NIH Public Access
Author Manuscript
Mol Microbiol. Author manuscript; available in PMC 2015 July 01.

Published in final edited form as:
Mol Microbiol. 2014 July ; 93(2): 276–290. doi:10.1111/mmi.12658.

The F pilus mediates a novel pathway of CDI toxin import

Christina M. Beck[1], **Elie J. Diner**[2,†], **Jeff J. Kim**[1], **David A. Low**[1,2], and **Christopher S. Hayes**[1,2,*]

[1]Department of Molecular, Cellular and Developmental Biology, University of California, Santa Barbara, Santa Barbara, CA 93106-9625, USA

[2]Biomolecular Science and Engineering Program, University of California, University of California, Santa Barbara, Santa Barbara, CA 93106-9625, USA

Summary

Contact-dependent growth inhibition (CDI) is a widespread form of inter-bacterial competition that requires direct cell-to-cell contact. CDI^+ inhibitor cells express CdiA effector proteins on their surface. CdiA binds to specific receptors on susceptible target bacteria and delivers a toxin derived from its C-terminal region (CdiA-CT). Here, we show that purified $CdiA\text{-}CT^{536}$ toxin from uropathogenic *Escherichia coli* 536 translocates into bacteria, thereby by-passing the requirement for cell-to-cell contact during toxin delivery. Genetic analyses demonstrate that the N-terminal domain of $CdiA\text{-}CT^{536}$ is necessary and sufficient for toxin import. The CdiA receptor plays no role in this import pathway; nor do the Tol and Ton systems, which are exploited to internalize colicin toxins. Instead, $CdiA\text{-}CT^{536}$ import requires conjugative F pili. We provide evidence that the N-terminal domain of $CdiA\text{-}CT^{536}$ interacts with F pilin, and that pilus retraction is critical for toxin import. This pathway is reminiscent of the strategy used by small RNA leviviruses to infect F^+ cells. We propose that $CdiA\text{-}CT^{536}$ mimics the pilin-binding maturation proteins of leviviruses, allowing the toxin to bind F pili and become internalized during pilus retraction.

Introduction

Contact-dependent growth inhibition (CDI) systems are distributed throughout α–, β– and γ–proteobacteria, where they function in competition between closely related bacteria (Aoki *et al.*, 2010, Aoki *et al.*, 2011, Ruhe *et al.*, 2013a, Ruhe *et al.*, 2013b). CDI is mediated by the CdiB/CdiA family of two-partner secretion proteins. CdiB is an outer-membrane β-barrel protein required for secretion of the CdiA effector. CdiA proteins are filamentous and very large, ranging from ~180 kDa in *Moraxella* to over 600 kDa in some *Pseudomonas* species (Aoki et al., 2010, Aoki et al., 2011). CdiA extends from the surface of CDI^+ inhibitor cells and interacts with receptors on susceptible target bacteria. CDI has been characterized most extensively in *Escherichia coli* EC93, and its $CdiA^{EC93}$ effector binds the highly conserved outer-membrane protein BamA as a receptor (Aoki *et al.*, 2008, Ruhe et al., 2013b). Upon binding the receptor, $CdiA^{EC93}$ appears to be cleaved to release a C-terminal toxin region ($CdiA\text{-}CT^{EC93}$), which is subsequently translocated into the target cell through a poorly

*Corresponding author: Ph: (805) 893-2028, Fax: (805) 893-4724, chayes@lifesci.ucsb.edu.
†Current address: Department of Chemistry, The Scripps Research Institute, La Jolla, CA 92037, USA

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

characterized pathway (Aoki et al., 2010, Aoki et al., 2008, Webb *et al.*, 2013). *E. coli* EC93 inhibitor cells also deliver toxins to one another, but are protected from inhibition by a small CdiI^{EC93} immunity protein encoded immediately downstream of *cdiA*^{EC93} (Aoki *et al.*, 2005). Thus, *E. coli* EC93 cells deploy CdiA-CT^{EC93} toxins to inhibit other non-immune strains of *E. coli*. Because CDI confers a significant growth advantage to inhibitor cells, these systems are thought to mediate inter-strain competition for environmental niches.

CDI loci encode a diverse group of CdiA-CT/CdiI toxin/immunity sequences. There are at least 18 CdiA-CT/CdiI sequence types distributed throughout *E. coli* strains, and *Burkholderia pseudomallei* strains carry 10 distinct sequence types (Nikolakakis *et al.*, 2012, Ruhe et al., 2013a). Thus, CDI toxin/immunity pairs are highly variable, even between strains of the same species. In accord with this sequence diversity, CDI toxins exhibit a number of distinct activities. For example, CdiA-CT^{EC93} dissipates the proton motive force and induces the phage-shock response (Aoki *et al.*, 2009), suggesting that the toxin forms pores in the inner membrane of target cells. Many other CDI toxins have nuclease activities. CDI toxins from *Dickeya dadantii* 3937 and *E. coli* EC869 are DNases that degrade target-cell genomic DNA (Aoki et al., 2010, Morse *et al.*, 2012, Webb et al., 2013). CDI$^+$ bacteria also deploy RNases that preferentially cleave tRNA molecules. CdiA-CT^{536} from uropathogenic *E. coli* 536 (UPEC 536) is a tRNA anticodon nuclease (Aoki et al., 2010, Diner *et al.*, 2012), and toxins from *B. pseudomallei* strains cleave tRNA in the anticodon loop, T-loop and aminoacyl-acceptor stem (Nikolakakis et al., 2012). Each CdiA-CT toxin is specifically neutralized by its cognate CdiI protein, but not the immunity proteins from other CDI systems (Aoki et al., 2010, Morse et al., 2012, Nikolakakis et al., 2012). Therefore, CDI toxin/immunity complexity provides the basis for self/non-self discrimination during inter-strain competition.

The CdiA-CT toxin region is typically demarcated by a short, highly conserved peptide sequence. Most CdiA-CT regions are defined by the VENN peptide motif, but CdiA proteins from *Burkholderia* species contain an analogous (Q/E)LYN sequence (Aoki et al., 2010, Zhang *et al.*, 2011, Nikolakakis et al., 2012, Anderson *et al.*, 2012). The function of this motif has not been explored, but it could play two important roles in CDI. First, the universally conserved Asn residue may catalyze auto-cleavage to release the CdiA-CT for subsequent translocation into target bacteria. Asn side-chains can undergo intra-molecular attack on the peptide backbone, thereby cleaving the peptide chain and producing a cyclic succinimide. Asn residue cyclization is a well characterized reaction that mediates auto-cleavage in several secreted proteins (Dautin *et al.*, 2007, Zarivach *et al.*, 2008). Second, the nucleotide sequence encoding VENN may be important for recombination and subsequent expression of new *cdiA-CT/cdiI* gene pairs acquired by horizontal transfer. CDI toxins are modular and can be readily exchanged between systems. For example, *E. coli* CdiA proteins can deliver heterologous toxins from *Yersinia pestis* CO92 and *Dickeya dadantii* 3937, provided the CdiA-CTs are fused at the common VENN motif (Aoki et al., 2010, Webb et al., 2013). Thus, bacteria might switch toxin/immunity types by recombining a new *cdiA-CT/cdiI* sequence onto the 3′-end of the *cdiA* gene, thereby displacing the original toxin/immunity pair. Moreover, many species carry fragmented *cdiA-CT/cdiI* gene pairs in tandem arrays downstream of the main *cdiBAI* gene cluster (Poole *et al.*, 2011). These “orphan”

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

toxin/immunity regions usually contain interspersed sequences that are homologous to transposases and integrases, implying recent acquisition through horizontal gene transfer (Poole et al., 2011, Ruhe et al., 2013a). Taken together, these observations suggest that CdiA proteins have the intrinsic ability to auto-cleave and deliver diverse toxins across the target-cell envelope.

Although CDI toxins are hypothesized to translocate autonomously, CdiA^{EC93} fragments released by *E. coli* EC93 cells do not inhibit the growth of other *E. coli* strains (Aoki et al., 2005). One possible explanation is that only specific CdiA-CT fragments – presumably those released in response to receptor-binding – are able to enter target bacteria. This model suggests that purified toxin may be active against bacteria if it mimics the naturally cleaved CdiA-CT fragment. Therefore, we tested whether purified CdiA-CT toxins inhibit the growth of bacterial cultures. We find that purified CdiA-CT^{536} (from UPEC 536) inhibits *E. coli* cell growth, whereas CdiA-CT^{3937} (from *D. dadantii* 3937) has no effect. Structure-function analyses show that the N-terminal domain of CdiA-CT^{536} is necessary and sufficient for cell import, but surprisingly toxin translocation does not require BamA. Instead, conjugative F pili are required for the import of purified CdiA-CT^{536} toxin. We provide evidence that CdiA-CT^{536} binds to F pilin and hypothesize that this interaction allows the toxin to be internalized during pilus retraction. F-dependent RNA phages exploit a similar pathway to transport their genomes into cells, suggesting that CdiA-CT^{536} mimics RNA phage to enter F$^+$ cells.

Results

Purified CdiA-CT^{536} inhibits E. coli growth

To test whether CdiA-CT toxins enter bacteria in the absence of cell-to-cell contact, we treated *E. coli* cultures with purified toxins from *Dickeya dadantii* 3937 (CdiA-CT^{3937}) and uropathogenic *E. coli* 536 (CdiA-CT^{536}). Each toxin was first purified as a complex with its cognate His$_6$-tagged CdiI protein and then separated from the immunity protein under denaturing conditions. CdiA-CT^{536} and CdiA-CT^{3937} refold efficiently and regain nuclease activity when denaturant is removed by dialysis (Aoki et al., 2010, Diner et al., 2012). We added each toxin (100 nM final concentration) to *E. coli* X90 cultures and monitored cell growth. Purified CdiA-CT^{3937} had no discernable effect on growth, but cells treated with CdiA-CT^{536} were inhibited compared to the buffer-treated control (Fig. 1A). We also tested the CdiA-CT/CdiI^{536} complex, and somewhat surprisingly found that cells treated with the toxin/immunity complex were inhibited to the same extent as cells treated with CdiA-CT^{536} toxin alone (Fig. 1A). This result suggests that only the toxin enters cells and immunity protein remains in the media. Alternatively, the CdiA-CT/CdiI^{536} complex might inhibit growth by a novel mechanism that is independent of the toxin's tRNase activity. To differentiate between these possibilities, we added purified CdiA-CT^{536} to cells that express plasmid-borne immunity genes. Cells expressing *cdiI*^{536} were not inhibited by added CdiA-CT^{536} toxin (Fig. 1B), indicating that immunity protein is protective when present in the cytoplasm. This protection is specific, because cells expressing non-cognate *cdiI*^{3937} were inhibited to the same extent as cells that carry no immunity gene (Fig. 1B). To confirm that purified CdiA-CT^{536} inhibits growth through its anticodon nuclease activity, we isolated

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

RNA from the toxin-treated cultures and examined transfer RNA by northern blot. This analysis revealed cleaved $tRNA_{ICG}Arg$ in all CdiA-CT536-treated cells except those expressing the cognate *cdiI*^{536} immunity gene (Fig. 1C). Together, these results show that exogenous CdiA-CT^{536} translocates into *E. coli* cells, where it cleaves tRNA to inhibit growth.

The N-terminal domain of CdiA-CT^{536} is required for cell import

Like many CdiA-CTs, CdiA-CT^{536} is composed of two domains (Diner et al., 2012, Morse et al., 2012). The tRNase activity of CdiA-CT^{536} is contained within the C-terminal 145 residues (Diner et al., 2012), which corresponds to Gly3098 – Ile3242 of full-length CdiA^{536} (Fig. 2A). However, *E. coli* cells were not inhibited when treated with the purified tRNase domain (Fig. 2B). This result suggests that the N-terminal domain of CdiA-CT^{536} is required for cell import; or alternatively, the C-terminal tRNase domain may be insufficient to inhibit cell growth. To test inhibition activity, we constructed a plasmid that produces the tRNase domain under control of the arabinose-inducible P_{BAD} promoter and asked whether this construct could be introduced into *E. coli* cells in the presence of L-arabinose. As a control, we also transformed the plasmid into *E. coli* Δ*cysK* cells, because CysK is required to activate the CdiA-CT^{536} nuclease (Diner et al., 2012). The construct expressing the tRNase domain yielded no stable transformants in the *cysK*$^{+}$ background, but was readily introduced into Δ*cysK* cells (Fig. 3). Furthermore, *cysK*$^{+}$ cells were transformed at high-efficiency with either the empty vector plasmid (pCH450) or a construct encoding CdiA-CT^{536} with the His178Ala mutation that abolishes tRNase activity (Diner et al., 2012) (Fig. 3). Together, these results demonstrate that the C-terminal tRNase domain is toxic when expressed inside cells and suggest that the N-terminal domain plays a role in cell import.

The VENN peptide motif is predicted to be part of a larger domain of unknown function, termed DUF638, which is found in many CdiA proteins (Aoki et al., 2010). Given the conservation of the VENN motif, we asked whether this sequence is required for CdiA-CT^{536} import. Cells treated with CdiA-CT^{536} lacking this sequence (VENN-less, residues Leu3023 – Ile3242) were inhibited to the same extent as cells treated with the full CdiA-CT^{536} containing residues Val3016 – Ile3242 (Fig. 2A & 2B). Surprisingly, a larger fragment (containing residues Gly2969 – Ile3242) that includes the entire DUF638 region had no effect on cell growth (Figs. 2A & 2B), even though this protein is toxic when produced inside *cysK*$^{+}$ cells (Fig. 3). Although CdiA-CT sequences are diverse, their N-terminal domains often contain paired cysteine residues that presumably form disulfide linkages. We reasoned that this predicted disulfide bond could be critical for toxin import, and therefore mutated Cys3028 and Cys3034 to serine residues to generate a “Cys-less” toxin (Fig. 2A). Cells treated with the Cys-less CdiA-CT^{536} were not inhibited (Fig. 2B), yet this toxin variant still inhibited growth when produced inside *E. coli* cells (Fig. 3). The preceding experiments results suggest a role for N-terminal domain of CdiA-CT^{536} in cell import. However, it also possible that some of the toxin variants are unstable in the growth medium and do not inhibit cell growth because they are rapidly degraded. To address this possibility, we monitored CdiA-CT^{536} antigen using immunoblot analysis, which revealed that each toxin was stable in shaking-broth cultures for up to five hours at 37 °C (Fig. S1). Together, these data indicate that the N-terminal domain of CdiA-CT^{536} is required for

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

import. However, the DUF368 region appears to block CdiA-CT^{536} translocation, perhaps by interacting with the N-terminal region and masking cell-import epitopes.

The N-terminal domain of CdiA-CT^{536} shares significant sequence identity with the corresponding region of CdiA^{ECL} from *Enterobacter cloacae* ATCC 13047 (ECL), but the C-terminal nuclease domains are not related in sequence (Fig. 4A). Moreover, the C-terminal domain of CdiA-CT^{ECL} has a distinct RNase activity that cleaves 16S ribosomal RNA (Beck *et al.*, 2014). We reasoned that if the N-terminus of CdiA-CT^{536} mediates cell import, then purified CdiA-CT^{ECL} (corresponding to residues Ala3087 – Asp3321) should also inhibit *E. coli* growth. We found that the purified CdiA-CT/CdiI^{ECL} complex had no effect on *E. coli* X90 cells (data not shown), but the isolated CdiA-CT^{ECL} toxin inhibited cell growth (Fig. 4B). This result may indicate that CdiI^{ECL} does not dissociate from CdiA-CT^{ECL} during translocation, or alternatively the bound immunity protein may prevent toxin import altogether. Regardless, the purified CdiA-CT^{ECL} toxin entered cells because the *cdiI*^{ECL} immunity gene prevented growth inhibition, whereas non-cognate *cdiI*^{536} provided no protection (Fig. 4B). Thus, the homologous N-terminal domains of CdiA-CT^{536} and CdiA-CT^{ECL} appear to function similarly in cell import.

The N-terminal domain of CdiA-CT^{536} is sufficient for cell import

The shared N-terminal domain of CdiA-CT^{536} and CdiA-CT^{ECL} is required for toxin import, suggesting that it carries tethered nuclease domains into the cell. We tested this hypothesis by asking whether the N-terminal domain still supports translocation when fused to a heterologous passenger domain. We fused residues Val1 – Tyr82 of CdiA-CT^{536} to the C-terminal nuclease domain of colicin E5 (ColE5-CT, residues Ala451 – Gln556) (Fig. 5A). We chose the ColE5-CT domain as a passenger because it is similar in size and activity to the CdiA-CT^{536} tRNase domain (Fig. 5A) (Ogawa *et al.*, 1999, Lin *et al.*, 2005). Although full-length colicin E5 enters and kills *E. coli* cells (Ogawa et al., 1999), the isolated ColE5-CT domain has no inhibition activity because it lacks receptor-binding and translocation domains (Figs. 5A & 5B). We fused the N-terminal domain of CdiA-CT^{536} to the ColE5-CT nuclease domain and tested the purified fusion protein on *E. coli* cultures. The fusion had weak activity against *E. coli* X90 cells (data not shown), but inhibited *E. coli* XL-1 cells more profoundly (Fig. 5B). This growth inhibition was blocked when cells express the *imE5* gene (Fig. 5B), which encodes an immunity protein that specifically neutralizes ColE5-CT activity (Ogawa et al., 1999, Lin et al., 2005). Because ColE5-CT cleaves the anticodon loops of tRNA^{His}, tRNA^{Tyr}, tRNA^{Asp} and tRNA^{Asn} molecules (Ogawa et al., 1999), we examined tRNA^{Tyr} by northern blot to detect nuclease activity. Cleaved tRNA^{Tyr} was detected in treated cells that either lack immunity or express *cdiI*^{536} (Fig. 5C). By contrast, there was less cleaved tRNA^{Tyr} in cells that express *imE5* (Fig. 5C), consistent with the ability of ImE5 to neutralize ColE5-CT activity. Together, these results indicate that the N-terminal domain of CdiA-CT^{536} is sufficient to translocate the colicin E5 nuclease domain into *E. coli* cells.

CdiA-CT^{536} import is independent of BamA, Tol and Ton translocation pathways

Cell mediated CDI requires specific receptors on target cells. Therefore, we asked whether CdiA-CT^{536} import requires the BamA receptor using *bamA101* mutant cells, which have

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

approximately five-fold less BamA on the cell surface and are significantly resistant to cell-mediated CDI (Aoki et al., 2008, Webb et al., 2013). *E. coli bamA101* cells were inhibited by purified CdiA-CT^{536} to the same extent as *bamA*$^{+}$ cells (Fig. 6A), suggesting the toxin is not imported through the usual CDI pathway. This result led us to consider other mechanisms for CdiA-CT^{536} import. Colicins exploit either the Tol or Ton systems to translocate their toxin domains into *E. coli* cells (Cascales *et al.*, 2007). Therefore, we generated X90 Δ*tolA* and Δ*tonB* strains and tested these cells for resistance to group A (colicin E5) and group B (colicin D) colicins. As expected from previous studies, we found that Δ*tolA* cells were completely resistant to colicin E5, but inhibited by colicin D (Fig. S2). Reciprocally, Δ*tonB* cells were resistant to colicin D and inhibited by colicin E5 (Fig. S2). Moreover, X90 Δ*tolA* cells were resistant to phage M13, but not phage R17 (Table 1), consistent with the established phenotype of this mutant. We then treated the Δ*tolA* and Δ*tonB* strains with purified CdiA-CT^{536} and found that the mutants were as sensitive as *tolA*$^{+}$ *tonB*$^{+}$ cells (Figs. 6B & 6C). We also tested a Δ*tolA* Δ*tonB* strain to test for possible redundancy and found that the double mutant was also inhibited by CdiA-CT^{536}, though it grew to slightly higher density than treated *tolA*$^{+}$ *tonB*$^{+}$ cells (Fig. 6D). Together, these findings suggest that purified CdiA-CT^{536} toxin enters *E. coli* cells through a novel pathway.

CdiA-CT^{536} import requires the F pilus

Although *E. coli* X90 and XL-1 strains are inhibited by purified CdiA-CT^{536}, *E. coli* strain MC4100 is resistant to the toxin (data not shown). There are several genetic differences between these strains, but both sensitive strains carry F′ episomes whereas the resistant stain does not. Therefore, we tested *E. coli* X90 cells that had been cured of F′ and found that this isolate is resistant to purified CdiA-CT^{536} (Fig. 7A). Reintroduction of a different F′ episome (from *E. coli* XL-1) into the cured X90 cells restored toxin sensitivity (Fig. 7A). Moreover, *E. coli* MC4100 cells that carry F′ were also sensitive to purified CdiA-CT^{536} (data not shown). Together, these results indicate that one or more genes on F are required for CdiA-CT^{536} import. As a first step to identify F-encoded import gene(s), we tested whether cells carrying a subset of F genes are sensitive to CdiA-CT^{536}. Plasmid pOX38*::gent* is derivative of F that contains the *tra* genes required for conjugation and a selectable gentamicin-resistance (*gent*) marker (Guyer *et al.*, 1981, Johnson & Reznikoff, 1984). We introduced pOX38*::gent* into *E. coli* X90 F^{-} and found that the resulting cells were sensitive to CdiA-CT^{536} (Fig. 7A), suggesting that *tra* gene(s) could mediate toxin import. We took a candidate approach and disrupted two genes, *traA* and *traT*, that encode abundant cell-surface proteins. The *traA* gene encodes F pilin, which polymerizes to form the conjugative pilus; and *traT* encodes an outer membrane β-barrel protein that functions to prevent mating between F^{+} cells (Achtman *et al.*, 1977, Manning *et al.*, 1980). The Δ*traT* mutant was still inhibited by CdiA-CT^{536}, but Δ*traA* cells were completely resistant to purified toxin (Fig. 7B). To exclude possible polar effects from the Δ*traA* mutation, we complemented the mutant with plasmid-borne *traA* and restored sensitivity to the toxin (Fig. 7C). These results suggest that the F pilus is required for the import of CdiA-CT^{536} toxin.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

CdiA-CT^{536} blocks F-mediated conjugation

We hypothesized that the N-terminal domain of CdiA-CT^{536} binds directly to F pili and exploits the organelle to enter cells. This import pathway could be analogous to that used by some F-dependent bacteriophages to infect *E. coli* F$^+$ cells. Therefore, we examined *traA* mutations that confer resistance to phage R17, reasoning that these alleles may also protect cells from purified CdiA-CT^{536}. We focused on the Asp74Gly and Gly120Cys mutations, because they produce functional conjugative pili, but interfere with phage R17 binding (Manchak *et al.*, 2002, Daehnel *et al.*, 2005). We introduced each mutation into plasmid-borne *traA* and used the constructs to complement X90 Δ*traA::cat* mutants. Each pilin variant supported conjugation, though the mating efficiency was somewhat reduced by the Asp74Gly mutation (Table 2). The mutant pilins also provided resistance to phage R17, but not phage M13 (Table 1). Cells expressing the Asp74Gly pilin were still inhibited by purified CdiA-CT^{536}, but the Gly120Cys mutation provided partial protection against the toxin (Figs. 7D & 7E). These results suggest that CdiA-CT^{536} binds F pili at a site that overlaps with the phage R17 binding site.

Because F-dependent bacteriophage bind directly to F pili, they can interfere with conjugation (Lin *et al.*, 2011). We reasoned that if CdiA-CT^{536} binds directly to the F pilus, then it may also reduce mating efficiency. We first tested the effects of M13 and R17 phages on mating efficiency. The phages were inactivated with ultraviolet radiation (to prevent infection and cell lysis), then added to conjugation co-cultures. The phage particles reduced mating efficiency to ~50% from about 88% for mock-treated cultures (Fig. 8). We next tested purified CdiA-CT^{536} carrying the His178Ala mutation so that the F$^+$ donor cells were not inhibited during culture. Purified CdiA-CT^{536} at 1 μM reduced mating efficiency approximately 2.5-fold compared to mock-treated cells (Fig. 8). By contrast, the purified tRNase domain (which lacks the N-terminal domain of CdiA-CT^{536}) had little effect on mating efficiency (Fig. 8). Similarly, the Cys-less and DUF638-containing toxins (see Fig. 2A) also had no substantive effect on conjugation (Fig. 8). These observations suggest that the tRNase domain and the latter CdiA-CT^{536} variants do not inhibit cell growth because they do not bind to F pili. Taken together with the protective effect of the *traA(G120C)* mutation, these results support a model in which the N-terminal domain of CdiA-CT^{536} binds to the F pilus.

trbI mutants are resistance to F-dependent bacteriophage and CdiA-CT^{536}

F pili are dynamic and undergo cycles of extension and retraction (Clarke *et al.*, 2008). Pilus retraction is critical for F-dependent phage infection and provides a possible mechanism to internalize CdiA-CT^{536} toxin. The *trbI* gene is thought to be required for pilus retraction (Maneewannakul *et al.*, 1992), so we generated an in-frame *trbI* deletion strain for analysis. We found that Δ*trbI* cells are resistant to both R17 and M13 phage (Table 1), but still capable of conjugation, albeit at lower efficiency than wild-type cells (Table 2). These observations indicate that the Δ*trbI* mutant produces functional F pili as reported previously (Maneewannakul et al., 1992). We next treated Δ*trbI* cells with purified CdiA-CT^{536} and found that the mutant was fully resistant to the toxin (Fig. 7F). We attempted to complement the mutant with plasmid-borne *trbI*, but were unable to do so with both IPTG- and arabinose-inducible *trbI* constructs (data not shown). We note that Maneewannakul *et al.*

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

could not complement *trbI* mutants with multi-copy plasmids. Moreover, they reported that *trbI* over-expression in wild-type cells phenocopies the *trbI* null mutation (Maneewannakul et al., 1992). We also found that *trbI* over-expression in the *trbI*$^+$ background conferred resistance to CdiA-CT^{536}, similar to the Δ*trbI* phenotype (Fig. 7F). Therefore, pili dynamics appear to be adversely affected by under- and over-expression of *trbI* (Maneewannakul et al., 1992). These results are consistent with a model in which pilus-bound toxin is internalized during retraction.

Discussion

The experiments presented here reveal an unexpected import pathway for CDI toxins. Purified CdiA-CT^{536} and CdiA-CT^{ECL} enter *E. coli* F$^+$ cells and inhibit growth. These toxins inhibit growth using different C-terminal nuclease domains, but they share a common N-terminal domain. The N-terminal domain is not necessary for nuclease activity *in vitro* (Diner et al., 2012), but is required for toxin translocation into F$^+$ cells. These results indicate that the N-terminal domain has autonomous import activity and carries tethered passenger domains into the cell. Indeed, the translocation domain is capable of transporting a heterologous nuclease domain from colicin E5 into F$^+$ cells. Several observations suggest that toxin import requires a binding interaction between the N-terminal domain and F pili. First, purified CdiA-CT^{536} interferes with mating when added to conjugation co-cultures. The same phenomenon is observed with phage R17 and M13 particles, both of which bind directly to F pili. Presumably, the binding of either phage or toxin to F pili disrupts the formation of conjugation bridges. This effect is specific because the C-terminal tRNase domain of CdiA-CT^{536} has little to no effect on mating efficiency, and mutations within the N-terminal domain that block cell-import also abrogate the toxin's effect on conjugation. Furthermore, cells expressing F pilin with the Gly120Cys mutation are significantly resistant to purified CdiA-CT^{536}. This mutation also disrupts the interaction between F pili and phage R17 (Daehnel et al., 2005), suggesting that phage and CdiA-CT^{536} share overlapping binding sites. Taken together, these results indicate that CdiA-CT^{536} and CdiA-CT^{ECL} contain a pilus-binding domain that facilitates entry into F$^+$ cells.

F pili are dynamic structures that extend in search of potential mating partners and retract to bring donor and recipient cells together to establish conjugation bridges (Novotny & Fives-Taylor, 1974, Clarke et al., 2008). This cycle of extension and retraction also provides a possible mechanism to import CdiA-CT^{536} toxin across the cell envelope. Retraction is driven by pilus disassembly into pilin monomers within the inner membrane (Moore *et al.*, 1981). Therefore, pilus-bound CdiA-CT^{536} could be carried directly to the inner membrane during retraction. If this model is correct, then the toxin must remain stably associated with the pilus as it retracts through the lumen of the type IV secretion assembly. Type IV secretion assemblies are large, barrel-shaped structures that span the entire cell envelope (Chandran *et al.*, 2009, Waksman & Fronzes, 2010). The F-pilus assembly is composed of a hetero-oligomeric complex of TraV, TraK and TraB, with a tetradecameric ring of TraV forming an aperture in the outer membrane through which the pilus emerges (Silverman & Clarke, 2010). Presumably, TraV forms a tight seal around the pilus to prevent the loss of periplasmic contents to the media. Therefore, the associated CdiA-CT^{536} must pass through the TraV aperture and not be stripped from the pilus surface. Pilus retraction is also critical

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

for F-dependent phage infection, and a number of phage-resistance mutations appear to disrupt pilus dynamics (Burke *et al.*, 1979, Willetts *et al.*, 1980). These resistance mutations are unmapped, and to our knowledge, *trbI* is the only F gene to be specifically linked to defects in pilus retraction (Maneewannakul et al., 1992). As predicted by the retraction-import model, in-frame deletion of *trbI* protects F^+ cells from CdiA-CT^{536}, just as it provides resistance to F-dependent phages (Maneewannakul et al., 1992). However, we note that pili dynamics have not been directly examined in Δ*trbI* mutants. The retraction-import model would be strengthened if Δ*trbI* retraction defects could be confirmed through real-time video microscopy as described by Silverman and colleagues (Clarke et al., 2008).

As outlined above, there are parallels between CdiA-CT^{536} import and infection by F-dependent phages. Two bacteriophage families exploit conjugative pili as host-cell receptors. The *Inoviridae* are filamentous, single-strand DNA viruses (e.g. phages fd and M13) that bind to the tip of the pilus using the g3p capsid protein (Daehnel et al., 2005, Manchak et al., 2002). These phages also require TolA as a co-receptor (Riechmann & Holliger, 1997). However, CdiA-CT^{536} import does not require TolA, suggesting that the toxin is internalized through another pathway. Moreover, CdiA-CT^{536} appears to bind F pili in a manner similar to phage R17 and other leviviruses. The *Leviviridae* are icosahedral, single-strand RNA viruses that attach to the side of conjugative pili using a single copy of the maturation (or assembly) protein present in the phage capsid (Roberts & Steitz, 1967, Steitz, 1968, Dent *et al.*, 2013). Upon binding the pilus, the maturation protein is released from the capsid and proteolytically processed into two fragments (Krahn *et al.*, 1972, Paranchych *et al.*, 1971, Paranchych *et al.*, 1970). These peptide fragments remain associated with the phage genome and are internalized with viral RNA during infection (Oriel, 1969, Osborn *et al.*, 1970, Krahn et al., 1972). The internalization process also requires TraD motor function and other plasmid-transfer initiation proteins (Lang *et al.*, 2014, Lang *et al.*, 2011, Schoulaker & Engelberg-Kulka, 1978, Willetts & Achtman, 1972). Together, these observations suggest that the maturation protein guides the tethered genome into the cytoplasm. A similar mechanism may underlie CdiA-CT^{536} import into F^+ cells. Although the N-terminal domain of CdiA-CT^{536} lacks homology with known maturation proteins, we note that these proteins are quite diverse between different phage (Rumnieks & Tars, 2012, Friedman *et al.*, 2009). For example, the maturation proteins from MS2, GA and Qβ phages are only 26 – 49% identical to one another, yet are all thought to bind along the shafts of F pili. Given this sequence diversity, it seems reasonable to posit that the N-terminal domain of CdiA-CT^{536} mimics a leviviral maturation protein.

In principle, pilus-mediated import provides an additional mechanism to deliver CDI toxins into target bacteria. However, it is not clear that this mode of delivery occurs naturally. We have previously reported that *E. coli* EC93 cells release CdiA fragments into the extracellular milieu, but these fragments are not inhibitory (Aoki et al., 2005). Similarly, $CdiA^{536}$ fragments from UPEC 536 culture supernatants do not inhibit the growth of F^+ cells (C.M.B & C.S.H., unpublished data). Another possibility is that the binding of $CdiA^{536}$ to F pili augments cell-cell interactions to potentiate CDI. We have found that F^+ cells are generally better CDI targets than F^- cells, but this is also true for CdiA effector proteins that lack the pilus-interaction domain (C.M.B & C.S.H, unpublished data). Thus, there is no

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

evidence that full-length CdiA^{536} on the surface of inhibitor cells binds to F pili on target cells. If the interaction with F pili plays no role in cell-mediated CDI, then what is the biological significance of these findings? We hypothesize that these phenomena reflect a critical function for the N-terminal domain in transport across the target-cell inner membrane during CDI. In this model, the unusual pilus-binding activity of CdiA-CT^{536} allows the toxin to by-pass the CDI receptor and gain entry into the periplasm. Once in the periplasm, the toxin is predicted to resume its normal translocation pathway, using the N-terminal domain to cross the inner membrane and enter the cytoplasm. Though this model has not been tested directly, there is evidence that the N-terminal domain of CdiA-CT^{536} is critical for CDI. Mutation of residues Cys3028/Cys3034 within full-length CdiA^{536} abrogates CDI, but still allows transfer of CdiA-CT^{536} toxin antigen to the surface of target bacteria (Julia S. Webb & D.A.L., unpublished data). These observations indicate that CDI is disrupted at a later stage in the pathway, as expected for a defect in toxin translocation. If this model is correct, then our results suggest that CDI toxins and leviviral genomes may use the same basic mechanism to cross the inner membrane of bacteria.

Experimental Procedures

Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 3. *E. coli* cells were grown in LB medium supplemented with antibiotics at the following concentrations: ampicillin (Amp), 150 μg mL^{-1}; chloramphenicol (Cm), 33 μg mL^{-1}; kanamycin (Kan), 50 μg mL^{-1}; rifampicin (Rif), 250 μg mL^{-1}; streptomycin (Str), 50 μg mL^{-1}; and tetracycline (Tet), 10 μg mL^{-1}. Bacteria were grown in baffled flasks at 37 °C with shaking (215 rpm) unless otherwise indicated. The F^{-} derivative of *E. coli* X90 (strain KW1070) was a generous gift from Dr. Kelly P. Williams. The Δ*tolA::kan* and Δ*tonB::kan* gene disruptions were obtained from the Keio collection (Baba *et al.*, 2006) and introduced into *E. coli* X90 by bacteriophage P1-mediated transduction. The Δ*traA::cat*, Δ*traT::cat* and Δ*trbI::cat* disruptions were generated by phage λ Red-mediated recombination as described (Datsenko & Wanner, 2000). The chloramphencol acetyltransferase (*cat*) open reading frame was amplified with oligonucleotides (listed in Table S1) containing homology to regions flanking *traA* (traA-cat-for/traA-cat-rev) and *traT* (traT-cat-for/traT-cat-rev). A similar procedure was used for the *trbI* gene, but the *cat* gene was amplified by two sequential reactions with trbI-cat-for1/trbI-cat-rev1 and trbI-cat-for2/trbI-cat-rev2 primer pairs. The resulting PCR products were electroporated into *E. coli* X90 cells expressing the Red proteins from plasmid pSIM6 (Datta *et al.*, 2006). The F′::Tn*10* (from strain XL-1) and pOX38::*gent* plasmids were transferred into *E. coli* strain KW1070 by conjugation. Donor and recipient cells were mixed and spotted onto LB-agar for 4 hr at 37 °C. Exconjugants were selected on LB-agar supplemented with: Tet/Rif for F′::Tn*10* transfer into *E. coli* KW1070, Gent/Rif for pOX38::*gent* transfer into KW1070, and Tet/Str for F′::Tn*10* transfer into *E. coli* MC4100.

Plasmids

Plasmids used in this study are listed in Table 3. Plasmids pET21::*cdiA-CT/cdiI*^{536} and pET21::*cdiA-CT/cdiI*^{3937} were used to over-produce CdiA-CT/CdiI^{536}-His$_6$ and CdiA-CT/

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

CdiI3937-His$_6$ complexes (respectively) (Aoki et al., 2010). All other CdiA-CT/CdiI^{536}-His$_6$ over-expression constructs were generated by PCR and ligated into plasmid pET21P using NcoI/XhoI restriction sites. The DUF-CT, VENN-less and tRNase domain constructs were amplified using primers DUF-536-Nco-for, VENN-less-Nco-for and tRNase-Nco-for (respectively) in conjunction with primer 536-Xho-rev (Table S1). Residues Cys13 and Cys19 of CdiA-CT^{536} were mutated to serine by mega-primer PCR (Aiyar & Leis, 1993). A fragment of plasmid pET21::*cdiA-CT/cdiI*^{536} was amplified using primers pET-Sph/Pst and 536-Cys(mut). This product was purified and used as a mega-primer in a second PCR with primer 536-Xho-rev. The final product was digested with NcoI/XhoI and ligated to plasmid pET21P. The *colE5-CT* and *imE5* coding sequences were amplified from plasmid ColE5-099 using primers colE5-M429-Nco and imE5-Spe, and the product digested with NcoI/SpeI and ligated to plasmid pET21P. The CdiA-CT/ColE5-CT fusion construct was generated by sequential ligation of *cdiA-CT*^{536} and *colE5-imE5* fragments into plasmid pET21P. The *colE5-CT/imE5* sequence was amplified with primers ColE5-Bam-for and ImE5-Xho-rev and ligated to plasmid pET21P. The coding sequence for Val1 – Tyr82 of CdiA-CT^{536} was amplified with primers 536-Nco-for and 536-Bam-rev and ligated to plasmid pET21::*colE5-CT/imE5* using NcoI and BamHI restriction sites. The CdiA-CT/CdiI^{ECL}-His$_6$ expression construct was generated in two steps. First, the T7 promoter region of pET21P was amplified with primers pET-Sph/Pst and pET-Kpn/Nco, and the fragment ligated to PstI/NcoI-digested pET21::*cdiA-CT/cdiI*^{3937}. The resulting pET21K::*cdiA-CT/cdiI*^{3937} plasmid contains a unique KpnI site upstream of NcoI. The *cdiA-CT/cdiI*^{ECL} region was then amplified with ECL-CT-Kpn-for and ECL-cdiI-Nhe-rev and ligated to KpnI/SpeI-digested plasmid pET21K::*cdiA-CT/cdiI*^{3937}.

The *cdiI*^{536}, *cdiI*^{ECL} and *imE5* immunity genes were amplified from pET21 expression plasmids using 536-cdiI-Eco-for, ECL-cdiI-Eco-for and imE5-Eco-for primers (respectively) in conjunction with the pET-Pst reverse primer. All PCR products were digested with EcoRI and PstI and ligated to plasmid pTrc99A. The *traA* coding sequence from the F plasmid was amplified with primers traA-Nco-for and traA-Spe-rev and the product ligated to NcoI/SpeI-digested plasmid pTrc99A::*rhsI*$_B$ (Poole et al., 2011). The Gly120Cys mutation in *traA* was generated by PCR using primers traA-Nco-for and traA-G120C-Spe. The Asp74Gly mutation in *traA* was generated by megaprimer PCR using primer traA-D74G in conjunction with the traA-Nco-for/traA-Spe-rev primer pair. The *trbI* gene was amplified with primers trbI-Kpn-for and trbI-Xho-rev and the product ligated to KpnI/XhoI-digested pTrc99KX and pCH450K to generate *trbI* expression constructs for complementation analysis.

Protein purification

All CdiA-CT/CdiI-His$_6$ complexes were over-produced in *E. coli* strain CH2016. Cultures were grown to OD_{600} ~ 0.7 and protein production induced with 1.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for three hr. Cells were harvested over ice and collected by centrifugation at 6,000 rpm for 10 min. Cells were resuspended in 10 mL of extraction buffer [20 mM sodium phosphate (pH 7.0), 150 mM sodium chloride, 0.05% Triton X-100, 10 mM β-mercaptoethanol (β-ME), 1 mM PMSF] and broken by french press passage at 20,000 psi. Cell lysates were clarified by two consecutive centrifugations at 14,000 rpm for

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

10 min (SS-34 rotor) and supernatants were incubated with Ni^{2+}-nitrilotriacetic acid (Ni^{2+}-NTA) resin for 1.5 hr at 4 °C. The resin was washed with 20 mM sodium phosphate (pH 7.0), 150 mM sodium chloride, 0.05% Triton X-100, 10 mM β-ME, 20 mM imidazole and loaded onto a Poly-Prep column (Bio-Rad) at 4 °C. CdiA-CT/CdiI-His_6 complexes were eluted from the resin with native elution buffer [20 mM sodium phosphate (pH 7.0), 150 mM sodium chloride, 10 mM β-ME, 250 mM imidazole], followed by dialysis into storage buffer [20 mM sodium phosphate (pH 7.0), 150 mM NaCl, 10 mM β-ME]. CdiA-CT toxins were isolated from CdiI-His_6 immunity proteins by Ni^{2+}-affinity chromatography under denaturing conditions [6 M guanidine-HCl, 20 mM sodium phosphate (pH 7.0), 10 mM β-ME] at room temperature. Purified CdiA-CT toxins were dialyzed against storage buffer and quantified by absorbance at 280 nm.

CdiA-CT inhibition assays

Mid-log phage cells were diluted to OD_{600} = 0.05 in fresh LB medium and incubated at 37 °C with shaking. After growth to OD_{600} = 0.1 – 0.15, purified CdiA-CT, ColE5-CT or CdiA-CT/CdiI complex was added to the culture at a final concentration of 100 nM. Cell growth was monitored by measuring OD_{600} every 30 min. Samples were collected periodically and total RNA isolated by guanidine isothiocyanate-phenol extraction as described (Garza-Sánchez *et al.*, 2006). Northern blot analysis was performed as described (Hayes & Sauer, 2003) using 5′-radiolabeled oligonucleotide probes to $tRNA_{ICG}$Arg and $tRNA_{GUA}$Tyr (Table S1). Expression of plasmid-borne *traA* alleles was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) prior to the addition of purified CdiA-CT^{536}. The stability of CdiA-CT^{536} in shaking broth cultures was determined by immunoblot analysis. Culture samples were removed at 0, 1, 3 and 5 hr and the media clarified by centrifugation at 14,000 μ*g* for 10 min. The supernatants (10 μL) were run on SDS-polyacrylamide gels and blotted onto PVDF membrane. Proteins were detected using polyclonal antibodies raised against CdiA-CT^{536} (Webb et al., 2013).

Bacteriophage plating and mating efficiency

Bacteriophage resistance was determined by measuring the efficiency of plating. X90 and derivatives were incubated with 10 and 100 plaque forming units (pfu) and then plated in soft agar. The number of plaques was divided by the number of infecting particles. Phage-resistant strains were also challenged with 10^3 pfu, but no plaques were detected. The *traA* and *trbI* mutants were tested for mating efficiency under the same conditions as CdiA-CT inhibition assays. F′::Tn*10* donor cells (Tet^R) were co-cultured with MC4100 recipient cells (Str^R) at a 1:10 ratio in LB for 3 hr at 37 °C. Samples were collected and plated onto LB-agar supplemented with Tet to enumerate F′::Tn*10* donor cells and Tet/Str quantify exconjugants. Mating efficiency was calculated by dividing the number of exconjugants by total donor cells and expressed as a percentage. The effect of purified CdiA-CT^{536} proteins on conjugation was determined using MC4100 F′::Tn*10* donors and MC4100 pTrc recipients. CdiA-CT proteins were added at 1 μM final concentration to conjugation co-cultures and mating efficiency determined as described above. Phage were inactivated with UV irradiation (64 mJ m^{-2}) in a Stratalinker. This dose resulted in a 10^6-fold reduction in R17 and M13 plaque-forming units (pfu). UV-inactivated phage (~10^5 particles mL^{-1}) were added to conjugation co-cultures to determine the effect on mating efficiency.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Beth Traxler for providing bacteriophages M13 and R17, and Brian Janssen for sequence analysis to determine the translation start site for colicin E5 (Genbank accession: KF925332). This work was supported by grants GM078634 (C.S.H.) and GM102318 (D.A.L & C.S.H.) from the National Institutes of Health.

References

Achtman M, Kennedy N, Skurray R. Cell–cell interactions in conjugating *Escherichia coli*: role of *traT* protein in surface exclusion. Proc Natl Acad Sci U S A. 1977; 74:5104–5108. [PubMed: 337311]

Aiyar A, Leis J. Modification of the megaprimer method of PCR mutagenesis: improved amplification of the final product. Biotechniques. 1993; 14:366–369. [PubMed: 8384463]

Anderson MS, Garcia EC, Cotter PA. The *Burkholderia bcpAIOB* genes define unique classes of two-partner secretion and contact dependent growth inhibition systems. PLoS Genet. 2012; 8:e1002877. [PubMed: 22912595]

Aoki SK, Diner EJ, de Roodenbeke CT, Burgess BR, Poole SJ, Braaten BA, Jones AM, Webb JS, Hayes CS, Cotter PA, Low DA. A widespread family of polymorphic contact-dependent toxin delivery systems in bacteria. Nature. 2010; 468:439–442. [PubMed: 21085179]

Aoki SK, Malinverni JC, Jacoby K, Thomas B, Pamma R, Trinh BN, Remers S, Webb J, Braaten BA, Silhavy TJ, Low DA. Contact-dependent growth inhibition requires the essential outer membrane protein BamA (YaeT) as the receptor and the inner membrane transport protein AcrB. Mol Microbiol. 2008; 70:323–340. [PubMed: 18761695]

Aoki SK, Pamma R, Hernday AD, Bickham JE, Braaten BA, Low DA. Contact-dependent inhibition of growth in *Escherichia coli*. Science. 2005; 309:1245–1248. [PubMed: 16109881]

Aoki SK, Poole SJ, Hayes CS, Low DA. Toxin on a stick: modular CDI toxin delivery systems play roles in bacterial competition. Virulence. 2011; 2:356–359. [PubMed: 21705856]

Aoki SK, Webb JS, Braaten BA, Low DA. Contact-dependent growth inhibition causes reversible metabolic downregulation in *Escherichia coli*. J Bacteriol. 2009; 191:1777–1786. [PubMed: 19124575]

Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol. 2006; 2 2006 0008.

Beck CM, Morse RP, Cunningham DA, Iniguez A, Low DA, Goulding CW, Hayes CS. CdiA from *Enterobacter cloacae* delivers a toxic ribosomal RNase into target bacteria. Structure. 2014; 22:707–718. [PubMed: 24657090]

Beckwith JR, Signer ER. Transposition of the *lac* region of *Escherichia coli*. I. Inversion of the *lac* operon and transduction of *lac* by phi80. J Mol Biol. 1966; 19:254–265. [PubMed: 5338856]

Bolivar F, Rodriguez RL, Greene PJ, Betlach MC, Heyneker HL, Boyer HW, Crosa JH, Falkow S. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene. 1977; 2:95–113. [PubMed: 344137]

Burke JM, Novotny CP, Fives-Taylor P. Defective F pili and other characteristics of F*lac* and Hfr *Escherichia coli* mutants resistant to bacteriophage R17. J Bacteriol. 1979; 140:525–531. [PubMed: 40959]

Casadaban MJ. Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. J Mol Biol. 1976; 104:541–555. [PubMed: 781293]

Cascales E, Buchanan SK, Duche D, Kleanthous C, Lloubes R, Postle K, Riley M, Slatin S, Cavard D. Colicin biology. Microbiol Mol Biol Rev. 2007; 71:158–229. [PubMed: 17347522]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Chandran V, Fronzes R, Duquerroy S, Cronin N, Navaza J, Waksman G. Structure of the outer membrane complex of a type IV secretion system. Nature. 2009; 462:1011–1015. [PubMed: 19946264]

Chang AC, Cohen SN. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J Bacteriol. 1978; 134:1141–1156. [PubMed: 149110]

Clarke M, Maddera L, Harris RL, Silverman PM. F-pili dynamics by live-cell imaging. Proc Natl Acad Sci U S A. 2008; 105:17978–17981. [PubMed: 19004777]

Daehnel K, Harris R, Maddera L, Silverman P. Fluorescence assays for F-pili and their application. Microbiology. 2005; 151:3541–3548. [PubMed: 16272377]

Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A. 2000; 97:6640–6645. [PubMed: 10829079]

Datta S, Costantino N, Court DL. A set of recombineering plasmids for gram-negative bacteria. Gene. 2006; 379:109–115. [PubMed: 16750601]

Dautin N, Barnard TJ, Anderson DE, Bernstein HD. Cleavage of a bacterial autotransporter by an evolutionarily convergent autocatalytic mechanism. EMBO J. 2007; 26:1942–1952. [PubMed: 17347646]

Dent KC, Thompson R, Barker AM, Hiscox JA, Barr JN, Stockley PG, Ranson NA. The asymmetric structure of an icosahedral virus bound to its receptor suggests a mechanism for genome release. Structure. 2013; 21:1225–1234. [PubMed: 23810697]

Diner EJ, Beck CM, Webb JS, Low DA, Hayes CS. Identification of a target cell permissive factor required for contact-dependent growth inhibition (CDI). Genes Dev. 2012; 26:515–525. [PubMed: 22333533]

Friedman SD, Genthner FJ, Gentry J, Sobsey MD, Vinje J. Gene mapping and phylogenetic analysis of the complete genome from 30 single-stranded RNA male-specific coliphages (family Leviviridae). J Virol. 2009; 83:11233–11243. [PubMed: 19710143]

Garza-Sánchez F, Janssen BD, Hayes CS. Prolyl-tRNA(Pro) in the A-site of SecM-arrested ribosomes inhibits the recruitment of transfer-messenger RNA. J Biol Chem. 2006; 281:34258–34268. [PubMed: 16968693]

Guyer MS, Reed RR, Steitz JA, Low KB. Identification of a sex-factor-affinity site in *E. coli* as gamma delta. Cold Spring Harb Symp Quant Biol. 1981; 45(Pt 1):135–140. [PubMed: 6271456]

Hayes CS, Sauer RT. Cleavage of the A site mRNA codon during ribosome pausing provides a mechanism for translational quality control. Mol Cell. 2003; 12:903–911. [PubMed: 14580341]

Johnson RC, Reznikoff WS. Copy number control of Tn5 transposition. Genetics. 1984; 107:9–18. [PubMed: 6327462]

Krahn PM, O'Callaghan RJ, Paranchych W. Stages in phage R17 infection. VI. Injection of A protein and RNA into the host cell. Virology. 1972; 47:628–637. [PubMed: 4551992]

Lang S, Gruber CJ, Raffl S, Reisner A, Zechner EL. Common Requirement for the Relaxosome of Plasmid R1 in Multiple Activities of the Conjugative Type IV Secretion System. J Bacteriol. 2014; 196:2108–2121. [PubMed: 24682328]

Lang S, Kirchberger PC, Gruber CJ, Redzej A, Raffl S, Zellnig G, Zangger K, Zechner EL. An activation domain of plasmid R1 TraI protein delineates stages of gene transfer initiation. Mol Microbiol. 2011; 82:1071–1085. [PubMed: 22066957]

Lin A, Jimenez J, Derr J, Vera P, Manapat ML, Esvelt KM, Villanueva L, Liu DR, Chen IA. Inhibition of bacterial conjugation by phage M13 and its protein g3p: quantitative analysis and model. PLoS One. 2011; 6:e19991. [PubMed: 21637841]

Lin YL, Elias Y, Huang RH. Structural and mutational studies of the catalytic domain of colicin E5: a tRNA-specific ribonuclease. Biochemistry. 2005; 44:10494–10500. [PubMed: 16060658]

Manchak J, Anthony KG, Frost LS. Mutational analysis of F-pilin reveals domains for pilus assembly, phage infection and DNA transfer. Mol Microbiol. 2002; 43:195–205. [PubMed: 11849547]

Maneewannakul S, Maneewannakul K, Ippen-Ihler K. Characterization, localization, and sequence of F transfer region products: the pilus assembly gene product TraW and a new product, TrbI. J Bacteriol. 1992; 174:5567–5574. [PubMed: 1355084]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Manning PA, Beutin L, Achtman M. Outer membrane of *Escherichia coli*: properties of the F sex factor *traT* protein which is involved in surface exclusion. J Bacteriol. 1980; 142:285–294. [PubMed: 6989806]

Moore D, Sowa BA, Ippen-Ihler K. Location of an F-pilin pool in the inner membrane. J Bacteriol. 1981; 146:251–259. [PubMed: 6111549]

Morse RP, Nikolakakis KC, Willett JL, Gerrick E, Low DA, Hayes CS, Goulding CW. Structural basis of toxicity and immunity in contact-dependent growth inhibition (CDI) systems. Proc Natl Acad Sci U S A. 2012; 109:21480–21485. [PubMed: 23236156]

Nikolakakis K, Amber S, Wilbur JS, Diner EJ, Aoki SK, Poole SJ, Tuanyok A, Keim PS, Peacock S, Hayes CS, Low DA. The toxin/immunity network of *Burkholderia pseudomallei* contact-dependent growth inhibition (CDI) systems. Mol Microbiol. 2012; 84:516–529. [PubMed: 22435733]

Novotny CP, Fives-Taylor P. Retraction of F pili. J Bacteriol. 1974; 117:1306–1311. [PubMed: 4591953]

Ogawa T, Tomita K, Ueda T, Watanabe K, Uozumi T, Masaki H. A cytotoxic ribonuclease targeting specific transfer RNA anticodons. Science. 1999; 283:2097–2100. [PubMed: 10092236]

Oriel PJ. The thermal and alkaline degradiation of MS2 bacteriophage. Arch Biochem Biophys. 1969; 132:8–15. [PubMed: 5792839]

Osborn M, Weiner AM, Weber K. Large scale purification of A-protein from bacteriophage R17. Eur J Biochem. 1970; 17:63–67. [PubMed: 5486584]

Paranchych W, Ainsworth SK, Dick AJ, Krahn PM. Stages in phage R17 infection. V. Phage eclipse and the role of F pili. Virology. 1971; 45:615–628. [PubMed: 4108185]

Paranchych W, Krahn PM, Bradley RD. Stages in phage R17 infection. Virology. 1970; 41:465–473. [PubMed: 4912821]

Poole SJ, Diner EJ, Aoki SK, Braaten BA, T'Kint de Roodenbeke C, Low DA, Hayes CS. Identification of functional toxin/immunity genes linked to contact-dependent growth inhibition (CDI) and rearrangement hotspot (Rhs) systems. PLoS Genet. 2011; 7:e1002217. [PubMed: 21829394]

Riechmann L, Holliger P. The C-terminal domain of TolA is the coreceptor for filamentous phage infection of E. coli. Cell. 1997; 90:351–360. [PubMed: 9244308]

Roberts JW, Steitz JE. The reconstitution of infective bacteriophage R17. Proc Natl Acad Sci U S A. 1967; 58:1416–1421. [PubMed: 5237875]

Ruhe ZC, Low DA, Hayes CS. Bacterial contact-dependent growth inhibition. Trends Microbiol. 2013a; 21:230–237. [PubMed: 23473845]

Ruhe ZC, Wallace AB, Low DA, Hayes CS. Receptor polymorphism restricts contact-dependent growth inhibition to members of the same species. MBio. 2013b; 4

Rumnieks J, Tars K. Diversity of pili-specific bacteriophages: genome sequence of IncM plasmid-dependent RNA phage M. BMC Microbiol. 2012; 12:277. [PubMed: 23176223]

Schoulaker R, Engelberg-Kulka H. *Escherichia coli* mutant temperature sensitive for group I RNA bacteriophages. J Virol. 1978; 25:433–435. [PubMed: 340711]

Silverman PM, Clarke MB. New insights into F-pilus structure, dynamics, and function. Integr Biol (Camb). 2010; 2:25–31. [PubMed: 20473409]

Steitz JA. Identification of the A protein as a structural component of bacteriophage R17. J Mol Biol. 1968; 33:923–936. [PubMed: 4178187]

Waksman G, Fronzes R. Molecular architecture of bacterial type IV secretion systems. Trends Biochem Sci. 2010; 35:691–698. [PubMed: 20621482]

Waterhouse AM, Procter JB, Martin DM, Clamp M, Barton GJ. Jalview Version 2--a multiple sequence alignment editor and analysis workbench. Bioinformatics. 2009; 25:1189–1191. [PubMed: 19151095]

Webb JS, Nikolakakis KC, Willett JLE, Aoki SK, Hayes CS, Low DA. Delivery of CdiA nuclease toxins into target cells during contact-dependent growth inhibition. PLoS ONE. 2013; 8:e57609. [PubMed: 23469034]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Willetts N, Achtman M. Genetic analysis of transfer by the *Escherichia coli* sex factor F, using P1 transductional complementation. J Bacteriol. 1972; 110:843–851. [PubMed: 4555413]

Willetts NS, Moore PM, Paranchych W. Variant pili produced by mutants of the Flac plasmid. J Gen Microbiol. 1980; 117:455–464. [PubMed: 6106661]

Zarivach R, Deng W, Vuckovic M, Felise HB, Nguyen HV, Miller SI, Finlay BB, Strynadka NC. Structural analysis of the essential self-cleaving type III secretion proteins EscU and SpaS. Nature. 2008; 453:124–127. [PubMed: 18451864]

Zhang D, Iyer LM, Aravind L. A novel immunity system for bacterial nucleic acid degrading toxins and its recruitment in various eukaryotic and DNA viral systems. Nucleic Acids Res. 2011; 39:4532–4552. [PubMed: 21306995]

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

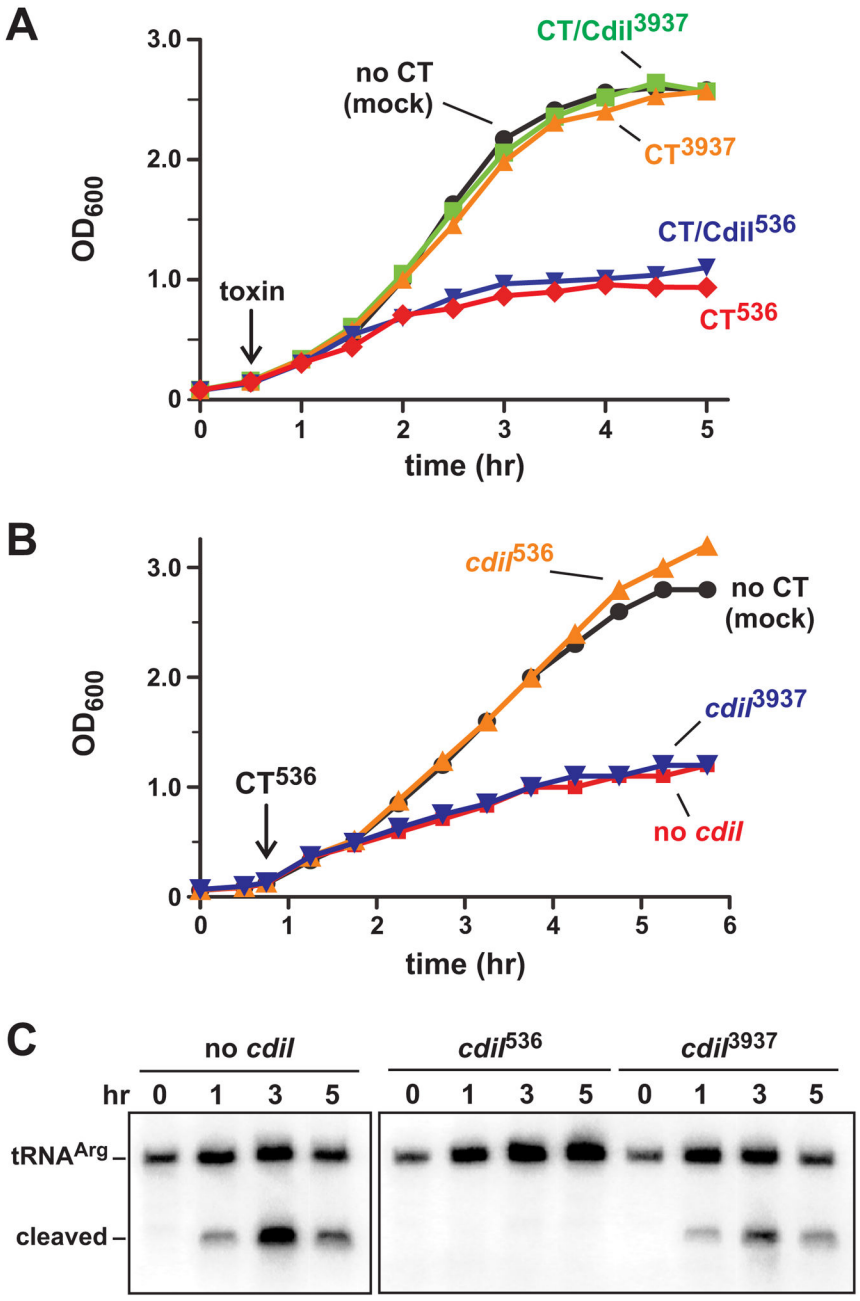

Figure 1. Purified CdiA-CT^{536} inhibits *E. coli* cell growth

A) Purified CdiA-CT (CT) or CdiA-CT/CdiI (CT/CdiI) complex was added to *E. coli* X90 cultures at 30 min (indicated by the arrow) and cell growth monitored by measuring the optical density at 600 nm (OD_{600}). **B**) *E. coli* X90 cells carrying plasmid-borne *cdiI*^{536} or *cdiI*^{3937} immunity genes were treated with purified CdiA-CT^{536} where indicated the arrow. **C**) Northern blot analysis of CdiA-CT^{536} treated cells. Total RNA was isolated from the cells in panel B and $tRNA_{ICG}Arg$ analyzed by northern blot. The migration positions of full-length and cleaved $tRNA_{ICG}Arg$ are indicated.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

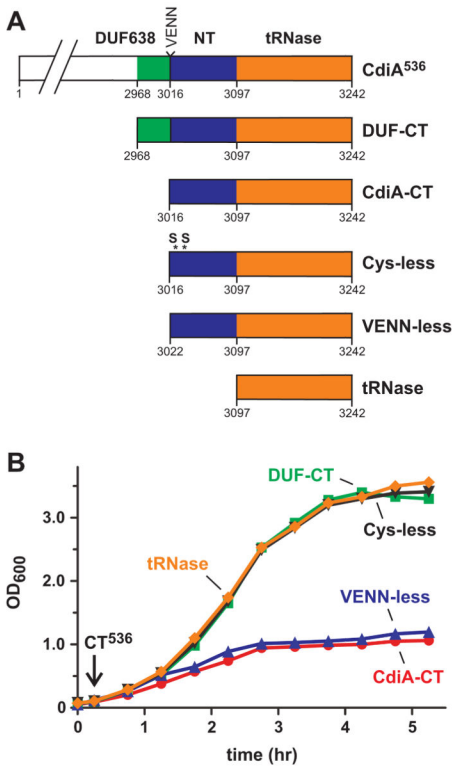

Figure 2. The N-terminal domain of CdiA-CT^{536} is required for import

A) CdiA-CT^{536} constructs. Predicted domain organization for the C-terminal region of CdiA^{536} is depicted with the corresponding residue numbers. Residues Val3016 – Asn3019 comprise the VENN peptide motif. DUF638 is a domain of unknown function corresponding to Pfam PF04829. **B**) *E. coli* X90 cells were treated with the indicated CdiA-CT^{536} fragments and cell growth monitored by OD_{600} measurements.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

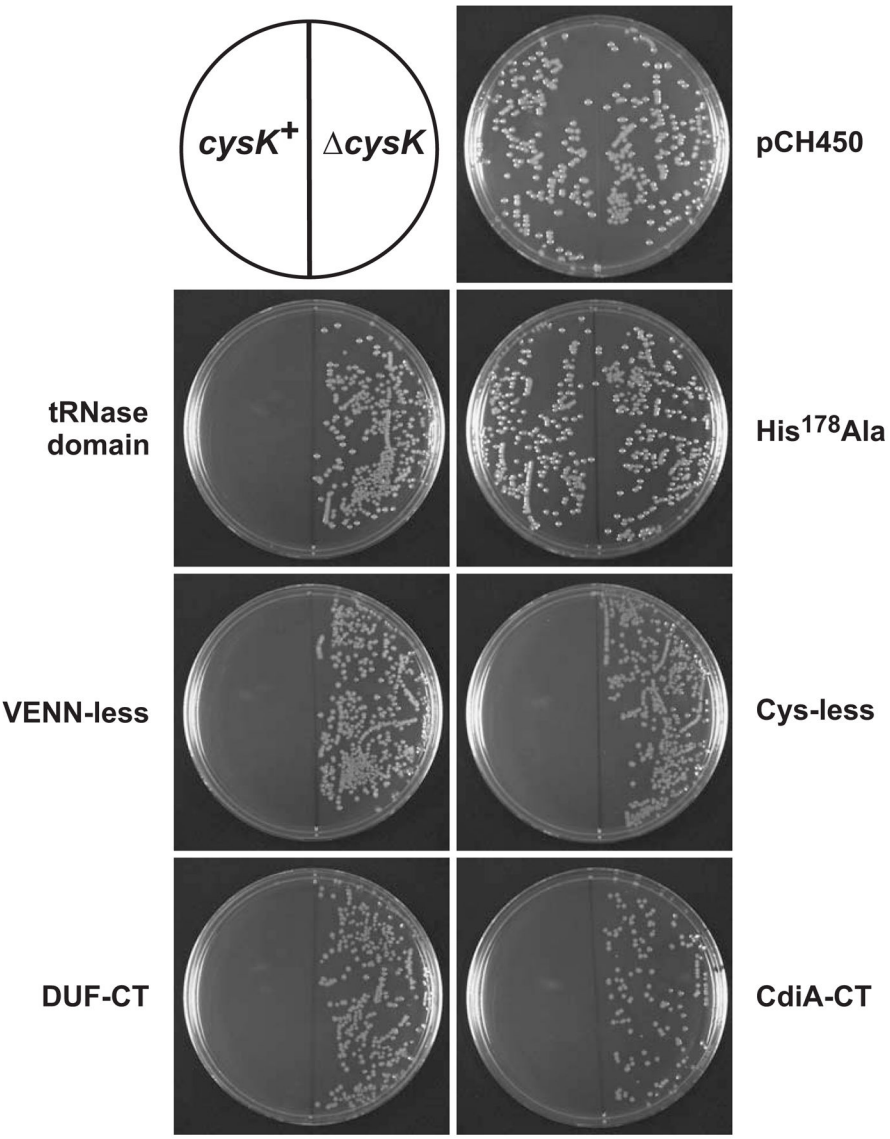

Figure 3. CdiA-CT^{536} toxicity

Arabinose-inducible *cdiA-CT*^{536} expression plasmids were introduced into *E. coli* X90 and transformants selected on LB-agar supplemented with tetracycline and L-arabinose. CdiA-CT^{536} requires activation by CysK, therefore *E. coli* Δ*cysK* cells were used to control for transformation efficiency. Construct nomenclature corresponds to that introduced in Fig. 2A. The His178Ala mutation ablates tRNase activity and plasmid pCH450 is the empty vector.

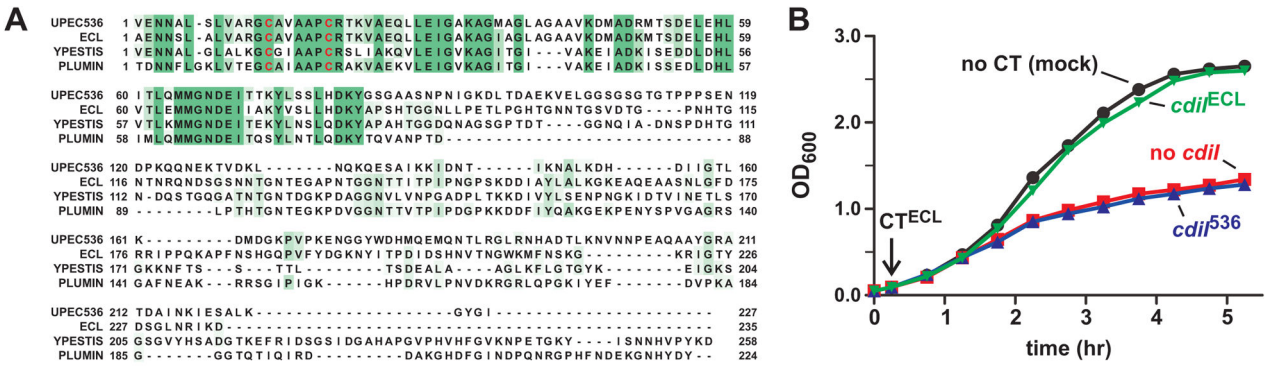

Figure 4. CdiA-CT^{ECL} from *Enterobacter cloacae* inhibits *E. coli* cell growth

A) Alignment of the CdiA-CT regions from UPEC 536 (Uniprot: Q0T963), *Enterobacter cloacae* ATCC 13047 (D5CBA0), *Yersinia pestis* 91001 (Q74T84) and *Photorhabdus luminescens* TT01 (Q7MB60). Proteins were aligned using Clustal-W and rendered with Jalview 2.8 (Waterhouse *et al.*, 2009) at 30% sequence identity. The conserved cysteine residues within the N-terminal domain are depicted in red. **B**) Purified CdiA-CT^{ECL} was added to *E. coli* X90 cultures when indicated (downward arrow) and cell growth monitored by OD_{600} measurements. Where indicated, cells carried plasmid-borne *cdiI*536 (*cdiI*536) or *cdiI*^{ECL} immunity genes.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

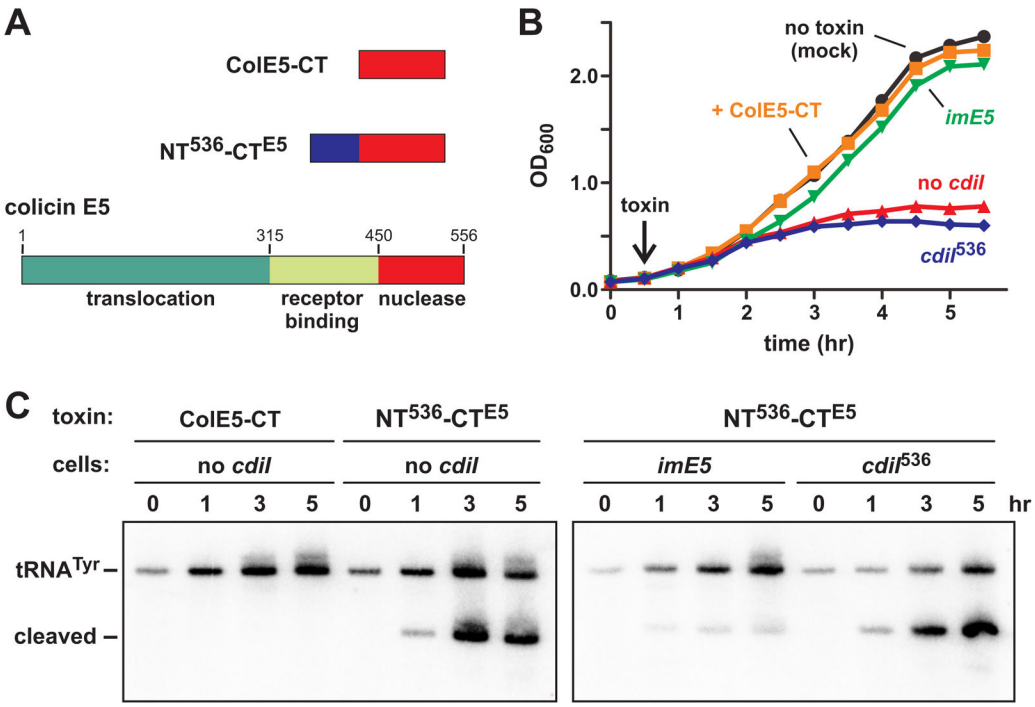

Figure 5. The N-terminal domain of CdiA-CT^{536} is sufficient for import

A) Colicin E5 is composed of translocation, receptor-binding and nuclease domains. The C-terminal domain (ColE5-CT) has anticodon nuclease activity. The NT^{536}-CT^{E5} protein contains residues Val3016 – Gly3097 of CdiA^{536} fused to the ColE5-CT nuclease domain. **B**) *E. coli* XL-1 cells were treated with purified ColE5-CT or NT^{536}-CT^{E5} fusion at 30 min (indicated by the arrow) and growth monitored by OD_{600} measurements. Where indicated, cells carried plasmid-borne *cdiI*^{536} (*cdiI*^{536}) or *imE5* immunity genes. **C**) Northern blot analysis of toxin-treated cells. Total RNA was isolated from the cells in panel B and tRNA^{Tyr} analyzed by northern blot. The migration positions of full-length and cleaved tRNA^{Tyr} are indicated.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

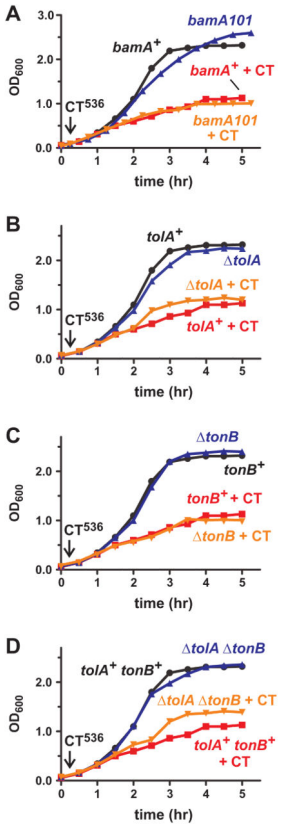

Figure 6. CdiA-CT^{536} is not translocated via known toxin-import pathways

Wild-type *E. coli* X90 and the indicated mutants were treated with purified CdiA-CT^{536} where indicated (+CT), and cell growth was monitored by OD_{600} measurements. **A**) *E. coli* X90 (*bamA*+) and *bamA101* mutants. **B**) *E. coli* X90 Δ*tolA* mutants. **C**) *E. coli* X90 Δ*tonB* mutants. **D**) *E. coli* X90 Δ*tolA* Δ*tonB* mutants.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

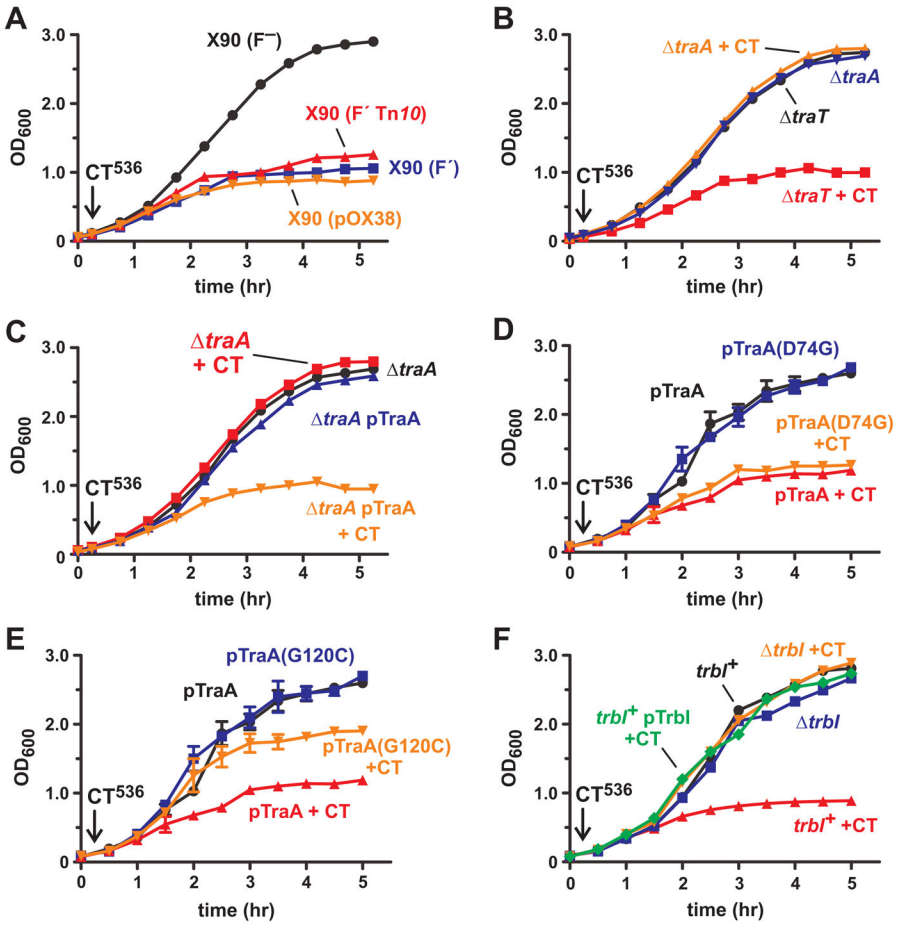

Figure 7. The F pilus is required for CdiA-CT^{536} import

E. coli X90 and its derivatives were treated with purified CdiA-CT^{536} where indicated (+CT), and cell growth was monitored by OD_{600} measurements. **A**) Wild-type X90 is indicated as F′ and cured cells as F^{-}. The F′::Tn*10* episome and pOX38::*gent* (pOX38) were introduced into cured X90 and the resulting cells treated with CdiA-CT^{536}. **B**) *E. coli* X90 Δ*traA::cat* and Δ*traT::cat* mutants. **C**) *E. coli* X90 Δ*traA::cat* cells complemented with plasmid-borne *traA*. **D**) and **E**) *E. coli* X90 Δ*traA::cat* cells complemented with plasmid-borne *traA(D74G)* and *traA(G120C)*. Error bars correspond to the standard error of the mean for three independent experiments. **F**) *E. coli* X90 Δ*trbI::cat* mutant. Wild-type (*trbI*$^{+}$) cells expressing *trbI* from a plasmid (pTrbI) were also tested for toxin-resistance.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

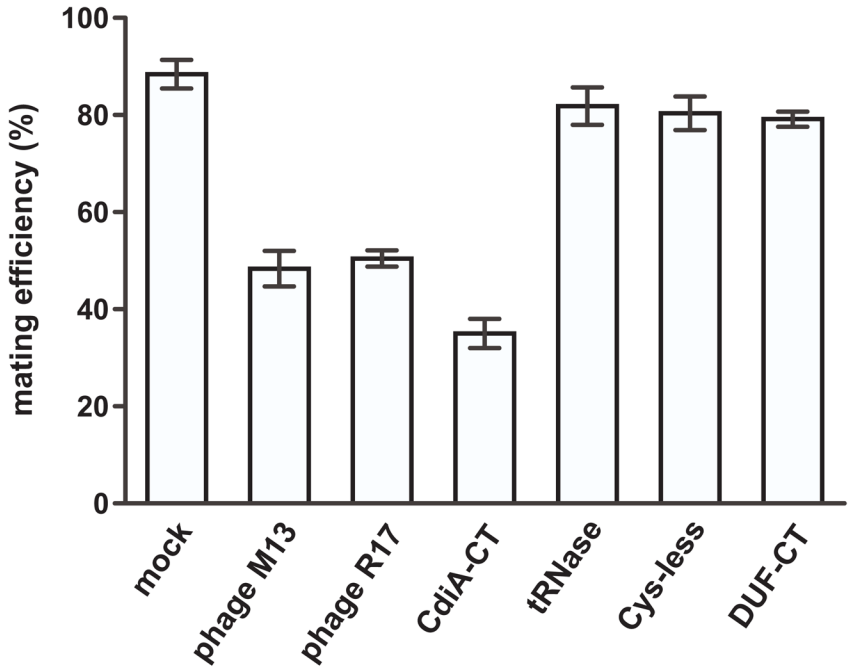

Figure 8. Purified CdiA-CT^{536} interferes with F-mediated conjugation

The efficiency of conjugation was determined using the F′::Tn*10* episome as described in methods. Cell suspensions were treated with UV-inactivated phage particles or the indicated CdiA-CT^{536} constructs (see Fig. 2A). The CdiA-CT^{536} construct contains the His178Ala mutation to ablate tRNase activity.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Table 1

Bacteriophage plating efficiency[a]

	Bacteriophage	
Bacterial strain	**R17**	**M13**
X90 Δ*tolA*	0.93 ± 0.12	0.0[b]
X90 Δ*tonB*	1.2 ± 0.26	1.0 ± 0.20
X90 Δ*tolA* Δ*tonB*	1.1 ± 0.15	0.0[b]
X90 Δ*traA::cat* pTrc	0.0[b]	0.0[b]
X90 Δ*traA::cat* pTrc::*traA*	1.2 ± 0.18	1.2 ± 0.15
X90 Δ*traA::cat* pTrc::*traA(D74G)*	0.0[b]	1.1 ± 0.09
X90 Δ*traA::cat* pTrc::*traA(G120C)*	0.09 ± 0.005[c]	0.77 ± 0.06[c]
X90 Δ*trbI::cat*	0.1 ± 0.04[c]	0.1 ± 0.03[c]

[a] The indicated bacterial strains were infected with 10 and 100 plaque forming units (pfu). The number of plaques for each strain was divided by the plaques obtained with wild-type X90 cells. The mean plating efficiency ± SEM is reported (n = 4).

[b] No plaques were detected when bacteria were incubated with 10^3 pfu of phage.

[c] Plaques were turbid.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Table 2

Mating efficiency[a]

F⁺ donor strain	**Percent exconjugants (%)**
CH11717	79 ± 2.8
CH11717 Δ*traA::cat*	0.0
CH11717 Δ*traA::cat* pTrc	0.0
CH11717 Δ*traA::cat* pTrc::*traA*	75 ± 5.4
CH11717 Δ*traA::cat* pTrc::*traA(D74G)*	58 ± 5.7
CH11717 Δ*traA::cat* pTrc::*traA(G120C)*	72 ± 0.5
CH11717 Δ*trbI::cat*	13 ± 3.6

[a]Donor strains were derived from strain CH11717, which is X90 transduced with the Tn*10* marker from *E. coli* XL-1. Donors were cultured at a 1:10 ratio with Str^R recipient cells as described in Methods. Mating efficiency was determined by dividing the number of Tet^R Str^R colonies by the number of total Tet^R colonies and expressed as a percent. The mean plating efficiency ± SEM is reported for three independent experiments.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

Table 3

Bacterial strains and plasmids

Strains or plasmids	Description[a]	Reference
Strain		
X90	F′ *lacI*^{q} *lac*′ *pro*′/*ara*Δ(*lac-pro) nal1 argE*(amb) *rif*^{r} *thi-1*, Rif^{R}	(Beckwith & Signer, 1966)
XL-1	F′::Tn*10 proA*$^{+}$*B*$^{+}$ *lacI*^{q} Δ*(lacZ)M15/recA1 endA1 gyrA96 thi hsdR17* (r_K^- r_K^+) *glnV44 relA1 lac*. Tet^{R}, Nal^{R}	Stratagene
MC4100	F^{-} *araD139* Δ*(argF-lac)U169 rpsL150 relA1 deoC1 rbsR fthD5301 fruA25* λ^{-}. Str^{R}	(Casadaban, 1976)
KW1070	X90 F^{-}	Kelly P. Williams
CH2016	X90 (DE3) Δ*rna* Δ*slyD::kan*, Kan^{R}	(Garza-Sánchez et al., 2006)
CH6479	X90 Δ*tolA::kan*, Kan^{R}	This study
CH6480	X90 Δ*tonB::kan*, Kan^{R}	This study
CH6680	X90 *bamA101::kan*, Kan^{R}	(Aoki et al., 2008) & this study
CH6866	KW1070 pOX38::*gent*, Gent^{R}	This study
CH6939	X90 Δ*traA::cat*, Cm^{R}	This study
CH7035	X90 Δ*traT::cat*, Cm^{R}	This study
CH9027	KW1070 F′::Tn*10*, Tet^{R}	This study
CH9354	X90 Δ*tonB tolA::kan*, Kan^{R}	This study
CH11476	X90 Δ*trbI::cat*, Cm^{R}	This study
CH11703	CH11717 Δ*trbI::cat*, Tet^{R} Cm^{R}	This study
CH11717	X90 Tn*10*, Tet^{R}	This study
CH11718	CH11717 Δ*traA::cat*, Tet^{R} Cm^{R}	This study
Plasmids		
pBR322	Cloning vector, Amp^{R} Tet^{R}	(Bolivar *et al.*, 1977)
pACYC184	Cloning vector, Cm^{R}, Tet^{R}	(Chang & Cohen, 1978)
pTrc99A	IPTG-inducible expression vector, Amp^{R}	GE Healthcare
pCH450	pACYC184 derivative containing *E. coli araC* and the L-arabinose-inducible P_{araBAD} promoter, Tet^{R}	(Hayes & Sauer, 2003)
pET21::*cdiA-CT/cdiI*^{3937}	Over-produces CdiA-CT^{3937} and CdiI^{3937}-His$_6$, Amp^{R}	(Aoki et al., 2010)
pET21::*cdiA-CT/cdiI*^{536}	Over-produces CdiA-CT^{536} and CdiI^{536}-His$_6$, Amp^{R}	(Aoki et al., 2010)
pET21::*DUF-CT/cdiI*^{536}	Over-produces DUF638-CdiA-CT^{536} and CdiI^{536}-His$_6$, Amp^{R}	This study
pET21::*VENN-less-CT/cdiI*^{536}	Over-produces VENNless-CdiA-CT^{536} and CdiI^{536}-His$_6$. Amp^{R}	This study
pET21::*Cys-less-CT/cdiI*^{536}	Over-produces CdiA-CT^{536} containing Cys13Ser and Cys19Ser mutations together with CdiI^{536}-His$_6$, Amp^{R}	This study
pET21::*tRNase/cdiI*^{536}	Over-produces the C-terminal tRNase domain of CdiA-CT^{536} together with CdiI^{536}-His$_6$, Amp^{R}	(Diner et al., 2012)
pET21::*cdiA-CT(H178A)/cdiI*^{536}	Over-produces catalytically inactive His178Ala variant of CdiA-CT^{536} toxin and CdiI^{536}-His$_6$, Amp^{R}	(Diner et al., 2012)

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Strains or plasmids	Description[a]	Reference
pET21K::*cdiA-CT*/*cdiI*^{ECL}	Over-produces CdiA-CT^{ECL} and CdiI^{ECL}-His$_6$ from *Enterobacter cloacae* ATCC 13047, Amp^R	This study
pCH450::*cdiA-CT*^{536}	Expresses *cdiA-CT*^{536} under control of P$_{BAD}$ promoter, Tet^R	This study
pCH450::*DUF-CT*^{536}	Expresses *DUF638-cdiA-CT*^{536} under control of P$_{BAD}$ promoter, Tet^R	This study
pCH450::*VENN-less-CT*^{536}	Produces VENNless-CdiA-CT^{536}, Tet^R	This study
pCH450::*Cys-less-CT*^{536}	Produes CdiA-CT^{536} containing Cys13Ser and Cys19Ser mutations, Tet^R	This study
pCH450::*tRNase*^{536}	C-terminal tRNase domain of CdiA-CT^{536}, Tet^R	This study
pCH450::*cdiA-CT(H178A)*^{536}	catalytically inactive His178Ala variant of CdiA-CT^{536}, Tet^R	This study
pDAL776	pBR322 derivative that constitutively expresses *cdiI*^{536}, Amp^R	(Aoki et al., 2010)
pDAL852	pBR322 derivative that constitutively expresses *cdiI*^{3937} from *Dickeya dadantii* 3937, Amp^R	(Aoki et al., 2010)
pOX38::*gent*	pOX38 with integrated gentamicin-resistance cassette, Gent^R	(Johnson & Reznikoff, 1984)
pTrc99A::*cdiI*^{536}	Expresses *cdiI*^{536} under control of the P$_{trc}$ promoter, Amp^R	This study
pTrc99A::*cdiI*^{ECL}	Expresses *cdiI*^{ECL} from *Enterobacter cloacae* ATCC 13047 under control of the P$_{trc}$ promoter, Amp^R	This study
pTrc99A::*imE5*	Expresses *imE5* under control of the P$_{trc}$ promoter, Amp^R	This study
pET21::*colE5-CT/imE5*	Over-produces ColE5-CT (residues Met429 - Gln556) and together with ImE5-His$_6$, Amp^R	This study
pET21::*NT*^{536}*-colE5-CT/imE5*	Over-produces a protein containing residues Val1 – Tyr82 of CdiA-CT^{536} fused to the nuclease domain of colicin E5 (colE5-CT) together with ImE5-His$_6$, Amp^R	This study
pTrc99A::*traA*	Expresses wild-type F-pilin under the control of the P$_{trc}$ promoter, Amp^R	This study
pTrc99A::*traA(D74G)*	Expresses the Asp74Gly variant of F-pilin under control of the P$_{trc}$ promoter, Amp^R	This study
pTrc99A::*traA(G120C)*	Expresses the Gly120Cys variant of F-pilin under control of the P$_{trc}$ promoter, Amp^R	This study
pTrc99A::*trbI*	Expresses *trbI* under the control of the P$_{trc}$ promoter, Amp^R	This study
pCH450::*trbI*	Expresses *trbI* under the control of the P$_{BAD}$ promoter, Tet^R	This study

[a] Abbreviations: Amp^R, ampicillin resistant; Cm^R, chloramphenicol resistant; Gent^R, gentamicin resistant; Kan^R, kanamycin resistant; Nal^R, nalidixic acid resistant; Rif^R, rifampicin resistant; Str^R, streptomycin resistant; Tet^R, tetracycline resistant