A widespread family of polymorphic contact-dependent toxin delivery systems in bacteria

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Summary paragraph

Bacteria have developed mechanisms to communicate and compete with one another in diverse environments \(^1\). A new form of intercellular communication, contact-dependent growth inhibition (CDI), was discovered recently in *Escherichia coli* \(^2\). CDI is mediated by the CdiB/CdiA two-partner secretion system. CdiB facilitates secretion of the CdiA ‘exoprotein’ onto the cell surface. An additional immunity protein (CdiI) protects CDI\(^+\) cells from autoinhibition \(^2\), \(^3\). The mechanisms by which CDI blocks cell growth and CdiI counteracts this growth arrest are unknown. Moreover, the existence of CDI activity in other bacteria has not been explored. Here we show that the CDI growth inhibitory activity resides within the carboxy-terminal region of CdiA (CdiA-CT), and that CdiI binds and inactivates cognate CdiA-CT, but not heterologous CdiA-CT. Bioinformatic and experimental analyses show that multiple bacterial species encode functional CDI systems with high sequence variability in the CdiA-CT and CdiI coding regions. CdiA-CT heterogeneity implies that a range of toxic activities are utilized during CDI. Indeed, CdiA-CTs from uropathogenic *E. coli* and the plant pathogen *Dickeya dadantii* have different nuclease activities, each providing a distinct mechanism of growth inhibition. Finally, we show that bacteria lacking the CdiA-CT and CdiI coding regions are unable to compete with isogenic wild-type CDI\(^+\) cells in both laboratory media and upon a eukaryotic host. Taken together, these results suggest that CDI systems constitute an intricate immunity network that plays an important role in bacterial competition.

Contact-dependent growth inhibition (CDI) is a phenomenon discovered in *Escherichia coli* strain EC93, which inhibits the growth of other *E. coli* strains upon direct cell-to-cell contact \(^2\). CDI is mediated by the CdiB/CdiA two-partner secretion (TPS) system. CdiB is required for assembly of the CdiA ‘exoprotein’ onto the outer membrane. Epitope insertion mutagenesis revealed the importance of the CdiA C-terminus (CdiA-CT) in CDI \(^2\). Genetic and antibody blocking experiments identified BamA, an essential protein required for outer membrane biogenesis, as the CDI receptor on target cells \(^3\). The inner membrane multidrug transporter AcrB may also play a role as acrB mutants, like bamA mutants, are resistant to CDI \(^3\). For EC93 mediated CDI, growth inhibition coincides with dissipation of the proton motive force across the cytoplasmic membrane, decreased aerobic respiration, and decreased ATP levels in the target cells \(^4\). EC93 is protected from autoinhibition by a small immunity
protein (CdiI), which is encoded immediately downstream of cdiA. These data suggest that CdiA binds BamA and delivers a signal, possibly a CdiA-derived toxin, which then inhibits target cell growth. CdiI could confer immunity to cells by binding to the CdiA peptide or otherwise neutralizing the growth inhibitory signal (Supplementary Fig. 1a).

Previous complementation analysis indicated the presence of functional cdiB and cdiA homologues in uropathogenic E. coli (UPEC), but no cdiI homolog was identified. Inspection of the cdi locus from E. coli UPEC 536 revealed a small open reading frame (ORF) in the same relative location as, but lacking significant sequence identity to, cdiIEC93. Expression of this ORF (cdiI536) protected E. coli from growth inhibition mediated by cells expressing CdiA536, but not from cells that express CdiAEC93 (Fig. 1a). Similarly, CdiIEC93 only provided immunity to cells expressing CdiAEC93 (Fig. 1a). Therefore, the protection conferred by CdiI appears to be limited to its cognate CDI system. Alignment of CdiAEC93 and CdiA536 showed that the N-terminal \( \sim 3,000 \) residues (up to and including a common VENN peptide motif) are 78% identical, but the C-terminal \( \sim 220 \) residues share no significant similarity. To determine if the dissimilar C-termini of CdiAEC93 and CdiA536 account for the specificity of CdiI-mediated immunity, we replaced the coding sequences for CdiA-CT536 and CdiI536 in UPEC 536 with the corresponding region from EC93. The resulting strain produces a chimeric CdiA protein, in which the C-terminal 223 residues of CdiAEC93 are fused to the N-terminal 3020 residues of CdiA536. UPEC 536 producing this chimeric CdiA inhibited target cells expressing CdiI536 but not cells expressing CdiIEC93, whereas the converse was true for wild-type UPEC 536 (Fig. 1c). These results show that CdiA-CTEC93 is functional when grafted onto the CdiA molecule from UPEC 536, and that the CdiA-CT sequence is important for specificity of immunity.

The observation that CdiI-mediated immunity is specific to the CdiA-CT suggests that growth inhibitory activity is also contained within the CdiA-CT. Expression of the C-terminal 268 residues of CdiAEC93 inside E. coli cells inhibited growth, and this inhibition was blocked by co-production of CdiIEC93, but not CdiI536 (Fig. 2a). Because the CdiA-CT lacks a secretion signal sequence, it is likely that this CdiA-CT-mediated growth inhibition and CdiI-mediated immunity occur within the cytoplasm. The minimal active region of CdiA-CTEC93 was determined by deletion analysis. Removal of up to 25 residues from the N-terminus of the 268 residue CdiA-CTEC93 construct did not significantly affect growth inhibitory activity (Fig. 2b). Removal of 45 residues from the N-terminus, which includes the conserved VENN motif, yielded a polypeptide with \( \sim 10 \)-fold greater inhibitory activity. Deletion of an additional 13 residues from the N-terminus completely abrogated activity (Fig. 2b). Deletion of as few as 12 residues from the C-terminus of the CdiA-CTEC93 construct also abolished inhibitory activity. Thus, the growth inhibitory activity resides within the C-terminal 223 amino acid residues of CdiAEC93.

We next searched for additional cdiBAI loci in other E. coli strains. Although several cdiBAI gene clusters are present in partially assembled E. coli genome sequences (Supplementary Table 1), we limited our analysis to the 33 fully assembled E. coli genomes currently available. Two E. coli isolates, UTI89 and CFT073, encode two-partner secretion systems related to EC93 CdiB/CdiA. The UTI89 CDI module is identical to that of UPEC 536 and is located within the same pathogenicity island (PAI II536) in both strains (Supplementary Fig. 4).
In contrast, the CFT073 CDI locus resides in an unrelated pathogenicity island (PAI-CFT073-aspV)\(^5\), and its predicted CdiA-CT and CdiI sequences show no similarity to the UPEC 536 or EC93 systems (Supplementary Figs. 2b, 2c, 3b). The EC93 CdiA-CT is unrelated to the CdiA-CTs identified in other fully sequenced \textit{E. coli} strains; however, it is 42% identical to the CdiA-CT from another species, \textit{Edwardsiella tarda} EIB202. This, together with the sporadic occurrence of \textit{cdi} loci in \textit{E. coli} strains and their association with genomic islands, suggests that these genes may be transferred horizontally.

Bioinformatic analyses showed that CDI systems are widespread amongst other Gram-negative bacteria, with representatives identified in a variety of \(\alpha\)-, \(\beta\)-, and \(\gamma\)-proteobacteria (Supplementary Table 1 and Supplementary Fig. 3). Although widely distributed, only certain strains of any given species contain \textit{cdiBAI} homologues. Some bacterial isolates encode multiple CDI modules, for example \textit{Bartonella grahamii} as4aup and \textit{Photorhabdus luminescens} \textit{laumondii} contain four and five CDI modules, respectively (Supplementary Fig. 3b). Based on their similarity to CdiA\textsubscript{EC93} each putative \textit{cdiA} homologue is likely to encode a TpsA family member with an N-terminal hemagglutination activity domain (Pfam PF05860, also called a TPS domain) and hemagglutinin repeats that are predicted to form a \(\beta\)-helical structure. Additionally, most \textit{cdiA} homologs encode the VENN peptide motif, which is part of the DUF638 domain of unknown function (Pfam PF04829) (Fig. 1b and Supplementary Fig. 3a). In general, significant variability between CdiA-CT/CdiI protein sequences was observed between different species and between different CdiA-CTs encoded within a single strain. There are instances where an extended genomic region, including the CDI module, is conserved between different strains of the same species (as in \textit{E. coli} UPEC 536 and UTI89, and most \textit{Y. pestis} strains); in such cases the entire CdiA protein and CdiI are highly conserved. \textit{Burkholderia} species also appear to encode CdiB/CdiA two-partner secretions systems, but these loci have a different gene organization (\textit{cdiAIB} rather than \textit{cdiBAI}), and the putative CdiA proteins lack the DUF638 domain. Instead of the VENN motif, the \textit{Burkholderia cdiA} homologues encode an NxxLYN motif that precedes variable C-terminal domains (Supplementary Fig. 3c). In all instances, the \textit{Burkholderia cdiA} genes are followed by short ORFs, which may be analogous to the \textit{cdiI} genes in \textit{E. coli}.

To determine whether the \textit{in silico}-identified \textit{cdi} loci encode functional CDI systems, we replaced the CdiA-CT and CdiI coding regions of UPEC 536 with the corresponding sequences from \textit{Yersinia pestis} CO92 and the region 2 CDI module from \textit{Dickeya dadantii} 3937 (Supplementary Table 1). UPEC 536 producing chimeric CdiAs inhibited target cells expressing heterologous CdiI proteins but not cells that express cognate CdiI (Fig. 1c), strongly suggesting that \textit{Y. pestis} CO92 and \textit{D. dadantii} 3937 encode CDI systems with allele-specific immunity proteins. These data also indicate that the N-terminal 3020 residues of CdiAs\textsubscript{36} are capable of delivering functional CdiA-CT domains from \textit{Y. pestis} CO92 and \textit{D. dadantii} 3937 into target cells.

How do CdiI immunity proteins protect against cognate CdiA-CT? We hypothesized that CdiI prevents CDI-mediated autoinhibition by specifically binding to the C-terminus of cognate CdiA. To test this hypothesis, we examined the interaction between CdiA-CT and hexahistidine-tagged CdiI (CdiI-His\textsubscript{6}) proteins using Ni\textsuperscript{2+}-affinity pull-down experiments.
Because the CdiA-CTs used in these experiments lack His\textsubscript{6} epitope tags, their retention on Ni\textsuperscript{2+}-nitrilotriacetic acid (Ni\textsuperscript{2+}-NTA) resin is dependent upon binding to CdiI-His\textsubscript{6}. CdiA-CT\textsubscript{536} was retained by the Ni\textsuperscript{2+}-NTA resin when pre-incubated with CdiI\textsubscript{536}-His\textsubscript{6}, but not with non-cognate CdiI\textsubscript{3937-2}-His\textsubscript{6} (Fig. 3a). Reciprocally, CdiA-CT\textsubscript{3937-2} bound to the resin only in the presence of cognate CdiI\textsubscript{3937-2}-His\textsubscript{6} (Fig. 3a). These data indicate that CdiI proteins bind to CdiA-CT in vitro in an allele-specific manner. To determine if CdiA-CT and CdiI bind one another in vivo, we used a modified bacterial two-hybrid system (BACTH, Euromedex). In this system, proteins of interest are fused to the T18 and T25 domains of adenylate cyclase, and binding of the two fusion proteins results in the production of cAMP, which is monitored indirectly through the expression of a β-galactosidase reporter. Co-expression of homologous T25-CdiA-CT\textsubscript{536} and CdiI\textsubscript{536}-T18 fusions, or T25-CdiA-CT\textsubscript{3937-2} and CdiI\textsubscript{3937-2}-T18 fusions yielded high β-galactosidase activities in both cases, over 300 units (Fig. 3b). Because CdiA-CTs are cytotoxic in the absence of cognate CdiI, we used green fluorescent protein (GFP) as a negative control to test binding specificity. Co-expression of T25-GFP and CdiI\textsubscript{536}-T18, or T25-GFP and CdiI\textsubscript{3937-2}-T18 resulted in very low β-galactosidase activities (Fig. 3b). Taken together, these data demonstrate that CdiI immunity proteins bind to their cognate CdiA-CTs in vitro and in vivo.

What is the mechanism(s) by which CDI systems inhibit target cell growth? Most CdiA-CTs are not similar to known proteins or protein domains. However, we found that the C-terminal 132 residues of CdiA\textsubscript{3937-2} from D. dadantii 3937 shares 35% identity with the pyocin S3 nuclease domain from Pseudomonas aeruginosa (Supplementary Fig. 4). Pyocin S3 is cytotoxic by virtue of its DNase activity, suggesting that CdiA\textsubscript{3937-2} may also use nuclease activity to inhibit target cell growth. We confirmed that purified CdiA-CT\textsubscript{3937-2} possesses a robust Mg\textsuperscript{2+}-dependent DNase activity, capable of completely digesting linear and supercoiled plasmid DNA (Fig. 3c, and data not shown). We also examined the activity of CdiA-CT\textsubscript{536}, which does not share sequence homology with other known toxins or colicins, and found that it has tRNase activity (Fig. 3d). Purified CdiA-CT\textsubscript{536} readily cleaved several E. coli tRNA species, but not ribosomal RNA or messenger RNA (Fig. 3d, and data not shown). For each CdiA-CT, the addition of purified cognate CdiI blocked nuclease activity, whereas addition of heterologous CdiI had no effect on activity (Fig. 3c, 3d). These results suggest that CDI systems use more than one mechanism to inhibit cell growth. If these DNase and tRNase activities are responsible for growth inhibition, then the CdiA-CTs must be translocated into the target cell cytoplasm (Supplementary Fig. 1). According to this model, CdiI proteins confer immunity to CDI by binding to cognate CdiA-CTs and blocking their enzymatic activities. Based on the diversity of CdiA-CT sequences (Fig. 1b, Supplementary Fig. 3), it seems likely that additional growth inhibitory mechanisms will be identified for other CDI systems.

It is not known when or where CDI systems are deployed in the environment, nor what precise biological function(s) they provide. UPEC 536 does not express the cdiBAI gene cluster under standard laboratory growth conditions (unpublished data). However, EC93 expresses cdiBAI constitutively. To determine if the CDI system in EC93 provides a selective advantage, we deleted the CdiA-CT and CdiI coding sequences of EC93 and mixed
the resulting mutant cells with wild-type EC93 at a one-to-one ratio in a growth competition experiment. After three hours of co-culture, EC93ΔcdiA-CTΔcdiI mutant bacteria were less than 1% of wild-type EC93 cells (competitive index <10^{-2}, Fig. 4a). However, EC93ΔcdiA-CTΔcdiI cells expressing CdiI_{EC93} from a plasmid were able to compete equally with wild-type EC93, indicating that the original loss of fitness was due to the mutant's susceptibility to CDI (Fig. 4a). These results indicate that CDI systems may play a significant role in intraspecies competition between bacteria occupying the same ecological niche. Further support for this conclusion came from analysis of D. dadantii. Previous work has shown that disruption of a putative cdiI gene, designated virA, reduces the virulence of D. dadantii EC16 on plant hosts. Our results indicate that VirA binds and inactivates the C-terminal domain of HecA_{EC16}, a CdiA homologue (Supplementary Table 1 and data not shown). These results suggest that D. dadantii may express cdi genes on plants. We used the fully sequenced D. dadantii 3937 strain, which contains two CDI regions (cdi_{3937-1} and cdi_{3937-2}, see Supplementary Table 1), to test the hypothesis that CDI plays a role in intrastrain competition. Each of the cdiA-CT/cdiI regions were deleted individually, and the resulting mutants were competed against wild-type D. dadantii 3937 on chicory. Although deletion of the cdi_{3937-2} region had no effect on competition, cells lacking cdi_{3937-1} were outcompeted by wild-type bacteria as evidenced by a competitive index of about 10^{-1} (Fig. 4b and data not shown). This competitive disadvantage was reversed by complementation with a chromosomal copy of the cognate cdiI_{3937-1} gene (Fig. 4b). Complementation was specific as non-cognate cdiI_{EC16} (virA) from D. dadantii EC16, had no effect on the competitive index (Fig. 4b). These results show that the region 1 CDI system in D. dadantii plays a role in growth competition on chicory. The role of the region 2 CDI system is unknown, but could function under different environmental conditions or target different bacterial species. Together, these results strongly indicate that CDI systems function in growth competition in the environment.

Methods Summary

The complete methods are presented in the Supplementary Information. Strains, plasmids, and oligonucleotides used in this study are shown in Supplementary Table 2. E. coli competition assays were carried out as previously described. D. dadantii competition assays on chicory were carried out as described in Supplementary Methods. EC93 and D. dadantii 3937 cdiA-CT-cdiI deletions and CdiA chimeras were constructed using allelic exchange as previously described. For chimera construction, the 3’ end of cdiA and all of cdiI from UPEC 536 ΔkpsK15 ΔaraCBA specRExBAD-cdiBAI (DL5646) were replaced with cdiA-CT (sequence immediately following VENNX) and cdiI from E. coli EC93, Y. pestis CO92 (accession number Q7CGD9), or D. dadantii 3937 region 2 (see Supplementary Methods). The ΔkpsK15 capsule mutation was used to increase the efficacy of CDI, based on our previous results showing that capsule production blocks CDI. Immunity plasmids were constructed by ligating PCR-amplified cdiI genes into plasmid pBR322 under tet promoter control (Fig. 1a, c). For D. dadantii 3937 the immunity plasmids were constructed by ligating PCR-amplified cdiI genes into the miniTn7 delivery plasmid pUC18R6KT-miniTn7T under tet promoter control (see Supplementary methods). Deletion mapping of E. coli EC93 cdiA-CT (Fig. 2b) was carried out by cloning specific sequences amplified by PCR into plasmid
pLAC11^14 under lac promoter control. All plasmids were propagated in EPI100 acrB mutant strain DL5154 to mitigate toxic effects. In vivo interactions between CdiA-CT and CdiI were determined using a modified BACTH bacterial two-hybrid system (Euromedex) \(^8\). β-galactosidase\(^1^4\) and fluorescence\(^3\) analyses were carried out as previously described. In vitro affinity pull-downs with His\(_6\)-tagged CdiI/CdiA-CT were carried out using Ni\(^{2+}\)-NTA resin (Qiagen) (Fig. 3a). CdiA-CT was released by denaturation in buffer containing 6 M guanidine-HCl, and His\(_6\)-tagged CdiI was released in native buffer supplemented with 250 mM imidazole. CdiA-CT activities were analyzed as described in supplementary methods.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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**References**


Figure 1. Analysis of CdiA chimeras

a, Target cells expressing CdiI<sub>EC93</sub> or CdiI<sub>536</sub> were co-cultured with inhibitor cells expressing CDI genes from either <em>E. coli</em> EC93 or UPEC 536. After 3 h, the number of viable target cells was determined as colony forming units (CFU) per ml (mean ± s.d., n = 4 experiments).

b, The C-terminal ~200 residues of the indicated CdiAs diverge following a conserved VENN peptide motif (shown by different colors). The CdiI proteins from each system are also highly variable.

c, Target cells expressing CdiI from <em>E. coli</em> UPEC 536, <em>E. coli</em> EC93, <em>Y. pestis</em> CO92 (accession Q7CGD9), and <em>D. dadantii</em> 3937 (CDI module 2, see Supplementary Table 1) were protected from CDI mediated by chimeric CdiA<sub>536</sub> proteins containing cognate, but not heterologous, CdiA-CT (mean ± s.d., n = 2 experiments).
Figure 2. CdiA-CT contains growth inhibitory activity

a, Growth of *E. coli* cells expressing CdiA-CT_{EC93} from plasmid pDAL778. Co-expression of cognate CdiI_{EC93}, but not heterologous CdiI_{536}, protected cells from growth inhibition. The mean ± s.d. is shown, n = 2 experiments. b, The 268 residue CdiA-CT_{EC93} peptide is depicted along with various truncation constructs indicating the number of residues deleted. Each CdiA-CT construct was tested for growth inhibitory activity when expressed in *E. coli* cells (“++” indicates that growth was blocked immediately after CdiA-CT_{EC93} induction, “+” indicates that growth was blocked after a 2-3 hr delay, and “-” indicates no growth inhibition).
Figure 3. CdiI immunity protein binds specifically to cognate CdiA-CT and blocks activity

a, Purified CdiA-CT and CdiI-His\textsubscript{6} proteins from UPEC 536 and D. dadantii 3937 (CDI module 2, 3937-2) were mixed \textit{in vitro} with Ni\textsuperscript{2+}-NTA resin. Aliquots of resin bound and unbound fractions were analyzed by SDS-PAGE and Coomassie blue staining.

b, A bacterial two-hybrid system (BACTH) based on adenylate cyclase activity was used to monitor CdiA-CT/CdiI binding \textit{in vivo}. A β-galactosidase reporter was used to measure adenylate cyclase activity. Expression of two-hybrid T25-ctdI-CT/cdiI-T18 fusion constructs resulted in significant β-galactosidase activity. T25-gfpmut3/cdiI-T18 fusions were used to control for background β-galactosidase activity. Fluorescence microscopy of cells expressing each D. dadantii 3937 construct confirms GFP expression of the control. \(P\) values were obtained using an unpaired, two-tailed \(t\)-test (mean ± s.d., \(n = 2\) experiments).

c, Purified CdiA-CT\textsubscript{3937-2} was incubated with linear pUC19 DNA in the presence and absence of cognate and heterologous CdiI. Reactions were analyzed by native agarose gel electrophoresis and ethidium bromide staining.

d, Purified CdiA-CT\textsubscript{536} was incubated with an S100 cell extract (100,000 x g supernatant), in the presence and absence of cognate and heterologous CdiI. Reactions were analyzed by denaturing gel electrophoresis. Top panel, ethidium bromide (EtBr) staining for total tRNA (arrow indicates tRNA; asterisk indicates...
degradation products); lower panels, Northern blot analyses of tRNA^{His} and tRNA^{Ala}_{1B}. Arrows indicate full-length tRNAs.
Figure 4. CDI systems function in intrastrain growth competition

**a.** StrR CDI+ EC93 cells were mixed 1:1 with rifR EC93 ΔcdiA-CTΔcdil cells that either contained no plasmid (cdi-), pBR322 plasmid (vector), or CdiI EC93-expressing plasmid (cdiI EC93). After 3 hr of co-culture, cells were plated onto LB medium, and rifR and strR CFU/ml were quantified to calculate the competitive index [(rifR CFU/strR CFU)3 hr / (rifR CFU/strR CFU)0 hr]. The mean ± s.d. is shown (n = 2 experiments), P value = 0.002.

**b.** GentR CDI+ D. dadantii 3937 cells were mixed 100:1 with nalR D. dadantii 3937 ΔcdiA-CT3937-1 Δcdil3937-1 alone (cdi-), or complemented with heterologous cdil from D. dadantii EC16 (cdil EC16), or cognate cdil (cdil3937-1). Cell mixtures were inoculated onto chicory leaves (see Supplementary methods), incubated for the indicated times, and viable counts were determined. The competitive index (CFU nalR /CFU gentR) was calculated as described for panel ‘a’ above at each time point. The mean ± s.d. is shown (n = 2 experiments). P value at 24 h = 0.00004. P values were obtained using an unpaired, two-tailed t-test.