PNAS COMMENTARY

Antitoxins within toxins: A new theme in bacterial antivirus defense

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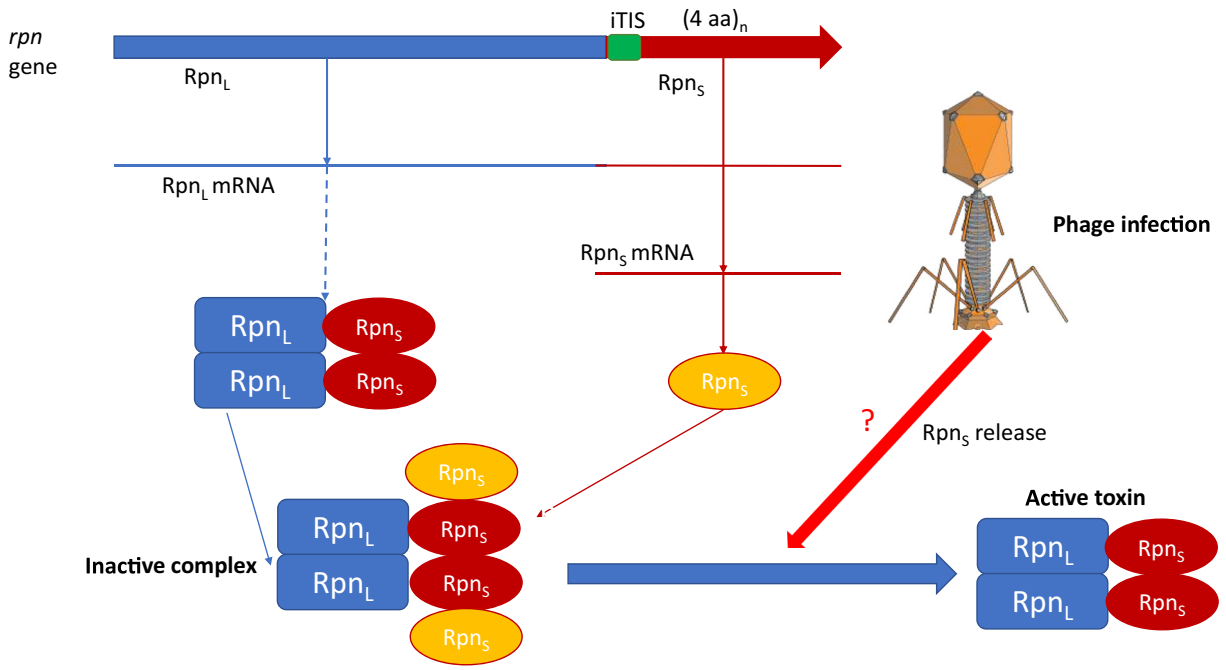

Fig. 1. A gene within gene yields the antitoxin in a bacterial TA system. iTIS, internal translation initiation site; (4 aa)n denotes the hypervariable four amino acid repeats in Rpn_S. The C-terminal domain of RpnL corresponding to RpnS is shown in dark red, and the free RpnS translated from the iTIS is shown in yellow. The active toxin appears to be an Rpn_L dimer, and the inhibition of the nuclease activity involves Rpn_S binding to each of the monomers. The mechanism of Rpn_S release triggered by phage infection (?) remains to be elucidated.

In the last few years, the study of antivirus defense went through a veritable microrevolution. The diversity of the discovered defense systems that often can be predicted through their colocalization within defense islands in microbial genomes is nothing short of astonishing (1–3). A key unifying theme emerging from these studies is abortive infection (Abi) whereby a broad variety of defense systems cause programmed cell death (PCD) or cell dormancy in response to infection, preventing virus spread across the microbial population. Most of the Abi systems are toxin–antitoxin (TA) modules. In the absence of infection, the toxin is inactive, often, as a result of complex formation with an antitoxin, but infection activates the toxin through a variety of mechanisms, triggering PCD (4). In PNAS, Zhong and colleagues report a novel class of Abi systems that employ a remarkably economical and elegant strategy of antitoxin formation (5).

This new class of Abi systems had been hiding in plain view in numerous bacterial genomes scattered across many phyla and some archaeal genomes, known for years as Rpn (Recombination-Promoting Nucleases). Rpn proteins contain the PD-(D/E)XK (named after the conserved pattern of catalytic amino acids) nuclease domain, which is an extremely abundant type of nuclease in prokaryotes, being present in restriction endonucleases, among others (6). The *rpn* genes have been considered as a type of transposable element (TE) because their content is highly variable even among closely related bacterial strains, some of which contain numerous *rpn* copies and because of the demonstrated DNA endonuclease activity of the Rpn protein (7). However, the argument in support of *rpn* genes being TE is weak as shown by Zhong et al. (5). Indeed, these genes are not flanked by repeats and/or target site duplications like most TE, and PD-(D/E)XK enzymes are not known to function as autonomous transposases. Furthermore, multiple copies of *rpn* genes appear to be products of tandem duplication rather than transposition. Zhong et al. thus sought to determine the actual function of Rpn and made several

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notable discoveries. First, it was demonstrated that, in addition to the full-length Rpn protein (denoted Rpn_L) containing the nuclease domain, the *rpn* genes direct the synthesis of small proteins (Rpn_S) that correspond to the C-terminal portions of RpnL proteins and are translated separately using an internal translation initiation site (iTIS) (Fig. 1). Previously, it has been shown that Rpn is toxic when overexpressed in *Escherichia coli* (7), but in those experiments, Rpn_L and Rpn_S were coexpressed, potentially, confounding the results. In the new study, Zhong et al. decouple RpnL from RpnS and show substantial toxicity of Rpn_L that can be abrogated by Rpn_S (5). Furthermore, Rpn_S was shown to form a complex with Rpn_L and inhibit the endonuclease activity of the latter (Fig. 1).

> **"Zhong et al. report a novel class of Abi systems that employ a remarkably economical and elegant strategy of antitoxin formation."**

The inhibitory effect of Rpn_S is specific to the particular Rpn_L from which the Rpn_S comes, and Zhong et al revealed a remarkable structural basis of such specificity. It turned out that the C-terminal region of Rpn that is shared by Rpn_L and Rpn_S contains a hypervariable region composed of four-amino acid repeats such that a mismatch in the number of repeats prevents the formation of the Rpn_L–Rpn_S complex. The crystal structure of Rpn_S was solved, and the hypervariable tetrad repeat region was shown to belong to a long α-helix that forms a dimerization interface.

The hypervariability of Rpn and the patchy distribution of the *rpn* genes amongst bacteria prompted the hypothesis that these genes comprise a distinct antiphage defense system. Indeed, Zhong et al. showed that expression of Rpn_{LS} inhibited the reproduction of several T-even phages in *E. coli* (5).

This study reveals a novel strategy for antitoxin formation in TA systems whereby the antitoxin is a portion of the toxin produced from a gene-within-gene. In principle, this is a variation on the general theme of dominant-negative inhibition of biological activities that is, in particular, a widespread antidefense strategy employed by viruses, in particular, large DNA viruses of eukaryotes. For example, many animal viruses encode derivatives of DEATH (named for their role in PCD) adaptor domains that form unproductive complexes with effector components of PCD networks and thus prevent apoptosis (8). However, this principle so far has not been known to apply to the regulation of toxin activity in TA modules, and the "ingenious" utilization of a gene within gene adds another unique feature. It is notable, in this context, that some *rpn* loci contain a second copy of Rpn_S indicating that the toxin and the antitoxin can be decoupled, perhaps, enhancing the flexibility of regulation (5).

As befits a truly novel discovery, additional questions abound. In particular, what is the mechanism of the release of the Rpn_L toxin upon infection? Zhong et al. provide a hint by showing that the Rpn_L–Rpn_S complex dissociates at alkaline pH, but obviously, definitive experiments remain to be performed. Further, what is the evolutionary driver of the fine-tuning of the Rpn_L inhibition by Rpn_S through the variable number of the amino acid tetrad repeats? It is natural to interpret this phenomenon within the context of the arms race between viruses and defense systems. In the many bacteria that harbor multiple *rpn* copies, repeat variation will prevent Rpn_L inhibition *in trans*, suggesting the intriguing possibility that different *rpn* copies specifically target distinct phages. And, perhaps, the most important and intriguing question: Is this discovery a tip of a proverbial iceberg, that is, are there many other TA and perhaps other systems that employ iTIS to produce dominant negative regulators? It would appear quite incongruous if such a simple and elegant regulation mechanism was unique to Rpn. Zhong et al. point to ribosome profiling data suggesting that expression of Rpn_S from iTIS indeed could be a widespread phenomenon in bacteria including at least one more TA module in *E. coli*, *yhaV-prlF*. Clearly, however, extensive additional work will be required to assess the generality of the gene within gene principle. While the new studies are eagerly anticipated, it is already clear that the Rpn system is a notable addition to the burgeoning armory of microbial mechanisms.

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1. S. Doron *et al.*, Systematic discovery of antiphage defense systems in the microbial pangenome. *Science* **359**, eaar4120 (2018).
2. L. Gao *et al.*, Diverse enzymatic activities mediate antiviral immunity in prokaryotes. *Science* **369**, 1077–1084 (2020).
3. A. Millman *et al.*, An expanded arsenal of immune systems that protect bacteria from phages. *Cell Host Microbe.* **30**, 1556–1569.e5 (2022).
4. D. Jurenas, N. Fraikin, F. Goormaghtigh, L. Van Melderen, Biology and evolution of bacterial toxin-antitoxin systems. *Nat. Rev. Microbiol.* **20**, 335–350 (2022).
5. A. Zhong *et al.*, Toxic antiphage defense proteins inhibited by intragenic antitoxin proteins. *Proc. Natl. Acad. Sci. U.S.A.* [Preprint] (2023). https://doi.org/10.1101/2023.05.02.539157 (Accessed 12 July 2023).
6. K. Steczkiewicz, A. Muszewska, L. Knizewski, L. Rychlewski, K. Ginalski, Sequence, structure and functional diversity of PD-(D/E)XK phosphodiesterase superfamily. *Nucleic Acids Res.* **40**, 7016–7045 (2012).
7. A. W. Kingston, C. Ponkratz, E. A. Raleigh, Rpn (YhgA-Like) proteins of *Escherichia coli* K-12 and their contribution to RecA-independent horizontal transfer. *J. Bacteriol.* **199**, e00787-16 (2017).
8. E. V. Koonin, V. V. Dolja, M. Krupovic, The logic of virus evolution. *Cell Host Microbe.* **30**, 917–929 (2022).