Interbacterial signaling via *Burkholderia* contact-dependent growth inhibition system proteins

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In prokaryotes and eukaryotes, cell–cell communication and recognition of self are critical to coordinate multicellular functions. Although kin and kind discrimination are increasingly appreciated to shape naturally occurring microbe populations, the underlying mechanisms that govern these interbacterial interactions are insufficiently understood. Here, we identify a mechanism of interbacterial signal transduction that is mediated by contact-dependent growth inhibition (CDI) system proteins. CDI systems have been characterized by their ability to deliver a polymorphic protein toxin into the cytoplasm of a neighboring bacterium, resulting in growth inhibition or death unless the recipient bacterium produces a corresponding immunity protein. Using the model organism *Burkholderia thailandensis*, we show that delivery of a catalytically active CDI system toxin to immune (self) bacteria results in gene expression and phenotypic changes within the recipient cells. Termed contact-dependent signaling (CDS), this response promotes biofilm formation and other community-associated behaviors. Engineered strains that are isogenic with *B. thailandensis*, except the DNA region encoding the toxin and immunity proteins, did not display CDS, whereas a strain of *Burkholderia dolosa* producing a nearly identical toxin-immunity pair induced signaling in *B. thailandensis*. Our data indicate that *bcpAIOB* loci confer dual benefits; they direct antagonism toward non-self bacteria and promote cooperation between self bacteria, with self being defined by the *bcpAIOB* allele and not by genealogic relatedness.

signal transduction | sociomicrobiology | contact-dependent competition | two-partner secretion | biofilm

Bacteria typically live in complex communities such as biofilms. To do so, they must cooperate and compete with neighboring microbes, which requires that they sense not only environmental conditions but also the neighboring microbes themselves. Understanding the underlying mechanisms that govern microbial community dynamics is critical because biofilms can cause or exacerbate human disease (1) and microbial community composition can affect immune system development and other aspects of human health (2).

Although the importance of diffusible quorum-sensing molecules in interbacterial communication has been established (3), our appreciation of how microbes sense and respond to their neighbors, particularly those with which they are in direct contact, is incomplete. In the social amoeba *Dictyostelium discoideum*, kin discrimination and cooperation between self cells is achieved by interactions between matching allelic pairs of surface proteins (4, 5). Similarly, allele-specific interactions between surface proteins restrict *Myxococcus xanthus* outer membrane protein exchange and multicellular activities to occur only between certain individuals (6, 7). Examples also exist of bacterial recognition that involves immunity to a self-produced toxin, including those delivered via type VI secretion (8, 9) and contact-dependent growth inhibition (CDI) systems (10, 11).

CDI systems are polymorphic toxin delivery systems that function in a contact-dependent manner. They are composed of two-partner secretion (TPS) pathway proteins and are widespread among Gram-negative bacteria (12). *Burkholderia*-type and *Escherichia coli*-type CDI systems are encoded by *bcpAIOB* and *cdiBAI* operons, respectively (13–15). The *bcpB*/*cdiB* gene encodes the outer membrane TpsB-family protein that translocates BcpA/CdiA, the TpsA-family protein, to the cell surface (16). The N-terminal ∼2,800 aa of BcpA/CdiA proteins are highly conserved whereas the C-terminal ∼300 aa (termed the BcpA-CT or CdiA-CT) are variable. A conserved motif, Nx(E/Q)LYN or VENN, delineates the conserved and variable regions (12). Most BcpA-CT and CdiA-CT polypeptides function as nucleases in vitro and are sufficient to mediate cell death when produced intracellularly (12–14). According to the current model, BcpA-CT or CdiA-CT toxins are delivered to recipient bacteria upon cell–cell contact, exploiting outer membrane and inner membrane proteins on the recipient cell for entry to the cytoplasm (17, 18). CDI^+ bacteria are protected from autoinhibition by production of an immunity protein, BcpI or CdiI. BcpI/CdiI proteins covary with their cognate BcpA-CT/CdiA-CT toxins, binding to and inactivating only cognate (encoded by the same allele), but not heterologous (encoded by a different allele), toxin (12, 14). CDI systems therefore provide a mechanism for elimination of non-self neighboring bacteria, with self being defined by the specific *bcpAIOB* (or *cdiBAI*) allele.

Emerging evidence suggests that, in addition to mediating interbacterial killing (12–15), CDI systems can promote cooperative behaviors. The CdiA proteins HrpA of *Neisseria meningitidis* (19) and HecA of *Dickeya dadantii* (20), as well as putative CdiA proteins in *Xyella fastidiosa* (21), *Xanthomonas fuscans* (22), and *Xanthomonas axonopodis* (23), are required for biofilm formation and/or virulence. *E. coli* EC93 CdiA was also recently shown to mediate biofilm formation and interbacterial adhesion, in a manner independent of the $CdiA\text{-}CT^{EC93}$ toxin (24).

Significance

How bacteria interact with one another has implications for human health and disease because complex bacterial communities like biofilms can impact agriculture, infection transmission, and disease progression. Contact-dependent growth inhibition systems are proteins produced by many bacteria that deliver toxins to neighboring bacteria, allowing the producing cell to inhibit competitors that do not make the correct antidote. Here, we show that these systems not only antagonize competitors but also mediate communication and cooperation between bacteria that produce identical toxin/antidote pairs. Antidote-containing bacteria that are targeted by toxins change their gene expression to promote biofilm formation. Leading to a more complete understanding of how these antibacterial protein systems function, this study will inform future development of therapeutics, vaccines, and decontaminants.

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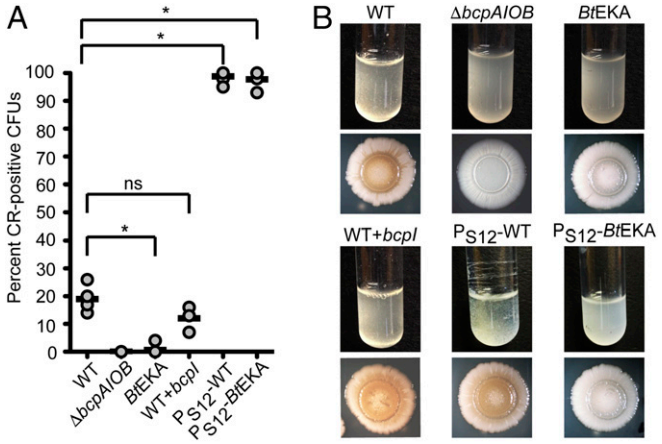

Fig. 1. Community-associated behaviors require BcpA catalytic activity independently of interbacterial growth inhibition. (*A*) Proportion of CR-binding colonies of wild-type bacteria, Δ*bcpAIOB* mutant, *B. thailandensis* producing catalytically inactive BcpA (*Bt*EKA), wild-type bacteria constitutively expressing *bcpI* (WT+*bcpI*), and *B. thailandensis* constitutively expressing WT *bcpAIOB* (P_{S12}-WT) or *bcpAIOB* in which *bcpA* contains toxin-inactivating mutations (P_{S12}-*Bt*EKA). Lines show the mean of at least three independent experiments per strain (n = 3–6). (*B*) Aggregation in minimal medium (*Upper*) and pigment production by colony biofilms (*Lower*) of the strains in *A*. *P < 0.05; ns, not significant.

We recently demonstrated that *Burkholderia thailandensis* also requires the *bcpAIOB* genes for biofilm formation (25). The BcpA-CT has DNA nickase activity in vitro, and amino acid substitutions that abolished its enzymatic activity also prevented bacteria producing this inactive protein from killing susceptible recipient cells (14, 25). Interestingly, bacteria producing inactive BcpA were also unable to form a biofilm, indicating that BcpA activity, and not simply the presence of BcpA on the bacterial surface, is required. Bacterial suicide or fratricide contributes to biofilm formation by some organisms (26, 27). However, prevention of interbacterial killing (i.e., CDI) among wild-type *B. thailandensis* by constitutive production of the immunity protein BcpI did not affect biofilm development, indicating that a similar fratricidal mechanism could not explain the requirement for BcpA enzymatic activity (25). Described here, we observed additional phenotypes correlating with BcpA activity and set out to test the hypothesis that CDI systems may also function in interbacterial signaling to mediate cooperative behaviors, such as biofilm development, when interacting with self bacteria (those expressing the same *bcpAIOB* or *cdiBAI* allele).

Results

CDI-Independent Phenotypes Require BcpA Activity. In other organisms, affinity for the dye Congo red (CR) often correlates with biofilm formation because CR binds polysaccharides (28), amyloid proteins (29), and other surface structures. Toward investigating the requirement of BcpA catalytic activity in biofilm development, we plated bacteria on medium containing CR. Approximately 20% of the colonies formed by wild-type *B. thailandensis* bound the dye whereas all colonies formed by Δ*bcpAIOB* bacteria remained white (Fig. 1*A*). A recombinase-based reporter containing the *bcpA* promoter region indicated that CR^+, but not CR^-, colonies had expressed *bcpAIOB* to high levels (*SI Appendix*, Fig. S1), consistent with the fact that high *bcpAIOB* gene expression is stochastic, occurring in only a small proportion of bacteria grown under laboratory conditions (13). A mutant (*Bt*EKA) that produces wild-type levels of catalytically inactive BcpA due to two amino acid substitutions (E3064A and K3066A) in the putative toxin active site (25) also did not bind CR (Fig. 1*A*), indicating that the enzymatic activity of the protein is required for this phenotype. However, interbacterial killing was not required because constitutive expression of the immunity-encoding *bcpI* (which prevents interbacterial growth inhibition) (13) did not alter CR binding. Constitutive expression of wild-type *bcpAIOB* from the *rpsL* promoter P_{S12} (P_{S12}-WT) resulted in 100% CR^+ colonies. Constitutive expression of *bcpAIOB* containing the toxin-inactivating mutations (P_{S12}-*Bt*EKA) also resulted in 100% CR^+ colonies (see, for example, Fig. 3*A*) although the amount of CR bound by this mutant was significantly less than that bound by P_{S12}-WT (*SI Appendix*, Fig. S1). These data suggest that CR binding to *B. thailandensis* is the sum of direct binding to BcpA

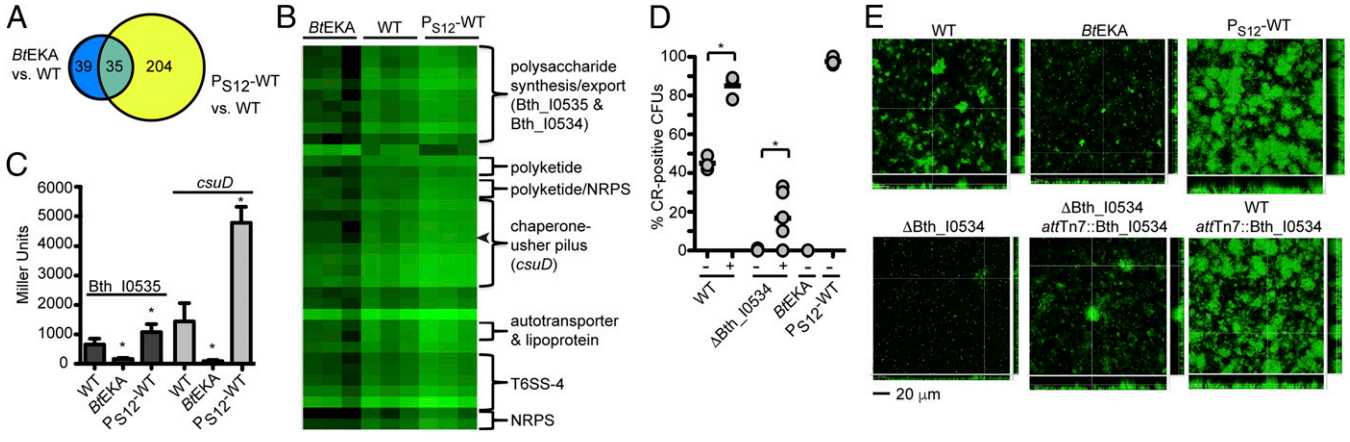

Fig. 2. Catalytically active BcpA affects expression of genes associated with community behaviors. (*A*) Genes identified by RNA-seq as significantly differentially expressed (P < 0.05) by *Bt*EKA mutants compared with wild-type bacteria (blue) and P_{S12}-WT mutants compared with the wild-type strain (yellow). (*B*) Heat map of RPKMs (reads per kilobase of transcript per million reads mapped) for the 35 genes that were oppositely expressed in P_{S12}-WT and *Bt*EKA mutants compared with wild-type *B. thailandensis* (WT). Shading indicates low (black) and high (green) RPKM values for each of triplicate samples of each strain. Genes are arranged in chromosomal order, and putative operons/multigene loci are annotated to the right. Arrowhead shows position of *csuD*. (*C*) β-Galactosidase activity (in Miller units) for wild-type, *Bt*EKA, and P_{S12}-WT bacteria carrying *lacZ* reporters within the coding regions of Bth_I0535 (*Left*, dark gray bars) or *csuD* (*Right*, light gray bars). Bars represent the mean of three independent experiments, and error bars show the SD. *P < 0.05 compared with wild-type cultures. (*D*) Proportion of CR-binding colonies of *Bt*EKA, P_{S12}-WT, and WT and ΔBth_I0534 mutant bacteria with (+) and without (–) P_{S12}-Bth_I0534 at a chromosomal *att*Tn7 site. Bars show the mean of three independent experiments (n = 3–6 per strain). *P < 0.05. (*E*) Z-stack images of 48-h biofilms formed by GFP-marked bacterial strains shown in *D*. (Scale bar: 20 μm.)

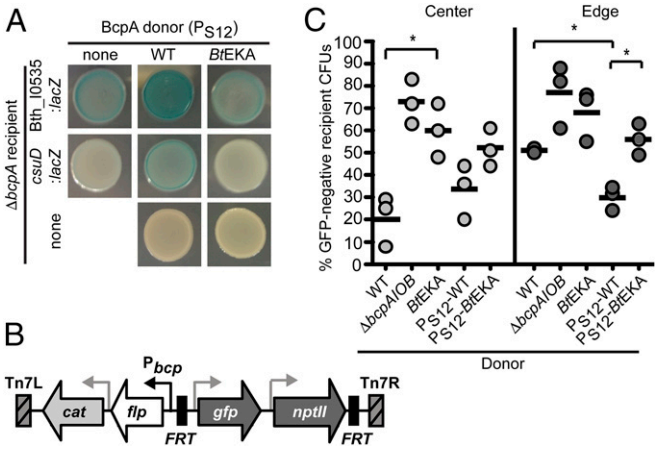

Fig. 3. Catalytically active BcpA acquired from neighboring bacteria affects gene expression in recipients. (*A*) Expression of Bth_I0535 (*Top*) and *csuD* (*Middle*) in Δ*bcpA* recipient bacteria containing *lacZ* reporters for these genes after incubation with no BcpA donor (first spot) or coincubation (at a 1:1 ratio) with *B. thailandensis* constitutively producing wild-type (P_{S12}-WT, middle spot) or inactive BcpA (P_{S12}-*Bt*EKA, last spot) on X-gal agar. Donor (*Bottom*) and recipient (first column) cells incubated alone are also shown. (*B*) Diagram of the modified P_{bcp}-*flp* reporter cassette. The putative *bcpA* promoter (black arrow) drives expression of *flp* recombinase. Gray arrows indicate constitutive promoters. Flp recombinase target (*FRT*) sites (black boxes) flank *nptII* (conferring kanamycin resistance) and *gfp* genes. A *cat* gene (conferring chloramphenicol resistance, Cm^R) is located outside the *FRT* sites. Tn7L and Tn7R (hatched boxes) sequences target the cassette to an *att*Tn7 site on one of the two *B. thailandensis* chromosomes. (*C*) Proportion of wild-type bacteria carrying the P_{bcp}-*flp* reporter (recipients) that expressed *bcpAIOB* when incubated with the indicated (Cm^S) mutants (donors) at a 1:100 ratio (recipient:donor) on M63 agar. The fraction of the reporter recipient population having expressed *bcpAIOB* is shown as the percentage of Cm^R colonies having lost GFP fluorescence (% GFP^- recipient cfus). Reporter recipient bacteria were recovered from both the center (*Left*) and edge (*Right*) of the coculture colony spot. $^*P < 0.05$.

(independently of its catalytic activity), as well as binding to an unknown structure, perhaps exopolysaccharide, that depends on BcpA activity.

During our studies, we also noticed that *B. thailandensis* colony biofilms developed a yellow-gold color when cultured on LSLB agar for extended periods (Fig. 1*B*). We refer to this phenotype as "pigment production" although it is not clear whether the gold color is indeed a pigment. Colony biofilms of Δ*bcpAIOB,* *Bt*EKA, and P_{S12}-*Bt*EKA mutants remained white, indicating that BcpA catalytic activity is required for this coloration. Expression of *bcpI* in wild-type bacteria did not alter pigment production.

Finally, as we previously described (13), *bcpAIOB* are required for *B. thailandensis* to autoaggregate in minimal medium, and constitutive expression of the genes results in hyperaggregation (Fig. 1*B* and *SI Appendix*, Fig. S2). Constitutive production of the immunity protein did not affect aggregation of wild-type bacteria, but the catalytic activity of BcpA was required (Fig. 1*B* and *SI Appendix*, Fig. S2). These data indicate that three distinct phenotypes—CR binding, pigment production, and aggregation—all require BcpA catalytic activity but not interbacterial growth inhibition.

BcpA Alters Expression of Genes Involved in Biofilm Formation. We hypothesized that BcpA-CT toxin exchange between wild-type bacteria, although not leading to cell death, triggers a change in gene expression that is responsible for these, and possibly other, phenotypes. To test this hypothesis, we compared transcript levels among triplicate samples of wild-type, *Bt*EKA, and P_{S12}-WT bacteria cultured in minimal medium using RNA-sequencing analysis. Transcript abundance was significantly different by more than twofold for 74 genes when wild-type and *Bt*EKA bacteria were compared (Fig. 2*A* and *SI Appendix*, Table S1) and for 239 genes when wild-type and P_{S12}-WT bacteria were compared (Fig. 2*A* and *SI Appendix*, Table S2). Comparison of P_{S12}-WT and *Bt*EKA mutants (strains displaying maximum and minimum biofilm, CR binding, and pigmentation phenotypes) revealed 841 genes with differential expression—410 that were greater in P_{S12}-WT than in *Bt*EKA (*SI Appendix*, Table S3) and 431 that were decreased in P_{S12}-WT compared with *Bt*EKA (*SI Appendix*, Table S4). To narrow our focus, we reasoned that the genes most strongly affected by BcpA activity should be oppositely expressed in P_{S12}-WT and *Bt*EKA mutants compared with wild-type bacteria, and 35 genes fit these stringent criteria (Fig. 2 *A* and *B* and *SI Appendix*, Table S5). The majority of these genes were located within several putative operons and multigene loci that are predicted to encode a type VI secretion system, putative polysaccharide synthesis proteins, nonribosomal peptide/polyketide synthesis enzymes, and a chaperone-usher pilus (*SI Appendix*, Fig. S3).

To determine gene expression changes revealed by the RNA-seq analysis using another method, we generated reporter strains by introducing promoterless *lacZ* genes within the coding sequence of Bth_I0535, which is predicted to encode a polysaccharide glycosyltransferase, and Bth_I2677 (*csuD*), which is predicted to encode a pilus usher protein. Expression of Bth_I0535-*lacZ* and *csuD*-*lacZ* was significantly higher in P_{S12}-WT and lower in *Bt*EKA than in wild-type *B. thailandensis* (Fig. 2*C*).

An ortholog of the putative transcriptional regulator Bth_I0534 (69% identity) is required by *Burkholderia cenocepacia* for biofilm formation and expression of polysaccharide synthesis genes (30, 31). Deletion of Bth_I0534 abrogated CR binding (Fig. 2*D*) and biofilm formation in *B. thailandensis* (Fig. 2*E* and *SI Appendix*, Fig. S4). These defects could be partially complemented by reintroduction of Bth_I0534 at a neutral chromosomal site, but not by coinoculation with wild-type bacteria (*SI Appendix*, Fig. S4). Overexpression of Bth_I0534 in wild-type bacteria led to increased CR binding (Fig. 2*D*) and biofilm biomass (Fig. 2*E* and *SI Appendix*, Fig. S4). Taken together, these data show that *B. thailandensis* BcpA catalytic activity affects expression of many genes, including at least one that is required for biofilm formation.

BcpA Delivered from Neighbor Bacteria Is Sufficient to Alter Gene Expression. We hypothesized that the gene expression changes observed by RNA-seq were due to the exchange of catalytically active BcpA molecules between bacteria. To test this hypothesis, we constructed a Δ*bcpA* mutant that, as expected, could no longer kill susceptible Δ*bcpAIOB* target cells, but was protected from growth inhibition (*SI Appendix*, Fig. S5). When incubated on X-gal agar, colony biofilms of Δ*bcpA* mutant bacteria containing Bth_I0535::*lacZ* or *csuD*::*lacZ* reporters were light colored, indicating that expression of these genes was low in the absence of a BcpA-derived signal (Fig. 3*A*). When these reporter bacteria were cocultured with *B. thailandensis* constitutively producing wild-type BcpA, the mixed colony biofilms were darker blue, indicating that Bth_I0535 and *csuD* expression was greater when the reporter strains were surrounded by wild-type neighbors. Coculture of the Δ*bcpA* reporters with a strain producing inactive BcpA did not cause the blue color change. These results indicate that interaction with bacteria producing active BcpA is sufficient to increase expression of Bth_I0535 and *csuD*, suggesting that recipient cells alter their gene expression in response to acquisition of catalytically active BcpA-CT from their neighbors.

BcpA Decreases *bcpAIOB* Gene Expression in Neighboring Bacteria. To further measure gene expression in recipient bacteria during interactions with BcpA-producing neighbors, we used a sensitive recombinase-based reporter system developed in our laboratory (Fig. 3*B*) (32). In this system, activation of a promoter of interest leads to Flp recombinase production, excision of FRT-flanked *gfp*

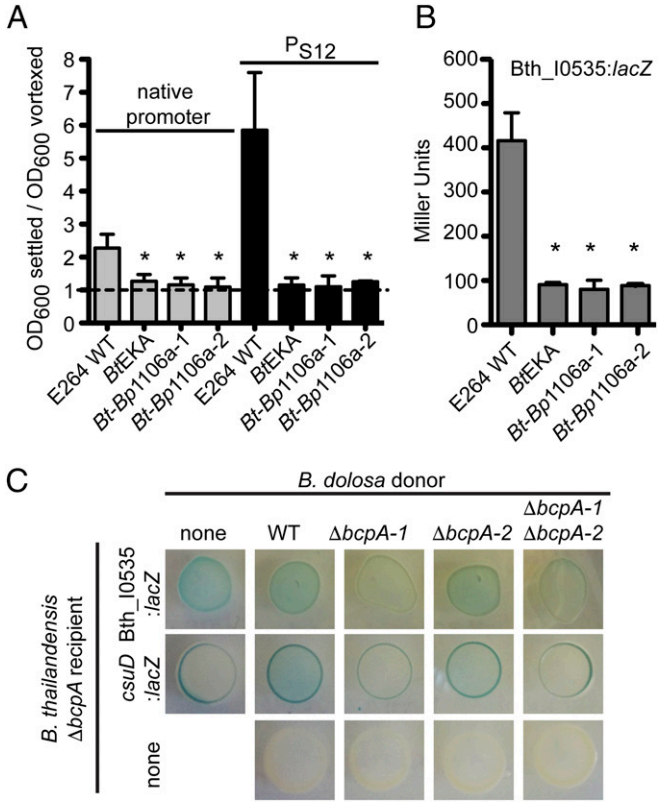

Fig. 4. *B. thailandensis* responds to self but not non-self BcpA. (*A*) Aggregation of wild-type *B. thailandensis*, *Bt*EKA mutants, and chimeric strains *Bt-Bp*1106a-1 and *Bt-Bp*1106a-2 (gray bars, "native promoter") or strains in which the *bcpAIOB* locus is driven by the constitutive promoter, P_{S12} (black bars, "P_{S12}"). The ratio of the OD_{600} of a settled culture to the OD_{600} of the same culture after vortexing is shown. Dashed line shows an OD_{600} ratio of 1, which indicates no aggregation. Bars represent the mean of four independent experiments, and error bars denote the SD. *$P < 0.05$ compared with WT or P_{S12}-WT aggregation. (*B*) β-Galactosidase activity (in Miller units) for WT and *Bt*EKA bacteria, and chimeric strains *Bt-Bp*1106a-1 and *Bt-Bp*1106a-2 carrying *lacZ* reporters within the coding region of Bth_I0535. Bars represent the mean of three independent experiments, and error bars show the SD. *$P < 0.05$ compared with WT cultures. (*C*) Expression of Bth_I0535::*lacZ* (*Top*) and *csuD*::*lacZ* (*Middle*) reporters in *B. thailandensis* Δ*bcpA* recipient bacteria after coincubation (at a 1:5 ratio) with *B. dolosa* wild-type or Δ*bcpA-1*, Δ*bcpA-2*, or Δ*bcpA-1*Δ*bcpA-2* mutant bacteria on X-gal agar. Donor (*Bottom*) and recipient (first column) cells incubated alone are also shown.

and *nptII* genes, and the permanent loss of GFP fluorescence and kanamycin resistance from the bacterium. Although our intention was to measure activation of the Bth_I0534 and *csu* promoters, as well as *bcpA* itself, we were unable to obtain *flp* reporters for Bth_I0534 and the *csu* operon in the "promoter off" state—all clones had already excised their *gfp* and *nptII* genes. Proceeding with the P_{bcp}-*flp* reporter, we tested the effect of BcpA-producing neighbors on recipient cell gene expression using a coculture assay. Recipients (carrying the P_{bcp}-*flp* reporter) were selected from the center and edge of coculture colony spots (which experience different interbacterial killing dynamics) (13) and the resulting colonies were analyzed for GFP fluorescence as a measure of the proportion of recipient bacteria that expressed *bcpAIOB*. After coculture with wild-type bacteria, ∼20% of recipient bacteria in the center of the colony spot and ∼50% of cells at the edge were GFP^- and therefore had expressed *bcpAIOB* (Fig. 3*C*). These numbers increased to 70–80% when the same recipient cells were incubated with either Δ*bcpAIOB* or *Bt*EKA mutant donors, indicating that significantly more bacteria expressed *bcpAIOB* when their neighbors did not produce catalytically active BcpA. During coculture with P_{S12}-WT, but not P_{S12}-*Bt*EKA, the number of reporter recipient bacteria having activated P_{bcpA} was significantly less than during coculture with wild-type bacteria, indicating that fewer recipients had expressed their *bcpAIOB* genes when their neighbors produced high levels of enzymatically active BcpA. Although the *bcpAIOB* genes were not identified in our RNA-seq analysis and autoregulation of *bcpAIOB* or *cdiBAI* has not been reported previously, these data (generated using a highly sensitive reporter) indicate that catalytically active BcpA produced by neighboring bacteria decreases *bcpAIOB* gene expression within recipient cells.

Taken together, our data thus far suggest that, in addition to interbacterial inhibition, the BcpAIOB proteins mediate interbacterial signaling in *B. thailandensis*. This process seems to depend on the delivery, likely to the cytoplasm, of a catalytically active enzyme from one bacterium to another. In contrast to interbacterial killing (i.e., CDI), signaling results in gene expression changes in the recipient cell, leading to an increase in community-associated behaviors, such as biofilm formation and the production of a CR-binding structure. To differentiate these outcomes from BcpAIOB-mediated CDI, we will refer to this response as contact-dependent signaling (CDS).

BcpA Toxins Encoded by Different *bcpAIOB* Alleles Do Not Induce CDS in *B. thailandensis*. BcpAIOB-mediated signaling could occur in recipient bacteria via several potential mechanisms. Toxin delivery into recipient cells requires translocation across both the outer and inner membranes (18), and one possibility is that the gene expression changes observed represent responses to membrane damage sustained during toxin entry. Indeed, envelope stress is known to trigger biofilm formation in other organisms (33, 34). To test this hypothesis, we examined *B. thailandensis* strains that express chimeric *bcpAIOB* genes, differing from wild-type E264 only within the regions encoding the BcpA-CT toxin and BcpI. Strains *Bt-Bp*1106a-1 and *Bt-Bp*1106a-2 produce chimeric BcpA proteins containing BcpA-CT (and BcpI) encoded by the alleles *Bp*1106a-1 and *Bp*1106a-2, respectively, of *Burkholderia pseudomallei* strain 1106a (*SI Appendix*, Fig. S6) (11). BcpA-$CT^{1106a\text{-}2}$ contains a putative toxin deaminase domain, but the precise enzymatic activities of both toxins are unknown.

We have previously demonstrated that chimeric strains *Bt-Bp*1106a-1 and *Bt-Bp*1106a-2 can inhibit the growth of susceptible *B. thailandensis* recipient bacteria, indicating that they must successfully deliver their chimeric BcpA toxins to recipient cells (11). However, whereas they can mediate CDI, chimeric strains *Bt-Bp*1106a-1 and *Bt-Bp*1106a-2 are defective for biofilm formation (11). Here, we show that these strains were also defective for aggregation (Fig. 4*A*) and pigment production, and yielded colonies that did not bind CR (*SI Appendix*, Fig. S6). These phenotypes correlated with low expression of BcpA-responsive genes identified in our RNA-seq analysis: Bth_I0535 (Fig. 4*B*) and *csuD* (*SI Appendix*, Fig. S6). Thus, the BcpA-CT toxins delivered by the chimeric strains are apparently capable of mediating CDI, but not CDS. That is, they are incapable of triggering the gene expression and phenotypic changes induced by $BcpA^{E264}$ in *B. thailandensis* E264. This result indicates that CDS is not a general response of the bacteria to any BcpA toxin, or simply to membrane perturbation or other consequences of protein translocation into the cytoplasm from an extracellular location. Moreover, these data show that BcpA-CT^{E264} and/or $BcpI^{E264}$ are required for BcpA-mediated signaling in *B. thailandensis* E264 and suggest the hypothesis that CDS represents a specific response to a bacterium's own BcpA toxin.

A Nearly Identical BcpA Toxin Produced by *Burkholderia dolosa* Induces CDS in *B. thailandensis*. We predicted that bacteria producing identical CDI system proteins could signal to each other and induce gene expression changes, even if the strains were otherwise genetically dissimilar. The genome of *B. dolosa* strain AU0158

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contains three *bcpAIOB* loci, including one, *bcpAIOB*$^{AU0158\text{-}1}$, that is nearly identical to the *bcpAIOB* locus in *B. thailandensis* E264. The predicted amino acid sequences of the BcpA proteins encoded by *bcpAIOB*$^{AU0158\text{-}1}$ and *bcpAIOB*^{E264} are 77% identical, with 86% identity within the BcpA-CT region, and the BcpI proteins are 82% identical (*SI Appendix*, Fig. S7). By contrast, BcpA-CT$^{AU0158\text{-}2}$ shares only 12% identity with BcpA-CT^{E264}. During coculture, wild-type *B. dolosa* and a Δ*bcpA-2* mutant induced increased *B. thailandensis* expression of Bth_I0535::*lacZ* and *csuD*::*lacZ* reporters whereas mutants lacking *bcpA-1* did not (Fig. 4*C*). This result suggests that both BcpA$^{AU0158\text{-}1}$ delivery to *B. thailandensis* and BcpA^{E264} exchange among *B. thailandensis* bacteria induce similar responses in recipient cells and indicate that BcpA-mediated signaling can occur between different *Burkholderia* species that express the same *bcpA*/*bcpI* allele—bacteria that are somewhat distantly related genealogically, but are "self" as defined by *bcpAIOB*. The results contrast with the lack of signaling observed between *B. thailandensis* strains that differed only within the region encoding BcpA-CT and BcpI (Fig. 4 *A* and *B*)—bacteria that are otherwise genetically identical, but "non-self" as defined by *bcpAIOB*.

Discussion

Contact-dependent growth inhibition was discovered based on the ability of CdiBAI/BcpAIOB proteins to inhibit the growth of neighboring bacteria unless those cells produce an appropriate immunity protein (12, 15). Our new data indicate that protection from growth inhibition is not the only consequence of BcpA toxin delivery to immune bacteria. Perhaps an equally important function, BcpA-CT delivery also leads to changes in gene expression in targeted cells that result in increased group behaviors, such as biofilm formation and production of a CR-binding structure and a pigment, as well as decreased expression of the *bcpAIOB* genes themselves. Thus, BcpAIOB (and possibly CdiBAI) proteins appear to play dual roles: antagonism of non-self cells that do not produce the correct immunity protein and promotion of community behaviors among self bacteria that produce identical proteins.

Our data suggest that BcpAIOB proteins enable bacteria to sense and respond to direct contact with sibling cells. In contrast to quorum sensing, CDS might allow bacteria to alter their behavior at relatively low cell densities or in high diffusion environments. Moreover whereas quorum-sensing pheromones are generally shared among all members of a bacterial genus or species (35), diversity among *bcpAIOB* alleles occurs within species, suggesting that CDS may relay information about the proximity of only very closely related or sibling neighbors. Because quorum sensing influences expression of both *bcpAIOB* and biofilm-related genes in *B. thailandensis* (36), CDS may function as a fine-tuning mechanism.

Neighbor-acquired BcpA could impact gene expression in BcpI-producing recipient bacteria by several possible mechanisms. It is not due to membrane perturbations associated with BcpA-CT delivery or a general response to CDI attack because it does not occur when catalytically inactive or *B. pseudomallei* BcpA-CTs are delivered. Instead, our data suggest that CDS requires that the BcpA-CT delivered to immune recipient cells performs a specific enzymatic reaction. One hypothesis is that BcpA-CT has sublethal DNase activity within an immune recipient cell, perhaps due to reversible or incomplete BcpI binding. If so, and the CDS phenotypes we observed (biofilm, CR binding, pigment) are a response to low-level DNA damage, this damage response must be occurring at a very low level because our RNA-seq data did not indicate significant differences in transcript levels of genes involved in DNA repair or stress responses. Another possibility is that, instead of cleaving DNA, the BcpA-BcpI complex has altered activity. The proteins could change gene expression by binding to a specific DNA site and functioning as a transcriptional repressor or activator. Alternatively, the BcpA/BcpI complex may display enzymatic activity with altered substrate specificity, such as cleavage of a small regulatory RNA or nucleotide second messenger like c-di-GMP or cAMP.

Communication systems evolve only when both the sender and receiver benefit from the communicated information (37). Biofilm production is likely an energetically costly behavior but provides desiccation tolerance, access to nutrients, and protection from antibiotics and predation (1). Use of polymorphic proteins like BcpAIOB to trigger biofilm development in only closely related cells (via CDS) and to maintain this community composition (via CDI) would restrict those benefits to kind (or kin) bacteria. Our data suggest that *B. thailandensis* must be able to both send and receive a BcpA-CT signal to participate in biofilms. Mutants that cannot send a BcpA-CT signal because they do not produce catalytically active BcpA^{E264} cannot participate in mixed biofilms with wild-type bacteria (25). A mutant that is partially defective in receiving a signal (ΔBth_I0534) is also unable to persist in biofilms with wild-type bacteria, suggesting that there is selection for recipient cells that respond appropriately to a BcpA-generated signal. The system may also elegantly provide its own policing mechanism because potential nonresponding cheaters that have lost BcpAIOB would experience toxicity upon BcpA-CT delivery and would be eliminated from the community.

Cooperative behavior among individuals is often explained by kin selection, in which a genetic allele directs benefits to others who are genealogically related and thus likely to share the same allele, or kind selection, in which an allele (a "greenbeard gene") causes help to others who share the same allele, regardless of their genealogical relationship (38). This study provides further evidence that *bcpAIOB* loci may function as greenbeard alleles (24). Moreover, our data indicate that *bcpAIOB* loci may function as both "helping" and "harming" greenbeards, simultaneously decreasing the fitness of non–greenbeard-bearing individuals (via CDI) and increasing the fitness of bacteria that share the same *bcpAIOB* allele (via CDS).

Rather than representing a cooperative behavior, an alternative theory is that biofilm formation is a response to stress or damage (39). There is increasing evidence that bacteria sense cell damage (40) or molecules associated with threats (41) as indications of ecological competition and respond by deploying counterattack and defense mechanisms, which may include forming biofilms. Antibiotic stress induces biofilm development (42), and biofilm growth is often enhanced when bacteria are cocultured with competitors (39), implying that biofilm formation might be a defensive strategy. Lysis of *Pseudomonas aeruginosa* cells similarly acts as a "danger signal" to the remaining population, inducing antibacterial factor production (43). Therefore, it is alternatively possible that CDS evolved as a mechanism to sense the close proximity of siblings, perhaps as a cue for the presence of more threatening nonkin competitors. The CDS response we observed here may then be interpreted to represent antagonistic (e.g., expression of polyketide- and T6SS-encoding genes) and protective (e.g., biofilm formation) mechanisms, rather than cooperative behaviors among sibling cells. However, at least one antibacterial factor appears to be suppressed by CDS: the CDI system encoded by the *bcpAIOB* genes themselves.

It remains to be investigated whether CDS is a function of all or a subset of CDI system proteins. Several groups have observed unexpected phenotypes upon mutation of CdiA homologs, consistent with the possibility that CDS occurs in other bacterial species. For example, mutation of the *X. axonopodis cdiA* homolog *XacFhaB* resulted in increased motility and exopolysaccharide production (23) and mutation of a *P. aeruginosa cdiA* homolog abolished hydrogen cyanide production and nematode virulence (44). It has also been reported that, like *B. thailandensis*, *E. coli* 536 requires catalytically active CdiA for biofilm formation (45), suggesting that this protein could mediate CDS. By contrast, Hayes and colleagues recently demonstrated that mutation of *cdiA* in *E. coli* EC93 impacted biofilm formation in a manner that did not require the CdiA-CT toxin (24). These studies suggest that some, but not all, CDI system proteins may perform CDS and imply that the

consequences of signaling are likely different in different bacterial species, depending on the genetic regulatory pathways present in each organism.

Materials and Methods

Bacterial strains and culture conditions are described in *SI Appendix*. Plasmids are listed in *SI Appendix*, Table S6. Bacterial mutants were analyzed for CR binding, autoaggregation, pigment production, and biofilm formation using established methods (*SI Appendix*). Competition assays were performed as described previously (13). An assay to measure *bcpAIOB* expression on a per cell basis was modeled after interbacterial competition assays used for *B. thailandensis* (13). Recipient wild-type E264 containing P_{bcp}-*flp* (Cm^R) were cocultured at a 1:100 ratio with Cm^S donor strains for ∼20 h on minimal medium agar and plated on Cm, and the number of resulting GFP^+ and GFP^- cfus was enumerated. For RNA-sequencing analysis, RNA was isolated from triplicate independent M63 cultures of wild-type E264, *Bt*EKA, and P_{S12}-WT bacteria cultured for 18 h at 37 °C with aeration. RNA was isolated as described (*SI Appendix*), and cDNA library preparation and sequencing were performed by the University of North Carolina High Throughput Sequencing Facility. Statistics were performed using Prism 5 (GraphPad Software), and significance was determined using the unpaired, two-tailed Student's *t* test. Additional experimental details are described in *SI Appendix*.

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