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The toxin/immunity network of *Burkholderia pseudomallei* contact-dependent growth inhibition (CDI) systems

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Summary

Burkholderia pseudomallei is a Category B pathogen and the causative agent of melioidosis – a serious infectious disease that is typically acquired directly from environmental reservoirs. Nearly all *B. pseudomallei* strains sequenced to date (>85 isolates) contain gene clusters that are related to the contact-dependent growth inhibition (CDI) systems of γ -proteobacteria. CDI systems from *Escherichia coli* and *Dickeya dadantii* play significant roles in bacterial competition, suggesting these systems may also contribute to the competitive fitness of *B. pseudomallei*. Here, we identify ten distinct CDI systems in *B. pseudomallei* based on polymorphisms within the *cdiA-CT/cdiI* coding regions, which are predicted to encode CdiA-CT/CdiI toxin/immunity protein pairs. Biochemical analysis of three *B. pseudomallei* CdiA-CTs revealed that each protein possesses a distinct tRNase activity capable of inhibiting cell growth. These toxin activities are blocked by cognate CdiI immunity proteins, which specifically bind the CdiA-CT and protect cells from growth inhibition. Using *Burkholderia thailandensis* E264 as a model, we show that a CDI system from *B. pseudomallei* 1026b mediates contact-dependent growth inhibition and is capable of delivering CdiA-CT toxins derived from other *B. pseudomallei* strains. These results demonstrate that *Burkholderia* species contain functional CDI systems, which may confer a competitive advantage to these bacteria.

Keywords

contact-dependent growth inhibition; intercellular communication; ribonuclease; toxin-immunity proteins; tRNase activity

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Introduction

Burkholderia pseudomallei (*Bp*) is an NIAID category B priority pathogen and the causative agent of melioidosis, a serious human disease endemic to southeast Asia and northern Australia and prevalent throughout tropical and subtropical regions (Limmathurotsakul & Peacock, 2011). Melioidosis can manifest as either acute or chronic disease and is often difficult to treat due to the bacterium's intrinsic resistance to multiple antibiotics. *Bp* infections are usually acquired from the environment, with soil and water constituting the main pathogen reservoir (DeShazer, 2004, Lazar Adler *et al.*, 2009). Indeed, the incidence of melioidosis increases after periods of extended rainfall (Lin *et al.*, 2011), consistent with a relationship between *Bp* infection and exposure to surface water. Given this mode of transmission, an understanding of the factors that contribute to pathogen growth and survival in the environment may provide strategies for eradication. Previous studies have shown that the distribution of *Bp* in the environment is complex (Chantratita *et al.*, 2008, Corkeron *et al.*, 2010). *Bp* often exhibits a discontinuous distribution with distinct sequence types (ST) dominating adjacent sites (Chantratita *et al.*, 2008). Although each niche tends to be dominated by a particular ST, there does not appear to be a fixed strain hierarchy. A given ST may dominate another in one sample, yet be outcompeted in an adjacent site (Chantratita *et al.*, 2008). Moreover, there is evidence that other *Burkholderia* species may exclude *Bp* strains from some environments (Lin *et al.*, 2011). These and other observations suggest that *Bp* strains compete with one another and other species for niche dominance, but the underlying mechanisms are not understood (Chantratita *et al.*, 2008).

Bacterial contact-dependent growth inhibition (CDI) provides one possible explanation for the complex distribution of *Bp* strains. CDI systems allow Gram-negative bacteria to inhibit the growth of neighboring bacteria upon direct cell-to-cell contact and are widely distributed throughout the α -, β - and γ -proteobacteria (Aoki *et al.*, 2005, Aoki *et al.*, 2010, Hayes *et al.*, 2010). CDI is mediated by the CdiB/CdiA family of two-partner secretion proteins (Aoki *et al.*, 2005). CdiB is an outer membrane protein required for the secretion and assembly of CdiA onto the inhibitor cell surface (Buscher *et al.*, 2006, Mazar & Cotter, 2006, Mazar & Cotter, 2007). CdiA proteins carry the growth inhibitory activity and are predicted to form elongated filaments that extend from inhibitor cells to interact with target bacteria. Each CDI system also encodes an immunity protein (CdiI), which protects CDI⁺ cells from autoinhibition (Aoki *et al.*, 2005, Aoki *et al.*, 2010). We recently localized the CDI toxin activity to the C-terminal region of CdiA (CdiA-CT). Remarkably, the CdiA-CT region is highly variable between different bacteria, suggesting that several distinct toxins are encoded by CDI systems. Indeed, the CdiA-CTs from uropathogenic *E. coli* 536 and *Dickeya dadantii* 3937 have toxic tRNase and DNase activities, respectively (Aoki *et al.*, 2010). CdiI immunity proteins are also highly variable and specifically bind their corresponding CdiA-CTs to block toxin activity. Thus, a given CdiI protein confers immunity to its cognate CdiA-CT toxin, but provides no protection from the toxins deployed by other CDI systems.

Nearly all *Bp* strains contain two-partner secretion proteins related to CdiB and CdiA, but in most instances the predicted CdiA proteins are annotated as hemolysins, adhesins or filamentous hemagglutinins. The putative CDI systems of *Burkholderia* species are distinct from those found in other proteobacteria. Firstly, the *Burkholderia* CDI genes are arranged as *cdiAIB* clusters in contrast to the *cdiBAI* organization found in all other genera for which genomic sequences are available. Additionally, *Burkholderia* CdiA proteins lack Pfam domain DUF638 (PF04829) and the associated VENN peptide motif that demarcates the variable CdiA-CT region in most other CDI systems (Aoki *et al.*, 2010). Here, we examine CdiA-CT/CdiI pairs from *B. pseudomallei* and *B. thailandensis* to determine whether these proteins function as toxin/immunity pairs in inter-bacterial competition. Expression of *Bp*

CdiA-CTs in *E. coli* cells revealed that these proteins have distinct tRNase activities capable of inhibiting cell growth. Each of these toxic activities is specifically blocked by cognate CdiI immunity protein, demonstrating the toxin/immunity functions of these proteins. Moreover, we show that the *cdiAIB* genes of *Bp* strains and *B. thailandensis* mediate contact-dependent growth inhibition. This growth inhibition is specifically blocked when target cells express the cognate *cdiI* immunity gene. These results indicate that *Burkholderia* CDI systems have the potential to contribute to the competition and survival of *Bp* strains in the environment.

Results

Analysis of *Burkholderia* *cdiAIB* gene clusters

Bioinformatic analysis of fully sequenced and assembled *Burkholderia* genomes shows that most species contain at least one gene cluster encoding proteins related to CdiB and CdiA from γ -proteobacteria. *B. cenocepacia* and *B. mallei* are notable exceptions, although *B. mallei* strains contain proteins with hemagglutinin repeats that are distantly related to CdiA proteins. In contrast to the typical *cdiBAI* gene organization found in other bacteria (Aoki et al., 2010), the putative CDI genes in *Burkholderia* species are generally arranged as *cdiAIB* clusters (Fig. 1). Examination of the publically available *B. pseudomallei* (*Bp*) genomes revealed that all strains contain one to three *cdiAIB* clusters, and in most instances the predicted *Bp* CdiA proteins have been annotated as either hemolysins or putative adhesins. Several *Bp* CDI systems are linked to *rhs* genes (Fig. 1; for example, the *Bp* K96243 *cdiA* gene is adjacent to *rhs* locus BPSS2054), which share several features with *cdiA* genes and may also be involved in intercellular growth competition (Poole et al., 2011). In general, *Burkholderia* CdiA proteins lack Pfam DUF638 domains and the associated VENN peptide motifs that demarcate the variable CdiA-CT region in most other bacteria. Instead, *Bp* CdiA-CT sequences diverge at varying positions after a conserved (Q/E)LYN peptide motif (Fig. S1). *Bp* CdiA proteins fall into two classes based on the sequence of the conserved N-terminal region. CdiA sequences N-terminal to the (Q/E)LYN motif are 78% – 98% identical within each class, but only share ~58% identity between classes (Fig. S1). *Bp* CdiA proteins can be further divided into ten different subclasses based on the variable CdiA-CT sequences (Fig. S1 and Table S1). In general, there is >98% amino acid sequence identity between *Bp* CdiA-CTs within each subclass; however the type X subclass is more variable with >88% sequence identity in pair-wise alignments. To determine whether these CDI sequence types are representative of *Bp* *cdiA* gene diversity, we analyzed CdiA-CT sequences encoded by a collection of 60 unassembled genomes from clinical and environmental *Bp* isolates (Table S2). We also amplified and sequenced the *cdiA-CT* coding regions from several environmental isolates described by Chantratita et al. (Chantratita et al., 2008). The additional CdiA-CT sequences all corresponded to one of the ten sequence types identified in the assembled *Bp* strain genomes (Tables S1 & S2), suggesting that the list of CdiA-CT sequence types is comprehensive. Additionally, the predicted *cdiI* immunity genes found immediately downstream of *cdiA* are also variable and fall into ten distinct sequence types that correspond to the CdiA-CT sequence types (Fig. 1). Intriguingly, some *Bp* CDI systems may encode multiple, distinct immunity proteins. For example, the presumed *cdiI* gene in *Bp* NCTC 13177 is followed by a short sequence encoding a protein related to the CdiI immunity protein from *Enterobacter aerogenes* KCTC 2190 (43% sequence identity, pairwise BLAST E value = $1e^{-16}$), as well as CdiI immunity proteins from other bacteria. In general, the *Bp* CDI systems contain one to four potential *cdiI* immunity genes between *cdiA* and *cdiB* (Fig. 1). Taken together, these observations suggest that the *Bp* CdiA-CT and CdiI proteins constitute a family of variable toxin-immunity pairs.

Bp CDI systems encode toxin/immunity pairs

To determine whether *Bp* CdiA-CTs possess growth inhibitory activity, we examined the type V CdiA-CT sequence encoded on chromosome II of *Bp* 1026b (hereafter referred to CdiA-CT_{II}^{1026b}). The region encoding CdiA_{II}^{1026b} residues Met2821 to Asn3122 was cloned into plasmid pCH450 under the control of the arabinose-inducible P_{BAD} promoter and introduced into *E. coli* cells. Induction of CdiA-CT_{II}^{1026b} synthesis with L-arabinose significantly inhibited *E. coli* cell growth compared to control cells carrying only the plasmid vector (Fig. 2A). This growth inhibition was completely alleviated by co-expression of CdiI_{II}^{1026b} from the IPTG-inducible P_{trc} promoter on a compatible pTrc plasmid (Fig. 2A). In contrast, the predicted CdiI^{E478} and CdiI^{E479} immunity proteins (from *Bp* isolates E478 and E479, respectively) did not rescue the growth of CdiA-CT_{II}^{1026b} inhibited cells when produced from the pTrc plasmid (Fig. 2A). These results indicate that CdiA-CT_{II}^{1026b} has growth inhibitory activity and suggest that CdiI_{II}^{1026b} confers specific immunity.

We next asked whether the same CdiA-CT regions from *Bp* E478 (CDI type I) and *Bp* E479 (CDI type II) also inhibit cell growth, but we were unable to clone the isolated *cdiA-CT*^{E478} and *cdiA-CT*^{E479} coding sequences without the linked *cdiI* genes, presumably due to CdiA-CT toxicity. Therefore, we introduced the *cdiA-CT/cdiI* modules into a pCH450 vector that fuses the *ssrA*(DAS) peptide coding sequence in-frame to the *cdiI* genes and found the resulting constructs were stable under conditions that repress transcription from the P_{BAD} promoter. The *ssrA*(DAS) peptide targets the tagged CdiI proteins for degradation by the ClpXP protease (Wah *et al.*, 2003, McGinness *et al.*, 2006, Poole *et al.*, 2011) and, upon induction with L-arabinose, both *cdiA-CT/cdiI*-DAS expression constructs inhibited the growth of *E. coli* cells (Figs. 2B & 2C). This growth inhibition was specifically blocked by the co-production of cognate CdiI from a compatible plasmid (Fig. 2B & 2C). Together, these results strongly suggest that *Bp* CdiA-CT/CdiI proteins constitute toxin/immunity pairs.

Bp CdiA-CTs have distinct tRNase activities

The C-terminal 96 residues of CdiA-CT^{E478} share sequence identity with the cytotoxic tRNase domain of colicin E5 (56% identity, pairwise BLAST E value = 5e⁻²²), suggesting that nuclease activity is responsible for CdiA-CT^{E478} mediated growth inhibition. Colicin E5 specifically cleaves in the anticodon loops of tRNA^{His}, tRNA^{Tyr}, tRNA^{Asn} and tRNA^{Asp} isoacceptors (Ogawa *et al.*, 1999). Therefore, we asked if CdiA-CT^{E478} exhibits a similar tRNase activity. For these experiments, we used the controlled proteolysis system of McGinness *et al.* to activate the CdiA-CT^{E478} toxin in *E. coli* cells (McGinness *et al.*, 2006). In this system, the CdiA-CT^{E478}/CdiI^{E478}-DAS complex was expressed constitutively in cells deleted for the *sspB* gene, which encodes an adaptor protein required to deliver *ssrA*(DAS)-tagged proteins to the ClpXP protease (Wah *et al.*, 2002, Wah *et al.*, 2003, Poole *et al.*, 2011). Degradation of the *ssrA*(DAS)-tagged CdiI^{E478} immunity protein was then initiated by the production of SspB from a plasmid-borne P_{BAD} promoter and total cellular RNA was isolated for analysis. Northern blot analyses revealed that tRNA₁^{Tyr} was cleaved upon activation of CdiA-CT^{E478} (Fig. 3). In contrast, full-length tRNA₁^{Tyr} persisted in control cells expressing SspB(Δ47), which binds the *ssrA*(DAS) peptide but delivers tagged proteins to ClpXP much less efficiently (Fig. 3) (Wah *et al.*, 2003). Other substrates for colicin E5 (tRNA^{His}, tRNA^{Asn} and tRNA^{Asp}) were cleaved to a lesser degree, but non-substrate tRNA₂^{Arg} appeared unaffected by toxin activation (Fig. 3). These results indicate that CdiA-CT^{E478} exhibits tRNase activity resembling that of colicin E5.

Bioinformatic analysis of the CdiA-CT sequences from *Bp* 1026b and *Bp* E479 failed to reveal clues to their biochemical activities. Given that several characterized CdiA-CTs are nucleases (Aoki *et al.*, 2010), we examined DNA and RNA from inhibited *E. coli* cells for

evidence of nuclease activity. Gel analysis of nucleic acids isolated from cells inhibited by the CdiA-CT^{E479}/CdiI^{E479}-DAS complex failed to reveal obvious nuclease activity (data not shown). However, over-expression of a truncated *cdiA-CT^{E479}/cdiI^{E479}* construct (encoding the C-terminal 160 residues of CdiA-CT^{E479}) from a strong bacteriophage T7 promoter resulted in profound growth arrest and led to the degradation of most cellular tRNAs (Fig. 4). Northern blot analysis revealed a cleavage product for tRNA₂^{Arg}, but similar degradation intermediates were not observed with other tRNA species such as tRNA₃^{Gly} (Fig. 4). We used S1 nuclease protection to identify a cleavage between the conserved thymidine (T55) and pseudouridine (Ψ56) residues in the T-loop of tRNA₂^{Arg} (Fig. S2). This cleavage is predicted to inactivate tRNA and appears to initiate tRNA degradation – both processes could account for growth inhibition. These results also indicate that the N-terminal 156 residues of CdiA-CT^{E479} are not required for RNase activity.

Nuclease activity was not readily observed by gel analysis of DNA and RNA isolated from CdiA-CT_{II}^{1026b}-inhibited *E. coli* cells (Fig. S3). Because the cleavage of one (or a few) tRNA species would be difficult to detect by gel analysis, we screened all 46 *E. coli* tRNA isoacceptors by northern blot hybridization. This analysis revealed that a single tRNA, tRNA_{1B}^{Ala}, was preferentially cleaved upon expression of CdiA-CT_{II}^{1026b} (Fig. S3). S1 nuclease protection analysis showed that this cleavage occurred in the aminoacyl acceptor stem near the 3'-end of the tRNA (data not shown). Cleavage at this position prevents tRNA charging and is presumably the underlying mechanism of growth inhibition. Together, these results show that the *Bp* CdiA-CTs have distinct tRNase activities, each providing a possible mechanism to inhibit cell growth.

CdiA-CT tRNase activities are required for growth inhibition

To determine whether the identified tRNase activities are responsible for growth inhibition, we inactivated each CdiA-CT with a single missense mutation and tested the resulting proteins for growth inhibition activity in *E. coli*. We altered residue Asp272 to Ala in CdiA-CT^{E478} (numbered from Gln1 of the (Q/E)LYN motif) based on previous work showing the corresponding residue in colicin E5 is important for nuclease activity (Lin *et al.*, 2005). The resulting Asp272Ala protein lacked both tRNase and growth inhibitory activities (Fig. S4 and data not shown). The biochemistry and structures of CdiA-CT_{II}^{1026b} and CdiA-CT^{E479} are uncharacterized, so we used multiple sequence alignments with distant protein homologues to identify conserved residues for mutagenesis. An Asp214Ala mutation in CdiA-CT_{II}^{1026b} ablated tRNase activity *in vivo* (Fig. S3) and rendered the domain non-toxic in *E. coli* (Fig. S4). Similarly, mutation of either Asp280 or Asp285 to alanine in CdiA-CT^{E479} eliminated tRNase and growth inhibitory activities (Figs. 4 & S4). Taken together, these results suggest that the tRNase activity of each CdiA-CT is required for growth inhibition.

CdiA-CTs bind to cognate CdiI immunity proteins

Our previous studies have shown that CdiI immunity proteins bind specifically to cognate CdiA-CTs to block nuclease activities (Aoki *et al.*, 2010, Poole *et al.*, 2011). To determine whether the *Bp* CdiA-CT/CdiI pairs also form specific complexes, we cloned each *cdiA-CT/cdiI* pair into an overexpression vector that appends a C-terminal His₆ epitope tag onto the CdiI immunity protein and over-produced the proteins for biochemical analyses. The CdiA-CT_{II}^{1026b}/CdiI_{II}^{1026b}-His₆ proteins were over-produced to high levels and co-purified by Ni²⁺-affinity chromatography, suggesting they form a stable complex. However, we found that the N-terminal region of CdiA-CT_{II}^{1026b} underwent extensive degradation during purification, so we generated a truncated construct that produced a stable CdiA-CT_{II}¹⁰²⁶ (beginning at residue Gly123 with respect to Gln1 in the QLYN motif) for subsequent analyses. This truncated CdiA-CT_{II}^{1026b} co-purified with His₆-tagged CdiI_{II}^{1026b} (Fig. 5),

indicating that the N-terminal region of CdiA-CT_{II}^{1026b} is not required to bind immunity protein. In contrast, the *Bp* E478 and *Bp* E479 CdiA-CT/CdiI-His₆ could not be over-produced to high levels, and induction of these expression constructs inhibited cell growth. We reasoned that low levels of free CdiA-CT were degrading cellular tRNA and thus limiting protein overproduction. Therefore, we used the inactive Asp272Ala and Asp285Ala versions of the *Bp* E478 and *Bp* E479 CdiA-CTs (respectively) and obtained much higher levels of protein overexpression. As observed for the *Bp* 1026b CdiA-CT/CdiI, the untagged CdiA-CTs from *Bp* E478 and *Bp* E479 co-purified with their cognate His₆-tagged CdiI proteins, suggesting complex formation. These results also indicate that the catalytically inactive CdiA-CT domains retain their native fold.

We next asked whether CdiA-CT/CdiI binding interactions are specific for cognate pairs. We isolated each CdiA-CT and CdiI-His₆ protein by Ni²⁺-affinity chromatography under denaturing conditions, and then refolded the purified proteins for *in vitro* binding studies. CdiI-His₆ immunity proteins were used as “bait” to test for the co-purification of untagged CdiA-CT “prey” by Ni²⁺-affinity chromatography. Each CdiI-His₆ protein retained its cognate CdiA-CT on the Ni²⁺-NTA resin, demonstrating that cognate pairs can re-establish their binding interactions after the denaturation and refolding protocol (Fig. 5). In contrast, non-cognate CdiA-CTs did not co-purify with the His₆-tagged CdiI immunity proteins (Fig. 5), demonstrating the specificity of CdiA-CT/CdiI binding interactions. These results are consistent with the specificity of CdiI-mediated immunity to growth inhibition in *E. coli* cells and suggest that CdiI blocks CdiA-CT nuclease activity.

CdiI_{II}^{1026b} specifically blocks CdiA-CT_{II}^{1026b} tRNase activity

We next tested whether purified CdiI immunity protein can block tRNase activity *in vitro*. Because we were unable to obtain appreciable quantities of active CdiA-CT^{E478} or CdiA-CT^{E479}, we used the CdiA-CT_{II}^{1026b}/CdiI_{II}^{1026b} complex as a model. We assayed the *in vitro* tRNase activities of the truncated CdiA-CT_{II}^{1026b} and its Asp214Ala variant using *B. thailandensis* tRNA as a substrate. In contrast to the activity in *E. coli* cells, where only tRNA_{1B}^{Ala} was cleaved (Fig. S3), we found that several tRNA species were cleaved *in vitro* (Fig. 6). The discrepancy in activities could reflect the different tRNA substrates used. However, CdiA-CT_{II}^{1026b} also cleaved most (if not all) *E. coli* tRNAs *in vitro* (data not shown), suggesting that conditions inside the cell limit tRNase activity. Substrate specificity notwithstanding, we found that the tRNase activity was specifically blocked by the addition of CdiI_{II}^{1026b}-His₆, but not by either of the other two non-cognate immunity proteins (Fig. 6). Together, these results show that tRNase activity is contained within the C-terminal 175 residues of CdiA-CT_{II}^{1026b} and support a model in which CdiI blocks the toxin activity of CdiA-CT through direct binding.

Burkholderia CDI systems are functional

The data presented thus far show that *B. pseudomallei* CdiA-CTs are toxic when expressed inside *E. coli* cells, raising the possibility that these systems function in bacterial competition. To test this hypothesis, we examined the predicted CDI system on chromosome I of *Burkholderia thailandensis* E264 (*Bt* E264), which is very closely related to *B. pseudomallei* (Yu *et al.*, 2006). We deleted the single *cdiAIB* gene cluster from *Bt* E264 and found that the resulting strain was inhibited by wild-type CDI⁺ bacteria when co-cultured together on LB agar plates (Fig. S5). Control experiments showed that co-culture of differentially marked wild-type *Bt* E264 cells resulted in no growth inhibition (Fig. S5). To determine whether this growth inhibition was due to CDI activity, we re-introduced the *cdiI*^{E264} immunity gene at the *glmS* locus (on chromosome I) of *Bt* E264 Δ *cdiAIB* cells using a Tn7-based vector (Choi *et al.*, 2005). The resulting strain was no longer inhibited by wild-type *Bt* E264 (Fig. S5), consistent with the predicted immunity function of the CdiI^{E264}

protein. In contrast, *Bt* E264 $\Delta cdiAIB$ cells carrying the *cdiI* genes from *Bp* 1026b, *Bp* E478 or *Bp* E479 were still inhibited by wild-type *Bt* E264 cells (Fig. S5). Taken together, these results strongly suggest that the *Bt* E264 *cdiAIB* genes are expressed and function in growth inhibition.

We next tested whether *Bp* CDI systems also mediate growth inhibition. We cloned the *cdiAIB* gene cluster from *Bp* 1026b (chromosome II) under control of the arabinose-inducible P_{BAD} promoter on a low-copy pVS1-based shuttle plasmid (Heeb *et al.*, 2000) and introduced the resulting construct into wild-type *Bt* E264. *Bt* E264 cells carrying the CDI_{II}^{1026b} system on a plasmid were co-cultured with *Bt* E264 target cells at a 100:1 ratio in both liquid media and on agar plates. Although no growth inhibition was observed in liquid culture (data not shown), viable target cell counts were reduced ~10-fold after 6 h of co-culture on solid media (Fig. 7A). Target cells were further inhibited upon prolonged incubation, with ~100-fold reduction in viable target cell counts after 48 h (Fig. 7A). These results indicated that the CDI_{II}^{1026b} system is functional in inter-species growth inhibition when expressed in *B. thailandensis* cells. To determine whether cell-to-cell contact is required for growth inhibition, we separated inhibitor and target cells with membranes containing pores that either allow (8 μ m) or restrict (0.45 μ m) cell passage (Fig. 7B). Target cell growth was arrested when cells could cross the membrane but not when cells were prevented from mixing (Fig. 7B). Taken together, these results indicate that inhibitor cells must be in close proximity to target cells to inhibit growth.

Bp CDI systems are modular

The CdiA proteins of *E. coli* and related γ -proteobacteria are modular, capable of delivering a variety of CdiA-CT toxins when fused at the VENN peptide sequence (Aoki *et al.*, 2010). Although *Burkholderia* CdiA proteins lack the VENN motif, the variability of *Bp* CdiA-CT/*CdiI* sequences suggests that these systems are also modular. Therefore, we replaced the *cdiA-CT* and *cdiI* coding sequences in the plasmid-borne CDI_{II}^{1026b} system with the corresponding sequences from *Bp* K96243 (CDI type I, 99.6% sequence identity to CdiA-CT^{E478}) and *Bp* E479 to construct chimeric CdiA proteins carrying different CdiA-CTs fused at the (Q/E)LYN peptide sequence. *Bt* E264 expressing the CdiA_{II}^{1026b}-CT^{E479} chimera inhibited target *Bt* E264 cells ~100-fold after 24 h of co-culture, but had no effect on target cells expressing the cognate CdiI^{E479} immunity protein (Fig. 8). Moreover, non-cognate CdiI proteins were unable to protect target cells from CdiA_{II}^{1026b}-CT^{E479} mediated growth inhibition (Fig. 8). These results indicate that the chimeric CdiA_{II}^{1026b}-CT^{E479} protein is functional and that the immunity function is specific for cognate CdiA-CT toxin. The CdiA_{II}^{1026b}-CT^{K96243} chimera also inhibited the growth of *Bt* E264 target cells, but cells expressing the cognate CdiI^{K96243} immunity protein were protected from inhibition (Fig. 8). Together, these results show that *Bp* CdiA proteins are modular and can deliver a variety of the CdiA-CT toxins when fused at the common (Q/E)LYN motif.

Dependence of CDI-mediated growth inhibition on CdiB and tRNase activity

By analogy with other two-partner secretion systems (Mazar & Cotter, 2006, Choi & Bernstein, 2010), the CdiB protein should be required for secretion of CdiA and growth inhibition of target cells. To test whether CdiB is required for CDI in *Burkholderia*, we deleted the *cdiB* gene from the plasmid-borne CDI_{II}^{1026b} system and found that this mutation abrogated growth inhibition (Fig. 9), consistent with the proposed role of CdiB in secretion. Finally, we tested whether the tRNase activities identified for the various *Bp* CdiA-CT proteins are required for cell-mediated growth inhibition. The Asp214Ala mutation that ablates CdiA-CT_{II}^{1026b} tRNase activity (Asp3039Ala in full-length CdiA_{II}^{1026b}) was introduced into the plasmid-borne CDI_{II}^{1026b} system, and the resulting strain was used in growth competition assays. *Bt* E264 carrying the CDI_{II}^{1026b} system

lacking tRNase activity was unable to inhibit target cell growth (Fig. 9), indicating that the mutant system lost all inhibitory activity. We also tested CdiA chimeras carrying the Asp272Ala mutation in CdiA-CT^{K96243} and the Asp285Ala mutation in CdiA-CT^{E479} and found that both mutations also abrogated growth inhibition (data not shown). These results indicate that CdiA-CT tRNase activities are responsible for the inhibition of target cell growth during CDI.

Discussion

The results presented here demonstrate that CDI systems of *B. thailandensis* and *B. pseudomallei* function in growth competition. Although the organization of the *cdi* gene clusters differs from that in most other bacteria, *Burkholderia* CDI systems retain tight linkage of the *cdiA* effector and *cdiI* immunity genes. Like other CDI systems, the C-terminal regions of *Burkholderia* CdiA proteins are variable and contain the growth inhibition activity. The *Bp* CdiA-CT toxins examined here all exhibit RNase activity and target distinct sites within tRNA molecules. Cleaved tRNAs are not functional in protein synthesis, and presumably this tRNA inactivation accounts for growth inhibition. However, we cannot exclude the possibility that cleavage of other critical RNAs contributes to inhibition. Molecular targets notwithstanding, it is clear that RNase activity accounts for the toxicity of *Bp* CdiA-CTs. Catalytically inactive *Bp* CdiA-CTs have no effect on growth when expressed in *E. coli* and do not inhibit *B. thailandensis* growth during cell-mediated CDI. These latter findings indicate that the CdiA-CT is the only toxin delivered by *Bp* CDI systems. The CdiA-CT from *Bt* E264 also has nuclease activity, but is a Mn²⁺-dependent DNA “nickase” rather than a tRNase (K.N. & C.S.H., unpublished results). Thus, all *Burkholderia* CdiA-CTs examined in this study possess nuclease activity capable of inhibiting bacterial growth. Additionally, we find that the predicted *Burkholderia* *cdiI* genes encode immunity proteins that neutralize the toxicity of corresponding cognate CdiA-CTs. Each CdiI immunity protein binds to its cognate CdiA-CT but not to heterologous toxins. This cognate binding interaction blocks CdiA-CT^{II1026b}-mediated tRNase activity *in vitro*, suggesting that the other CdiI proteins also block nuclease activity upon binding cognate CdiA-CT. Based on these results, it seems likely that other *Burkholderia* CdiA-CT domains are toxins and are specifically neutralized by their cognate CdiIs. Our results indicate that CDI systems constitute a complex toxin/immunity network amongst *Burkholderia* species.

Intriguingly, each *Bp* CdiA-CT examined here appears to target tRNA to inhibit cell growth. The CdiA-CT deployed by several uropathogenic *E. coli* strains displays yet another tRNase activity that cleaves within the anticodon loops of many isoacceptors (Aoki et al., 2010). There are a number of possible reasons why tRNA molecules are common targets for CDI systems. Firstly, tRNAs play a critical role in translation, and their inactivation provides an efficient means to interfere with protein synthesis. At least three bacteriocins (colicin E5, colicin D and pyocin S4) are known to cleave specific tRNA isoacceptors to kill susceptible bacteria (Ogawa et al., 1999, Tomita et al., 2000, Michel-Briand & Baysse, 2002). The CdiA-CTs from *Bp* E478 and *Bp* K96243 share significant sequence identity with the nuclease domain of colicin E5 (Aoki et al., 2010), implying exchange of the toxin between these systems. Additionally, the conserved architecture of tRNA molecules could facilitate the evolution of CdiA-CT toxin diversity. According to this model, the CdiA-CT domain would accumulate changes allowing it to recognize different tRNA isoacceptors, while retaining the same overall fold and intrinsic RNase activity. Of course, the associated CdiI immunity protein must also co-evolve to maintain cognate binding interactions and provide immunity to the new toxin. The CdiI proteins from related CDI systems typically exhibit lower sequence identity than the CdiA-CT regions, suggesting that the immunity proteins are evolving more rapidly than the toxins and could be the force driving diversification. Finally, because tRNAs are ubiquitous, these toxins have the potential to inhibit all cells

provided they could be delivered into the cytosol. In accord, we find that the *Bp* CdiA-CTs inhibit growth when produced inside *E. coli* cells. In fact, the molecular targets for all characterized CDI systems are universal and include membranes, DNA and RNA (Aoki *et al.*, 2009, Aoki *et al.*, 2010). This feature of CDI is consistent with the distribution of related toxin/immunity pairs in diverse bacteria (Aoki *et al.*, 2010, Poole *et al.*, 2011). For example, sequences related to CdiA-CT_{II}^{1026b} are found not only in other *Burkholderia* species (*B. ambifaria* IOP40-10, *B. phymatum* DSM 17167 and *B. gladioli* BSR3), but also in the CDI systems of other β - and γ -proteobacteria – including *Ralstonia solanacearum* strains, *Serratia proteomaculans* 568 and *Pseudomonas aeruginosa* PA7 (see Fig. S6). Additionally, two δ -proteobacteria (*Myxococcus xanthus* and *Haliangium ochraceum*) contain uncharacterized proteins with C-terminal domains related to the CdiA-CT_{II}^{1026b} nuclease. This wide distribution indicates that the tRNase domain functions in a variety of proteobacteria, where it presumably mediates inter-cellular growth competition.

Although CdiA-CTs can be exchanged between related CDI systems (Aoki *et al.*, 2010), fusion of the *Bp* CdiA-CT_{II}^{1026b} at the VENN motif of the *E. coli* EC93 CdiA protein does not produce a functional chimera (S.K.A. & D.A.L., unpublished results). Because the toxic nuclease domain must be delivered into target cells, it is possible that specific sequences within the CdiA-CT are required for translocation across the target cell envelope. Comparative sequence analysis indicates that many CdiA-CTs are bipartite. For example, an alignment of *Bp* CdiA-CT_{II}^{1026b} with CdiA-CTs from other bacteria shows two distinct regions of homology (see Fig. S6). As described above, the C-terminal 130 residues of CdiA-CT_{II}^{1026b} share sequence identity with the C-terminal regions of CdiA proteins from other species. This extreme C-terminal region likely represents the actual toxic effector domain, because it contains the RNase activity and is sufficient to bind to immunity protein. The N-terminal region of CdiA-CT_{II}^{1026b} shares significant sequence identity with the corresponding regions from other *Bp* CdiA proteins, yet the C-terminal toxin sequences for each protein are distinct (see Fig. S6). These observations suggest that CdiA-CTs themselves are modular and built from C-terminal toxin domains and N-terminal regions of unknown function. Related N-terminal regions are generally restricted to phylogenetically similar bacteria, suggesting that these domains function in a limited number of species. The N-terminal region could contribute to cell-surface receptor binding, or perhaps help to mediate CdiA-CT translocation through the target cell envelope.

Are the polymorphic CdiA-CT regions of *Bp* CDI systems sufficient to account for environmental distributions? The results presented here clearly demonstrate that *Burkholderia* CDI systems mediate inter-cellular growth competition. Moreover, because the *Bp* 1026b CDI_{II} system is capable of inhibiting *B. thailandensis* growth, it appears likely that CDI⁺ *Burkholderia* cells are able to target other species within the genus. Our analysis shows that there are at least 10 CdiA-CT sequence types in *Bp* strains. There is additional CdiA-CT sequence diversity in the predicted CDI systems of *B. ambifaria*, *B. glumae* and *B. phymatum*. If all of these different CdiA-CTs can be delivered to every *Burkholderia* species, then the systems would constitute an elaborate toxin/immunity network that could perhaps shape species distribution in the environment. Of course, other factors are also likely to contribute to inter-strain competition. For example, bacterial type VI secretion systems have recently been shown to inject toxic effectors into competing bacteria to inhibit their growth (Hood *et al.*, 2010, Schwarz *et al.*, 2010). The proposed interactions are complex and it is not yet possible to predict the winner of a head-to-head competition between strains deploying different CDI and/or type VI systems. Do competing strains inhibit each other equally, such that no strain dominates? Are some toxins more potent than others (for example having a higher tRNase turnover rate), giving a decided advantage to some strains? These questions are further complicated by the fact that most *Bp* strains contain multiple CDI systems, increasing the complexity of any given cell-cell interaction.

Moreover, there appear to be additional *cdiI* genes located in the *cdiI-cdiB* intergenic regions of some CDI systems, which could confer immunity to other bacterial strains.

Another unresolved question is whether the CDI mechanism can account for the observed distributions of *Bp* strains in the environment. Soluble CdiA fragments are not inhibitory and therefore each CDI⁺ inhibitor cell has a very limited “sphere of inhibition”. Therefore, micro-colonies of different *Burkholderia* strains should coexist provided they are segregated. CDI could influence the intersection zones of different strains. *Burkholderia* cells are motile (Tomich *et al.*, 2002, Boonbumrung *et al.*, 2006) and this property should allow different strains to encounter one another periodically. Additionally, the environment is subject to significant seasonal rains that could disrupt micro-niches and facilitate mixing of different *Burkholderia* species (Wuthiekanun *et al.*, 2009), allowing CDI to play a role in niche competition. Alternatively, it is possible that CDI systems perform other functions that are distinct from growth inhibition. Bacteria typically exist as clonal micro-aggregates, and therefore CDI⁺ cells probably deliver toxins to their isogenic siblings. If CDI systems are solely dedicated to growth inhibition, then this delivery of CdiA-CT toxins to immune sibling cells would appear to be futile. However, the exchange of CdiA-CT domains between isogenic cells could serve an unappreciated signaling function. According to this model, the delivered CdiA-CT would have a distinct signaling function when it forms a complex with its cognate CdiI immunity protein.

Experimental procedures

Bacterial strains and plasmids

All bacterial strains used in this study are presented in Table 1. Bacteria were grown in Luria-Bertani (LB) broth or M9 minimal media supplemented with 20 mM D-glucose. Antibiotics were used at the following concentrations: *B. thailandensis* – 500 µg ml⁻¹ kanamycin (Kan); 300 µg ml⁻¹ trimethoprim (Tp); 0.1% *p*-chlorophenylalanine (cPhe); 100 µg ml⁻¹ polymyxin B (PxB); *E. coli* – 150 µg ml⁻¹ ampicillin (Amp); 40 µg ml⁻¹ kanamycin (Kan); 12.5 µg ml⁻¹ tetracycline (Tet); and 50 µg ml⁻¹ trimethoprim (Tp). All bacterial cultures were incubated at 37 °C with aeration unless otherwise indicated. *E. coli* strain X90 and its derivatives were used for analysis of *Bp* CdiA-CT activities. All plasmids used in this study are listed in Table S3 and the details of all plasmid constructions are presented in the Supplement. All *cdiA-CT/cdiI-DAS* plasmid constructs were generated in either *E. coli* CH4180 or CH7157, and then transformed into strain X90 to assess CdiA-CT toxicity. The *cdiA-CT*_{II}^{1026b} and *cdiA-CT/cdiI-DAS* expression plasmids (for CdiA-CT^{E478} and CdiA-CT^{E479}) were co-transformed into *E. coli* X90 along with compatible plasmids encoding the *Bp* CdiI immunity proteins. Overnight cultures of each strain were resuspended at OD₆₀₀ = 0.05 in fresh LB medium supplemented with Tet, Amp and 1 mM IPTG to induce expression of CdiI immunity proteins. After 30 min, CdiA-CT (and CdiI-DAS) expression was induced by addition of L-arabinose to 0.2%, and culture growth monitored at 30 min intervals over the following 5 h. Overproduction of CdiA-CT/CdiI-His₆ complexes was performed in strain CH2016 (Garza- Sánchez *et al.*, 2006).

The *cdiA* (BTH_I2723) and *cdiI/cdiB* (BTH_I2722/BTH_I2721) genes were deleted sequentially from *B. thailandensis* E264 using allelic exchange as described (Kang *et al.*, 2011). Regions upstream and downstream of *cdiA*^{E264} were amplified by PCR using primer pairs 2130/2265 and 2266/2267 (oligonucleotides used in this study are presented in Table S4), and the resulting PCR products combined by overlapping extension PCR (OE-PCR) using primers 2130 and 2267 (Aiyar *et al.*, 1996). The resulting product was digested with HindIII/KpnI and ligated to pEX18Tp-pheS (Kang *et al.*, 2011) to construct plasmid pDAL917. DNA regions encompassing the *cdiI-cdiB* DNA region were amplified by PCR using primer pairs 2130/2004 and 2005/2131, and the resulting products were combined by

OE-PCR using primers 2130 and 2131. The resulting DNA product was digested with HindIII/KpnI and ligated to plasmid pEX18Tp-pheS to construct plasmid pDAL908. The *cdiA*^{E264} gene was first deleted using plasmid pDAL917, followed by deletion of the *cdiI*^{E264} and *cdiB*^{E264} genes using plasmid pDAL908. The *cdiI* immunity genes from *B. pseudomallei* and *B. thailandensis* were placed under control of the *Bt* E264 P_{S12} promoter and inserted into chromosome I of *B. thailandensis* E264 Δ *cdiAIB* cells using a Tn7-based integration vector (Choi *et al.*, 2006, Choi *et al.*, 2008). A cassette formed by oligonucleotides SpeI-S12-UP and S12-XmaI-DN was ligated into SpeI/XmaI-digested pUC18T-miniTn7T-Gm to construct plasmid pUC18T-miniTn7T-Gm-P_{S12}. The *cdiI*_{II}^{1026b} immunity gene was amplified using primers CdiI^{1026b}-XmaI-FWD and CdiI^{1026b}-XhoI-REV and ligated to SmaI-digested pUC18T-miniTn7T-Gm-P_{S12} to construct pUC18T-miniTn7T-Gm-P_{S12}-*cdiI*_{II}^{1026b}. Due to the intrinsic resistance of *Bt* E264 to gentamicin, a fragment containing the P_{S12}-*cdiI*_{II}^{1026b} sequence was excised by HindIII/SpeI digestion and ligated to pUC18T-miniTn7-Kn-FRT (Choi *et al.*, 2008). The resulting plasmid, pAMB7 (pUC18T-miniTn7T-Kan-FRT-P_{S12}-*cdiI*_{II}^{1026b}) was transformed into *E. coli* DH5 α , and the resulting strain used in a four-parent mating with SM10 λ pir/pTNS3 (Miller & Mekalanos, 1988, Choi *et al.*, 2005), HB101 (pRK2013) (Ditta *et al.*, 1980) and *Bt* E264. *Bt* E264 exconjugants were selected with PxB and Kan and clones screened for mini-Tn7 insertions by whole-cell PCR using primer pairs Tn7L/Tn7R and pglmS1/glmS1-DN. The *cdiI*^{K96243} gene was amplified with primers CdiI^{K96243}-XmaI-UP/CdiI^{K96243}-HindIII-DN, the product digested with XmaI/HindIII and ligated to pAMB7 to generate plasmid pAMB8. The *cdiI*^{E479} gene was amplified with primers CdiI^{E479}-KpnI-FWD/CdiI^{E479}-PstI-REV, the product digested with PstI and ligated to PstI/SmaI-digested pAMB7 to generate plasmid pAMB10. The *cdiI* gene from *Bt* E264 was amplified using primer pair 2536/2537 and the resulting product ligated pAMB7 between SmaI and KpnI to generate plasmid pDAL940. Plasmids pAMB8, pAMB10 and pDAL940 were introduced into *E. coli* DH5 α and the resulting strains used in four-parent matings as described above. The *cdi* gene clusters from environmental *Bp* isolates were amplified using oligonucleotides *cdiA-con* and *cdiB-con*, and the resulting products sequenced using the initial primers and then by sequence walking.

RNA analysis

RNA was isolated from *E. coli* and *B. thailandensis* cells as previously described (Garza-Sánchez *et al.*, 2006). Northern blot hybridization was performed as described (Hayes & Sauer, 2003), using 5'-radiolabeled oligonucleotides *Bt* tRNA_{ICG}^{Arg} and *Bt* tRNA_{UGC}^{Ala} probes. S1 nuclease protection assays were conducted essentially as described (Hayes & Sauer, 2003). Oligonucleotide tRNA₂^{Arg} S1 probe was radiolabeled at the 5'-end with polynucleotide kinase to map 3'-cleavage fragments and at the 3'-end with terminal transferase to map 5'-cleavage fragments. The marker oligonucleotides were radiolabeled at the appropriate termini and used as gel migration standards.

In vitro analysis of CdiA-CT/CdiI proteins

CdiA-CT/CdiI-His₆ complexes were purified by Ni²⁺-affinity chromatography and the proteins separated under denaturing conditions as described (Aoki *et al.*, 2010, Poole *et al.*, 2011). Isolated CdiA-CTs were dialyzed against 10 mM acetic acid and stored at 4 °C. All CdiI-His₆ proteins were overproduced in the absence of CdiA-CT and purified by Ni²⁺-affinity chromatography. CdiI-His₆ proteins were dialyzed against storage buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM β -mercaptoethanol (β -ME)]. CdiA-CT/CdiI-His₆ binding studies were conducted in binding buffer [50 mM Tris-HCl (pH 7.5), 400 mM NaCl, 10 mM β -ME]. Purified CdiA-CT (5 μ M) was incubated with CdiI-His₆ (5 μ M) for 30 min at 23 °C and an aliquot removed for gel analysis. Ni²⁺-NTA resin was then added and incubated for 1 hr. The resin was then collected by centrifugation and the supernatant removed as the unbound or free fraction. The resin was washed with binding buffer, and the

bound proteins eluted in binding buffer supplemented with 250 mM imidazole. Samples were analyzed by SDS-PAGE and staining with Coomassie R-250.

CdiA-CT_{II}^{1026b} activity was examined *in vitro* using RNA isolated from *Bt* E264 cells. Purified CdiA-CT_{II}^{1026b} or the inactive Asp214Ala variant (at 1 μ M final concentration) were incubated with 2 μ g of total cellular RNA (isolated from *Bt* E264) in storage buffer supplemented with 1 mM MgCl₂ for 1 hr at 37 °C. Where indicated, purified CdiI-His₆ proteins (at 2 μ M final concentration) were also included. Reactions were extracted with guanidinium isothiocyanate-phenol as described (Garza-Sánchez et al., 2006), and 0.5 μ g of each RNA sample was analyzed by polyacrylamide gel electrophoresis and northern blot hybridization as described above.

CDI growth competition

Wild-type *B. thailandensis* E264 was used in competitions with various target cells derived from *Bt* E264. Target cells (Bt6, Bt7, Bt8, Bt9, Bt36 and Bt56) were grown to early log phase (OD₆₀₀ $\approx$ 0.2–0.5) in LB medium. Cultures were adjusted to OD₆₀₀ = 0.2, mixed at an inhibitor to target cell ratio of 1000 to 1, and 150 μ L of the co-culture was plated onto LB agar followed by incubation at 37 °C. At the indicated time points, cells were harvested from the plates with 1 mL of M9 salts, and viable cell counts determined. For competitions with the plasmid-borne CDI_{II}^{1026b} system, the *Bp cdiAIB* gene cluster was induced with 0.2% L-arabinose in M9 minimal medium (M9-Ara) prior to co-culture with target cells at a 100:1 inhibitor to target ratio. After different periods of incubation, cells were collected and viable cell counts determined by plating on selective media. Proximity-dependence of growth inhibition was determined using a modified filter assay (Aoki et al., 2005). Inhibitor cells (*Bt* E264 carrying plasmid pJSW1-6) or mock inhibitor cells lacking the CDI_{II}^{1026b} CDI system (*Bt* E264 carrying plasmid pJSW2) were plated onto M9-Ara agar and nitrocellulose filters placed on top of the cells. Bt6 target cells were then plated on top of the filter, and the plate incubated at 37°C. After 24 h of incubation, the cells were recovered and plated onto LB-agar supplemented with Kan to quantify viable target cells. Three technical replicates were conducted for each growth competition time point, and all experiments were repeated (biological replicate) at least once.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Genomic organization of *B. pseudomallei* *cdi* gene clusters

The genomic neighborhoods of representative *Bp* CDI systems encoding the ten different CdiA-CT/CdiI sequence types (CDI types **I** through **X**) are presented. The *Bp* strain and encoding chromosome (if known) for each representative CDI system is given in parentheses. *Bp* 1106a carries two predicted CDI systems on chromosome I (types **VII** and **X**); these loci are labeled **i** and **ii** to distinguish the two systems. The *cdiA* and *cdiB* genes are shown in olive and yellow (respectively); and the *cdiA*-CT regions (corresponding to the 3'-end of *cdiA* genes) and *cdiI* genes are depicted in various colors. Genes related to transposases are shown in orange and all other coding sequences, including *rhs* (rearrangement hotspot) genes are white. Some systems contain additional small coding sequences between the *cdiI* and *cdiB* genes (white triangles). The asterisk indicates a putative immunity gene that is related to *cdiI* from *Enterobacter aerogenes* KCTC 2190.

Figure 2. *B. pseudomallei* CdiA-CTs inhibit cell growth

The CdiA-CT/CdiI toxin/immunity network was examined in *E. coli* cells using a two-plasmid strategy. CdiA-CT_{II}^{1026b} and the CdiA-CT^{E478}/CdiI^{E478}-DAS and CdiA-CT^{E479}/CdiI^{E479}-DAS complexes were produced from plasmid pCH450 upon induction with L-arabinose (indicated by the downward arrows). Each culture was also induced at 0 min with IPTG to produce *Bp* CdiI immunity protein (indicated in the inset legend) from a compatible pTrc plasmid. **A**) CdiA-CT_{II}^{1026b} production was induced at 30 min, and growth monitored by measuring the optical density at 600 nm (OD₆₀₀). **B**) CdiA-CT^{E478}/CdiI^{E478}-DAS complex production was induced at 30 min and growth monitored by OD₆₀₀. The CdiA-CT^{E478} is liberated in these cells through degradation of ssrA(DAS)-tagged CdiI^{E478} immunity protein. **C**) CdiA-CT^{E479}/CdiI^{E479}-DAS complex production was induced at 30 min and growth monitored by OD₆₀₀. The CdiA-CT^{E479} is liberated in these cells through degradation of ssrA(DAS)-tagged CdiI^{E479} immunity protein. In each panel, the black curve represents the growth of control cells carrying empty pCH450 and pTrc plasmid vectors.

Figure 3. CdiA-CT^{E478} has tRNase activity similar to colicin E5
 The CdiA-CT^{E478}/CdiI^{E478}-DAS complex was produced in *E. coli* Δ *sspB* cells and degradation of the *ssrA*(DAS)-tagged CdiI^{E478} was initiated at 0 hr by induction of the SspB adaptor protein. RNA samples were taken at the indicated time points for northern blot analysis. Cells expressing SspB(Δ47), which does not efficiently deliver CdiI^{E478}-DAS to the ClpXP protease, were used as a control. Each gel lane contains 10 μg of total RNA, which was hybridized against oligonucleotide probes specific for *E. coli* tRNA₁^{Tyr}, tRNA^{Asn}, tRNA^{His} and tRNA₂^{Arg}. The gel migration positions for full-length and cleaved tRNA are indicated.

Figure 4. CdiA-CT^{E479} has tRNase activity

CdiA-CT^{E479}/CdiI^{E479} production was induced by IPTG in *E. coli* cells, and RNA samples taken for gel and northern blot analyses. RNA from cells expressing CdiA-CT^{E479} with the Asp285Ala mutation was also analyzed. RNA was hybridized against oligonucleotide probes specific for *E. coli* tRNA^{2Arg} and tRNA^{3Gly}. The gel migration positions for ribosomal RNA (rRNA) and tRNA are indicated.

Figure 5. *B. pseudomallei* CdiI immunity proteins bind specifically to cognate CdiA-CT
Purified CdiA-CTs and His₆-tagged CdiI immunity proteins were incubated together, then subjected to Ni²⁺-affinity chromatography to bind CdiI-His₆ and any associated CdiA-CT. All fractions were run on SDS-polyacrylamide gels and stained with Coomassie blue. The lanes labeled **input** show the unfractionated CdiA-CT/CdiI-His₆ mixtures, the lanes labeled **free** show proteins that did not bind Ni²⁺ resin, and the lanes labeled **bound** show proteins that bound to the resin.

Figure 6. CdiI_{II}¹⁰²⁶ immunity protein specifically blocks CdiA-CT_{II}^{1026b} tRNAse activity *in vitro*

Burkholderia thailandensis tRNA was incubated with purified CdiA-CT_{II}^{1026b} and analyzed by gel electrophoresis and northern blot hybridization. Different CdiI immunity proteins were included in reactions where indicated, and CdiA-CT_{II}^{1026b} containing the Asp214Ala mutation was also assayed for tRNAse activity. RNA was hybridized against oligonucleotide probes specific for *B. thailandensis* tRNA_{ICG}^{Arg} and tRNA_{UGC}^{Ala}. The gel migration positions of full-length tRNA are indicated.

Figure 7. Growth inhibition mediated by the CDI_{II}^{1026b} system is contact-dependent
A) *Bt* E264 inhibitor cells carrying the *Bp* CDI_{II}^{1026b} system or empty cosmid vector (mock inhibitors) were co-cultured with *Bt* E264 target cells as described in Experimental Procedures. Cells were collected at the indicated time points, and viable counts for both inhibitor and target cells were determined as colony forming units per mL (CFU/mL). **B)** A filter competition assay was carried out as described in Experimental Procedures. Inhibitor cells expressing the CDI_{II}^{1026b} system, or mock inhibitor cells lacking the CDI_{II}^{1026b} system were separated from *Bt*6 target cells by filters with the indicated pore sizes, and viable target cell counts were determined after 24 h incubation. CFU/mL values were determined from three technical replicates and are expressed as the mean $\pm$ SEM.

Figure 8. *B. pseudomallei* CDI systems are modular

Bt E264 target cells were co-cultured with inhibitor cells expressing the *Bp* CDI_{II}^{1026b} system or chimeric CDI systems that deploy the CdiA-CTs from *Bp* K96243 or *Bp* E479. Target cells were provided with *Bp* *cdiI* immunity genes as indicated. The competitive index was calculated as the inhibitor cell CFU/target cell CFU ratio at $t = 0$ h divided by the inhibitor cell CFU/target cell CFU ratio at $t = 24$ h. Competitive indices were determined from three technical replicates and are expressed as the mean $\pm$ SEM.

Figure 9. CDI-mediated growth inhibition requires CdiB and tRNase activity

Bt24 cells (*B. thailandensis* E264 $\Delta cdiAIB$) carrying a plasmid-borne copy of the CDI_{II}^{1026b} system or its mutant derivatives that either lack the *cdiB* gene or encode the Asp3039Ala variant of CdiA_{II}^{1026b} (corresponding to the catalytically inactive Asp214Ala change in CdiA-CT_{II}^{1026b}) were incubated with Bt6 target cells. Bt7 target cells (expressing the *cdiI*_{II}^{1026b} immunity gene) were used as a negative control. Growth competition analysis was carried out as described in Experimental Procedures. CFU/mL values were determined from three technical replicates and are expressed as the mean $\pm$ SEM

Table 1

Bacterial strains used in this study.

Strain	Description ^a	Reference
X90	F' <i>lacI^q lac' pro' ara Δ(lac-pro) nalI argE(amb) rif^r thi-1</i>	(Beckwith & Signer, 1966)
CH2016	X90 (DE3) <i>Δrna ΔslyD::kan</i> , Kan ^R	(Garza-Sánchez et al., 2006)
CH4180	X90 <i>ΔsspB</i>	(Poole et al., 2011)
CH7157	X90 <i>ΔclpX ΔclpA::kan</i> , Kan ^R	(Poole et al., 2011)
<i>Burkholderia thailandensis</i> E264	<i>Burkholderia thailandensis</i> wild-type isolate	(Brett <i>et al.</i> , 1998)
Bt6	<i>B. thailandensis</i> E264 <i>attTN7::miniTn7T-Kan</i>	This study
Bt7	<i>B. thailandensis</i> E264 <i>attTN7::miniTn7T-kan-cdiI^{1026b}</i> , Kan ^R	This study
Bt8	<i>B. thailandensis</i> E264 <i>attTN7::miniTn7T-kan-cdiI^{K96243}</i> , Kan ^R	This study
Bt9	<i>B. thailandensis</i> E264 <i>attTN7::miniTn7T-kan-cdiI^{E479}</i> , Kan ^R	This study
Bt23	<i>B. thailandensis</i> E264 <i>ΔcdiA</i>	This study
Bt24	<i>B. thailandensis</i> E264 <i>ΔcdiAIB</i>	This study
Bt36	<i>B. thailandensis</i> E264 <i>ΔcdiAIB attTN7::miniTn7-kan</i> , Kan ^R	This study
Bt56	<i>B. thailandensis</i> E264 <i>ΔcdiAIB attTN7::miniTn7-P_{S12}-cdiI^{E264}</i> , Kan ^R	This study

^a Abbreviations used: Kan^R, kanamycin resistant