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

Stephanie K. Aoki,
Department of Molecular, Cellular, and Developmental Biology, University of California–Santa Barbara (UCSB), Santa Barbara, CA 93106-9625, USA

Elie J. Diner,
Biomolecular Science and Engineering Program, University of California–Santa Barbara (UCSB), Santa Barbara, CA 93106-9625, USA

Claire t'Kint de Roodenbeke,
Department of Molecular, Cellular, and Developmental Biology, University of California–Santa Barbara (UCSB), Santa Barbara, CA 93106-9625, USA

Brandt R. Burgess,
Department of Molecular, Cellular, and Developmental Biology, University of California–Santa Barbara (UCSB), Santa Barbara, CA 93106-9625, USA

Stephen J. Poole,
Department of Molecular, Cellular, and Developmental Biology, University of California–Santa Barbara (UCSB), Santa Barbara, CA 93106-9625, USA

Bruce A. Braaten,
Department of Molecular, Cellular, and Developmental Biology, University of California–Santa Barbara (UCSB), Santa Barbara, CA 93106-9625, USA

Allison M. Jones,
Department of Molecular, Cellular, and Developmental Biology, University of California–Santa Barbara (UCSB), Santa Barbara, CA 93106-9625, USA

Julia S. Webb,

Correspondence and requests for materials should be addressed to D.A.L. (low@lifesci.ucsb.edu).
*Current address: Department of Microbiology and Immunology, University of North Carolina - Chapel Hill School of Medicine, Chapel Hill, NC 27599-7290
‡Co-contributing senior authors

Author Contributions: D.A.L., S.K.A., P.A.C., and C.S.H. designed research. The manuscript was prepared by D.A.L., C.S.H., P.A.C., and S.J.P., and S.K.A.. S.J.P. and B.R.B. carried out bioinformatic analyses. B.R.B., A.M.J., and P.A.C. obtained initial evidence for the toxic nature of CdiA-CT, variability of CdiA-CTs and CdiIs, and binding between CdiA-CTs and cognate CdiIs in studies of *Burkholderia pseudomallei cdi* genes. S.K.A. cloned and performed competition assays and growth curves involving *cdiBAI*, *cdiA-CT*, and *cdiA* chimeras and deletions. B.A.B. and J.S.W. conducted the deletion mapping study with assistance from S.K.A. B.A.B. and S.K.A. conducted the bacterial two-hybrid study. C. T. R. constructed *D.dadantii cdi* mutants and plasmids and performed growth competition assays on chicory. E.J.D. cloned, purified protein, and performed the *in vitro* protein interaction and CdiA-CT activity studies.

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Department of Molecular, Cellular, and Developmental Biology, University of California–Santa Barbara (UCSB), Santa Barbara, CA 93106-9625, USA

Christopher S. Hayes,
Biomolecular Science and Engineering Program, University of California–Santa Barbara (UCSB), Santa Barbara, CA 93106-9625, USA

Peggy A. Cotter[‡,*], and
Biomolecular Science and Engineering Program, University of California–Santa Barbara (UCSB), Santa Barbara, CA 93106-9625, USA

David A. Low[‡]
Biomolecular Science and Engineering Program, University of California–Santa Barbara (UCSB), Santa Barbara, CA 93106-9625, USA

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* [2]. 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 [2, 5]. 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, $cdiI_{EC93}$. Expression of this ORF ($cdiI_{536}$) protected *E. coli* from growth inhibition mediated by cells expressing $CdiA_{536}$, but not from cells that express $CdiA_{EC93}$ (Fig. 1a). Similarly, $CdiI_{EC93}$ only provided immunity to cells expressing $CdiA_{EC93}$ (Fig. 1a). Therefore, the protection conferred by CdiI appears to be limited to its cognate CDI system. Alignment of $CdiA_{EC93}$ and $CdiA_{536}$ showed that the N-terminal ~3,000 residues (up to and including a common VENN peptide motif) are 78% identical, but the C-terminal ~220 residues share no significant similarity [6] (Fig. 1b). To determine if the dissimilar C-termini of $CdiA_{EC93}$ and $CdiA_{536}$ account for the specificity of CdiI-mediated immunity, we replaced the coding sequences for CdiA-CT_{536} and $CdiI_{536}$ 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 $CdiA_{EC93}$ are fused to the N-terminal 3020 residues of $CdiA_{536}$. UPEC 536 producing this chimeric CdiA inhibited target cells expressing $CdiI_{536}$ but not cells expressing $CdiI_{EC93}$, whereas the converse was true for wild-type UPEC 536 (Fig. 1c). These results show that CdiA-CT_{EC93} 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 $CdiA_{EC93}$ inside *E. coli* cells inhibited growth, and this inhibition was blocked by co-production of $CdiI_{EC93}$, but not $CdiI_{536}$ (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-CT_{EC93} was determined by deletion analysis. Removal of up to 25 residues from the N-terminus of the 268 residue CdiA-CT_{EC93} 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 ~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-CT_{EC93} construct also abolished inhibitory activity. Thus, the growth inhibitory activity resides within the C-terminal 223 amino acid residues of $CdiA_{EC93}$.

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 II_{536}) in both strains (Supplementary Fig.

2a)[7]. 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 *E. coli* strains; however, it is 42% identical to the CdiA-CT from another species, *Edwardsiella tarda* EIB202. This, together with the sporadic occurrence of *cdi* loci in *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 α-, β-, and γ-proteobacteria (Supplementary Table 1 and Supplementary Fig. 3). Although widely distributed, only certain strains of any given species contain *cdiBAI* homologues. Some bacterial isolates encode multiple CDI modules, for example *Bartonella grahamii* as4aup and *Photorhabdus luminescens* subsp. *laumondii* contain four and five CDI modules, respectively (Supplementary Fig. 3b). Based on their similarity to $CdiA_{EC93}$ each putative *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 β-helical structure. Additionally, most *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 *E. coli* UPEC 536 and UTI89, and most *Y. pestis* strains); in such cases the entire CdiA protein and CdiI are highly conserved. *Burkholderia* species also appear to encode CdiB/CdiA two-partner secretions systems, but these loci have a different gene organization (*cdiAIB* rather than *cdiBAI*), and the putative CdiA proteins lack the DUF638 domain. Instead of the VENN motif, the *Burkholderia cdiA* homologues encode an NxxLYN motif that precedes variable C-terminal domains (Supplementary Fig. 3c). In all instances, the *Burkholderia cdiA* genes are followed by short ORFs, which may be analogous to the *cdiI* genes in *E. coli*.

To determine whether the *in silico*-identified *cdi* loci encode functional CDI systems, we replaced the CdiA-CT and CdiI coding regions of UPEC 536 with the corresponding sequences from *Yersinia pestis* CO92 and the region 2 CDI module from *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 *Y. pestis* CO92 and *D. dadantii* 3937 encode CDI systems with allele-specific immunity proteins. These data also indicate that the N-terminal 3020 residues of $CdiA_{536}$ are capable of delivering functional CdiA-CT domains from *Y. pestis* CO92 and *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 ($CdiI\text{-}His_6$) proteins using Ni^{2+}-affinity pull-down experiments.

Because the CdiA-CTs used in these experiments lack His_6- epitope tags, their retention on Ni^{2+}-nitrilotriacetic acid (Ni^{2+}-NTA) resin is dependent upon binding to CdiI-His_6. CdiA-CT_{536} was retained by the Ni^{2+}-NTA resin when pre-incubated with $CdiI_{536}$-His_6, but not with non-cognate $CdiI_{3937\text{-}2}$-His_6 (Fig. 3a). Reciprocally, CdiA-$CT_{3937\text{-}2}$ bound to the resin only in the presence of cognate $CdiI_{3937\text{-}2}$-His_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 [8]. Co-expression of homologous T25-CdiA-CT_{536} and $CdiI_{536}$-T18 fusions, or T25-CdiA-$CT_{3937\text{-}2}$ and $CdiI_{3937\text{-}2}$-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 $CdiI_{536}$-T18, or T25-GFP and $CdiI_{3937\text{-}2}$ -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 $CdiA_{3937\text{-}2}$ 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 [9], suggesting that $CdiA_{3937\text{-}2}$ may also use nuclease activity to inhibit target cell growth. We confirmed that purified CdiA-$CT_{3937\text{-}2}$ possesses a robust Mg^{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_{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_{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 [2]. 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, EC93Δ*cdiA-CT*Δ*cdiI* mutant bacteria were less than 1% of wild-type EC93 cells (competitive index $<10^{-2}$, Fig. 4a). However, EC93Δ*cdiA-CT*Δ*cdiI* 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 [10]. 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 ($cdi_{3937\text{-}1}$ and $cdi_{3937\text{-}2}$, 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 [11, 12]. Although deletion of the $cdi_{3937\text{-}2}$ region had no effect on competition, cells lacking $cdi_{3937\text{-}1}$ 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 $cdiI_{3937\text{-}1}$ 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 [2]. *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 [13]. For chimera construction, the 3′ end of *cdiA* and all of *cdiI* from UPEC 536 Δkps_{K15} Δ*araCBA spec*RExBAD-*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 Δkps_{K15} capsule mutation was used to increase the efficacy of CDI, based on our previous results showing that capsule production blocks CDI [3]. 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[14] 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

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Acknowledgments

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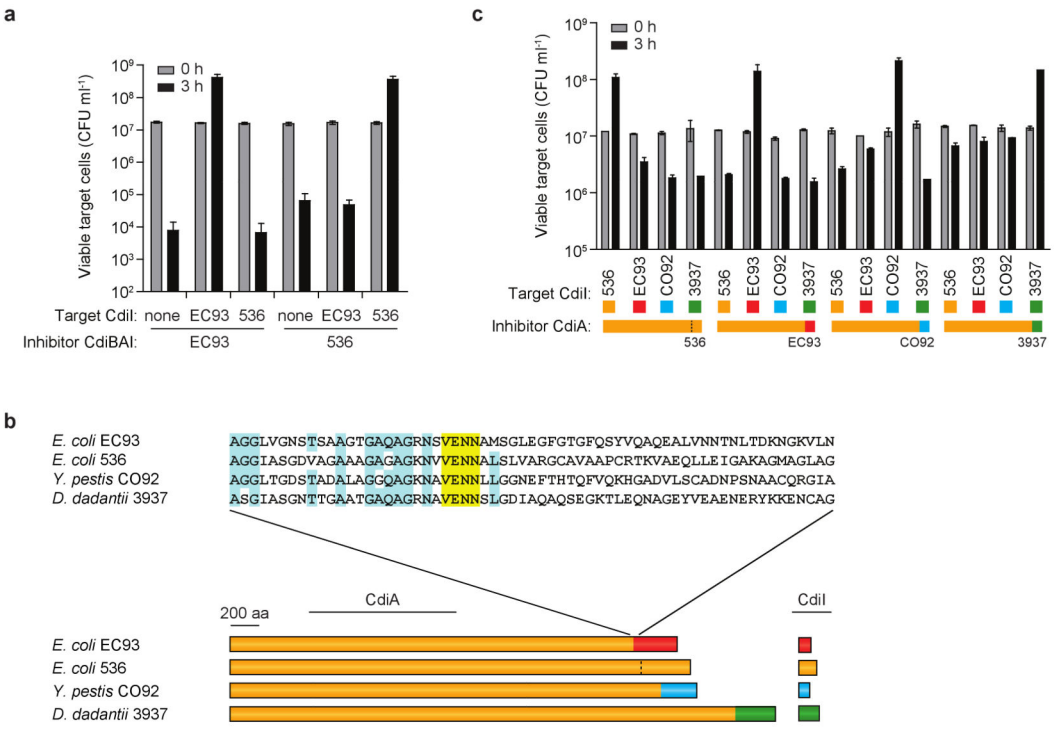

Figure 1. Analysis of CdiA chimeras

a, Target cells expressing $CdiI_{EC93}$ or $CdiI_{536}$ were co-cultured with inhibitor cells expressing *cdi* genes from either *E. coli* 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 *E. coli* UPEC 536, *E. coli* EC93, *Y. pestis* CO92 (accession Q7CGD9), and *D. dadantii* 3937 (CDI module 2, see Supplementary Table 1) were protected from CDI mediated by chimeric $CdiA_{536}$ proteins containing cognate, but not heterologous, CdiA-CT (mean ± s.d., n = 2 experiments).

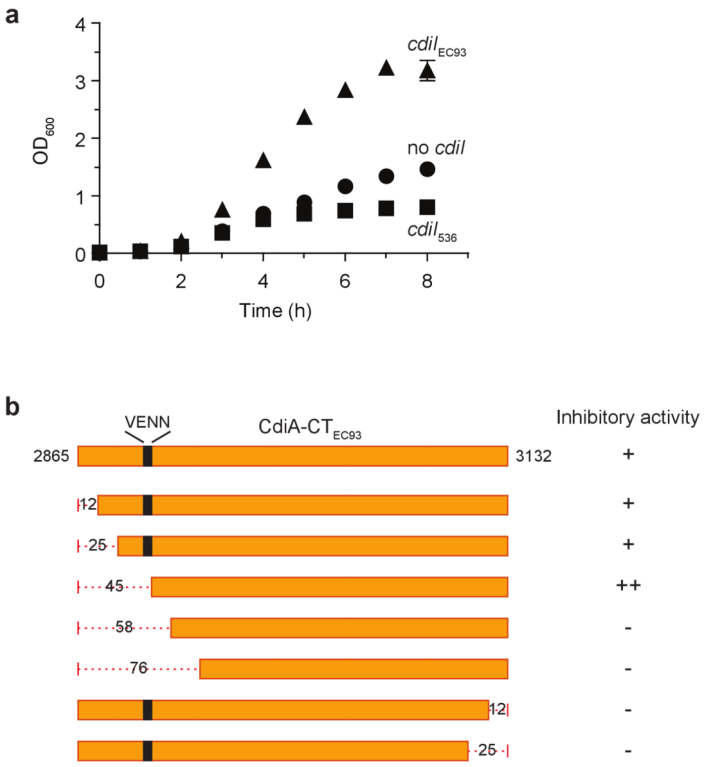

Figure 2. CdiA-CT contains growth inhibitory activity

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).

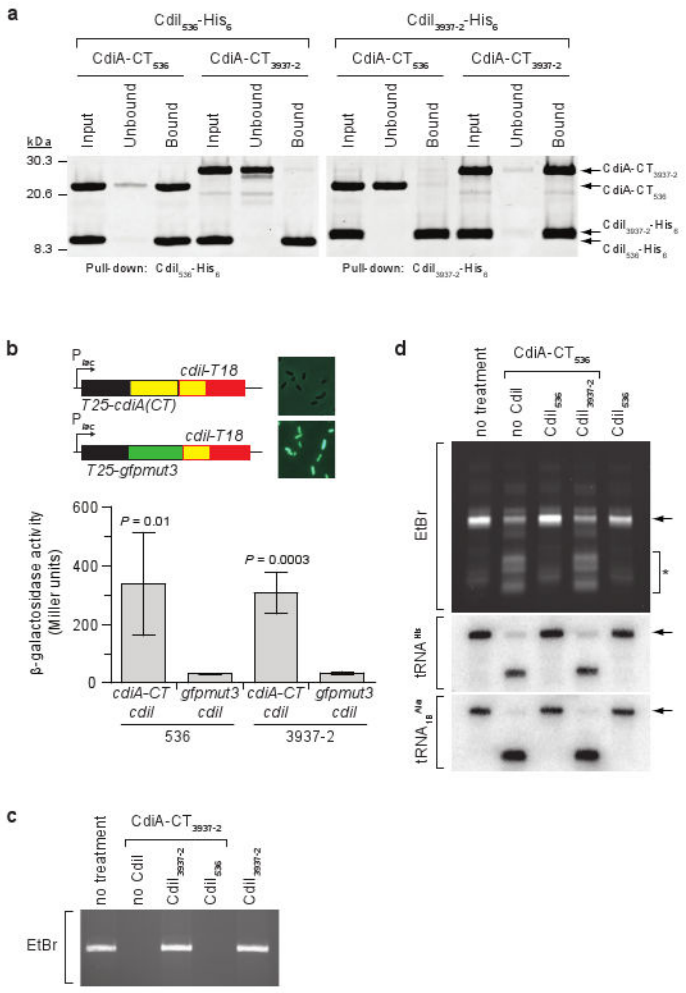

Figure 3. CdiI immunity protein binds specifically to cognate CdiA-CT and blocks activity

a, Purified CdiA-CT and CdiI-His_6 proteins from UPEC 536 and *D. dadantii* 3937 (CDI module 2, 3937-2) were mixed *in vitro* with Ni^{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 *in vivo*. A β-galactosidase reporter was used to measure adenylate cyclase activity. Expression of two-hybrid *T25-cdiA-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 $CdiA\text{-}CT_{3937\text{-}2}$ 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 $CdiA\text{-}CT_{536}$ 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 $tRNA_{1B}^{Ala}$. Arrows indicate full-length tRNAs.

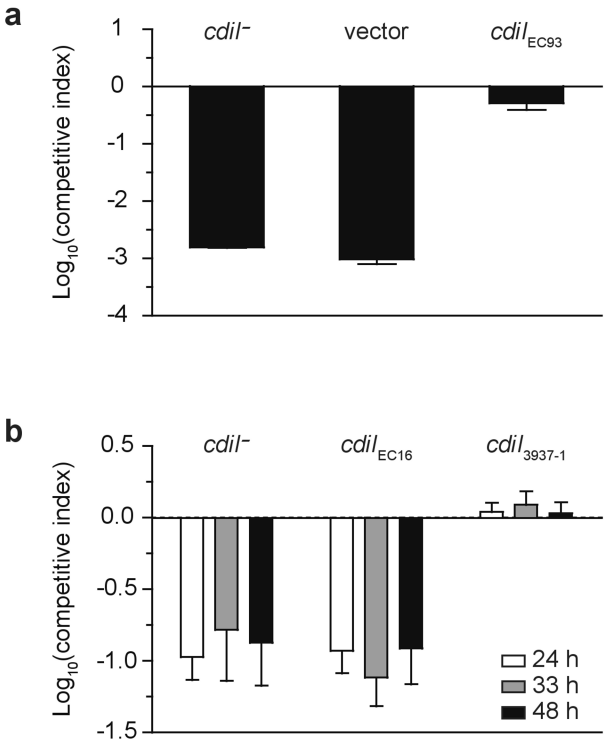

Figure 4. CDI systems function in intrastrain growth competition

a, Str^R CDI^+ EC93 cells were mixed 1:1 with rif^R EC93 Δ*cdiA-CT*Δ*cdiI* cells that either contained no plasmid (*cdiI*$^-$), pBR322 plasmid (vector), or $CdiI_{EC93}$-expressing plasmid (*cdiI*$_{EC93}$). After 3 hr of co-culture, cells were plated onto LB medium, and rif^R and str^R CFU/ml were quantified to calculate the competitive index [$(rif^R$ CFU/str^R CFU$)_{3\ hr}$ / (rif^R CFU/str^R CFU$)_{0\ hr}$]. The mean ± s.d. is shown (n = 2 experiments), *P* value = 0.002. **b.** $Gent^R$ CDI^+ *D. dadantii* 3937 cells were mixed 100:1 with nal^R *D. dadantii* 3937 Δ*cdiA-CT*$_{3937\text{-}1}$ Δ*cdiI*$_{3937\text{-}1}$ alone (*cdiI*$^-$), or complemented with heterologous *cdiI* from *D. dadantii* EC16 (*cdiI*$_{EC16}$), or cognate *cdiI* (*cdiI*$_{3937\text{-}1}$). Cell mixtures were inoculated onto chicory leaves (see Supplementary methods), incubated for the indicated times, and viable counts were determined. The competitive index (CFU nal^R /CFU $gent^R$) 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.