

Dissection of the *Burkholderia* intracellular life cycle using a photothermal nanoblade

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Burkholderia pseudomallei and *Burkholderia thailandensis* are related pathogens that invade a variety of cell types, replicate in the cytoplasm, and spread to nearby cells. We have investigated temporal and spatial requirements for virulence determinants in the intracellular life cycle, using genetic dissection and photothermal nanoblade delivery, which allows efficient placement of bacterium-sized cargo into the cytoplasm of mammalian cells. The conserved Bsa type III secretion system (T3SS_{Bsa}) is dispensable for invasion, but is essential for escape from primary endosomes. By nanoblade delivery of *B. thailandensis* we demonstrate that all subsequent events in intercellular spread occur independently of T3SS_{Bsa} activity. Although intracellular movement was essential for cell–cell spread by *B. pseudomallei* and *B. thailandensis*, neither BimA-mediated actin polymerization nor the formation of membrane protrusions containing bacteria was required for *B. thailandensis*. Surprisingly, the cryptic (*fla2*) flagellar system encoded on chromosome 2 of *B. thailandensis* supported rapid intracellular motility and efficient cell–cell spread. Plaque formation by both pathogens was dependent on the activity of a type VI secretion system (T6SS-1) that functions downstream from T3SS_{Bsa}-mediated endosome escape. A remarkable feature of *Burkholderia* is their ability to induce the formation of multinucleate giant cells (MNGCs) in multiple cell types. By infection and nanoblade delivery, we observed complete correspondence between mutant phenotypes in assays for cell fusion and plaque formation, and time-course studies showed that plaque formation represents MNGC death. Our data suggest that the primary means for intercellular spread involves cell fusion, as opposed to pseudopod engulfment and bacterial escape from double-membrane vacuoles.

The robust Gram-negative bacillus *Burkholderia pseudomallei* (*Bp*) is endemic to warm, fecund soils of tropical regions (1, 2). Infections acquired from the environment can lead to melioidosis, a serious and sometimes fatal human disease. Accumulating evidence suggests that adaptations selected in the rhizosphere are responsible for “accidental virulence” in mammals (3). *Bp* has a large (7.2 Mb) genome that has been shaped by extensive horizontal exchange (4). *Burkholderia mallei* (*Bm*) is a clonal descendant of *Bp* that has undergone genome decay and has lost the capacity for environmental survival. *Bm* is the agent of equine glanders and it can also cause fatal human infections (2). Resistance to antibiotics and their low infectious dose have led to the classification of *Bp* and *Bm* as biowarfare threats.

The geographic distribution of *Bp* overlaps with that of *B. thailandensis* (*Bt*) and their genomes are highly similar and syntenic (5). Although *Bt* is rarely associated with human disease and is considered relatively nonpathogenic, this assessment is not absolute. Following aerosol challenge of mice, *Bt* causes fulminant, lethal infections that are dependent on virulence determinants shared with *Bp* and *Bm* (2, 6). *Bp*, *Bt*, and *Bm* invade and replicate in a wide range of cell types and exhibit nearly identical intracellular life cycles (1, 2). Following invasion and escape from endosomes, replication in the cytoplasm is accompanied by actin-based motility and cell–cell spread, analogous to *Shigella*

flexneri and *Listeria monocytogenes* (7–9). Actin motility is mediated by BimA, a polarly localized surface protein that binds actin and promotes polymerization (9). An unusual feature of infection is the induction of cell fusion and the formation of multinucleate giant cells (MNGCs) (10). For *Bp* and *Bm* this requires BimA and has been observed with multiple cell types in vitro and in tissues from patients with melioidosis (2).

Bp possesses a generous endowment of specialized export systems including three “injection” type III secretion systems (T3SS), two of which are similar to those in phytopathogenic bacteria. The third, T3SS_{Bsa} is homologous to the *Shigella* Mxi-Spa and *Salmonella* SPI-1 T3SSs and is highly conserved in *Bp*, *Bt*, and *Bm* (1, 2). T3SS_{Bsa} is required for virulence in hamster and murine models of pathogenesis (2) and has been implicated in invasion of epithelial cells, escape from endosomes, intracellular survival, and evasion of autophagy (11). In addition, *Bp* encodes six type VI secretion systems (T6SSs) (12). Using the nomenclature of Schell et al. (13), T6SS-1 (also referred to as T6SS-5) (14) is critical for virulence in the *Bt* murine model of acute melioidosis and contributes to the lethality of *Bm* in hamsters (13). Recently, T6SS-1 mutants in *Bp* were shown to be capable of endosome escape in RAW264 cells but were defective in MNGC formation (15).

For intracellular pathogens, understanding the roles of virulence determinants is complicated by their involvement in temporally and spatially staged events. T3SS_{Bsa} has been proposed to be required for late events associated with cell–cell spread, but direct investigation has been difficult since mutants are defective in earlier steps in the intracellular life cycle. To address this conundrum, we have used a photothermal nanoblade to deliver live bacteria directly into the cytoplasm of mammalian cells (16). The photothermal nanoblade device uses a laser pulse to excite a thin titanium coating on the tip of a glass capillary pipette. Rapid thermal excitation of the metallic nanostructure produces an explosive nanoscale vapor bubble that creates a small incision in the cell membrane at the point of pipette contact. This incision provides a transient delivery portal through which variably sized cargo—from molecules to bacteria—can be efficiently delivered with high cell viability. We have combined the use of this technology with traditional genetic ablation techniques and infection analysis to probe virulence mechanisms participating in the intracellular life cycle of *Burkholderia*.

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Results

T3SS_{Bsa} Is Required for Plaque Formation and Endosome Escape but Is Dispensable for Invasion. HEK293 cells are efficiently invaded by *Bp* and *Bt* and are highly amenable to photothermal nanoblade-mediated cytosolic delivery. In the experiment in Fig. 1A, HEK293 monolayers were infected with *B. thailandensis* E264 (17), *B. pseudomallei* Bp340 (18), or derivatives containing in-frame deletions in *sctN*, which encodes the ATPase required for activity of the Bsa T3SS. Plaque formation, a hallmark of cell-cell spread, was eliminated in $\Delta sctN$ mutants and restored by complementation *in trans*. These observations are consistent with previous reports that plaque formation by *Bp* is dependent on T3SS_{Bsa} (19).

It has been suggested that T3SS_{Bsa} is required for invasion of nonphagocytic cells, a prerequisite for plaque formation (20). For Bp340 and BtE264, invasion was inhibited by cytochalasin D as expected, but it was unaffected by $\Delta sctN$ alleles (Fig. 1B). These results indicate that whereas invasion requires actin polymerization, it occurs independently of T3SS_{Bsa} activity. Analogous results were obtained using HeLa cells (Fig. S1A). To determine whether T3SS_{Bsa} facilitates endosome escape in HEK293 cells, monolayers were stained 8 h after infection for F-actin. As shown in Fig. S1B, BtE264 and Bp340 formed actin tails, indicating successful escape, whereas $\Delta sctN$ mutants and $\Delta bimA$ control strains did not. Moreover, $\Delta sctN$ mutants colocalized with the late endosomal marker LAMP1 (Fig. S1C). At later time points, only scattered bacterial debris was detected for $\Delta sctN$ mutants, suggesting they were killed and degraded due to endosomal entrapment (Fig. S1D). Although our results do not support a role for T3SS_{Bsa} in invasion, they are consistent with previous reports of its essential contribution to endosome escape (2, 21).

Intercellular Spread Following Cytoplasmic Delivery by a Photothermal Nanoblade. The inability of $\Delta sctN$ mutants to escape from primary endosomes confounds efforts to understand the involvement of T3SS_{Bsa} in subsequent steps required for cell-cell spread. Solutions to this dilemma require the ability to bypass early events that are otherwise essential during infection. To accomplish this, we exploited the capabilities of our recently developed photothermal nanoblade to place bacteria directly into the cytoplasm of host cells (Fig. 2A) (16). Because a nanoblade has not yet been customized and approved for use with *Bp* in a select agent BSL-3 facility, we focused our analysis on *Bt*, which can be safely manipulated under BSL-2 conditions.

In Fig. 2, wild-type (*WT*) BtE264 or mutant derivatives were introduced into HEK293 cells by infection or by photothermal nanoblade delivery. Plaque formation following infection was

absolutely dependent on T3SS_{Bsa} activity (Fig. 2B). When $\Delta sctN$ mutants were delivered into the cytosol using the nanoblade, they divided and polymerized actin as well as wild-type bacteria (Fig. 2C), demonstrating that actin motility functions independently of T3SS_{Bsa} and does not require passage through the endosomal environment. We were surprised, however, to find that $\Delta sctN$ mutants were capable of forming plaques following nanoblade delivery that were indistinguishable in size and morphology from those formed by *WT Bt* following infection or nanoblade delivery (Fig. 2D–F). The ability to bypass early events in the intracellular life cycle allows us to conclude that the *only* requirement for T3SS_{Bsa} in cell-cell spread and plaque formation is for escape from primary endosomes following invasion. It had been assumed that for cell-cell spread to occur, T3SS_{Bsa} would be required to lyse double-membrane secondary vacuoles formed during penetration of adjacent cells (1). Our observations indicate that either some other factor(s) performs this function, or intercellular spread occurs by an entirely different mechanism.

Two Distinct Motility Systems Facilitate Plaque Formation. A deletion mutant in *bimA* was included as a control for plaque formation in photothermal delivery experiments. As shown in Fig. S1B and Fig. 2C, the $\Delta bimA$ allele eliminates actin polymerization as previously described (22), and it also eliminates the formation of membrane protrusions containing *Burkholderia* at their tips. Although we assumed that actin motility would be required as a driving force for cell-cell spread, this notion was clearly incorrect. Following infection or nanoblade delivery, $\Delta bimA$ mutants formed plaques that were similar in size and morphology to those of their *WT* parent (Fig. 2B, D, and E). BimA-mediated actin motility and the formation of membrane protrusions are therefore dispensable for cell-cell spread by *B. thailandensis*.

Intrigued by these results, we examined live infected cells by microscopy and discovered that *Bt* exhibits remarkably rapid intracellular motility. Bacteria move at speeds of $>20 \mu\text{m/s}$ and often reverse course and change direction (Fig. S2A and Movies S1, S2, and S3). Because rapid intracellular motility was independent of BimA (Fig. 3A), we explored the possibility that flagellar motility was involved. Deletion of *motA1*, a motor component locus in the chromosome 1 flagellar biosynthesis gene cluster (*fla1*), eliminated swarming in soft agar but had no effect on intracellular motility or plaque formation (Fig. S2, Fig. 3A and B, and Table S1). Previous studies identified potential chemotaxis and flagellar loci on chromosome 2 in *Bt* (23), and on closer inspection we found a full complement of flagellar structural and regulatory genes that could encode a second, functional motility system (*fla2*; Fig. S3). Deletion of *motA2* from the *fla2* flagellar cluster had no effect on swarming in agar or actin polymerization following invasion (Fig. S2B and Fig. 3C), but it eliminated rapid intracellular motility (Fig. 3A), as did deletion of *fliC2*, which is predicted to encode flagellin (Table S1). Although significant differences were not observed in plaquing efficiency (Fig. 3B), $\Delta motA2$ mutants formed plaques that were smaller than those produced by *WT* or $\Delta bimA$ strains (Fig. 3D and Table S1). When *motA2* was deleted from a $\Delta bimA$ background, plaque formation was almost completely abolished, and the defect was reversed by complementation with *motA2* (Fig. 3B).

These observations demonstrate that MotA2-dependent flagellar motility can drive intercellular spread independently of BimA-mediated actin polymerization, and at least one of the two motility systems must be active for plaque formation. Although flagellar motility does not affect invasion or endosome escape (Fig. 3E and Table S1), an interesting phenotype is observed in intracellular growth assays. As shown in Fig. 3F, *WT*, $\Delta bimA$, and $\Delta motA2$ strains multiply, plateau at 12 h, and decrease in num-

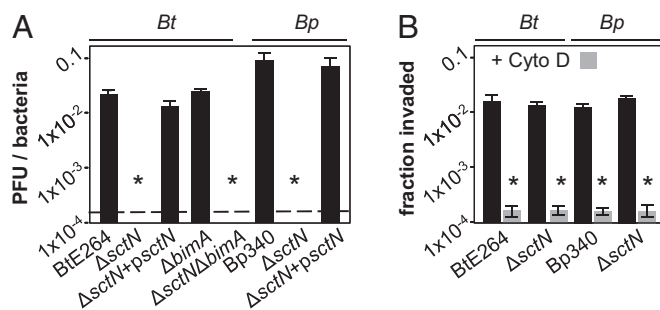


Fig. 1. T3SS_{Bsa} is required for endosome escape but not invasion. (A) Plaque-forming units (pfu) per bacteria 18 h after infection of HEK293 cells with BtE264, Bp340, $\Delta sctN$ mutants, or complemented derivatives (psctN). Dashed line, limit of detection. (B) Colony forming units (cfu) recovered per bacteria in 2-h invasion assays. HEK293 cells were untreated (solid bars) or treated with 2 $\mu\text{g/mL}$ cytochalasin D (shaded bars) before infection. Assays were performed in triplicate and error bars represent $\pm\text{SEM}$. * $P < 0.005$.

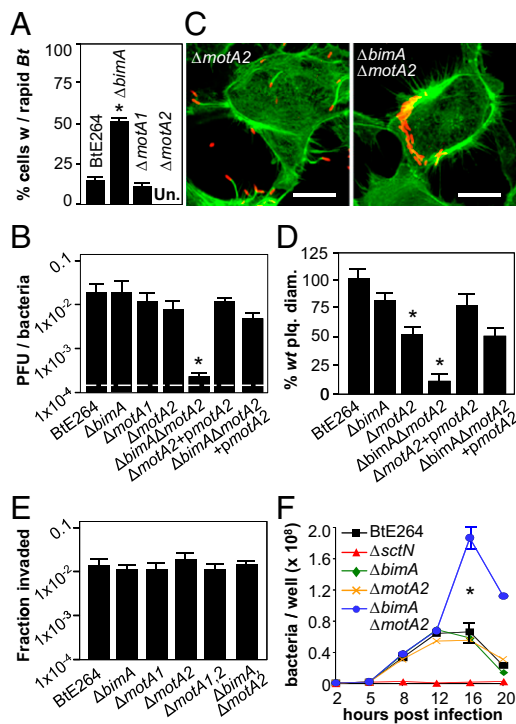


Fig. 3. *fla2*-mediated flagellar motility facilitates plaque formation. (A) Fraction of HEK293 cells containing rapidly motile bacteria 8 h after infection with BtE264 and derivatives. Approximately 300 cells were monitored per strain. (B) Plaque-forming efficiency on HEK293 cell monolayers 18 h after infection. (C) Representative infected cells stained for bacteria (red) and actin (green). (D) Plaque diameters 24 h after infection of HEK293 cell monolayers. (E) HEK293 cell invasion efficiencies by BtE264 or mutant strains 2 h postinfection. (F) Time course of intracellular replication in HEK293 cells. All assays were performed in triplicate and error bars represent \pm SEM. * P < 0.005; Un, undetectable.

Discussion

Our results are incorporated into a model for the *Burkholderia* intracellular life cycle shown in Fig. S4. This model is based on observations with HEK293 cells and may not take into account factors that are specifically required for survival and replication in professional phagocytes.

Invasion. Invasion of HEK293 cells by *Bp* or *Bt* requires host-cell actin polymerization but not the activity of T3SS_{Bsa}, contrasting with a clear requirement for the Mxi-Spa and SPI-1 T3SSs in invasion by *Shigella* (7) and *Salmonella* (26), respectively, and with expectations for *Burkholderia* based on analyses of BopE, a T3SS_{Bsa} substrate homologous to *Salmonella* SopE and SopE2 (20). In an experiment often cited as supporting a role for T3SS_{Bsa} in invasion (20), insertion mutations in *bipD* (a T3SS_{Bsa} translocon gene) or *bopE* conferred modest decreases in invasion (35–40%) 6 h after infection of HeLa cells. The discrepancy between these data and ours is likely due to the use of a late time point where endosomally trapped mutants lose viability (6 h in ref. 20 vs. 2 h in Fig. 1), giving the appearance of an invasion defect. A similar conclusion was previously reported by Haraga et al., using BtE264 and HeLa cells (21). Their study and ours support the conclusion that for *Bp* and *Bt*, invasion of non-phagocytic cells can occur by mechanisms that are independent of T3SS_{Bsa}. Virulence determinants that mediate invasion await discovery.

Bsa T3SS. T3SS_{Bsa} is required for escape from endosomes following invasion of HEK293 cells by *Bp* or *Bt*, consistent with

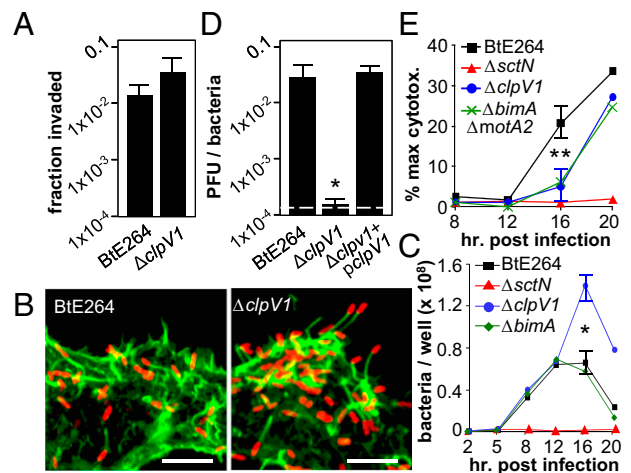


Fig. 4. T6SS-1 is critical for efficient intercellular spread. (A) Invasion efficiencies by BtE264 or $\Delta clpV1$ mutants 2 h postinfection in HEK293 cells. (B) HEK293 cells were infected and stained for bacteria (red) and actin (green) 8 h postinfection. (Scale bar, 20 μ m.) (C) Time course of intracellular replication in HEK293 cells. (D) Plaque-forming efficiency on HEK293 cell monolayers 18 h after infection. (E) Cytotoxicity assays in HEK293 cells. All assays in A and C–E were performed in triplicate and error bars represent \pm SEM. ** P < 0.05; * P < 0.005.

numerous prior studies with a variety of cell types (1, 2). Cytosolic delivery of *Bt* using a photothermal nanoblade allowed us to bypass endosome escape and directly examine the role of T3SS_{Bsa} in downstream events: actin polymerization, cell–cell spread, and MNGC formation. In all cases the results were remarkably clear; plaque formation following infection was absolutely dependent on T3SS_{Bsa}, whereas plaque formation following nanoblade delivery was independent of its activity. The same was true for MNGC formation. We also show that replication and actin polymerization occur normally when $\Delta sctN$ mutants are placed directly into the cytosol.

These observations have several implications; first, they allow us to conclude that in our system, the *only* role for T3SS_{Bsa} is to facilitate escape from primary endosomes of initially infected cells. Next, our results help explain a perplexing lack of phenotypes associated with known or putative effectors. In contrast to obvious requirements for the Bsa T3SS in vitro and in vivo (2), to our knowledge no effectors have been *definitively* shown to be required for invasion, replication in nonphagocytic cells, cell–cell spread, MNGC formation, or virulence in animals. BopA, a suspected T3SS_{Bsa} substrate, is reported to facilitate survival and evasion of autophagy in phagocytic cells (11), but *bopA* mutants are not significantly attenuated in mice (2). The precise mechanism of T3SS-mediated endosome escape is unknown for any intracellular pathogen; however, it could conceivably be a function of translocon insertion in the endosomal membrane and occur in the absence of additional effectors. Finally, there is an instructive contrast between the roles of the *Burkholderia* Bsa and *Shigella* Mxi-Spa T3SSs. The *Shigella* system is essential for both endosome escape and escape from double-membrane vacuoles formed during the process of cell–cell spread (7). For *Burkholderia*, our results support a model for intracellular spread that obviates the need for membrane lysis after the primary endosome has been breached.

Intracellular Motility. Polarized, unidirectional actin polymerization is a hallmark of cell–cell spread and plays an essential role for *Shigella*, *Listeria*, and other intracellular pathogens (9). The discovery that *Bt* remains fully capable of plaque formation in the absence of BimA was quite unexpected. Even more sur-

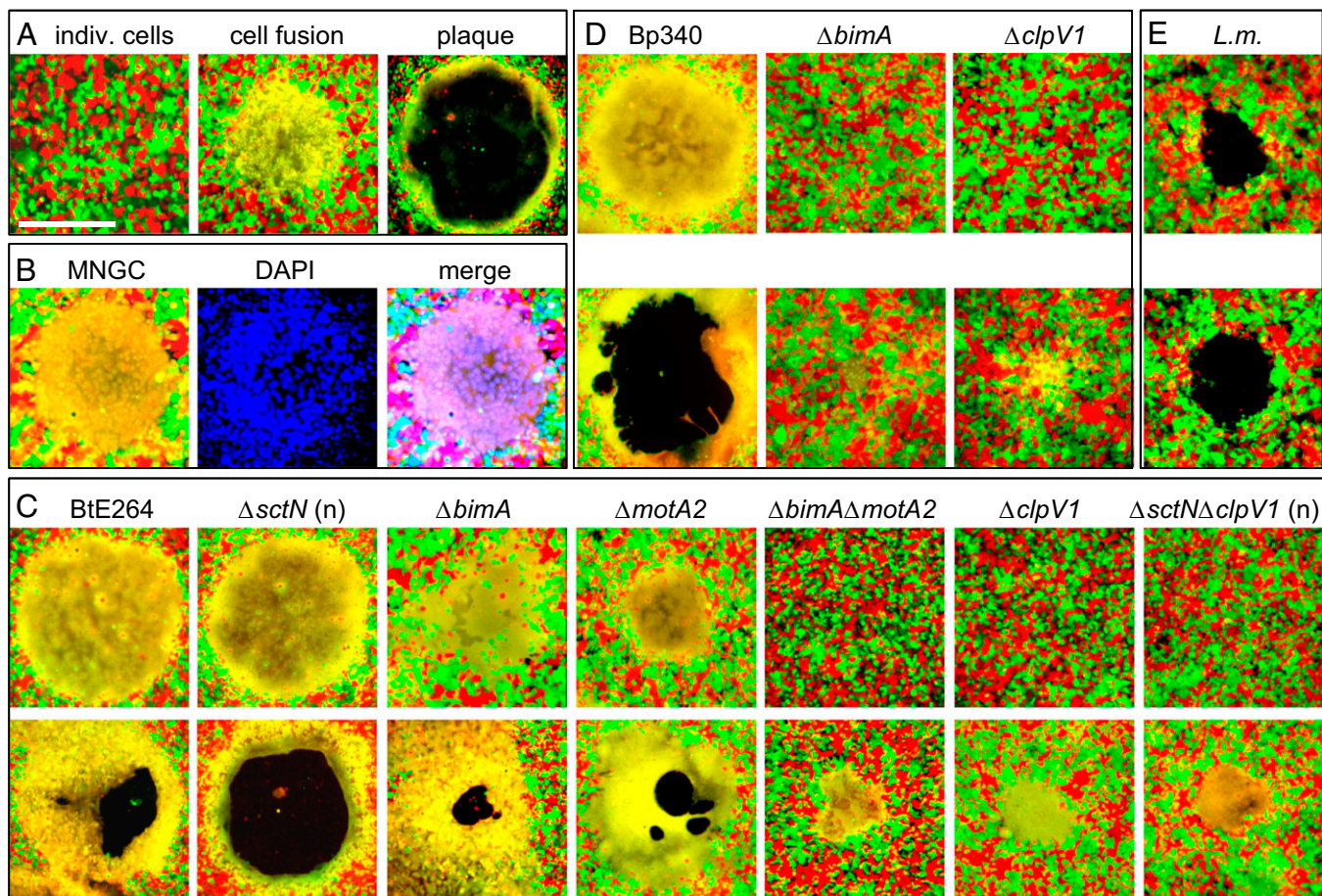


Fig. 5. Intercellular spread and plaque formation occur through cell fusion. (A) Progression of events leading to plaque formation. (Left) HEK293-RFP (red) and -GFP (green) cells immediately after infection. Twelve hours later, a MNGC is formed (yellow, Center), which undergoes lysis and forms a plaque at 24 h (Right). (Scale bar, 500 μ m.) (B) A MNGC (Left) was stained with DAPI (blue, Center). (Right) Triple-color merged image. (C) MNGC and plaque formation by BtE264 and mutants on HEK293 RFP + GFP monolayers 12 h (Upper) or 24 h (Lower) following infection or nanoblade delivery ("n", Δ sctN, Δ sctN Δ clpV1). (D) MNGC and plaque formation 12 h (Upper) and 24 h (Lower) after infection with Bp340 and mutants. (E) Plaque formation 56 h following infection with *L. monocytogenes* 104035. The slight appearance of yellow at the edge of plaques is due to physical overlap of red and green cells and does not indicate cell fusion. Images are representative of multiple independent experiments.

prising was the observation that a predicted flagellar system on chromosome 2 (*fla2*) can compensate for the lack of actin motility and drive intercellular spread and MNGC formation.

The *Bt* and *Bp* *fla1* flagellar gene clusters on chromosome 1 are highly conserved. *fla1* encodes polar flagella, which in *Bp* have been implicated in invasion of epithelial cells and virulence in animal models (2). In *Bt*, mutation of *motA1* (*fla1*) or *motA2* (*fla2*) individually or in combination had no effect on invasion. Although this is a first characterization of dual flagellar motility systems in an intracellular pathogen, their occurrence and functions have been described in *Vibrio* and *Aeromonas* spp., where they facilitate motility in response to different environmental signals (27). Not surprisingly, *fla1* and *fla2* in *Bt* were observed to function under different conditions; deletion of *motA1* eliminated swarming in soft agar but had no effect on motility following infection. Conversely, deletion of *motA2* had no effect in soft agar but eliminated rapid intracellular motility.

Our sequence analysis suggests that *fla2* encodes lateral flagella (Fig. S3B), but this prediction awaits direct confirmation. It is also unknown how the system is regulated or whether intracellular bacteria are simultaneously capable of BimA-mediated actin polymerization and *fla2*-dependent motility. Because flagellin monomers are known to activate assembly of the NLRC4 inflammasome, resulting in cytokine production and inflamma-

tory cell death (28), the use of flagella for intra- and intercellular motility is surprising. It is presently unknown whether *fla2* flagellin induces inflammasome-dependent cytoplasmic responses or whether mechanisms to overcome them exist. Perhaps the most important question involves the relevance of our observations with *Bt* to pathogenesis in *Bp* and *Bm*. A recent analysis of the global population structure of *Burkholderia* species pathogenic for mammals predicts an Australian origin for *Bp*, with a single introduction event leading to the expansion of Southeast Asian isolates (SEA *Bp*) and *Bm* (29). Interestingly, the *fla2* gene cluster is absent in SEA *Bp* isolates such as Bp340, which is dependent on BimA for plaque formation (Fig. 5D), but it is highly conserved in sequenced genomes from Australian strains (Fig. S3) (23). The potential role of the *fla2* locus in pathogenesis by Australian *Bp* remains to be investigated.

T6SS-1. T6SSs are widely distributed among pathogenic and nonpathogenic Gram-negative species; they have broad roles in survival and fitness and have been linked to virulence in numerous pathogens, including *Bp*, *Bm*, and *Bt* (2, 14). In our analysis, T6SS-1 was found to facilitate intercellular spread following infection or photothermal delivery. Consistent with recent reports (14, 15, 19), deletion of *clpV1* resulted in a defect in plaque formation in HEK293 cells and a delay in the formation

of MNGCs. $\Delta clpVI$ mutants exhibited robust actin-polymerization and flagellar-mediated motility inside cells, and genetic dissection using the photothermal nanoblade established that T6SS-1 functions downstream of invasion and T3SS_{Bsa}-mediated endosome escape. Concomitant with decreased efficiency of MNGC formation, we observed an increase in cell survival following infection with $\Delta clpVI$ mutants and an accumulation of intracellular bacteria. These data are consistent with the hypothesis that T6SS-1 participates in events that can alternatively facilitate intercellular spread by fusing cell membranes or kill cells by compromising their integrity.

Cell Fusion and Intercellular Spread. *Burkholderia* efficiently induce MNGC formation in both phagocytic and nonphagocytic cells (10). We propose that cell fusion represents the primary path for intercellular spread and plaque formation by *Bt* and *Bp* (Fig. S4), and the same is likely to hold true for *Bm*. These observations are consistent with the results of time course experiments showing that the formation of MNGCs and their eventual lysis give rise to the open cores of plaques, and with the lack of a requirement for T3SS_{Bsa} in cell–cell spread following cytosolic delivery of bacteria using our photothermal nanoblade. Whereas *Bt* T3SS_{Bsa} mutants are incapable of endosome escape following infection, *Bp* T3SS_{Bsa} mutants are reported to exhibit delayed escape that eventually leads to MNGC formation (30), consistent with our proposal that cell–cell spread occurs independently of T3SS_{Bsa} activity for both *Bt* and *Bp*. Furthermore, mutations that eliminate intracellular motility or inactivate T6SS-1 have analogous effects on MNGC formation and cell–cell spread following photothermal delivery or infection. Our model also explains the lack of published reports demonstrating double-membrane vacuoles following engulfment of protrusions with *Burkholderia* at their tips, as observed with *L. monocytogenes*, *S. flexneri*, and other

pathogens with similar lifestyles (7, 8). Although membrane protrusions are readily formed by wild-type *Bt*, they are not observed with $\Delta bimA$ mutants. The ability of $\Delta bimA$ strains that retain *fla2* motility to efficiently form plaques shows that membrane protrusions are not required for intercellular spread.

Our results, along with a recent report from Stevens et al. (22), demonstrate a clear link between motility and the efficiency of cell fusion by intracellular *Burkholderia*. We hypothesize that flagellar and/or actin-mediated motility increases the frequency of contact between bacteria and host cell membranes and that contact is prerequisite for membrane fusion through a process facilitated by T6SS-1. It is tempting to speculate that a bacterially encoded fusogenic factor is involved in this unique mechanism of cell–cell spread.

Materials and Methods

Detailed experimental procedures are found in *SI Materials and Methods*. BtE264 (17) and Bp340 (SEA *Bp* 1026b $\Delta amrRAB-oprA$) (18) mutants were constructed using allelic exchange as described (31). Nanoblade delivery was performed as described in *SI Materials and Methods* and in ref. 16.

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