Published in final edited form as:
Mol Microbiol. 2013 September ; 89(6): 1213–1225. doi:10.1111/mmi.12339.

Burkholderia BcpA mediates biofilm formation independently of interbacterial contact dependent growth inhibition

Erin C. Garcia, **Melissa S. Anderson**, **Jon A. Hagar**, and **Peggy A. Cotter***
Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, NC 27599

SUMMARY

Contact dependent growth inhibition (CDI) is a phenomenon in which Gram-negative bacteria use the toxic C-terminus of a large surface-exposed exoprotein to inhibit the growth of susceptible bacteria upon cell-cell contact. Little is known about when and where bacteria express the genes encoding CDI system proteins and how these systems contribute to the survival of bacteria in their natural niche. Here we establish that, in addition to mediating interbacterial competition, the *Burkholderia thailandensis* CDI system exoprotein BcpA is required for biofilm development. We also provide evidence that the catalytic activity of BcpA and extracellular DNA are required for the characteristic biofilm pillars to form. We show using a *bcpA-gfp* fusion that within the biofilm, expression of the CDI system-encoding genes is below the limit of detection for the majority of bacteria and only a subset of cells express the genes strongly at any given time. Analysis of a strain constitutively expressing the genes indicates that native expression is critical for biofilm architecture. Although CDI systems have so far only been demonstrated to be involved in interbacterial competition, constitutive production of the system's immunity protein in the entire bacterial population did not alter biofilm formation, indicating a CDI-independent role for BcpA in this process. We propose, therefore, that bacteria may use CDI proteins in cooperative behaviors, like building biofilm communities, and in competitive behaviors that prevent non-self bacteria from entering the community.

INTRODUCTION

Within both eukaryotic hosts and environmental niches, bacteria communicate, cooperate, and compete with other microorganisms. Contact dependent growth inhibition (CDI) has been characterized primarily in *Escherichia coli* as an interbacterial competition phenomenon. Mediated by Two Partner Secretion (TPS) system proteins, CDI is thought to occur when the toxic C-terminal ~300 residues of the large (>3000 aa) exoprotein (called CdiA in *E. coli*, CdiA-CT for the C-terminal ~300 aa) is delivered to the cytoplasm of a susceptible target bacterium (Aoki *et al.*, 2005; Aoki *et al.*, 2010; Webb *et al.*, 2013). Bacteria are protected from CDI if they produce a small immunity protein (called CdiI in *E. coli*).

Characterization of TPS family proteins in *Burkholderia pseudomallei* led to the discovery that genes encoding CDI systems are present in a broad range of proteobacteria and that the C-terminal regions of the large exoproteins and the immunity proteins are highly variable (Aoki *et al.*, 2010). CDI system-encoding genes fall into two main categories: *E. coli*-type, which have the gene order *cdiBAI*, and *Burkholderia*-type, which have the locus structure *bcpAIOB*. The first CdiA protein examined (in *E. coli* EC93) affects the target cell

*Corresponding author: peggy_cotter@med.unc.edu.
The authors have no conflict of interest to declare.

membrane (Aoki *et al.*, 2009), resulting in growth inhibition rather than cell death (Aoki *et al.*, 2005). All other CdiA-CTs examined so far display nuclease activity *in vitro*, cleaving tRNA or DNA substrates (Aoki *et al.*, 2010; Nikolakakis *et al.*, 2012; Morse *et al.*, 2012), likely killing the target cells. Consistent with this hypothesis, cells targeted by a CdiA-CT DNase have been shown to lose DAPI staining (Webb *et al.*, 2013). Immunity proteins bind specifically to their cognate CdiA-CTs or BcpA-CTs (Aoki *et al.*, 2010; Nikolakakis *et al.*, 2012; Morse *et al.*, 2012) and, when produced intracellularly, protect a bacterium from CdiA-CT- or BcpA-CT-mediated toxicity (Aoki *et al.*, 2010; Nikolakakis *et al.*, 2012; Anderson *et al.*, 2012; Morse *et al.*, 2012).

Although CDI has been clearly demonstrated *in vitro*, whether interbacterial competition is the main or only function performed by CDI systems in nature is unknown. In the *E. coli* strain in which CDI was first described, the *cdiBAI* genes are expressed constitutively (Aoki *et al.*, 2005). In all other strains examined, within and outside the *Escherichia* genus, *cdiBAI* gene expression appears to be tightly regulated and, in many cases, genetic manipulation to constitutively express *cdiBAI* genes is required for CDI to occur (Aoki *et al.*, 2010). Furthermore, CDI has only been observed between wildtype bacteria (or bacteria constitutively expressing the *cdiBAI* or *bcpAIOB* genes from a multicopy plasmid) and mutants lacking the *cdiBAI* or *bcpAIOB* genes (Aoki *et al.*, 2010; Nikolakakis *et al.*, 2012; Anderson *et al.*, 2012), a situation that is unlikely to occur in nature. Moreover, while mutation of the *cdi* or *bcp* locus in *Dickeya* (Aoki *et al.*, 2010) or *Burkholderia* (Nikolakakis *et al.*, 2012; Anderson *et al.*, 2012), respectively, is sufficient to render these bacteria susceptible to CDI *in vitro*, the lack of the conserved surface structures capsule (Aoki *et al.*, 2008) or P pili (Aoki *et al.*, 2005) on target cells is also required for efficient CDI in *E. coli*. Thus, the biological relevance of CDI remains unclear.

Bacteria often grow as biofilms, communities of microbes enclosed within an extracellular polymeric matrix (Davey and O'Toole, 2000; Vlamakis *et al.*, 2013). Biofilms can form on a wide variety of surfaces in diverse environments, including plant rhizospheres, indwelling medical devices, and mammalian mucosal surfaces and can exacerbate human disease. Especially within environmental niches, biofilms are frequently polymicrobial, although the mechanisms utilized by bacteria to cooperate and/or compete in these complex sociomicrobiological communities are not well understood.

Here, using *B. thailandensis* as a model, we demonstrate that the CDI system-encoding genes *bcpAIOB* are essential for the development of multicellular biofilm communities. We additionally show that although the activity of BcpA-CT is required for this process, the role of BcpA in biofilm formation is independent of interbacterial growth inhibition. These data suggest a model in which in the *Burkholderia* CDI proteins function to mediate both competitive and cooperative bacterial behaviors.

RESULTS

B. thailandensis biofilm formation requires *bcpAIOB*

Previous work from our laboratory demonstrated that the CDI system-encoding genes in *B. thailandensis, bcpAIOB*, are required for autoaggregation during culture in minimal medium, suggesting a role for these genes in biofilm formation (Anderson *et al.*, 2012). To investigate this possibility, we used confocal laser scanning microscopy (CLSM) to measure *B. thailandensis* E264 static, submerged biofilm formation on glass coverslips (Fig. 1A). Six hours after inoculation, wildtype bacteria, a mutant lacking the entire *bcpAIOB* locus (Δ*bcpAIOB*), and a mutant lacking the *bcpB* gene that encodes the outer membrane channel for surface localization of BcpA (Δ*bcpB*) adhered similarly to the coverslip in small aggregates. The mutants were defective, however, in several subsequent steps of biofilm

development, resulting in significantly less biomass than biofilms formed by wildtype bacteria (Fig. 1A, 1B). The mutants formed slightly sparser monolayers at 16 h and their biofilms at 24 h contained fewer microcolonies than those formed by the wildtype strain. The microcolonies that the mutants did develop were an average of 10 μm shorter than those formed by wildtype bacteria. Furthermore, while wildtype microcolonies developed into large pillar structures extending over 35 ± 2 μm above the coverslip by 72 h, the sparse Δ*bcpAIOB* microcolonies attained a maximum height of only 23 ± 2 μm.

Extracellular DNA contributes to *B. thailandensis* biofilms

For many bacterial species, extracellular DNA (eDNA) is an essential component of the biofilm matrix (Whitchurch *et al.*, 2002; Lappann *et al.*, 2010; Seper *et al.*, 2011). To test the role of eDNA in *B. thailandensis* biofilms, DNase I was added during biofilm inoculation. After 6 h of growth, no difference was observed between DNase-treated and control biofilms (Fig. S1), indicating that DNA is not required for the initial attachment step. However, after 16 h of growth, monolayers of both wildtype and Δ*bcpAIOB* mutant bacteria in DNase-treated biofilms contained fewer substrate-associated cells than in samples treated with buffer alone (Fig. 2A), demonstrating that eDNA is required for this process. While necessary for biofilm formation, however, addition of excess eDNA was not sufficient to rescue the biofilm defect of Δ*bcpAIOB* mutant bacteria (Fig. 2B). Interestingly, however, while the addition of chromosomal DNA generally had no effect on wildtype biofilms (Fig. 2B), it occasionally appeared to enhance biofilm formation (Fig. S1).

Native expression of *bcpAIOB* is necessary for biofilm architecture

Our previous studies demonstrated that when *B. thailadensis* was cultured in liquid medium, *bcpAIOB* gene expression was stochastic, with only approximately one in 1000 bacteria expressing a *bcpA-gfp* fusion at a high level at a given time (Anderson *et al.*, 2012). When grown on a solid surface, such as in the colony biofilm competition assay, *bcpA-gfp* was not expressed highly enough to see GFP^+ bacteria by microscopy, bout our data indicated that most, if not all, bacteria had expressed the *bcpAIOB* genes highly enough to mediate interbacterial competition (Anderson *et al.*, 2012). To investigate *bcpAIOB* expression in a biofilm, we constructed a strain containing a P_{bcpA}-*gfp* reporter and a constitutive *rfp* gene, each in single copy at one of the two *att*Tn7 sites in the *B. thailandensis* genome. Examination of this strain indicated that, similar to what was observed during liquid culture (Anderson *et al.*, 2012), only a small subset of bacteria expressed the *bcpAIOB* genes to high levels at a given time within the biofilm (Fig. 3A). In addition to individual GFP^+ bacteria within the monolayer of cells adhering to the coverslip, 72 h biofilms also rarely contained entire pillar structures composed almost exclusively of GFP^+ bacteria. However, the majority of pillars contained bacteria that were not highly expressing *bcpAIOB* (*ie.* GFP^-), and the GFP^+ pillars were never observed at earlier timepoints. Strong constitutive expression of the *bcpAIOB* genes from the *rpsL* promoter, P_{S12}, resulted in an abnormally dense, flat biofilm that lacked discrete pillar structures (Fig. 3A). This was particularly evident at 72 h, when biofilms formed by wildtype bacteria were significantly thicker than those made by the bcp^C strain (Fig. 3B). Furthermore, when biofilm heterogeneity was quantified, 72 h biofilms produced by the bcp^C mutant were significantly ($p<0.0001$) more uniform in thickness (roughness coefficient of 0.14±0.01) than the highly textured biofilms formed by wildtype *B. thailandensis* (roughness coefficient of 0.36±0.03). These data indicate that native expression of *bcpAIOB*, which appears to include strong expression by a small subpopulation of cells, is critical for biofilm formation.

BcpA shows homology to Holliday junction resolvases and conserved residues are required for interbacterial competition

Other TPS system exoproteins, including FHA of *Bordetella* species, have been shown to mediate autoaggregation (Menozzi *et al.*, 1994) and biofilm formation (Irie *et al.*, 2004; Serra *et al.*, 2011) via intermolecular interactions that lead to interbacterial adhesion. To assess a putative role for interbacterial growth inhibition during biofilm formation and/or a potential role for BcpA functioning as an interbacterial adhesin, we constructed a strain producing BcpA protein with single amino acid substitutions that rendered it defective for CDI. A 50 amino acid region within the C-terminal 130 residues of BcpA is predicted by PHYRE2 protein fold recognition (Kelley and Sternberg, 2009) analysis to share structural similarity with archaeal Holliday junction resolvases and endonucleases. Protein sequence alignment of the putative structurally conserved region with the PHYRE2 hits and their homologs showed striking conservation of several amino acids. Interestingly, three of these residues (corresponding to D3051, E3064, and K3066 in BcpA) have been shown to be required for DNA substrate cleavage by the *Pyrococcus furiousus* Holliday junction resolvase, Hjc (Komori *et al.*, 2000) (Fig. 4A).

To determine whether these conserved amino acids are important for BcpA function, we mutated two of the corresponding codons to alanine codons on the *B. thailandensis* chromosome, generating a strain (*Bt*EKA) with E3064A and K3066A substitutions in BcpA. To measure the BcpA protein produced by this strain, nucleotides encoding an HA epitope were introduced into *bcpA* (immediately 3' to the F2633 codon) in both the wildtype and *Bt*EKA strains (resulting in E264BcpA-HA and *Bt*EKABcpA-HA, respectively). Because *bcpAIOB* expression *in vitro* is insufficient to detect BcpA-HA protein production (Anderson *et al.*, 2012), the locus's native promoter was replaced with the strong constitutive promoter P_{S12} in the HA-tagged strains. Western blot analysis of cell lysates prepared from these strains showed that *Bt*EKABcpA-HA produced total BcpA-HA similar to the wildtype (E264BcpA-HA) strain (Fig. 4B). To examine BcpA-HA localization, the HA-tagged strains were further modified to carry a copy of the *E. coli phoA* gene at a neutral chromosomal site. Whole cell dot blots indicated that both E264BcpA-HA::*phoA* and *Bt*EKABcpA-HA::*phoA* produced similar levels of surface-exposed BcpA-HA (Fig. 4C), indicating that the E3064A and K3066A substitutions do not affect BcpA protein production or localization.

It is well-established that BcpA-CT (and CdiA-CT) proteins bind to their cognate BcpI (or CdiI) proteins *in vitro* (Aoki *et al.*, 2010; Nikolakakis *et al.*, 2012). To determine whether $BcpA\text{-}CT_{EKA}$ retained the ability to bind BcpI, His-tagged BcpA-CT proteins from wildtype or *Bt*EKA bacteria were co-produced in *E. coli* with untagged BcpI. In pulldown purification experiments, native, but not denatured BcpI bound to His-BcpA-CT proteins from both wildtype and *Bt*EKA bacteria (Fig. S2), suggesting that the mutant BcpA protein folds properly.

To measure the competitive fitness of the *Bt*EKA mutant, the untagged *Bt*EKA strain was tested in interbacterial competition assays. While wildtype bacteria outcompeted a Δ*bcpAIOB* mutant more than 100-fold, the *Bt*EKA mutant failed to outcompete the CDI-susceptible strain (Fig. 4D), demonstrating the requirement of E3064 and K3066 for CDI. Because *bcpI* is unaltered in this strain, however, *Bt*EKA bacteria remain protected from interbacterial killing (Fig. 4D).

E3064 and K3066 are conserved in other BcpA/CdiA proteins

Interestingly, the residues required for $BcpA_{E264}$ interbacterial killing (E3064 and K3066) appear to be conserved in other putative CDI system exoproteins (Fig. S3). This E/DxK

motif is found in approximately the same location (>100 amino acids from the C-terminus) in exoproteins of both *Burkholderia*- and *E. coli*-type CDI systems, including those found in *Burkholderia multivorans*, *Acinetobacter baumannii*, and *Neisseria meningitidis*. While several of the proteins containing the E/DxK motif appear to be full-length BcpA/CdiA proteins (for example, sequences from *Burkholderia dolosa* and *N. meningitidis* alpha14), others seem to be orphan BcpA-CTs/CdiA-CTs (for example, sequences from *N. meningitidis* H44/76 and G2136). These truncated *bcpA/cdiA* genes are encoded downstream of an intact locus, have been shown in *E. coli* to mediate interbacterial killing when fused to the N-terminal portion of CdiA, and are thought to represent a pool of toxin diversity (Poole *et al.*, 2011). Other E/DxK-containing proteins appear to be orphan BcpA-CTs/CdiA-CTs unassociated with an intact *bcpAIOB/cdiBAI* locus and one example (the protein from *Solitalea canadensis*) seems to be a putative Rhs protein. Analogous to CDI system proteins, Rhs proteins have recently been shown to mediate interbacterial competition (Poole *et al.*, 2011; Koskiniemi *et al.*, 2013). Although the sequences of the entire variable C-terminal domains of all these proteins (BcpA-CTs/CdiA-CTs), as well as their associated immunity proteins, do not share striking similarity, localized homology exists in the region surrounding the putative catalytic region, suggesting that this predicted activity of BcpA-CT_{E264} may be conserved in other systems.

Biofilm development requires BcpA activity

To define the role of BcpA-CT activity in *B. thailandensis* biofilm development, we tested the ability of the *Bt*EKA mutant to form a biofilm. Like Δ*bcpAIOB* mutant bacteria, the *Bt*EKA mutant adhered to the glass coverslip similarly to wildtype bacteria, but was defective for biofilm development, forming a thin biofilm with sparse microcolonies (Fig. 5A) that reached a maximum height of only 22 μm by 72 h (Fig. 5B). Thus, activity of BcpA, not simply surface-localized protein utilized for interbacterial adhesion, is required for biofilm development. BcpAIOB-dependent autoaggregation of *B. thailandensis* (Anderson *et al.*, 2012) also appears to require BcpA activity, as *Bt*EKA mutant bacteria did not aggregate when cultured in minimal medium (data not shown).

Role for BcpA activity is CDI-independent

Although it has not been demonstrated that BcpA-mediated CDI results in death of target bacteria, our data suggested a model in which biofilm development requires that some *bcpAIOB*-expressing bacteria in the community kill other bacteria that express *bcpAIOB* at a much lower level (or not at all), causing them to release a key nutrient, signaling molecule, or matrix component (perhaps eDNA) that is required for the biofilm to form. This model predicts that constitutive expression of the immunity-encoding gene, *bcpI*, in the entire population will prevent BcpA-mediated toxicity and, therefore, biofilm formation. Surprisingly, although *bcpI* protects from interbacterial inhibition in a competition assay (Anderson *et al.*, 2012), its constitutive expression in wildtype *B. thailandensis* only slightly decreased monolayer formation (16 h) and had no effect on biofilm pillar development (Fig. 5A), biomass, or thickness (Fig. 5B). These results indicate that biofilm formation does not require interbacterial killing or growth inhibition, a finding that is inconsistent with the paradigm that CDI systems are dedicated to interbacterial competition.

Wildtype bacteria cannot rescue *bcpAIOB* mutants in mixed strain biofilms

As an alternative approach to determine the role of BcpA activity during biofilm formation, we inoculated biofilm chambers with 1:1 mixtures of RFP- and GFP-labeled bacteria. Mixtures of differentially labeled wildtype *B. thailandensis* demonstrated that biofilm pillar structures are predominantly clonal, as solely GFP- or RFP-positive cells composed most large microcolonies (Fig. 6A). Twenty-four percent of pillars appeared sectored, suggesting that they formed from the merging of neighboring GFP^+ and RFP^+ populations. Planktonic

bacteria entering pillar structures was apparently an infrequent event, as only 24% of pillars appeared to contain a mixture of GFP- and RFP-labeled cells. While some "mixed" structures were indeed nearly homogenous mixtures of GFP- and RFP-labeled bacteria, many were predominantly composed of bacteria with one fluorescent label, containing oppositely labeled cells attached to the pillar surface.

The presence of wildtype bacteria in a biofilm was not sufficient to rescue the biofilm defects of Δ*bcpAIOB* (Fig. 6B), Δ*bcpB* (Fig. 6C), or *Bt*EKA (Fig. 6D) mutant bacteria, supporting the conclusions that the purpose of BcpAIOB is not simply to release a factor from a subset of cells that is required for biofilm formation and that to participate in the biofilm, each bacterium must produce an active BcpA protein. As with monoculture biofilms, constitutive expression of *bcpI* did not alter mixed strain biofilms (Fig. 6E, 6F).

CDI occurs in the biofilm monolayer, but does not impact pillar development by Δ*bcpAIOB* mutant bacteria

While it appears to have no effect on the ability of Δ*bcpAIOB* mutant cells to develop pillar structures, CDI does occur in the submerged biofilm environment at later timepoints. At 72 h, mixtures containing RFP-labeled wildtype bacteria co-inoculated with GFP-labeled wildtype cells (Fig. 7A) or wildtype bacteria expressing constitutive *bcpI* (Fig. 7C) appeared to contain approximately 50% of each strain, as observed after 24 h of growth (Fig. 6A). Unlike earlier timepoints (Fig. 6B, 6E), while mixtures of wildtype *B. thailandensis* and Δ*bcpAIOB* mutant bacteria again contained fewer Δ*bcpAIOB* mutant cells (Fig. 7B), constitutive *bcpI* expression in Δ*bcpAIOB* allowed more mutant bacteria to persist with wildtype cells on the substratum (Fig. 7D). This result indicates that Δ*bcpAIOB* mutants were eliminated from the monolayer by wildtype bacteria via CDI. However, while it could persist in the monolayer, BcpI production did not allow the Δ*bcpAIOB* mutant to grow up into pillar structures that were composed solely of Δ*bcpAIOB* bacteria nor did it substantially affect the ability of Δ*bcpAIOB* bacteria to coexist in wildtype pillars. Similar results were seen with 72 h mixed biofilms consisting of wildtype and *Bt*EKA mutant bacteria (data not shown), further supporting the conclusion that each biofilm pillar participant requires active BcpA on its surface. Additionally, as these experiments demonstrate that BcpA-CT molecules must be delivered into target cells within mixed strain biofilms, they suggest that delivery of BcpA-CT to a target cell, either producing (Fig. 6C–F, Fig. 7D) or not producing (Fig. 6B, Fig. 7B) BcpI, does not function to signal the recipient cell to develop a biofilm pillar structure. Together, these data indicate a distinct role for BcpA catalytic activity that is independent of both interbacterial growth inhibition and the presence of intracellular BcpI, implying that this activity occurs extracellularly.

DISCUSSION

The current paradigm is that CDI systems mediate competition between bacterial cells. Although CDI system-dependent interbacterial competition clearly occurs, it has thus far only been observed between wildtype inhibitor cells (or cells that overproduce the CDI system proteins) and mutant target cells (CDI^- and sometimes $capsule^-$ $pili^-$) (Aoki *et al.*, 2005; Aoki *et al.*, 2010; Nikolakakis *et al.*, 2012; Anderson *et al.*, 2012). While CdiA-CT and BcpA-CT domains are toxic to a range of bacterial species when produced intracellularly (Aoki *et al.*, 2010; Nikolakakis *et al.*, 2012; Anderson *et al.*, 2012) or delivered on chimeric CdiA or BcpA proteins (Aoki *et al.*, 2010; Nikolakakis *et al.*, 2012), interspecies or even inter-strain competition between wildtype strains, as is expected to occur in nature, has not yet been observed, raising the question of the biological role of CDI. Here we demonstrate that the BcpAIOB proteins, and specifically BcpA activity, are required for biofilm formation, functioning within a genetically identical population to facilitate sociomicrobiological community development.

Although BcpA activity was indispensible for biofilm development, suggesting that CDI was also required, expression of the immunity-encoding *bcpI* gene had no effect on biofilm growth. As BcpI production protects target *B. thailandensis* bacteria from CDI-meditated toxicity (Anderson *et al.*, 2012), we must conclude that the role for BcpA activity during biofilm formation is CDI-independent. This surprising result implies that BcpA facilitates CDI and biofilm construction through distinct mechanisms. In contrast, the fact that the *Bt*EKA mutant was defective for both CDI and biofilm formation suggests that, while distinct in their requirement for BcpI-mediated immunity, these two mechanisms require similar enzymatic activities and/or substrates. Resolving the apparently intertwined roles for BcpAIOB in biofilm development and CDI is our next goal.

While there are examples of regulated cell death and eDNA release affecting biofilm development in some bacterial species (Rice *et al.*, 2007; Thomas *et al.*, 2009; Lopez *et al.*, 2009), in others eDNA release during biofilm formation is not dependent on cell lysis (Barnes *et al.*, 2012). Our data provide evidence that CDI system protein biofilm-promoting activity in *B. thailandensis* is independent of interbacterial killing. Combined with our finding that transcomplementation of Δ*bcpAIOB* mutant biofilms by wildtype *B. thailandensis* does not occur, indicating that each biofilm participant must produce active BcpA on its surface, this result implies that the activity of BcpA during biofilm formation occurs extracellularly. The similarity of $BcpA_{E264}$-CT to Holliday junction resolvases and its possible DNA nickase activity (Nikolakakis *et al.*, 2012) suggest the hypothesis that $BcpA_{E264}$ and related proteins may perform an enzymatic function on eDNA within the biofilm matrix, perhaps crosslinking it or covalently anchoring bacteria to the eDNA network. In *Staphylococcus aureus*, there is evidence for a mechanism of this nature. Beta toxin, a secreted protein that contributes to both virulence and biofilm formation, was shown to form crosslinks in the presence of DNA *in vitro*, forming a nucleoprotein complex independent of its well-characterized sphingomyelinase activity (Huseby *et al.*, 2010). However, although CDI systems are widespread among Gram-negative bacteria (Aoki *et al.*, 2010), whether this putative biofilm mechanism is common to all CDI systems may be unlikely, as it is difficult to imagine how the BcpA/CdiA proteins that have been shown to have tRNase activity (Aoki *et al.*, 2010; Nikolakakis *et al.*, 2012; Morse *et al.*, 2012) could function in this way.

The failure of the strain constitutively expressing the *bcpAIOB* genes to form pillar structures demonstrates that native *bcpAIOB* gene expression, perhaps involving turning *bcpAIOB* both on and off at appropriate stages, is necessary for proper biofilm development. Within biofilms, however, the majority of cells did not express the *bcpA-gfp* fusion strongly enough to be detected by fluorescence microscopy. However, our data from biofilms co-inoculated with wildtype and mutant strains suggest that each bacterium must produce active BcpA to participate in the biofilm structure. Thus, it seems that, similar to our previous findings on *bcpAIOB* expression in colony biofilms (Anderson *et al.*, 2012), *bcpAIOB* expression in the majority of biofilm-associated cells is too low or too transient for *bcpA-gfp* to be detected by microscopy, but is sufficient to mediate biofilm development. In addition to this low-level expression, in both liquid culture (Anderson *et al.*, 2012) and biofilms, a small subpopulation of bacteria expresses *bcpAIOB* very strongly, suggestive of a bistable regulation phenomenon. The finding that occasionally all *B. thailandensis* cells within a pillar structure appear to express *bcpAIOB* simultaneously suggests that these bacteria employ a sensing/signaling mechanism to coordinate BcpAIOB production. Quorum sensing, which has been demonstrated to affect *B. thailandensis* autoaggregation (Chandler *et al.*, 2009) and is well-established to contribute to biofilm development in other species (Davies *et al.*, 1998; Hammer and Bassler, 2003), provides one possible mechanism. Interestingly, these bcpAIOB-expressing pillar structures were only observed at later

timepoints (72 h), suggesting that this expression pattern may play a role at a later stage of biofilm development or maturation, such as dispersal.

We show here that the putative catalytic region of $BcpA_{E264}$, including the residues required for CDI and biofilm formation (E3064 and K3066), is conserved in other BcpA and CdiA proteins, suggesting that the biofilm-promoting activity of BcpA may be conserved. Moreover, *cdi* loci in *Neisseria meningitidis* (Neil and Apicella, 2009), *Xanthomonas axonopodis* (Gottig *et al.*, 2009), and *Dickeya dadantii* (Rojas *et al.*, 2002) have been found previously to function in biofilm formation and autoaggregation, although they were not identified as CDI system-encoding genes at the time. Similarly, a TPS exoprotein with characteristics of CdiA proteins was found to be the fifth most abundant protein in the extracellular matrix of *Pseudomonas aeruginosa* colony biofilms (Toyofuku *et al.*, 2012). These observations support the hypothesis that contribution to biofilm development is a common function for at least a subset of both *Burkholderia*-type and *E. coli*-type CDI systems.

Paradoxically, while CDI systems have so far been described in terms of their ability to mediate interbacterial competition, our data indicate that these proteins can facilitate a cooperative bacterial behavior. In nature, it is possible that both of these seemingly conflicting activities occur, perhaps under different environmental conditions. Indeed, while not required for biofilm development, our findings indicate that CDI does occur within the biofilm monolayer. The hallmark diversity of CdiA-CT/BcpA-CT proteins (Aoki *et al.*, 2010; Nikolakakis *et al.*, 2012; Anderson *et al.*, 2012) and the observation that *B. pseudomallei bcpAIOB* genes expressed in *B. thailandensis* can inhibit *B. thailandensis* (Nikolakakis *et al.*, 2012) support the hypothesis that interspecies CDI occurs in the environment. An intriguing possibility is that CDI systems function both to build biofilms, as well as exclude non-self competitors from the community.

EXPERIMENTAL PROCEDURES

Bacterial strains and culture conditions

Burkholderia thailandensis E264 is an environmental isolate (Brett *et al.*, 1998). All plasmids were maintained in *E. coli* DH5α and DH5αλpir and mated into E264 using *E. coli* donor strain RHO3 (López *et al.*, 2009). Unless otherwise stated, bacteria were cultured overnight with aeration at 37°C in low salt Luria broth (LSLB, 0.5% NaCl) supplemented with 250 μg/ml (for *B. thailandensis*) or 50 μg/ml (for *E. coli*) kanamycin, 100 μg/ml ampicillin, 20 μg/ml tetracycline, or 200 μg/ml diaminopimelic acid as appropriate. M63 minimal medium (110 mM KH_2PO_4, 200 mM K_2HPO_4, 75 mM $(NH_4)_2SO_4$, 16 nM $FeSO_4$) supplemented with 1 mM $MgSO_4$, 0.2% glucose, and 0.4% glycerol (Thongdee *et al.*, 2008) was used where indicated. All constructed plasmids and bacterial strains were confirmed by DNA sequence analysis.

B. thailandensis strains E264Cm^R, Δ*bcpAIOB*, Δ*bcpB*, Δ*bcpAIOB*::*bcpI*, WT::*bcpI*, and bcp^C (P_{S12}-*bcpAIOB*) were constructed previously (Anderson *et al.*, 2012). *Bt*EKA was constructed by allelic exchange. A DNA fragment corresponding to ~700 bp of internal *bcpA* sequence containing three nucleotide changes (A9191C, A9196G, A9197C) to generate the E3064A and K3066A substitutions, as well as a single nucleotide change (T9186A) to introduce a translationally-silent *SnaB*I restriction site, was generated by overlap PCR and cloned into pEXKm5, which was used for allelic exchange as described (López *et al.*, 2009). For competition assays, a gene encoding kanamycin or chloramphenicol resistance was delivered to *Bt*EKA at one of the strain's two *att*Tn7 sites using pUC18T-miniTn7T-Kan (Choi *et al.*, 2008), generating the strains *Bt*EKA(Km) and *Bt*EKA(Cm), respectively.

Strains for biofilm assays were marked with green or red fluorescent protein using the miniTn7-*kan-gfp* and miniTn7-*kan-rfp* plasmids (Norris *et al.*, 2010) to deliver a constitutive (expression driven by P_{S12}) *gfp* or *rfp* gene to one of the two *att*Tn7 sites in the *B. thailandensis* genome (one site on each of two chromosomes). The P_{bcpA}-*gfp* reporter strain was constructed previously (Anderson *et al.*, 2012) and contains ~500 bp immediately upstream of the *bcpA* start codon cloned 5' to a promoterless *gfp* and delivered to the *att*Tn7 site on *B. thailandensis* chromosome 2. The *kan* cassette was removed from this strain and from Δ*bcpAIOB* by Flp-*FRT* recombination as described (Choi *et al.*, 2008) using pFlpTet, a pFlpe4-derived plasmid modified to carry tetracycline, rather than kanamycin, resistance. The resulting kanamycin-sensitive P_{bcpA}-*gfp* reporter was marked at the *att*Tn7 site on *B. thailandensis* chromosome 1 with constitutive *rfp*, as described above. The *kan* cassettes were also similarly excised from the wildtype P_{S12}-*gfp* and Δ*bcpAIOB* strains. The miniTn7-*kan-gfp* construct was used to deliver a *gfp* gene to the kanamycin-sensitive Δ*bcpAIOB* strain at the *att*Tn7 site on chromosome 1. Plasmid pECG22 (Anderson *et al.*, 2012), which contains the first ~500 bp of *bcpA* immediately downstream of the strong constitutive *rpsL* promoter (P_{S12}), was cointegrated into the kanamycin-sensitive wildtype P_{S12}-*gfp* strain to generate the *gfp*-marked constitutive strain bcp^{C}.

For protein analyses, the wildtype strain that encodes an HA tag located after F2633 in BcpA (E264BcpA-HA) was constructed previously (Anderson *et al.*, 2012) and the HA-tagged *Bt*EKA strain (*Bt*EKABcpA-HA) was constructed in the same manner, by allelic exchange using pEXKm5 (López *et al.*, 2009) to introduce HA-encoding sequence. For dot blot analysis, the *phoA* gene was PCR-amplified from *E. coli* RHO3 and cloned into pUC18T-miniTn7T-Km (Choi *et al.*, 2008) immediately downstream of P_{S12}. This *phoA* construct was integrated into one of the two *att*Tn7 sites in the genomes of the HA-tagged strains. The *kan* cassette was removed from the *phoA*-expressing strains by Flp-*FRT* recombination as described above and plasmid pECG22 (Anderson *et al.*, 2012), was cointegrated into all HA-tagged strains (both those with and without *phoA*) to generate strains that constitutively expressed *bcpAIOB* from P_{S12}.

For purification of BcpA-CT, DNA fragments containing the last 1146 bp of *bcpA*, encoding G2766 to the C-terminus, and *bcpI* (including the stop codon) were PCR-amplified from wildtype and *Bt*EKA bacteria. Each fragment was cloned into pET28a (Novagen) in-frame with the vector's N-terminal His_6 tag and including a 5' ATG, resulting in plasmids pECG20 (encoding BcpA-CT_{WT}) and pECG21 (encoding BcpA-CT_{EKA}).

Static biofilm assay

Overnight cultures were washed in M63 medium and inoculated to an OD_{600} of 0.02 in 400 μl M63 in chambered coverglass dishes (Thermo Scientific). For mixed strain biofilms, GFP- and RFP-producing strains were mixed at a 1:1 ratio and inoculated as above. For DNase treatment, M63 medium was supplemented with 2mM $MgCl_2$ and 1mM $CaCl_2$ and 20 U DNase I (Ambion) was added during inoculation. For exogenous DNA addition, 3 μg chromosomal DNA, prepared from an overnight culture of E264 using Gentra Puregene reagents (Qiagen, according to the manufacturer's instructions), was added during biofilm inoculation. Biofilms were incubated in humidified chambers at 37°C for 6–72 h, washed 4–5 times with 400 μl PBS, overlaid with 400 μl PBS, and imaged by confocal laser scanning microscopy with a Zeiss LSM 700 using a 63x objective lens. Z stacks were processed with Imaris x64 v7.5.2 (Bitplane Scientific Software) and analyzed with COMSTAT (Heydorn *et al.*, 2000).

Protein alignments

Approximately 100 residues near the BcpA C-terminus were submitted to the PHYRE2 Protein Fold Recognition Server (Kelley and Sternberg, 2009) and the resulting high confidence/identity hits (*Rp*, *Rhodopseudomonas palustris* NP_945676; *Ss*, *Sufolobus solfataricus* AAK41424; *Af*, *Archaeoglobus fulgidus* NP_07377) were aligned to the BcpA region of homology (K3018-L3068). Also included in the alignment were homologs (determined by pBLAST) of each PHYRE2 hit (*Bj*, *Bradyrhizobium japonicum* YP_005605519; *Bm*, *Brucella melitensis* AAL52950; *Mc*, *Metallosphaera cuprina* YP_004459243; *Pf*, *Pyrococcus furiosus* NP_579232; *Cm*, *Cyclobacterium marinum* YP_004776468; *Hm*, *Hippea maritima* YP_004340293). Protein sequences were aligned using Vector NTI Advance 11and alignments analyzed with Jalview 2.7 with ClustalX residue coloring.

For identification of the "E/DxK" motif in other BcpA-CTs/CdiA-CTs, the region of $BcpA_{E264}$ showing homology to Holliday junction resolvases (K3018-L3068) and the entire amino acid sequence of the putative truncated BcpA in *B. thailandensis* TXDOH (ZP_02369362) were used as pBLAST queries against the NCBI genome database. The resulting protein sequences, truncated to include solely the C-terminal 300–400 residues, were aligned as described above.

Immunoblots

Overnight cultures were resuspended to an OD_{600} of 5 in 2x SDS PAGE loading buffer, boiled 5 min, separated on a 5% SDS PAGE gel, and transferred to nitrocellulose. Membranes were blocked in 5% milk in PBS with 0.01% Tween and probed with mouse monoclonal anti-HA.11 antibody (Covance) at 1:1000, followed by goat anti-mouse IgG conjugated to IRDye680 (Odyssey) at 1:15,000. Blots were imaged on a LiCor (Odyssey) with Odyssey v3.0 software.

Dot blots

Overnight cultures were washed in PBS and duplicate samples were resuspended to an OD_{600} of 4.0. One set of samples was boiled 5 min (lysate). Five microliters of each sample (whole cells and lysate) were pipetted onto nitrocellulose and air-dried. Membranes were processed as described above or probed with mouse monoclonal anti-bacterial alkaline phosphatase (Sigma) at 1:1000 followed by secondary antibody and detection as described.

BcpA-CT/BcpI pull-down

BcpA-CT/BcpI complexes were purified as described previously (Aoki *et al.*, 2010; Nikolakakis *et al.*, 2012). Cultures (200 ml) of *E. coli* BL21(DE3) cells carrying plasmids pECG20 and pECG22 (encoding His-BcpA-CT and BcpI from wildtype and *Bt*EKA bacteria, respectively) were induced with 200 μM isopropyl-β-D-1-thiogalactopyranoside, resuspended in 10 ml sodium phosphate extraction buffer (20 mM sodium phosphate buffer pH 7, 150 mM NaCl, 0.05% Triton X-100, 100 mM β-mercaptoethanol) containing 1 mM phenylmethylsulfonyl fluoride and lysed by French press at 20,000 psi. Lysate was centrifuged twice at 3000×*g* at 4°C for 10 min and cleared lysate incubated with Ni^{2+}NTA agarose resin (Qiagen) for 3 h at 4°C. Resin was washed twice with 10 ml sodium phosphate extraction buffer containing 20 mM imidazole and resuspended in 1 ml denaturing wash buffer (20 mM sodium phosphate buffer pH 7, 6 M guanidine-HCl, 10 mM β-mercaptoethanol). After incubation at room temperature for 5 min, flow through was collected and resin was washed 8–10 times. Column material was resuspended in 300 μl denaturing elution buffer (20 mM sodium phosphate buffer pH 7, 6 M guanidine-HCl, 10 mM β-mercaptoethanol, 240 mM imidazole), incubated 5 min at room temperature, flow

through collected, and elutions repeated 5 times. Fractions were analyzed on a 12% SDS-PAGE gel.

Competition assay

Interbacterial competition was determined as previously described (Anderson *et al.*, 2012). Bacteria (E264Cm^R, Δ*bcpAIOB*, *Bt*EKA(Km), and *Bt*EKA(Cm)) were cultured overnight with appropriate antibiotics, washed, and diluted to OD_{600} 0.2 in fresh LSLB without antibiotics. Strains were mixed at a 1:1 ratio and 20 μl of culture was plated on solid LSLB (1.5% agar) without antibiotic selection. The culture inoculum was plated on LSLB with antibiotic selection to determine the ratio at 0 h. Agar plates were incubated at room temperature (~25°C) for 24 h. Bacteria were picked from the colony biofilms with a sterile pipette tip, diluted in PBS, and plated on LSLB with antibiotic selection to determine the cfu of each strain in the competition. The competitive index (CI) was calculated as the ratio of the inhibitor strain to the target strain at time 24 h divided by the ratio at time 0 h.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank C. Robert Bagnell, Steven Ray, and Victoria Madden at the UNC Microscopy Services Laboratory for microscopy assistance, Herbert Schweizer (Colorado State University) and Tung Hoang (University of Hawaii) for bacterial strains and plasmids, and John Leong (Tufts University) for insightful discussion. Research reported in this publication was supported by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under Award Numbers U54 AI065359 (P.A.C.), R21 AI093154 (P.A.C.), and F32 AI096728 (E.C.G.). The content is solely the responsibility of the authors and does not represent the official views of the National Institutes of Health.

REFERENCES

Anderson MS, Garcia EC, Cotter PA. The *Burkholderia bcpAIOB* genes define unique classes of Two-Partner secretion and contact dependent growth inhibition systems. PLoS Genet. 2012; 8:e1002877. [PubMed: 22912595]

Aoki SK, Diner EJ, de Roodenbeke CT, Burgess BR, Poole SJ, Braaten BA, et al. A widespread family of polymorphic contact-dependent toxin delivery systems in bacteria. Nature. 2010; 468:439–442. [PubMed: 21085179]

Aoki SK, Malinverni JC, Jacoby K, Thomas B, Pamma R, Trinh BN, et al. Contact-dependent growth inhibition requires the essential outer membrane protein BamA (YaeT) as the receptor and the inner membrane transport protein AcrB. Mol Microbiol. 2008; 70:323–340. [PubMed: 18761695]

Aoki SK, Pamma R, Hernday AD, Bickham JE, Braaten BA, Low DA. Contact-dependent inhibition of growth in *Escherichia coli* . Science. 2005; 309:1245–1248. [PubMed: 16109881]

Aoki SK, Webb JS, Braaten BA, Low DA. Contact-dependent growth inhibition causes reversible metabolic downregulation in *Escherichia coli* . J Bacteriol. 2009; 191:1777–1786. [PubMed: 19124575]

Barnes AMT, Ballering KS, Leibman RS, Wells CL, Dunny GM. *Enterococcus faecalis* produces abundant extracellular structures containing DNA in the absence of cell lysis during early biofilm formation. MBio. 2012; 3 e00193-12-e00193-12.

Brett PJ, DeShazer D, Woods DE. *Burkholderia thailandensis* sp. nov., a *Burkholderia pseudomallei*-like species. Int J Sys Bacteriol. 1998; 48:317–320.

Chandler JR, Duerkop BA, Hinz A, West TE, Herman JP, Churchill MEA, et al. Mutational analysis of *Burkholderia thailandensis* quorum sensing and self-aggregation. J Bacteriol. 2009; 191:5901–5909. [PubMed: 19648250]

Choi K-H, Mima T, Casart Y, Rholl D, Kumar A, Beacham IR, Schweizer HP. Genetic tools for select-agent-compliant manipulation of *Burkholderia pseudomallei* . Appl environ microbiol. 2008; 74:1064–1075. [PubMed: 18156318]

Davey ME, O'Toole GA. Microbial biofilms: from ecology to molecular genetics. Microbiol Mol Bio Rev. 2000; 64:847–867. [PubMed: 11104821]

Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, Greenberg EP. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science. 1998; 280:295–298. [PubMed: 9535661]

Gottig N, Garavaglia BS, Garofalo CG, Orellano EG, Ottado J. A filamentous hemagglutinin-like protein of *Xanthomonas axonopodis* pv. *citri*, the phytopathogen responsible for citrus canker, is involved in bacterial virulence. PLoS ONE. 2009; 4:e4358. [PubMed: 19194503]

Hammer BK, Bassler BL. Quorum sensing controls biofilm formation in *Vibrio cholerae* . Mol Microbiol. 2003; 50:101–104. [PubMed: 14507367]

Heydorn A, Nielsen AT, Hentzer M, Sternberg C, Givskov M, Ersbøll BK, Molin S. Quantification of biofilm structures by the novel computer program COMSTAT. Microbiology. 2000; 146:2395–2407. [PubMed: 11021916]

Huseby MJ, Kruse AC, Digre J, Kohler PL, Vocke JA, Mann EE, et al. Beta toxin catalyzes formation of nucleoprotein matrix in staphylococcal biofilms. Proc Natl Acad Sci U S A. 2010; 107:14407–14412. [PubMed: 20660751]

Irie Y, Mattoo S, Yuk MH. The Bvg virulence control system regulates biofilm formation in *Bordetella bronchiseptica* . J Bacteriol. 2004; 186:5692–5698. [PubMed: 15317773]

Kelley LA, Sternberg MJE. Protein structure prediction on the Web: a case study using the Phyre server. Nat Protoc. 2009; 4:363–371. [PubMed: 19247286]

Komori K, Sakae S, Daiyasu H, Toh H, Morikawa K, Shinagawa H, Ishino Y. Mutational analysis of the *Pyrococcus furiosus* Holliday junction resolvase Hjc revealed functionally important residues for dimer formation, junction DNA binding, and cleavage activities. J Biol Chem. 2000; 275:40385–40391. [PubMed: 11005813]

Koskiniemi S, Lamoureux JG, Nikolakakis KC, de Roodenbeke CT, Kaplan MD, Low DA, Hayes CS. Rhs proteins from diverse bacteria mediate intercellular competition. Proc Natl Acad Sci U S A. 2013; 110:7032–7037. [PubMed: 23572593]

Lappann M, Claus H, van Alen T, Harmsen M, Elias J, Molin S, Vogel U. A dual role of extracellular DNA during biofilm formation of *Neisseria meningitidis* . Mol Microbiol. 2010; 75:1355–1371. [PubMed: 20180907]

Lopez D, Vlamakis H, Losick R, Kolter R. Cannibalism enhances biofilm development in *Bacillus subtilis* . Mol Microbiol. 2009; 74:609–618. [PubMed: 19775247]

López CM, Rholl DA, Trunck LS, Schweizer HP. Versatile dual-technology system for markerless allele replacement in *Burkholderia pseudomallei* . Appl environ microbiol. 2009; 75:6496. [PubMed: 19700544]

Menozzi FD, Boucher PE, Riveau G, Gantiez C, Locht C. Surface-associated filamentous hemagglutinin induces autoagglutination of *Bordetella pertussis* . Infect Immun. 1994; 62:4261–4269. [PubMed: 7927683]

Morse RP, Nikolakakis KC, Willett JLE, Gerrick E, Low DA, Hayes CS, Goulding CW. Structural basis of toxicity and immunity in contact-dependent growth inhibition (CDI) systems. Proc Natl Acad Sci U S A. 2012; 109:21480–21485. [PubMed: 23236156]

Neil RB, Apicella MA. Role of HrpA in biofilm formation of *Neisseria meningitidis* and regulation of the *hrpBAS* transcripts. Infect Immun. 2009; 77:2285–2293. [PubMed: 19289515]

Nikolakakis K, Amber S, Wilbur JS, Diner EJ, Aoki SK, Poole SJ, et al. The toxin/immunity network of *Burkholderia pseudomallei* contact-dependent growth inhibition (CDI) systems. Mol Microbiol. 2012; 84:516–529. [PubMed: 22435733]

Norris MH, Kang Y, Wilcox B, Hoang TT. Stable, site-specific fluorescent tagging constructs optimized for *Burkholderia* species. Appl environ microbiol. 2010; 76:7635–7640. [PubMed: 20851961]

Poole SJ, Diner EJ, Aoki SK, Braaten BA, t'Kint de Roodenbeke C, Low DA, Hayes CS. Identification of functional toxin/immunity genes linked to contact-dependent growth inhibition (CDI) and rearrangement hotspot (Rhs) systems. PLoS Genet. 2011; 7:e1002217. [PubMed: 21829394]

Rice KC, Mann EE, Endres JL, Weiss EC, Cassat JE, Smeltzer MS, Bayles KW. The *cidA* murein hydrolase regulator contributes to DNA release and biofilm development in *Staphylococcusaureus* . Proc Natl Acad Sci U S A. 2007; 104:8113–8118. [PubMed: 17452642]

Rojas CM, Ham JH, Deng W-L, Doyle JJ, Collmer A. HecA, a member of a class of adhesins produced by diverse pathogenic bacteria, contributes to the attachment, aggregation, epidermal cell killing, and virulence phenotypes of *Erwinia chrysanthemi* EC16 on *Nicotiana clevelandii* seedlings. Proc Natl Acad Sci U S A. 2002; 99:13142–13147. [PubMed: 12271135]

Seper A, Fengler VHI, Roier S, Wolinski H, Kohlwein SD, Bishop AL, et al. Extracellular nucleases and extracellular DNA play important roles in *Vibrio cholerae* biofilm formation. Mol Microbiol. 2011; 82:1015–1037. [PubMed: 22032623]

Serra DO, Conover MS, Arnal L, Sloan GP, Rodriguez ME, Yantorno OM, Deora R. FHA-mediated cell-substrate and cell-cell adhesions are critical for *Bordetella pertussis* biofilm formation on abiotic surfaces and in the mouse nose and the trachea. PLoS ONE. 2011; 6:e28811. [PubMed: 22216115]

Thomas VC, Hiromasa Y, Harms N, Thurlow L, Tomich J, Hancock LE. A fratricidal mechanism is responsible for eDNA release and contributes to biofilm development of *Enterococcus faecalis* . Mol Microbiol. 2009; 72:1022–1036. [PubMed: 19400795]

Thongdee M, Gallagher LA, Schell M, Dharakul T, Songsivilai S, Manoil C. Targeted mutagenesis of *Burkholderia thailandensis* and *Burkholderia pseudomallei* through natural transformation of PCR fragments. Appl environ microbiol. 2008; 74:2985–2989. [PubMed: 18310423]

Toyofuku M, Roschitzki B, Riedel K, Eberl L. Identification of proteins associated with the *Pseudomonas aeruginosa* biofilm extracellular matrix. J Proteome Res. 2012; 11:4906–4915. [PubMed: 22909304]

Vlamakis H, Chai Y, Beauregard P, Losick R, Kolter R. Sticking together: building a biofilm the *Bacillus subtilis* way. Nat Rev Micro. 2013; 11:157–168.

Webb JS, Nikolakakis KC, Willett JLE, Aoki SK, Hayes CS, Low DA. Delivery of CdiA nuclease toxins into target cells during contact-dependent growth inhibition. PLoS ONE. 2013; 8:e57609. [PubMed: 23469034]

Whitchurch CB, Tolker-Nielsen T, Regas PC, Mattick JS. Extracellular DNA required for bacterial biofilm formation. Science. 2002; 295:1487–1487. [PubMed: 11859186]

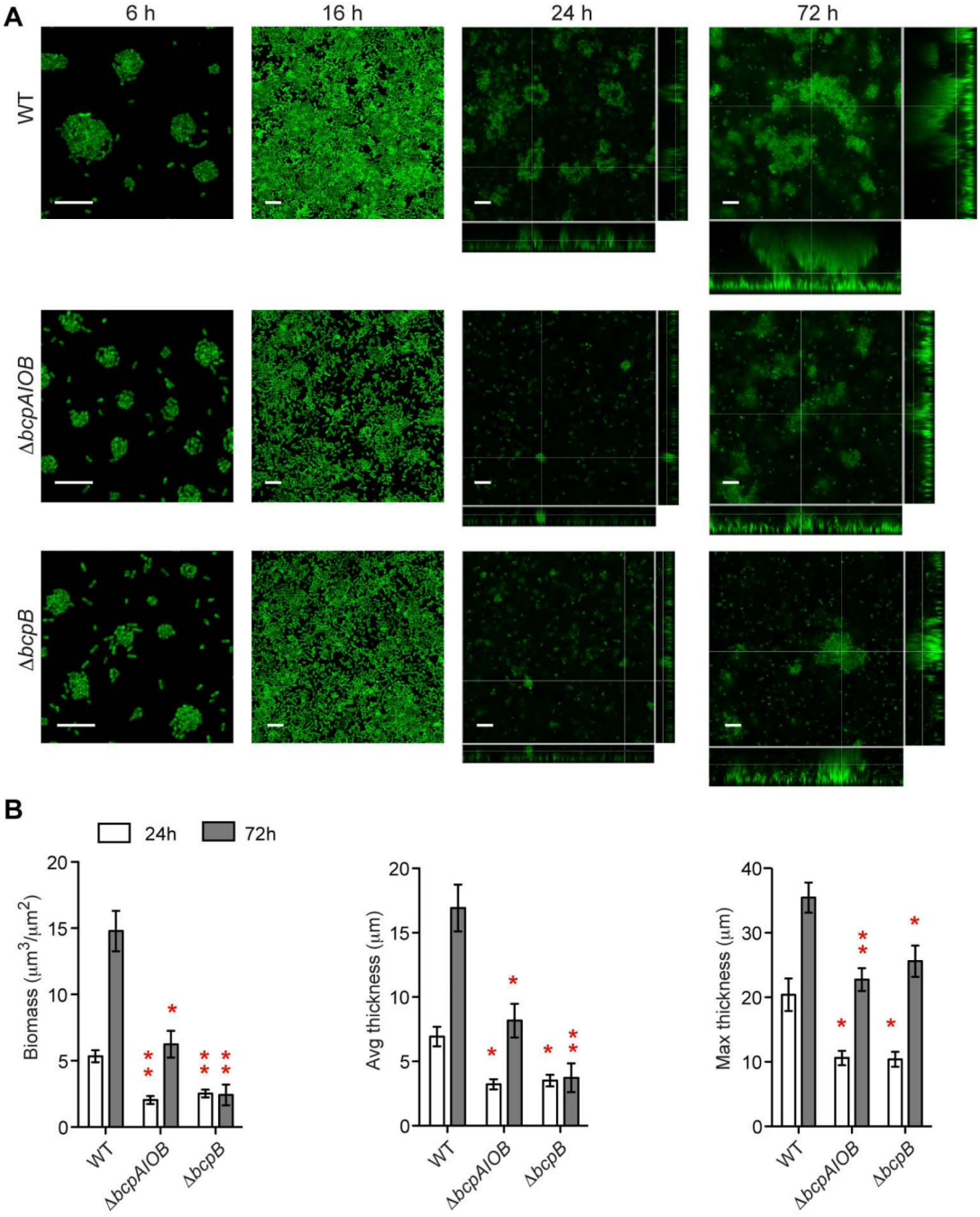

Fig. 1. Biofilm development by *B. thailandensis bcpAIOB* mutants

(A) CLSM of biofilms formed by wildtype *B. thailandensis* (top row), Δ*bcpAIOB* mutant bacteria (middle row), and Δ*bcpB* mutant bacteria (bottom row) at 6, 16, 24, and 72 h post-inoculation. All strains carry a *gfp* gene expressed constitutively from P_{S12} located at one of the two *att*Tn7 sites in the *B. thailandensis* genome. In Z stack renderings (24 h and 72 h), cross-sections through the plane parallel to the coverslip (shown by large center image) were set at 6 μm above the substratum for 24 h and 10 μm for 72 h. Scale bars represent 10 μm. (B) For each of the strains in (A), COMSTAT analysis of Z stacks collected after 24 (white bars) or 72 h (gray bars) of biofilm development was used to determine biofilm total biomass (left panel), average thickness (middle panel), and maximum thickness (right

panel). Bars represent the mean of at least three independent experiments and error bars indicate the SEM. Significance was determined by two-tailed *t*-tests, comparing each mutant to the wildtype strain at the indicated timepoint. *$p<0.05$; **$p<0.001$.

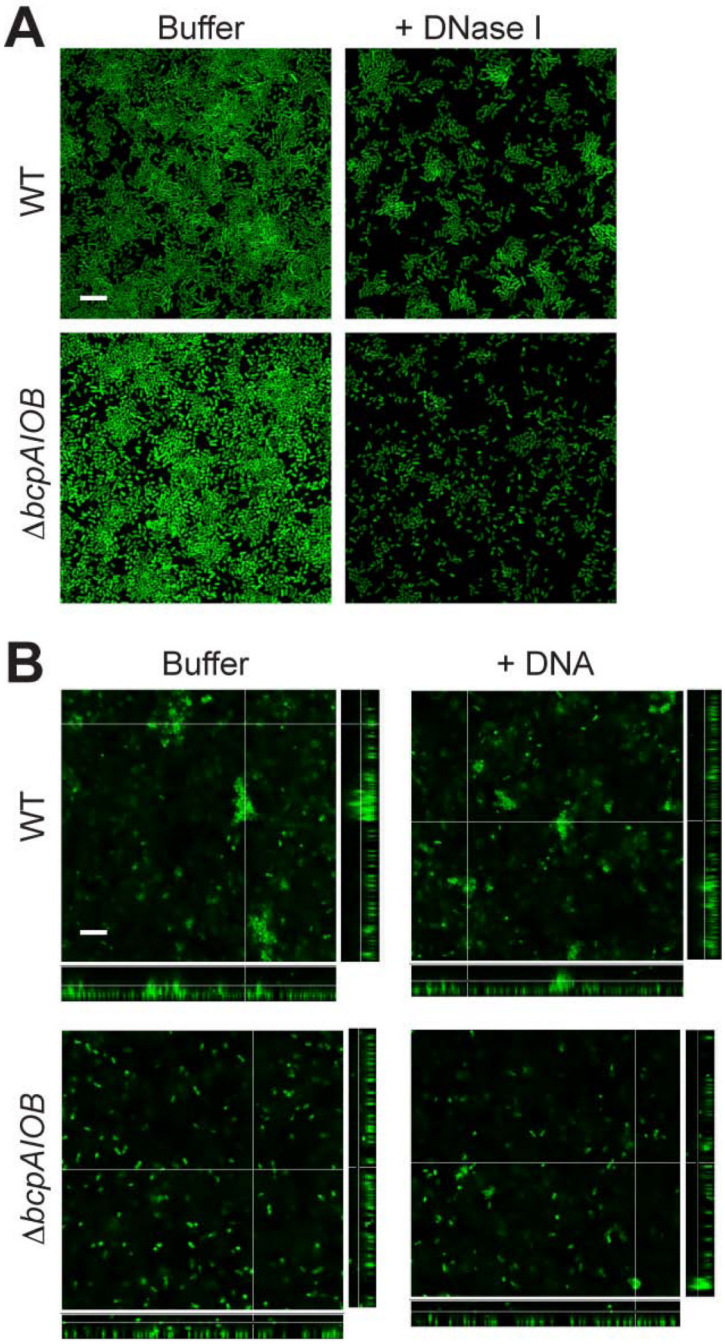

Fig. 2. Contribution of extracellular DNA to *B. thailandensis* biofilms

(A) CLSM of 16 h biofilms formed by wildtype (top) and Δ*bcpAIOB* mutant (bottom) bacteria that were co-inoculated with 20 U DNase I (right) or buffer alone (left). (B) CLSM of 24 h biofilms formed by wildtype (top) and Δ*bcpAIOB* mutant (bottom) bacteria that were co-inoculated with 3 μg purified *B. thailandensis* chromosomal DNA (right) or buffer alone (left). Cross-sections through the plane parallel to the coverslip (shown by large center image) were set at 6 μm above the substratum. The wildtype strain carries a *gfp* gene expressed constitutively from P_{S12} at the *att*Tn7 site of *B. thailandensis* chromosome 2 and the Δ*bcpAIOB* mutant carries the same construct at the *att*Tn7 site on chromosome 1. Scale bars represent 10 μm.

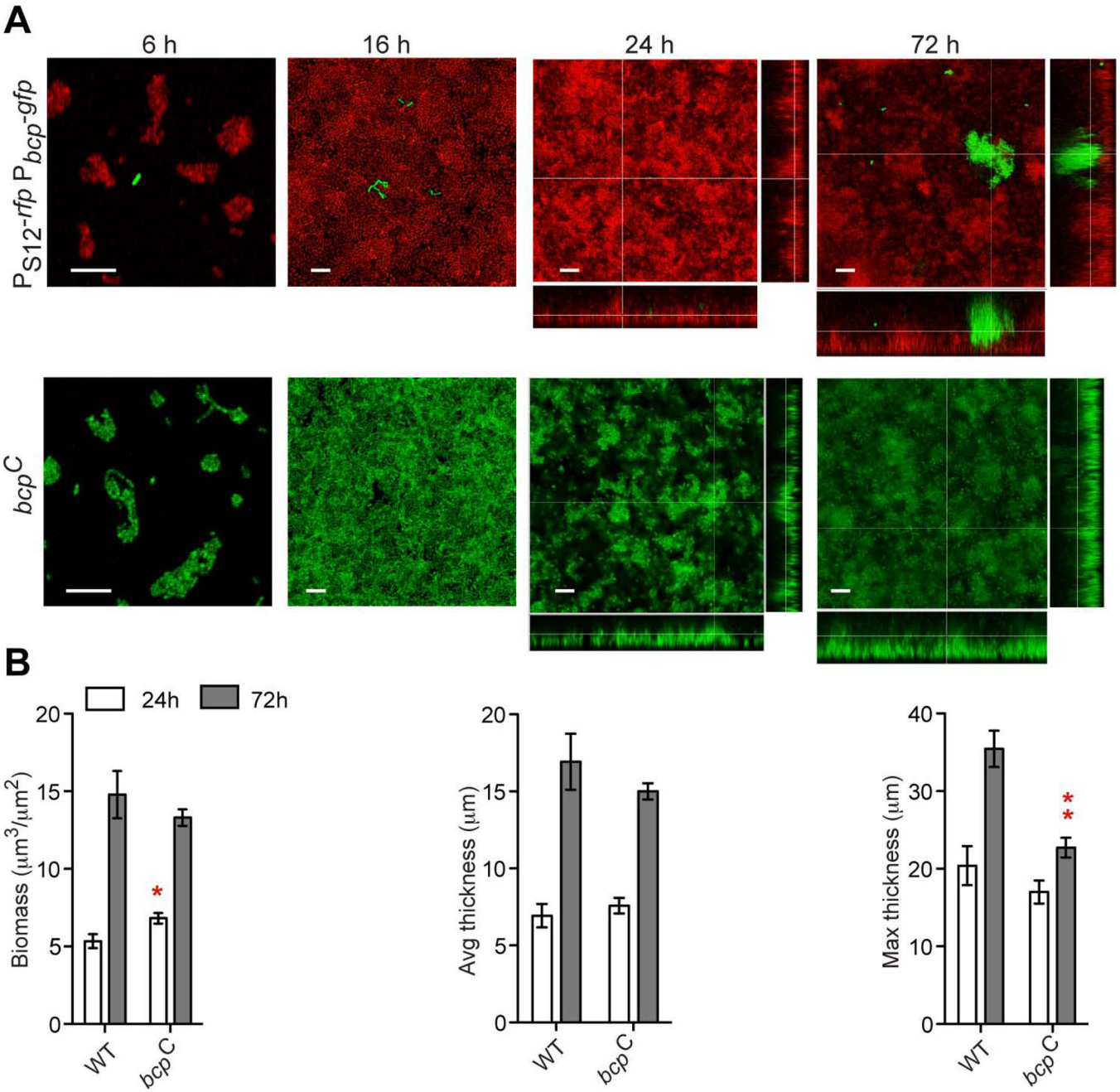

Fig. 3. *bcpAIOB* gene expression during *B. thailandensis* biofilm formation and effect of constitutive expression on biofilm architecture

(A) CLSM of biofilms formed by a *B. thailandensis* fluorescent reporter strain carrying a *gfp* gene driven by P_{bcpA} located at the *att*Tn7 site on *B. thailandensis* chromosome 2 and an *rfp* gene expressed constitutively from P_{S12} at the *att*Tn7 site on *B. thailandensis* chromosome 1 (top row), and mutant bacteria constitutively expressing *bcpAIOB* (*bcp*^{C}) (bottom row). In Z stack renderings (24 h and 72 h), cross-sections through the plane parallel to the coverslip (shown by large center image) were set at 6 μm above the substratum for 24 h and 10 μm for 72 h. Scale bars represent 10 μm. (B) For each of the strains in (A), COMSTAT analysis of Z stacks collected after 24 (white bars) or 72 h (gray bars) of biofilm development was used to determine biofilm total biomass (left panel), average thickness (middle panel), and

maximum thickness (right panel). Wildtype data from Fig. 1 are re-plotted here for comparison. Bars represent the mean of at least three independent experiments and error bars indicate the SEM. Significance was determined by two-tailed *t*-tests, comparing each mutant to the wildtype strain at the indicated timepoint. *$p<0.05$; **$p<0.001$.

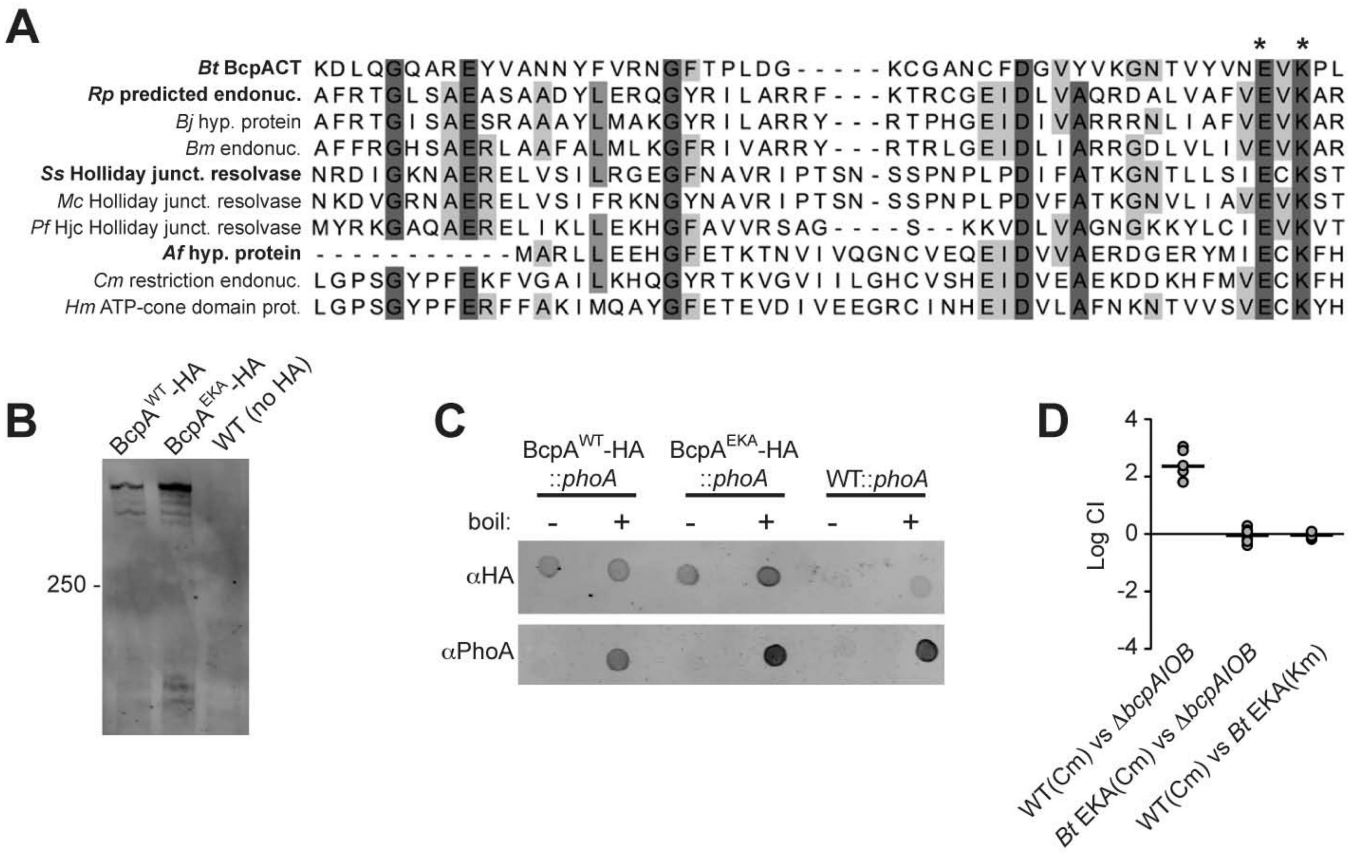

Fig. 4. Identification of BcpA$_{E264}$ putative catalytic residues E3064 and K3066 and their role in interbacterial competition

(A) Alignment of BcpA residues 3018–3068 (top line) with PHYRE2 secondary structure prediction hits (bolded) and their homologs. See Methods for accession numbers. Highlighted residues are conserved in at least 5/10 sequences and asterisks indicate amino acids (E3064 and K3066) mutated in strain *Bt*EKA. (B) Anti-HA western blot of whole cell lysates prepared from wildtype and *Bt*EKA bacteria constitutively producing HA-tagged BcpA (BcpA^{WT}-HA and BcpA^{EKA}-HA, corresponding to strains E264BcpA-HA::pECG22 and *Bt*EKABcpA-HA::pECG22, respectively) or wildtype bacteria constitutively producing untagged BcpA [WT (no HA), corresponding to strain *bcp*^{C}]. HA-encoding sequences were inserted after the F2633 codon of *bcpA* on the chromosomes of wildtype and *Bt*EKA bacteria. (C) Dot blot of the strains in (B) that have been modified to also contain *phoA* driven by P_{S12} at one of the two *att*Tn7 sites in the *B. thailandensis* genome. Boiled (+) and unboiled (−) cells were probed wnd anti-PhoA antibodies to assess surface exposure of BcpA-HA. (D) Competitive indices (CI) (inhibitor:target) after 24 h of interbacterial competition. Inhibitor bacteria [WT(Cm) (E264Cm^{R}) or *Bt*EKA(Cm)] were mixed at a 1:1 ratio with the indicated target bacteria [Δ*bcpAIOB* or *Bt*EKA(Km)], spotted onto agar. After 24 h of incubation, samples from the center of the colony spot were collected and plated with antibiotic selection to determine the CFUs of each strain and calculate CI (shown as log CI). Data represent two independent experiments, each performed in triplicate.

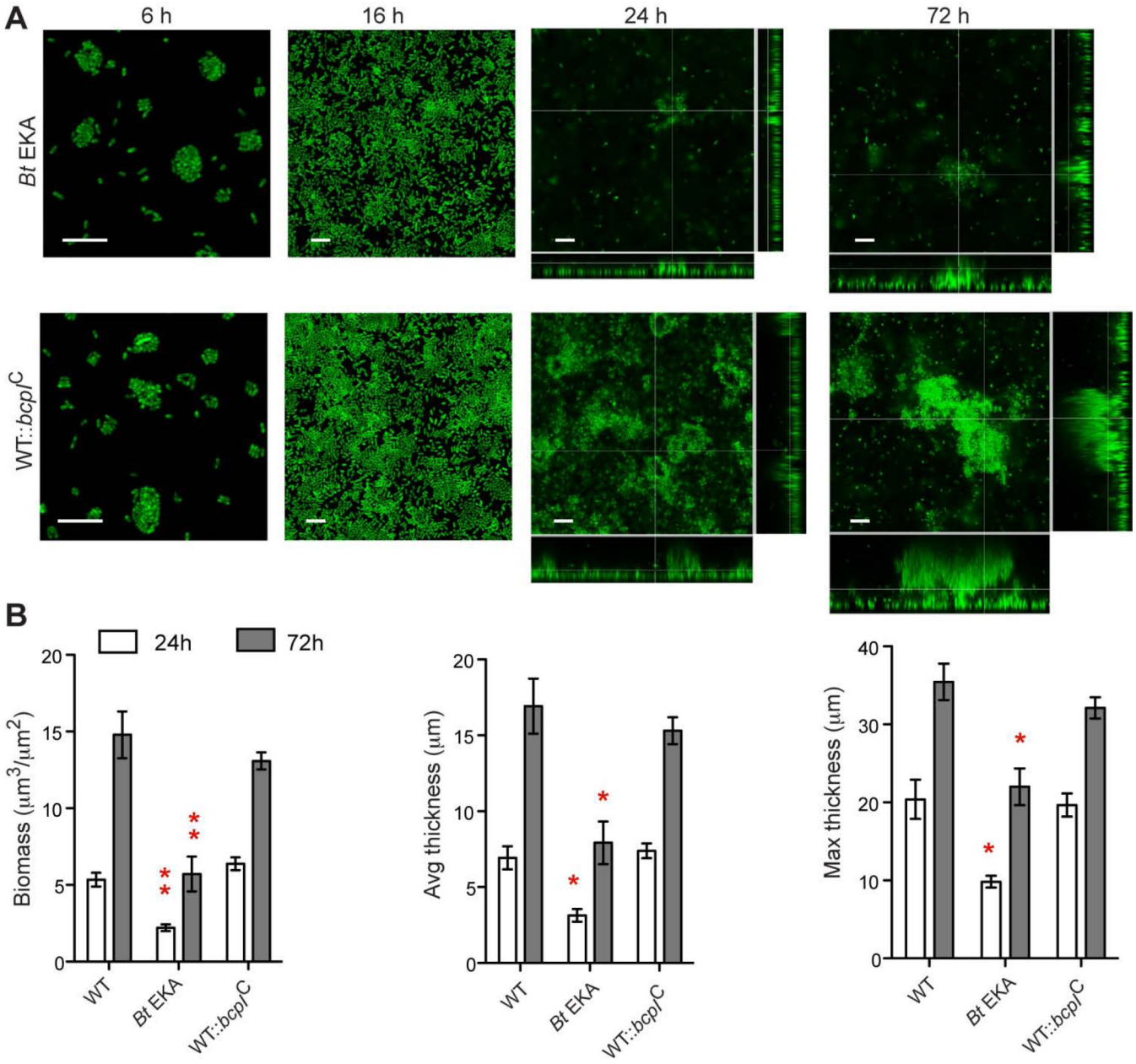

Fig. 5. Biofilm development by the CDI-inactive *Bt*EKA mutant and effect of constitutive *bcpI* expression on biofilm formation

(A) CLSM of biofilms formed by *Bt*EKA mutant bacteria (top row) and wildtype bacteria constitutively expressing *bcpI* (WT::*bcpI*^{C}) (bottom row) at 6, 16, 24, and 72 h post-inoculation. Strains carry a *gfp* gene expressed constitutively from P_{S12} located at the *att*Tn7 site on *B. thailandensis* chromosome 1. In Z stack renderings (24 h and 72 h), cross-sections through the plane parallel to the coverslip (shown by large center image) were set at 6 μm above the substratum for 24 h and 10 μm for 72 h. Scale bars represent 10 μm. (B) For each of the strains in (A), COMSTAT analysis of Z stacks collected after 24 (white bars) or 72 h (gray bars) of biofilm development was used to determine biofilm total biomass (left panel), average thickness (middle panel), and maximum thickness (right panel). Wildtype data from Fig. 1 are re-plotted here for comparison. Bars represent the mean of at least three independent experiments and error bars indicate the SEM. Significance was determined by

two-tailed *t*-tests, comparing each mutant to the wildtype strain at the indicated timepoint. *$p<0.05$; **$p<0.001$.

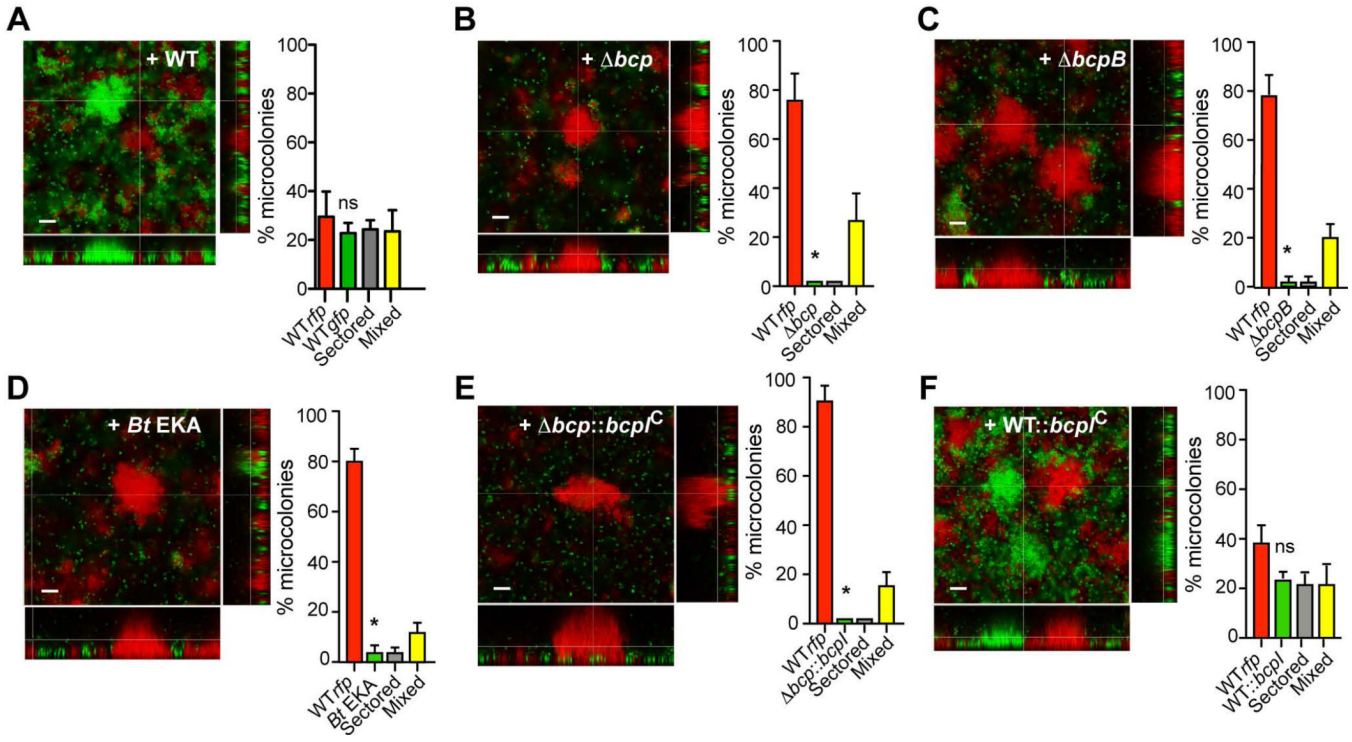

Fig. 6. Analysis of biofilms formed by mixtures of wildtype *B. thailandensis* and *bcpAIOB* mutants

RFP-producing wildtype *B. thailandensis* was mixed and inoculated at a 1:1 ratio with GFP-producing (A) wildtype bacteria, (B) Δ*bcpAIOB* mutant (Δ*bcp*), (C) Δ*bcpB* mutant, (D) *Bt*EKA mutant, (E) Δ*bcpAIOB* mutant constitutively expressing *bcpI* (Δ*bcp::bcpI*^{C}) or (F) wildtype bacteria constitutively expressing *bcpI* (WT::*bcpI*^{C}). Images represent 48 h biofilms of each mixture. Each graph depicts the percent of microcolonies observed that were composed of only RFP^{+} bacteria (WT, red bars), only GFP^{+} bacteria (mutant, green bars), half RFP^{+} and half GFP^{+} bacteria (sectored, gray bars), or containing a mixture RFP^{+} and GFP^{+} bacteria (mixed, yellow bars). It should be noted that most "mixed" microcolonies were predominantly RFP^{+} with fewer GFP^{+} cells adhered to their surfaces. Microcolony composition was scored at 24 h in at least 10 randomly-selected microscope fields in each of three independent experiments. Bars represent the mean and error bars show the standard error of the mean. Significance was determined by two-tailed *t*-test, comparing each proportion of GFP^{+} microcolonies (mutant, green bars) to the proportion of RFP^{+} structures (WT, red bars). *p<0.05; ns, not significant.

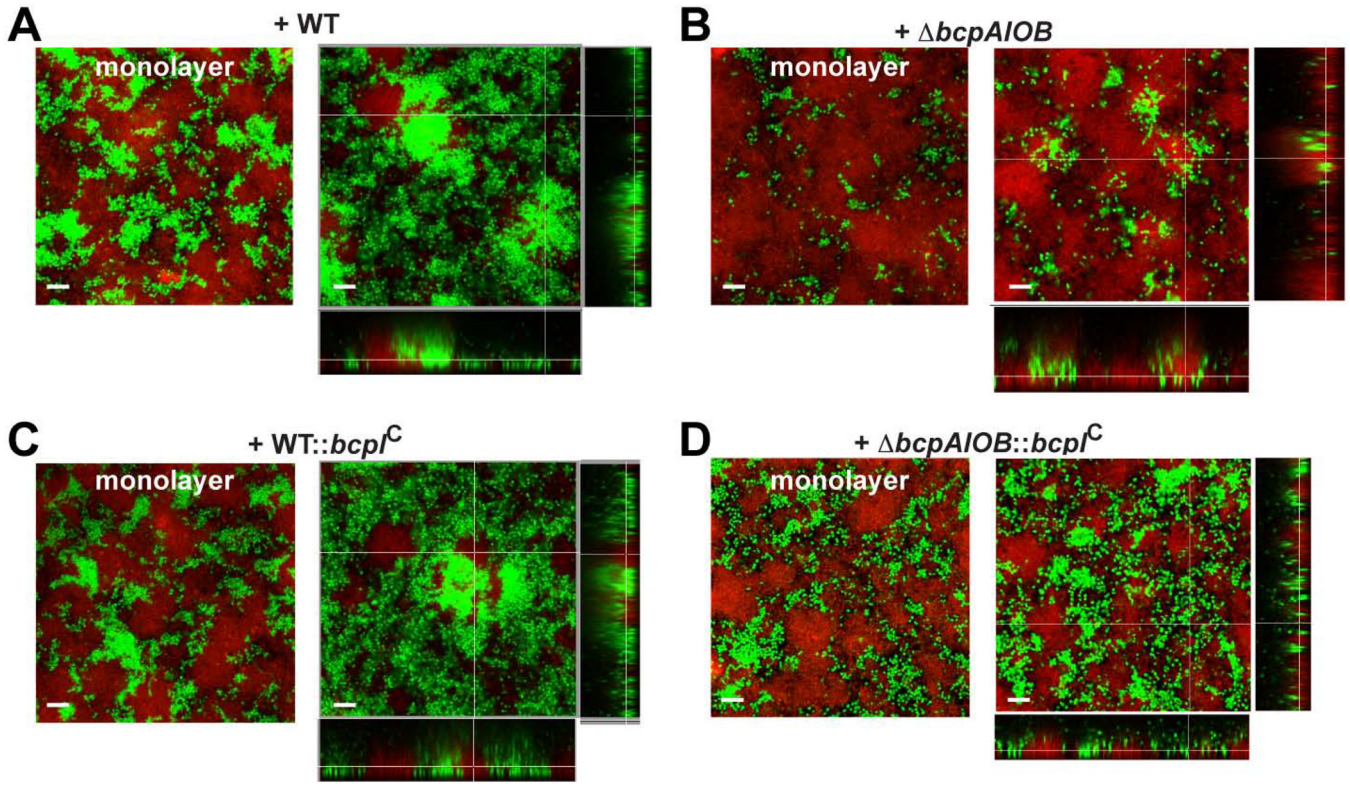

Fig 7. Interbacterial competition within *B. thailandensis* biofilms

RFP-marked wildtype *B. thailandensis* was mixed and inoculated at a 1:1 ratio with GFP-marked (A) wildtype bacteria, (B) Δ*bcpAIOB* mutant, (C) wildtype bacteria constitutively expressing *bcpI* (WT::*bcpI*^{C}), or (D) Δ*bcpAIOB* mutant constitutively expressing *bcpI* (Δ*bcpAIOB::bcpI*^{C}) and imaged at 72 h. For each mixture, the left image depicts the biofilm monolayer and the right image shows Z stack rendering. Scale bars represent 10 μm.