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Contact-dependent interbacterial toxins deliver a message

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Abstract

Both Gram-negative and Gram-positive organisms harbor systems for delivering toxins to neighboring bacteria upon direct cell contact. These systems, typified by type VI secretion (T6S) and contact-dependent growth inhibition (CDI) systems, are defined by their ability to mediate interbacterial competition *in vitro*, while their biological roles have remained uncertain. Recent research into the mechanisms of toxin delivery and activity, as well as investigation of contact-dependent toxin function during relevant biological processes, has offered insight into how interbacterial competition might work outside of the laboratory. Furthermore, non-competitive roles for contact-dependent toxin delivery systems, including interbacterial signal transduction, have been described. This review suggests that contact-dependent toxin delivery systems that exhibit functions beyond interbacterial competition are probably more common than currently appreciated.

Graphical abstract

Introduction

Whether in the soil rhizosphere or the mammalian gastrointestinal tract, bacteria encounter fierce competition for limited resources and must respond quickly and appropriately to changing conditions, including the presence of other bacteria. Diffusible toxic compounds produced by bacteria, such as bacteriocins and antibiotics, act at a distance to damage susceptible cells, providing the producing cell with a competitive edge over its neighbors

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[1]. Similarly, quorum sensing molecules released into the environment facilitate cooperation by allowing bacteria to sense and respond to other bacteria [2]. In addition to this network of diffusible signals and toxins, we are beginning to appreciate that bacteria harbor equally complex systems to sense and respond to direct contact with neighboring cells. Many contact-dependent mechanisms of bacterial interactions are defined by their ability to mediate interbacterial antagonism, but key differences exist among these systems. Moreover, recent research has begun to explore the biological consequences of contact-dependent toxin exchange between bacteria, raising questions about the physiological role of these interactions.

Mechanisms of bacterial contact-dependent toxin delivery

Contact-dependent growth inhibition

Contact-dependent growth inhibition (CDI) is a phenomenon in which Gram-negative bacteria deliver the toxic C-terminus of a polymorphic surface-exposed protein to the cytoplasm of neighboring cells upon cell-cell contact (Fig. 1) [3,4]. CDI systems are composed of Two-Partner secretion pathway (type V secretion) proteins [5]. CdiB (BcpB in *Burkholderia* and related species) is an outer membrane beta-barrel protein that facilitates the secretion of a large (>3000 aa) CdiA (BcpA) exoprotein to the bacterial cell surface. The majority of the CdiA N-terminus is predicted to adopt a beta-helical structure that extends several hundred angstroms from the cell surface [6]. While this N-terminal region is highly conserved across species, the CdiA C-terminal ~300 amino acids are variable. Termed the CdiA-CT (BcpA-CT), at least a portion of this region is translocated into the target bacterium, although the precise identity of the delivered molecule is unknown. Unlike type VI secreted effectors (see below), which target a wide range of conserved bacterial molecules [7], the majority of characterized CdiA-CT/BcpA-CT toxins have nuclease activity [4,8]. Bacteria producing CDI system proteins protect themselves from self-intoxication by producing small immunity proteins (CdiI/BcpI) that bind to and inactivate their cognate CdiA-CT/BcpA-CT toxins [4,9].

CDI systems were first described based on their ability to mediate interbacterial competition, and growth inhibition has defined this family of proteins [3,9]. CDI system-mediated interbacterial antagonism has been observed for systems found in *Escherichia coli* [3], *Burkholderia thailandensis* [9], *Neisseria meningitidis* [10], *Pseudomonas aeruginosa* [11], and *Acinetobacter baumannii* [12], and functional CdiA-CT toxins have been characterized from many other species [4,13]. However, the relevance of this competition is not clear, and evidence is increasing that CDI system proteins play other roles in bacterial biology.

Type VI secretion

The type VI secretion system (T6SS) is a contractile nanomachine that delivers effector protein toxins directly from the cytoplasm of Gram-negative bacteria into neighboring cells [7]. With structural homology to contractile bacteriophages, the T6SS consists of a multi-protein core complex that spans the inner and outer membranes, a cytoplasmic tube, a puncturing tip, and an outer sheath that surrounds the tube. Upon cell-cell contact, the outer sheath contracts, forcing the inner tube and puncturing device into the neighboring cell,

where effectors carried within the inner tube or attached to the puncturing tip are released. Although T6SSs were first recognized for injection of toxic effectors into eukaryotic host cells [14], these systems perhaps primarily target prokaryotic cells and deploy dedicated antibacterial effectors [15], including nucleases, peptidoglycan-degrading amidases, and membrane-targeting lipases [7]. As with CDI systems, bacteria elaborating T6SSs protect themselves from self-intoxication by producing immunity proteins specific to each deployed effector toxin. T6SS activity has been demonstrated to deliver toxins between numerous Gram-negative bacteria, mediating intra- and inter-species competition [16,17].

Other mechanisms

In addition to type VI secretion and CDI, other mechanisms of contact-dependent toxin delivery have recently been described. A unique class of type IV secretion systems in *Xanthomonas* species delivers a peptidoglycan hydrolase to neighboring cells upon cell-cell contact, mediating interbacterial killing unless the toxin is bound by a specific immunity protein [18]. Additionally, cell-associated aggregates of bacteriocin-like glycine zipper proteins in *Caulobacter crescentus* mediate interbacterial killing through direct contact with neighboring bacteria [19]. In Gram-positive organisms, the YD repeat-containing protein WapA and LXG toxin family proteins have been shown to mediate interbacterial antagonism in *Bacillus subtilis* and *Streptococcus intermedius*, respectively [20,21]. Similar to CDI systems, these surface proteins deliver polymorphic toxins derived from their C-termini to neighboring bacteria and specific immunity proteins prevent self-intoxication. Thus, both Gram-negative and Gram-positive organisms are capable of deploying interbacterial toxins upon cell-cell contact and many, if not most, species likely utilize several mechanisms of contact-dependent antagonism.

Specificity of toxin translocation into recipient cells

Specificity associated with toxin delivery, or lack thereof, may provide insights into the biological roles of bacterial contact-dependent toxin delivery systems. In general, T6SSs do not appear to require specific target cell receptors for effector translocation, instead relying on physical force to disrupt the target cell outer membrane [22]. Phylogenetically diverse bacteria can, therefore, be targeted by a single T6SS. An exception may be the recently-described requirement of the *P. aeruginosa* NAD⁺/NADP⁺-degrading toxin, Tse6, for translation elongation factor Tu (EF-Tu) in the target cell [23]. Findings suggest that EF-Tu facilitates Tse6 delivery to the target cell cytoplasm. Interestingly, Tse6 can interact with EF-Tu orthologs from diverse bacteria, suggesting that this effector would retain function when deployed during interspecies interactions.

By contrast, CDI system toxin translocation is mediated by specific receptors on the surface of the target cell. The essential outer membrane protein BamA is required for entry of a subset of CdiA variants [24,25], while another group of toxins requires heterotrimers of the outer membrane proteins OmpC and OmpF for target cell entry [26]. Strikingly, polymorphisms in BamA and OmpC surface-exposed loops limit CdiA-CT delivery to bacteria that produce the “correct” receptor variant [25,26]. The most efficient receptor-toxin pairs are encoded by the same bacterial strain, suggesting that CdiA is delivered

preferentially to “self” bacteria. Still other *E. coli* CdiA-CT toxins utilize the outer membrane protein Tsx, although it is not known whether specific *tsx* alleles are required [24]. The target cell surface receptors for BcpA-CT toxins are not known, although data indicate that altered lipopolysaccharide structure affects susceptibility to a *Burkholderia pseudomallei* BcpA toxin [27].

Specific target cell proteins are also required for CdiA/BcpA translocation across the cytoplasmic membrane. Data suggest that the N-terminal region of the CdiA-CT or BcpA-CT toxin functions as a translocation domain, interacting with one of several conserved inner membrane proteins to mediate transfer to the target cell cytoplasm [27,28]. In general, target cell cytoplasmic membrane proteins required for toxin translocation appear to be conserved within species, but absent from more distantly-related bacteria [27,28].

Together, these studies suggest that, unlike T6SS effector proteins, which are successfully delivered across species [7], CDI system toxins are exclusively exchanged between closely related bacteria that possess the necessary receptors for toxin translocation. Thus, the T6SS may be a more effective generalized weapon for interbacterial competition, while CDI may have evolved to target specific bacteria. Closely related bacteria competing for the same niche might represent a greater threat than more distantly related species. Additionally, within-species or within-strain exchange of CdiA-CT/BcpA-CT proteins may perform other biological functions.

Recipient cell response to toxin delivery

Contact-dependent signaling

In a population of bacteria assembling contact-dependent toxin delivery systems, a large amount of produced toxin is likely exchanged among “self” cells, rather than delivered to foreign competitors. Paradoxically, sibling bacteria also produce the cognate immunity protein(s) and do not represent a competitive threat. One proposed hypothesis is that this self-exchanged toxin could perform an interbacterial signaling function [7,29].

Recently, it was demonstrated that a CDI system in *B. thailandensis* functions in this manner to transmit a signal between bacteria of the same strain. BcpA received from neighboring cells induced gene expression changes in immune target cells (those producing cognate BcpI) [30]. These changes included upregulation of genes encoding pili and polysaccharide synthesis machinery and led to an increase in biofilm formation. While the molecular mechanism underlying these gene expression changes is not known, the process, termed contact-dependent signaling (CDS) (Fig. 1), requires the catalytic activity of BcpA-CT. Thus, signaling may involve limited or residual BcpA-CT DNase activity in a cell producing BcpI, altered kinetics or substrate specificity upon BcpI binding, or interactions of target cell proteins with the BcpA-CT/BcpI complex. However, it appears that having a cognate BcpA-CT/BcpI pair is not sufficient for CDS; other target cell proteins are required and may be unique to each toxin-immunity pair.

Whether *bcpA* or *cdiA* alleles in other bacterial species mediate signaling or whether CDS is unique to *B. thailandensis* is not known. If CDS is a conserved function of CDI system

proteins, the molecular mechanism may vary among BcpA/CdiA variants. While some BcpA-CT/CdiA-CT proteins are DNases like the *B. thailandensis* BcpA-CT, many others have tRNase activity [8,31]. It is conceivable that alterations in the levels of specific tRNAs could affect translation rates, similar to the activity of toxin-antitoxin systems in growth regulation and stress adaptation [32]. Fluctuations in tRNA levels also affect cellular processes such as the stringent response, metabolite synthesis, and macromolecule modification [33], all of which could be exploited by CDI systems for interbacterial signaling. Additionally, in eukaryotic cells, the tRNA fragments themselves that result from tRNA cleavage affect gene expression [34].

It is intriguing to speculate that CDI systems could facilitate interbacterial communication, with each toxin variant transmitting information that would be interpreted into gene expression changes only by neighboring cells that possessed the correct immunity protein. Conveniently, recipient cells not producing the cognate immunity protein would be killed. The magnitude of the signal (or number of bacteria receiving a signal) might be expected to increase with cell density as densely packed bacteria with numerous cell-cell contacts would receive increased doses of toxin. In *B. thailandensis*, interbacterial signaling appears to induce cooperative behaviors such as biofilm formation [30], but CDS could also be used to respond defensively to the stresses of a dense bacterial community.

CdiA toxin activation

For several CDI system toxins, the CdiA-CT enzyme requires additional recipient cell-derived proteins for activity. The CdiA-CT toxin of uropathogenic *E. coli* 536 requires CysK, an O-acetylserine sulfhydrylase involved in cysteine biosynthesis, for tRNase activity [29]. CysK binds to CdiA-CT^{EC536}, increasing its thermostability and allowing for interaction with tRNA substrates [35]. Interestingly, CysK binding also increases the affinity of CdiA-CT^{EC536} for CdiI^{EC536}, suggesting that this interaction may contribute to toxin immunity [36]. Similarly, the CdiA-CT toxin of enterohemorrhagic *E. coli* 869 requires the translation elongation factors EF-Ts and EF-Tu for tRNase activity *in vivo* [37]. Unlike the T6SS effector Tse6, which appears to require EF-Tu for target cell entry [23], CdiA-CT binds to EF-Tu and cleaves its tRNA substrates in the context of GTP-EF-Tu-tRNA ternary complexes [37].

It is not clear why some CDI system toxins have evolved to depend on recipient cell factors for toxicity, as this may further restrict target cell susceptibility and introduces opportunities for evolved resistance to CDI. One possibility is that interactions of CdiA-CT/BcpA-CT toxins with recipient cell proteins could contribute to an interbacterial signaling function of CDI systems. Sequestration of these proteins by binding to CdiA-CT/CdiI complexes in immune sibling cells or modification of their function could alter cellular processes or gene regulation. Modulation of cysteine metabolism and loss of *cysK* in *Vibrio fischeri*, for example, are known to alter biofilm formation [38].

Biological roles for contact-dependent toxin delivery systems

Despite considerable research on contact-dependent toxin delivery systems, the role of these proteins in bacterial biology is still unclear. While T6S and CDI systems can clearly mediate

interbacterial competition in the laboratory, recent research has provided insight into the physiological role for this phenomenon and has suggested that competition is not the only function of these protein complexes (Fig. 2). For example, during *in vitro* biofilm growth, *Vibrio cholerae* T6SS-mediated killing of neighboring nonimmune *V. cholerae* may provide source DNA for horizontal gene transfer [39].

One likely role for contact-dependent toxin delivery systems is competition for specific environmental niches, which may be achieved by preventing territory encroachment by competing bacteria. Indeed, CDI system-mediated interbacterial competition facilitates defense of biofilms against non-immune invading bacteria [40]. In *Proteus mirabilis*, T6SS activity mediates interbacterial killing between approaching swarming populations, preventing strain mixing and resulting in a macroscopic boundary termed a Dienes line [41,42]. Secreted effector proteins transferred to neighboring cells via T6SS bind to cognate immunity proteins in an allele-specific manner [43,44], and differences in the complement of effector-immunity pairs between *P. mirabilis* strains presumably results in kin discrimination. Similarly, the contact-dependent polymorphic toxin WapA contributes to the partitioning of swarming *B. subtilis* strains [45].

Contact-dependent toxin delivery systems have also been suggested to play roles in defense against non-cooperators in a population. In *B. thailandensis*, quorum sensing co-regulates expression of genes encoding a T6SS and secreted “public goods” [46], and it was recently shown that T6SS activity against quorum sensing mutants limits their growth, serving as a “policing” mechanism to protect the population from exploitation by these “cheaters” [47]. Similarly, CDI has been demonstrated to provide sufficient selective pressure for maintenance of an exogenous plasmid in *E. coli*, suggesting that CDI system-mediated interbacterial killing could serve as a way to prevent mobile genetic element loss [48].

There is increasing evidence that CDI systems facilitate interbacterial cooperation as well as competition. In *B. thailandensis*, interbacterial signaling promotes cooperative behaviors among sibling bacteria and results in biofilm formation by CDI⁺ cells [30,49]. CdiA similarly mediates biofilm formation in *E. coli* 536, although this process likely involves interactions between CdiA molecules and is independent of the CdiA-CT catalytic activity [50]. Contribution to cell-cell aggregation appears to be a conserved function of CDI system proteins, as CdiA proteins in other bacterial species have also been implicated in biofilm formation [51–53].

Contact-dependent toxin delivery systems likely have roles during interbacterial interactions in the environment, but recent reports suggest that some systems are also important during bacterial exchanges that occur within host organisms. T6SS antagonism against co-colonizing bacteria or members of the intestinal microbiota was implicated in colonization by several pathogens, including *Salmonella*, *Vibrio cholerae*, and *Shigella sonnei* [54–56]. CdiA proteins in several plant pathogens were also required for colonization or virulence, perhaps due to their role in bacterial aggregation [51,57]. Furthermore, *Burkholderia dolosa* *bcpA* was found to be under positive selection during an outbreak among patients with cystic fibrosis, suggesting a role for CDI system proteins in lung colonization or transmission [58].

Conclusions

Contact-dependent toxin delivery systems such as type VI secretion and CDI systems are defined by their ability to mediate interbacterial competition *in vitro*. As we begin to appreciate the mechanisms of these systems, including how toxins are translocated into target cells, understanding the physiological relevance of the observed competition has become complicated. Recent studies have offered insight into the biological context for contact-dependent interbacterial toxicity and have provided the first concrete evidence that some toxin delivery systems have roles beyond antagonism. Given the diversity of contact-dependent toxin delivery systems and their prevalence in bacterial genomes, future research will likely uncover additional examples of interbacterial signal transduction or other complex processes that are controlled by interbacterial toxin exchange.

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Highlights

- Type VI secretion (T6S) and contact-dependent growth inhibition (CDI) systems mediate interbacterial competition *in vitro*
- Unlike T6S effectors, CDI system toxins are only delivered to closely-related or sibling bacteria and may need additional recipient cell proteins for toxicity
- The CDI system in *Burkholderia* mediates interbacterial signaling
- T6S and CDI systems function in horizontal gene transfer, community defense, cooperative behaviors, and host colonization

Figure 1. Model for contact-dependent growth inhibition (CDI) and contact-dependent signaling (CDS)

During CDI (left), a portion of the BcpA-CT (or CdiA-CT) is translocated into target cells via specific outer and inner membrane receptors. Once in the cytoplasm, BcpA-CT degrades target cell DNA or tRNA, causing growth inhibition and cell death. When BcpA-CT is delivered to another cell producing the same CDI system proteins (right), delivered BcpA-CT binds to its cognate BcpI immunity protein, preventing cell death. Additionally, interaction of the BcpA-CT/BcpI complex with target cell nucleic acids and/or proteins induces gene expression changes in a process termed CDS. OM, outer membrane; IM, inner membrane.

Figure 2. Biological roles for contact-dependent toxin delivery systems

Functions of type VI secretion systems (left, in blue shaded region), contact-dependent growth inhibition (CDI) systems (right, in green shaded region), or both (center, in blue-green shaded region) that are supported by experimental evidence. Blocked arrows indicate antagonistic interactions between the toxin-producing cell and neighboring cells and arrows indicate non-antagonistic or cooperative interactions. Gray cells indicate closely-related kin or 'self' bacteria and colored cells indicate 'non-self' bacteria. HGT, horizontal gene transfer; QS, quorum sensing.