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Contact-dependent growth inhibition (CDI) and CdiB/CdiA two-partner secretion proteins

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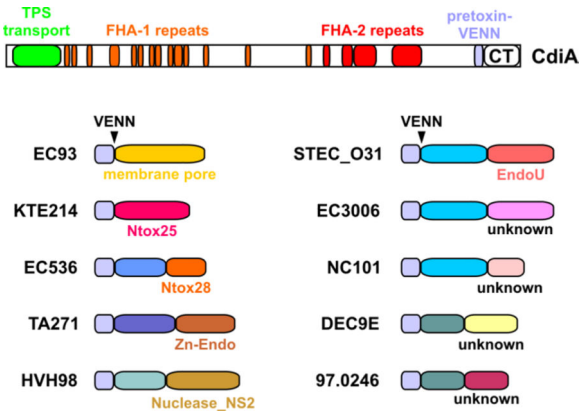
Abstract

Bacteria have developed several strategies to communicate and compete with one another in complex environments. One important mechanism of inter-bacterial competition is contact-dependent growth inhibition (CDI), in which some Gram-negative bacteria use CdiB/CdiA two-partner secretion proteins to suppress the growth of neighboring target cells. CdiB is an Omp85 outer-membrane protein that exports and assembles CdiA exoproteins onto the inhibitor-cell surface. CdiA binds to receptors on susceptible bacteria and subsequently delivers its C-terminal toxin domain (CdiA-CT) into the target cell. CDI systems also encode CdiI immunity proteins, which specifically bind to the CdiA-CT and neutralize its toxin activity, thereby protecting CDI^+ cells from auto-inhibition. Remarkably, CdiA-CT sequences are highly variable between bacteria, as are the corresponding CdiI immunity proteins. Variations in CDI toxin/immunity proteins suggest that these systems function in bacterial self/nonself recognition and thereby play an important role in microbial communities. In this review, we discuss recent advances in the biochemistry, structural biology and physiology of CDI.

Graphical abstract



Keywords

biofilms; self/nonself recognition; toxin/immunity proteins; type V secretion

Introduction

Bacteria generally live in mixed species communities, forming intricate networks in nearly all environments[1]. These bacterial networks play important host-associated roles in insects, plants and animals[2]. Recent studies show that microbial communities influence host physiology and behavior, affecting sexual preference, host development, endocrine signaling, brain/nervous system diseases, immunity and other complex traits[3; 4; 5; 6; 7; 8]. Within each niche, bacteria communicate and compete with one another for colonization and survival. Much is known about the secreted factors that govern inter-cellular communication through quorum sensing[9; 10] and the soluble siderophores used in the competition for iron acquisition[11; 12; 13]. Similarly, the functions of diffusible antibiotics and bacteriocins, which directly inhibit or kill surrounding cells, have been examined in detail over the past 90 years[14]. By contrast, we know less about inter-cellular communication/competition systems that rely on direct physical contact. Here, we discuss recent insights into bacterial contact-dependent growth inhibition (CDI) – a mechanism by which growth inhibitory toxin domains are translocated into neighboring target bacteria following direct cell-to-cell contact.

The discovery of contact-dependent growth inhibition (CDI)

CDI was discovered in 2005 during analysis of *Escherichia coli* strain EC93, which was identified as a dominant isolate from rat intestine[15]. As with many natural isolates, *E. coli* EC93 has a significant growth advantage over domesticated *E. coli* strains and reduces *E. coli* K-12 viability several thousand-fold after a few hours of co-culture. However, unlike previously characterized inhibitory strains, *E. coli* EC93 does not secrete diffusible microcin or colicin toxins. Instead, EC93 cells must make direct contact with target cells to inhibit growth[15]. This new mode of bacterial competition was termed "contact-dependent growth inhibition" or CDI[15]. The CDI locus was isolated from a cosmid library of *E. coli* EC93 genomic DNA and found to contain three genes, *cdiB, cdiA* and *cdiI* (Fig. 1a), which are

sufficient to convert *E. coli* K-12 into a CDI$^+$ inhibitor strain[15]. The *cdiB* and *cdiA* genes encode a two-partner secretion (TPS) system. TPS proteins constitute a sub-family of type V secretion systems and include a number of important adhesins that are used by bacterial pathogens to adhere to eukaryotic host cells[16; 17; 18; 19; 20]. As indicated by its name, TPS systems are characterized by two proteins, generically known as TpsB (CdiB) and TpsA (CdiA), that are secreted across the Gram-negative envelope. TpsB partners are outer-membrane proteins of the Omp85 family, and export TpsA exoprotein cargos through the central lumen of their β-barrels[21]. In many instances, the TpsA partner is thought to remain tethered non-covalently to TpsB, thereby projecting the exoprotein outward from the surface of the secreting bacterium (Fig. 1b). CdiA and other TpsA exoproteins are often quite large and probably form long β-helical filaments that extend several hundred angstroms from the cell surface[22; 23]. Therefore, the *E. coli* EC93 CdiB/CdiA proteins represent a specialized TpsB/TpsA pair used to bind and inhibit the growth of neighboring *E. coli* target cells (Fig. 1c). The *cdiI* gene encodes a small hydrophobic peptide that acts as an immunity protein to protect *E. coli* EC93 cells from auto-inhibition. The CdiI protein is also sufficient to confer immunity to *E. coli* K-12 target strains, allowing them to grow unabated in co-culture with *E. coli* EC93[15; 24]. Together, these findings led to the hypothesis that CDI is used to compete with other bacteria for environmental resources.

When CDI was first reported, there were only a handful of known TpsA proteins with significant homology to CdiA^{EC93} [15]. As more bacterial genomes were sequenced, it became apparent that *cdi* gene clusters are found in several α-, β- and γ-proteobacteria[25]. Probable *cdi* loci are also found in Fusobacteria and the Negativicutes, which are a class of didermal Firmicutes. Comparative analysis of CdiA proteins from a given species typically shows significant sequence variability over the C-terminal region (CdiA-CT). Moreover, most of the predicted *cdiI* immunity genes downstream of *cdiA* do not share significant sequence identity with one another. Together, these observations suggest that CDI growth inhibition activity resides in the CdiA-CT region and that many different toxins are carried by CdiA effectors. Both hypotheses are supported by biochemical analyses of CdiA-CT/CdiI pairs. The CdiA-CT^{EC93} toxin from *E. coli* EC93 appears to form pores in membranes, because it dissipates the proton-motive force and reduces ATP synthesis[24]. Most other characterized CDI toxins have nuclease activities that are blocked by their cognate CdiI immunity proteins[25; 26; 27; 28]. Thus, CdiA effectors can be armed with an array of C-terminal toxin domains, each associated with a specific CdiI immunity protein to prevent self intoxication. Because immunity proteins do not protect against non-cognate toxins, CDI is thought to mediate inter-strain competition and contribute to self/non-self recognition.

Architecture of CdiA effector proteins

CdiA effectors exhibit considerable diversity in sequence and size depending on bacterial species and strain. The smallest recognizable CdiA protein is from *Moraxella catarrhalis* O35E (MhaB1, Uniprot: A5JFL2) at ~182 kDa[29], and the largest is a ~636 kDa protein encoded by the PSPTO_3229 locus of *Pseudomonas syringae* DC3000 (Fig. 2a). Despite this heterogeneity, most CdiA proteins have an architecture similar to that of the filamentous hemagglutinin (FHA/FhaB) adhesins of *Bordetella* species (Fig. 2a). These proteins share N-terminal elements that guide secretion across the cell envelope. All CdiA proteins carry

an N-terminal signal sequence for Sec-dependent secretion, though many have an unusual extended signal peptide region (ESPR, Pfam: PF13018) in common with FHA/FhaB (Fig. 2a)[30; 31]. The N-terminal region also contains the TPS transport domain (also known as the hemagglutinin activation domain, PF05860), which is required for export across the outer membrane. CdiB recognizes the TPS transport domain as it emerges into the periplasm and transports CdiA through the central pore in its β-barrel domain[21; 32; 33]. CdiA proteins also share FHA-1 (PF05594) and FHA-2 (PF13332) peptide repeats with FHA/FhaB (Fig. 2a). FHA peptide repeats are degenerate 20-residue sequences characterized by alternating polar and non-polar residues[34]. These sequences are predicted to form right-handed parallel β-helices with a pitch of 4.8 Å per repeat[22; 35]. Given this conformation, CdiA proteins could extend 40 to 140 nm from the surface of CDI^+ bacteria. Domain composition is much more variable over the C-terminal region of CdiA proteins. CdiA proteins from the Pseudomonadaceae and Moraxellaceae often contain the predicted α-helical DUF637 domain of unknown function (PF04830); and those from the Enterobacteriaceae, Moraxellaceae, Pasteurellaceae and *Neisseria meningitidis* strains usually contain the pretoxin-VENN domain (PF04829), which demarcates the variable CdiA-CT toxin region (Fig. 2a). Although CdiA proteins from other species lack the pretoxin-VENN domain, the CdiA-CT is identifiable as a sequence-variable region at the extreme C-terminus. For example, *Burkholderia* CdiA proteins show abrupt divergence of the C-terminal region after a conserved (E/Q)LYN peptide motif[28; 36]. CdiA-CT regions also vary in domain organization. The $CdiA\text{-}CT^{EC93}$ pore-forming toxin appears to be a composed of a single domain, as is the predicted Ntox25 RNase toxin from *E. coli* strain KTE214 (Fig. 2b)[37]. Many other CdiA-CTs are composed of two domains. The C-terminus of MhaB1 from *M. catarrhalis* O35E contains an unannotated domain between the pretoxin-VENN and HNH nuclease domains, and the CdiA-CT region from *P. syringae* DC3000 contains a predicted hedgehog-intein domain (PF07591) followed by a C-terminal domain of unknown function (Fig. 2a). Analysis of CdiA-CTs from *E. coli* strains shows that the two constituent domains can be rearranged in new combinations. For example, the CdiA-CT regions from *E. coli* strains STEC_O31, 3006 and NC101 all share the same N-terminal domain, but have different C-terminal domains (Fig. 2b). The same is true of CdiA-CTs from *E. coli* strains DEC9E and 97.0246, which contain related N-terminal domains, but divergent C-terminal domains (Fig. 2b). The C-terminal domain of the CdiA-CT region contains the actual toxin activity and these domains are typically nucleases. For example, the C-terminal domain of $CdiA\text{-}CT^{STECO31}$ is a predicted RNase related to eukaryotic EndoU RNA processing enzymes[37; 38] (Fig. 2b), and the C-terminal domains of other experimentally characterized CdiA-CTs have toxic nuclease activities[26; 28; 39; 40]. Recent genetic studies suggest that the N-terminal domain mediates transport of the tethered nuclease toxin into target bacteria[41]. Perhaps the most remarkable feature of the CdiA-CT region is its modularity. CdiA-CT sequences can be exchanged between CdiA proteins to generate chimeric effectors. Heterologous CdiA-CTs from *Yersinia pestis*, *Dickeya dadantii*, *Enterobacter cloacae* and *Photorhabdus luminescens* are all functional when fused at the VENN sequence of *E. coli* CdiA proteins[25; 26; 41; 42]. Similarly, functional chimeras have been generated by fusing different *Burkholderia* CdiA-CT sequences at the (E/Q)LYN motif of $CdiA_{II}^{Bp1026b}$ [28]. This modularity allows *cdi* loci to acquire new toxins through horizontal gene transfer and recombination. In fact, CdiA-CT related toxins are commonly found at the C-terminus of

Rhs, LXG and MafB proteins, which represent different classes of secreted effectors that also transfer toxin domains between bacteria[37; 40; 43; 44; 45]. Together, these observations indicate that different bacterial competition systems share the same pool of toxin/immunity genes through horizontal exchange.

CdiA-CT activities and structures

The first CDI toxin to be characterized was the pore-forming CdiA-CT^{EC93} from *E. coli* EC93[24]. DNase and RNase toxins were subsequently identified through sequence homology to bacteriocin nuclease domains. CdiA-CTs encoded by Dda3937_02098 of *Dickeya dadantii* 3937 and BPSS2053 of *Burkholderia pseudomallei* K96243 share significant sequence identity with the C-terminal nuclease domains from pyocin S3 and colicin E5, respectively[25]. Aravind and colleagues have conducted comprehensive sequence analyses of prokaryotic toxin/immunity pairs and predict that CdiA proteins also carry toxin domains with RNA deaminase and peptidase activities[37; 38]. However, despite these combined experimental and informatic analyses, the activities of most CDI toxins remain unknown. For example, there are at least 20 distinct CdiA-CT sequence types present in *E. coli* strains, yet only five of these toxin domains currently have Pfam designations (Table 1). Biochemical screens have uncovered the activities of some toxins like CdiA-CT^{EC869} from *E. coli* EC869 (ECH7EC869_5848), which cleaves near the 3′-end of tRNA^{Gln} (Table 1) and CdiA-CT^{Bp1655} from *Burkholderia pseudomallei* 1655 (DP51_5554), which cleaves tRNA T-loops[28; 46]. Structural studies have informed the activities of other CDI toxins. Crystal structures of toxin/immunity protein complexes from *B. pseudomallei* 1026b (CdiA-CT/CdiI$_{II}^{Bp1026b}$) and *E. coli* TA271 (CdiA-CT/CdiI^{TA271}) show that each CdiA-CT contains a C-terminal nuclease domain with a type IIS restriction endonuclease fold[27] (Fig. 3). Though these nuclease domains are similar in structure, they only share ~18% sequence identity and have distinct substrate specificities. CdiA-CT^{TA271} is a Zn^{2+}-dependent DNase, whereas CdiA-CT$_{II}^{Bp1026b}$ is an RNase that preferentially cleaves the aminoacyl acceptor stem of tRNA^{Ala} molecules[27; 28]. The CdiA-CT^{TA271} and CdiA-CT$_{II}^{Bp1026b}$ nuclease domains also interact with their immunity proteins in distinct manners. The Bp1026b toxin and immunity proteins interact through complementary shape and electrostatics, with the immunity protein binding directly over the toxin active site. In contrast, the CdiA-CT/CdiI^{TA271} complex is formed through an unusual β-augmentation interaction in which the toxin extends a β-hairpin into a binding pocket within the immunity proteins to complete a six-stranded anti-parallel sheet[27]. The nuclease active site remains exposed in the inactive CdiA-CT/CdiI^{TA271} complex, and it is remains unclear how toxin activity is neutralized by the immunity protein.

Though the nuclease domains of CdiA-CT$_{II}^{Bp1026b}$ and CdiA-CT^{TA271} share the same fold, other CDI toxins do not (Fig. 3). The C-terminal Ntox21 domain of CdiA-CT^{ECL} from *Enterobacter cloacae* ATCC 13047 has a BECR (barnase/EndoU/colicin E3/RelE) fold and cleaves 16S rRNA between residues A1493 and G1494 (*E. coli* numbering)[26; 37]. This activity is identical to that of colicin E3 and the structures of the two nuclease domains superimpose with rmsd of 2.1 Å. However, the CdiA-CT^{ECL} active site is distinct from that of colicin E3, suggesting the toxins do not share the same catalytic mechanism. Moreover, CdiI^{ECL} shares no sequence or structural homology with the ImmE3 immunity protein.

CdiI^{ECL} is structurally similar to the Whirly family of single-stranded DNA binding proteins[26; 47], but was recently proposed to have evolved from an ancestral SUKH family immunity protein[38; 48]. Intriguingly, the CdiI$_{o2}^{MC58-1}$ immunity protein encoded by locus NMB0503 of *Neisseria meningiditis* MC58 also shares some structural homology with Whirly proteins[49]. The cognate toxin for this latter immunity protein is a predicted EndoU RNase[37; 49]. Because EndoU and BECR toxin folds are related[37], it appears that the Ntox21 and EndoU nuclease families evolved from a common ancestor that was associated with an SUKH immunity protein.

Genomic organization and orphan toxin/immunity modules

The simplest *cdi* loci contain only three genes: *cdiB*, *cdiA* and *cdiI* (Fig. 1a), but many systems are linked to additional toxin/immunity protein coding sequences. In *E. coli* EC93, the *cdiI* immunity gene is closely followed by sequences that encode a toxin/immunity protein pair that closely resembles CdiA-CT/CdiI from uropathogenic *E. coli* 536[40]. The toxin open reading frame (ORF) lacks an initiation codon and begins with Val of the VENN motif (Fig. 4a). These toxin/immunity gene pairs have been termed "orphans" because they resemble the displaced 3′-ends of *cdiA* cistrons with their associated immunity genes. Orphan regions usually contain predicted transposase and integrase genes as well as insertion sequence (IS) elements[40]. These observations suggest that orphan regions are hotspots for horizontal gene flow. In this model, new toxin/immunity pairs are collected and eventually undergo recombination with the full-length *cdiA* gene to deploy a new toxin type on the cell surface. Moreover, CDI systems are typically encoded on genomic and pathogenicity islands, indicating that entire *cdi* loci are transferred horizontally between bacteria. Many uropathogenic *E. coli* strains carry a *cdi* gene cluster on pathogenicity associated island-2 (PAI-2)[50]. Examination of *E. coli* strains from the Broad Institute UTI genome sequencing initiatives has revealed another family of genomic islands that contain *cdi* genes flanked by a putative surfactin biosynthesis operon and prophage sequences (Fig. 4b). These islands are inserted at six different tRNA genes in over 50 *E. coli* strains (Fig. 4b). Moreover, the *cdiA* genes on these latter islands encode 11 different CdiA-CT toxins, indicating that smaller scale horizontal gene transfer events can abruptly replace *cdiA-CT/cdiI* sequences. The *cdi* genes in *B. pseudomallei* strains are also found on genomic islands. The *cdi* genes of *B. pseudomallei* K96243 are within genomic island 16 (GI-16), and the three *cdi* clusters of *B. pseudomallei* 1106a are found in GI-5a.1, GI-11.1 and GI-16.1[51; 52].

In contrast to the *cdiBAI* gene organization common in γ-proteobacteria and *Neisseria*, the cluster is arranged in an alternative *cdiAIB* order in other β-proteobacteria. The latter arrangement dominates in *Burkholderia*, *Commodus* and *Variovorax*, whereas *Ralstonia* species have both types of loci. The toxin and immunity protein coding sequences remain closely linked, but the *cdiAIB* organization appears to preclude the accumulation of extensive orphan gene pair arrays. *B. pseudomallei* serves as a good model for *cdi* genetic organization in the Burkholderiales because more than a hundred isolates have been sequenced and each strain carries at least one *cdi* locus[28]. *B. pseudomallei* strains contain 10 distinct *cdi* locus types, each characterized by a unique *cdiA-CT/cdiI* sequence[28; 36], though the type VII and VIII loci encode closely related RNA deaminase toxin/immunity protein pairs[37]. Most *B. pseudomallei* clusters contain additional small ORFs between *cdiI* and *cdiB*,

and many of these sequences encode putative or known immunity proteins (Fig. 5). For example, the intervening gene in the type locus of *B. pseudomallei* K96243 has weak homology to a predicted CdiI immunity protein from *Serratia* sp. DD3 (SRDD_21370), and the ORFs from strains NTCC 13179 (class IV) and 576 (class VI) systems are distantly related to the CdiI protein from *Enterobacter aerogenes* ATCC 13048 (EAE_10270) (Fig. 5). *B. pseudomallei* type V loci contain a copy of the type IV *cdiI* immunity gene together with 43 codons of the upstream type IV *cdiA-CT* coding sequence (Fig. 5). This orphan *cdiA-CT/cdiI* fragment is flanked by 125-bp direct repeats, strongly suggesting that the immunity gene was transferred horizontally into the type locus. Another interesting example is the type IX locus, which contains two *cdiI* immunity genes that share 42% sequence identity with each other. These observations indicate that the *cdiAIB* genetic organization still allows limited transfer and retention of orphan immunity genes.

Several *Burkholderia cdi* loci, including the *B. pseudomallei* type VII, VIII, IX and X clusters contain an unusual intervening gene that is not related to known immunity genes (Fig. 5). Cotter and colleagues have termed this gene, *bcpO* (*B*urkholderia *c*ompetition *p*rotein O), and have characterized its deletion phenotype in *Burkholderia thailandensis*[36]. BcpO is a predicted lipoprotein required for the full inhibition effect of the *B. thailandensis* CDI system. Closely related homologs of *bcpO* are associated with *cdi* loci in other *Burkholderia* species including *B. gladioli*, *B. glumae* and *B. phymatum*. There are also genes encoding more distantly related lipoproteins in *B. phytofirmans*, *B. ambifaria*, *B. cenocepacia* and *Pseudomonas* species, though these latter homologues are not linked to *cdi* gene clusters. The precise function of BcpO remains unclear, but given its presumed localization in the outer membrane, perhaps this lipoprotein collaborates with CdiB to export CdiA onto the inhibitor-cell surface. Many other CDI^+ bacteria contain yet another accessory gene that encodes a predicted hemolysin activator (HlyC) or RTX toxin acyltransferase. This gene is found between *cdiB* and *cdiA* in strains of *E. coli* (e.g. ECIG_03035 locus of *E. coli* M605), *Enterobacter cloacae*, *Ralstonia solanacearum*, *Pseudomonas fluorescens* and other bacteria. The HlyC gene product is related to fatty acyl transferases that modify lysyl residues within hemolysin[53; 54]. The role of HlyC acyltransferases in CDI has not been explored, but presumably this enzyme modifies either CdiB or CdiA to promote their association with membranes.

Regulation of *cdi* gene expresssion

The expression of most CDI systems is tightly regulated, with the notable exception of *E. coli* EC93, which constitutively expresses one of its two *cdi* gene clusters[15]. The *cdi* loci of soft-rot pathogens appear to be expressed only when the bacteria are grown on plant hosts. Collmer and colleagues first characterized $cdiI^{EC16}$ from *Erwinia (Dickeya) chrysanthemi* EC16 as a virulence gene (*virA*) because its disruption significantly reduced virulence on plant hosts[55]. Now that the EC16 *hecBA-virA* cluster is recognized as $cdiBAI^{EC16}$ [25; 26], an alternative explanation for these findings is that the locus is only expressed during infection. Thus, the immunity gene is not required under laboratory growth conditions when *cdiA* is not expressed, but the bacteria either auto-inhibit or inhibit neighboring sibling cells when inoculated onto the host. Similar findings have been obtained with a related plant pathogen, *D. dadantii* 3937, which expresses its *cdi* gene cluster on chicory but not in laboratory

media[25]. Cotter and colleagues have shown differential *cdi* expression within regions of an individual colony[36], suggesting stochastic activation of the gene cluster. Greenberg and colleagues have shown further that *cdi* expression in *B. thailandensis* E264 is induced in response to acylhomoserine signaling[56]. However, quorum sensing does not regulate the two *cdiA* genes (BP1026B_I2481 and BP1026B_II2207) in closely related *B. pseudomallei* 1026b[57]. Given the energy required to synthesize large CdiA exoproteins and their exposure to immune surveillance systems, most bacteria probably regulate expression to ensure that *cdi* genes are only expressed at high cell densities when cell-cell contact is favored.

Recognition of target cells and toxin delivery

CDI requires direct interaction between inhibitor and target bacteria, suggesting that CdiA proteins recognize receptors on the surface of target cells. Aoki *et al*. used genetic selections to identify the receptor for $CdiA^{EC93}$, reasoning that disruption of the receptor gene would confer resistance to CDI (CDI^{R})[58]. This approach led to the isolation of the *E. coli bamA101* mutant, which carries a transposon insertion that decreases *bamA* expression approximately five-fold. BamA is an essential outer membrane β-barrel protein that forms the core of the β-barrel assembly machine (BAM) complex. The BAM complex is required for the biogenesis of all β-barrel outer-membrane proteins (OMP) in Gram-negative bacteria and eukaryotic plastids[59; 60; 61; 62; 63]. Several observations indicate that BamA is the receptor for $CdiA^{EC93}$. First, other components of the BAM complex are not required for CDI, and a biogenesis inactive version of BamA lacking its POTRA-3 domain still complements the *bamA101* mutation with respect to CDI sensitivity[58]. Second, antibodies to BamA block the binding of CDI^{EC93} inhibitor cells to target bacteria and also protect target cells from growth inhibition[58]. Finally, allelic exchange of *E. coli bamA* with the *bamA* genes of other enterobacterial species renders *E. coli* cells completely resistant to CDI^{EC93} [46; 64; 65]. The latter BamA-replacement strategy facilitated localization of the $CdiA^{EC93}$ binding site to extracellular loops L6 and L7 of BamA[64]. These loop sequences vary considerably between different species, restricting the CDI^{EC93} target-cell range to *E. coli*. Because most cell-surface epitopes are variable due to strong positive selection, these observations suggest that other CdiA proteins probably have narrow target-cell ranges. However, there are two examples where CDI acts across species. *Enterobacter cloacae* ATCC 13047 can inhibit *E. coli* K-12 derivatives using its CDI system[26], and the $CDI_{II}^{Bp1026b}$ system from *B. pseudomallei* 1026b inhibits closely related *Burkholderia thailandensis* E264[28; 66]. The receptors for these latter CdiA proteins have not been identified, but there is some evidence that lipopolysaccharide (LPS) may serve as a receptor for $CdiA_{II}^{Bp1026b}$. Disruption of the BTH_I0986 locus in *B. thailandensis* target bacteria confers resistance to $CDI_{II}^{Bp1026b}$ [66]. The BTH_I0986 gene encodes a predicted LPS glycosyltransferase, and ΔBTH_I0986 mutants have altered LPS and show decreased binding to inhibitor cells that express $CdiA_{II}^{Bp1026}$ [66]. Thus, it seems likely that CdiA effectors exploit several different cell-surface receptors to identify susceptible target cells.

Many CDI toxins are nucleases and therefore must translocate into the target-cell cytoplasm to reach their substrates. CdiA-CT fragments have been detected inside target bacteria directly using immunofluorescence microscopy[42] and indirectly by monitoring nuclease activities[26; 46]. Thus, C-terminal toxin domains must be transported across both outer- and

inner-membranes of target bacteria. Recent work shows that CDI toxin translocation requires the proton gradient across the inner membrane of target bacteria[46]. This gradient generates an electrochemical potential called the proton motive force (pmf), which is used to power diverse processes in bacteria including the import of small molecules and flagellum rotation. Colicins also require the pmf to enter bacteria, using pmf-energized Tol or Ton systems for transport across the outer membrane[14]. However, *tol* and *ton* mutants are not resistant to CDI, and there is evidence that the pmf is not required for CdiA-CT translocation across the target-cell outer membrane[46]. CdiA proteins are easily removed from the surface of inhibitor cells with extracellular protease; but the CdiA-CT toxin is protected from protease when inhibitor cells are bound to target bacteria in the presence of uncoupling agents, which dissipate the pmf. Under these conditions, much of the CdiA filament is still degraded by extracellular protease, converting the inhibitor-target cell aggregates into a suspension of individual cells. Remarkably, toxin activity is detectable within target cells shortly after the cells are washed to remove the uncoupling agent. This result suggests that the CdiA-CT is transferred into the periplasm of target bacteria, protecting it from extracellular protease. Further, toxin can apparently dwell in the target-cell periplasm for several minutes and remain competent to resume translocation once the pmf is reestablished. Together, these findings indicate that the pmf is required for the final translocation step across the target-cell inner membrane.

Contact-dependent growth inhibition in Gram-positive bacteria

CdiB/CdiA proteins have evolved to transport cargo exoproteins across the Gram-negative outer membrane and therefore are not found in Gram-positive bacteria, which are surrounded by a single membrane and thick peptidoglycan layer. However, there is an analogous toxin-delivery system commonly found in *Bacillus* and *Listeria* species. Wall-associated protein A (WapA) was first identified in *Bacillus subtilis* as an abundant secreted protein that associates non-covalently with the peptidoglycan wall[67]. WapA exhibits a number of features in parallel with CdiA effector proteins. Both proteins are cell-surface associated and very large. WapA from *B. subtilis* 168 has a predicted molecular mass of 258 kDa, though the full-length chain is difficult to detect and cleavage products are far more abundant[67; 68]. WapA also contains peptide-repeat regions, but the repeats are similar to YD-peptide/Rhs sequences rather than the FHA repeats of CdiA. Most importantly, the C-terminal region of WapA is highly variable between different strains of *B. subtilis* and these domains have toxic nuclease activities[44]. All of the characterized WapA-CT toxins have tRNase activities. WapA-CT^{168} from *B. subtilis* 168 cleaves four nucleotides from the 3´-end of tRNA molecules, whereas the toxins from *B. subtilis natto* and *B. subtilis* T-UB-10 specifically cleave near the anticodon loops of tRNA^{Glu} and tRNA^{Ser}, respectively[44]. Like *cdi* loci, each *wapA* gene is closely linked to a downstream immunity gene that is responsible for protecting the cell from WapA-CT toxin activity. *wapAI* genes are found in many different strains of *B. subtilis, B. cereus, B. thringiensis, B. anthracis, B. amyloliquefaciens, Geobacillus sp., Anoxybacillus sp.* and *Listeria monocytogenes*, suggesting that these systems play important roles in self/nonself recognition in Gram-positive bacteria. Because this growth inhibition appears to require direct contact between

inhibitor and target cells[44], WapA presumably binds to cell-surface receptors and translocates its C-terminal nuclease domains into the cytoplasm.

CDI and cooperative behavior

Although much of the work on CDI has focused on inter-cellular competition, these systems also contribute to cooperative group activities. Collmer and colleagues provided the first evidence for this function before the growth inhibition activity of CdiA proteins was appreciated. They found that HecA (CdiA^{EC16}) is required for the pathogenesis of *E. chrysanthemi* EC16 on plant hosts[69]. *hecA* mutants have defects in adherence to plant epidermal cells and do not auto-aggregate like wild-type cells. Similar findings have been reported for *Neisseria meningitidis*, *Xylella fastidiosa* and *Xanthomonas axonopodis*[70; 71; 72; 73; 74], suggesting that inter-cellular adhesion between isogenic cells contributes to host colonization and infection. These latter reports also show that *cdiA* mutants are defective for biofilm formation, a finding that has been confirmed and extended in *B. thailandensis* E264 and *E. coli* EC93 (Fig. 6)[36; 65; 75; 76]. Using *Burkholderia thailandensis* E264 as a model, Cotter and colleagues have shown that *cdiA* and *cdiB* mutants have profound biofilm defects[36]. Intriguingly, point mutations that ablate the nuclease activity of the CdiA-CT^{E264} toxin domain also interfere with biofilm formation[76]. Although toxin activity is required for biofilm formation, cell killing is not, suggesting that the exchange of the CdiA-CT^{E264} between isogenic (and immune) cells could influence gene expression to promote biofilms[76]. Of course, the cell-cell adhesion activity of CdiA proteins also promotes the biofilm lifestyle. Recent work with *E. coli* EC93 shows that CdiA binding to the BamA receptor is important for the formation of mature biofilms. However, EC93 cells that express a heterologous *bamA* gene, and therefore lack a suitable receptor for CdiA^{EC93}, still form biofilm structures[65]. CdiA^{EC93} contains an additional adhesin domain that probably mediates homotypic interactions and facilitates auto-aggregation independently of the BamA receptor[65]. Together, these reports show that CDI promotes collective behaviors directly through cell-cell adhesion, but also indirectly through uncharacterized signaling pathways. Thus, CDI promotes the establishment of kin communities, where isogenic cells collaborate to form biofilms, and the growth inhibition activity of these systems excludes non-identical bacteria from participating in the group behavior[75].

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Highlights

Contact-dependent growth inhibition (CDI) systems mediate inter-bacterial competition.

CdiA effector proteins carry a variety of C-terminal toxin domains.

CDI toxins are specifically neutralized by cognate immunity proteins.

CDI systems also contribute to community structure by promoting biofilm formation.

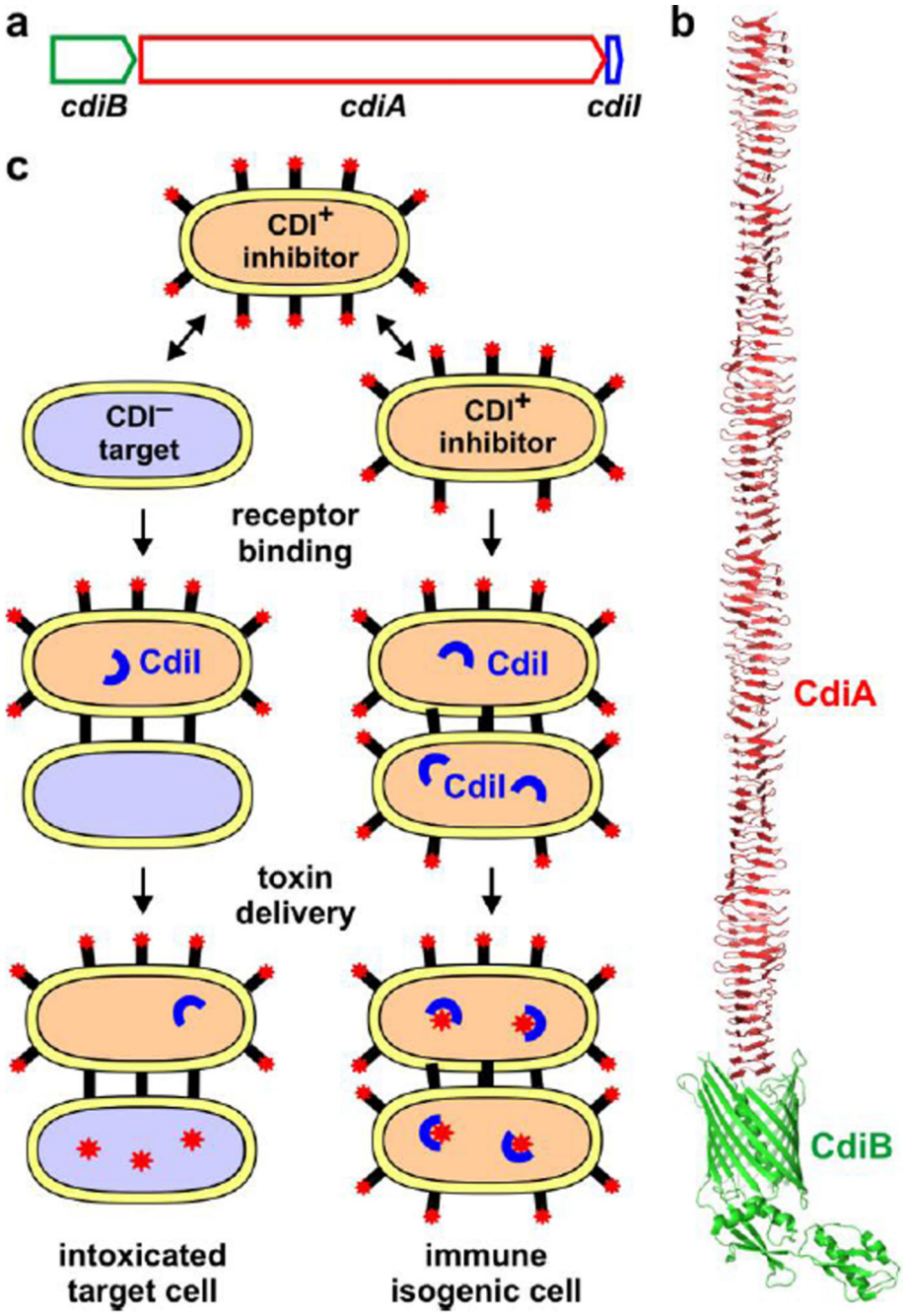

Fig. 1. Contact-dependent growth inhibition (CDI)

a) CDI$^+$ bacteria carry *cdiBAI* gene clusters that encode CdiB-CdiA two-partner secretion proteins and CdiI immunity proteins. **b**) Model for the CdiB/CdiA complex. CdiB is represented by the crystal structure of *B. pertussis* FhaC (PDB: 3NJT), and CdiA is modeled as concatenated β-helices from *E. coli* Ag43 (PDB: 4KH3). **c**) CdiA binds to receptors on neighboring bacteria and delivers its C-terminal toxin domain (red star) into the target cell. If target cells lack immunity (left pathway), then their growth is inhibited. In contrast, CdiI

neutralizes the toxin in isogenic CDI^+ bacteria, preventing growth inhibition (right pathway).

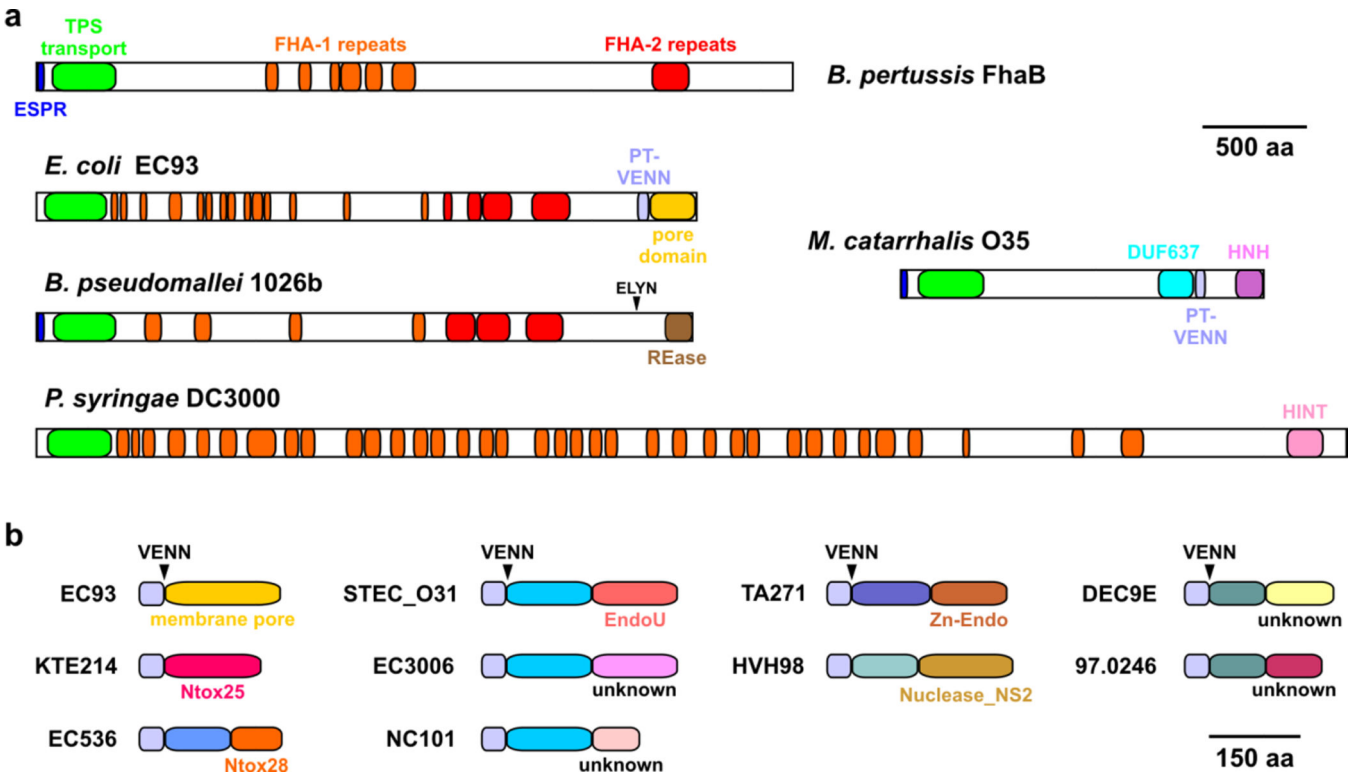

Fig. 2. Domain structure of CdiA

a) Architectures of putative CdiA proteins. Predicted domain structures are presented for CdiA proteins from *E. coli* EC93 (Uniprot: Q3YL96), *M. catarrhalis* O35E (A5JFL2), *B. pseudomallei* 1026b (I1WVY3) and *P. syringae* DC3000 (Q880E1). The domain structure of *B. pertussis* FhaB is also presented for comparison. The extended signal peptide region (ESPR) and TPS transport domain are required for TpsA/CdiA secretion. FHA-1 (PF05594) and FHA-2 (PF13332) peptide repeats were first identified in FhaB and are predicted to form β-helical structures[34; 35]. In many species, the pretoxin-VENN domain (PF04829) demarcates the variable C-terminal (CT) region, which contains the CDI toxin activity. DUF637 (PF04830) is a domain of unknown function found in a subset of CdiA proteins. HINT indicates the pretoxin-HINT domain (hedgehog intein; PF07591), and HNH indicates a predicted colicin DNase domain (PF12639). The restriction endonuclease (REase) domain at the C-terminus of $CdiA^{Bp1026b}$ was determined though X-ray crystallography[27]. **b**) Domain structures of CdiA-CT regions from different *E. coli* CdiA proteins. $CdiA\text{-}CT^{EC93}$ and $CdiA\text{-}CT^{KTE214}$ are formed from single domains, but many other CdiA-CTs are composed of two domains. The extreme C-terminal domain usually contains nuclease activity, whereas the function of the variable N-terminal domain is not known. *E. coli* strains are indicated as superscripts and domains are color-coded to indicate sequence variation. The position of the common VENN peptide motif is indicated.

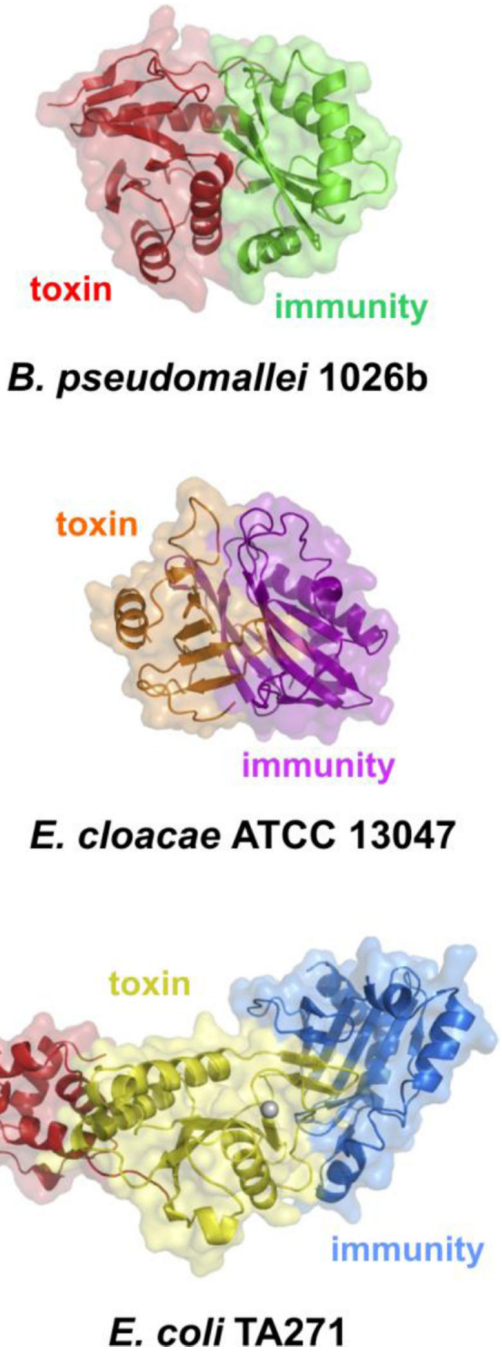

Fig. 3. Crystal structures of selected CdiA-CT/CdiI complexes

Toxin/immunity protein complexes from *B. pseudomallei* 1026 (PDB: 4G6V), *E. cloacae* ATCC 13047 (PDB: 4NTQ) and *E. coli* TA271 (PDB: 4G6U) are presented[26; 27]. All CdiA-CT structures contain only the C-terminal nuclease domain, with the exception of CdiA-CT^{TA271}, for which a portion of the N-terminal domain (shown in red) has been resolved.

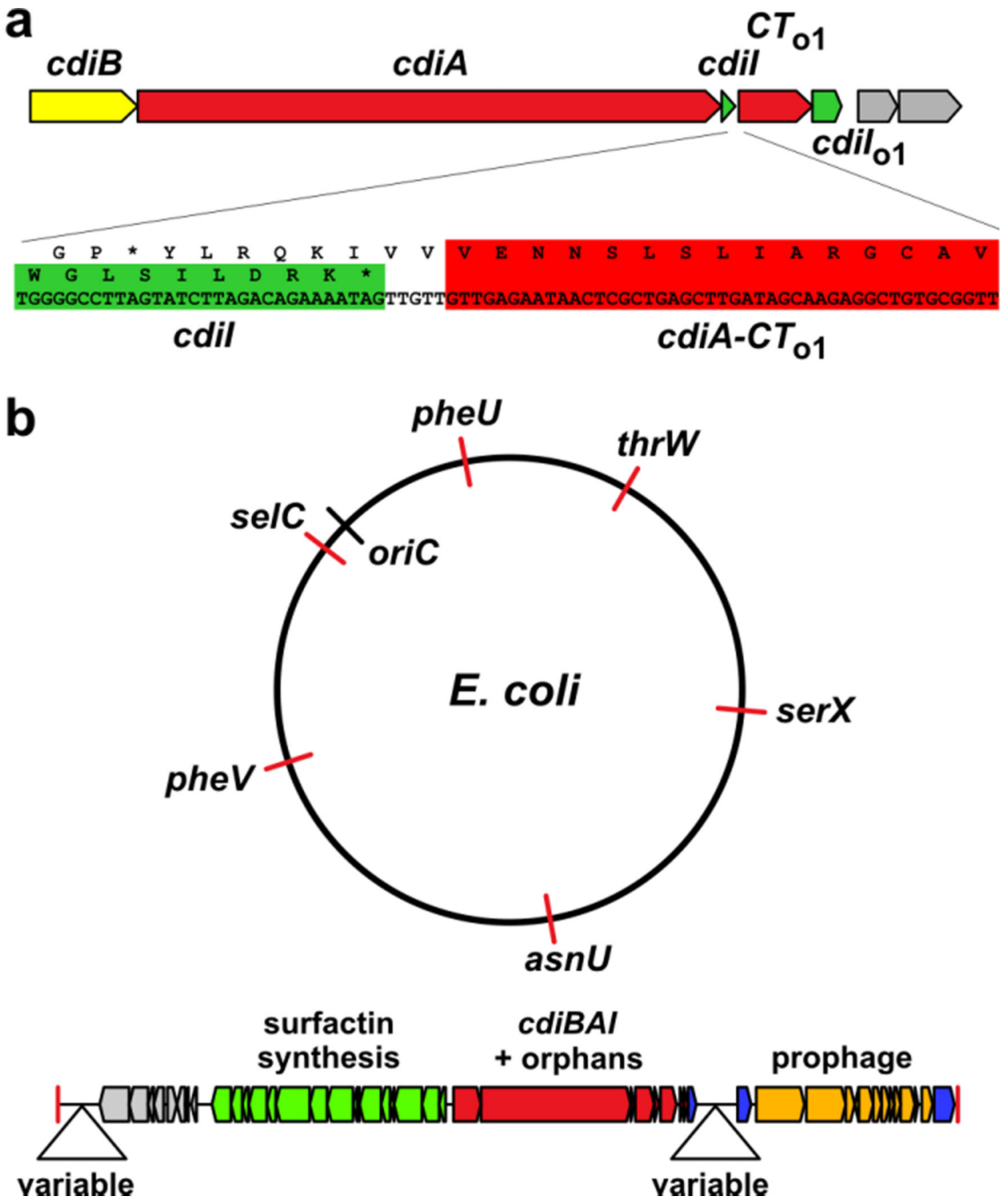

Fig. 4. Genomic organization and horizontal gene transfer

a) The *cdi* gene cluster from *E. coli* EC93 is depicted with the *cdiA-CT/cdiI*$_{o1}$ orphan gene pair. The genes rendered in gray encode a predicted IS3-family transposase. The *cdiA-CT*$_{o1}$ toxin coding sequence is outlined in red and lacks an initiating methionine codon. **b**) *E. coli cdi* genes are found on genomic islands. A family of *cdi* gene containing islands is inserted at several different tRNA genes in *E. coli* strains.

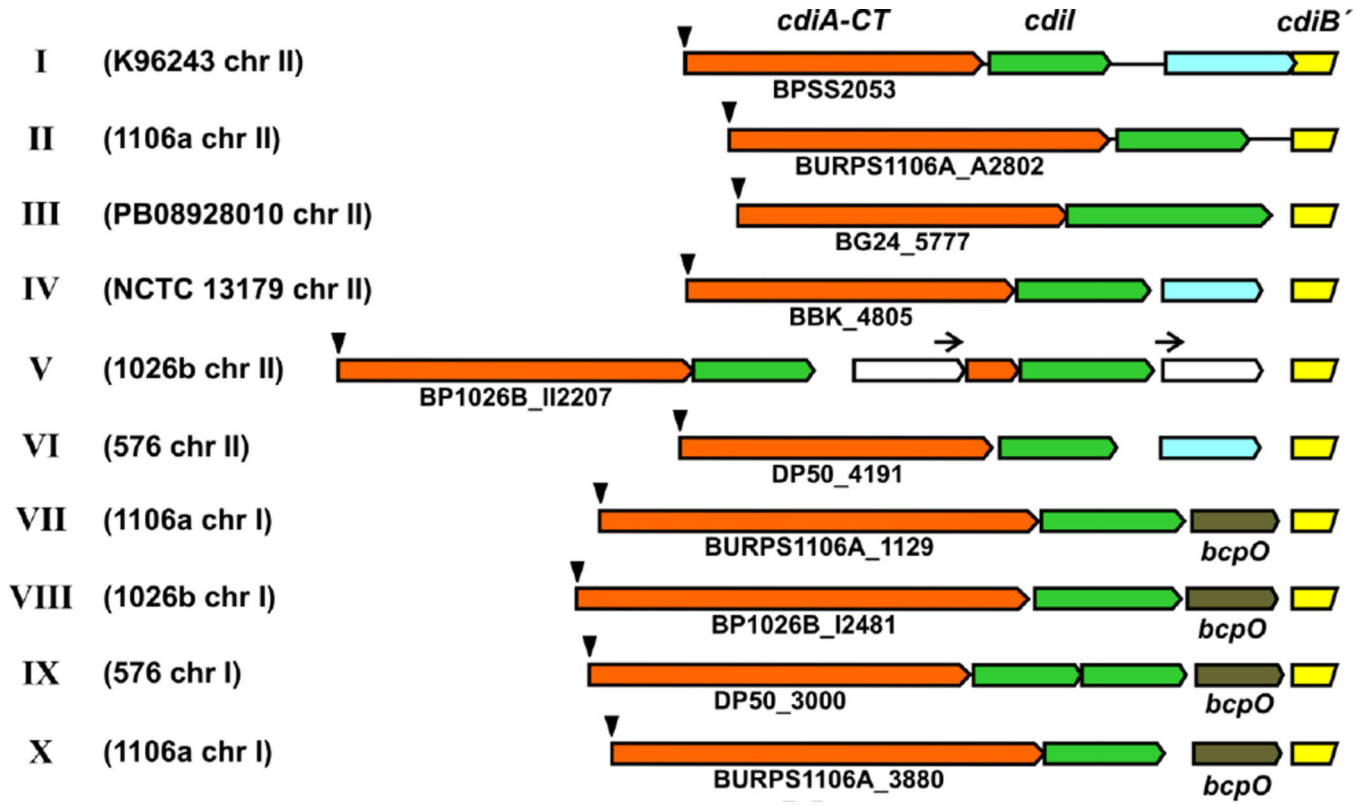

Fig. 5. *Burkholderia pseudomallei cdi* loci

Organization of the 10 *cdi* sequence types found in strains of *B. pseudomallei*. The *cdiA-CT* to *cdiB* regions are shown from representative strains, with *cdiA* genes identified by their ordered locus tags. Immunity genes are shown in green, *bcpO* in brown and predicted orphan immunity genes in light blue. The direct repeats surrounding the orphan *cdiI* immunity gene within the type locus are shown as arrows.

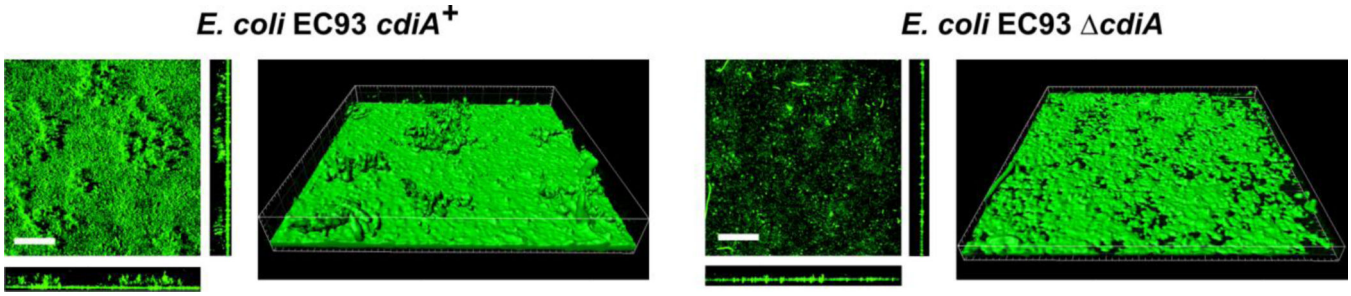

Fig. 6. *E. coli* EC93 Δ*cdiA* mutants are defective for biofilm formation

Biofilms of wild-type (*cdiA*$^+$) and Δ*cdiA* strains of *E. coli* EC93 after 24 h incubation in a flow-cell. Inset scale bars equal 40 μm. The left panels show three-dimensional reconstructions of biofilm structures. Images courtesy of Loni Townsley and Fitnat Yildiz (UC Santa Cruz).

Table 1

E. coli CdiA-CT toxin families.

Class	Strain	Genbank ID	Activity	Pfam designation
1	*E. coli* DEC9E	EHW54111.1	-	-
2	*E. coli* 97.0246	EIG93024.1	-	-
3	*E. coli* EC1738	EIP59427.1	-	-
4	*E. coli* STEC_O31	EJK94116.1	RNase	EndoU (PF14436)
5	*E. coli* NC101	EFM55009.1	-	-
6	*E. coli* HVH 98	ESK02023.1	DNase	Endonuclease_NS_2 (PF13930)
7	*E. coli* O32:H37	EIF16908.1	-	-
8	*E. coli* 96.154	EIH97650.1	-	DUF4258 (PF14076)
9	*E. coli* 3006	EKI34460.1	-	-
10	*E. coli* EC93	AAZ57198.1	pore-forming	-
11	*E. coli* 536	ABG72516.1	tRNA anticodon nuclease	Ntox28 (PF15605)
12	*E. coli* EC869	EDU89581.1	cleaves $tRNA^{Gln}$/$tRNA^{Asn}$	-
13	*E. coli* TA271	EGI36612.1	DNase	-
14	*E. coli* 0.1288	EKJ55728.1	-	-
15	*E. coli* B088	EFE64162.1	-	-
16	*E. coli* KTE214	ELD41871.1	RNase	Ntox25 (PF15530)
17	*E. coli* M605	EGI12780.1	-	-
18	*E. coli* B799	EIG47241.1	-	-
19	*E. coli* KTE75	ELE47736.1	-	-
20	*E. coli* 3-267-03_S3_C2	KDU01818.1	-	-