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Permalink
https://escholarship.org/uc/item/1x68z5h4

Journal
mBio, 8(2)

ISSN
2150-7511

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Publication Date
2017-03-28

DOI
10.1128/mbio.00290-17

Peer reviewed
CdiA Effectors Use Modular Receptor-Binding Domains To Recognize Target Bacteria

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ABSTRACT Contact-dependent growth inhibition (CDI) systems encode CdiA effectors, which bind to specific receptors on neighboring bacteria and deliver C-terminal toxin domains to suppress target cell growth. Two classes of CdiA effectors that bind distinct cell surface receptors have been identified, but the molecular basis of receptor specificity is not understood. Alignment of BamA-specific CdiAE93 from Escherichia coli EC93 and OmpC-specific CdiAE536 from E. coli 536 suggests that the receptor-binding domain resides within a central region that varies between the two effectors. In support of this hypothesis, we find that CdiAE93 fragments containing residues Arg1358 to Phe1646 bind specifically to purified BamA. Moreover, chimeric CdiAE93 that carries the corresponding sequence from CdiAE536 is endowed with OmpC-binding activity, demonstrating that this region dictates receptor specificity. A survey of E. coli CdiA proteins reveals two additional effector classes, which presumably recognize distinct receptors. Using a genetic approach, we identify the outer membrane nucleoside transporter Tsx as the receptor for a third class of CdiA effectors. Thus, CDI systems exploit multiple outer membrane proteins to identify and engage target cells. These results underscore the modularity of CdiA proteins and suggest that novel effectors can be constructed through genetic recombination to interchange different receptor-binding domains and toxic payloads.

IMPORTANCE CdiB/CdiA two-partner secretion proteins mediate interbacterial competition through the delivery of polymorphic toxin domains. This process, known as contact-dependent growth inhibition (CDI), requires stable interactions between the CdiA effector protein and specific receptors on the surface of target bacteria. Here, we localize the receptor-binding domain to the central region of E. coli CdiA. Receptor-binding domains vary between CdiA proteins, and E. coli strains collectively encode at least four distinct effector classes. Further, we show that receptor specificity can be altered by exchanging receptor-binding domains, demonstrating the modularity of this domain. We propose that novel CdiA effectors are naturally generated through genetic recombination to interchange different receptor-binding domains and toxic payloads.

KEYWORDS bacterial competition, cell-cell adhesion, self/nonself discrimination, toxin immunity proteins, type V secretion system

Reveived 21 February 2017. Accepted 1 March 2017. Published 28 March 2017
Citation Ruhe ZC, Nguyen JY, Xiong J, Koskiniemi S, Beck CM, Perkins BR, Low DA, Hayes CS. 2017. CdiA effectors use modular receptor-binding domains to recognize target bacteria. mBio 8:e00290-17. https://doi.org/10.1128/mBio.00290-17
Editor Michael T. Laub, Massachusetts Institute of Technology
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This article is a direct contribution from a Fellow of the American Academy of Microbiology. External solicited reviewers: Thomas Bernhardt, Harvard Medical School; Michael Ibba, The Ohio State University.
mechanism has been termed contact-dependent growth inhibition (CDI). Related CDI systems have since been identified and characterized in other Gram-negative bacteria, where they play important roles in interstrain competition and self/nonself discrimination (7–11). CDI is mediated by the CdiB/CdiA family of two-partner secretion proteins. CdiB is an Omp85 transport protein that exports and presents toxic CdiA proteins on the cell surface. CdiA effectors range from 180 to 630 kDa depending on bacterial species (12), but each is predicted to form an elongated filament projecting from the inhibitor cell. Upon binding a specific receptor, CdiA transfers its C-terminal toxin domain (CdiA-CT) into the target bacterium through an incompletely understood translocation pathway (13, 14). CdiA-CT sequences vary considerably between effector proteins, but most toxins either degrade nucleic acids or form pores in target cell membranes (9, 15–19). To prevent self-intoxication, CDI+ bacteria produce CdiI immunity proteins, which bind the CdiA-CT domain and neutralize its activity. Because CdiA-CT sequences are highly variable and the domains have distinct three-dimensional structures (16, 20–23), immunity proteins protect against only cognate toxins deployed by sibling cells. Thus, CDI confers a selective advantage against nonisogenic competitors, and toxin immunity polymorphism provides a mechanism for self/nonself discrimination.

CdiA-receptor-binding interactions also play a critical role in self/nonself recognition. Using a genetic approach, Aoki et al. identified BamA as the receptor for the CdiAEC93 effector from E. coli EC93 (24). BamA is an essential outer membrane β-barrel protein found in all Gram-negative bacteria (25). Though BamA proteins share a high degree of sequence identity between enterobacteria, the surface-exposed extracellular loops vary dramatically between species (26, 27). Thus, CdiAEC93 effectively targets E. coli strains but does not recognize closely related bacteria like Enterobacter cloacae, even though BamA from E. cloacae (BamAECCL) and BamA from E. coli (BamAECO) share ~95% sequence identity (28). Variations in bacterial surface antigens are thought to reflect the selective pressures exerted by bacteriophages and adaptive immune systems (29, 30). Because surface epitopes vary dramatically between bacteria, the CdiA effector family must collectively recognize many distinct receptors. Accordingly, we recently found that CdiAEC536 from uropathogenic E. coli 536 uses heterotrimeric OmpC-OmpF as a receptor (31). The E. coli pan-genome encodes at least 220 distinct OmpC porins, with much of the sequence variability localized to extracellular loops L4 and L5 (31–33). These loops appear to be recognition epitopes for CdiAEC536, and consequently, several E. coli OmpC variants are not recognized by this effector. Notably, CdiAEC536 binds OmpC and OmpF from E. cloacae ATCC 13047 (31), suggesting that E. coli 536 could use CDI to inhibit other species. Thus, CDI-target cell interactions are complex and idiosyncratic. However, one common and perhaps universal feature is the recognition of “self” receptors, which promotes the autoaggregation of CDI+ sibling cells (3, 34). CDI-dependent cell-cell adhesion also contributes significantly to biofilm formation (10, 35–38), and recent work suggests that CDI-mediated toxin exchange is exploited for intercellular communication between siblings (39). These findings indicate that CDI plays an important role in bacterial cooperation, and its toxin delivery activity excludes nonisogenic cells from group activities.

Though CDI recognition epitopes have been localized on BamA and OmpC (28, 31), the corresponding receptor-binding domains in CdiA have not yet been identified. In this report, we use a combination of biochemical and molecular genetic approaches to map the receptor-binding region within E. coli CdiA effectors. Previous work with truncated CdiAEC93 indicates that its BamAECO-binding domain resides within the N-terminal 2,000 residues (38). We find that CdiAEC93 fragments containing residues Arg1358 to Phe1646 interact stably with BamAECO but not with the closely related BamAECCL protein. These CdiAEC93 residues comprise an unannotated central segment located between the FHA-1 and FHA-2 peptide repeat regions. Notably, E. coli CdiA proteins can be divided into four major classes based on sequence variation in the putative receptor-binding region. Using chimeric effectors, we show that CdiAEC93 is redirected to bind OmpC when residues Ser1347 to Tyr1636 are replaced with the corresponding region from CdiAEC536. Given the variability between effector classes, we
reasoned that a third class of CdiAs likely recognizes an uncharacterized receptor. Using the CDISTECO31 system from *E. coli* STEC_O31 as a model, we identify the outer membrane nucleoside transporter Tsx as the receptor for class III effectors. Thus, CdiA proteins collectively recognize diverse receptors, and their receptor-binding domains are interchangeable between effectors. This modularity suggests that receptor-binding and toxin domains are actively recombined to generate novel effectors.

RESULTS

Localization of the BamA\textsuperscript{Eco}\_binding region in CdiA\textsuperscript{EC93}. We previously reported that truncated CdiA\textsuperscript{EC93} lacking residues Ala1931 to Lys3242 is exported to the cell surface and retains BamA\textsuperscript{Eco}-binding activity (38). Further truncation abrogates adhesin function, but these smaller proteins are not exported efficiently and appear to be degraded rapidly. Therefore, we took a biochemical approach to define the receptor-binding region more precisely. We generated a series of His\textsubscript{6}-tagged CdiA\textsuperscript{EC93} fragments (Fig. 1A) and tested them for binding interactions with purified BamA. Using Ni\textsuperscript{2+}-affinity chromatography, we found that BamA\textsuperscript{Eco} copurifies with CdiA\textsuperscript{EC93} fragments containing residues Arg1358 to Phe1646 and Arg1358 to Arg2123 (Fig. 1B). In contrast, CdiA\textsuperscript{EC93} fragments containing the FHA-2 repeat region and the pretoxin-VENN domain did not interact stably with BamA\textsuperscript{Eco} (Fig. 1A and B). To ascertain binding specificity, we also tested for stable interactions with BamA\textsuperscript{ECL} from *E. cloacae*. Though the two BamA proteins share 94.8% sequence identity, BamA\textsuperscript{ECL} did not copurify with any of the His\textsubscript{6}-tagged CdiA\textsuperscript{EC93} fragments (Fig. 1B). Thus, the in vitro binding specificity conforms to prior work showing that BamA\textsuperscript{ECL} is not recognized as a receptor by CdiA\textsuperscript{EC93} (28). These data strongly suggest that the BamA\textsuperscript{Eco}-binding domain resides between residues Arg1358 and Phe1646 of CdiA\textsuperscript{EC93}.
CdiA receptor-binding domains are modular. The putative receptor-binding region of CdiAEC93 (from Ser1379 to Tyr1636) shares only ~24% sequence identity with the corresponding region of CdiAEC536 (Gln1377 to Trp1668) from uropathogenic E. coli 536 (Fig. 2; see also Fig. S1 in the supplemental material). Therefore, we reasoned that sequence divergence over this region could determine receptor specificity. To test this hypothesis, we replaced residues Ser1347 to Tyr1636 of CdiAEC93 with Ala1345 to Trp1668 from CdiAEC536 (Fig. 2) and determined the receptor-binding specificity of the resulting chimera using a flow cytometry-based cell-cell adhesion assay (40). In this assay, CdiA-expressing inhibitor cells are labeled with green fluorescent protein (GFP) and mixed at a 5:1 ratio with DsRed-labeled target bacteria. After the populations are allowed to interact, the cell suspension is analyzed by flow cytometry for events that exhibit both GFP and DsRed fluorescence (Fig. 3A), which are quantified as inhibitor-target cell aggregates. Control experiments show that ~5% of target bacteria adhere nonspecifically to mock (CDI−) inhibitor cells (Fig. 3A and B). In contrast, cells that express CdiA bind to target bacteria in a receptor-dependent manner. CdiAEC93-expressing cells bound ~80% of bamAEC93-target bacteria but failed to aggregate with bamAEC536 targets (Fig. 3A and C). Similarly, inhibitor cells that express CdiAEC536 bind a substantial fraction of ompCEC536 target bacteria but exhibited only background adhesion with ΔompC targets (Fig. 3A and D). As predicted, chimeric CdiAEC93 containing the putative receptor-binding region from CdiAEC536 recognized target cells in an ompC-dependent manner (Fig. 3A and E). Moreover, because this chimera was expressed at approximately the same level as CdiAEC536 (Fig. S2), the grafted domain appears to bind OmpF-OmpC with the same avidity as in its native context. These data, together with the in vitro binding results, indicate that the central regions of CdiAEC93 and CdiAEC536 are responsible for receptor recognition.

The CVR is required for OmpC-dependent toxin delivery. Though the receptor-binding region chimera supported robust OmpC-dependent adhesion, this effector protein did not inhibit target cell growth in competition cocultures (Fig. 4D). In contrast, inhibitors expressing CdiAEC93 outcompeted target cells approximately 105-fold in cocultures (Fig. 4B), and cells that deploy CdiAEC536 exhibited a greater than 100-fold advantage (Fig. 4C). In each instance, CDI+ inhibitors only outcompeted target cells that express the appropriate CdiA receptor (Fig. 4B and C). Together, these results suggest that the chimera is defective for toxin delivery. We noted that the CdiAEC93 region spanning Ala1910 to Gly2205 also diverges significantly with CdiAEC536 and that CdiAEC536 contains a 69-residue insertion in this region (Fig. 2 and Fig. S1). Because this region is conserved between CdiAEC536 homologues that bind OmpC-OmpF, we refer to this sequence as the “covarying region” (CVR). We explored the function of this
FIG 3 Cell-cell adhesion. (A) Flow cytometric analysis of CDI-dependent cell-cell adhesion. GFP-labeled inhibitor cells were mixed at a 5:1 ratio with DsRed-labeled target bacteria, and the cell suspension was (Continued on next page)
sequence by replacing CdiAEC93 residues Ser1347 to Gly2205 with the receptor-binding and covarying regions of CdiAEC536 (Ala1345 to Gly2310) (Fig. 2). The resulting chimera was expressed stably (Fig. S2) and supported ompC-dependent cell-cell adhesion comparably to wild-type CdiAEC536 (Fig. 3A and G). Moreover, CdiAEC93 carrying the

**FIG 3** Legend (Continued)

analyzed by flow cytometry for dual green/red fluorescent events. CdiA protein identity is indicated schematically in the left margin, and target cell genetic backgrounds are indicated along the top. (B to G) CdiA-dependent cell-cell adhesion was quantified for each effector protein: mock CDI− (A), CdiAEC93 (B), CdiAEC536 (C), CdiAEC93 with the receptor-binding region (RBR) from CdiAEC536 (D), CdiAEC93 with the covarying region (CVR) from CdiAEC536 (E), and CdiAEC93 with both RBR and CVR from CdiAEC536 (F). Viable inhibitor and target cells were enumerated as CFU milliliter−1, and the competitive index was calculated as final inhibitor-to-target cell ratio divided by the initial ratio for each coculture. Competitive indices are reported as averages ± standard errors for two independent experiments. UPEC, uropathogenic E. coli.
heterologous receptor-binding and covarying regions also inhibited target bacteria in an ompC-dependent manner (Fig. 4F). In contrast, another CdiAEC93 chimera containing only the covarying region from CdiAEC536 (Pro1669 to Gly2310) retained BamA-binding specificity (Fig. 3A and F) and inhibited target cells in a bamAECo-dependent manner (Fig. 4E). Collectively, these data suggest that CdiAEC536 residues Pro1669 to Gly2310 may be important for toxin delivery through the OmpC-OmpF receptor pathway.

**Identification of the receptor for CdiASTECO31.** We surveyed all identifiable *E. coli* CdiA effectors in the NCBI protein database and found that they segregate into at least four classes based on receptor-binding region sequences. CdiAEC93 defines the BamA-binding class I effectors, and CdiAEC536 is the paragon for class II effectors that recognize OmpC-OmpF. Class III CdiA proteins are closely related to class I and II over the two-partner secretion (TPS) transport domain and filamentous hemagglutinin (FHA)-peptide repeat regions but diverge significantly at the receptor-binding region (Fig. 5; see also Fig. S1). Class IV CdiA proteins appear to be more distantly related and share little sequence identity with the other three classes over the FHA-1 peptide repeat region (Fig. S3). To determine whether class III CdiA recognizes a unique receptor, we cloned the *cdiBAI* gene cluster from *E. coli* STEC_E031 and tested its activity in growth competitions. Class III CdiASTECO31 (NCBI reference sequence WP_001385946.1) carries a C-terminal EndoU RNase toxin domain, and cells deploying this effector outcompeted target bacteria after 4 hours of coculture (Fig. 6A). Having established growth inhibition activity, we used a genetic approach to identify the receptor for CdiASTECO31. We subjected target cells to mariner transposon mutagenesis and selected for CDISTECO31-resistant (CDIr) mutants. We isolated 12 CDiIr mutants from three independently prepared mutant pools and identified transposon insertion sites. Four of the CDiIr mutants carried independent insertions in the *ptsG* gene, which encodes the fused IIB and IIC components of the glucose phosphotransferase system (PTS). We previously reported that *ptsG* null alleles also confer resistance to Cdi toxins from *E. coli* 3006 and NC101 (14). CdiA-CTSTECO31, CdiA-CT3006, and CdiA-CTNC101 all share the same “translocation” domain, which is thought to bind PtsG and mediate toxin transport from the periplasm into the target cell cytosol (14). The remaining eight CDiIr mutants were disrupted in *tsx* (Fig. 6A), which encodes an outer membrane nucleoside transporter. To confirm the role of Tsx in CDI STECO31, we tested target cells carrying a nonpolar Δtsx mutation and found that they were resistant to growth inhibition (Fig. 6A). Moreover, complementation of Δtsx target cells with plasmid-borne *tsx* restored sensitivity to growth inhibition (Fig. 6A). Finally, we used flow cytometry to demonstrate that Tsx is required for cell-cell adhesion between target bacteria and CdiASTECO31-expressing inhibitor cells (Fig. 6B). Together, these results show that the distinct receptor-binding region of class III CdiASTECO31 recognizes Tsx.
DISCUSSION

The results presented here show that CdiA effectors from E. coli strains bind target bacteria using at least three cell surface receptors. Receptor specificity for the different CdiA classes is determined by an unannotated stretch of ~300 residues located between the FHA-1 and FHA-2 peptide repeat regions (see Tables S1, S2, S3, and S4 in the supplemental material). CdiAEC93 is the paragon for class I effectors, which bind specifically to BamA from E. coli (28). Class II effectors recognize heterotrimeric OmpC-OmpF osmoporins (31), and here, we show that class III effectors bind the outer membrane nucleoside transporter Tsx. The receptor-binding regions of class I, II, and III effectors share 24 to 27% pairwise sequence identity, and each domain contains a conserved central FHA-1 element (Fig. S1). These observations suggest that the classes diverged from a common ancestral sequence and that the three receptor-binding domains adopt similar conformations. The domains also exhibit some mutational drift within each class, though it is not clear whether these minor sequence variations influence receptor specificity. Class I effectors carry nine different receptor-binding

FIG 6 CdiA^{STECO31} uses Tsx as a receptor to bind target bacteria. (A) E. coli Δtsx mutants are resistant to CdiA^{STECO31}-mediated growth inhibition. Eight independent transposon insertions within tsx were identified in selections for CDI-resistant mutants. Inhibitor cells (CDI^{STECO31} or CDI^{−} mock) were cocultured with tsx^{+} or Δtsx target bacteria in shaking broth for 4 hours as described in Materials and Methods. Viable inhibitor and target cells were enumerated as CFU, and the competitive index was calculated as the final inhibitor-to-target cell ratio divided by the initial ratio. Competitive indices are presented as averages ± standard errors. (B) Tsx is required for CdiA^{STECO31}-dependent cell-cell adhesion. The fraction of red fluorescent target bacteria bound to green fluorescent inhibitor cells was quantified for two independent experiments. Data are presented as averages ± standard errors.
sequences that arise from combinations of 17 missense changes (Table S1). Class II receptor-binding regions are less polymorphic with only nine missense variations (Table S2), and there are 14 distinct class III receptor-binding sequences (Table S3). Class IV E. coli CdiA effectors have diverged from the other three classes but still share the same overall domain structure (Fig. S3). Given the similarities in effector architecture, we predict that the receptor-binding domain of class IV effectors also resides between the FHA-1 and FHA-2 regions. Notably, class IV cdi gene clusters also encode a predicted HlyC-type lysyl acyltransferase (14). The functional significance of this acyltransferase has not yet been explored, but it presumably modifies CdiA and/or CdiB to promote cell adhesion and growth inhibition activities. Additionally, the database contains three CdiA proteins from E. coli isolates SWW33 (WP_001764992.1), upec-172 (WP_052432358.1), and 696_ECOL (WP_049080366.1) that do not fit into the four major classes. These proteins are most closely related to class IV CdiA but have longer FHA-1 repeat regions and diverge over the central (putative) receptor-binding region (Fig. S4). Moreover, the three effectors are encoded by cdi gene clusters that lack the characteristic hlyC acyltransferase of class IV loci. These observations suggest that these three CdiA proteins constitute a fifth effector class that probably recognizes yet another cell surface receptor.

Sequence alignments of class I, II, and III CdiA effectors predict that their receptor-binding domains are interchangeable. This hypothesis is supported by data showing that chimeric CdiAEC93 carrying a class II receptor-binding region is endowed with OmpC-binding activity. Though this chimera appears to bind OmpC-OmpF with the same avidity as wild-type CdiAEC536, it does not inhibit target bacteria, indicating that toxin delivery function is compromised. Growth inhibition activity is restored when the covarying region is grafted together with the receptor-binding domain. The latter result suggests that the covarying region of CdiAEC536 is critical for toxin delivery through the OmpC pathway. However, chimeric CdiAEC93 carrying the covarying region from CdiAEC536 inhibits target bacteria in a BamA/Eco-dependent manner, showing that this sequence is not solely dedicated to the OmpC-OmpF pathway. In the context of domain modularity, these observations raise the possibility that specific toxin families may be excluded from certain receptor-mediated delivery pathways. Surveys of CdiA proteins show that some toxin families tend to be paired with specific receptor-binding domains. For example, Ntox28 RNase domains are found only on class II effectors in E. coli (Table S2). However, Ntox28 toxins are very effective at killing target bacteria when experimentally grafted onto class I CdiAEC93 (13, 14, 41). Thus, the apparent bias in naturally occurring effectors does not necessarily reflect domain incompatibility. In fact, we have shown that CdiAEC93 can deploy over a dozen heterologous toxin domains (9, 13, 14, 19–21, 41, 42), even though many of the grafted CdiA-CT sequences are not found on class I effectors. Moreover, the database shows significant combinatorial flexibility for some toxin families. For example, endonuclease NS_2 toxin domains are associated with each of the four major effector classes in E. coli (Tables S1 to S4). Comprehensive analysis of E. coli CdiA proteins reveals that each effector class is associated with multiple different toxin families (Tables S1 to S4). Taken together, these observations suggest that new CdiA effectors are assembled through genetic recombination to exchange receptor-binding domains and toxic payloads.

The location of the receptor-binding region has implications for the structure and presentation of CdiA on the inhibitor cell surface. CdiA effectors are thought to be structurally similar to the FhaB adhesins of Bordetella species because the protein families share related domain architectures (3, 12). FhaB is synthesized initially as a 370-kDa precursor, from which the C-terminal “prodomain” is removed to yield a mature filamentous hemagglutinin (FHA) fragment of ~220 kDa (43, 44). FHA is monomeric and extends about 50 nm in length (45). The central shaft of FHA corresponds to the FHA-1 peptide repeat region, which is predicted to form a right-handed β-helix with a 4.8-Å pitch per 20-residue repeat (45, 46). According to this model, the FHA-1 repeat region of CdiAEC93 should form a filament 30 to 35 nm in length. It is less clear that the CdiA FHA-2 repeat region is homologous to the FhaB prodomain. Though
the prodomain contains a short FHA-2 region (Phe2927 to Gly3086), CdiA and FhaB share little sequence identity over this region. Moreover, FhaB lacks a C-terminal toxin domain and does not mediate interbacterial competition (47). Nevertheless, FhaB and CdiA are processed in a similar manner, with immunoblot analysis revealing a stable N-terminal CdiA fragment of ~230 kDa (see Fig. S2). This processed fragment almost certainly retains adhesin activity, because truncated CdiAEC93 lacking the FHA-2 and CdiA-CT regions is still exported to the cell surface and mediates BamAEC93-dependent adhesion (38). These observations raise questions about the location of the FHA-2/CdiA-CT regions relative to the cell surface. Current models assume that the CdiA C terminus projects away from the inhibitor cell to facilitate toxin transfer to target bacteria. However, we propose that the receptor-binding domain should be positioned at the distal tip of the filament (Fig. 7), where it can easily interact with target bacteria. Studies on FhaB biogenesis from the Cotter lab provide further support for the topological model presented in Fig. 7 (48). Their work indicates that the C-terminal prodomain is retained in the periplasm during FhaB export and processing. Thus, both N and C termini of FhaB remain within an intracellular compartment while the FHA-1 region assembles into a β-helix on the cell surface (48). It is not clear how the FhaB chain is exported as a tethered loop; however, their work predicts that the CdiA receptor-binding domain should be located at the distal tip of the FHA-1 repeat filament. Further, the model suggests that the FHA-2/CdiA-CT region is sequestered within the inhibitor cell periplasm prior to receptor recognition (Fig. 7). The latter prediction is consistent with unpublished data from our laboratories showing that the C-terminal region is protected from extracellular protease, whereas the N-terminal TPS domain and FHA-1 region are rapidly degraded by this treatment. If this model is correct, then receptor recognition must induce an extraordinary change in CdiA conformation to transfer the CdiA-CT toxin domain from the inhibitor cell periplasm into the target bacterium.

**MATERIALS AND METHODS**

**Bacteria, growth conditions, and competition cocultures.** Bacteria used in this study are listed in Table 1. All strains were grown at 37°C in lysogeny broth (LB) or on LB agar unless otherwise indicated. Media were supplemented with antibiotics at the indicated concentrations: ampicillin (Amp), 150 μg ml⁻¹; chloramphenicol (Cm), 33 μg ml⁻¹; kanamycin (Kan), 50 μg ml⁻¹; rifampin (Rif), 200 μg ml⁻¹; and tetracycline (Tet), 15 μg ml⁻¹. The ΔompC:kan allele from the Keio collection (49) was amplified using

![FIG 7 Topological model for CdiA. The constituent domains of cell surface CdiA are labeled and color coded as in Fig. 1. CdiB is represented as a barrel in the outer membrane (OM). The proposed model is based on unpublished protease protection data and the work of Noël et al. (48).](image-url)
oligonucleotides ompC(UPECswap)-Sac (5'-TTT GAG CTC ACT AGT GGT GAC AGC GTG TTA) and cloned in the same manner. The resulting PCR products were digested with SpeI/XhoI and ligated to plasmids pET21b and pCH450.

Table 2

| Strain | Description | Source or reference |
|--------|-------------|---------------------|
| EP1100 | F- mcrA Δmr-hsdRM5-mcrBC ΔpadXZ ΔpadXZ | Epicentre |
| MFDpir | MG1655 RR4-2-TcΔ:[ΔMul1::lacZ ΔpadXZ ΔpadXZ-zeocin] | 55 |
| F | W3110 ΔcI857 Δ(cro-bioA) | 50 |
| DL4295 | E. coli MC4100 that expresses GFP-mut3 | 16 |
| CH2016 | X90 (DE3) ΔslyD::kan Rif R | 56 |
| CH175 | EP1100 Δwzb Strr | 19 |
| CH8119 | DH5α pir | Biomedal s.l. (Spain) |
| CH9597 | EP1100 ΔbamA::cat pZS21amp-bamA'Cm' Amp' | 28 |
| ZR343 | EP1100 ΔbamA::cat ΔompC pZS21-bamA'Cm' Kan' | This study |
| ZR344 | EP1100 ΔbamA::cat ΔompC pZS21-bamA'Cm' Kan' | This study |
| ZR345 | EP1100 ΔbamA::cat ΔompC pZS21-bamA'Cm' Kan' | This study |
| ZR346 | EP1100 ΔbamA::cat ΔompC pZS21-bamA'Cm' Kan' | This study |
| ZR372 | EP1100 Δwzb Δtsx::kan Strr Kan' | This study |
| ZR376 | EP1100 Δwzb Δtsx Strr | This study |
| ZR412 | EP1100 ΔbamA::cat ΔompC::kan pZS21amp-bamA'Cm' Kan' Amp' | This study |
| ZR413 | EP1100 ΔbamA::cat ΔompC::kan pZS21amp-bamA'Cm' Kan' Amp' | This study |

*Abbreviations: Ampr, ampicillin resistant; Apr, apramycin resistant; Cmr, chloramphenicol resistant; Ermr, erythromycin resistant; Kanr, kanamycin resistant; Rifr, rifampin resistant; Strr, streptomycin resistant; Zeor, zeocin resistant.*
TABLE 2 Plasmids

| Plasmid          | Descriptiona                  | Source or reference |
|------------------|-------------------------------|---------------------|
| pWEB-TNC         | Cosmid cloning vector; Amp’ Cm’ | Epicentre           |
| pCP20            | Heat-inducible expression of FLP recombinase; Cm’ Amp’ | 57                  |
| pSC189           | Mobilizable plasmid with R6Ky replication origin; carries the mariner transposon containing kanamycin resistance cassette; Amp’ Kan’ | 58                  |
| pRE118           | Vector plasmid for allelic exchange; Kan’ | 52                  |
| pSIM5            | Heat-inducible expression of the phage λ Red recombinase proteins; Cm’ Amp’ | 59                  |
| pSIM6            | Heat-inducible expression of the phage λ Red recombinase proteins; Cm’ Amp’ | 59                  |
| pDisRedExpress2  | Constitutive expression of DisRed; Amp’ | Clontech            |
| pZS21-bamA+      | pZS21 derivative that expresses bamAEC536; Kan’ | 60                  |
| pZS21-bamA-ECL   | Expresses bamAEC536 from Enterobacter cloacae ATCC 13047; Kan’ | 28                  |
| pZS21amp-bamA+   | pZS21amp derivative that expresses E. coli bamA; Amp’ | 24                  |
| pDA860Δ1-39      | Constitutively expresses the cdIAEC536 gene cluster; Amp’ Cm’ | 3                   |
| pDAL7720         | pDA860Δ1-39 derivative that expresses chimeric CdIAEC536 with residues Ser1347 to Tyr1636 replaced with Ala1345 to Trp1668 from CdIAEC536; Amp’ Cm’ | This study          |
| pDAL7912         | pDA860Δ1-39 derivative that expresses chimeric CdIAEC536 with residues Ser1347 to Gly2205 replaced with residues Ala1345 to Gly2310 from CdIAEC536; Amp’ Cm’ | This study          |
| pDAL7936         | pDA860Δ1-39 derivative that expresses chimeric CdIAEC536 with residues Pro1637 to Gly2508 replaced with residues Pro1669 to Gly2310 from CdIAEC536; Amp’ Cm’ | This study          |
| pCH450           | pACYC184 derivative with E. coli araBAD promoter for arabinos-inducible expression; Tet’ | 51                  |
| pCH7277          | pSH21::araA, contains a Spel site to generate in-frame N-terminal His6 fusion proteins; Amp’ | 61                  |
| pCH8619          | pSH21::cdiA(Q2681–A2909); Amp’ | This study          |
| pCH9216          | pSH21::(Asl)bamAEC536; overproduces E. coli BamA lacking the signal sequence peptide; Amp’ | This study          |
| pCH9231          | pSH21::(Asl)bamAEC536; overproduces E. cloacae BamA lacking the signal sequence peptide; Amp’ | This study          |
| pCH9674          | pShIM6 derivative in which the phage λ red genes have been replaced with E. coli recA; Amp’ | This study          |
| pCH9718          | pSH21::cdiA(R1358–R1646); Amp’ | This study          |
| pCH10235         | pSH21::cdiA(R1358–R2123); Amp’ | This study          |
| pCH10316         | pSH21::cdiA(N2556–V2727); Amp’ | This study          |
| pCH10319         | pSH21::cdiA(G1953–N2556); Amp’ | This study          |
| pCH12352         | pCH450::cdiBAEC536; Tet’ | This study          |
| pCH13602         | pCH450::cdiBA-TEC531; Tet’ | This study          |
| pCH13603         | pCH450::txs; Tet’ | This study          |
| pCH13604         | pET21b::cdiBA-TEC531; Amp’ | This study          |

aAbbreviations: Amp’, ampicillin resistant; Spc’, spectinomycin resistant; Kan’, kanamycin resistant; Tmp’, trimethoprim resistant; Tet’, tetracycline resistant.

to plasmid pCH450 using NotI and Xhol restriction sites. The E. coli recA gene was amplified with recA-BglII (5′-TGA AGT TCT TGC GAC ACC TAA TCT TAC) and recA-Xma (5′-GGC ACC CGG GTG TAT CAA ACA AGA CGC) and ligated to BglII/XmaI-digested plasmid pSIM6 to generate pCH9674.

Plasmid pDAL7912 was constructed by introducing Kan resistance and counterselectable sacB genes into pDAL660Δ1-39. The sacB and kan genes were amplified from plasmid pRE118 (52) with primers EC93-1953-ssaC-for (5′-GGC CCT TTC TGC CGG T CCC GTC GTA TGC TGG AAA TCC) and EC93-1972-sacB-rev (5′-CCG GTA TCA GTG ATG ACT GGC CCC TGC CAT) and E. coli recA

Chimeric cdIAEC536 genes were constructed by Red-mediated recombination and suroce counterselection. The receptor-binding region of cdIAEC536 (encoding residues Ala1345 to Trp1668) was replaced with UPEC1345A (5′-GGC CCT TTC TGC CGG T CCC GTC GTA TGC TGG AAA TCC) and UPEC1668W (5′-TGT TAC CGG ATG CAA GGC TGG GTG TAT CAA ACA AGA CGC) and ligated to BglII/XmaI-digested plasmid pSIM6 to generate pCH9674.

Homology regions from cdIAEC536 were amplified with EC93-Chimera-for/ UPEC1345-REV (5′-TGT TAC CGG ATG CAA GGC TGG GTG TAT CAA ACA AGA CGC) and ligated to pSIM6 digested with BglII/XmaI to generate pCH9674.
recombination, and chimeric recombinants were selected on no-salt LB agar supplemented with Amp and 5% sucrose. The identities of all plasmid constructs were identified by DNA sequence analysis.

**Protein purification.** BamA<sup>EcO</sup> and BamA<sup>EcL</sup> were overproduced in *E. coli* CH2160 grown at 37°C in LB medium supplemented with Amp and 1.5 mM IPTG (isopropyl-β-D-thiogalactoside). Though the plasmid constructs encode N-terminal His<sub>6</sub> epitopes, we found that these tags were removed, precluding the use of Ni<sup>2+</sup>-affinity chromatography. Therefore, we isolated BamA proteins from insoluble inclusion bodies. Bacteria were harvested by centrifugation, and the cell pellets were suspended in 5 ml of BugBuster reagent and incubated on a rotisserie at room temperature for 20 min. Next, cells were harvested by centrifugation at 6,000 × g for 10 min, and the supernatant was decanted to remove soluble proteins. Cells were then resuspended in 5 ml of BugBuster reagent and broken by three passages through a French press. Lysates were diluted with 25 ml deionized water and vortexed vigorously. Inclusion bodies were collected by centrifugation at 15,000 × g for 20 min and washed three times with 5 ml of 0.1× BugBuster solution. Isolated inclusion bodies were dissolved in 0.5 ml of urea lysis buffer (8 M urea, 10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 20 mM imidazole, 0.05% Triton X-100). Residual His<sub>6</sub>-tagged BamA was removed by overnight incubation with urea lysis buffer-equilibrated Ni<sup>2+</sup>-nitrilotriacetic acid (NTA) agarose resin. Unbound protein was collected by centrifugation through an 0.45-μm nylon Costar Spin-X column and diluted into 10 mM Tris-HCl (pH 8.0)-0.5% Triton X-100. Refolding reaction mixtures were incubated on a rotisserie for 3 days at ambient temperature, followed by 3 weeks at 4°C. More than 95% of BamA was refolded as determined by heat-modifiable gel mobility as previously described (33).

His<sub>6</sub>-tagged CdiA<sup>EcS</sup> fragments were overproduced in *E. coli* CH2160 grown at 37°C in LB medium supplemented with Amp and 1.5 mM IPTG. Bacteria were harvested by centrifugation, and the cell pellets were frozen at −80°C and then resuspended directly in 15 ml urea lysis buffer. Cells were disrupted by two freeze-thaw cycles. The debris was removed through two rounds of centrifugation at 15,000 × g for 20 min. Ni<sup>2+</sup>-NTA agarose resin was equilibrated in urea lysis buffer and added to each clarified lysate, and the mixtures were incubated on a rotisserie for 90 min at ambient temperature. The resin was collected by centrifugation at 3,000 × g for 30 s, resuspended in 5 ml urea lysis buffer, and transferred to a fresh tube. After three washes with 5 ml urea lysis buffer, resin-bound His<sub>6</sub>-CdiA<sup>EcS</sup> fragments were refolded with three washes of 5 ml native lysis buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 30 mM imidazole, 0.05% Triton X-100). After the final wash, resins were resuspended in 250 μl of binding buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 30 mM imidazole, 0.5% Triton X-100) for use in BamA-binding experiments.

**BamA-binding assays.** Refolded BamA<sup>EcO</sup> and BamA<sup>EcL</sup> were diluted to 10 μM in 1 ml of binding buffer. Ni<sup>2+</sup>-NTA agarose resin containing His<sub>6</sub>-CdiA<sup>EcS</sup> fragments was added to the BamA solutions, and the mixtures were incubated on a rotisserie for 90 min at 4°C. Resins were collected by centrifugation at 3,000 × g for 30 s, resuspended in 1 ml of binding buffer, and transferred to a fresh tube. The resins were washed three times with binding buffer and then eluted with binding buffer supplemented with 25 mM EDTA. Eluted proteins were run on SDS-10% polyacrylamide gels and blotted onto nitrocellulose for immunoblot analysis using polyclonal antibodies raised against BamA<sup>EcO</sup>. Blots were visualized using IRDye 680 (Li-Cor)-labeled anti-rabbit secondary antibodies and an Odyssey infrared imager as described previously (54).

**Cell-cell adhesion assays.** BamA<sup>EcO</sup>-binding studies were conducted using *E. coli* strain DL4259, which expresses gfp-mut3 from the papA promoter (19). *E. coli* DL4259 cells were transformed with pDL6606l1-39 (CdiA<sup>EcS</sup>), pCH12352 (CdiA<sup>EcL</sup>), pDL7770 (CdiA<sup>EcL</sup>,RBB), pDAL7718 (CdiA<sup>EcS</sup>,RBB), pDAL7936 (CdiA<sup>EcL</sup>,CdiA<sup>EcS</sup>), pCH13604 (CDI<sup>EcS</sup>), or pCH13604 (CDI<sup>EcL</sup>,RBB<sup>EcS</sup>), and the resulting strains were grown at 37°C in LB medium (supplemented with Amp or Tet) until the cells developed fluorescence. The expression of each CdiA effector was assessed by immunoblot analysis of total urea-soluble protein using polyclonal antibodies raised against BamA<sup>EcO</sup>. Bacteria were fluorescently labeled with DsRed using plasmid pDsRedExpress2. For Ttx-dependent adhesion experiments, the target cells were grown overnight in LB supplemented with Tet, Amp, 0.4% arabinose, and 1 mM IPTG to induce expression of Ttx and DsRed prior to mixing with inhibitors. GFP-labeled inhibitor cells were mixed at a 5:1 ratio with DsRed-labeled target bacteria at a final OD<sub>600</sub> of 0.2. Cell suspensions were shaken at 30°C for 20 min, diluted into 1× PBS, vortexed briefly, and then analyzed on an Accuri C6 flow cytometer using FL1 (533/30 nm, GFP) and FL2 (585/40 nm, DsRed) fluorophore filters. The fraction of target bacteria bound to inhibitor cells was calculated as the number of dual green/red fluorescent events divided by the total number of red fluorescent events.

**Transposon library construction and selection for CDI<sup>D</sup> mutants.** The *mariner* transposon was introduced into *E. coli* CH7175 cells through conjugation with *E. coli MFDpir* donor cells carrying plasmid pSC189. Donor cells were grown to mid-log phase in LB medium supplemented with Amp and 30 μM dianaminopimelic acid. Donors and recipients were mixed and incubated on LB agar for 4 h at 37°C. Cell mixtures were harvested with a sterile swab, diluted into 1× M9 medium, and plated onto Kan-supplemented LB agar to select transposon mutants. The following day, the mutant pool (~100,000 Kan<sup>R</sup> colonies) was harvested into 1 ml of 1× M9 medium. Mutant pools were co-cultured with *E. coli* EPI100 cells carrying plasmid pCH13604 in LB medium overnight at 37°C, and target bacteria were selected on Kan-supplemented LB agar. The surviving target cell colonies were harvested into 1× M9 medium and subjected to two additional cycles of coulture selection. Four CDI<sup>D</sup> clones were randomly selected from each of the three independently prepared transposon mutant pools. Transposon insertion sites were identified by rescue cloning. Chromosomal DNA from each CDI<sup>D</sup> mutant was digested with NspI overnight at 37°C. The restriction endonuclease was inactivated at 65°C for 20 min, and reaction mixtures were supplemented with ATP and T4 DNA ligase for overnight incubation at 16°C. The ligated DNA was...
electroporated into E. coli DH5α pir⁺ cells. Plasmids were isolated from resulting Kan-resistant transformants, and transposon insertion junctions were identified by DNA sequencing using primer mariner-seq (5'-CAAT GCT TGT CAT CGT CAT CC).

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at https://doi.org/10.1128/mBio.00290-17.

FIG S1, PDF file, 0.1 MB.
FIG S2, PDF file, 0.9 MB.
FIG S3, PDF file, 0.1 MB.
FIG S4, PDF file, 0.05 MB.
TABLE S1, PDF file, 0.04 MB.
TABLE S2, PDF file, 0.05 MB.
TABLE S3, PDF file, 0.02 MB.
TABLE S4, PDF file, 0.04 MB.

ACKNOWLEDGMENTS
We are grateful to Thomas Silhavy for providing antibodies to BamA. Z.C.R. was supported by grants from the Tri-Counties Blood Bank and the California Nanosystems Institute (CNSI). This work was supported by grants GM117930 (C.S.H.) from the National Institutes of Health and MCB1545720 (C.S.H. and D.A.L.) from the National Science Foundation. The funding agencies had no role in study design, data collection and interpretation, or the decision to submit this work for publication.

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