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Polymorphic toxins and their immunity proteins: diversity, evolution and mechanisms of delivery

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Abstract

All bacteria must compete for growth niches and other limited environmental resources. These existential battles are waged at several levels, but one common strategy entails the transfer of growth inhibitory protein toxins between competing cells. These antibacterial effectors are invariably encoded with immunity proteins that protect cells from intoxication by neighboring siblings. Several effector classes have been described, each designed to breach the cell envelope of target bacteria. Although effector architectures and export pathways tend to be clade specific, phylogenetically distant species often deploy closely related toxin domains. Thus, diverse competition systems are linked through a common reservoir of toxin-immunity pairs that is shared via horizontal gene transfer. These toxin-immunity protein pairs are extraordinarily variable in sequence, and this polymorphism underpins an important mechanism of self/nonself discrimination in bacteria. This review focuses on the structures, functions and delivery mechanisms of polymorphic toxin effectors that mediate bacterial competition.

Keywords

colicins; contact-dependent growth inhibition (CDI); MafB; type VI secretion system (T6SS); Esx secretion system (ESS); outer-membrane exchange (OME)

INTRODUCTION

Toxin-immunity protein pairs play a prominent role in prokaryotic biology. In this context, a “toxin” is a small protein, or protein domain, that inhibits bacterial cell growth. Bacteriocins were the first antibacterial toxins to be characterized (27). Many species release bacteriocins to kill competitors in the environment. Bacteriocinogenic cells also produce specific immunity proteins, which neutralize toxicity and protect against auto-inhibition. In this manner, bacteriocin release provides a competitive growth advantage to some strains. In the 1980s, a different class of small toxin-antitoxin gene pairs were found to contribute to plasmid maintenance. The antitoxins of these systems are rapidly degraded by cytoplasmic

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proteases, which in turn exerts a selective pressure to retain the plasmid for continued antitoxin synthesis. These latter toxin-antitoxin systems are also commonly encoded in the genomes of eubacteria and archaea, where they are thought to be important regulators of cell growth (53; 113).

In 2005, a new competitive mechanism that requires direct contact between toxin-producing and target bacteria was discovered. This “contact-dependent growth inhibition” (CDI) phenomenon is mediated by CdiA proteins exported by a subclass of type V secretion systems (T5SS) (4). Like bacteriocins, CdiA effectors are encoded with specific immunity proteins that neutralize toxicity. With time, it became clear that bacteria utilize a broad spectrum of toxin delivery strategies. The potent bactericidal activity of the type VI secretion system (T6SS) was first reported in 2010 (57; 83); followed rapidly by predictions of other antibacterial mechanisms (56; 105; 157; 158). These predictions have been verified for *Neisserial* MafB proteins (6; 65) and Esx-like secretion systems (ESS) in Gram-positive bacteria (25; 146). Further, some type IV secretion systems (T4SS) deliver toxins directly into target bacteria (129), and antibacterial WapA and SitA effectors have been characterized from *Bacillus subtilis* and *Myxococcus xanthus*, respectively (73; 139). It is now clear that inter-cellular toxin transfer is a fundamental and ubiquitous aspect of prokaryotic biology.

POLYMORPHIC EFFECTOR FAMILIES

Polymorphic effectors are a diverse assemblage of proteins linked by their common function in inter-bacterial competition. Effector classes vary in size, sequence and structure, but share similarly organized multi-domain architectures. Each class is characterized by conserved N-terminal regions that guide export and/or mediate delivery into target bacteria (Fig. 1a). *Neisseria* MafB (6; 65), *Myxococcus* SitA (139) and *Bacillus* WapA (73) proteins rely on Sec-dependent secretion, whereas CdiA carries an additional transport domain required for T5SS-dependent export across the outer membrane (52). T6SSs export valine-glycine repeat G (VgrG) and hemolysin-coregulated proteins (Hcp), which sometimes carry toxin domains (34). Several other T6SS effectors have Pro-Ala-Ala-Arg (PAAR) repeat domains, which enable export via direct interactions with VgrG (125). The ESS selects substrates through N-terminal coiled-coil domains that contain Trp-Xaa-Gly (WXG) or Leu-Xaa-Gly (LXG) motifs (48; 157). For each effector class, the extreme C-terminal domain is responsible for bactericidal activity. Toxin domain sequences can vary dramatically, even between closely related strains, and therefore the “polymorphic” appellation describes the diversity of toxic cargos. Thus, all polymorphic effectors have the capacity to deliver a variety of toxin domains. This modularity was first described for E-type colicins, which use homologous receptor-binding (R) and translocation (T) domains to transfer distinct DNase, rRNase and tRNase nucleases into target bacteria (Fig. 1b) (27).

Because polymorphic effectors are antibacterial, they are invariably produced with immunity proteins that prevent self-intoxication. Immunity proteins are usually encoded immediately downstream of the effector gene, and this organization enables toxin-immunity pairs to be fused to various effector classes. For example, CdiA proteins from *Chromobacterium violaceum* (NCBI ID: WP_011136418.1) and *Burkholderia ubonensis* (WP_080434302.1) carry toxins that are >50% identical to the RNase domains of ColE4 and ColE5,

respectively. Similarly, *Paracoccus saliphilus* encodes a PAAR-Rhs effector-immunity pair (WP_141225802.1/WP_084203033.1) that shares ~49% identity with the ColE8 DNase domain and immunity protein. These observations suggest that horizontally acquired toxin-immunity pairs are integrated as modules into secretion systems. Comparison of the *maf* genomic island-2 from *N. meningitidis* MC58 and *N. gonorrhoeae* FA 1090 illustrates this phenomenon (65). The nucleotide sequence encoding the MafB toxin domain and MafI immunity protein in FA 1090 is the same as that of a *cdiA-cdiI* derived gene pair in the MC58 island (Fig. 1c). Thus, MafB and CdiA effectors can apparently deliver identical EndoU RNase domains. The MC58 island contains additional fragmented effector-immunity pairs (6; 65), which are also a common feature of *cdiA, rhs* and *wapA* loci (7; 73; 105). These "orphaned" toxin-immunity sequences likely represent ancestral modules displaced by more recently acquired cassettes. Horizontal gene transfer also enables closely-related bacteria to deploy distinct T6SS effector arsenals (37; 122; 136). The versatility of polymorphic toxins is exemplified by the aforementioned EndoU (PF14436) family, which is found at the C-terminus of CdiA, MafB, Rhs, WXG/LXG and several uncharacterized effectors across diverse phyla (Fig. 1d) (65; 88; 157).

TOXIN ACTIVITIES

The identification of polymorphic toxin activities remains a significant challenge. Aravind and colleagues have predicted biochemical activities for dozens of toxin families (62; 157; 158), but the C-terminal domains of many effectors remain unannotated. Despite their extraordinary variability, polymorphic toxins use only a handful of activities to disrupt critical molecular structures in target bacteria.

Ionophores: pore-forming toxins

Bacterial cells maintain a proton gradient across the cytoplasmic membrane, and this electrochemical proton-motive force (pmf) powers nutrient uptake, flagellar rotation and ATP synthesis. Therefore, pore-forming toxins that dissipate the pmf are potent inhibitors of bacterial growth. Many bacteriocins carry a stereotypical pore-forming domain composed of a hydrophobic α8-α9 hairpin core surrounded by eight amphipathic α-helices (100; 148). This domain remains soluble as it diffuses to target cells, but upon encountering a polarized membrane, the α8-α9 hairpin everts from the core and inserts into the lipid bilayer to initiate pore formation. Diphtheria toxin uses a related 10-helix domain to breach the eukaryotic plasma membrane (99), and the VasX T6SS effector from *V. cholerae* apparently uses it to form pores in both eukaryotic and prokaryotic membranes (89; 90). Though unannotated, these pore-forming domains are commonly carried by PAAR proteins in enterobacteria (e.g. WP_095441387.1) and *Pseudomonas* species (e.g. WP_087089637.1). T6SSs also deploy pore-forming toxins that are distinct from the 10-helix domain. Tse4 from *P. aeruginosa* contains four transmembrane helices, with the C-terminal helix containing a predicted glycine-zipper motif (74). Ssp6 from *S. marcescens* is yet another novel pore-forming toxin built from two short transmembrane helices (86). Finally, CdiA from *E. coli* EC93 dissipates the pmf in target cells (5). This latter domain contains two predicted glycine-zipper motifs, but lacks hydrophobic α-helices, suggesting a mechanism distinct from other pore-forming toxins.

Phospholipases

Phospholipase toxins are commonly delivered by T6SSs, but are largely absent from other delivery systems (157). Russell *et al.* identified and characterized five families of T6SS lipase effectors (Tle1 - Tle5) (119). The first four clades use a common nucleophilic elbow motif to hydrolyze acyl chains from the A1 and/or A2 position. The Tle5 family contains duplicated HxKxxxxD motifs found in phospholipase D enzymes (119). Phospholipases are packaged for T6SS-dependent delivery in several ways. Tle1 toxins are fused to VgrG in *Pseudomonas* species (60; 157), and to Hcp in many *Salmonella* strains (80). *Dickeya, Pectobacterium, Aeromonas*, and *Shewanella* all encode PAAR proteins with predicted phospholipase A1 domains (73), and T6SS-associated Rhs effectors from *Vibrio vulnificus* (e.g. POB69978.1) carry Tle1 family toxins. Lastly, lipase toxins can be linked to VgrG indirectly through adaptor proteins (135).

Peptidoglycan: blocking synthesis and promoting its degradation

Polymorphic effectors use two strategies to disrupt the peptidoglycan cell wall and lyse target bacteria. ColM and related bacteriocins degrade the lipid II precursor of peptidoglycan biosynthesis. This activity generates dephosphorylated undecaprenol, which cannot be recycled to support further polymerization of the cell wall (49; 124; 156). *Streptococcus intermedius* deploys an LXG effector with the same phosphatase activity, though it has a novel fold unrelated to ColM (146). The second strategy entails enzymatic degradation of peptidoglycan. Pesticin is a muramidase bacteriocin produced by *Yersinia pestis*. Like lysozyme, pesticin hydrolyzes the β−1,4-glycosidic linkage between *N*-acetyl-muramic acid and *N*-acetyl-glucosamine (103; 143). Lytic muramidases have also been described for T6SSs. VgrG-3 from *V. cholerae* carries a C-terminal lysozyme-like domain (20), and Tse3 from *Pseudomonas aeruginosa* has similar activity (118). In addition, T6SSs deploy a variety of amidases that cleave peptide cross-links in the cell wall (118; 120). The C-terminal domain of *P. aeruginosa* VgrG2b is a metalloprotease that cleaves cross-links only at the division septum (151). T6SS Tse1 and Tae4 effectors from *Pseudomonas aeruginosa* and *Enterobacter cloacae* are NlpC/P60 family amidases that cleave peptide cross-links between D-Glu and *m*-diaminopimelic acid residues (120). The latter amidases lack the canonical multi-domain architecture of most polymorphic effectors, but they are sequence-diverse and rely on a specialized delivery system.

Nucleases and deaminases

Nucleases are perhaps the most common and versatile of the polymorphic toxin payloads (157). Most DNase toxins are members of either the HNH/EndoVII or PD-(D/E)*X*K superfamilies (157). HNH/EndoVII DNases contain a ββα-metal active-site motif first identified in phage T4 endonuclease VII and colicins (71; 72; 111). Zhang *et al.* have predicted that polymorphic effectors deploy 14 distinct clades of HNH/EndoVII DNases (157; 158). Few of these have been tested, though DNase activities have been verified for Tox-GHH2 (PF15635) (104) and Endonuclease NS_2 (PF13930) domains (25; 73; 97). PD-(D/E)*X*K phosphodiesterase domains are common in restriction endonucleases and DNA repair enzymes, but their extraordinarily plastic active sites make them difficult to identify through informatics (131). Zhang *et al.* identified 10 novel toxic restriction endonuclease

(Ntox-REase) clades associated with polymorphic toxin systems (157), though only five are listed in the current Pfam database. Other PD-(D/E)*X*K toxins have been discovered through structure-function analyses (66; 92), suggesting that more family members remain to be characterized. The structure of the Tde DNase from *Agrobacterium tumefaciens* remains unknown. This T6SS effector was originally thought to have a novel RNase fold (157), but has since been shown to be specific for DNA (81).

Polymorphic RNase domains adopt a greater diversity of folds than the DNase toxins. Zhang *et al.* predicted that several novel toxin (Ntox) families have RNase activities (157; 158), though few have been examined experimentally (2; 13). Ntox21 adopts the barnase-EndoU-colicin D/E5-RelE (BECR) superfamily fold and cleaves 16S rRNA at the same position as ColE3 (13; 157). Ntox28 is an α-helical metal-dependent tRNA anticodon nuclease (42; 67). Ntox41 adopts the vertebrate RNase A fold, though it lacks the characteristic disulfide bonds of the superfamily (11). EndoU toxins contain a partial duplication the BECR core and are structurally similar to RNA processing enzymes found in vertebrates and coronaviruses (88; 158). Lastly, some *Burkholderia* CdiA proteins carry PD-(D/E)*X*K phosphodiesterase domains that cleave specific sites within tRNA molecules (68; 92; 139).

Deaminases are another major class of toxins associated with polymorphic effectors (157). These toxins have undergone a major radiation into at least nine clades (62). Surprisingly, this important class of enzymes has not yet been tested experimentally, and substrate specificities for the various clades remain unknown. In principle, these enzymes could act on adenine or cytosine in the context of DNA, RNA or nucleotides.

Nucleotide specific toxins

Mougous and colleagues first described the structure and activity of an Ntox46 (PF15538) domain that adopts the same ADP-ribosyltransferase (ART) fold as diphtheria toxin (147). However, this toxin does not ADP-ribosylate target proteins, but rather acts as an $NAD(P)^+$ glycohydrolase (NADase). Thus, these enzymes block biosynthesis and greatly reduce ATP production by disrupting the flow of electrons to the respiratory chain. There are likely to be more unrecognized NADase toxins, because recent work from Whitney and coworkers has uncovered a new class associated with Rhs and LXG effectors (132). Moreover, the widespread Arg-Glu-Ser (RES) toxin domain (PF08808) is now known to degrade nicotinamide dinucleotides (128). However, there are toxic ART domains that ADP-ribosylate protein targets. Tre1 is a T6SS effector from *Serratia proteamaculans* that covalently modifies FtsZ to block cell division (133). Finally, RelA/SpoT hydrolases (RSH) are associated with some polymorphic effector classes (157). These enzymes classically synthesize and degrade the stringent factor alarmone (p)ppGpp, but recent work shows that toxic RSH domains inhibit target-cell growth by producing a novel (p)ppApp nucleotide (1).

TOXIN-ACTIVATING FACTORS

Polymorphic toxins typically hijack cell-envelope proteins to gain entry into target cells, but a subset also require co-factors to support enzymatic activity. ColM is unable to kill *E. coli fkpA* mutants, because they lack a peptidyl prolyl isomerase that refolds the phosphatase domain after translocation into the periplasm (54; 61). Similarly, Ssp2 and Ssp4 effectors of

the *Serratia marcescens* T6SS rely on periplasmic oxidoreductases in target bacteria for proper disulfide formation after delivery (85). Thus, toxins that unfold during delivery can become reliant on chaperones. In addition, several CdiA toxins have evolved interactions with target-cell proteins to promote tRNase activities. The Ntox28 domain from uropathogenic *E. coli* is active only when bound to CysK, an enzyme that catalyzes the final step of L-cysteine biosynthesis (42; 67). This interaction is intriguing because the toxin mimics CysE – the physiological binding partner of CysK (42). Other BECR-fold tRNases collaborate with translation factors EF-Tu and EF-Ts to cleave substrate (51; 69; 87). These latter toxins recognize substrate only in the context of tRNA•EF-Tu•GTP ternary complexes, the formation of which is greatly accelerated by EF-Ts (69). The selective pressures driving interactions with EF-Tu could stem from biophysical constraints that limit the size of toxin payloads. In this model, economized domains are so small that they depend on binding partners to stabilize the nuclease fold and/or organize the catalytic center.

IMMUNITY PROTEINS

Immunity proteins prevent auto-intoxication, but are also required to protect bacteria from toxins delivered by neighboring siblings. Immunity proteins typically bind directly over the active sites of enzymatic toxins, though some nuclease toxins are neutralized by antitoxins that bind to “exosites” adjacent to the catalytic center (26; 71; 92). The Tri1 immunity protein is unique in that it actively hydrolyzes ADP-ribosyl groups added to FtsZ by its cognate toxin Tre1 (133; 157). The molecular basis of immunity to pore-forming toxins is unknown as there are no high-resolution structures of membrane-embedded pores. However, the latter immunity proteins are usually small and very hydrophobic, suggesting that they localize to the cytoplasmic membrane, where they could block pore opening or interfere with glycine-zipper oligomerization.

Structures of polymorphic toxin-immunity protein complexes have revealed that individual families are rapidly diversifying. As an example, colicin DNase domains generally share >50% pairwise identity, and their immunity proteins are ~47% identical on average. Much of this sequence divergence is concentrated at the complex binding interface (Fig. 2); and consequently, immunity proteins exhibit significantly lower affinity for “near-cognate” DNase domains (76). Because immunity function is essential to prevent auto-intoxication, this evolution must proceed through a series of reciprocal mutations that gradually sculpt the interface without catastrophically perturbing binding affinity (112). Nuclease colicins bind cognate immunity proteins with femtomolar dissociation constants, and this extraordinarily high affinity may facilitate divergence. Substitutions that reduce binding 1000-fold still yield complexes with nanomolar affinity, which is sufficient to protect against intoxication (76). Although this phenomenon has been explored most extensively with colicins, rapid evolution at the protein binding interface is a hallmark of all toxin-immunity pairs that mediate bacterial competition (51; 88; 93).

MECHANISMS OF TOXIN DELIVERY

Protein bacteriocins/colicins

Bacteriocins are diverse ribosome-synthesized antimicrobials that include small modified peptides and multi-domain proteins (27). The protein bacteriocins of γ-proteobacteria – and in particular the colicins of *E. coli* – are a paradigm for polymorphic effectors. Colicins are usually plasmid-encoded and are only expressed in response to extensive DNA damage and other stresses (Fig. 3a) (27; 79). In contrast, the downstream immunity genes are transcribed constitutively. Colicins lack signal sequences and typically require bacteriocin release proteins for export (106; 137). These small lipoproteins activate OmpLA phospholipase, which disrupts envelope integrity and allows cytoplasmic contents to leak from the cell (Fig. 3b) (39; 107). Nuclease colicins are released in complex with immunity proteins, because the latter are produced constitutively and have very high affinity for their cognate toxin domains. Notably, colicin release is lethal, and therefore the toxin producing cell obtains no competitive advantage. However, non-expressing sibling cells are immune and gain a significant growth advantage over susceptible competitors.

Colicins bind to target bacteria through specific cell-surface receptors, which are usually outer membrane proteins (OMP) that function in nutrient uptake. ColIa, ColD, and ColM recognize the iron-siderophore transporters Cir, FepA and FhuA (respectively), whereas E colicins use the vitamin B12 transporter BtuB as a receptor (27). In principle, these receptors could also be exploited as portals for toxin import, but colicins recruit a second OMP that serves as a "translocator" (Fig. 3B). Most E colicins thread the unstructured N-terminal segment of their T domains through the lumen of OmpF translocators (58; 153). The analogous region of ColIa appears to mimic a siderophore, enabling it to recruit a second Cir molecule for the translocation step (64). In each case, the colicin inserts an N-terminal peptide motif into the periplasm where it interacts with components of the Tol or Ton machineries. Tol maintains OM integrity and is particularly important during cell division (28). TolQ and TolR form an inner membrane complex that harnesses the proton gradient and conveys that energy to the OM through TolA. The Ton system powers the import of cobalamin and siderophores through TonB-dependent OM transporters (149). ExbB and ExbD convert pmf energy into conformational changes in TonB, which spans the periplasm to control transporters in the OM. Colicin translocation across the outer membrane depends on pmf-driven conformational changes in TolA and TonB, but it remains unclear whether the entire colicin molecule is imported to the periplasm. Cramer and coworkers have suggested that the ColE3 nuclease domain becomes destabilized after dissociation of the immunity protein, which in turn enables the toxin to translocate through the OmpF translocator (35; 154). Despite this uncertainty, it is clear that colicins unfold during import, and that immunity proteins must be actively dissociated from nuclease domains before translocation can proceed (59).

Once inside the periplasm, pore-forming domains insert into the cytoplasmic membrane and dissipate the proton gradient. Colicin nuclease domains must be further transported into the cytosol. This final translocation step depends on FtsH, which is an ATP-dependent metalloprotease that normally extracts misfolded proteins from the inner membrane for

degradation (36). E colicin nuclease domains are strongly electropositive and bind to anionic phospholipids. Membrane binding destabilizes these domains, rendering them into molten globules that are presumably recognized as defective membrane proteins and retro-translocated into the cytosol by FtsH (94; 95; 144). The tRNase domain of ColD does not associate with membranes and instead relies on interactions with the LepB signal peptidase for cell entry (91). Nevertheless, ColD also depends on FtsH for import (91), suggesting that LepB brings the nuclease into close proximity to the membrane for delivery to FtsH. The final step of colicin nuclease delivery entails proteolytic cleavage to release the toxin domain into the cytosol (30; 38). FtsH appears to mediate this proteolytic processing (30), though it remains unclear how the nuclease domains avoid complete degradation during import.

Contact-dependent growth inhibition (CDI)/type V secretion systems (T5SS)

CdiB/CdiA two-partner secretion (TPS) systems are most common in β- and γ-proteobacteria, though several α-proteobacteria, fusobacteria and Negativicutes also contain *cdi* genes (2; 157). CdiB is an Omp85 β-barrel protein that transports CdiA across the outer membrane. CdiA proteins span from ~200 to over 700 kDa, but all share the same general domain architecture (Fig. 4a). The N-terminal half of CdiA is dominated by filamentous hemagglutinin-1 (FHA-1; PF05594) peptide repeats, which form a β-helical filament that projects from the inhibitor-cell surface (70; 116). FHA-1 domains range from ~300 to 4,600 residues, which corresponds to filament lengths of ~15 to over 100 nm (70). A second repeat domain composed of distinct FHA-2 (PF13332) repeats comprises much of the C-terminus. The two repeat regions surround a central receptor-binding domain (RBD), which functions in target-cell recognition (115; 117). The variable C-terminal (CdiA-CT) region is demarcated by a conserved peptide sequence, exemplified by the VENN motif in enterobacterial effectors (2). The CdiA-CT is frequently composed to two domains with distinct functions. The N-terminal “cytoplasm entry” domain mediates translocation across the target-cell inner membrane, and the extreme C-terminal domain has toxic activity (150).

Newly synthesized CdiA is directed to the periplasm by a Sec-dependent signal sequence (Fig. 4b). The TPS transport domain of CdiA is then bound by CdiB, which uses N-terminal polypeptide transport-associated (POTRA) domains to guide the effector through its outer membrane β-barrel (12). The lumen of CdiB is too narrow to accommodate a β-helix, so CdiA must be exported in an unfolded state. Thus, the filament likely forms as the FHA-1 peptide repeats emerge from CdiB, with the free energy of β-helix folding to power secretion (52). Shortly after the CdiA RBD is exported, secretion is halted at a Tyr/Pro-enriched region. As a result, the N-terminal half of CdiA forms a cell-surface filament, while the FHA-2 and CdiA-CT regions remain sequestered in the periplasm (Fig. 4b) (116). Because the RBD forms the distal tip of the filament, the TPS transport domain presumably remains associated with CdiB at the cell surface. Thus, CdiA forms a hairpin with the Tyr/Pro-enriched region adopting an extended conformation to span the length of the filament, linking the RBD to the periplasmic FHA-2 domain (116).

As with colicins, CDI toxin delivery is initiated by specific interactions with cell-surface receptors. Different classes of *E. coli* CdiA effectors recognize BamA (3), OmpC/OmpF (14; 142) and Tsx (115) as receptors. Receptor binding alleviates the export arrest through an

unknown mechanism, and the FHA-2 domain is secreted directly onto the target-cell surface (Fig. 4c). FHA-2 becomes stably associated with the target-cell outer membrane and is thought to form a transmembrane conduit for toxin translocation (116). The energetics of outer membrane transport are not understood, but Tol and Ton have no role in CDI toxin delivery (114). Once inside the target-cell periplasm, the CdiA-CT is cleaved near the VENN motif for delivery to the cytosol (116). This final translocation step is mediated by the N-terminal cytoplasm entry domain of the CdiA-CT. Genetic evidence suggests that entry domains exploit inner membrane proteins to transfer nuclease domains into the cytosol (150). Entry domains are highly variable, and each class recognizes a specific integral membrane protein. Some of the hijacked proteins, like PtsG, MetI, RbsC and GltK/GltJ, are metabolite transporters, but transport function is not required for toxin import (150). CdiA-CT entry domains have low thermodynamic stability and are thought to adopt a molten globule-like conformation (9), which could facilitate insertion into the membrane. Finally, the target-cell pmf is required for CdiA-CT delivery into the cytoplasm (114), suggesting that the electrochemical gradient drives transport.

Type VI secretion systems (T6SS)

The T6SS is structurally and evolutionarily related to contractile bacteriophages. The T6SS ejects a spear shaped projectile that impales neighboring cells to deliver a payload of effector proteins. These systems were first characterized as virulence determinants (109), but many T6SSs function in inter-bacterial conflict. The T6SS apparatus is built from three sub-complexes that assemble sequentially during the secretion duty cycle (Fig. 5a). TssJ, TssL, and TssM form a multimeric, membrane-embedded complex that spans the cell envelope and provides the secretion conduit. TssJ is an outer-membrane lipoprotein (8), and TssL is an integral membrane protein that also anchors the sub-complex to the peptidoglycan cell wall (82). TssM spans the periplasm and binds TssJ near the inner leaflet of the outer membrane (46; 82). This trans-envelope complex serves as the docking site for the phage-like baseplate, which assembles independently in the cytoplasm (21). Trimeric VgrG is homologous to the gp27-gp5 tailspike phage T4 (75; 108; 134), and it forms the central hub around which the baseplate assembles. TssE, TssF and TssG form a hexagonal array surrounding VgrG, with TssK oligomers forming a bowl-shaped ring about the outer edge of the baseplate (45). TssK cavity interfaces with the cytoplasmic domains of TssM and TssL to connect the baseplate to the membrane complex (78; 159). Once engaged with the trans-envelope complex, the baseplate serves as the origin for assembly of the contractile sheath and inner tube complex (Fig. 5b). Hexameric Hcp rings stack onto VgrG to form a tube, which is surrounded by a latticed sheath of TssBC heterodimers (10; 19). Sheath and tube polymerization is coordinated by TssA, which caps the growing end of the tail (160). Once the sheath-tube complex extends across this cell (123), it rapidly contracts through a rigid-body rotation of TssBC heterodimers that causes the sheath to reduce its length in half with an attendant increase in diameter (29; 145). Because the sheath is anchored to the baseplate and trans-envelope complexes, contraction causes the distal end of the tail to move toward the membrane complex rapidly. The baseplate releases VgrG, freeing the spike complex to exit through open pore of the trans-envelope complex. In addition, the TssM ring must open from its resting diameter of 15 Å to allow passage of the Hcp tube (44). Following

contraction, cytosolic components are disassembled and recycled for further rounds of secretion (18).

All known T6SS effectors are exported through interactions with either VgrG or Hcp (Fig. 5a). In some instances, toxin domains are fused to VgrG as C-terminal extensions from the β-spike (20; 55; 108; 151). Other toxic effectors are tethered to the VgrG spike through PAAR-repeat domains. PAAR and related DUF4150 domains form pyramidal structures that cap the end of the spike through β-complementation interactions (125). PAAR-containing effectors exhibit many different architectures, ranging from relatively small two-domain effectors to the large (~160 kDa) rearrangement hotspot (Rhs) proteins (17; 22; 33; 73; 104; 125). The latter effectors are characterized by RHS/YD-peptide repeats that form an expansive β-cage that encapsulates the C-terminal toxin domain (24). Other T6SS effectors are linked to the β-spike indirectly through DUF4123 adaptor proteins, which are encoded in operons with *vgrG* and effectors genes (77; 121; 135; 155). Because VgrG is sequestered within a ~17.5 nm chamber formed by TssK (32), effectors must be loaded onto the β-spike before the baseplate docks onto the trans-envelope complex. Electron cryotomography reveals that this chamber contains a mass consistent with VgrG-tethered effectors (29). Recent structural studies suggest that toxins are also loaded into the cavity formed by the N-terminal gp27-like domains of VgrG trimers (152). Hcp associated effectors are packaged within the lumen of the hexameric ring (127). Given that only a short segment of the tube is likely to be delivered into target bacteria, it is unclear whether every Hcp hexamer is loaded with an effector. Hcp associated effectors could also have affinity for the VgrG hub, which would ensure that toxin is preferentially loaded into the first hexamer of the nascent tube. It is also possible that effectors become depleted early in assembly, such that distal portions of the tube would be devoid of toxin. A subset of Hcp proteins carry fused C-terminal toxin domains with lipase, DNase and cell wall hydrolase activities (80). Given that the tube lumen can only accommodate one toxin domain, these latter effectors must form mixed hexamers with canonical Hcp.

T6SSs use mechanical force to breach the outer membranes of target bacteria (Fig. 5c). This process is presumably facilitated by the PAAR domain, which forms a sharp, metal-reinforced apex at the tip of the VgrG β-spike (125). However, VgrG also carries various globular toxin domains that dangle from the spike and potentially hinder translocation across the outer membrane. The initial impact of PAAR could create a membrane disruption, perhaps forming a pore large enough for toxin passage. Alternatively, the membrane could become hyper-fluidic as lipids are displaced by the PAAR domain. Regardless of the precise mechanism, non-covalently tethered toxins must resist shear forces associated with outer membrane passage. This is particularly important for PAAR-Rhs effectors, which auto-cleave their toxins and hold the released domains inside the β-cage structure (24; 43). Hcp encapsulated toxins are shielded from these forces, but the tube must presumably dissociate into individual rings after delivery to release its payload.

Transfer into the target-cell periplasm is sufficient for T6SS phospholipase and peptidoglycan amidase effectors, but nucleases and NADases must be delivered into the cytosol (Fig. 5c). Studies from the Mekalanos and Basler laboratories indicate that VgrG and Hcp are delivered directly into the cytoplasm of *Vibrio* (55; 141), but studies with VgrG

associated phospholipase toxins from other bacteria suggest that T6SS effectors are generally deposited into the periplasm (47; 119). Work on the PAAR-containing Tse6 effector from *P. aeruginosa* indicates that its N-terminal transmembrane helices insert into the inner membrane of target bacteria, resulting in translocation of the NADase domain into the cytosol (110). Presumably the entire effector remains membrane-bound, perhaps positioning it to more effectively disrupt electron flow to the respiratory chain. Some PAAR-Rhs effectors carry similar N-terminal transmembrane segments, which could initiate fusion of the RHS β-cage with the membrane to release the encapsulated nuclease toxin into the cytosol. These hydrophobic helices must be prevented from inserting into membranes prior to export. Dimeric effector-associated gene (Eag) chaperones bind these helices and presumably help guide the effectors into the T6SS apparatus (33; 43; 147). Notably, some evolved VgrG (e.g. WP_000371003.1) and Hcp (e.g. WP_000502504.1) nuclease effectors contain central pyocin S translocation domains (PF06958) (15; 80). These translocation domains are not found in similar effectors that carry phospholipase and muramidase toxins (55; 80), suggesting they may be specifically required to deliver nucleases into the cytosol.

Neisserial MafB effectors

MafB proteins are polymorphic effectors encoded on five genomic islands in strains of *N. meningitidis* and *N. gonorrhoeae* (6; 65; 157). MafB carries a signal peptide and a conserved N-terminal DUF1020 (PF06255) domain that defines the family. MafB can be detected in culture supernatants when over-produced (65), but no mechanism has been established for export across the outer membrane. Tommassen and coworkers have proposed that MafA lipoproteins encoded on a subset of the *maf* islands may constitute a novel secretion pathway (6), though Jamet *et al.* found that MafA is not required for MafB secretion (65). The Nassif and Tommassen labs have both demonstrated MafB-dependent competition, and it appears that inhibition only occurs on solid media when effectors are expressed at endogenous levels (6; 65). This latter finding may indicate that toxin transfer is cell-contact or proximity dependent, though this has yet to be tested explicitly.

Esx secretion systems (ESS) in Gram-positive bacteria

The ESS was discovered in *Mycobacterium tuberculosis*, which uses the pathway to export EsxA and EsxB, which are ~100 residue peptides that form α-hairpins with a conserved WXG motif in the connecting turn. These particular WXG100 proteins are critical for *M. tuberculosis* pathogenesis (130), but related systems to function in iron uptake and competence (50; 126). Mark Pallen first recognized that *Bacillus, Clostridium* and *Staphylococcus* species encode WXG100 homologs, and hypothesized that Gram-positive bacteria harbor ESSs (98). These predictions were quickly verified for *Staphylococcus aureus* and *Bacillus anthracis* (23; 48). It was also recognized early on that the Gram-positive WXG100-like proteins carry extended C-terminal domains with homology with CdiA, Rhs and MafB proteins (48; 98). Zhang *et al.* formally hypothesized that these latter extended WXG proteins, and similar LXG-motif containing proteins, mediates inter-bacterial competition (157; 158).

ESS mediated export relies on at least six proteins. EsaA, EssB and EssA are integral membrane proteins that likely form the core export channel, and EsaB is a required cytosolic

factor (23). Substrate selection is governed by membrane-bound EssC, which is an FtsK/SpoIIIE family AAA+ ATPase that also powers secretion (23; 63). LXG/WXG effectors heterodimerize with EsxA through their N-terminal α-hairpin domains in order to be exported through the ESS (25; 146). *S. aureus* also encodes a peptidoglycan hydrolase, EssH, which is required for effector release into the supernatant (16). This suggest that a channel must be excavated through the cell wall in some species. There are intervening cistrons between the chaperone and effector genes in *Streptococcus intermedius* (146), raising the possibility that additional proteins guide effectors to the core export machinery. The ESS of *S. intermedius* is capable of inhibiting various Gram-positive species during co-culture on solid media (146). There are conflicting reports on the role of the *S. aureus* ESS in bacterial competition. Missiakas and coworkers found that EssD/EsaD DNase effector was principally used against host cells to modulate immune responses (97), whereas Palmer and coworkers reported that over-expression of this effector provides a competitive advantage over non-immune target bacteria (25). The mechanistic details of WXG/LXG effector transfer between bacteria are almost completely unexplored. Mougous and coworkers showed that purified LXG effectors have no effect when added to susceptible bacteria, and that direct cell-to-cell contact is required for toxin transfer (146). It remains to be determined how ESS effectors are conducted across the thick cell wall of Gram-positive target bacteria, but some WXG/LXG effectors contain central domains that may mediate translocation across the cell membrane (146).

Myxobacterial outer-membrane exchange (OME)

Myxobacteria are soil bacteria that exhibit several cooperative behaviors, including social motility and fruiting body formation (96). These bacteria also have the remarkable ability to share outer membrane lipids and OMPs through outer-membrane exchange (OME) (101; 102). This process requires TraA and TraB proteins in both partners, and gliding motility is critical to properly align the cells for exchange. TraA is a cell-surface protein with a variable N-terminal domain that mediates homotypic interactions with compatible TraA receptors on partner cells (Fig. 6) (102). TraB contains a predicted β-barrel and C-terminal OmpA-like domain that presumably anchors it to the cell wall. Molecular flux is remarkably rapid during OME, with bi-directionally exchanged lipoproteins equilibrating between partner cells within minutes. The biophysical basis of exchange remains poorly understood, but the efficient transfer of lipid is consistent with membrane fusion. Although prodigious quantities of lipid and protein cargo are exchanged, TraA itself is not transferred, presumably due to its interaction with peptidoglycan-tethered TraB. Wall and co-workers have demonstrated that healthy bacteria can use OME to help siblings repair damage to their cell envelopes (138). Thus, *traA* is an example of a ‘greenbeard’ gene – a theoretical locus that imparts the ability to recognize and direct benefits toward other individuals that carry the same allele (84). Because OME only requires congruence at a single locus, valuable resources could be donated to non-sibling cells that happen to share compatible *traA* alleles. *Myxobacteria* mitigate this risk by delivering toxic SitA lipoproteins during OME (Fig. 6) (40; 139). SitA effectors carry several different classes of C-terminal nuclease domains (139; 140), which must ultimately be transferred into the cytoplasm. Translocation to cytoplasm has not been examined in detail, but it appears to require a specific integral membrane protein OmrA (41). Mutants lacking OmrA participate in OME, but are resistant to SitA-mediated swarm

inhibition, suggesting that this integral membrane protein is hijacked for toxin import. Remarkably, SitA lipoproteins can be serially exchanged between multiple cells before they enter the cytoplasm (139), indicating that cell entry is slow relative to the frequency of OME. This time delay may help ensure that SitA toxins do not engage in futile auto-delivery. It is not known whether the lipid anchor must be removed before the toxin can be transported to the cytoplasm, but this could provide a mechanism to control the kinetics of toxin delivery.

CONCLUDING REMARKS

Polymorphic toxin delivery systems have emerged as important mediators of inter-bacterial competition and self/nonself recognition. In addition to the obvious ecological implications, polymorphic effector systems have garnered interest as platforms for novel antibacterial therapies. Indiscriminant use of antibiotics has fueled the emergence of multidrug resistant bacteria, and antibiotic-induced dysbioses can lead to catastrophic *Clostridium difficile* infections (31). New approaches are clearly needed to treat bacterial infections. While challenging to implement, strategies that utilize phages, bacteriocins or polymorphic toxin delivery systems may be the key to target specific bacterial pathogens. In principle, non-pathogenic strains could be armed to edit microbial communities precisely, removing unwanted residents, while leaving commensals unmolested.

Many toxins carried by polymorphic effectors can be delivered into both Gram-negative and Gram-positive target bacteria. The seeming ease with which these domains traverse cell envelopes belies the formidability of these barriers. The Gram-negative outer membrane excludes a host of small hydrophobic and hydrophilic compounds, yet five of the effector classes reviewed here are capable of translocating 15 kDa enzymatic domains across this structure. Toxin domains are clearly modular, but there is often bias in the cargoes carried by effector classes. E colicins are limited to three types of nuclease domains, presumably because the translocation pathway imposes strict biophysical constraints on the cargo. By contrast, CdiA effectors deliver the same DNase, rRNase and tRNases as colicins, but also carry more than 100 other toxin sequence types. Perhaps the advent of the cytoplasm entry domain allowed CdiA to expand its toxin repertoire. More work is needed to understand the mechanisms that govern protein retro-translocation into bacterial cells. Once these rules are established, perhaps we can fully engineer polymorphic effectors to shape microbial communities.

Finally, it is certain that more toxin delivery mechanisms remain to be discovered. Aravind and coworkers proposed that PrsW, TcdB/TcaC and phage head morphogenesis proteins function in inter-bacterial competition (157), but there are still no experimental studies that test these hypotheses. Database queries with known toxin sequences often recover proteins of unknown function, suggesting they are the effector arms of novel toxin-delivery systems. For example, EndoU RNase domains are fused to PAAR and VgrG-like proteins in *Clostridium* and *Paenibacillus*, though these species encode no other T6SS components. These genes may be non-functional dead-end products of horizontal gene transfer. Alternatively, perhaps Gram-positive bacteria harbor an unrecognized phage-derived competition apparatus. Given the rapidly accelerating pace of research into polymorphic

effectors, we anticipate that several more inter-bacterial competition mechanisms will be described in the near future.

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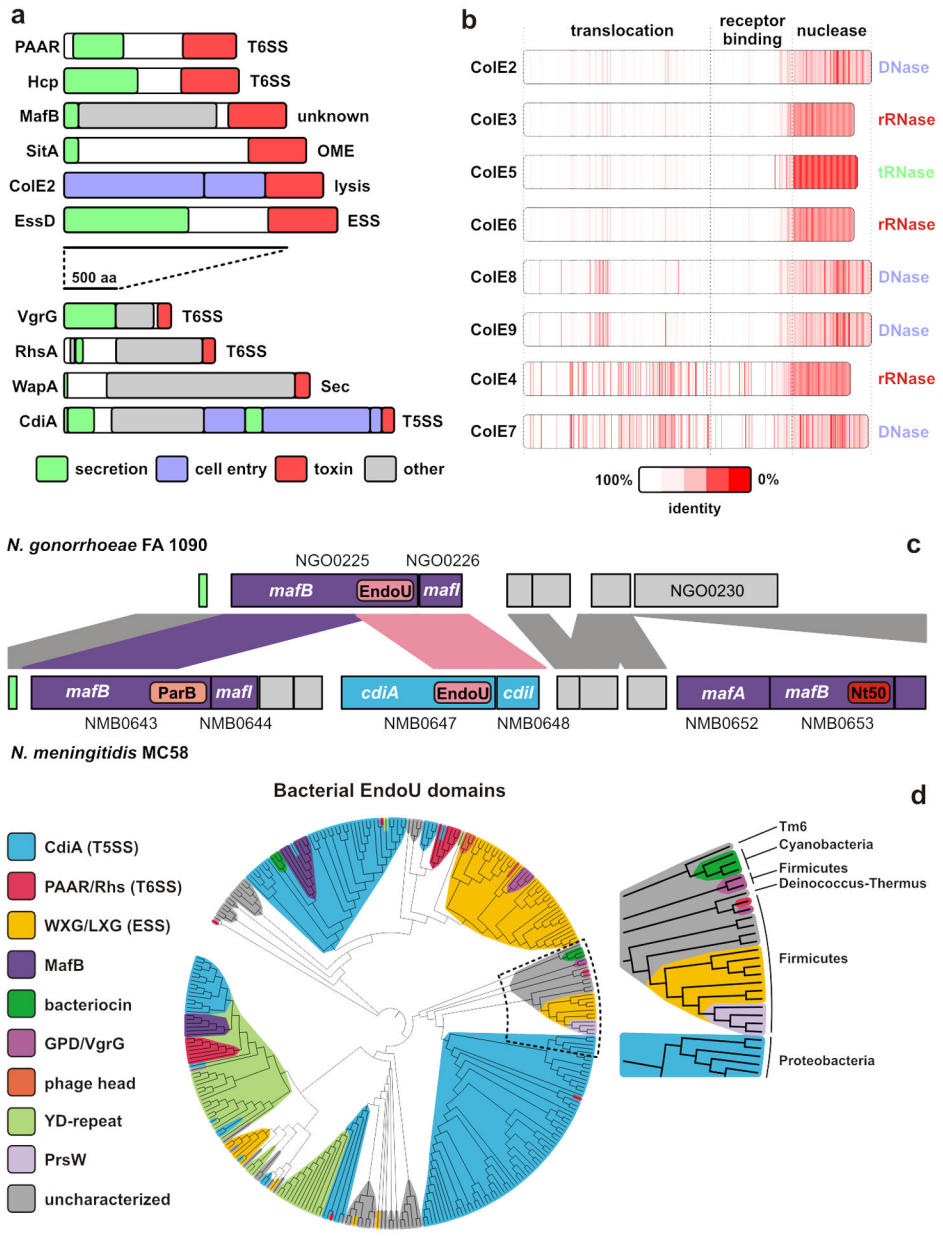

Figure 1. Polymorphic effector families.

a) Polymorphic effector classes that function in bacterial competition. **b**) E-type colicin domain structure and homology. **c**) Alignment of *maf* genomic islands from *N. gonorrhoeae* FA 1090 and *N. meningitidis* MC58. Ordered locus identifiers are given and sequences encoding predicted ParB, EndoU and Ntox50 (Nt50) toxin domains are indicated. **d**) Distribution of EndoU (PF14436) RNase domains across effector classes. Abbreviations: ESS, Esx-like secretion system; Hcp, hemolysin-coregulated protein; OME, outer membrane exchange; T5SS, type V secretion system; T6SS, type VI secretion system.

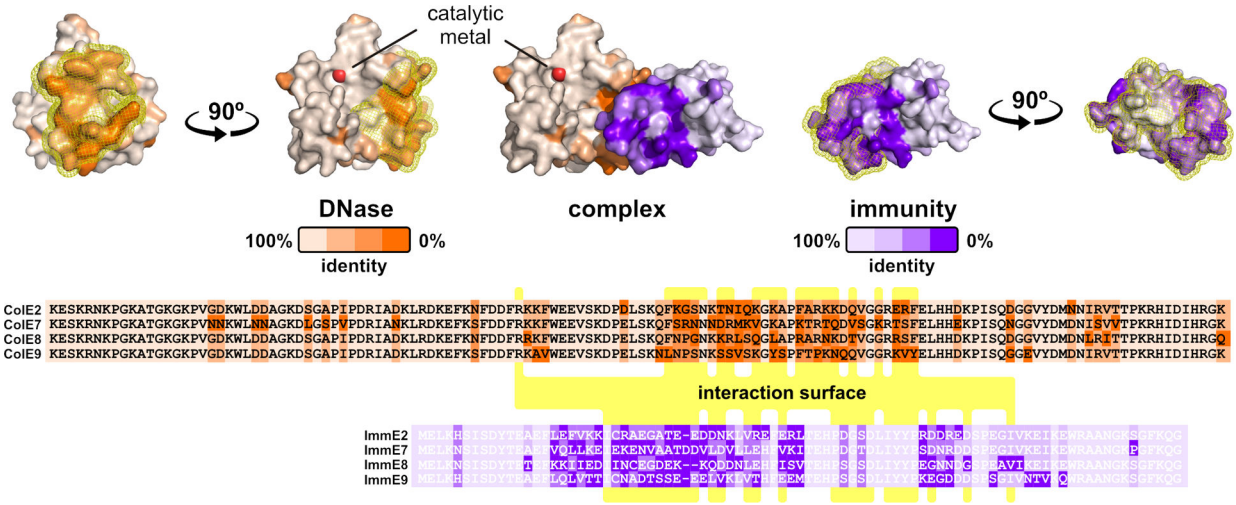

Figure 2. Diversification of toxin-immunity pairs.

Sequence variation is mapped onto the crystal structure of the ColE2 DNase domain bound to its immunity protein. The interaction surface is highlighted with yellow mesh, and residues making direct contact are indicated with yellow in the alignments. The DNase catalytic center is indicated by the divalent metal ion.

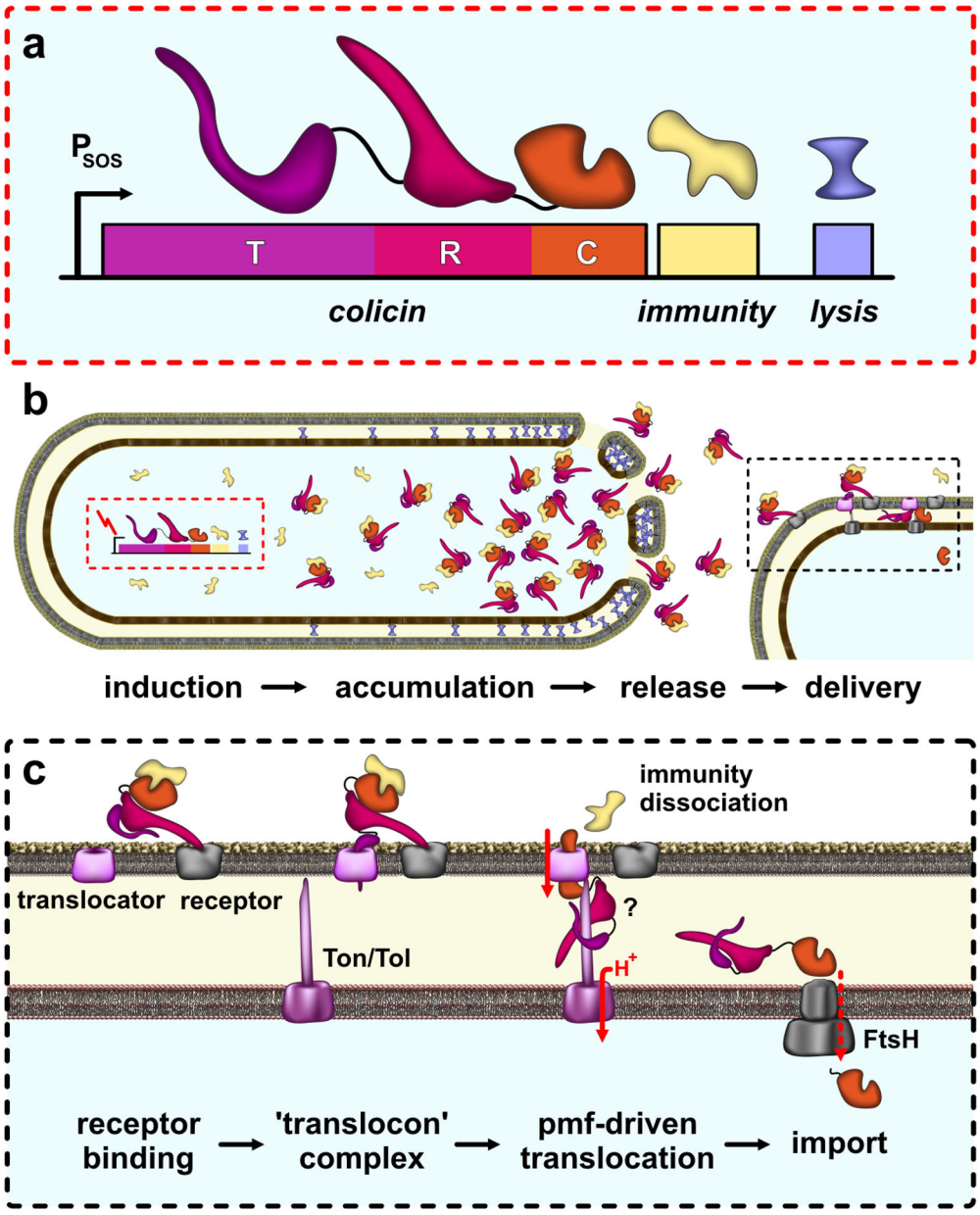

Figure 3. Colicins.

a) Colicin operon organization. The bacteriocin release protein is encoded by the *lysis* gene, which is separated from the upstream colicin and immunity cistrons by a transcription terminator. **b**) Colicin release. Induction of colicin expression allows read-through of the terminator, producing release proteins that disrupt the cell envelope to release colicin-immunity complexes. **c**) Colicin delivery. Colicins use R domain to recognize OMP receptors on target bacteria, then recruit a second OMP translocator using the T domain. Translocation is energized by the pmf through Tol or Ton systems. Nuclease domains require FtsH for import to the cytosol.

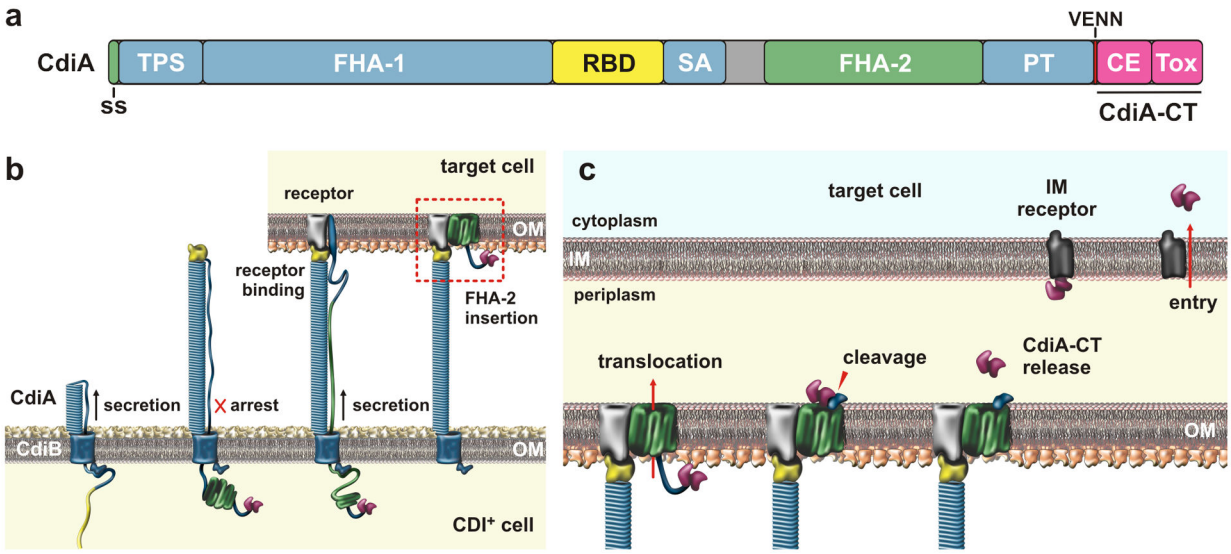

Figure 4. Contact-dependent growth inhibition (CDI).

a) CdiA domain architecture. The CdiA-CT is composed of cytoplasm entry (CE) and toxin domains; Abbreviations: ss, signal sequence; TPS, two-partner secretion transport domain; RBD, receptor-binding domain; SA, secretion arrest domain; PT, pretoxin domain; and CE, cytoplasm entry domain. **b**) CdiA biogenesis. CdiB guides CdiA export across the outer membrane (OM). The C-terminal half of CdiA remains sequestered in the periplasm due to a programmed secretion arrest. CdiA export resumes upon binding receptor, and the FHA-2 domain integrates into the target-cell OM to form a toxin translocation conduit. **c**) Toxin delivery. Once transferred into the periplasm, the CdiA-CT is released for a second translocation step across the inner membrane (IM). The final step is mediated by cytoplasm entry domains, which interact with membrane proteins and translocate the C-terminal toxin domain in a pmf-dependent manner. Abbreviations: CE, cytoplasm entry domain; CT, C-terminal region; IM, inner membrane; OM, outer membrane; PT, pretoxin domain; RBD, receptor-binding domain; SA, secretion arrest domain; ss, signal sequence; Tox, toxin domain; TPS, two-partner secretion transport domain.

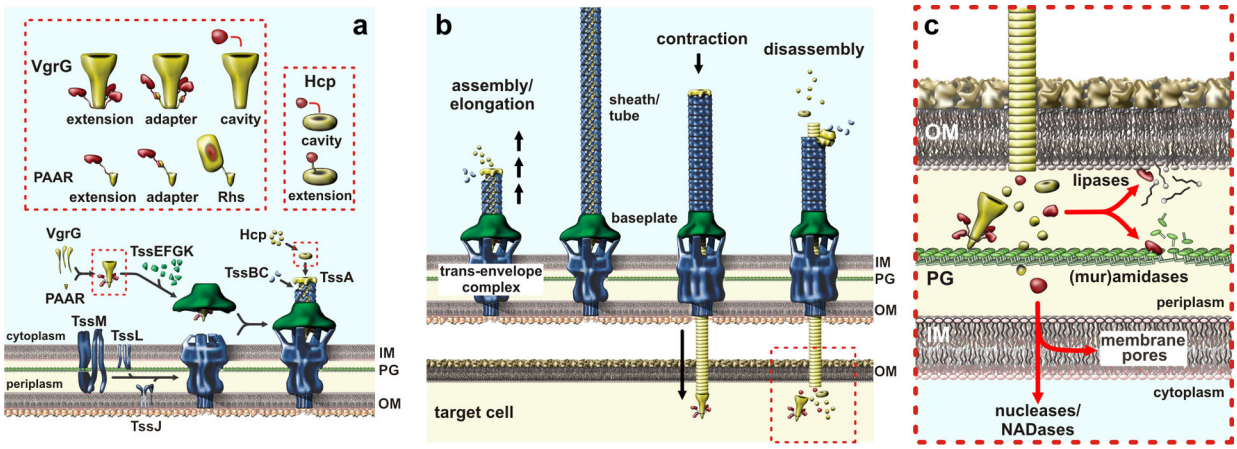

Figure 5. Type VI secretion systems.

a) T6SS sub-complex assembly and effector packaging onto VgrG and Hcp. **b**) Sheath-tube assembly initiates at the baseplate and extends across the width of the cell. The sheath rapidly contracts, expelling the Hcp tube through the baseplate and trans-envelope complexes. The PAAR-tipped VgrG protein penetrates the OM of target bacteria and releases effector proteins. **c**) T6SS effector proteins activities. Nucleases and NADases are translocated into the cytoplasm through poorly understood pathways. Abbreviations: Hcp, hemolysin-coregulated protein; IM, inner membrane; NADase, $NAD(P)^+$ glycohydrolase; OM, outer membrane; PG, peptidoglycan; Rhs, rearrangement hotspot; T6SS, type VI secretion system; VgrG, valine-glycine repeat G protein.

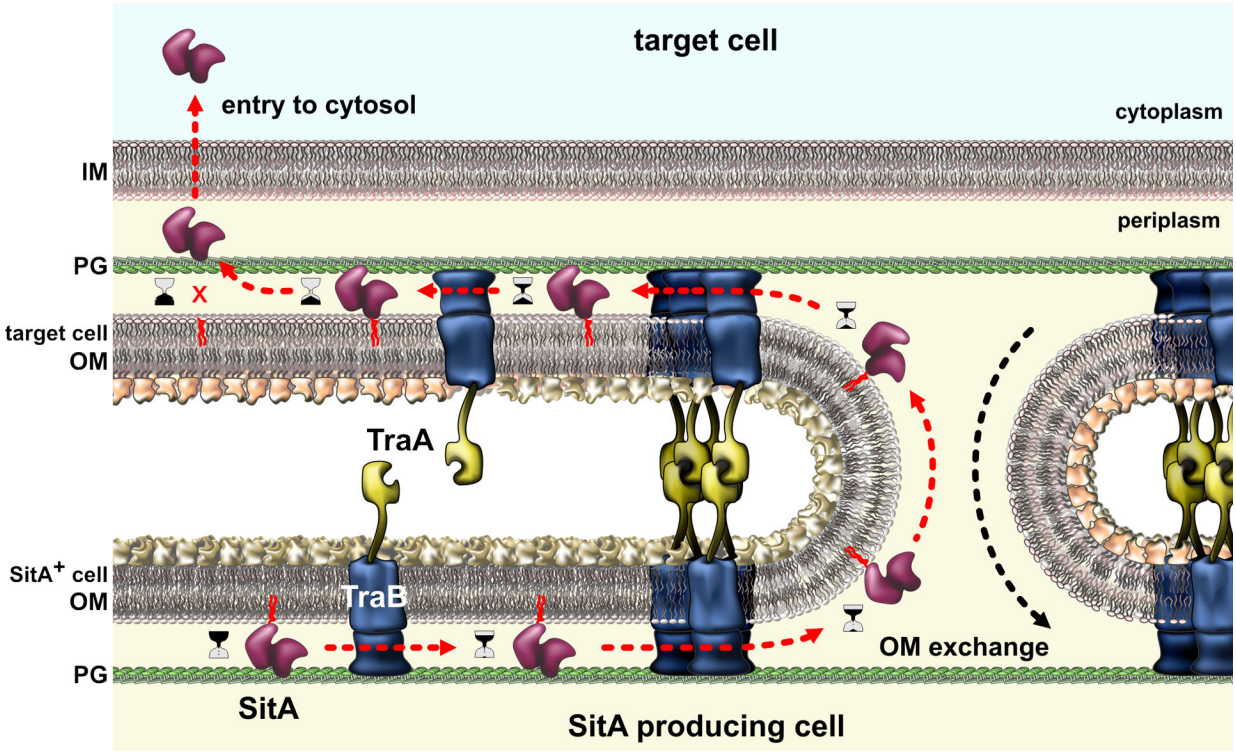

Figure 6. Outer-membrane exchange.

Myxobacteria with compatible TraA proteins engage in homotypic interactions that bring the cells into close approximation for outer-membrane fusion. OM lipids and proteins are freely exchanged between the cells, but TraA itself is not transferred due to interactions with TraB, which is tethered to the peptidoglycan (PG) cell wall. Polymorphic SitA effectors are among the many lipoproteins transferred during OME. SitA nucleases ultimately disengage from the OM and enter the cytoplasm through an uncharacterized mechanism.

Abbreviations: IM, inner membrane; OM, outer membrane; PG, peptidoglycan.