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Genetic Analysis of the CDI Pathway from *Burkholderia pseudomallei* 1026b

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‡ These authors also contributed equally to this work.
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**Abstract**

Contact-dependent growth inhibition (CDI) is a mode of inter-bacterial competition mediated by the CdiB/CdiA family of two-partner secretion systems. CdiA binds to receptors on susceptible target bacteria, then delivers a toxin domain derived from its C-terminus. Studies with *Escherichia coli* suggest the existence of multiple CDI growth-inhibition pathways, whereby different systems exploit distinct target-cell proteins to deliver and activate toxins. Here, we explore the CDI pathway in *Burkholderia* using the CDIII_1026b system encoded on chromosome II of *Burkholderia pseudomallei* 1026b as a model. We took a genetic approach and selected *Burkholderia thailandensis* E264 mutants that are resistant to growth inhibition by CDIII_1026b. We identified mutations in three genes, BTH_I0359, BTH_II0599, and BTH_I0986, each of which confers resistance to CDIII_1026b. BTH_I0359 encodes a small peptide of unknown function, whereas BTH_II0599 encodes a predicted inner membrane transport protein of the major facilitator superfamily. The inner membrane localization of BTH_II0599 suggests that it may facilitate translocation of CdiA-CTII_1026b toxin from the periplasm into the cytoplasm of target cells. BTH_I0986 encodes a putative transglycosylase involved in lipopolysaccharide (LPS) synthesis. ΔBTH_I0986 mutants have altered LPS structure and do not interact with CDI+ inhibitor cells to the same extent as BTH_I0986+ cells, suggesting that LPS could function as a receptor for CdiA-II_1026b. Although ΔBTH_I0359, ΔBTH_II0599, and ΔBTH_I0986 mutations confer resistance to CDIII_1026b, they provide no protection against the CDIE264 system deployed by *B. thailandensis* E264. Together, these findings demonstrate that CDI growth-inhibition pathways are distinct and can differ significantly even between closely related species.
Introduction

Contact-dependent growth inhibition (CDI) is a mechanism of inter-cellular competition used by some Gram-negative species to inhibit the growth of neighboring bacteria [1–3]. CDI is mediated by the CdiB/CdiA family of two-partner secretion proteins, which are distributed through α-, β- and γ-proteobacteria [4]. CdiB is an outer-membrane β-barrel protein that exports the CdiA toxic effector. CdiA proteins are very large (180–650 kDa depending on the species) and are predicted to form long β-helical filaments that extend from the surface of inhibitor cells [2,5]. During CDI, CdiA binds to specific receptors on susceptible bacteria and delivers a toxin domain derived from its C-terminal region (CdiA-CT). CdiA-CT sequences are highly variable between bacterial species and strains, but the N-terminal boundary of this region is typically delineated by a highly conserved VENN peptide motif [1,6]. CdiA-CT sequence diversity suggests a variety of toxin activities, and indeed most characterized CDI toxins are nucleases with different cleavage specificities for DNA, tRNA or rRNA [1,7–9]. Additionally, CdiA-CTEC93 from Escherichia coli EC93 appears to form pores in target-cell membranes [10], and sequence analysis suggests that other CDI toxins may have RNA deaminase and protease/peptidase activities [11]. CDI+ bacteria protect themselves from auto-inhibition by producing CdiI immunity proteins, which bind to CdiA-CT toxins and neutralize their activities.

CDI has been characterized most extensively in γ-proteobacteria, with E. coli EC93 and uropathogenic E. coli 536 (UPEC 536) serving as model systems. Studies with those systems have revealed that CDI exploits specific target-cell proteins to deliver growth inhibitory toxins [12,13]. Selections for mutants that are resistant to the E. coli EC93 system (CDIEC93) identified bamA and acrB mutations that protect target cells from growth inhibition [12]. BamA is an essential outer-membrane protein required for the assembly of all β-barrel proteins [14–17], and is specifically recognized as a target-cell receptor by CdiAEC93 [12,18]. AcrB is a trimeric integral membrane protein that functions together with AcrA and TolC as a multi-drug efflux pump [19]. However, the efflux function of AcrB is not required for CDIEC93 because ΔacrA and ΔtolC mutants are both fully sensitive to CDIEC93 [12]. Though the role of AcrB during CDIEC93 is not known, its localization suggests that it could facilitate assembly of the CdiA-CTEC93 pore-forming toxin into the target-cell inner membrane. Biochemical studies on CdiA-CT536 from UPEC 536 have shown that this toxin is a latent tRNase that only exhibits nuclease activity when bound to the cysteine synthase, CysK [13]. In accord with in vitro studies, E. coli ΔcysK mutants are completely resistant to inhibition by CDIUPEC536. Collectively, these findings indicate that CDI pathways can encompass at least three distinct steps: i) receptor-binding to identify target bacteria, ii) translocation of CdiA-CT toxin across the target-cell envelope, and iii) activation of the toxin in the target-cell cytoplasm. Notably, the protective effects of cysK and acrB mutations are specific to the CDIUPEC536 and CDIEC93 pathways, respectively [13]. These findings raise the possibility that each CDI system/toxin exploits a unique set of proteins to inhibit target-cell growth.

CdiB and CdiA share significant homology across the proteobacteria, but the CDI systems of Burkholderiales exhibit a number of differences compared to other bacteria. Firstly, the variable toxin region in Burkholderia CdiA is typically demarcated by the (E/Q)LYN peptide motif rather than the VENN sequence found in most other bacteria [9,20]. Burkholderia toxins are modular and can be exchanged readily between Burkholderia CdiA proteins [9], but chimeric E. coli CdiA proteins carrying Burkholderia CdiA-CTs fused at the VENN sequence are not functional in CDI [1]. Secondly, CDI genes are arranged as cdiAIB clusters in Burkholderia, Variovorax and Cupriavidus species rather than the cdiBAI order found in other bacteria. This alternative gene arrangement is also correlated with a lack of "orphan" cdiA-CT/cdiI gene pairs. Orphan modules resemble the displaced 3´-fragments of full-length cdiA genes together with
their cognate cdil immunity genes [3,21]. Tandem arrays of orphan cdIA-CT/cdil gene pairs are commonly found downstream of cdIBAI loci in γ-proteobacteria, and all strains of Neisseria meningitidis also carry well-defined orphan toxin/immunity clusters [21,22]. Finally, many Burkholderia CDI systems encode a small predicted lipoprotein, BcpO, between the cdil and cdIB genes [20]. The function of BcpO is not understood completely, but it is required for CdiA secretion in Burkholderia thailandensis E264 [20]. Collectively, these observations suggest that the mechanisms of CDI in Burkholderia species are fundamentally distinct from other bacteria.

Here, we begin exploring Burkholderia CDI pathways using the CDIII Bp1026b system encoded on chromosome II of Burkholderia pseudomallei 1026b as a model. We took a genetic approach and isolated transposon mutants of B. thailandensis E264 that are resistant to inhibition by the CDIII Bp1026b system. Independent selections identified multiple transposon insertions in three genes—BTH_I0359, BTH_I10599, and BTH_I0986, each of which confers resistance to CDIII Bp1026b. BTH_I0359 encodes a small cystolic protein of unknown function, BTH_I10599 encodes an integral membrane protein from the major facilitator superfamily (MFS), and BTH_I0986 encodes a predicted lipopolysaccharide (LPS) transglycosylase. We find that LPS structure is altered in BTH_I0986 mutants, suggesting that LPS may function as a receptor or co-receptor for CdiAII Bp1026b. These results demonstrate that the CDIII Bp1026b is distinct from previously described E. coli pathways, suggesting that multiple pathways exist to translocate CDI toxins into target bacteria.

Materials and Methods

Bacterial strains and growth conditions

Bacterial strains were derived from Burkholderia thailandensis E264 and are listed in Table 1. Bacteria were routinely cultured in LB media supplemented with the following antibiotics where appropriate: kanamycin (Kan) 500 μg/mL; tetracycline (Tet) 25 μg/mL; trimethoprim (Tp) 100 μg/mL; chloramphenicol (Cam) 34 μg/mL; and polymyxin B (PB) 100 μg/mL. CDIII Bp1026b competitions used Bt81 inhibitors, which are B. thailandensis E264 cells that express cdIABII from plasmid pJSW1–6 (Table 2) [9]. Bt81 inhibitors and target cells were grown individually for at least 48 h (to OD600 > 0.6) in M9-minimal media supplemented with 0.2% L-arabinose. Approximately 10⁷ colony-forming units (cfu) of Bt81 inhibitors and 10⁸ cfu of target cells were mixed in 150 μL of M9-minimal medium supplemented with 0.2% L-arabinose, 1 μg/mL thiamine and 0.3 μg/mL ferric citrate, and aliquots plated onto LB agar supplemented with Tet or Kan to enumerate viable inhibitors and targets (respectively) at time 0 h. The remaining cell mixture (100 μL) was spread onto M9-minimal medium agar supplemented with 0.2% L-arabinose, 1 μg/mL thiamine and 0.3 μg/mL ferric citrate and incubated for 24 h at 30°C. Cells were then harvested from the agar surface, and viable inhibitor and target cell counts were determined as total cfu on Tet and Kan (respectively) supplemented LB agar. The competitive index (C.I.) was calculated as the ratio of target cells to inhibitor cells at 24 h divided by the target to inhibitor ratio at time 0 h. CDI E264 competitions were conducted in a similar manner, except inhibitor and target cells were co-cultured on tryptone broth agar. For these latter competitions, the target cells were derived from strain Bt36, which carries a deletion of the entire cdIABII gene cluster [9]. CdiA-CTI Bp1026b toxicity was tested by expressing the toxin domain inside B. thailandensis cells. Plasmid pSCBAD-CTII1026b was introduced into E. coli DH5α and the resulting strain used in a four-parent mating with SM10λpir/pTNS3 [23,24], HB101 (pRK2013) [25] and B. thailandensis E264. Conjugation mixtures were split into two equal portions and plated on LB agar supplemented with PB, Tp and 0.2% D-glucose and LB agar supplemented with PB, Tp and 0.2% L-arabinose. The presence of exconjugants on plates supplemented with D-glucose and the simultaneous absence of exconjugant colonies...
Table 1. Bacterial strains used in this study.

<table>
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<th>Strains</th>
<th>Description</th>
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<td>[54]</td>
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<td>E264</td>
<td></td>
<td></td>
</tr>
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<td>Bt5</td>
<td>B. thailandensis E264 (pJSW2)</td>
<td>This study</td>
</tr>
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<td>Bt6</td>
<td>glmS1::Tn7-Kan, KanR</td>
<td>[9]</td>
</tr>
<tr>
<td>Bt7</td>
<td>glmS1::Tn7-cdli^{1036b}::Kan, KanR</td>
<td>[9]</td>
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<td>This study</td>
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<td>T. Hoang</td>
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<tr>
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<td>This study</td>
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<td>Bt134</td>
<td>ΔcdiAIB glmS1::Tn7-kan ΔBTH_I0359, KanR</td>
<td>This study</td>
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<tr>
<td>Bt137</td>
<td>glmS1::Tn7-kan ΔBTH_I0099, pSCBAd::Io0599, KanR TpR</td>
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<td>Bt138</td>
<td>glmS1::Tn7-kan ΔBTH_I0359, pSCBAd::Io0359, KanR TpR</td>
<td>This study</td>
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<tr>
<td>Bt143</td>
<td>glmS1::Tn7-kan ΔBTH_I0359, pSCBAd::DsRed, KanR TpR</td>
<td>This study</td>
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</table>

Abbreviations: KanR, kanamycin-resistant; TetR, tetracycline-resistant; TpR, trimethoprim-resistant

doi:10.1371/journal.pone.0120265.001
Selection of CDIR mutants

A library of random T23-TpR transposon-insertion mutants (>38,000 unique insertions) [26] was co-cultured with B. thailandensis Bt81 inhibitor cells [9]. Inhibitors and mutant target cells were mixed at a 10:1 ratio and plated onto M9 minimal agar medium supplemented with 0.2% L-arabinose 1 μg/mL thiamine and 0.3 μg/mL ferric citrate. After 24 h co-culture at 37°C, cells were harvested from the agar surface and surviving target cells isolated on Tp-supplemented LB agar. The target cells were pooled and subjected to two additional rounds of selection against CDIII_Bp1026b expressing inhibitor cells. After enrichment, individual clones were selected and tested for CDI-resistance in competition co-cultures with Bt81 inhibitor cells. Transposon-insertion junctions were amplified by arbitrary PCR using primers LacZ-124L2, LacZ-148 and CEKG 2E/K/L (Table 3). The resulting products were sequenced with primers LacZ211 and CEKG4 to identify insertion sites (Table 3).

Construction of Plasmids and Chromosomal Deletions

Plasmid pSCBAD is a derivative of pSCRhaB2 [27]. The araC gene and araBAD promoter were excised from plasmid pCH450 [28] by NsiI/NcoI digestion and ligated to plasmid pSCRhaB2. This sub-cloning step replaces the original rhamnose-inducible promoter with an arabinose-inducible promoter. Plasmid pCH450 was amplified with primers CH1730/CH2799 and the resulting product cloned into pSCRhaB2 using NsiI/NcoI restriction sites to generate
plasmid pSCBAD-KX. The BTH_II0599 and BTH_I0986 genes were amplified from chromosomal DNA using primers 3258/2725 and 3259/2729 (respectively), and the resulting products ligated to plasmid pSCBAD using EcoRI and XmaI restriction sites. BTH_I0359 was amplified using primers CH2059/CH2800 and ligated into pSCBAD using KpnI and PstI restriction sites. The region encoding CdiA-CTII_{Bp1026b} (residues Met2821—Asn3122 of full-length CdiAII_{Bp1026b}) was subcloned from plasmid pCH450-CTII_{1026b} [9] into pSCBAD using NcoI and PstI restriction sites. The DsRed coding sequence was subcloned from plasmid pTrc-DsRed [8] into pSCBAD using NcoI and PstI restriction sites.

Gene deletions were constructed by allelic exchange as described previously [29]. DNA sequences upstream and downstream of the target gene were amplified and the two PCR products combined into one fragment using overlapping end PCR (OE-PCR) [30]. The OE-PCR products were ligated to plasmid pEX18-Tp (Table 2) using HindIII and KpnI/XbaI restriction sites. The BTH_I0359 deletion construct was generated using primer pairs 3296/3297 and 3298/3299; the BTH_II0599 deletion construct was generated using primer pairs 3182/3183.

Table 3. Oligonucleotides used in this study.

<table>
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<td>2729</td>
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<td>3103</td>
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<tr>
<td>3104</td>
<td>5‘—GAT CGT ACT GGA TCG CTGC ACG CAA AAA ACC AAC AGC CGG ACC C</td>
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<td>3105</td>
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</tr>
<tr>
<td>3106</td>
<td>5‘—CAA CAA ggt acc CGT GTC GCC GAG CAA CAG ATG A</td>
<td>This study</td>
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<td>CEKG 4</td>
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aRestriction endonuclease sites are in lowercase; N indicates equal mixture of all four deoxyribonucleotides.

doi:10.1371/journal.pone.0120265.t003
and 3184/3185; and the BTH_I0986 deletion construct generated using primer pairs 3103/3104 and 3105/3106 (Table 3).

Cell-cell adhesion

GFP-labeled *B. thailandensis* E264 [24] carrying plasmid pJSW1–6 (Bt121) or pJSW2 (Bt5) were grown overnight in tryptone broth, then diluted 1:50 in fresh tryptone broth and grown to OD_{600} ~0.5. DsRed-labeled target strains (Bt101, Bt123, Bt124 and Bt143) were grown in minimal M9-media supplemented with 0.2% L-arabinose for at least 48 h to OD_{600} ~ 0.5. Inhibitor and target cells were mixed at a 1:1 ratio and incubated for 30 min at room temperature to allow cell-cell binding. Cell suspensions were then diluted 1:50 into sterile filtered 1X phosphate buffered saline and analyzed by flow cytometry. Samples were run on an Accuri C6 flow cytometer using FL1 (533/30nm, GFP) and FL2 (585/40nm, DsRed) fluorophore filters. Cell-cell binding was measured as the percent of target cells in aggregates with inhibitor cells divided by the total number of target cells. Binding data were normalized to the level of cell-cell binding between wild-type target cells (Bt101) and CDI_{II}^{B. p1026b} expressing inhibitors (Bt121).

Lipopolysaccharide (LPS) analysis

Bacteria were grown to OD_{600} > 0.6 in M9-minimal medium supplemented with 0.2% L-arabinose and LPS was harvested from an equivalent of 10 mL of OD_{600} = 1 culture using the LPS Extraction Kit (Boca Scientific, USA). Purified LPS was resolved on a 4–20% polyacrylamide Tris-glycine SDS gel (Thermo Scientific) and visualized using ProQ 300 Emerald LPS stain (Molecular Probes, USA).

Results

Isolation of CDIR mutants

To gain insight into the CDI pathways in *Burkholderia* species, we used a genetic approach to identify target-cell genes that are required for growth inhibition. We reasoned that mutants with disruptions in the genes encoding the CDI receptor, toxin translocators and toxin activators would be CDI-resistant (CDIR). *B. thailandensis* E264 cells were subjected to random mutagenesis using a Tn5-based T23 transposon. Two independent T23 mutant pools were then co-cultured on solid media with *B. thailandensis* inhibitor cells that express the *B. pseudomallei* CDI_{II}^{Bp1026} system from a plasmid vector (Bt81, Table 1). CDIR mutants were enriched through three cycles of co-culture with inhibitor bacteria, and 20 clones were selected for the identification of transposon insertion sites. Each mutant contained a T23 insertion within BTH_I0359, BTH_II0599 or BTH_I0986; corresponding to eleven unique insertion sites (Fig. 1A and Table 1). BTH_I0359 is located upstream of the genes for methionine biosynthesis and encodes a hypothetical protein of 85 amino acid residues (Fig. 1A). BTH_II0599 encodes a predicted major facilitator superfamily (MFS) protein and is likely to be an inner-membrane localized transporter. BTH_I0986 is annotated as lipooligosaccharide (LOS) glycosyltransferase G and is located within an LPS biosynthesis operon on chromosome I (Fig. 1A).

We picked two mutants for each disrupted gene and confirmed that each was 10- to 100-fold more resistant to the CDI_{II}^{Bp1026} system than wild-type *B. thailandensis* (Fig. 1B). Because multiple independent insertions were identified for each gene, it is likely that these mutations are directly responsible for the CDIR phenotype. However, it is possible that the mutant strains carry additional unidentified mutations that contribute to resistance. To ascertain the roles of BTH_I0359, BTH_I0986 and BTH_II0599 in CDI, we constructed in-frame deletions of each gene and tested the resulting mutants for CDIR. As expected, the deletion mutants
Fig 1. Selection of CDIΔ mutants of *B. thailandensis* E264. A) T23 transposon insertion sites were identified by semi-arbitrary PCR as described in Methods. Orange arrows indicate T23 insertions in the same transcriptional orientation of the disrupted gene and blue arrows indicate insertions in the opposite orientation. The corresponding CDIΔ mutant strain number is given above each arrow. Automated gene annotations are given below each ordered locus designation. GT-1, GT-2 and GT-9 indicate predicted glycosyltransferase
each had CDIR phenotypes that were very similar to the originally isolated transposon-insertion mutants (Figs. 1B and 2). ΔBTH_I0986 and ΔBTH_II0599 mutants were fully resistant to CDIII \textit{Bp1026b}, whereas the ΔBTH_I0359 mutant was only partially protected from inhibition (Fig. 2). These results strongly suggest that each gene is required for the CDIII \textit{Bp1026b} inhibition pathway. We also showed that each deletion mutant was rendered sensitive to CDIII \textit{Bp1026b} when complemented with a plasmid-borne copy of the appropriate gene (Fig. 2). These latter data exclude effects from transcriptional polarity and indicate that BTH_I0359, BTH_II0599 and BTH_I0986 are required for full sensitivity to the CDIII \textit{Bp1026b} system.

Resistance mutations are specific for the CDII\textit{Bp1026b} system

\textit{B. thailandensis} E264 carries its own CDI system (CDIE264) and the CdIA\textit{E264} protein shares approximately 53\% sequence identity with CdIA\textit{Bp1026b}. However, the CdIA-CTE264 and CdIA-CT\textit{Bp1026b} toxins are not homologous and have different nuclease activities [9],

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**Fig 2. Complementation of CDIR mutations.** The indicated \textit{B. thailandensis} strains were co-cultured with Bt81 inhibitors (Table 1) that express the CDII\textit{Bp1026b} system for 24 h on solid medium, and the competitive index was calculated as described in Materials and Methods. The strain labeled cdII\textit{Bp1026b} expresses the cognate CdiIII\textit{Bp1026b} immunity protein. Plasmid-borne copies of BTH_I0359, BTH_I0986 and BTH_II0599 genes were expressed from an L-arabinose inducible promoter. Data represent the mean ± SEM for three independent experiments. Sample values that were statistically different from one another (p < 0.05) are shown by bars with an asterisk (see Fig. 1).
suggesting that the two toxin-delivery pathways could be distinct. Therefore, we asked whether mutations in BTH_I0359, BTH_II0599 and BTH_I0986 also provide resistance to CDIE264. We first confirmed that ΔcdiAIB E264 mutants, which lack immunity to CDIE264, are inhibited by wild-type CDI+ B. thailandensis cells as reported previously [9,20]. B. thailandensis ΔcdiAIB E264 targets were inhibited approximately 105-fold during co-culture with CDI+ cells (Fig. 3). This growth inhibition is attributable to CDIE264, because the target cells were fully protected when complemented with the cdiIE264 gene on a Tn7-based vector (Fig. 3). We then introduced ΔBTH_I0359, ΔBTH_II0599 and ΔBTH_I0986 mutations into the ΔcdiAIB E264 background and found that each of the resulting strains was still sensitive to CDIE264 (Fig. 3). These results demonstrate mutations in BTH_I0359, BTH_II0599 and BTH_I0986 specifically confer resistance to the CDIII Bp1026b system.

**CDI R genes are not required to activate the CdiA-CTII Bp1026b toxin**

Work with the CDI536 system from UPEC 536 has shown that some CDI toxins must be activated by so-called "permissive" factors. CdiA-CT536 only has tRNase activity when bound to CysK, and therefore E. coli ΔcysK mutants are completely resistant to the toxin, even when produced at high levels inside the cell [13,31]. Based on the CDI536 paradigm, we asked whether any of the Burkholderia CDI R genes encode proteins with permissive factor function. We placed the cdiA-CTII Bp1026b coding sequence under control of an arabinose-inducible PbAD promoter and moved the construct onto a mobilizable plasmid. This plasmid can be stably maintained in E. coli cells under conditions that repress transcription from PbAD [9]. We then tested whether the cdiA-CTII Bp1026b plasmid could be introduced into B. thailandensis cells through tri-parental mating. No exconjugants were produced from matings to introduce the toxin plasmid into wild-type cells, but dozens of exconjugants were obtained when recipient cells expressed the cognate cdiIE264 immunity gene (Fig. 4). These results indicate that
Fig 4. Toxicity of CdiA-CTIIBp1026b expressed inside B. thailandensis cells. Plasmids pSCBAD and pSCBAD::cdiA-CTII Bp1026b were introduced into the indicated B. thailandensis strains by conjugation as described in Materials and Methods. The mating mixtures were split into equal portions and plated onto LB agar with Polymyxin B and Trimethoprim supplemented with either D-glucose (left panels) or L-arabinose (right panels). See Materials and Methods.

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CdiA-CTII \textsuperscript{Bp1026b} is toxic when expressed inside \textit{B. thailandensis} cells and that CdiIII \textsuperscript{Bp1026b} neutralizes the toxin to allow cell growth. We next performed matings with \( \Delta \text{BTH}_I0359 \), \( \Delta \text{BTH}_II0599 \) and \( \Delta \text{BTH}_I0986 \) recipient strains, each of which produced no exconjugants (Fig. 4). Together, these results show that none of the CDI\(^R\) mutations protect the cell from intracellular CdiA-CTII \textsuperscript{Bp1026b}, indicating that the corresponding gene products do not function as CDI permissive factors.

\textbf{BTH}_I0986 influences the binding of inhibitor and target cells}

We next considered the possibility that the CDI\(^R\) genes may influence the recognition of target cells. The BTH\(_{I0986}\) mutation is of particular interest because this gene belongs to the GT1 family of glycosyltransferases and is predicted to function in lipopolysaccharide (LPS) biosynthesis. Thus, the BTH\(_{I0986}\) mutation could alter LPS structure, thereby preventing CdiA-CTII \textsuperscript{Bp1026b} inhibitor cells from recognizing and/or binding to target bacteria. To determine whether BTH\(_{I0986}\) influences LPS structure, we used SDS-PAGE to analyze LPS isolated from wild-type and \( \Delta \text{BTH}_I0986 \) cells. Surprisingly, we found that LPS isolated from wild-type \textit{B. thailandensis} E264 cells lacked polymeric O antigen (Fig. 5), in contrast to previous reports \cite{32,33}. The LPS from \( \Delta \text{BTH}_I0986 \) mutants also lacked an O-antigen ladder, but migrated more rapidly during electrophoresis than LPS from BTH\(_{I0986}^+\) cells (Fig. 5).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Fig5.png}
\caption{Lipopolysaccharide (LPS) analysis. LPS was isolated from the indicated \textit{B. thailandensis} strains and analyzed by SDS-PAGE using fluorescent detection. The LPS standard is from \textit{Escherichia coli} serotype 055:B5.}
\label{fig5}
doi:10.1371/journal.pone.0120265.g005
\end{figure}
Complementation with plasmid-borne BTH_I0968 restored mutant LPS to the wild-type mobility (Fig. 5). Therefore, disruption of BTH_I0986 alters the target-cell surface by changing LPS structure.

In the *E. coli* EC93 system, inhibitor cells bind stably to target bacteria and the resulting cell aggregates can be detected and quantified using flow cytometry [12,18]. Therefore, we used the same approach to examine the binding of CDII\(^{Bp1026b}\) inhibitors to different target cell strains. We mixed GFP-labeled CDII\(^{Bp1026b}\) inhibitors at a 1:1 ratio with DsRed-labeled target cells and analyzed the suspensions by flow cytometry to detect events with both green and red fluorescence, which correspond to aggregates containing both inhibitor and target cells. This analysis showed that approximately 40% more target cells bind to CDII\(^{Bp1026b}\) inhibitors compared to CDI\(^{–}\) mock inhibitors (Fig. 6).

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**Discussion**

The results presented here show that at least three genes, BTH_I0359, BTH_I0986 and BTH_II0599, are required for *B. thailandensis* cells to be fully inhibited by the CDII\(^{Bp1026b}\) system. We identified each gene in two independent selection experiments, suggesting that they represent the major non-essential genes required for the CDII\(^{Bp1026b}\) pathway. Indeed, BTH_I0599 and BTH_I0986 are particularly critical because deletion of either gene provides full resistance to target bacteria. Notably, the three *B. thailandensis* genes identified here are distinct from those previously identified in *E. coli* within the CDI\(^{EC93}\) growth inhibition pathway [12]. These results suggest that the CDII\(^{Bp1026b}\) and CDI\(^{EC93}\) systems deliver toxins...
through different pathways. CDI is initiated through direct binding interactions between CdiA and receptors on the surface of target bacteria. CdiAEC93 uses the E. coli BamA protein as a receptor and appears to bind specific epitopes within extracellular loops eL6 and eL7 [12,18]. Our results here suggest that B. pseudomallei CdiA\textsubscript{Bp1026b} may exploit LPS as a target-cell receptor. BTH\textsubscript{I0986} is a predicted transglycosylase and mutants lacking this enzyme have altered LPS structure (Fig. 5). Moreover, the ΔBTH\textsubscript{I0986} mutant shows defects in binding to CdiA\textsubscript{Bp1026b} inhibitor cells (Fig. 6), consistent with a role in receptor function. Surprisingly, we also found that our B. thailandensis E264 isolate lacks a detectable O-antigen ladder. This could account for the fact that we did not identify any additional LPS biosynthesis genes in independent selections. It is unclear whether the rough LPS phenotype reflects phase variation [34–36], or whether a rough-strain mutant was selected through laboratory passage. In either event, it will be important to determine how O-antigen influences CDI susceptibility in Burkholderia species. Although our results do not support a role for BamA in Burkholderia CDI, we acknowledge that CDI\textsuperscript{R} alleles of the essential bamA gene would be difficult to isolate using a transposon mutagenesis approach. If Burkholderia BamA does function as a CDI receptor, then the interactions must be distinct from the Cdi\textsuperscript{EC93} system, because BamA loops eL6 and eL7 loops differ significantly between E. coli and Burkholderia species (Fig. 7) [37].

Because CdiA-CT\textsubscript{Bp1026b} is a tRNase, this toxin must be transported into the target-cell cytoplasm to reach its substrate. CDI toxin translocation is poorly understood, but our recent work with E. coli indicates that transport across the target-cell outer membrane is energy-independent, whereas translocation into the cytoplasm requires the proton-motive force [38]. These findings raise the possibility that BTH\textsubscript{I0599}, a predicted MFS transporter, is co-opted to translocate the tRNase domain across the target-cell inner membrane. In this model, periplasmic toxin would bind to BTH\textsubscript{I0599} and be driven into the cytoplasm by either the chemical or electrical potential of the pmf. These interactions are specific because the ΔBTH\textsubscript{I0599} mutation provides no protection against the B. thailandensis CDI\textsubscript{E264} system, suggesting that the CdiA-CT\textsubscript{E264} toxin must exploit another entry pathway. Although MFS proteins harness chemiosmotic gradients to transport a variety of metabolites [39,40], it seems unlikely that the transporter could translocate a folded nuclease domain in the same manner as small solutes. One possibility is that CdiA-CT\textsubscript{Bp1026} has an autonomous membrane translocation activity, but requires BTH\textsubscript{I0599} as a receptor to facilitate insertion into the inner membrane. This model is similar to that proposed by Kleanthous and colleagues for the translocation of colicin nuclease domains, some of which interact with phospholipids and form pores in membranes [41–43].

The role of BTH\textsubscript{I0359} in the Cdi\textsubscript{Bp1026b} pathway remains enigmatic, in part because the function of this gene is unknown. BTH\textsubscript{I0359} encodes a DUF3567 family member (PF12091, http://pfam.xfam.org/family/PF12091), which is only found within the order Burkholderiales. The gene neighborhood of BTH\textsubscript{I0359} includes the downstream metH\textsubscript{a} and metH\textsubscript{b} (which encode a split methionine synthase) and an upstream DUF3108 family member. DUF3567 and DUF3108 genes are linked throughout all the Burkholderiales, whereas linkage to metH is limited to Burkholderia, Ralstonia and Cupriavidus species. DUF3108 genes encode outer-membrane β-barrel proteins with a characteristic YmcC fold (PDB: 3FZX). Although strong genetic linkage is often indicative of a functional relationship, we did not isolate BTH\textsubscript{I0360} mutations in our CDI\textsuperscript{R} selections, even though this gene is not essential for B. thailandensis growth [26]. We have also excluded a "permissive" factor function for BTH\textsubscript{I0359}. Permissive factors are target-cell proteins that are required to activate CdiA-CT toxins in the target-cell cytoplasm [13]. This conclusion is also supported by previous studies showing that purified CdiA-CT\textsubscript{Bp1026b} has tRNase activity in vitro, and therefore does not require an additional factor for activation [8].
All *B. pseudomallei* strains contain at least one CDI system, and some isolates carry up to three loci [9]. Each system can be placed into one of 10 different toxin/immunity groups [9,20], suggesting that CDI mediates competition between different *B. pseudomallei* strains. Using *B. thailandensis* as a model, Cotter and colleagues have recently demonstrated that such competition does in fact occur in mixed-strain biofilms, and that CDI influences the composition of these communities [20,44]. Additionally, there are indications that *B. pseudomallei* and *B. thailandensis* do not co-inhabit the same environmental niches [45], again suggesting that anti-bacterial competition systems shape their environmental distributions. If *Burkholderia* species do in fact directly antagonize one another in the environment, then type VI secretion systems (T6SS) are more likely to effect this competition. *B. thailandensis* and *B. pseudomallei* strains all carry multiple T6SS, which have been shown to deploy toxins against both bacteria and eukaryotic targets [46–49]. Moreover, a given T6SS is capable of killing many different species of Gram-negative bacteria [50–52]. In contrast, CDI is a receptor-mediated process, and therefore variations in the cell-surface receptor epitopes restrict inhibition activity to a subset of bacteria [18]. In accord with this general model, data presented here show that CDI is significantly more effective against *B. thailandensis* targets than CDI in *B. pseudomallei*. Together, these...
observations indicate that CDI is used primarily to differentiate sibling cells from other closely related bacteria.

**Author Contributions**
Conceived and designed the experiments: SK CSH DAL. Performed the experiments: SK FGS NE SC. Analyzed the data: SK FGS NE SJP CSH DAL. Contributed reagents/materials/analysis tools: SJP CM. Wrote the paper: SK FGS CM CSH DAL.

**References**


20. Anderson MS, Garcia EC, Cotter PA. The Burkholderia bcpAI/OB genes define unique classes of two-partner secretion and contact dependent growth inhibition systems. PLOS Genet 2012; 8: e1002877. doi: 10.1371/journal.pgen.1002877 PMID: 22912595


46. Schwarz S, West TE, Boyer F, Chiang WC, Carl MA, Hood RD. Burkholderia type VI secretion systems have distinct roles in eukaryotic and bacterial cell interactions. PLOS Pathog 2010; 6: e1001068. doi: 10.1371/journal.ppat.1001068 PMID: 20865170
