-RM phage defence protein that binds msDNA-Sen2, inhibiting AT and liberating active T Ec48 senses phage-encoded RecBCD inhibitors	Retron-based defence weaker in absence of RNase H1 Eco48 proposed to impair membrane integrity	Various, RcaT toxin directly inhibited by *racC*	[8,33,34]
RnlAB			Unknown Theorised transcriptional shutoff	Unknown	Direct inhibition by phage Dmd	[27,53]
RosmerTA			Unknown	Unknown	None shown	[54]
SanaTA			Unknown	Dependent on presence of Lon	T7Δ4.5 is sensitive to SanaTA whilst WT is not	[55]

Table 1 (*continued*)

System	Demonstrated in		Activation mechanism	Toxicity mechanism	Escape strategies	Refs.
	Hosts	Phages				
ShosTA	*Novosphingobium aromaticivorans* DSM 12444		Unknown	Unknown	Mutations in SSB protein, inhibitor of host RNA polymerase and DNA primase/helicase genes	[46,56]
ToxIN	*E. coli* *P. atrosepticum*		Labile RNA antitoxin is degraded, liberating free toxin	Prevention of virion formation via blockage of viral protein production	Partial transcriptional shutoff, RNA antitoxin mimics, Phage ϕM1; mutation in *m1-23*	[10,15,25,26,47–49]
TAC systems	*E. coli* K12	T4	Unknown	Unknown	None shown	[5]
PD-T4-5		Lambda				
PD-T4-7						
PD-T4-9						
PD-lamdba-2						
PD-T4-10						

considering whether toxin levels are native or artificially over-expressed. This review aims to address this complexity by summarising recent discoveries in mechanisms of phage defence provided by TA systems. We will consider the contributions of TA systems in cell killing as a response to phage, and how best to combine our growing molecular understanding of toxicity with consideration of the classic Abi phenotype.

Activation of toxin–antitoxin mediated phage defence

Whilst the number of TA loci implicated in phage defence continues to increase, mechanistic details that underpin their activation are comparably elusive. As of Dec 2022, only a handful of specific routes to activation have been identified in the context of phage defence (Table 1).

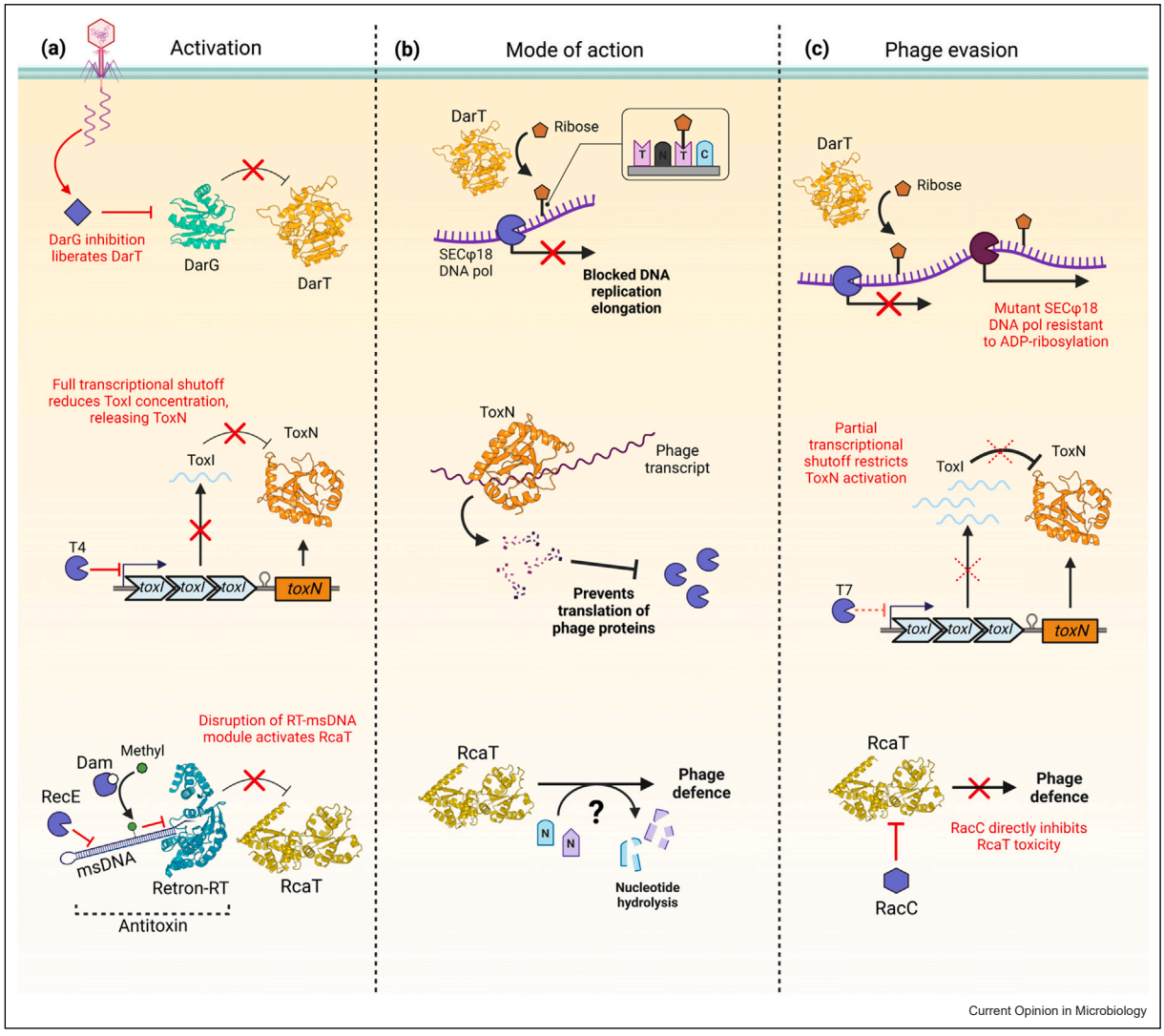
Direct toxin release

Our canonical understanding of type II TA systems is that the toxin protein is held inert by the antitoxin protein, before activation due to antitoxin degradation. The DNA-modifying *E. coli* DarTG system appears to be rapidly activated post-infection, with ~fivefold lower DNA synthesis rates in cells harbouring direct toxin release (DarTG) compared with DarGΔT deletion strains as early as 5 min after exposure to phage RB69 [9]. It is proposed that the fast onset of toxicity is reliant on constitutively active DarT toxin being sequestered by DarG antitoxin, before phage-mediated liberation of DarT (Figure 1a), though further work is required to demonstrate complex dissociation and whether there is degradation of DarG [9]. DarTG homologues have also been shown to confer immunity in a phage-specific manner, likely due to individual recognition of specific phage factors to allow release of DarT, rather than through a conserved trigger for complex dissociation [9]. Similarly, activation of the monocistronic CapRel^{SJ46} TA module, encoded by numerous *E. coli* prophages, has been linked to direct binding of phage capsid protein Gp57, and its homologues, to the catalytically active conformation of the fused CapRel protein [21]. Crucially, these findings suggest that type II TA systems can be activated in response to phage infection without antitoxin degradation, in agreement with work suggesting that not all type II systems are activated as a result of antitoxin proteolysis [22].

Transcriptional shutoff

Many bacteriophages, such as T4, trigger rapid shutdown of host transcriptional and translational machinery to facilitate the production of phage progeny [23,24]. The type III TA phage defence system ToxIN, first characterised in *P. atrosepticum* [15,25,26], was hypothesised to be activated due to destabilisation of the ToxI:ToxN ratio following phage infection [15]. This has now been demonstrated for

Figure 1



Phage defence by selected TA systems DarTG, ToxIN, and Retron-Sen2. Whilst many examples are present in the literature (see main text), these systems were selected due to recent progress in determining the molecular processes involved. **(a)** Activation of TA systems can occur through DarTG, transcriptional shutoff (ToxIN), or activation of the toxin (Retron-Sen2), **(b)** Once released, toxins prevent phage replication either by ADP-ribosylating phage DNA to prevent replication elongation (DarT), degradation of phage transcripts (ToxN), or (as hypothesised for RcaT), hydrolysis of nucleotides and nucleosides. **(c)** Phages can escape TA-mediated phage defence by mutating polymerases to circumvent template nucleic acid modifications (SECϕ18 versus DarTG), reducing their activity to evade detection (T7 versus ToxIN), or encoding a direct toxin inhibitor (RacC versus RcaT).

E. coli ToxIN, as due to phage-induced transcriptional inhibition, infected cells were unable to synthesise sufficient ToxI RNA antitoxins to inhibit the ToxN ribonuclease toxin (Figure 1a) [10]. Similar mechanisms of activation have been proposed for RnlAB and Hok-Sok TA systems, with auxiliary degradation of labile antitoxic proteins and RNAs attributed to toxin liberation, respectively [13,27]. Interestingly, there is new evidence demonstrating how TA system activation through depletion of antitoxin expression might also actively promote the phage life-cycle by allowing cell lysis, as observed for *hokW-sokW* in *E. coli* O157 [28]. This builds on similar recent evidence of prophage-encoded TA systems controlling phage immunity and production [29,30]. Furthermore, a newly discovered widespread tripartite type VII [31] TA system, Kinase-Kinase-Phosphatase (KKP), is proposed to provide phage defence against lytic phages whilst also regulating prophage lysogeny [32].

Retron activation

Retron TA systems encode an effector toxin, a non-coding RNA, and a reverse transcriptase (RT) responsible for generating multicopy single-stranded DNA (msDNA) [8]. Toxin activation appears to be intrinsically coupled to disruption of the msDNA-RT antitoxin complex (Figure 1a) [8,33]. It is not currently fully understood, however, how the toxin is antagonised in the absence of infection [34]. In *S. enterica*, activation of Retron-Sen2 was demonstrated by induction of prophage-encoded components that resulted in either degradation or methylation of mature msDNA, causing release of the RcaT toxin (Figure 1a) [8], though it remains to be demonstrated whether these are effective triggers during a phage infection. Activation of *E. coli* retrons Ec48, Ec73 and Ec86 has been attributed to recognition of phage proteins by the msDNA within the msDNA-RT complex, as each retron offers defence against a different range of phages, though the exact recognition mechanisms remain to be identified [33]. This again demonstrates that activation is likely multifaceted and that as studies continue, we will discover additional layers of complexity.

Mode of action for toxin–antitoxin mediated phage defence

Once activated, toxins have varied means of impacting cell growth. Reduced cell growth is thought to then lead to phage defence through an Abi mechanism, though the direct and causal links have not always been clear. New studies are suggesting more specific mechanisms by which TA systems provide defence (Table 1).

Interference with DNA replication

Modification of ssDNA by DarT was first described in 2016, with the cognate antitoxin DarG shown to reverse this modification [35]. Induction of DarT leads to an SOS response and bacteriostasis [35]. DarTG has since been shown to provide phage defence, supposedly through an Abi mechanism, as it was demonstrated that DarT prevents phage DNA replication elongation, though not initiation (Figure 1b), leading to an altered protein production profile, prevention of virion formation, and cell death [9].

Whilst the involvement of retrons in phage defence has previously been investigated [36], only recently have they been described as tripartite TA systems responding to phage infection [8,33,34]. Modes of action have not been fully investigated, but the Ec48 retron system appears to inhibit bacterial DNA repair, thereby blocking cell growth and phage propagation, though this remains to be demonstrated conclusively [33]. Similarly, the RcaT toxin from Retron-Sen2 is hypothesised to hydrolyse nucleosides and nucleotides, and thereby interfere with cellular DNAs and RNAs, though this activity is yet to be confirmed (Figure 1b) [8].

Building on work showing RelA-SpoT Homologues (RSHs) can mediate phage defence, such as mycobacterial prophage Phrann *gp29* [30], induction of the recently identified *Cellulomonas marina* FaRel RSH TA system rapidly depletes cellular GTP and ATP, with an accompanying increase in ppApp and ppGpp alarmones [37]. This leads to decondensation of the nucleoid, inhibition of transcription, and later translation and DNA replication [37].

Interference with translation

Sequence-specific endoribonuclease toxin PemK was initially implicated in plasmid maintenance in *S. aureus* [38]. More recently, PemK has also been implicated in phage defence in clinical strains of *Klebsiella pneumoniae* [39]. Post-infection, PemK expression increased, and cell growth was inhibited due to mRNA cleavage leading to metabolic arrest, which would reduce phage replication [39]. In contrast, the sequence-specific endoribonuclease ToxN appears to directly inhibit the expression of phage genes and ultimately prevents phage propagation (Figure 1b) [10]. As per PemK, the previous hypothesis for ToxN had been that toxin release causes cell arrest leading to death and therefore protects from phage by Abi; however, these new data show that ToxN prevents virion formation rather than impacting cell fate *per se* [10]. Therefore, as per ToxIN, the hypothesis should now be developed, in that phage infection itself might directly block host transcription, leading to a loss of cellular homoeostasis and ultimately growth arrest or cell death [10]. Furthermore, whilst the newly identified CrlTA system in *P. aeruginosa* shows dose-dependent bacterial mRNA cleavage, leading to growth arrest as seen in other endoribonuclease TA systems, again it is not suggested that this is responsible for an Abi phenotype [40]. Instead, as antitoxin CrlA is a Cro-like DNA-binding protein, it was hypothesised that the antiphage activity is a result of direct binding to incoming phage DNA resulting in reduced replication.

Recent studies on AbiE homologues have further probed the bacteriostatic nucleotidyltransferase activity of AbiEii [17]. AbiEii homologues found in *Serratia* sp. and *Mycobacterium tuberculosis* were demonstrated to perturb tRNA charging [41,42]. Consequently, the common model would suggest that reduced translation results in cell growth arrest and death, with concomitant reduction in phage replication. This should perhaps be reconsidered based on the new ToxIN hypothesis, wherein the phage infection leads to host cell death, but phage replication is prevented through the action of AbiEii [10].

Global responses

The prophage-encoded tripartite kinase-kinase-phosphatase (KKP) TA system of *P. aeruginosa* encodes two toxin kinases, PfpA and PfpB [32]. Targets of the kinases include cell division, transcription, and translation-associated proteins, with phosphorylation thought to disrupt cellular processes and result in phage defence through Abi [32]. Whilst the mechanistic link for KKP is unclear, Hok/Sok TA systems are known to disrupt the cell membrane and cause global loss of proton motive force, thereby directly preventing phage replication [13,43,44].

Escape from toxin–antitoxin mediated phage defence

As bacteria have evolved new TA system modalities, phages have evolved to counteract or circumvent these defences. This relationship is the driving force behind the incredible complexity and multiplicity of phage defence systems, and concurrently TA systems, across the microbiome. Though many studies omit TA system escape, scientists have utilised phage escape to provide valuable insight (Table 1).

Mutations in early phage genes prevent toxin–antitoxin system activation

An extensive study used 19 different phage defence systems to isolate escape phages and identify escape mutations, to help determine which phage components are activating host defences [45]. Of the systems assayed, only ShosTA [46] and the Eco8, Se72 and Ec67 retron systems [33] are confirmed as having TA functionality. Phages that escape these systems predominantly contained mutations in early phage genes; for ShosTA, ssDNA binding (SSB) proteins, an inhibitor of host RNA polymerase and a DNA primase/helicase; for Eco8, SSB family proteins; for Se72, a RecBCD inhibitor protein, a ssDNA annealing protein and an exonuclease; for Ec67, the DNA transfer protein, A1 [45]. Further study is needed to ascertain whether these phage escape mutations directly or indirectly affect activation of TA systems.

Prophages can bootstrap host toxin–antitoxin systems

A high-throughput reverse genomics approach successfully identified proteins that act as either blockers or activators of the *Salmonella enterica* retron Sen2 [8]. Perhaps unsurprisingly, TA blockers and activators were enriched within prophages. Of the five prophage genes identified as blockers (*racC*, *dicC*, *ydaW*, *yfjH*, *yjhC*), only *racC* was further investigated [8]. Interestingly, *racC* is conserved adjacent to an activator gene, *recE*. Together, these two genes form a linked blocker/activator pair in which *racC* directly inhibits the RcaT toxin and *recE* actively degrades msDNA (Figure 1a and c) [8]. Thus, lysogenic phages can evolve ways to bootstrap host TA systems and escape by applying their own mechanisms of control, adding an additional layer of complexity to TA system function.

Phages can evolve different escape mechanisms for a single toxin–antitoxin system

Further documentation of phage escape is sporadic in recent studies (Table 1). Nevertheless, identification of differing escape mechanisms for a single TA system may provide additional mechanistic insight. RB69 phages were shown to escape DarTG by mutations to a native DarT1 inhibitor, 61.2 [1]. The mutation was sufficient to abolish DNA ribosylation and restore plaquing efficiency of WT RB69 when coexpressed in host cells. A second population of SECφ18 escape phages found several individual mutations in the SECφ18 DNA polymerase which allowed the phage to replicate despite DNA ribosylation (Figure 1c) [9].

ToxIN phage escape via RNA antitoxin mimics has previously been documented [47,48]. Phage ϕM1 was also shown to produce a single mutation in a non-essential gene, *m1-23*, which provided protection against two type III TA systems — ToxIN and TenpIN — through preventing early TA system activation [49]. More recently, phage T7 has been shown to avoid full activation of ToxIN through only partially shutting off host transcription, maintaining plaquing efficiency but displaying a much smaller burst size (Figure 1c) [10]. Such measures illustrate how escape mutations can often decrease short-term phage fitness whilst allowing infection of otherwise resistant bacteria. The interaction of ToxIN and T7 suggests that the phage-bacteria arms race should perhaps be viewed as a gradient of phage infectivity rather than the black-and-white view of bacteria either being resistant or susceptible.

Concluding remarks

Multiple TA systems have recently been shown to provide phage defence (Table 1). Whilst encouraging, most studies are largely exploratory, describing the initial discovery of the TA system. We suggest it is important to use known systems for deeper exploration. To our knowledge, only a few TA phage defence systems have been described more deeply, including DarTG, ToxIN and AbiE, but more work is needed to fully characterise activating triggers, molecular details of toxicity and phage defence, and how phages can escape.

Experimental design should be carefully considered. Recent work examining the impact of stress-induced transcription on toxin activation demonstrated that levels of transcription do not necessarily correlate with degree of toxicity and should not be used as a proxy measurement [50]. This finding can now also be applied to examine the impact of stress-induced TA locus transcription on TA-mediated phage defence. A further

experimental factor to consider is how infection at low multiplicity of infection (MOI) may result in growth arrest, whilst high MOI may result in cell death [9]. This detail is important to consider whilst determining if a system acts through Abi, or temporary inhibition of growth followed by death as a result of phage infection.

As described elsewhere [51], future assessments of toxicity and target identification might be more effective when performed closer to *in vivo* conditions, for example, by working under the native promoter [10,20,51] via a proteomics approach [32] or via deletion of system components in the native host [14]. Of course, it is noted that this may not always be possible especially in the case of plasmid-borne systems [39]. Assessment of the critical matter of cell growth arrest or cell death could be probed more rigorously, for example, via measurements of NADPH activity [39], cell proliferation assays [39], or live/dead staining [40].

Finally, greater precision and consistency in language use may help to clarify conclusions with regards to outcomes of phage infection under physiological conditions. Previous commentaries have noted that TA systems likely allow for a tactical reduction in growth rate that prevents phage replication, but death is a result of the phage infection itself, rather than toxin activity [20,51]. Much of the current literature refers to cell death upon TA system induction as occurring by Abi. On the one hand, use of 'Abi' could strictly refer to processes wherein a host system has been shown to directly cause cell death in response to phage infection, thereby preventing phage replication. Using that definition, many TA systems can be said to prevent phage replication but should not be labelled as operating as Abi systems. On the other hand, using an evolving definition of Abi, we could consider TA systems as causing Abi if the defence mechanism prevents phage replication with concomitant cell death due to irreversible damage caused by the initial phage infection or prolonged growth arrest. As noted elsewhere [51], it is difficult to differentiate between these two outcomes; either cell death caused by a cell-produced effector (i.e. a toxin) responding to phage infection, or cell death as a direct consequence of the original phage infection. Using an evolved definition of Abi could solve this complexity. We therefore suggest that reductions in cell growth are not conflated with Abi, where the aetiology of cell death is unknown, until the field can reach a consensus on the definition of Abi itself. These efforts will be bolstered by deeper molecular understanding of how cell death occurs post-infection. Nevertheless, recent studies into TA systems and their roles in phage defence have highlighted dazzling layers of complexity. Clearly, extensive interactions remain to be discovered between diverse phages, hosts, plasmids, and defence systems across the microbiome.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

No data were used for the research described in the article.

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