Contact-dependent growth inhibition toxins exploit multiple independent cell-entry pathways

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Contact-dependent growth inhibition (CDI) systems function to deliver toxins into neighboring bacterial cells. CDI$^+$ bacteria export filamentous CdiA effector proteins, which extend from the inhibitor-cell surface to interact with receptors on neighboring target bacteria. Upon binding its receptor, CdiA delivers a toxin derived from its C-terminal region. CdiA C-terminal (CdiA-CT) sequences are highly variable between bacteria, reflecting the multitude of CDI toxin activities. Here, we show that several CdiA-CT regions are composed of two domains, each with a distinct function during CDI. The C-terminal domain typically possesses toxic nuclease activity, whereas the N-terminal domain appears to control toxin transport into target bacteria. Using genetic approaches, we identified *ptsG*, *metI*, *rbsC*, *gltK/gltJ*, *yciB*, and *ftsH* mutations that confer resistance to specific CdiA-CTs. The resistance mutations all disrupt expression of inner-membrane proteins, suggesting that these proteins are exploited for toxin entry into target cells. Moreover, each mutation only protects against inhibition by a subset of CdiA-CTs that share similar N-terminal domains. We propose that, following delivery of CdiA-CTs into the periplasm, the N-terminal domains bind specific inner-membrane receptors for subsequent translocation into the cytoplasm. In accord with this model, we find that CDI nuclease domains are modular payloads that can be redirected through different import pathways when fused to heterologous N-terminal "translocation domains." These results highlight the plasticity of CDI toxin delivery and suggest that the underlying translocation mechanisms could be harnessed to deliver other antimicrobial agents into Gram-negative bacteria.

bacterial cell envelope | DNase activity | genetic selection | toxin/immunity genes | tRNase activity

Bacteria are constantly in competition for environmental resources and have evolved a number of systems to suppress the growth of competing cells. Research during the past decade has revealed that Gram-negative bacteria commonly use type V and type VI secretion systems to deliver protein toxins into neighboring cells (1, 2). The type V mechanism was the first to be identified and has been termed contact-dependent growth inhibition (CDI) because inhibitor cells must make direct contact with target bacteria to transfer toxins (3, 4). CDI$^+$ bacteria express CdiB/CdiA two-partner secretion (TPS) systems, which assemble as a complex on the cell surface. CdiB is an outer-membrane β-barrel protein required for the export and presentation of toxic CdiA effectors. CdiA proteins are very large (180–630 kDa depending on bacterial species) and are presented as individual β-helical filaments that emanate several hundred angstroms from the inhibitor-cell surface (5). CdiA binds to specific outer-membrane receptors on susceptible bacteria and transfers its C-terminal toxin domain (CdiA-CT) into the target cell (6, 7). CDI$^+$ bacteria also produce CdiI immunity proteins to protect themselves from toxin delivered by neighboring sibling cells. The immunity protein binds to the CdiA-CT and neutralizes its toxin activity (6, 8). Notably, CdiA-CT/CdiI sequences are highly variable between bacteria and even between different strains of the same species (6, 8). For example, isolates of *Escherichia coli* contain at least 20 CDI toxin/immunity sequence types. These toxin/immunity protein families are distinct from one another and form specific CdiA-CT/CdiI cognate pairs. Because CdiI immunity proteins do not protect against noncognate toxins, CDI provides a mechanism for self/nonself recognition between bacteria.

A remarkable feature of CDI is the modularity of CdiA-CT toxins, which can be exchanged between different CdiA proteins to generate functional chimeras. All CdiA proteins have a similar architecture consisting of an N-terminal TPS transport domain, an extended central region of filamentous hemagglutinin peptide repeats, and the CdiA-CT toxin region (Fig. 1*A*). In many bacteria, the variable CdiA-CT region is demarcated by the VENN peptide motif, which forms the C-terminal boundary of the pretoxin-VENN domain (Fig. 1*A*) (6, 9). Heterologous CdiA-CTs can be delivered into *E. coli* target cells when fused to the VENN sequence of CdiA^{EC93} from *E. coli* EC93 (6, 10–13). Closer examination of the CdiA-CT region reveals that it is often composed of two variable domains that assort independently to form CdiA-CT composites (Fig. 1*B*). For example, the CdiA-CT^{EC536} from uropathogenic *E. coli* 536 and CdiA-CT^{ECL} from *Enterobacter cloacae* American Type Culture Collection 13047 (ECL) share nearly identical N-terminal domains but carry different C-terminal nucleases (10, 14). The function of the CdiA-CT N-terminal domain has not been examined, but biochemical studies show this region is not required for nuclease activities in vitro (8, 11, 14). Here, we provide evidence that the N-terminal domain of the CdiA-CT region plays a critical role in toxin translocation during CDI. Using a genetic approach, we identified a

Significance

Gram-negative bacteria use contact-dependent growth inhibition (CDI) systems to bind neighboring bacteria and deliver diverse nuclease toxins that inhibit target-cell growth. This process requires toxin transport across the outer and inner membranes of target bacteria to reach DNA and RNA substrates in the cytoplasm. Our data indicate that CDI toxins contain a variable domain that specifies the entry pathway into target bacteria. These "translocation domains" exploit specific integral membrane proteins to deliver linked nuclease domains into the cytoplasm. We also find that CDI translocation domains can be exchanged between CdiA C-terminal toxin domains to deliver nucleases via different routes. These findings reveal a versatile protein-transport mechanism that could potentially be harnessed to deliver other antimicrobial cargoes into Gram-negative bacteria.

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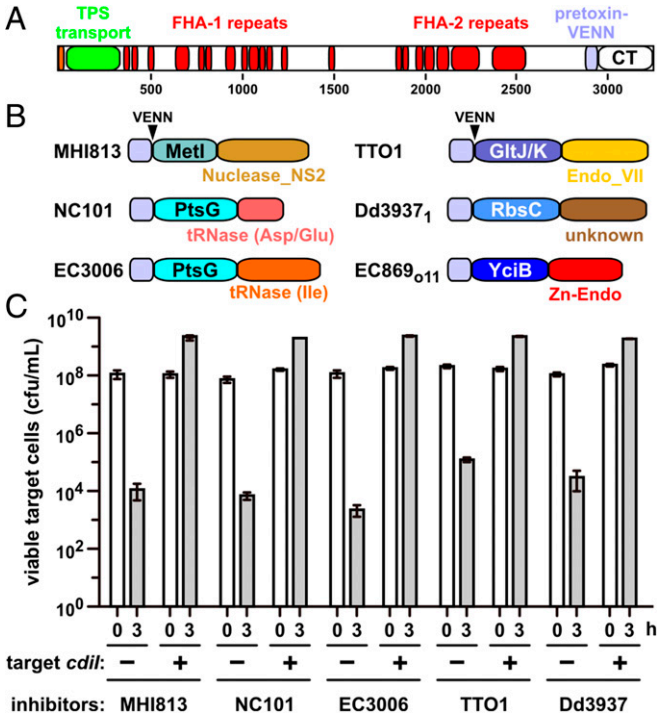

Fig. 1. Activity of CdiA chimeras. (*A*) CdiA proteins contain an N-terminal TPS transport domain and two filamentous hemagglutinin (FHA)-peptide repeat regions. The pretoxin-VENN domain is adjacent to and demarcates the variable CdiA-CT region. (*B*) Predicted CdiA-CT domain structures. Toxins from *E. coli* MHI813 and *Photorhabdus luminescens* TTO1 carry predicted C-terminal Nuclease_NS2 (Pfam database ID: PF13930) and Endonuclease_VII (PF14411) domains, respectively. The C-terminal nuclease domains from *E. coli* NC101 and 3006 cleave tRNA^{Asp}/tRNA^{Glu} and tRNA^{Ile}, respectively. The nuclease domain from *E. coli* EC869 is a Zn^{2+}-dependent DNase, and the activity of the *Dickeya dadantii* 3937 toxin is unknown. N-terminal domains are labeled according to their putative membrane receptors. The pretoxin-VENN domain and the conserved VENN motif are also depicted. (*C*) CDI competitions. *E. coli* target cells were cocultured with the indicated CDI inhibitors. Average target-cell counts (±SEM) are presented for three independent experiments. Where indicated, target cells were provided with the cognate *cdiI* immunity gene.

collection of CDI-resistance (CDI^R) mutations that protect *E. coli* target cells from specific CDI toxins. Each CDI^R mutation disrupts expression of an inner-membrane protein (IMP) and confers resistance to CdiA-CTs that share homologous N-terminal domains. We also demonstrate that the N- and C-terminal domains of CdiA-CT regions can be recombined to produce novel hybrids that are functional in cell-mediated CDI. We propose that the N-terminal domain of the CdiA-CT region binds to specific IMP receptors and mediates toxin transport across the inner membrane.

Results

We performed a series of selections for CDI-resistant (CDI^R) *E. coli* mutants, reasoning that protective mutations would disrupt genes required for toxin import and/or activation. Plasmid-borne chimeric CDI systems were constructed in which heterologous *cdiA-CT*/*cdiI* coding sequences were fused at the VENN encoding region of *cdiA*^{EC93} (Fig. 1*A*). Each chimeric fusion was functional in CDI, reducing target-cell viability between 10^3- and 10^6-fold during coculture (Fig. 1*C*). Moreover, target bacteria were protected when provided with the appropriate cognate *cdiI* immunity gene (Fig. 1*C*), indicating that the grafted CdiA-CTs are responsible for growth inhibition. Inhibitor strains were then used to enrich CDI^R target cells from a pool of *mariner* transposon-insertion mutants. CDI^R mutants were selected with iterative cycles of competition coculture until the target-cell population was fully resistant. We isolated individual target-cell clones from independent experiments and tested CDI^R phenotypes in competitions. Linkage of CDI^R to each transposon insertion was confirmed by transduction. Identification of the transposon-insertion sites revealed that resistance to a given CdiA-CT toxin was due to disruption of one or two genes. For example, CDI^{MHI813}-resistant mutants contained independent insertions in *metI*, whereas the nine CDI$_1^{Dd3937}$-resistant mutants had multiple insertions within *rbsC* (Fig. 2*A*). CDI^{TTO1}-resistant mutants were disrupted in *gltK* or *gltJ*, and CDI$_{o11}^{EC869}$-resistant mutants carried insertions in *yciC* or *yciB* (Fig. 2*A*). Interestingly, *ptsG* mutations were isolated from selections for resistance to CDI^{NC101} and CDI^{EC3006} (Fig. 2*A*). Notably, each disrupted gene encodes an integral membrane protein. MetI, RbsC, and GltJ/GltK are ABC transporter membrane permeases for D/L-methionine, D-ribose, and L-glutamate/L-aspartate, respectively (15–17).

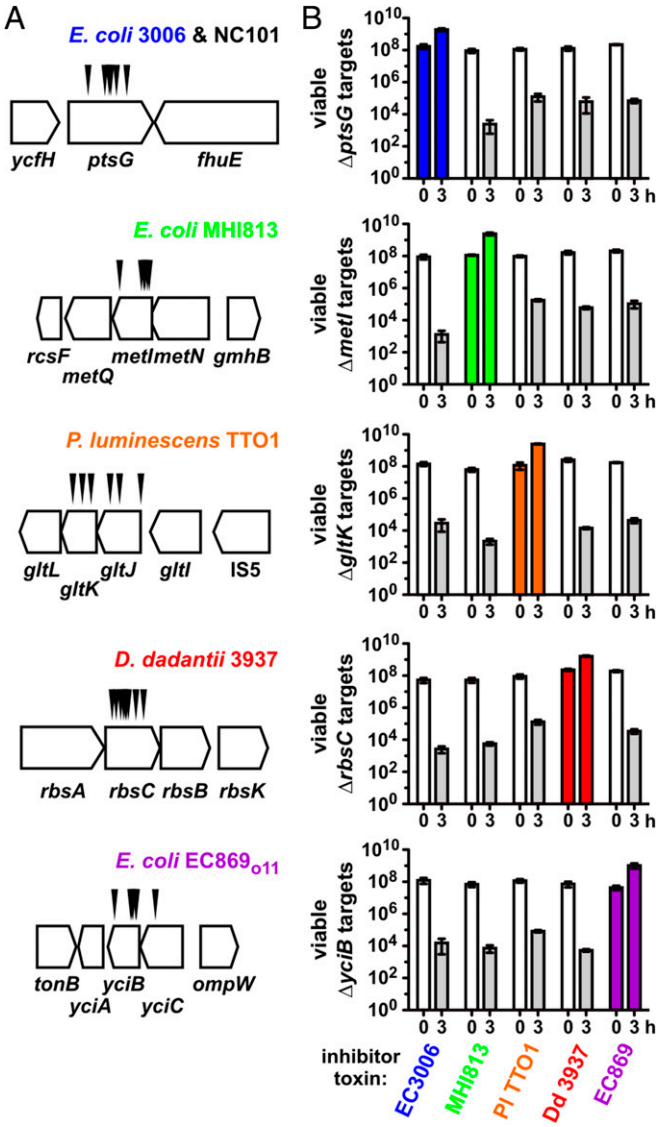

Fig. 2. Specificity of CDI^R mutations. (*A*) Transposon-insertion sites are shown for each selection toxin. No other verified CDI^R mutations were identified during the selections. (*B*) CDI^R mutations are toxin-specific. The indicated target cell strains were cocultured with inhibitors that deploy CdiA-CT^{EC3006}, CdiA-CT^{MHI813}, CdiA-CT^{TTO1}, CdiA-CT$_1^{Dd3937}$, or CdiA-CT$_{o11}^{EC869}$ toxins. Average target-cell counts (±SEM) per mL are presented for three independent experiments.

PtsG is the main phosphotransferase system permease for D-glucose (18). The functions of YciC and YciB are unknown, but both are predicted integral IMPs. In-frame deletions were constructed for each gene to confirm its role in CDI^{R} (Fig. 2*B*). This analysis showed that Δ*yciC* mutants are not resistant to CDI$_{o11}^{EC869}$ (Fig. S1), indicating that the original *yciC* insertion exerts a polar effect on *yciB* (Fig. 2*A*). Complementation analysis confirmed the role of *yciB* in the CDI$_{o11}^{EC869}$ pathway, and showed the *metI*, *rbsC*, *gltK*, and *ptsG* are required for their respective CDI pathways (Fig. S1). We also tested each in-frame deletion strain in competitions against other inhibitor strains and found that resistance was specific, such that Δ*metI* cells were resistant to CDI^{MHI813} but susceptible to other CDI systems (Fig. 2*B*). Thus, each CDI system requires a specific IMP to inhibit target cells.

Given that CDI^{R} was invariably associated with disruption of IMPs, we hypothesized that CDI toxins exploit these proteins to enter target bacteria. We tested whether CDI toxins are delivered into CDI^{R} target cells by monitoring nuclease activities in competition cocultures. CdiA-CT$_{o11}^{EC869}$ toxin has a potent DNase activity that produces anucleate target cells (Fig. 3*A*) (11). However, Δ*yciB* mutants retained normal nucleoid morphology during coculture with CDI$_{o11}^{EC869}$ inhibitors and appeared similar to immune target cells that express the CdiI$_{o11}^{EC869}$ immunity protein (Fig. 3*A*). We also examined the tRNase activity of CdiA-CT^{EC3006}, which specifically cleaves tRNA$_1^{Ile}$. Cleaved tRNA was detected in *ptsG*$^{+}$ cells after 1 h incubation with CDI^{EC3006} inhibitors, but no tRNase activity was observed in cocultures with Δ*ptsG* targets (Fig. 3*B*). These results suggest that toxin is excluded from the cytoplasm of CDI^{R} target cells. Alternatively, the IMPs could function as so-called permissive factors, which activate CDI toxins after entry into target bacteria (14). This latter model predicts that CDI^{R} mutants should also be resistant to toxin produced internally. To test this model, we used controllable proteolysis to degrade ssrA(DAS)-tagged immunity proteins and thereby activate toxins inside the cell (8, 19, 20). CdiA-CT$_{o11}^{EC869}$ activation was slow in *yciB*$^{+}$ cells, with growth inhibition and in vivo DNase activity observed after 3 h (Fig. 4*A*). In contrast, Δ*yciB* cell growth was inhibited immediately upon toxin activation, and DNase activity was apparent within 1 h (Fig. 4*A*). Similar results were obtained when CdiA-CT^{EC3006} was activated in *ptsG*$^{+}$ and Δ*ptsG* cells, in which growth inhibition was immediate and tRNase activity was identical in both backgrounds (Fig. 4*B*). These results show that CdiA-CT$_{o11}^{EC869}$ and CdiA-CT^{EC3006} retain full toxicity when expressed inside CDI^{R} mutant strains, excluding the toxin-activation model. Together, these data support a role for IMPs in the delivery of CDI toxins into the target-cell cytoplasm.

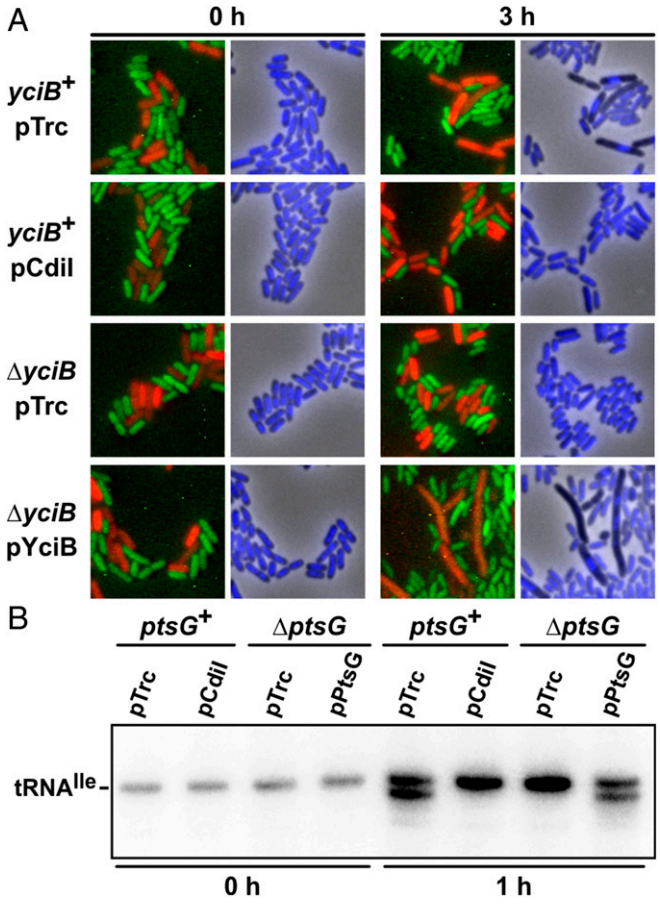

Fig. 3. Toxin nuclease activities inside target bacteria. (*A*) Fluorescence microscopy of CDI$_{o11}^{EC869}$ competition cocultures. Inhibitor cells (YFP-labeled) were incubated with the indicated *yciB*$^{+}$ or Δ*yciB* target cells (mRFP-labeled), and nucleoids were visualized with DAPI staining. (*B*) Northern blot analysis of CDI^{EC3006} competition cocultures. Target cells (*ptsG*$^{+}$ or Δ*ptsG*) were incubated with CDI^{EC3006} inhibitor cells and RNA isolated for Northern blot analysis of tRNA$_1^{Ile}$. The migration position of uncleaved tRNA$_1^{Ile}$ is indicated.

Most of the CDI^{R} mutations disrupt metabolite permeases, raising the possibility that transport activity could play a general role in CDI toxin import. We tested mutants lacking the cytoplasmic ATP-binding components of the Met (Δ*metN*), Rbs (Δ*rbsA*), and Glt (Δ*gltL*) ABC transporters and found that each strain was still sensitive to CDI (Fig. S2). We also tested PtsG proteins that carry the Cys421Ser mutation and lack the entire cytoplasmic IIB domain, both of which are unable to transport D-glucose (18, 21). Each transport-defective PtsG protein rendered Δ*ptsG* cells sensitive to CDI^{EC3006} (Fig. S2). Therefore, the membrane permeases are required for CDI-mediated growth inhibition, but their metabolite transport activities are not.

ptsG mutants were isolated in selections for resistance to CDI^{NC101} and CDI^{EC3006} (Fig. 2*A*). These CdiA-CT sequences are 73.7% identical over the first 167 residues, but the C-terminal nuclease domains are unrelated (Fig. S3*A*). The CdiI immunity proteins also share no significant homology (Fig. S3*B*). In accord with this divergence, neither immunity protein protects against inhibition by the heterologous system (Fig. S3*C*). Together with previous analyses of CDI toxins (8, 11, 14), these observations indicate that CdiA-CT regions are often composed of two domains, with the extreme C-terminal domain containing the actual growth inhibition activity. Moreover, the genetic interaction between PtsG and the N-terminal sequences of CdiA-CT^{NC101} and CdiA-CT^{EC3006} suggests that the shared domain specifies the cell-entry pathway. The CdiA-CTs from uropathogenic *E. coli* 536 (EC536) and ECL also share N-terminal domains, but carry different C-terminal RNase domains and have distinct immunity proteins (Fig. S4 *A* and *B*) (10, 14). Based on reports that *E. coli* Δ*ftsH* mutants are resistant to multiple colicin nucleases (22–24), we screened Δ*ftsH* cells in CDI competitions and discovered that they are resistant to CDI^{EC536} and CDI^{ECL}, but sensitive to inhibition by CDI^{EC93} (Fig. S4*C*). FtsH is a hexameric AAA+ unfoldase/protease that is tethered to the inner membrane through two transmembrane helices, again suggesting that each CDI system exploits a specific IMP. Δ*ftsH* mutants are also resistant to the CdiA-CT^{PestA} toxin from *Yersinia pestis* Pestoides A, which shares the N-terminal domain with CdiA-CT^{EC536} and CdiA-CT^{ECL} (Figs. S4 *A* and *B*). Together, these data show that CdiA-CT regions are commonly composed of two variable domains and suggest that the N-terminal domain may dictate the cell-entry pathway.

Analyses of naturally occurring CdiA-CTs suggest that the N- and C-terminal domains can be rearranged to deliver nucleases through different pathways. We tested this prediction with novel CdiA-CT hybrid constructs. We fused the N-terminal domain of

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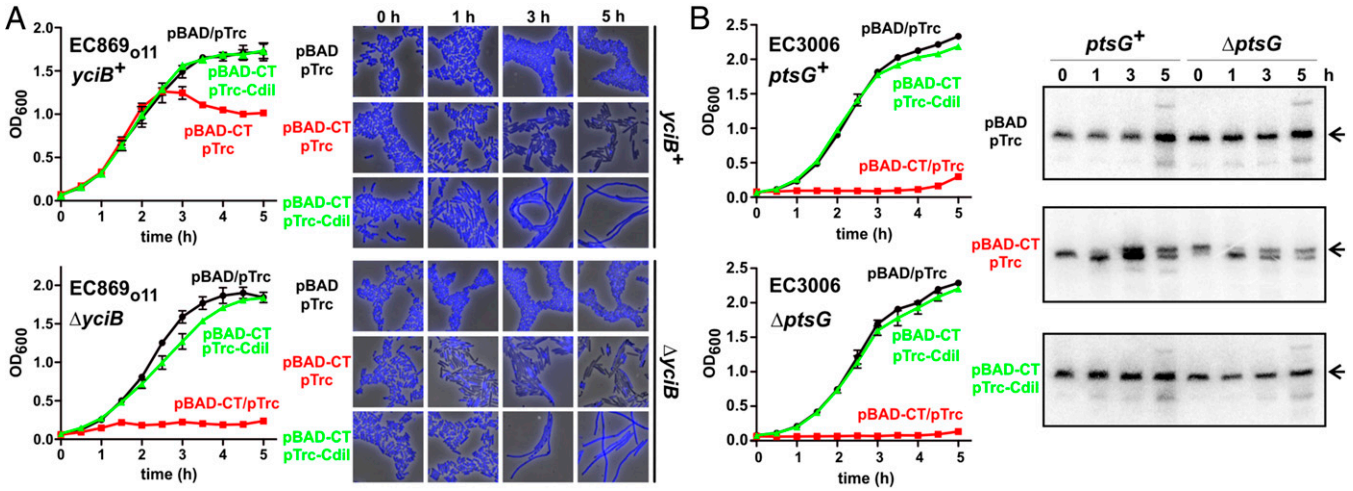

Fig. 4. Toxin expression inside CDI^R mutants. (*A*) CdiA-CT$_{o11}^{EC869}$ was induced at 0 h from a pBAD vector in *yciB*$^+$ and Δ*yciB* cells as described previously (20). Cell growth was monitored by measuring the OD at 600 nm of the culture. CdiI$_{o11}^{EC869}$ immunity protein was coexpressed from a pTrc vector where indicated. (*Right*) DAPI-stained cells sampled at 0, 1, 3, and 5 h of culture. (*B*) CdiA-CT^{EC3006} was induced at 0 h from a pBAD vector in *ptsG*$^+$ and Δ*ptsG* cells, and growth was monitored by measuring the OD$_{600}$ of the culture. CdiI^{EC3006} immunity protein was coexpressed from a pTrc vector where indicated. (*Right*) Northern blot analysis of RNA isolated at 0, 1, 3, and 5 h. The arrows indicate the migration position of full-length tRNA$_1^{Ile}$.

CdiA-CT^{EC3006} (Val1–Leu167, numbered from Val1 of the VENN motif; Fig. S3*A*) to the DNase domain of CdiA-CT$_{o11}^{EC869}$ (Ala154–Lys297) (11) (Fig. 5), and then grafted the hybrid onto CdiA^{EC93} to generate a chimeric CDI system. The resulting triple chimera reduced target-cell viability ∼100-fold in coculture, and target cells were protected when they expressed *cdiI*$_{o11}^{EC869}$ but not the *cdiI*^{EC3006} immunity gene (Fig. 5). We then tested the EC3006/EC869$_{o11}$ hybrid against Δ*yciB* and Δ*ptsG* target cells and found that only Δ*ptsG* mutants were resistant (Fig. 5). We used the same approach to deliver the CdiA-CT^{EC536} tRNase domain (Lys127–Ile227) into target cells with the CdiA-CT^{EC3006} N-terminal domain (Fig. S5). As expected, CdiI^{EC536} protein protected target cells from the EC3006/EC536 hybrid, and growth inhibition required PtsG (Fig. S5). These results show that the two CdiA-CT domains are modular, and nucleases can be delivered through different pathways specified by the N-terminal domain.

CDI systems are generally conserved between bacteria, but *Burkholderia* systems have an alternative gene order, and the CdiA-CT region is demarcated by a distinct ELYN peptide motif (8, 25). We found that fusion of CdiA-CT$_{II}^{Bp1026b}$ (Glu1–Asn297, numbered from Glu1 of ELYN) from *Burkholderia pseudomallei* 1026b to CdiA^{EC93} produces a nonfunctional chimera (Fig. 6*A*). However, the C-terminal tRNase domain from CdiA-CT$_{II}^{Bp1026b}$ (Thr162–Asn297) can be delivered efficiently when fused to the N-terminal domain of CdiA-CT$_{o11}^{EC869}$ (Fig. 6*A*). Moreover, as predicted from the delivery domain model, *E. coli* Δ*yciB* mutants are resistant to the EC869$_{o11}$/Bp1026b hybrid CdiA-CT (Fig. 6*A*). This latter result shows that the *Burkholderia* tRNase domain can be delivered into *E. coli* target cells, raising the possibility that *E. coli* lacks the pathway required for native CdiA-CT$_{II}^{Bp1026b}$ import. We recently discovered that *Burkholderia thailandensis* ΔBTH_II0599 mutants are resistant to the CDI$_{II}^{Bp1026b}$ system (26). BTH_II0599 encodes a member of the major facilitator superfamily (MFS), which are integral membrane transporters of small metabolites and antibiotics (27). BTH_II0599 is highly conserved among *Burkholderia* species, but homologs are absent from enterobacteria. Therefore, we provided *E. coli* cells with plasmid-borne BTH_II0599 and tested them as targets in competitions against inhibitors that deploy the native CdiA-CT$_{II}^{Bp1026b}$. Remarkably, cells that express BTH_II0599 became sensitized to growth inhibition, and showed a ∼15-fold decrease in viable cell counts after 3 h (Fig. 6*B*). Moreover, sensitized target cells were protected when they expressed the cognate *cdiI*$_{II}^{Bp1026b}$ immunity gene (Fig. 6*B*), indicating that the CdiA-CT$_{II}^{Bp1026b}$ tRNase domain mediated growth inhibition. Collectively, these data reveal a genetic interaction between the N-terminal domain of CdiA-CT$_{II}^{Bp1026b}$ and BTH_II0599 and

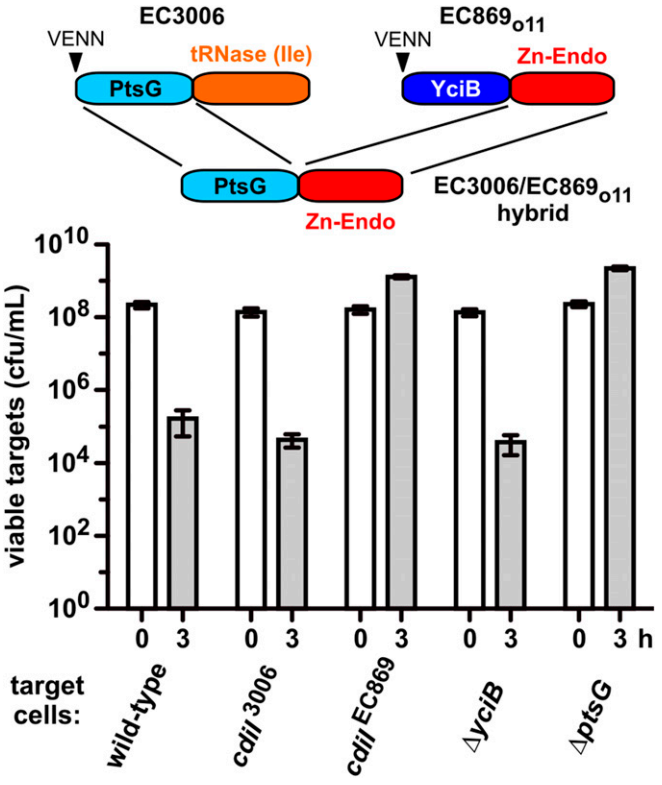

Fig. 5. CdiA-CT constituent domains are modular. The N-terminal domain of CdiA-CT^{EC3006} was fused to the C-terminal DNase domain of CdiA-CT$_{o11}^{EC869}$. EC3006-EC869$_{o11}$ hybrid inhibitors were cocultured with the indicated target strains. Average target-cell counts (±SEM) are presented for three independent experiments.

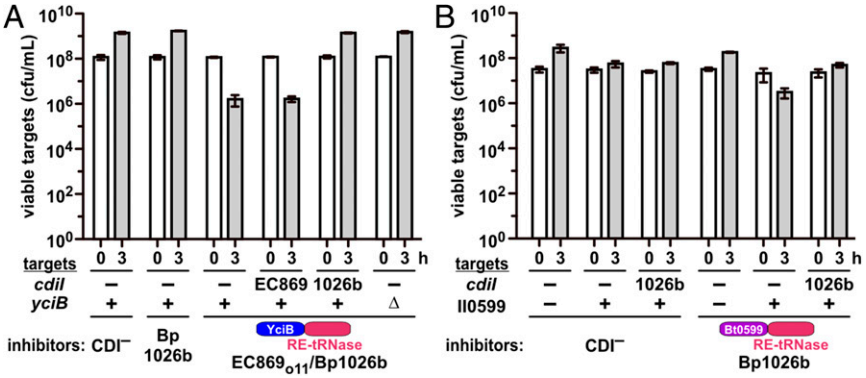

Fig. 6. BTH_II0599 allows CdiA-CT$_{II}^{Bp1026b}$ delivery into *E. coli* cells. (*A*) Inhibitor strains (mock CDI$^-$, Bp1026b, and hybrid EC869$_{o11}$-Bp1026b) were cocultured with target bacteria of the indicated *cdiI* and *yciB* genotypes. Viable target bacteria were quantified at 0 and 3 h. (*B*) Inhibitor strains (mock CDI$^-$ and Bp1026b) were cocultured with target cells that express BTH_II0599 and *cdiI*$_{II}^{Bp1026b}$ where indicated. Average target-cell counts (±SEM) are presented for three independent experiments.

suggest that this MFS protein is required for toxin translocation into target bacteria.

Discussion

We previously reported that variable CdiA-CT regions are often composed of two domains (8, 10, 11, 14). The extreme C-terminal domain typically has nuclease activity and is sufficient to inhibit growth when expressed inside *E. coli* cells (10, 11, 14). In contrast, the N-terminal domain of the CdiA-CT has no inhibition activity, and its function has not been explored. The findings presented here suggest that the N-terminal domain is critical for nuclease toxin translocation during CDI. This model is based on the identification of multiple CDI^R mutations that disrupt integral membrane proteins and concomitantly protect target bacteria from specific CdiA-CT toxins. In principle, these membrane proteins could function as permissive factors that bind and activate CdiA-CT toxins after delivery (14). However, CdiA-CT$_{o11}^{EC869}$ and CdiA-CT^{EC3006} nuclease domains have full activity when expressed inside CDI^R mutants, excluding permissive factor function. Moreover, *ptsG*, *yciB*, and *ftsH* mutants are resistant to CdiA-CTs based on the identity of the nontoxic N-terminal domains. These genetic interactions suggest that N-terminal domains use specific IMPs as receptors during CDI. Although our data do not demonstrate direct toxin–IMP interactions, this model is supported by experiments showing that BTH_II0599 expression sensitizes *E. coli* to the native CdiA-CT$_{II}^{Bp1026b}$ toxin. Because BTH_II0599 is completely heterologous, with no homologs in γ-proteobacteria, the simplest explanation is that CdiA-CT$_{II}^{Bp1026b}$ binds directly to this IMP to translocate into the cytoplasm. Moreover, the N-terminal domain of CdiA-CT$_{II}^{Bp1026b}$ is limited to *B. pseudomallei* systems, arguing that these effectors target only other *Burkholderia* that contain BTH_II0599 homologs.

Crystal structures are available for three CDI toxin/immunity protein complexes, but the N-terminal domain is resolved in only one model (10, 11). Residues Met86–Thr153 of CdiA-CT$_{o11}^{EC869}$ form a small helical bundle that packs against the C-terminal DNase domain (11). The tertiary contacts with the nuclease domain probably facilitated the resolution of this domain. For many other CdiA-CT regions, the N- and C-terminal domains are connected by flexible peptide linkers, suggesting the domains have few or no tertiary contacts and move independently of one another. Additionally, the N-terminal domains do not make direct contacts with CdiI immunity proteins (10, 11, 14). Together, these observations suggest that the N- and C-terminal domains are autonomous units that can be recombined in virtually any combination. Although this hypothesis is supported by the functional hybrid CdiA-CTs constructed in this work, we note that the reengineered toxins are less effective than their naturally occurring counterparts. Thus, a given nuclease domain may have a preferred translocation pathway that is more efficient than others.

CdiA-CT nuclease domains are delivered into the target-cell cytoplasm (10–13), but the molecular details of CDI toxin translocation remain obscure. Most of the IMPs identified here are metabolite transporters, but our data indicate that transporter activity is not required for toxin import. Moreover, it seems unlikely that protein toxin domains could be transported in the same manner as small molecules. Instead, we hypothesize that CDI toxins exploit IMPs as receptors to bring nuclease domains into close proximity with the membrane, thereby allowing the toxin to enter and penetrate the lipid bilayer. Further, because the target-cell proton motive force is required for CDI (12), we postulate that this electrochemical gradient provides the driving force to transport toxins into the cytosol. This mechanism is similar to that proposed for colicin E3 and E9 nuclease toxins, which spontaneously enter lipid micelles and mediate their own transport across membranes (28, 29). However, colicins do not appear to require IMP receptors, and nearly all CDI^R mutations provide no protection against colicins (30). The one exception is *ftsH*, which was originally identified as the *tolZ* mutation and confers resistance to nuclease toxins of group A and B colicins (22, 31). FtsH is a membrane-associated AAA+ superfamily member with ATP-dependent metalloprotease activity. Two models have been proposed for the role of FtsH in colicin import. De Zamaroczy and coworkers have shown that FtsH is required for the release of colicin nucleases into the cell, and they hypothesize that the protease directly cleaves the domain (23, 32). Kleanthous and coworkers have proposed that the ATP-dependent unfoldase activity of FtsH is used to pull the nuclease domain into the cell (24). AAA+ proteases are processive enzymes that actively unfold and cleave proteins into small peptides, so, in these models, the colicin nuclease domain must resist complete degradation during transport. Intriguingly, bacteriophages are also known to exploit IMPs to transfer their genomes into host cells. Phage λ requires the ManY component of the mannose phosphotransferase system to infect *E. coli* cells (33), and it was recently reported that PtsG is required for infection by *E. coli* phage HK97 (34). Thus, CDI and phages may use similar strategies to transport macromolecules into bacterial targets.

Other toxin-delivery systems, including *Neisseria* MafB proteins (35), type VI secretion-associated Rhs proteins (9, 20, 36), and predicted type VII secretion toxins from *Bacillus* and *Mycobacteria* (37), carry C-terminal nuclease domains that are related to those in CdiA proteins. Like CDI, the genetic organization of these other toxin-delivery systems is modular, allowing toxin interchange at the C terminus of conserved delivery proteins. These observations imply that toxin/immunity coding sequences are subject to frequent horizontal gene transfer between systems. Therefore, widely distributed toxin domains must be active against multiple clades of bacteria. Perhaps

this explains why so many of these toxins are nucleases, which should be effective against any bacterium provided the domain can be delivered into the cytoplasm. It seems likely that each competition system uses a different mechanism to deliver toxins into target bacteria. For example, type VI secretion is thought to mechanically penetrate the target cell envelope, which could explain why analogous translocation domains are not found adjacent to the C-terminal toxin domains of Rhs effectors. The physical basis for CDI toxin translocation is unknown, but the mechanism appears to be quite versatile, allowing a nuclease domain to be transported through multiple independent pathways. Further, CDI exploits several membrane protein families, suggesting that, in principle, any IMP could be hijacked as a translocation receptor. Given this plasticity, we speculate that the mechanism could be harnessed to transport other cargos into Gram-negative bacteria and perhaps form the basis of novel antibacterial therapies.

Materials and Methods

Bacterial strains are listed in Table S1. *E. coli* EPI100 cells carrying plasmid-borne *cdi* gene clusters were used as inhibitors, and *E. coli* MC4100 and MG1655 derivatives were used as target cells. *E. coli* MC4100 was subjected to *mariner*-mediated mutagenesis by using plasmid pSC189 (38). Gene disruptions were from the Keio collection (39) and were transferred into *E. coli* MC4100 by using phage P1-mediated general transduction. Plasmids and oligonucleotides are listed in Tables S2 and S3, respectively. The details of all plasmid constructions are provided in *SI Materials and Methods*. Competition cocultures were performed at a 1:1 inhibitor to target cell ratio in shaking lysogeny broth medium at 37 °C as described in *SI Materials and Methods*. Competitions with Δ*ftsH* target cells were performed at 30 °C, and chimeric EC93-Bp1026b inhibitors were used in 10-fold excess over target bacteria. Viable target cells were enumerated as cfu counts per milliliter and expressed as the average ± SEM for three independent experiments. RNA was isolated by guanidinium isothiocyanate-phenol extraction (40). Northern blots were performed with 10 μg of total RNA using a probe for *E. coli* $tRNA_1^{Ile}$. In vivo DNase activity was assessed by fluorescence microscopy of DAPI-stained bacteria as described in *SI Materials and Methods*.

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