A hyperpromiscuous antitoxin protein domain for the neutralization of diverse toxin domains

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Toxin–antitoxin (TA) gene pairs are ubiquitous in microbial chromosomal genomes and plasmas as well as temperate bacteriophages. They act as regulatory switches, with the toxin limiting the growth of bacteria and archaea by compromising diverse essential cellular targets and the antitoxin counteracting the toxic effect. To uncover previously uncharted TA diversity across microbes and bacteriophages, we analyzed the conservation of genomic neighborhoods using our computational tool FlaGs (for flanking genes), which allows high-throughput detection of TA-like operons. Focusing on the widespread but poorly experimentally characterized antitoxin domain DUF4065, our in silico analyses indicated that DUF4065-containing proteins serve as broadly distributed antitoxin components in putative TA-like operons with dozens of different toxin domains with multiple different folds. Given the versatility of DUF4065, we have named the domain Panacea (and proteins containing the domain, PanA) after the Greek goddess of universal remedy. We have experimentally validated nine PanA-neutralized TA pairs. While the majority of validated PanA-neutralized toxins act as translation inhibitors or membrane disruptors, a putative nucleotide cyclotase toxin from a Burkholderia prophage compromises transcription and translation as well as inducing RelA-dependent accumulation of the nucleotide alarmone (pppGpp). We find that Panacea-containing antitoxins form a complex with their diverse cognate toxins, characteristic of the direct neutralization mechanisms employed by Type II TA systems. Finally, through directed evolution, we have selected PanA variants that can neutralize noncognate TA toxins, thus experimentally demonstrating the evolutionary plasticity of this hyperpromiscuous antitoxin domain.

Significance

Toxin–antitoxin systems are enigmatic and diverse elements of bacterial and bacteriophage genomes. We have uncovered remarkable versatility in an antitoxin protein domain that has evolved to neutralize dozens of different toxin domains. We find that antitoxins carrying this domain—Panacea—form complexes with their cognate toxins, indicating a direct neutralization mechanism, and that Panacea can be evolved to neutralize a noncognate and nonhomologous toxin with just two amino acid substitutions. This raises the possibility that this domain could be an adaptable universal or semi-universal protein neutralizer with significant biotechnological and medical potential.

TN toxin–antitoxin systems (TAs) are diverse two-gene elements that are widespread in plasmas and chromosomes of bacteria and archaea (1, 2) as well as in genomes of temperate bacteriophages that prey on these microbes (3–6). The various protein toxins target different core processes of the encoding cell to dramatically inhibit growth while their cognate antitoxins efficiently neutralize the toxicity. Known TA toxins exert their toxicity in a variety of ways (1), often targeting translation through modification or cleavage of the ribosome, translation factors, transfer RNAs (tRNAs), or messenger RNAs (mRNAs). Similarly, antitoxins counteract the toxins through diverse mechanisms (1, 7). The means of neutralization are often classified into four or more subtypes, the main four being base pairing of the antitoxin RNA with the toxin mRNA (Type I TA systems), direct protein–protein binding and inhibition (Type II), inhibition of the protein toxin by the antitoxin RNA (Type III), or indirect nullification of toxicity (Type IV).
transcription followed by the inhibition of translation and replication (4, 13). The synthesis of (pp)pApp is not the only mechanism of toxicity employed by toxSAS enzymes; we have found that the majority of experimentally explored toxSASs, such as PhRel2 from Bacillus subtilis strain 1a1a, act as specific protein synthesis inhibitors that pyrophosphorylate the 3′ CCA end of tRNA to abrogate aminoacylation (12). In the case of Mycobacterial phase Phthrom protein Gp29, a translation-inhibiting toxSAS in the PhRel subfamily that likely pyrophosphorylates tRNA (12), the biological function appears to be defense against phage superinfection (5).

ToxSASs are neutralized by several different antitoxins that act via Type II and Type IV mechanisms. The cognate antitoxin of B. subtilis 1a1a PhRel2 (a tRNA-modifying toxSAS) belongs to a widespread domain family of unknown function designated by the Pfam database as DUF4065, in which DUF stands for domain of unknown function (14). Clues about the roles of DUF4065 are limited; however, it is found in so-called genetic elements of unknown function (14), associated with TA loci (15, 16), and is also present in the proteolysis-promoting SocA antitoxin of the replication-inhibiting SocB toxin (17). This unusual mechanism of neutralization by an antitoxin is referred to as Type VI. We have earlier identified the DUF4065 domain in a putative alternative antitoxin to the ribonuclease (RNase) MqsR, but this was not tested experimentally (15).

We asked whether, given the broad distribution of DUF4065 across multiple phyla of bacteria and archaea, the analysis of the genomic neighborhood of DUF4065 can enable the prediction of novel TA systems. Using our tool FlaGs (for flanking genes) (18) to analyze diverse genomes across the tree of life, we find that DUF4065 is the predicted antitoxin counterpart of at least 1,268 different putative TA system families corresponding to at least 88 distinct putative toxin–DUF4065 domain combinations, found in diverse bacteria, archaea, and bacteriophages. While many of the toxins of these systems are related to classical TA toxins such as various mRNA interferases (19, 20), Fic/Doc-type protein modification enzymes (21), and toxSASs (4), others have little similarity to known domains or proteins with solved structures. We have experimentally verified nine DUF4065-containing antitoxins as neutralizers of their cognate toxin partners. These toxins include translation inhibitors, membrane disruptors, and a putative nucleotide cyclase that pleiotropically affects metabolism, compromising transcription and translation, as well as inducing RelA-dependent accumulation of the guanosine tetraphosphate alarmone nucleotide (pp)pGpp. Complex formation indicates DUF4065-containing antitoxins neutralize toxins via direct protein–protein interaction [that is, act as Type II TA systems (1, 2)], and we have identified substitutions that confer the ability of one antitoxin to neutralize a noncognate toxin. Given the versatility of the antitoxin function of DUF4065, we have named the domain Panacea after the Greek goddess of universal remedy.

Results

The Domain DUF4065 Is Found in Diverse TA-Like Loci across Bacteria, Archaea, and Bacteriophages. As DUF4065 has previously been associated with TA systems (15–17), we asked whether it may constitute a widespread antitoxin domain paired in operons with novel toxin domains. To answer this, we used sensitive sequence searching combined with an analysis of gene neighborhoods using our tool FlaGs (18) (see SI Appendix, Fig. S1 for a graphical overview of the procedure). Using the hidden Markov model (HMM) of the DUF4065 domain (14) to scan 20,209 genomes across cellular life and viruses, we identified 2,281 hits (Dataset S1) in prokaryotes and bacteriophages comprising 27 phyyla of bacteria, 3 phyyla of archaea, and 17 different bacteriophages (Dataset S1). Of those 2,281, 76 are present in complete prokaryotic genomes, allowing the determination of whether they are chromosome or plasmid encoded according to the genome annotations. All but two of our identified DUF4065 homologs in complete genomes are chromosome localized. The two exceptions annotated as plasmid encoded (but may be minichromosomes) are archaeal, found in Haloarchaea (protein accessions WP_049834827.1 and WP_049834827.1). Most DUF4065 carrying taxa only carry a single homolog; 217 taxa have two, 45 have three, 14 have four, 12 have five, and 5 have more than five. Of these five taxa, the taxon with the most DUF4065 homologs is the Mollicute bacterium “strawberry lethal yellows phytoplasma” strain NZSb11. This genome contains 25 DUF4065 homologs, of which three are predicted as being encoded in TA-like loci by our in silico analysis pipeline.

Adapting FlaGs for analyzing gene neighborhood conservation (SI Appendix, Fig. S1), we find that around half of the identified DUF4065-containing proteins can be detected as being encoded in two- or more genomes across multiple species, reminiscent of TA systems (Dataset S1, representatives in Fig. 1, Dataset S2, and SI Appendix, Fig. S2). In total, we predicted 1,313 preliminarily TA (pTA)-like loci using the criteria 1) that there should be a maximum distance of 100 nucleotides between the two genes, 2) that this architecture is conserved in two or more species, and 3) the conservation of the gene neighborhood does not suggest longer operons than the rule of three (SI Appendix, Fig. S1). We allowed three-gene architectures into our analysis, as TA-encoding genes sometimes can be found with a conserved third gene, such as mazG in the case of mazEF (22), chaperones in the case of tripartite TA–chaperone modules (23), or transcriptional regulators in the case of the paaR-paaA-parE system (3). By allowing three-part clusters, we have identified 25 clusters that are conserved as a third gene in a subset of genomes that encode a particular predicted TA pair (Dataset S1). We call these accessory proteins, annotations of which include DNA/nucleotide and protein/peptide acidification enzymes, helicases, proteases, and nuclease. Each detected accessory third gene was only present in a small fraction of the genomes in which the main TA pair was identified, suggesting that whatever the role of these third genes, they probably do not play a general role in toxicity and neutralization.

We receivable thDUF4065 homologous genes are found adjacent to DUF4065-encoding genes in multiple genomes purely by chance and are not part of genuine TA systems. Therefore, we used a BlastP-based (24) reciprocal consistency test to filter out putative “toxins” that are at risk as being spurious hits (SI Appendix, Fig. S1C). From the 1,313 pTA-like loci, we determined that 67 proteins (of which 39 are predicted toxins and 28 are accessory proteins) are likely spurious hits (Dataset S1). Major classes of these spurious hits are transposas/Integrases that are commonly found in TA-encoding neighborhoods and various ATPases that are captured into homologous clusters because of their well-conserved ATP-binding motifs (Dataset S1).

The remaining 1,268 putative TA loci that we predict to be relatively reliable correspond to 88 clusters of potential toxins. We number these clusters with a T prefix; for example, SocB is in cluster T10. The vast majority of these are annotated as “hypothetical protein,” as they share only weak similarity to proteins of known function. Therefore, we searched the putative toxin protein sequences against the National Center for Biotechnology Information Conserved Domains Database (NCBI CDD) to detect the presence of known domains (Dataset S1). Of the 1,268 putative toxins, 938 sequences (belonging to 41 clusters) had no hit to a domain, and of the others, the most predominant domains were MqsR like (n = 90), Fic/Doc like (n = 32), and toxSAS like (domain names NT_Pol-beta-like, RelA_SpoT, and NT_Rel-Spo-like; n = 31). Other known toxin domains that were represented in the CDD results were PmK (mRNAase) and ParE (DNA gyrase inhibitor). For clusters that
failed to find a hit in the CDD database, HHPred (25) was run with one to two representative sequences per cluster, revealing additional potential homology to proteins of known structures for 30 clusters (Dataset S1; see the following sections of Results for examples among our verified TAs).

The variety in the potential toxin domains suggests that the DUF4065 domain may be a universal or semi-universal antitoxin domain capable of neutralizing various different toxic proteins. In light of this, we suggest renaming DUF4065 to Panacea and abbreviate each Panacea-containing putative antitoxin and putative toxin protein as PanA and PanT, respectively. We refer to this kind of domain-level partner swapping as hyperpromiscuity, to distinguish from the promiscuity that can be seen when one single antitoxin sequence can nullify multiple cognate but homologous toxins (26–28).

Maximum likelihood phylogenetic analysis shows the PanA tree largely does not follow taxonomic relationships, reflecting a high degree of mobility (Fig. 1, SI Appendix, Fig. S2, and Dataset S2). While the deepest branches are poorly supported (not surprising for a small protein), there are a number of groups with medium to strong (over 60 to 100%) bootstrap support that include different bacterial—and sometimes archaeal—phyla. While Panacea is present broadly across prokaryotes, it does not appear to be present in eukaryotes. The only PanA we discovered in eukaryotes was in the Pharaoh ant (Monomorium pharaonic; XP_028045404.1), and this appears to be a case of contamination, as an identical sequence is found in the bacterium Stenotrophomonas maltophilia. Surprisingly, a strongly supported clade of PanA sequences does not necessarily mean they all share the same PanT as shown by the inner ring in Fig. 1 and the toxin partner swapping in focus in Fig. 2A and SI Appendix, Fig. S2. Indeed, the exchange of toxin partners within a clade appears to be frequent. We refer to this kind of domain-level partner swapping as hyperpromiscuous, to distinguish from the promiscuity that can be seen when one single antitoxin sequence can nullify multiple cognate but homologous toxins (26–28).

Some—but not all—PanAs carry additional N-terminal domain regions (Fig. 1). Often, these match a known helix-turn-helix (HTH) domain, of which a number of variations exist in eukaryotes was in the Pharaoh ant (Monomorium pharaonic; XP_028045404.1), and this appears to be a case of contamination, as an identical sequence is found in the bacterium Stenotrophomonas maltophilia. Surprisingly, a strongly supported clade of PanA sequences does not necessarily mean they all share the same PanT as shown by the inner ring in Fig. 1 and the toxin partner swapping in focus in Fig. 2A and SI Appendix, Fig. S2. Indeed, the exchange of toxin partners within a clade appears to be frequent. We refer to this kind of domain-level partner swapping as hyperpromiscuous, to distinguish from the promiscuity that can be seen when one single antitoxin sequence can nullify multiple cognate but homologous toxins (26–28).

Some—but not all—PanAs carry additional N-terminal domain regions (Fig. 1). Often, these match a known helix-turn-helix (HTH) domain, of which a number of variations exist in eukaryotes. We aligned all the identified regions with hits to HTH models to make our own updated HTH model. From this, we identified HTH domains in the N-terminal regions of 343 PanA sequences (Dataset S1). HTH domains are often predicted associated toxin groups, respectively, according to the left-hand keys. Colored circles between the rings indicate putative TA pairs that have been tested in toxicity neutralization assays and the results of those assays. “TA” means the expression of the toxin compromises E. coli growth, and coexpression of the antitoxin either fully or partially counteracts the toxicity. “Toxic” means toxicity is confirmed, but the cognate PanA sequence does not rescue in this E. coli system. “Stuck in cloning” refers to cases in which the putative toxin genes could not be successfully chemically synthesized and plasmid subcloned, potentially because of the toxicity being too severe. Gray circles on the branches indicate branch support from IQTree ultrafast bootstrapping (53). Tree annotation was carried out with iTOL (54).

![Diagram](https://doi.org/10.1073/pnas.2102212119)
Antitoxins can in some cases also regulate the TA function at the level of transcription. Apart from HTH domains, the only widely conserved N-terminal extension appears to correspond to a new domain, which we refer to as PanA-associated domain 1 (PAD1) (SI Appendix, Fig. S3). All but two of the TA-predicted PAD1 containing PanAs are paired with toxSAS-like toxins (the exception being putative ATPases from Clostridia [PanT group T62; Dataset S1]). The position at the N terminus and the presence of conserved histidines may indicate that PAD1 is a new DNA-binding domain, although it has no detectable homology with any known domain. PAD1 is also present in nine Panacea-containing proteins that do not meet the criteria for TA-like loci (Dataset S1). In all cases in which PanA contains the PAD1 domain and is in a TA-like locus, the toxin is encoded upstream of the antitoxin, the less common arrangement in the data set as a whole and in TA systems in general (1, 2).

PanA is a Hyperpromiscuous Antitoxin Domain. Sampling broadly across PanA diversity, we selected 25 of the putative novel TAs for experimental validation in toxicity neutralization assays (Figs. 1 and 2A, Table 1, SI Appendix, Table S1, and Dataset S2). Putative toxins and antitoxins were expressed in E. coli strain BW25113 under the control of arabinose- and isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible promoters, respectively. For a gene pair to classify as a bona fide TA, two criteria need to be fulfilled: 1) the expression of the toxin should compromise E. coli growth, and 2) coexpression with the antitoxin should—either fully or partially—restore growth inhibition.

**Fig. 2.** PanA antitoxins form stable complexes with evolutionarily diverse TA toxins. (A) The maximum likelihood tree of PanA sequences, annotated with conserved gene neighborhoods generated with FlaGs (18). Numbers on branches show IQtree ultrafast bootstrap support (53). Genes belonging to homologous clusters are colored the same; the PanA antitoxin is universally shown in black. Numbers on genes preceded by a T indicate toxin clusters. (B) Validation of panAT TA pairs by toxicity neutralization assays. Overnight cultures of E. coli strains transformed with pBAD33 and pKK223-3 vectors or derivatives expressing putative panT toxins and panA antitoxins, correspondingly, were adjusted to OD600 1.0, serially diluted, and spotted on LB medium supplemented with appropriate antibiotics and inducers (0.2% arabinose for panT induction and 1 mM IPTG for panA induction). (C–G) A pull-down assay demonstrates complex formation between PanA antitoxins and PanT toxins. Untagged PanA representatives were coexpressed in the E. coli BL21 DE3 strain together with N-terminally affinity-tagged (His10-SUMO in the case of Burkholderia prophage phi52237 PanT; His6 in all other cases) cognate PanT toxin. Filtered lysate was incubated with buffer-equilibrated Ni-beads, and PanAT complexes were eluted with 300 mM imidazole and resolved on 15% SDS-PAGE. The theoretical molecular weights for tagged toxins and antitoxins are indicated in red and green, respectively.
Table 1. Summary of experimentally characterized PanAT pairs

| Organism                          | Description | Toxicity Modality | Toxin Accession | Antitoxin Accession |
|-----------------------------------|-------------|-------------------|-----------------|---------------------|
| *Escherichia coli* STEC O31       | PanA<sub>Esc</sub>:PanT<sub>Esc</sub> | Membrane         | WP_00019185.1   | WP_00028725.1       |
| *Helicobacter* sp. 13500482-2     | PanA<sub>Hel</sub>:PanT<sub>Hel</sub> | Membrane         | WP_09529540.3   | WP_09529540.1       |
| *Burkholderia* sp.                | PanA<sub>Bu</sub>:PanT<sub>Bu</sub> | Membrane         | WP_07793242.1   | WP_07793240.1       |
| *Burkholderia* prophage phi52237 | PanA<sub>Bu</sub>:PanT<sub>Bu</sub> | Nucleotide       | YP_293707.1     | YP_863932.1         |
| *Bifidobacterium* ruminantium     | PanA<sub>Bi</sub>:PanT<sub>Bi</sub> | RNase             | WP_02664888.1   | WP_08181566.1       |
| *Pseudomonas* moraviensis         | PanA<sub>Pa</sub>:PanT<sub>Pa</sub> | RNase             | WP_08321920.1   | WP_08335436.5       |
| *Vibrio* harveyi                  | PanA<sub>Vi</sub>:PanT<sub>Vi</sub> | toxSAS             | WP_06105447.1   | WP_06105448.1       |
| *Bacillus subtilis* ia1a          | PanA<sub>Bi</sub>:PanT<sub>Bi</sub> | toxSAS             | WP_09055840.1   | WP_09055840.1       |
| *Lactobacillus* animalis          | PanA<sub>La</sub>:PanT<sub>La</sub> | toxSAS             | WP_05200634.1   | WP_03544770.1       |
| *Corynebacterium* doosanense      | PanA<sub>Co</sub>:PanT<sub>Co</sub> | Fic/Doc           | WP_01802280.1   | WP_01802280.3       |

†The NBCI sequence of PanA from *P. moraviensis* appeared to be truncated at the N terminus relative to its homologues, and therefore we took an upstream start codon, equivalent to adding ten amino acids, MIFEQKVAQ, to the N terminus.

PanAT Pairs Are Type II TA Systems. The Panacea domain–containing SoCA antitoxin of *Caulobacter crescentus* acts as a proteolytic adapter, bringing the toxin SocB into contact with the protease ClpXP (17). To test whether other PanAs act as such adapters, we repeated our neutralization assays in *E. coli* strains lacking ClpXP and Lon proteases. These proteases are not necessary for neutralization by PanA (*SI Appendix*, Fig. S7). We therefore hypothesized that the general neutralization mechanism of PanA is through direct binding and inhibition typical of classical Type II systems. To test this, we carried out pull-down assays using coexpressed cognate native PanA antitoxins together with N-terminally affinity-tagged PanT toxins (with either His<sub>6</sub> or His<sub>6</sub>-SUMO tags). We validated stable complex formation for five PanAT pairs: *L. animalis* PanA<sub>La</sub>:PanT<sub>La</sub> (Fig. 2C), *P. moraviensis* PanT<sub>Pa</sub>:PanA<sub>Pa</sub> (Fig. 2D), *Burkholderia* prophage phi52237 PanT<sub>Bu</sub>:PanA<sub>Bu</sub> (Fig. 2E), *C. doosanense* Fic/Doc<sub>Co</sub>:PanA<sub>Co</sub> (Fig. 2F), and *V. harveyi* CapRel<sub>Vi</sub>:PanA<sub>Vi</sub> (Fig. 2G).

Protein Synthesis Is a Major Target of PanT Toxins. To address the molecular mechanisms of PanT toxicity, we assayed the effects of PanT expression on macromolecular synthesis by following the incorporation of 14C urethane in proteins, 3H uridine in RNA, and 3H thymidine in DNA, comparing to the effects of *E. coli* MazF RNase as a positive control (*SI Appendix*, Fig. S8). As predicted, three of the identified PanT—specific inhibit protein synthesis (Fig. 3 A–E). The mechanism of action of all the protein synthesis–inhibiting toxins can be predicted by homology. ToxSAS *L. animalis* PanT<sub>La</sub> and *V. harveyi* CapRel<sub>Vi</sub> are closely related to other representatives we have characterized earlier (12) and almost certainly phophorylate the CCA end of tRNA. The *C. doosanense* Fic/Doc toxin Fic/Doc<sub>Co</sub>—presumably modifies EF-Tu as observed for other Doc enzymes (32). Predicted RNases PanT<sub>Bi</sub>:PanA<sub>Bi</sub> and PanT<sub>Pa</sub>:PanA<sub>Pa</sub> likely inhibit translation by cleaving mRNA or tRNA as their—albeit distant—relatives (33).
**Burkholderia Prophage phi52237 PanT Is a Pleiotropic Toxin that Induces the RelA-Mediated Stringent Response.** The *Burkholderia* prophage PanT<sub>Bur</sub> toxin is unique among our verified toxins in that it predominantly inhibits transcription, with weaker effects on translation and even weaker on replication (Fig. 4A). The mode of inhibition is reminiscent of that of *C. marina* FaRel toxSAS (4) and *P. aeruginosa* Type VI secretion system RSH effector Tas1 (12, 13) that act though production of the toxic nucleotide alarmone (pp)pApp, leading to dramatic depletion of ATP and GTP. Therefore, we used our high performance liquid chromatography (HPLC)-based approach to study the effects of PanT<sub>Bur</sub> toxin expression on *E. coli* nucleotide pools (34). In contrast to the drastic drop in GTP and ATP seen upon expression of *C. marina* FaRel toxSAS (4), expression of PanT<sub>Bur</sub> results

**Fig. 3.** Protein synthesis is a major target of PanT toxins. Metabolic-labeling assays following the incorporation of 35S methionine (black traces), 3H uridine (red), and 3H thymidine (blue) upon expression of translation-inhibiting PanT representatives: (A) *L. animalis* PhRel2<sub>Lac. ani.</sub> and (B) *V. harveyi* CapRel<sub>Vib. har.</sub> and (C) *B. ruminantium* RNAse PanT<sub>Bif. rum.</sub>; putative RNases (C) PanT<sub>Bif. rum.</sub> and (D) PanT<sub>Pse. mor.</sub>, and (E) *C. doosanense* Fic/Doc toxin, Fic/Doc<sub>Cor. doo.</sub> Expression of PanTs in *E. coli* BW25113 was induced with 0.2% L-arabinose.

**Fig. 4.** PanT<sub>Bur</sub> phage toxin from *Burkholderia* prophage phi52237 compromises transcription and translation as well as inducing the RelA-mediated stringent response. (A) Metabolic-labeling assay using wild-type *E. coli* BW25113 expressing PanT<sub>Bur</sub> phage toxin. (B and C) Guanosine nucleotide pools in either wild-type (B) or ΔrelA (C) *E. coli* BW25113 expressing PanT<sub>Bur</sub> phage toxin. Cell cultures were grown in defined minimal MOPS medium supplemented with 0.5% glycerol at 37°C with vigorous aeration. Expression of PanT<sub>Bur</sub> phage toxin was induced with 0.2% L-arabinose at the OD<sub>600</sub> 0.2. Intracellular nucleotides are expressed in pmol per OD<sub>600</sub> × mL as per inset. Error bars indicate the SE of the arithmetic mean of three biological replicates.
in only a slight decrease in GTP (Fig. 4B) without affecting the ATP levels (SI Appendix, Fig. S9A). Surprisingly, despite having no detectable sequence or structural homology with RSH protein family members, PanT

phage expression causes an accumulation of the alarmonic nucleotide ppGpp (Fig. 4B). This suggests that either 1) the toxin activates cellular RSH enzymes—given the strength of the effect, likely the most potent E. coli (ppGpp synthetase RelA—or 2) the PanT

phage toxin itself is capable of producing the alarmon. To distinguish between the two scenarios, we analyzed nucleotide levels upon toxin expression in an E. coli strain lacking relA. No accumulation of ppGpp is detected upon PanT

phage expression in the relA-deficient strain (Fig. 4C), and just as in the case of wild type, there is no effect on ATP levels (SI Appendix, Fig. S9B). Therefore, we conclude that expression of this toxin directly or indirectly induces ppGpp production by RelA. To deconvolute the direct effects of Burkholderia prokaryotic PanT

phage toxin on 35S methionine, 3H uridine, and 3H thymidine incorporation from the secondary effects caused by RelA-dependent ppGpp accumulation, we performed metabolic labeling in the ΔrelA E. coli strain (SI Appendix, Fig. S9C). Just as in the wild-type strain, the main target is transcription, closely followed by translation. Thus, the growth inhibition and metabolic-labeling effects observed upon PanT

phage expression are not related to ppGpp accumulation.

The Cell Membrane Is Another Major Target of PanT Toxins. Next, we performed 35S methionine, 3H uridine, and 3H thymidine metabolic labeling experiments with the predicted transmembrane domain harboring toxins PanT

E. coli (Fig. 5A), PanT

apS, and PanT

Hel. sp. (Fig. 5B). Unlike the toxins of the previous two sections of Results that predominantly target translation or transcription, expression of these toxins indiscriminately inhibited transcription, translation, and DNA replication, consistent with a more general shutdown of metabolic activities caused by membrane disruption. Indeed, a comparable response was observed with the induction of membrane-depolarizing E. coli HokB TA toxin (35) (SI Appendix, Fig. S8B) and treatment with the membrane-targeting inhibitor of oxidative phosphorylation carbonyl cyanide 3-chlorophenylhydrazone (SI Appendix, Fig. S8C).

To directly test this hypothesis, we analyzed the integrity of cell membranes upon toxin induction using a combination of the membrane potential sensitive dye DiSC3(5) (36) and intact membrane permeability indicator SYTOX Green (37). A strong membrane depolarization combined with an increased SYTOX Green permeability was observed for PanT

apS, and PanT

E. coli (Fig. 5D–F). Expression of PanT

apS, in contrast, triggered strong depolarization without an increase in SYTOX Green permeability. Thus, we conclude PanT

E. coli, PanT

Hel. sp., and PanT

apS exert their toxic activity through membrane depolarization which, in the case of PanT

E. coli, and PanT

apS, is caused by large pore formation. Finally, weak membrane depolarization was also observed for PanA

Hel. nam. and PanA

Pre. nov., although these are not predicted to contain transmembrane helices and are instead predicted to be RNases. Therefore, the effect of these toxins on cell membranes is more likely to be indirect through disturbances in respiration or central carbon metabolism. A potential membrane-spanning region is predicted for PanA

Hel. nam. phage, albeit with relatively weak support (55%) (SI Appendix, Fig. S4D). As this protein does not appear to affect membrane integrity, its toxicity that is particularly striking in its effect on transcription as described above is more likely to result from its enzymatic activity, putatively cyclic nucleotide synthesis.

While PanAs Are Naturally Specific for their Cognate PanT Toxins, Their PanT Neutralization Spectrum Can Be Exploited through Directed Evolution. We have earlier shown that Type II antitoxins neutralizing toxSAS toxins—such as B. subtilis PanA

bac. sub. neutralizing PhRel2
bac. sub.—are specific for their cognate toxins (4). PanA is clearly a versatile domain that can evolve to neutralize—and become specific for—a range of different toxin domains. Therefore, we performed exhaustive cross-inhibition testing, resulting in a 10 × 10 cross-neutralization matrix (Fig. 6A and SI Appendix, Fig. S10). A clear diagonal signal is indicative of PanA antitoxins naturally efficiently protecting only from cognate toxins—even within groups of evolutionary related toxic effectors such as toxSAS CapRel

bac. hua. PhRel2
bac. hua., and PhRel2
bac. sub. Conversely, on the evolutionary timescale, Panacea changes its toxin specificity and swaps partners, which raises the questions of what the structurally important regions for neutralization are and how a new specificity profile can be evolved.

The Panacea domain is not identifiably homologous to any protein with a known structure. Therefore, we have de novo predicted the structure of PanA

hau. using trRosetta, a deep learning–based method (31) (Fig. 6B). The model has a confidence categorized as “very high,” with an estimated template modeling (TM) score of 0.704. Independent structural prediction with AlphaFold2 (10) indicates the same overall fold, with an RMSD of 1.05 Å as calculated by PyMol (SI Appendix, Fig. S11). The structure is comprised of a central helix (α2) surrounded by five further helices and a small three-strand β-sheet that contains a strongly conserved GPV amino acid sequence motif in the β2 strand proximal to the central helix α2 (Fig. 6B and SI Appendix, Fig. S12). The β3 and α2 elements are particularly well conserved in the sequence alignment (SI Appendix, Fig. S12). We probed the functional importance of the GPV motif in toxicity neutralization assays. While individual G62A and V64S substitutions did not affect the ability of PanA

bac. sub. to neutralize its cognate toxin capRel
bac. hua., the G62A V64S double substitution resulted in the loss of neutralization activity (Fig. 6C), supporting that the GPV motif is, indeed, functionally important.

Next, we subjected a pair of toxSAS:PanA TA systems with effectors belonging to two distinct toxSAS subfamilies—PhRel2 and CapRel—to directed evolution experiments and screened for mutant variants of PanA

hau. that are able to neutralize B. subtilis PhRel2
bac. sub. Even though the amino acid identity between PanA

bac. hua. and PanA

bac. sub. proteins is only 30% to 40%, just two substitutions—T36M and Q131L—were sufficient for cell viability as judged by colony-counting experiments (Fig. 6D and SI Appendix, Fig. S13A). Individual T36M and Q131L substitutions are not sufficient to elicit cross-reactivity (SI Appendix, Fig. S13B). T36 is part of the well-conserved central helix α2, while Q131 is located in a small, variable strand containing the conserved GPV motif. Notably, the T36M Q131L PanA

bac. sub. variant is still capable of protecting from the cognate CapRel
bac. hua. toxin. However, the protection from PhRel2
bac. sub. toxicity is less efficient than that conferred by the cognate PanA
bac. sub. antitoxin: the bacterial colonies are smaller, indicative of incomplete detoxification (SI Appendix, Fig. S13C). Therefore, we hypothesized that the T36M Q131L double substitution does not result in specificity switching in a strict sense but rather relaxes the specificity, thus allowing the neutralization of noncognate toxins. To probe this hypothesis, we tested if T36M Q131L PanA

hau. could protect from other noncognate PanTs (Fig. 6E and SI Appendix, Fig. S13B). We found that T36M Q131L PanA

hau. can protect from the noncognate cell membrane–targeting PanT

E. coli (Fig. 6E), although incompletely, as evident from the smaller colony size (SI Appendix, Fig. S13B); no increased protection from other noncognate PanTs was detected (SI Appendix, Fig. S13C).

Discussion

Type II TA systems are highly specific at the sequence level; however, small changes can result in promiscuous intermediates allowing the neutralization of additional homologous but noncognate toxins...
Through selection experiments, we have demonstrated that via just two amino acid substitutions, Panacea-containing anti-toxins can be made to neutralize not just noncognate but nonhomologous noncognate toxins that have different cellular targets and mechanisms of action. This reveals a remarkable versatility of the Panacea domain. We suggest describing the ability of an antitoxin domain to evolve to neutralize different toxin domains as hyper-promiscuity, distinguishing from promiscuity, in which one individual antitoxin can neutralize noncognate but homologous toxins sharing the same structural fold (Fig. 7). A naturally occurring

(28, 38, 39). Through selection experiments, we have demonstrated that via just two amino acid substitutions, Panacea-containing anti-toxins can be made to neutralize not just noncognate but nonhomologous noncognate toxins that have different cellular targets and mechanisms of action. This reveals a remarkable versatility of the Panacea domain. We suggest describing the ability of an antitoxin domain to evolve to neutralize different toxin domains as hyper-promiscuity, distinguishing from promiscuity, in which one individual antitoxin can neutralize noncognate but homologous toxins sharing the same structural fold (Fig. 7). A naturally occurring
example of the latter can be seen in the bacteriophage T4 antitoxin Dmd that neutralizes the homologous mRNase toxins RnlA and LsoA (26, 27).

Other versatile antitoxin domains have also previously been observed in computational analyses to be associated with multiple toxin-like domains (16, 40, 41), indicating potentially similar plasticity and hyperpromiscuity. One example is the Phd-related antitoxin domain found in proteins that can neutralize RelE-like mRNases, in addition to those that neutralize the related antitoxin domain found in proteins that can neutralize plasticity and hyperpromiscuity. One example is the Phd-like toxin-like domains (16, 40, 41), indicating potentially similar observed function of the Panacea domain–containing antitoxin (17). While our results are most consistent with a Type II direct mechanism of inactivation rather than the indirect Type VI–like mechanism of inactivation rather than the indirect T36M Q131L antitoxin. To quantify the effects of PanA/PanT coexpression on bacterial viability, the overnight cultures of strains transformed with pBAD33 and pKK223-3 vectors or derivatives thereof expressing toxin and PanA antitoxins was adjusted to 1.0, cultures serially diluted from 101- to 106-fold and spotted on LB agar medium supplemented with appropriate antibiotics as well as inducers (0.2% arabinose for toxin induction and 1 mM IPTG for induction PanA variants); 10-fold dilution is shown. (B) trRosetta-predicted structure (TM score 0.70, “very high confidence”) of the PanA_{Vib. har.} antitoxin colored by degree of conservation as per SI Appendix, Fig. S11. (C) Effect of amino acid substitutions in conserved GPV motif on of PanA_{Vib. har.} antitoxin functionality. (D) Neutralization of PhRel2 bact. sub. toxin by evolved noncognate PanA_{Vib. har.} T36M Q131L antitoxin. To quantify the effects of PanA/PanT coexpression on bacterial viability, the overnight cultures were subdiluted and spread on the LB agar, and individual colonies were counted. Analogous experiments with single-substituted T36M and Q131L PanA_{Vib. har.} variants are shown in SI Appendix, Fig. S11. (E) Neutralization of PanT bact. sub. toxin by wild-type and T36M Q131L PanA_{Vib. har.} variants.

A number of other outstanding questions about PanA remain. Firstly, how is one single domain able to neutralize so many different toxins while insulating itself against noncognate interactions? The answer to this will come from structural analyses of multiple PanAs—both alone and in complex with cognate toxins—combined with additional directed evolution experiments using different PanA pairs and an analysis of sequence coevolution. A structural analysis of complexes will also reveal the molecular function of the conserved GPV motif that is a signature of the Panacea domain and is critical for neutralization. The second question is just how much of a role proteases play in the function of PanA in some species—given the previously observed function of the Panacea domain–containing antitoxin SocA in the proteolytic degradation of toxin SocB in Caulobacter (17). While our results are most consistent with a Type II direct mechanism of inactivation rather than the indirect Type VI–like
One antitoxin domain evolves to neutralize multiple unrelated toxin domains. Each individual antitoxin may be promiscuous, or may evolve multiple cognate toxin domains.

Fig. 7. Antitoxin promiscuity versus hyperpromiscuity. (A) A promiscuous antitoxin has relaxed neutralization specificity toward its target toxin and can neutralize a range of related toxins which all share the same structural fold. Examples include cross regulation of RelBE-like modules in Mycobacterium tuberculosis (55) and promiscuous ParD antitoxins generated through directed evolution that neutralize noncognate ParE toxins (28). (B) A hyperpromiscuous antitoxin domain, as exemplified by Panacea, can evolve to neutralize unrelated toxins that share neither structural fold nor mechanism of action.

One antitoxin can neutralize multiple related toxins that all share the same structural fold.
an OD600 of 0.2. For maintaining the plasmids, all cultures were grown in the presence of 34 μg/mL chloramphenicol. Toxic production was induced by the addition of arabinose (0.2%) for 30 min followed by staining with 200 nM of the membrane permeability indicator SYTOX Green (37) alongside the induction and 250 nM membrane potential–sensitive dye DiSC3(5) (49) for the last 3 min (56, 30). The samples were immobilized on microscope slides covered with a thin layer of H2O/1.2% agarose and imaged immediately. As a positive control for pore formation, BW25113 E. coli cells transformed with the empty pBAD33 vector were incubated with 10 μg/mL polymyxin B for 15 min (51). Microscopy was performed using a Nikon Eclipse Ti equipped with a Nikon Plan Apo 100×/1.40 Oil Ph3 objective, CoolLED e-4000 light source, Photo- metrics BSI iCMOS camera, and Chroma 49002 (excitation [EX] 470/40, dichroic mirror [DM] 495 lpix, and emission [EM] 525/50) and Semrock Cy5-4040C (EX 628/40, DM 660 lp, EM 692/240) filter sets. The images were acquired with Metamorph 7.7 (MolecularDevices) and analyzed with Fiji (52).

Selection of Cross-Infesting PanA Mutants. An error-prone PCR mutant library of Vibrio Harveyi PanA antitoxin was created as described in SI Appendix, Methods: Selection of cross-neutralizing PanA preparations of the antitoxin mutant library. A total of 5 × (around 1 μg) antitoxin mutant library was transformed into the BW25113 E. coli strain carrying a noncognate toxin expression plasmid pRefE_proE.Pri::pBAD with toxA&S toxin from B. subtilis (1α [Wp3003]). The transformants were let to recover for 1 h in 1 mL SOC media at 37 °C and added to 20 mL LB media supplemented with ampicillin (100 μg/mL), chloramphenicol (25 μg/mL), 0.2% L-arabinose, and 1 mM IPTG. The bacteria were grown overnight at 37 °C while expressing both toxin and antitoxin. Next day, the plasmid was extracted from 3 mL culture using a Favorprep Plasmid Extraction Mini Kit and cleaved with FastDigest Sac I restriction enzyme (Thermo Scientific) to eliminate the toxin plasmids. The transformed culture was used for infesting the E. coli DH5α strain, and the plasmids were extracted from the offspring of a single colony. The E. coli BW25113 strain expressing the cognate or noncogonate toxin was then transformed with 500 ng mutated plasmid. Again, 100 μL recovery culture was spread onto LB supplemented with corresponding antibiotics as well as 0.2% glucose agar plates, and the rest of the culture was collected by centrifugation and spread on an LB agar plate containing corresponding antibiotics. Colonies growing in the presence of arabinose (0.2%) for 30 min followed by staining with 200 nM of fireRed DNA Polymerase (Solis BioDyne); antitoxins were tested with the combination of pK223_rev_CPEC and STEC_ToxnCtrl1, VhPanA_ctrl1, or BspuPanA_ctrl1 primers and toxins with the combination of pBAD_fwd and STEC_ToxnCtrl1, Vh_Toxn Ctrl1, and Bspu_Toxn Ctrl1 (Dataset S3).

Data Availability. Python code and text files of alignments, trees, and HMMs have been deposited in GitHub (https://github.com/GCA-VH-lab/Panacea) (56). All other study data are included in the article and/or supporting information.

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