

# New Players in the Toxin Field: Polymorphic Toxin Systems in Bacteria

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**ABSTRACT** Bacteria have evolved numerous strategies to increase their competitiveness and fight against each other. Indeed, a large arsenal of antibacterial weapons is available in order to inhibit the proliferation of competitor cells. Polymorphic toxin systems (PTS), recently identified by bioinformatics in all major bacterial lineages, correspond to such a system primarily involved in conflict between related bacterial strains. They are typically composed of a secreted multidomain toxin, a protective immunity protein, and multiple cassettes encoding alternative toxic domains. The C-terminal domains of polymorphic toxins carry the toxic activity, whereas the N-terminal domains are related to the trafficking mode. *In silico* analysis of PTS identified over 150 distinct toxin domains, including putative nuclease, deaminase, or peptidase domains. Immunity genes found immediately downstream of the toxin genes encode small proteins that protect bacteria against their own toxins or against toxins secreted by neighboring cells. PTS encompass well-known colicins and pyocins, contact-dependent growth inhibition systems which include CdiA and Rhs toxins and some effectors of type VI secretion systems. We have recently characterized the MafB toxins, a new family of PTS deployed by pathogenic *Neisseria* spp. Many other putative PTS have been identified by *in silico* predictions but have yet to be characterized experimentally. However, the high number of these systems suggests that PTS have a fundamental role in bacterial biology that is likely to extend beyond interbacterial competition.

Colonization of niches implies a struggle for space and nutrients. Secretion of proteinaceous toxins is a strategy widely used by prokaryotic and eukaryotic organisms to restrict growth of competitors. The structures, the mechanisms of action, and the genomic organizations of loci encoding toxic proteins are highly diverse. Toxic proteins (i.e., cholera, anthrax, or botulinum toxins) can be deployed by bacterial pathogens against their hosts or used to target neighboring bacterial cells. In this review, we focus on a recently described family of secreted multidomain toxins, termed polymorphic toxins, which are primarily involved in interbacterial competition between related strains. A hallmark of loci encoding an antibacterial toxin is that they also encode an immunity protein (see Fig. 1A) able to protect the producing cell both from autointoxication and from toxin produced by clone-mates. Polymorphic toxins (PT) are typified by a modular organization. The N-terminal region is related to secretion, and the C-terminal region harbors a toxic domain. In addition, filamentous toxins exhibit repeats of several domains from the same family, such as the RHS/YD or filamentous hemagglutinin repeats. A striking feature of polymorphic toxins is that a given N-terminal region can be found fused to distinct toxic domains and a given toxic domain can be found fused to distinct N-terminal regions (Fig. 1B) (1).

In addition to the toxin gene, the typical locus of polymorphic toxin systems (PTS) includes an immunity gene that confers protection against the toxin. The locus may also contain additional genes involved in the secretion of the toxin and genes encoding alternative toxic domains associated with cognate immunity genes (Fig. 1A). Genes encoding alternative toxin cassettes do not encode N-terminal trafficking modules but might recombine with the 3' extremity of the full-length toxin gene to generate new toxins with the same N-terminal region but different C-terminal toxin domains. These genes encoding alternative toxic domains with their cognate immunity genes are named orphan modules (Fig. 1A) (2).

The sets of toxin domains harbored by different isolates may be highly divergent even among strains of the same species.

In most PTS, regardless of the toxin delivery mode, the gene encoding the toxin is found immediately upstream of a gene encoding the cognate immunity protein (1, 3). Indeed, numerous new toxin genes have been discovered recently by computational analysis based on the fact that they were located in close proximity of immunity genes (Fig. 1C). Toxic domains found at the C-terminal regions of polymorphic toxins encompass nuclease domains targeting genomic DNA, tRNA, or rRNA, nucleic acid-modifying enzymes such as deaminases and peptidases, and protein-modifying enzymes such as ADP-ribosyltransferases (1).

## THE SUKH SUPERFAMILY OF IMMUNITY PROTEINS AND THE DISCOVERY OF PTS

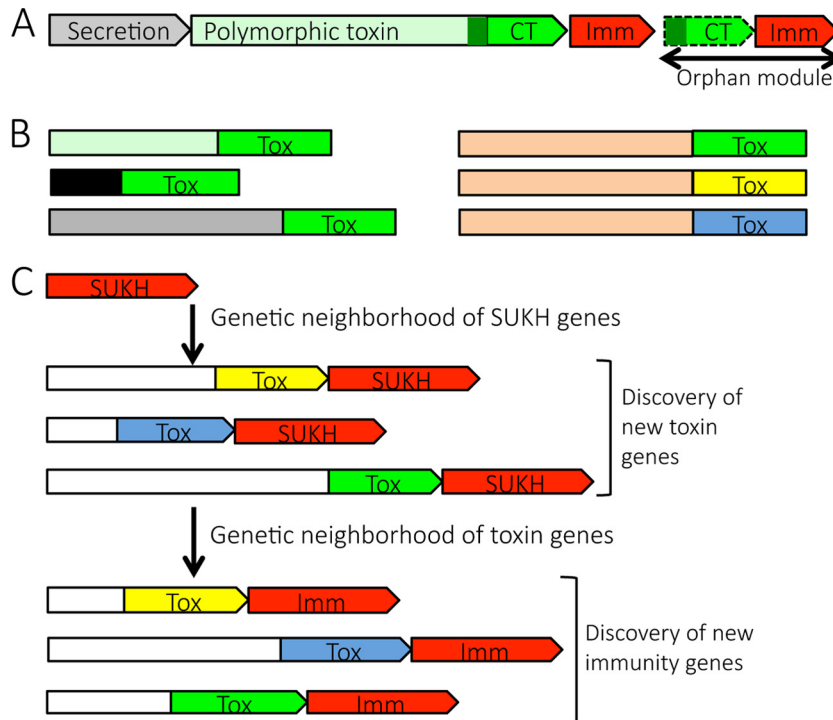
The SUKH superfamily was reported in 2011 by Zhang et al. (3). The name SUKH stands for Syd, US22, Knr4 homology domain superfamily. This superfamily is prototyped by baker's yeast *Saccharomyces cerevisiae* protein Knr4. However, SUKH domains are also widespread in bacteria. Despite the low sequence similarity across the whole SUKH superfamily, the structural core of the SUKH domain consisting of four conserved helices and six strands is predicted in all the representative proteins (3, 4). With the aim to further understand the role of the SUKH domains, Zhang et al. performed an *in silico* analysis of the genomic neighborhoods of hundreds of bacterial genes encoding a protein with a SUKH domain. Indeed, searching for conserved genomic neighborhoods is

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**FIG 1** Definition and discovery of polymorphic toxins. (A) General organization of a typical locus encoding a polymorphic toxin with its cognate immunity protein (Imm). A gene encoding a protein involved in the secretion of the toxin can be found upstream of the toxin gene (such as *cdiB*) and orphan modules encoding alternate toxic domains (CT), and their cognate immunity proteins can be found downstream of the toxin gene. A homologous region possibly involved in recombination between the toxin gene and the orphan module is colored in dark green. (B) Schema illustrating the possibility for a given N-terminal region to be fused to distinct toxic domains (Tox) and for a given toxic domain to be fused to distinct N-terminal regions. (C) *In silico* discovery of polymorphic toxins and their immunity proteins. By analyzing genetic neighborhood of SUKH genes, Zhang et al. (3) discovered that genes encoding various nucleic toxic activities were located immediately upstream of SUKH genes. SUKH genes are depicted as red arrows labeled SUKH, and toxin genes encoding various toxic activities are depicted as white arrows with ends of different colors labeled Tox (upper panel). The lower panel shows that the analysis of the genetic neighborhood of toxin genes enabled the discovery of additional genes encoding immunity proteins which are not part of the SUKH superfamily. These immunity genes are depicted as red arrows labeled “Imm.” Immunity proteins are encoded by small ORFs that may contain a domain of the SUKH superfamily (e.g., SUKH-1, SUKH-2, SUKH-3. . .) or a domain of another family (e.g., Imm1, Imm2, Imm3. . .).

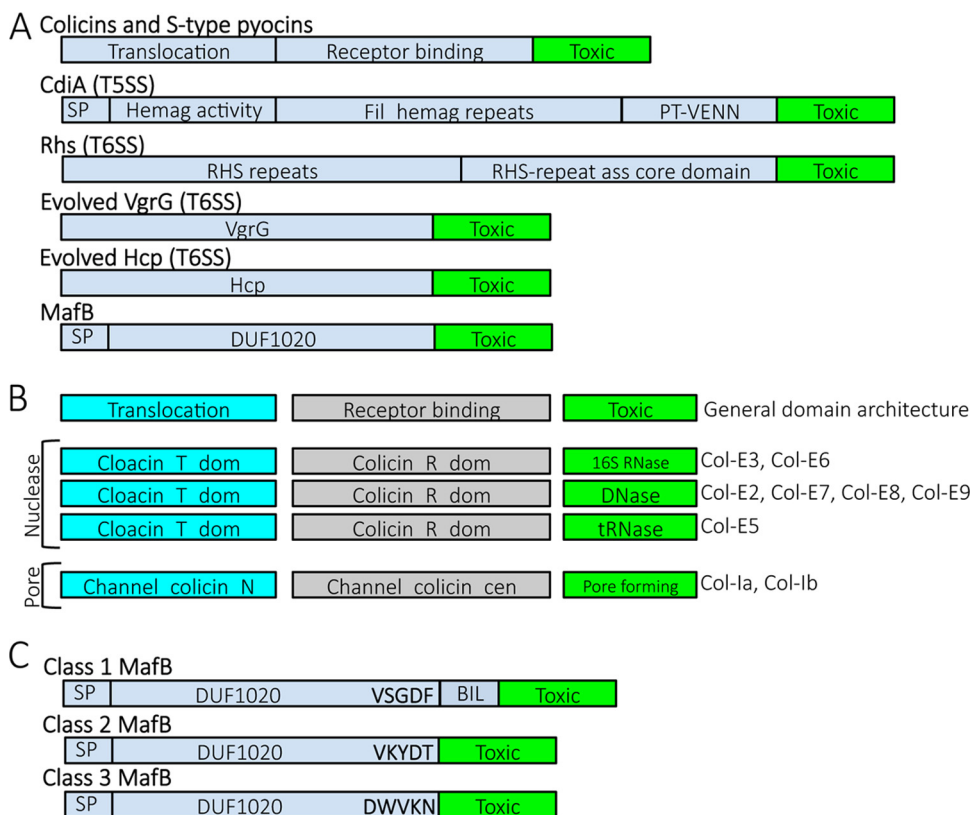
a powerful means of functional inference for proteins of unknown function. The results of the analysis of neighboring genes showed that there is a clear linkage of SUKH genes with genes encoding different types of nucleases (Fig. 1C, upper panel). Therefore, SUKH genes were believed to encode immunity proteins able to neutralize the toxicity of the nuclease encoded by the gene found upstream in the same operon. This strategy allowed the identification of more than 10 distinct families of putative nucleic acid-targeting toxins encoded by genes immediately upstream of SUKH genes.

Using the same contextual approach, and starting from nucleic acid-targeting toxin sequences, Zhang and coworkers predicted that the function of gene products encoded immediately downstream of these newly described toxin domains was as immunity proteins (Fig. 1C, lower panel) and therefore identified hundreds of new toxins and immunity proteins across all major bacterial genera (1). These toxins, together with the corresponding immunity proteins, defined the widespread polymorphic toxin systems.

### COLICINS AND S-TYPE PYOCINS

Colicins and colicin-like bacteriocins can also be included in PTS family since they exhibit the same typical features. In contrast, microcins and Gram-positive bacteriocins, which are small proteins with a molecular mass of below 10 kDa, are not polymorphic toxins.

Colicins produced by *Escherichia coli* and S (soluble)-type pyocins produced by the pseudomonads are the most extensively studied of the Gram-negative bacteriocins (reviewed in references 5 and 6). A large proportion of *E. coli* strains isolated from the human gastrointestinal tract produce colicins (7, 8), thus suggesting an important ecological role in the control of intestinal microbiota. Colicins have a narrow killing spectrum, targeting mainly strains of the same or of closely related species. The colicin gene cluster is located on pCol plasmids and consists in a colicin gene (*cx<sub>a</sub>*, where “*x*” is different for each colicin) and a cognate immunity gene (*cx<sub>i</sub>*). In many cases, a third gene in the same locus (*cx<sub>l</sub>*) encodes a lysis protein involved in the release of colicin by cell wall leakage, resulting in the death of the producing cell. Most colicins have either a pore-forming or a nuclease activity. The orientation of the immunity gene depends on the toxic activity of the colicin; i.e., the immunity gene is in an operon with nuclease colicins and located on the opposite DNA strand of pore-forming colicins (5). Nuclease colicins include colicins E3, E4, and E6 that cleave rRNA, colicins E5 and D that cleave tRNA, and colicins E2, E7, E8, and E9 that cleave DNA. Pore-forming colicins include colicins A, B, E1, Ia, and Ib. Colicins are modular multidomain proteins of typically less than 100 kDa. They contain an N-terminal translocation domain, a central receptor binding do-



**FIG 2** Overview of the polymorphic toxin families. (A) Simplified schematic representation of the domain organization found in several well-characterized families of polymorphic toxins. Domains constituting the conserved N-terminal region of polymorphic toxins are depicted as blue boxes irrespective of their family. Toxic domains of the variable C-terminal region are depicted as green boxes irrespective of their toxic activity. The secretion system involved in the secretion of the toxin is indicated. T5SS, type V secretion system; T6SS, type VI secretion system; SP, signal peptide; Hemag activity, hemagglutination activity domain, also called a “TPS domain” (PF05860); fil hemag repeats, filamentous hemagglutinin repeats (PF13332); RHS repeat ass core domain, RHS repeat-associated core domain (IPR022385). (B) Domain architectures found in colicins. Colicins usually contain three domains: an N-terminal translocation domain allowing translocation into target cells (blue boxes), a central domain involved in receptor recognition (gray boxes), and a C-terminal cytotoxic domain (green boxes). The short name of the corresponding domain from the Interpro database is written inside the blue and gray boxes. Cloacin\_T\_dom, cloacin translocation domain (IPR016128); colicin\_R\_dom, colicin receptor domain (IPR024566); channel\_colicin\_N, channel-forming colicin, N-terminal domain (IPR014739); channel\_colicin\_cen, channel-forming colicin, central receptor recognition domain (IPR014740). The toxic activity of the C-terminal domain is indicated in the green boxes as follows: 16S RNase (IPR009105), DNase (IPR024622), tRNase (IPR021964), Pore forming (pore-forming domain; IPR000293). Col, colicin. (C) Simplified schematic representation of the 3 classes of MafB toxins. Class 1 MafBs contain a VSGDF motif at the end of the N-terminal conserved domain and an optional bacterial intein-like (BIL) domain. Class 2 MafBs contain a VKYDT motif at the end of the N-terminal conserved domain. Class 3 MafBs contain a DWVKN motif at the end of the N-terminal conserved domain. Domains in panels A, B, and C are not depicted to scale.

main, and a C-terminal cytotoxic domain (Fig. 2A and B). The modularity of colicin domains suggests that colicin evolution is driven by exchange and mixing of domains from heterologous colicins. For instance, the 410 N-terminal residues of colicin E3 and E8 are 95% identical, whereas the C-terminal toxic domains are less than 35% identical.

The release of group A colicins (e.g., colicin A, E1 to E9, K, L, N, S4, U, and Y) is accompanied by the death of the colicinogenic cell due to the production of the lysis protein. No lysis gene has been identified in the vicinity of other polymorphic toxin genes. In addition, group B colicins (e.g., colicin B, D, H, Ia, Ib, M, 5, and 10) are usually not associated with a lysis gene. Whether or how group B colicins are released into the extracellular medium is currently not clear.

Like other polymorphic toxin systems, colicin systems have a modular multidomain architecture of the toxins with a toxic C-terminal region (Fig. 2B). Several homologous toxin domains are shared with other polymorphic toxin systems such as the nu-

lease domains of the HNH family. In contrast to the Cdi, Rhs, and Maf systems described below, colicin loci do not contain orphan modules. However, some colicin loci contain several immunity genes downstream of the colicin gene (9).

As mentioned above, colicins are encoded by plasmids, whereas other polymorphic toxin genes are found on chromosomes. Interestingly, S-type pyocins share many features with colicins except that they are encoded by chromosomes and that there is no lysis gene found in the same cluster. They are also modular proteins composed of three domains (the translocation, receptor binding, and toxic domains). It was suggested that S-type pyocins could be released by a phage-like lytic system (6).

#### **CdiA AND CdiB: TWO-COMPONENT SYSTEMS INVOLVED IN CONTACT-DEPENDENT INHIBITION OF GROWTH**

Members of the David Low laboratory first reported the phenomenon of contact-dependent growth inhibition (CDI) in 2005 in *E. coli* strain EC93 (10). The predominance of *E. coli* EC93 in rat

intestine and the ability of this strain to inhibit the growth of the *E. coli* K-12 laboratory strain suggested that EC93 was able to produce a colicin or a similar toxin against its competitors. However, the requirement for a direct contact between EC93 and target *E. coli* cells contrasted with the mode of action of a diffusible factor such as colicins. Hence, genes involved in this inhibitory phenomenon were named *cdiB*, *cdiA*, and *cdiI* (for “contact-dependent growth inhibition”). The introduction of the *cdiBAI* locus into *E. coli* K-12 was sufficient to confer the contact-dependent inhibitory phenotype, and the *cdiI* gene was sufficient to confer protection (10). CdiA and CdiB belong to the family of two-partner secretion proteins. Two-partner secretion systems (SS) constitute a subclass of type V secretion systems (type Vb) in which an outer membrane transporter of the TpsB family allows the translocation of a TpsA family protein (11). One of the best-studied TpsA/TpsB pairs is FhaA/FhaC of *Bordetella pertussis* where FhaC exports the filamentous hemagglutinin named FHA (12, 13). TpsA proteins are very large proteins (more than 3,000 amino acids [aa]) that can carry adhesive (i.e., FHA) (13), hemolysis (i.e., the ShIA hemolysin from *Serratia marcescens*) (14), or toxic (i.e., CdiA toxins) properties (10).

The CdiA/CdiB pairs constitute a distinct subfamily of two-partner secretion proteins (15). CdiB is an outer membrane pore-forming protein allowing secretion of the CdiA polymorphic toxin. CdiA is a large filamentous protein whose N-terminal region is homologous to that of *B. pertussis* FHA. Indeed, the N-terminal regions of CdiA and FHA contain typical hemagglutinin repeats (PF13332) and a hemagglutination activity domain also called a “TPS domain” (PF05860) (Fig. 2A) (16). In contrast to FHA, the CdiA N-terminal region ends with a conserved domain designated PT-VENN (2, 16). PT-VENN domains are located immediately upstream of the CdiA C-terminal toxin (CdiA-CT) region (Fig. 2A). The function of VENN motif is not yet known, but it may play a role in autocleavage to release the CdiA-CT toxin for delivery into target bacteria. While the first 3,000 aa of CdiA proteins are highly similar, the polymorphic CdiA-CT domains are about 100 to 200 residues long and highly variable (16). The CdiA-CT domain of CdiA interacts directly with the CdiI protein, encoded by a gene immediately downstream of *cdiA*, thus counteracting the toxic activity of CdiA and preventing autoinhibition (16–18).

The CdiA toxins form long filaments extending from the surface of the cell and are usually compared to a stick with a toxic tip (19). CdiA<sup>EC93</sup> is likely to be a pore-forming toxin (20), while CdiA<sup>EC869</sup> is a nuclease that degrades target-cell genomic DNA (16, 17, 21) and CdiA<sup>536</sup> is a tRNA anticodon nuclease able to cleave all tRNA isoacceptors (16, 22).

**Additional partners required for CdiA activity.** Using transposon mutagenesis, Aoki and coworkers identified BamA (also known as YaeT or Omp85) and AcrB as important partners of CDI systems (23). However, whereas BamA seems to be a universal receptor of CdiA toxins in *E. coli*, AcrB is involved in only the CDI pathway of strain EC93 (24). BamA is required to transport and fold beta-barrel proteins in the outer membrane of Gram-negative bacteria (25). It has been demonstrated that CdiA<sup>EC93</sup> binds at extracellular loops 6 and 7 of the BamA protein of *E. coli* target cells. The extracellular loops are the most variable regions of this protein in *Enterobacteriaceae* species (26). However, the extracellular loops of BamA among *E. coli* strains are identical. The variability of these loops between *Enterobacteriaceae* species is re-

sponsible for the resistance of species such as *Salmonella* spp. or *Enterobacter* spp. to CdiA produced by *E. coli* (23, 27).

In the UPEC 536 isolate, an additional partner of the CDI pathway has been identified (22). Indeed, CdiA<sup>536</sup> is inactive in target cells until it binds to the CysK protein, which is then considered a “permissive factor.” CysK is an enzyme involved in cysteine biosynthesis that is necessary to allow CdiA<sup>536</sup> to cleave tRNA (22).

**Burkholderia CDI systems are involved in biofilm formation.** CDI systems of *Burkholderia* species have also been the focus of several studies (16, 28–31). *Burkholderia* CDI systems differ from similar systems found in other proteobacteria in several aspects. In *Burkholderia* species, the demarcation between the N-terminal region and the toxic CT domain consists of the (Q/E)LYN conserved motif instead of the VENN motif (28, 30). Furthermore, *Burkholderia cdi* genes (named “*bcp*,” for *Burkholderia CDI* proteins) are arranged in the *bcpA-bcpI-bcpO-bcpB* gene order instead of being in the typical *cdiB-cdiA-cdiI* gene order. Besides, orphan *bcpA*-CT and *bcpI* modules are absent from *Burkholderia cdi* loci (30). The *bcpO* gene found between *bcpI* and *bcpB* is unique to *Burkholderia cdi* loci and is required to induce inter-bacterial competition in *B. thailandensis* (28). Anderson et al. have suggested that BcpO could contribute to BcpA maturation. Another study focusing on three *B. pseudomallei* BcpA/BcpI pairs showed that the three toxins inhibit cell growth by cleaving tRNA at three distinct positions (30).

In addition to mediating competition between bacterial cells, the *B. thailandensis* BcpA toxin is required for biofilm formation and plays a role in biofilm architecture (29). This role in biofilm development involves the BcpA-CT region but is independent of its toxic activity. The exact molecular mechanism by which BcpA-CT modulates biofilm formation is unknown. Garcia et al. have suggested that BcpA-CTs, which target nucleic acids, could have a role on modifying extracellular DNA (eDNA), an important constituent of the biofilm matrix (29). The latter hypothesis suggests that BcpA-producing cells impede the growth of non-immune bacterial cells, i.e., cells lacking the cognate immunity gene, and that they participate, in conjunction with immune bacterial cells, in the formation of biofilm. Thus, CDI systems are involved both in competitive behaviors that include growth inhibition of competitors and in cooperative behaviors via biofilm formation.

**CDI systems in other bacterial species.** Several CdiA toxins outside species of the *Escherichia* and *Burkholderia* genera, such as a DNase from the plant pathogen *Dickeya dadantii* 3937, have also been characterized (16). CdiA/CdiB systems have also been experimentally characterized in *Enterobacter cloacae* (18) and in *Neisseria meningitidis* (32). Since these systems are widespread, their characterization in other species will undoubtedly be the focus of future studies.

## THE RHS FAMILY OF TOXINS

Rearrangement hot spot (RHS) repeats (PF05593) were first described in *E. coli* proteins (33) and were later found to be widespread in both Gram-negative and Gram-positive bacterial proteins (34–36). RHS repeats harbor a YDxxGRL(I/T) consensus sequence (37) and are also named YD (tyrosine/aspartate) repeats. Rhs proteins are large filamentous proteins of typically more than 1,500 residues. Their N-terminal core regions exhibit RHS repeats, whereas their C termini (or tip regions) are highly variable (36, 38) (Fig. 2A). In species of the *Enterobacteriaceae*, N-terminal core regions are separated from the C-terminal tip by the con-



served peptide motif PxxxxDPxGL (38). RHS repeats are also found in the C protein of the tripartite ABC insecticidal toxins of entomopathogen bacteria such as *Yersinia entomophaga*. The C-terminal regions of the C proteins found in *Y. entomophaga* are predicted to exhibit distinct toxic domains belonging to the cytotoxic necrotizing factor family and to the YwqJ-like deaminase family (39). The C protein possesses a self-cleavage site between the conserved N-terminal region and the variable C-terminal region in a domain called the RHS repeat-associated core domain (IPR022385) (39) (Fig. 2A). Determination of the structure of the complex formed by B and C subunits of ABC insecticidal toxin revealed that the N-terminal RHS repeat-containing region of C protein forms a large shell inside of which the cytotoxic portion of the C protein is sequestered, protecting the producing bacterial cell from the toxicity of the C protein. Another Rhs toxin with an activity against eukaryotic cells has been described in *Pseudomonas aeruginosa* PSE9. Indeed, RhsT protein (A6N5U6) is translocated into phagocytic cells where it activates the inflammasome (40).

Low and coworkers discovered that some loci encoding proteins with RHS repeats share many features with CdiBAI systems and are involved in contact-dependent growth inhibition during bacterial competition (2, 35). Their findings are in line with previous work of Vlazny and Hill (41) showing that the C-terminal tip of a Rhs protein is toxic in *E. coli* and that the coexpression of the downstream open reading frame (ORF) suppresses the toxic effect. Rhs and CdiA share similar architectures, with repeats in their central region. The two protein families share similar genetic organizations, with the presence of orphan modules carrying alternative *rhs-CT* and encoding cognate immunity proteins (RhsI) in many *rhs* loci. In addition, some Rhs-CTs are homologous to the toxin modules of CdiAs (2, 38). It has been shown that recombination between an orphan *rhs-CT* and a full-length *rhs* gene occurs *in vivo* and can provide a competitive advantage, as shown for *Salmonella enterica* serovar Typhimurium strain LT2 (42).

Even if Rhs proteins share similarities with CdiA proteins, they are secreted not through a type V secretion system but via a type 6 secretion system (T6SS). Indeed, in *P. aeruginosa* PAO1, a Rhs toxin named Tse5 (43) requires ClpV1 for its secretion, and a deletion of *clpV1* is known to inactivate the H1-T6SS (44). In addition, some T6SS effectors required an association with a protein named VgrG for their secretion (43). VgrG (for “valine-glycine repeat protein G”) proteins are predicted to form a trimeric complex located at the tip of the syringe-like injection apparatus of T6SS (45) and are also able to bind other effectors and to play a chaperone role during secretion (43). A requirement for an association with VgrG has been demonstrated for RhsA and RhsB of *D. dadantii* 3937, for RhsA and RhsB of *E. cloacae*, and for Tse5 of *P. aeruginosa* (35, 43).

As previously mentioned, RHS repeat-containing proteins are also found in Gram-positive bacteria (36). For instance, WapA (wall-associated protein A), an RHS repeat-containing protein of *Bacillus subtilis*, carries variable C-terminal toxic domains that are neutralized by a cognate immunity protein, WapI (35). WapA of *B. subtilis* strain 168 (BSU39230) is a tRNase and mediates contact-dependent growth inhibition (35). The CDI phenotype observed with *B. subtilis* is similar to that observed with Rhs and CdiA toxins of Gram-negative bacteria. However, WapA proteins exhibit canonical peptide signals and are likely to be exported by the general secretory pathway (35).

## THE Maf TOXINS IN PATHOGENIC NEISSERIA SPECIES

Maf proteins are encoded by genes belonging to the multiple adhesin family (*maf*) and were first described in the gonococcal strain MS11 (46). MafA has been shown to interact with a specific glycolipid (GgO4) and is believed to subsequently be an adhesin. The gene immediately downstream of *mafA* was then designated *mafB*, with *mafA* and *mafB* being organized in an operon (47). The function of *mafB* genes and of the numerous small ORFs found in the same loci was unknown. We recently analyzed loci containing *maf* genes in both *N. meningitidis* and *N. gonorrhoeae* and demonstrated that *mafB* genes encode secreted polymorphic toxins neutralized by specific immunity proteins encoded by *mafI* genes found immediately downstream of *mafB* genes (48). *maf* loci have conserved chromosomal locations in *Neisseria* genomes and were named MGI (for “*maf* genomic island”). Numerous orphan modules encoding alternative MafB-CTs are present in most MGIs. The number of MGIs ranges from 3 (in *N. meningitidis*) to 5 (in *N. gonorrhoeae*) in pathogenic *Neisseria* spp., whereas they are virtually absent from nonpathogenic species (48).

Toxic domains identified in MafB-CTs include “toxin 64” (PF15542, a predicted RNase), “MafB19” (PF14437, a predicted nucleotide deaminase), “toxin 45” (PF15524, a predicted RNase), and “EndoU” (PF14436, a predicted RNase). As usual in polymorphic toxins, toxic domains of MafB-CTs share homology with CT extremities of other families of polymorphic toxins. Indeed, a “toxin 45” domain can be found in a CdiA putative toxin (G0K2D7) in *Stenotrophomonas maltophilia* JV3 and a “MafB19” domain can be found in a Rhs putative toxin (E1IMF2) in *E. coli* MS 145-7.

MafB proteins exhibit a signal peptide sequence, an N-terminal conserved domain of unknown function named DUF1020 (PF06255), and a C-terminal variable region. In addition, a bacterial intein-like domain can be inserted between the conserved and variable regions. DUF1020 domains are restricted to species of the *Neisseria* genus and can be divided in 3 classes based on sequence homology (Fig. 2C) (48). MafB proteins are detected in culture supernatant of pathogenic *Neisseria* spp.; however, how they are secreted remains unknown (48). Interestingly, a proteomic study has demonstrated that both MafA and MafB toxins can be found in OMV released by several gonococcal strains (49). Since MafA has been previously shown to bind cellular glycolipids (46), MafA could mediate attachment of OMVs to eukaryotic cells via GgO4 or to bacterial cells via an as-yet-unknown receptor. This suggests that the secretion of OMVs could be a means for delivery of MafB toxins to neighboring bacteria or to eukaryotic cells.

## EFFECTORS OF TYPE VI SECRETION SYSTEMS WITH A MODULAR ARCHITECTURE

First described in *Vibrio cholerae* (50), type VI secretion systems (T6SS) have a broad distribution and are found in one-third of Gram-negative bacterial species (51) whose genomes have been sequenced. The T6SS machinery resembles a tail of contractile phage with a syringe-like structure that allows the export of effector proteins. Type 6 effectors belong to distinct families of proteins, and only some antibacterial effectors secreted by these systems are polymorphic toxins. We have excluded from this section Rhs toxins secreted via a T6SS, which are discussed above.

“Evolved” VgrG and Hcp proteins are polymorphic toxins. The first T6SS effector to have been described is the VgrG1 protein

of *V. cholerae* strain V52. The injection of VgrG1 in eukaryotic host cells is responsible for the remodeling of the actin cytoskeleton (52). VgrG1 is defined as an “evolved” VgrG because it carries an N-terminal region identical to that of “regular” VgrG proteins fused with a C-terminal domain (CTD) of 300 to 400 aa (52). “Regular” VgrGs contain two domains also found in phage proteins: a domain of phage late control gene D (PF05954) and a domain of phage base plate assembly proteins (PF04717). In “evolved” VgrG, this N-terminal region is fused with polymorphic CTD with various toxic activities (53) (Fig. 2A) directed against eukaryotic or bacterial cells.

VgrG1 from *V. cholerae* strain V52 exhibits a CTD with an actin cross-linking domain able to covalently cross-link G-actin (52). In *V. cholerae* strain N16961, another evolved VgrG protein, designated VgrG3, exhibits a peptidoglycan-binding domain able to hydrolyze the cell wall of Gram-negative competitors. The producing bacterium is protected from self-intoxication by an immunity protein, the product of the *tsaB* gene (type VI secretion anti-toxin B, also named *tsiV3*), located downstream of *vgrG3* (54, 55). VgrG1 and VgrG3 share 67% identity over their first 600 residues, whereas their C-terminal extremities share less than 23% identity. A VgrG protein of *Aeromonas hydrophila* (A0KHA9, named VgrG1 [<http://www.uniprot.org/uniprot/A0KHA9>]) carries a CTD catalyzing ADP ribosylation of actin at its C terminus (PF03494), and it has been shown that this domain could result in apoptosis of HeLa cells (56).

Similarly, in *Salmonella* genomes, Blondel and coauthors also observed unusually long Hcp proteins with C-terminal extensions (57). These proteins were designated “evolved” Hcp proteins. Hcp (for “hemolysin coregulated protein”) proteins are major components of the tail tube of T6SS (45). Like VgrG, Hcp proteins also play a chaperone role during secretion of some effectors (58). “Evolved” Hcp proteins exhibit the Hcp domain (PF05638) at their N termini (~150 aa) and distinct putative toxic domains at their C termini (Fig. 2A) (57). Using HHpred, we identified a zincin-like metallopeptidase domain (PF15639 Tox-MPTase3) with more than 90% probability in the C-terminal region of SARI\_03217, a protein of *Salmonella enterica* Arizonae. In some uropathogenic *E. coli* strains, a protein called Usp (for “uropathogenic-specific protein”) harbors an N-terminal Hcp domain and a C-terminal colicin nuclease domain (PF12639). The Usp protein from UPEC strain Z42 has been purified, and the recombinant protein has a nuclease activity able to degrade plasmid DNA in a nonspecific manner (59). Nipič and coauthors demonstrated that the exposure of human cell lines to an *E. coli* strain expressing the *usp* locus or to purified Usp resulted in a loss of human cell viability (60). The activation of caspases 3 and 7 and the cell apoptosis observed are likely to be the consequence of Usp-provoked DNA damage (60). The C-terminal domain of Usp shares more than 40% identity with the DNase-like domain of DNase-type colicins of *E. coli* (60), thus demonstrating that a bacteriocin toxic domain has a toxic activity toward a eukaryotic host cells. However, experimental evidence supporting the hypothesis of Usp secretion is lacking.

**The diversity of type VI effectors.** Several other toxic effectors have been reported in *P. aeruginosa*. These effectors have been named Tse1 to Tse6 (type VI secretion exported). Among Tse1 to Tse6, only Tse5 and Tse6 are polymorphic toxins. Tse5 and Tse6 harbor a PAAR domain at their N terminus and an inhibitory domain at their C terminus (43). In addition, Tse5 harbors

Rhs/YD repeats in its central region (43). With the discovery of additional new effectors of T6SS, a classification based on the toxic activity has been chosen by some authors and has led to the clustering of proteins from divergent origins in a same group. For instance, effectors with amidase activity are named Tae (type VI secretion amidase effector) (61), effectors with lipase activity are named Tle (type VI secretion lipase effector) (62), and effectors with DNase activity are named Tde (type VI secretion DNase effector) (63). Most of these effectors are small nonpolymorphic toxins. However, some Tde effectors contain an N-terminal domain (e.g., a DUF4150, DUF4157, or PAAR domain) fused to the DNase toxin 43 domain (PF15604) (63) and are polymorphic.

## CONCLUSION

The aim of this short review is to highlight the striking similarities shared by all families of polymorphic toxins. A conserved N-terminal region fused to variable toxic domains defines a polymorphic toxin family. This bipartite architecture is found in colicins, Rhs, CdiA and MafB toxins, and some effectors of T6SS. A toxic domain of the same family can be found fused with N-terminal regions of distinct polymorphic toxin families, suggesting a shared pool of toxic domains. Interestingly, some N-terminal regions can be associated with either antibacterial or antieukaryotic toxic domains as in the case of evolved VgrG or Rhs toxins. It is plausible that additional antieukaryotic toxins will be discovered among members of polymorphic toxin families such as MafB or evolved Hcp. The high prevalence and diversity of polymorphic toxins in both Gram-negative and Gram-positive bacteria have just begun to be uncovered. Hundreds of putative toxic domains have been predicted by *in silico* analysis and require experimental validation. It is highly probable that some of these toxic domains will have therapeutic or biotechnological applications.

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