Receptor Polymorphism Restricts Contact-Dependent Growth Inhibition to Members of the Same Species

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ABSTRACT **Bacteria that express contact-dependent growth inhibition (CDI) systems outcompete siblings that lack immunity, suggesting that CDI mediates intercellular competition. To further explore the role of CDI in competition, we determined the target cell range of the CDI^{EC93} system from *Escherichia coli* EC93. The CdiA^{EC93} effector protein recognizes the widely conserved BamA protein as a receptor, yet *E. coli* EC93 does not inhibit other enterobacterial species. The predicted membrane topology of BamA indicates that three of its extracellular loops vary considerably between species, suggesting that loop heterogeneity may control CDI specificity. Consistent with this hypothesis, other enterobacteria are sensitized to CDI^{EC93} upon the expression of *E. coli bamA* and *E. coli* cells become CDI^{EC93} resistant when *bamA* is replaced with alleles from other species. Our data indicate that BamA loops 6 and 7 form the CdiA^{EC93}-binding epitope and their variation between species restricts CDI^{EC93} target cell selection. Although BamA loops 6 and 7 vary dramatically between species, these regions are identical in hundreds of *E. coli* strains, suggesting that BamA^{Ecoli} and CdiA^{EC93} play a role in self-nonself discrimination.**

IMPORTANCE **Contact-dependent growth inhibition (CDI) systems are widespread among Gram-negative bacteria, enabling them to bind to neighboring bacterial cells and deliver protein toxins that inhibit cell growth. In this study, we tested the role of CDI in interspecies competition using intestinal isolate *Escherichia coli* EC93 as an inhibitor cell model. Although *E. coli* EC93 inhibits different *E. coli* strains, other bacterial species from the intestine are completely resistant to CDI. We show that resistance is due to small variations in the CDI receptor that prevent other species from being recognized as target cells. CDI receptor interactions thus provide a mechanism by which bacteria can distinguish siblings and other close relatives (self) from more distant relatives or other species of bacteria (nonself). Our results provide a possible means by which antimicrobials could be directed to one or only a few related bacterial pathogens by using a specific receptor "zip code."**

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Bacterial contact-dependent growth inhibition (CDI) was discovered and characterized in *Escherichia coli* strain EC93. This enteric isolate uses the CdiB/CdiA two-partner secretion system to inhibit the growth of other *E. coli* strains upon direct cell-to-cell contact (1). Based on other two-partner systems, CdiB^{EC93} is localized to the outer membrane and mediates the export of CdiA^{EC93} (1, 2). CdiA^{EC93} is a hemagglutinin (HA) repeat protein that is predicted to form a long β-helical filament extending from the surface of *E. coli* EC93 cells (1, 3). CDI^{EC93} toxin activity is contained within the C-terminal 224 residues of CdiA^{EC93} (CdiA-CT^{EC93}), and this domain inhibits growth when expressed inside *E. coli* cells (4, 5). CdiA-CT^{EC93}-mediated inhibition is associated with dissipation of the proton motive force and low ATP levels (5), suggesting that the toxin forms a pore in the inner membrane of target bacteria. *E. coli* EC93 protects itself from this activity by producing a small CdiI^{EC93} immunity protein that is encoded immediately downstream of *cdiA*^{EC93} (1, 5). The precise mechanism of immunity is unknown, but CdiI^{EC93} contains two predicted transmembrane regions, suggesting that it localizes to the inner membrane, where it blocks toxin activity. CDI systems are also found in other *E. coli* isolates and a variety of alpha-, beta-, and gammaproteobacteria (4, 6). Within a given genus, CdiA proteins are typically conserved throughout much of their length but the CdiA carboxy-terminal toxin regions (CdiA-CTs) are highly variable (4, 6). Additionally, the predicted CdiI immunity proteins are also diverse, suggesting that *cdi* loci constitute a family of polymorphic toxin/immunity pairs (6, 7). This hypothesis is supported by studies showing that many CdiA-CTs have distinct nuclease activities that are specifically neutralized by their cognate CdiI proteins (4, 8, 9).

CDI toxin delivery has been studied most extensively in the CDI^{EC93} system. Genetic selections for CDI^{EC93}-resistant mutants revealed two target cell proteins that are required for growth inhibition. A transposon insertion in the *bamA* promoter region renders *E. coli* partially resistant to CDI^{EC93} because of decreased BamA expression (10). BamA is an essential outer membrane protein (OMP) that forms the core of the β-barrel assembly machine (BAM) complex (11–16). The BAM complex is required for assembly of β-barrel proteins into the outer membrane, but notably, the biogenesis function of BamA is not required for susceptibility

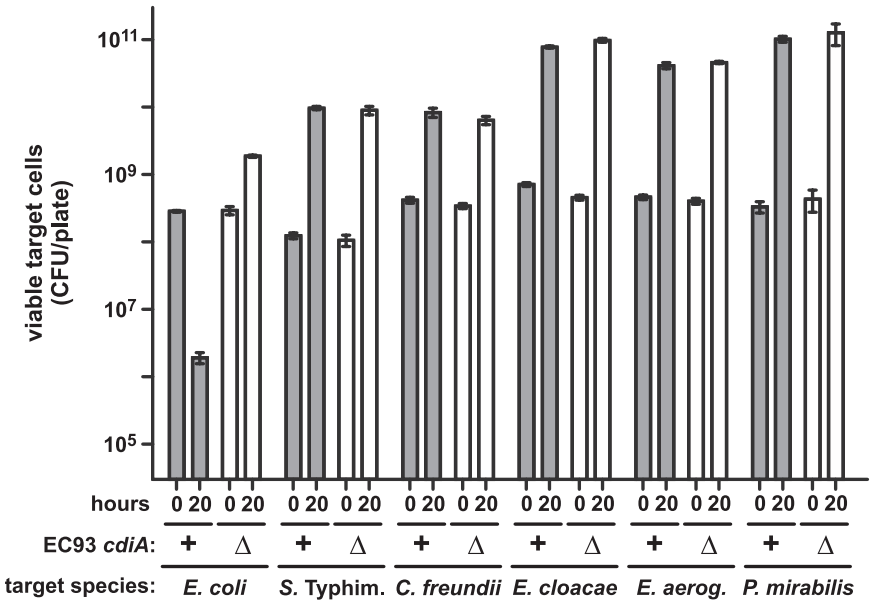


FIG 1 Enterobacteria are resistant to CDI^{EC93}. Wild-type *E. coli* EC93 (*cdiA*$^+$) or *E. coli* EC93 Δ*cdiA* mutant cells were mixed with the indicated target species at a 10:1 ratio, and the suspension were incubated on LB agar for 20 h. Cells were harvested, washed, and replated on LB agar supplemented with streptomycin to enumerate viable target cells as CFU. All competitions were conducted at least twice, and the reported values are the mean ± the standard error of the mean.

to CDI^{EC93} (10). BamA plays a critical role in cell-cell adhesion during CDI^{EC93}, and binding interactions between inhibitor and target cells are blocked by anti-BamA antibodies (10). Anti-BamA antibodies also protect target cells from CDI^{EC93}-mediated growth inhibition. These observations suggest that BamA is the receptor for CdiA^{EC93}. Additionally, *acrB* null mutations confer resistance to CDI^{EC93} (10). AcrB is a trimeric inner membrane protein that functions together with AcrA and TolC as a multidrug efflux pump (17, 18). However, *acrA* and *tolC* mutants have no resistance phenotype (10), indicating that the role of AcrB in CDI^{EC93} is distinct from its efflux function. The localization of AcrB suggests that this protein could facilitate CdiA-CT^{EC93} insertion into the membrane or perhaps stabilize the pore once assembled. Together, these studies have led to a model postulating that the CdiA^{EC93} effector exploits specific cell envelope proteins to deliver and activate its toxin domain.

E. coli EC93 inhibits a variety of *E. coli* strains (1, 5, 10), but its activity against other bacterial species has not been examined. BamA and AcrB are both well conserved among enterobacteria, raising the possibility that *E. coli* EC93 could inhibit other species. However, we find that the growth of *Salmonella enterica* serovar Typhimurium, *Citrobacter freundii*, *Enterobacter aerogenes*, *Enterobacter cloacae*, and *Proteus mirabilis* cells is not inhibited during coculture with *E. coli* EC93. BamA from these enterobacteria has 73% to 93% sequence identity with *E. coli* BamA, but sequence differences are concentrated within three of the predicted extracellular loops. These observations suggest that CdiA^{EC93} is unable to bind BamA from other species because of surface residue variability. In accord with this hypothesis, expression of *E. coli bamA* sensitizes other species to CDI^{EC93}. Furthermore, replacement of the *E. coli bamA* gene with alleles from other species renders cells resistant to inhibition. Using chimeric receptors, we localized the CdiA^{EC93}-binding region to extracellular loops 6 and 7 of *E. coli* BamA. These findings demonstrate that CDI^{EC93} is restricted at the level of target cell selection and suggest that CDI may function more broadly in kin selection.

RESULTS

Diverse enterobacteria are resistant to CDI^{EC93}. To test whether CDI^{EC93} exhibits cross-species inhibition activity, we examined the growth of *Enterobacter aerogenes* (ATCC 13048), *Enterobacter cloacae* (ATCC 13047), *Citrobacter freundii* (ATCC 8090), *Proteus mirabilis* (ATCC 7002), and *Salmonella* Typhimurium LT2 cells in cocultures with *E. coli* EC93. *E. coli* EC93 cells were suspended with target bacteria at a 10:1 ratio, and the suspension was plated onto LB agar at high density to facilitate cell-cell contact. Under these conditions, viable *E. coli* MC4100 target cell counts decreased ~150-fold after 20 h of coculture with *E. coli* EC93 (Fig. 1). This inhibition is attributable to CDI^{EC93} because *E. coli* MC4100 cell counts increased 6-fold in mock competitions with *E. coli* EC93 Δ*cdiA* mutant cells (Fig. 1). In contrast, all other target species grew to about the same cell density when cocultured with wild-type *E. coli* EC93 (CDI$^+$) or the *E. coli* EC93 Δ*cdiA* mutant (CDI$^-$) (Fig. 1). Thus, other species are resistant to CDI^{EC93}, suggesting that *E. coli* EC93 only inhibits related *E. coli* strains. We also measured *E. coli* EC93 growth during interspecies competition and found that cell counts increased 9-fold to 50-fold in all cocultures except those with *E. cloacae*, in which *E. coli* EC93 cells did not increase significantly (see Fig. S1 in the supplemental material). Because the cultures were seeded with 10-fold more *E. coli* EC93 cells, the latter data suggest that *E. cloacae* ATCC 13047 inhibits *E. coli* EC93.

***E. coli bamA* sensitizes other enterobacterial species to CDI^{EC93}.** Mutations that decrease *bamA* or *acrB* expression confer CDI^{EC93} resistance on *E. coli* cells (10). Each of the tested target species contains *bamA* and *acrB* genes that are homologous to but distinct from those of *E. coli* (see Fig. S2 and S3 in the supplemental material). Therefore, we tested whether *E. coli* alleles of *bamA* (*bamA*^{Ecoli}) and *acrB* (*acrB*^{Ecoli}) are required for susceptibility to CDI^{EC93}. We introduced plasmids (see Text S1 in the supplemental material) carrying either *bamA*^{Ecoli} (pZS21-*bamA*$^+$) or *acrB*^{Ecoli} (p*acrAB*$^+$) into *S.* Typhimurium and cultured the resulting strains with *E. coli* EC93 on LB agar. *S.* Typhimurium targets harboring pZS21-*bamA*$^+$ grew to a 5-fold lower level than cells with the empty plasmid vector (Fig. 2A). In principle, this growth inhibition could reflect toxicity from heterologous *bamA*^{Ecoli} expression. However, *S.* Typhimurium cells carrying pZS21-*bamA*$^+$ were not inhibited in cocultures with *E. coli* EC93 Δ*cdiA* mutant cells (Fig. 2A), indicating that inhibition is due to CDI^{EC93}. In contrast, *S.* Typhimurium cells carrying plasmid p*acrAB*$^+$ were not inhibited during coculture with *E. coli* EC93 (Fig. 2A). These results suggest that other species are resistant to CDI^{EC93} because they lack the BamA receptor allele from *E. coli*. We extended this analysis to *E. aerogenes* and *C. freundii* and found that plasmid

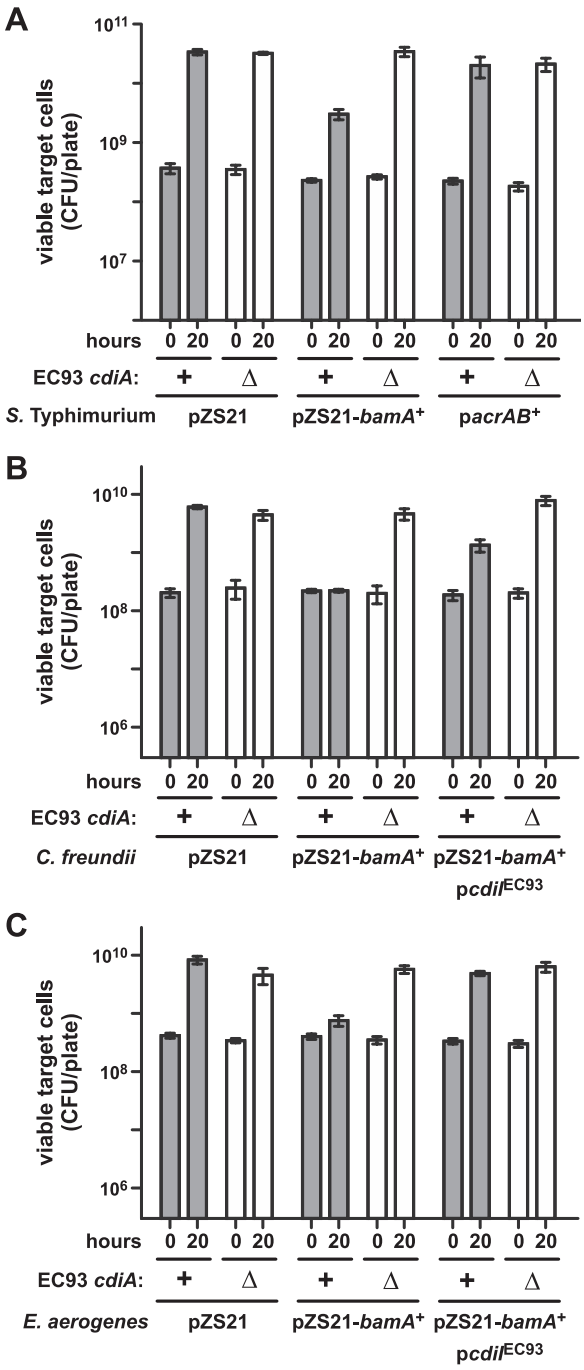


FIG 2 Expression of $bamA^{Ecoli}$ sensitizes enterobacteria to CDI^{EC93}. (A) Competitions between *E. coli* EC93 and *S.* Typhimurium target cells carrying plasmid pZS21 or plasmids that express *E. coli bamA* or *acrAB*. (B) Competitions between *E. coli* EC93 and *C. freundii* cells carrying plasmid pZS21 or pZS21-$bamA^+$. Plasmid $pcdiI^{EC93}$ corresponds to pDAL741 and constitutively expresses the *cdiI* immunity gene from *E. coli* EC93. (C) Competitions between *E. coli* EC93 and *E. aerogenes* cells carrying plasmid pZS21, pZS21-$bamA^+$, and/or $pcdiI^{EC93}$, as indicated. For all competitions, wild-type *E. coli* EC93 ($cdiA^+$) or *E. coli* EC93 Δ*cdiA* mutant cells were cocultured with target bacteria at a 10:1 ratio on LB agar for 20 h. Cells were harvested, washed, and replated on LB agar supplemented with streptomycin for enumeration of viable target cells as CFU. All competitions were conducted at least twice, and the reported values are the mean ± the standard error of the mean.

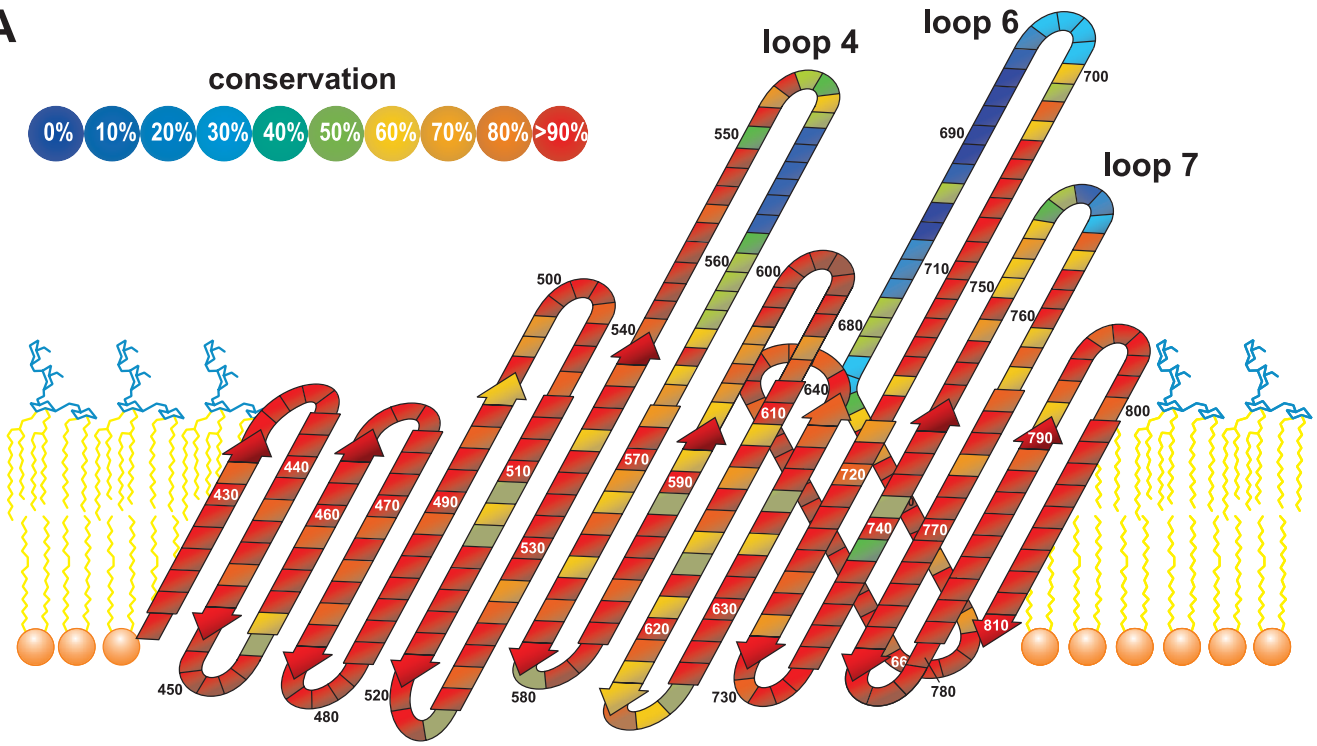
pZS21-$bamA^+$ also sensitizes these bacteria to CDI^{EC93} (Fig. 2B and C). In each instance, growth inhibition was dependent upon a functional CDI^{EC93} system and the sensitized bacteria were protected by the expression of a plasmid-borne $cdiI^{EC93}$ immunity gene (Fig. 2B and C). Thus, expression of $BamA^{Ecoli}$ in normally resistant enterobacterial species renders them susceptible to growth inhibition by *E. coli* EC93.

Heterologous *bamA* confers CDI^{EC93} resistance on *E. coli*. A previous study proposed that the extracellular regions of BamA vary between different species (19), but the structure of the BamA β-barrel domain is unknown. Therefore, we generated a topological model of BamA based on the crystal structure of the homologous FhaC protein from *Bordetella pertussis* (2). We then aligned 41 enterobacterial BamA sequences and projected the results onto the topology model as a conservation heat map (Fig. 3A). Strikingly, the regions of lowest conservation (≤30% identity) lie within loops 4, 6, and 7, which are the longest of the predicted extracellular loops (Fig. 3A). In accord with these observations, BamA proteins from *E. coli*, *S.* Typhimurium, *C. freundii*, *E. aerogenes*, *E. cloacae*, and *P. mirabilis* have very diverse loop 4, 6, and 7 regions (see Fig. S2 in the supplemental material). In general, each species has a unique set of BamA loop sequences, but *Shigella* species and an isolate of *Salmonella arizonae* have the same loops as *E. coli* strains.

The predicted BamA topology suggests that extracellular loop polymorphism restricts the *E. coli* EC93 target cell range. If so, then exchange of $bamA^{Ecoli}$ with *bamA* genes from other species should protect *E. coli* cells from CDI^{EC93}. BamA function is essential for viability (11, 14); therefore, we first asked whether such chimeric *E. coli* strains could be generated. We deleted $bamA^{Ecoli}$ in cells that carry plasmid pZS21-$bamA^+$ and then used plasmid exchange to test whether *bamA* genes from other enterobacteria support *E. coli* cell viability. *E. coli* Δ*bamA*::*cat* mutant cells harboring pZS21-$bamA^+$ were transformed with plasmid pZS21*amp* derivatives that contain the same replication origin as pZS21-$bamA^+$ but confer resistance to ampicillin. Ampicillin-resistant (Amp^r) transformants were selected and tested for kanamycin resistance (Kan^r) to determine whether plasmid pZS21-$bamA^+$ was retained. Retention of the Kan^r phenotype indicates that the introduced Amp^r plasmid is unable to complement the chromosomal $\Delta bamA^{Ecoli}$ mutation. As a proof of principle, we tested plasmid pZS21*amp*-$bamA^+$ and found that all Amp^r transformants were sensitive to kanamycin (see Table S1 in the supplemental material), indicating that plasmid pZS21-$bamA^+$ is readily displaced if the incoming plasmid provides BamA function. In contrast, cells transformed with either the empty pZS21*amp* vector or a construct encoding inactive $BamA^{Ecoli}$ (lacking the POTRA-3 domain) retained plasmid pZS21-$bamA^+$ (see Table S1). We then tested pZS21*amp* constructs carrying *bamA* from other enterobacteria and found that the genes from *S.* Typhimurium LT2, *E. cloacae* ATCC 13047, and *Dickeya dadantii* 3937 all support *E. coli* Δ*bamA* cell viability (see Table S1). However, *bamA* from *Yersinia pseudotuberculosis* YPIII is unable to complement the *E. coli* Δ*bamA* mutant (see Table S1). From these results, we conclude that many, but not all, BamA proteins from other enterobacteria are functional for the biogenesis of *E. coli* OMPs.

We next tested whether *E. coli* cells that express heterologous *bamA* are resistant to CDI^{EC93}. Target cells complemented with pZS21*amp*-$bamA^+$ were sensitive, sustaining a 3,600-fold decrease in viable cells after 4 h of coculture with *E. coli* EC93

A



B

BamA	Description
4-HA	*E. coli* Glu^{554} – Asp^{562} replaced with HA epitope
6-HA	*E. coli* His^{677} – Asp^{685} replaced with HA epitope
7-HA	*E. coli* Tyr^{754} – Ser^{755} replaced with HA epitope
Δ4	*E. coli* Pro^{556} – Ser^{564} replaced with Gly-Ser
Δ6	*E. coli* Phe^{675} – Ser^{702} replaced with Gly-Ser

FIG 3 Predicted membrane topology and conservation of the BamA β-barrel domain. (A) Predicted topology of the BamA β-barrel based on the crystal structure of *B. pertussis* FhaC (Protein Data Bank code 2QDZ). Residues are numbered according to *E. coli* BamA. The percent identity for each residue is indicated as a heat map. Predicted extracellular loops 4, 6, and 7 are indicated. (B) Description of the $BamA^{Ecoli}$ loop variants used in this study. The mutated residues are indicated, and the HA peptide epitope sequence is Val-Asp-Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala.

(Fig. 4A). In contrast, targets expressing *bamA* from *S.* Typhimurium, *E. cloacae*, or *D. dadantii* 3937 were resistant to CDI^{EC93} (Fig. 4A). However, the latter target strains were inhibited by CdiA-CT^{EC93} when the toxin was expressed inside the cells from an inducible promoter (see Fig. S4 in the supplemental material). These results argue that CdiA-CT^{EC93} is not delivered into cells that express heterologous *bamA*, presumably because CDI^{EC93} is disrupted at the level of receptor binding. Our previous work has shown that target cells form stable aggregates with inhibitor cells, provided the inhibitors overexpress the CDI^{EC93} system from a cosmid (1). Furthermore, this aggregation is proportional to $BamA^{Ecoli}$ surface expression on target cells (1), suggesting that cell-cell adhesion reflects the binding interaction between $BamA^{E.coli}$ and $CdiA^{EC93}$. Therefore, we examined adhesion between targets that express the various *bamA* alleles and inhibitor cells that overexpress CDI^{EC93} from cosmid pDAL660Δ1-39 (1). Inhibitor cells were labeled with green fluorescent protein (GFP), mixed with DsRed-labeled targets at a 5:1 ratio, and then analyzed by flow cytometry to detect aggregates with both green and red fluorescence. Control experiments showed that 83 ± 1.4% of the targets expressing $bamA^{Ecoli}$ formed aggregates with inhibitor cells (Fig. 4B). Moreover, this cell-cell adhesion is dependent upon CDI^{EC93} because mock inhibitors carrying an empty cosmid vector did not aggregate with target cells (Fig. 4B). Conversely, cells expressing *bamA* from *S.* Typhimurium, *E. cloacae*, or *D. dadantii* 3937 showed only background levels of aggregation (4 to 6%) with inhibitor cells (Fig. 4B). Together, these results strongly suggest that $CdiA^{EC93}$ binds specifically to $BamA^{Ecoli}$.

Disruption of $BamA^{Ecoli}$ extracellular loops confers resistance to CDI^{EC93}. To determine whether the predicted extracellular loops are exposed on the cell surface, we examined BamA topology. Three *bamA* constructs were generated in which nine

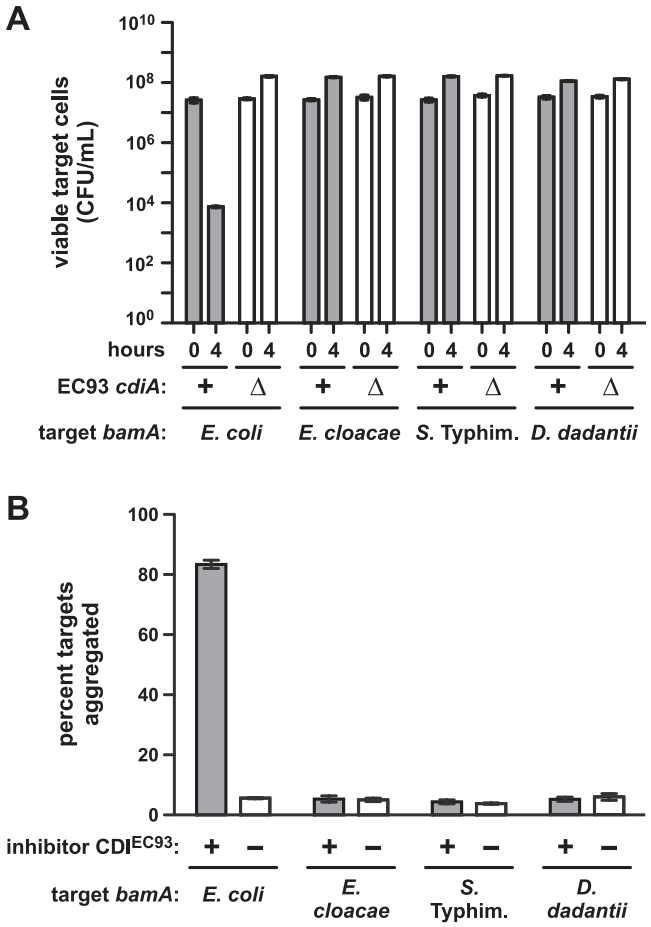


FIG 4 Heterologous *bamA* confers CDI^{EC93} resistance on *E. coli*. (A) Competitions between *E. coli* EC93 and *E. coli* Δ*bamA*::*cat* mutant cells complemented with plasmid-borne copies of *bamA* from the indicated species. For all competitions, wild-type *E. coli* EC93 (*cdiA*$^+$) or *E. coli* EC93 Δ*cdiA* mutant cells were cultured with target bacteria at a 1:1 ratio in LB broth for 4 h. Cultures were sampled at 0 and 4 h and plated onto LB agar supplemented with ampicillin to enumerate viable target cells as CFU per milliliter. (B) Adhesion between CDI^{EC93} inhibitor and target cells. GFP-labeled *E. coli* MC4100 cells carrying cosmid pDAL660Δ1-39 (CDI^{EC93} +) or pWEB::TNC (CDI^{EC93} −) were used as inhibitors and mixed in 5-fold excess with DsRed-labeled target bacteria expressing *bamA* genes from the indicated species. Cell suspensions were analyzed by flow cytometry, and the percentage of red-fluorescent target cells aggregated with green-fluorescent inhibitors was calculated. The reported values are the mean ± the standard error of the mean from two independent experiments. Typhim., Typhimurium.

residues within loops 4, 6, and 7 were replaced with the HA peptide epitope (Fig. 3B). Each of the HA epitope constructs could displace plasmid pZS21-*bamA*$^+$ from *E. coli* Δ*bamA*::*cat* cells (see Table S1 in the supplemental material), indicating that the modified BamA proteins are functional and presumably assembled correctly into the outer membrane. We then used immunofluorescence microscopy to examine the surface exposure of HA epitopes. Cells were fixed without permeabilizing the outer membrane, which allows the detection of cell surface antigens but prevents antibody access to periplasmic and cytoplasmic antigens (20). Immunostaining with anti-HA antibody detected the epitopes within loops 4 and 6 (see Fig. S5 in the supplemental material), indicating that these regions are accessible to antibody and therefore could potentially interact with CdiA^{EC93}. However, no signal from the loop 7 epitope was detected (see Fig. S5). The latter result could indicate that loop 7 is not extracellular, but it is also possible that this region is occluded by another extracellular loop. We next tested whether HA mutations provide resistance to CDI^{EC93}. Cells expressing *bamA*$_{4\text{-HA}}{}^{Ecoli}$ and *bamA*$_{6\text{-HA}}{}^{Ecoli}$ were still sensitive to CDI^{EC93}, but *bamA*$_{7\text{-HA}}{}^{Ecoli}$ provided nearly full protection against growth inhibition (Fig. 5A). This resistance presumably results from a defect in CdiA-CT^{EC93} delivery, because the strain expressing *bamA*$_{7\text{-HA}}{}^{Ecoli}$ is sensitive to CdiA-CT^{EC93} toxin produced internally (see Fig. S4).

We next generated in-frame deletions to remove loop regions that are unique to the BamA^{Ecoli} receptor (Fig. 3B). Notably, we were unable to generate the loop 7 deletion, suggesting that this loop may be required for function of the BAM complex. The *bamA*$_{\Delta 4}{}^{Ecoli}$ and *bamA*$_{\Delta 6}{}^{Ecoli}$ alleles each supported *E. coli* Δ*bamA*::*cat* cell viability (see Table S1 in the supplemental material), indicating that these loop regions are not required for OMP biogenesis. In competitions with *E. coli* EC93 cells, targets expressing *bamA*$_{\Delta 4}{}^{Ecoli}$ were slightly more resistant than cells expressing wild-type *bamA*^{Ecoli}, but *bamA*$_{\Delta 6}{}^{Ecoli}$ conferred complete resistance (Fig. 5B). The 7-HA and Δloop 6 mutations also disrupted adhesion to inhibitors that overproduce the CDI^{EC93} system, whereas target cells that express *bamA*$_{\Delta 4}{}^{Ecoli}$ still formed aggregates with inhibitors (Fig. 5C). The latter results correlate with CDI^{EC93} resistance and implicate BamA^{Ecoli} loops 6 and 7 as important determinants of target cell recognition.

BamA^{Ecoli} extracellular loops 6 and 7 are sufficient for CDI^{EC93}. The cell-cell adhesion data suggest that BamA^{Ecoli} loops 6 and 7 interact with CdiA^{EC93}. If so, then these loops should restore sensitivity to CDI^{EC93} when grafted onto BamA from another species. Chimeric *bamA*^{Ecloacae} genes were constructed in which individual loop-coding sequences were replaced with the corresponding region from *bamA*^{Ecoli} (see Fig. S6 in the supplemental material). Each of the single-loop chimeras (*bamA*$_{Ec4}{}^{Ecloacae}$, *bamA*$_{Ec6}{}^{Ecloacae}$, and *bamA*$_{Ec7}{}^{Ecloacae}$) supported *E. coli* Δ*bamA*::*cat* cell viability (see Table S1). Cells expressing these chimeras were as resistant to CDI^{EC93} as cells that express *bamA*^{Ecloacae} (Fig. 6B), indicating that no single *E. coli* loop is sufficient for target cell recognition. Similarly, cells expressing *bamA*^{Ecloacae} with *E. coli* loops 4 and 7 were also CDI^{EC93} resistant. However, cells expressing *bamA*$_{Ec6/7}{}^{Ecloacae}$, which encodes loops 6 and 7 of *E. coli*, were almost fully sensitive (Fig. 6B). The loop 6/7 chimera also supported CDI^{EC93}-dependent cell-cell adhesion, whereas the other chimeric *bamA*^{Ecloacae} alleles did not (Fig. 6C). Together, these results indicate that BamA^{Ecoli} loops 6 and 7 are sufficient for target cell selection during CDI^{EC93}.

Previous studies have shown that CdiA^{EC93} is modular and capable of delivering different CdiA-CTs from other systems (8, 20, 21). This heterologous toxin delivery is dependent upon BamA^{Ecoli} (20), suggesting that specific CdiA-CT sequences are not required for target cell selection. Therefore, we asked whether the chimeric BamA$_{Ec6/7}{}^{Ecloacae}$ receptor supports the delivery of other CdiA-CTs into target cells. We tested the previously characterized CdiA^{EC93}-CT$_{o1}{}^{EC93}$ effector protein, in which the original CdiA-CT^{EC93} membrane-pore toxin is replaced with a tRNase toxin encoded by the "orphan-1" *cdiA-CT*$_{o1}{}^{EC93}$ gene from *E. coli* EC93 (21). CdiA-CT$_{o1}{}^{EC93}$ is an anticodon nuclease (21, 22); therefore, we assayed toxin activity by Northern blotting with a probe for *E. coli* tRNA$_{ICG}{}^{Arg}$. We first examined RNA from inhibitor and target cells that had been cultured separately and detected no

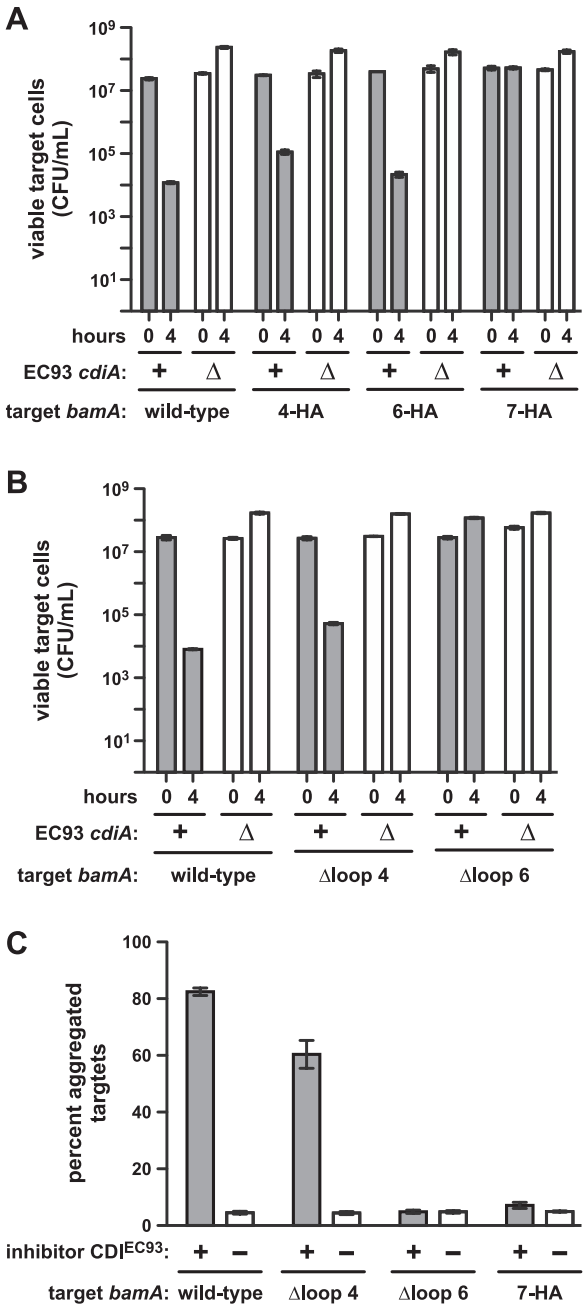


FIG 5 BamA^{Ecoli} loops 6 and 7 are required for CDI^{EC93}. (A and B) Competitions between *E. coli* EC93 and *E. coli* Δ*bamA*::*cat* targets that express the indicated *bamA*^{Ecoli} alleles. For all competitions, wild-type *E. coli* EC93 (*cdiA*$^+$) or *E. coli* EC93 Δ*cdiA* mutant cells were cultured with target bacteria at a 1:1 ratio in LB broth for 4 h. Cultures were sampled at 0 and 4 h and plated on LB agar supplemented with ampicillin to enumerate viable target cells as CFU per milliliter. (C) Adhesion between CDI^{EC93} inhibitor and target cells. GFP-labeled *E. coli* MC4100 cells carrying cosmid pDAL660Δ1-39 (CDI^{EC93} +) or pWEB::TNC (CDI^{EC93} −) were used as inhibitors and mixed in 5-fold excess with DsRed-labeled target bacteria expressing the indicated *bamA*^{Ecoli} alleles. Cell suspensions were analyzed by flow cytometry, and the percentage of red-fluorescent target cells aggregated with green-fluorescent inhibitors was calculated. The reported values are the mean ± the standard error of the mean from two independent experiments.

tRNase activity in either population (Fig. 7A). However, tRNase activity was observed when inhibitors were cocultured with target cells that express *bamA*^{Ecoli} (Fig. 7B). This nuclease activity was not detected when target cells carried a plasmid-borne copy of the cognate *cdiI*$_{o1}$^{EC93} immunity gene (Fig. 7B), verifying that the nuclease activity is due to the CdiA-CT$_{o1}$^{EC93} toxin. We next tested targets that express *bamA*^{Ecloacae} and the various *bamA* chimeras and detected tRNase activity only in cocultures with inhibitor cells that express *bamA*$_{Ec6/7}$^{Ecloacae} (Fig. 7B). These results demonstrate that chimeric BamA$_{Ec6/7}$^{Ecloacae} supports the delivery of other CDI toxins into *E. coli* cells.

DISCUSSION

The results presented here show that the CDI system from *E. coli* EC93 is ineffective against other enteric gammaproteobacterial species. Because CDI-mediated inhibition requires the translocation of protein toxins across the target cell envelope, multiple steps could potentially be disrupted to prevent toxin import into other species. However, our results indicate that cross-species inhibition is blocked primarily at the level of target cell recognition because *S.* Typhimurium, *E. aerogenes*, and *C. freundii* cells become susceptible to CDI^{EC93} upon the expression of the BamA^{Ecoli} receptor. BamA is conserved between enterobacteria, but three of the predicted extracellular loops contain regions of sequence variability. Two of these variable loops play an important role in target cell selection by *E. coli* EC93. Perturbations of BamA^{Ecoli} loops 6 and 7 prevent adhesion to inhibitor cells and protect target cells from CDI^{EC93}-mediated growth inhibition. Moreover, these loops are sufficient for cell-cell adhesion and growth inhibition when grafted onto BamA from another species. The latter observation indicates that loops 6 and 7 together form the CdiA^{EC93}-binding epitope on *E. coli* target cells. These data also demonstrate that, given the appropriate receptor, CdiA-CT^{EC93} toxin can be delivered into other species. This finding underscores previous work showing that CdiA-CTs from *Yersinia pestis* CO92 and *Dickeya dadantii* 3937 can be delivered into *E. coli* cells provided the toxins are fused onto *E. coli* CdiA proteins (4, 20). Thus, although the target cell range of a given CDI system may be restricted, CdiA-CTs appear to possess a general import activity sufficient for translocation into a variety of Gram-negative bacteria.

The clustering of sequence variation within the BamA extracellular loops is striking, but such variability is fairly common with OMPs. These surface-exposed β-barrel proteins interact directly with the environment and, as a consequence, are under selective pressure to diversify (23). OMPs are recognized as antigens by adaptive immune systems and are often exploited as receptors by bacteriophage and bacteriocins. These forces can drive the diversification of a given OMP within an individual bacterial species. For example, extracellular loops 4, 5, and 7 of OmpC vary considerably between *E. coli* strains, even those isolates that have similar niches and pathogenic lifestyles (24). In contrast, BamA is almost completely conserved across the hundreds of *E. coli* strains that have been sequenced to date. Although the extracellular loops of BamA^{Ecoli} are identical in nearly all strains, these regions are subjected to the same selective forces as other OMPs. Shiga-toxin-producing STX phage use BamA as a receptor to infect *E. coli* cells (19), and the *E. coli* EC93 CDI system itself represents a force that could conceivably shape BamA^{Ecoli} evolution. Therefore, BamA conservation between *E. coli* strains implies that the extracellular loops may be critical for function. Indeed, portions of BamA loop 6 are highly conserved between species and these residues are important for OMP biogenesis (25, 26). However, our data show that

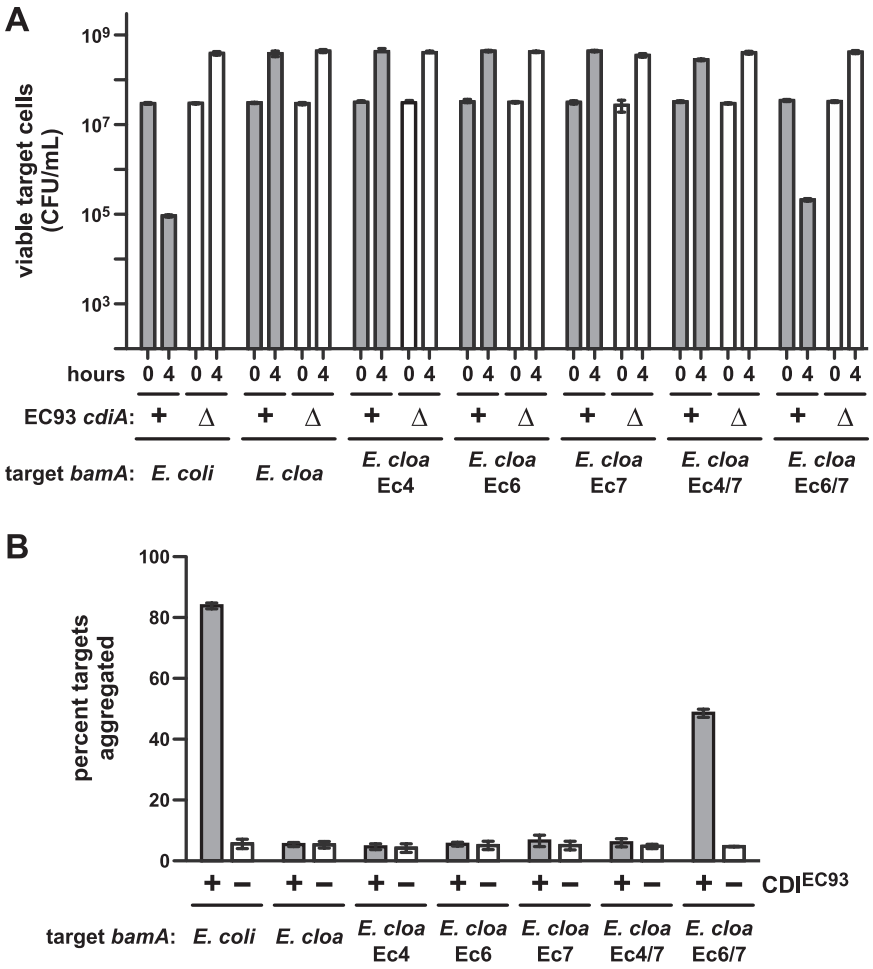


FIG 6 BamA^{Ecoli} loops 6 and 7 are sufficient for CDI^{EC93}. (A) Competitions between *E. coli* EC93 and *E. coli* Δ*bamA*::*cat* targets that express the indicated *bamA* alleles. For all competitions, wild-type *E. coli* EC93 (*cdiA*$^+$) or *E. coli* EC93 Δ*cdiA* mutant cells were cultured with target bacteria at a 1:1 ratio in LB broth for 4 h. Cultures were sampled at 0 and 4 h and plated on LB agar supplemented with ampicillin to enumerate viable target cells as CFU per milliliter. (B) Adhesion between CDI^{EC93} inhibitor and target cells. GFP-labeled *E. coli* MC4100 cells carrying cosmid pDAL660Δ1-39 (CDI^{EC93} +) or pWEB::TNC (CDI^{EC93} −) were used as inhibitors and mixed in 5-fold excess with DsRed-labeled target bacteria expressing the indicated *bamA* alleles. Cell suspensions were analyzed by flow cytometry, and the percentage of red-fluorescent target cells aggregated with green-fluorescent inhibitors was calculated. The reported values are the mean ± the standard error of the mean from two independent experiments.

regions within each loop can be mutated extensively and even deleted without loss of viability. In fact, heterologous BamA proteins with alterations in all three loops still support *E. coli* growth at nearly wild-type rates under laboratory conditions. Therefore, the *E. coli*-specific extracellular loops are clearly not required for OMP biogenesis, suggesting that other forces act to prevent BamA^{Ecoli} diversification. Though the significance of BamA^{Ecoli} conservation is unclear, *E. coli* EC93 exploits this invariant antigen to target and inhibit closely related competitors.

CDI systems are typically encoded within pathogenicity/genomic islands and are associated with transposable elements. This linkage to mobile genetic elements suggests that *cdi* genes are transferred horizontally between bacteria. The finding that certain CdiA-CTs from disparate bacterial species have high sequence identities supports this hypothesis (21). If such a transfer occurred between unrelated bacteria, then the recipient bacterium would gain an inhibition system that is probably targeted to the donor species. In principle, the newly acquired system could be deployed immediately, but because the donor bacteria are immune to their own CDI toxin, the recipients would gain no competitive advantage over the donor cells. Furthermore, if all CDI systems are self-targeting, then all horizontally transferred *cdiA* genes must eventually evolve to recognize receptors on the surface of the recipient species. The selective pressures that drive adaptation to self-receptors are unknown, but perhaps CDI-mediated cell-cell adhesion provides a benefit to bacteria. It is also unclear how *cdi* genes could be retained during the adaptation process. It is possible that other genes within the transferred genomic island provide a selective advantage and the linked *cdi* genes could be retained as a result. Alternatively, the toxin/immunity activities encoded by the *cdiA-CT*/*cdiI* pair may stabilize the genomic island in a manner similar to that described for some type II toxin/antitoxin (TA) modules (27, 28). In the absence of intercellular CdiA-CT exchange, the latter model would require cryptic translation initiation within *cdiA* to produce cytoplasmic toxin. If *cdi* genes do indeed stabilize genomic regions in the absence of selection, then it raises the possibility that CDI systems, like TA modules, are encoded by selfish genetic elements that propagate through horizontal transfer.

The target cell range of *E. coli* EC93 suggests that CDI is used for self-recognition and could help coordinate multicellular activities. Because CDI^{EC93} mediates adherence between *E. coli* cells exclusively, it provides a mechanism for increasing the relatedness of the local population and thus represents a form of "kin selection." Direct interaction between close kin is a common mechanism to increase the fitness of related cells (29). In accord with this idea, CDI systems enhance biofilm formation in both *Burkholderia thailandensis* E264 (30) and *E. coli* EC93 (Z.C.R. and C.S.H., unpublished data). Biofilms require collaboration between individual cells and provide fitness benefits such as protection from predation and antimicrobial agents. These observations indicate that CDI$^+$ siblings recognize and cooperate with one another. But CDI$^+$ cells also harm CDI$^-$ kin and therefore also act to exclude related cells that lack *cdi* genes. These phenomena are evocative of so-called "greenbeard" cooperativity (29, 31). Greenbeard genes give rise to a trait and simultaneously recognize and direct cooperation to other individuals with that same trait (32). Moreover, greenbeard genes are also sometimes capable of harming others that lack the allele. Individuals that have the same greenbeard alleles need not be related at other genetic loci, and

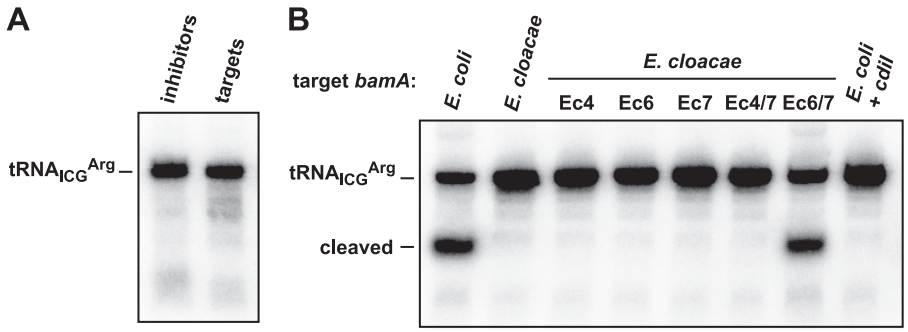


FIG 7 BamA$_{Ec6/7}^{Ecloacae}$ allows delivery of another CdiA-CT. (A) Northern blot analysis of tRNA$_{ICG}^{Arg}$ from *E. coli* EPI100 carrying cosmid pDAL879 (inhibitors) and *E. coli* CH9350 (targets). (B) Northern blot analysis of RNA isolated from competition cocultures. Inhibitors were mixed with target cells expressing the indicated *bamA* alleles at a 1:1 ratio and incubated for 1 h. RNA was isolated from the mixed cultures and analyzed by Northern hybridization using a radiolabeled probe to tRNA$_{ICG}^{Arg}$. Target cells from the sample on the far right also expressed the cognate *cdiI*$_{o1}^{EC93}$ immunity gene from plasmid pDAL867.

therefore, cooperation occurs between those of the same "kind" and not necessarily between kin. However, different strains of a given species typically harbor distinct CDI toxin/immunity pairs (6), so only closely related bacteria that have identical toxin/immunity alleles are likely to associate and cooperate. Thus, CDI^{EC93} discriminates kin at two levels—potential collaborators are recognized through specific receptor-binding interactions, and favored siblings are selected by virtue of their immunity gene allele.

MATERIALS AND METHODS

Bacteria and growth conditions. The bacterial strains and plasmids used in this study are listed in Table S2 in the supplemental material. Bacteria were grown in LB broth or LB agar unless otherwise noted. Media were supplemented with antibiotics at the following concentrations: ampicillin, 150 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; chloramphenicol, 66 μg ml^{-1}; spectinomycin, 50 μg ml^{-1}; rifampin, 200 μg ml^{-1}; streptomycin, 100 μg ml^{-1}. The *bamA* gene was replaced with a chloramphenicol acetyltransferase (*cat*) resistance cassette in *E. coli* X90 and EPI100 cells carrying plasmids pZS21-*bamA*$^{+}$ (13) and pSIM6 (33). The *cat* gene was amplified with oligonucleotides bamA-cat-for and bamA-cat-rev (see Table S3 in the supplemental material), and the resulting product was electroporated into cells as previously described (33). Transformants were selected on LB agar supplemented with chloramphenicol at 33 μg ml^{-1}.

For interspecies competitions, inhibitors and targets were first grown to mid-log phase at 37°C in LB broth supplemented with the appropriate antibiotics. Cells were harvested in M9 salts, and cell densities were determined by measuring optical density at 600 nm (OD$_{600}$). Inhibitors and targets were then mixed at a 10:1 ratio in M9 salts (to a final OD$_{600}$ of ~33.0), and 100 μl of the cell suspension was plated onto LB agar for incubation at 37°C. After 20 h, cells were harvested into 2 ml of M9 salts. The cell suspensions were serially diluted and plated on LB agar containing the appropriate antibiotics to enumerate viable inhibitor and target cells as colony-forming units (CFU). Intraspecies *E. coli* competitions were performed in LB broth. The inhibitor and target cells were mixed at a 10:1 ratio (final OD$_{600}$, ~0.330) in LB without antibiotics and cocultured for 3 h at 37°C with vigorous shaking. Samples of the cocultures were removed, serially diluted, and plated onto selective LB agar to enumerate target cells as CFU ml^{-1}. CdiA-CT^{EC93} toxicity was assessed by expression inside *E. coli* cells from an L-arabinose-inducible promoter on plasmid pCH450 (34). Plasmids pCH450, pCH450-*cdiA*-CT^{EC93}, and pCH450-*cdiA-CT*/*cdiI*^{EC93} were introduced into *E. coli* X90 Δ*bamA*::*cat* strains carrying the various pZS21-*bamA* constructs, and the resulting cells were cultured at 37°C in LB medium supplemented with tetracycline and 0.2% L-arabinose. Cell growth was monitored by measuring the OD$_{600}$ as a function of time.

Plasmid exchange. *E. coli* X90 Δ*bamA*::*cat* carrying plasmid pZS21::*bamA*$^{+}$ (Kan^{r}) was transformed with pZS21*amp* (Amp^{r}) or pZS21*amp* derivatives carrying the *bamA* alleles described above (35). Transformed cells were incubated for 1 h at 37°C in LB broth, plated onto LB agar supplemented with ampicillin, and then incubated at 37°C for 20 h. Amp^{r} colonies were picked at random and streaked onto fresh LB agar supplemented with ampicillin. The resulting Amp^{r} colonies were then cross-streaked onto separate LB agar plates supplemented with kanamycin or ampicillin. Cell growth on kanamycin and ampicillin was scored after 20 h at 37°C.

Cell-cell adhesion. Overnight cultures of *E. coli* strain DAL4905 carrying cosmid pDAL660Δ1-39 (CDI^{+}) or pWEB::TNC (CDI^{-}) were diluted into fresh tryptone broth (TB) and grown to mid-log phase at 30°C. These cells were then mixed at a 5:1 ratio with *E. coli* EPI100 Δ*bamA*::*cat* target cells carrying pZS21-*bamA* constructs and pDsRedExpress2. The cell suspension was incubated with aeration for 15 min at 30°C, diluted 1:50 into filtered 1× phosphate-buffered saline (PBS), and then analyzed on an Accuri C6 flow cytometer using FL1 (533/30 nm, GFP) and FL2 (585/40 nm, DS-Red) fluorophore filters (Becton Dickinson).

Whole-cell immunofluorescence. Overnight cultures were inoculated into fresh TB containing the appropriate antibiotics and grown to an OD$_{600}$ of ~0.8. Cells were harvested and fixed for 15 min in 1× PBS supplemented with 4% formaldehyde. Fixation reactions were quenched with 125 mM glycine (pH 7.4). Cells were washed once with 1× PBS supplemented with 125 mM glycine (pH 7.4) and then washed twice with 1× PBS. Washed cells were resuspended in 1× PBS supplemented with 1% bovine serum albumin (BSA) containing a 1:50 dilution of anti-HA monoclonal antibody (Sigma-Aldrich). Cells were incubated for 30 min at room temperature and washed three times with 1× PBS–1% BSA. Washed cells were resuspended in 1× PBS–1% BSA containing a 1:500 dilution of anti-mouse monoclonal antibody conjugated to Alexa Fluor 488 (Invitrogen). Cells were incubated for 30 min on ice and washed thrice with 1× PBS supplemented with 1% BSA. Cells were resuspended in 1× PBS and allowed to adhere to poly-D-lysine-coated slides for 5 min at room temperature. Nonadhering cells were removed with deionized water. Adhering cells were covered and fixed with FluoroGel solution and imaged by fluorescence microscopy.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00480-13/-/DCSupplemental.

Text S1, DOCX file, 0.1 MB.
Figure S1, PDF file, 0.1 MB.
Figure S2, PDF file, 0.1 MB.
Figure S3, PDF file, 0.1 MB.
Figure S4, PDF file, 0.1 MB.

Figure S5, PDF file, 0.2 MB.
Figure S6, PDF file, 0.1 MB.
Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.
Table S3, DOCX file, 0.1 MB.

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