Supporting Information

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SI Materials and Methods

Bacterial Strains, Growth, and Mutant Construction. BtE264 (1) and Bp340 (SEA *Bp* 1026b Δ*amrRAB-oprA*) (2) were routinely grown in L-medium with or without NaCl (LB or LB-NS). In-frame mutations were constructed using allelic exchange with the *pheS** negative selection marker on M9 agar containing 0.1% cholorophenylalanine (cPhe) (3). Gene loci that were targeted for mutational analysis are as follows: In BtE264, *sctN* (Bth_II0832), *bimA* (Bth_II0875), *clpV1* (Bth_II0864), *motA1* (Bth_I3185), *motA2* (Bth_II0153), and *fliC2* (Bth_II0151); and in Bp340, *sctN* (BPSS1541), *bimA* (BPSS1492), and *clpV1* (BPSS1502). Complementation of mutants was performed using derivatives of the pBBR1-MCS2 broad host-range plasmid (4) containing the *nptII* kanamycin resistance gene. For infection studies and cytosolic delivery with the photothermal nanoblade, 2 mL of LB-NS was inoculated with a small amount of bacteria swabbed from a fresh L-NS agar plate and grown with shaking to an OD_{600} of ∼0.5.

Cell Culture, Infection, and Cytotoxicity Assays. HEK293 (ATCC CRL-1573) and HeLa (ATCC CCL-2) cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% bovine growth serum (BGS; HyClone) and 5% CO_2. Before experiments, plate wells were incubated at room temperature for 30 min with a 1:40 dilution of Matrigel liquid (BD) in serum-free medium to enhance retention of cells during washing and manipulation. For infection studies, cells were seeded at 1.8–2.0 × 10^6 cells per well in six-well plates. Following the addition of bacteria, plates were gently centrifuged to bring bacteria into contact with cells (200 × *g* for 5 min).

Cells were infected using a multiplicity of infection (MOI) of 1 for cytotoxicity and intracellular replication experiments, an MOI of 10 for invasion, and an MOI of 3×10^{-4} for plaque assays. One hour after infection, cells were washed thoroughly with Hank's balanced salts and extracellular bacteria were eliminated by the addition of 1,000 μg/mL Km. In invasion and replication experiments, infected cells were washed with Hank's, harvested by trypsinization, and lysed with 0.2% Triton X-100 + 20 mM $MgSO_4$ and 50 μg/mL DNase I (to reduce lysate viscosity). Assays for intracellular colony-forming units (cfu) were performed on serial dilutions of the lysate. Intracellular growth and replication time-course experiments were done similarly, except cells were harvested at indicated time points. For plaque assays, cells were infected as described above and overlaid with DMEM containing 0.2% $NaHCO_3$, 50mM Hepes (pH 7.4), 10% BGS, and antibiotics (see below) in 1.2% low melting temperature agarose (BioRad). Plaques were counterstained for photography by adding 200 μL DMEM containing 0.1% neutral red onto the agarose 24 h later. For immunofluorescence microscopy, agarose and neutral red stainings were not used. Antibiotics to suppress growth of extracellular bacteria were used as follows: 125 μg/mL kanamycin (BtE264 and Bp340), 150 μg/mL gentamicin (pBBR-MCS Km-complemented *Bp* strains), or 10 μg/mL pipericillin/tazobactam (Zosyn) (complemented *Bt* and *Bp* strains).

Cell Fusion Assays. HEK293 cells were transduced with third-generation recombinant lentivirus (5) encoding enhanced green fluorescent protein (GFP) or mStrawberry red fluorescent protein (RFP). Stable cell lines were isolated by limiting dilution and clonal expansion in DMEM + BGS containing 3 μg/mL puromycin. RFP and GFP cells were seeded for experiments as described above. Starting at 12 h after infection or nanoblade delivery, live cells were examined by fluorescence microscopy for the appearance of MNGCs or plaques or were fixed and stained as described below and in refs. 6 and 7.

Cytosolic Delivery of *B. thailandensis* Using a Photothermal Nanoblade. Photothermal delivery was performed essentially as described (7). The photothermal nanoblade uses a laser pulse to induce ultrafast bubble cavitation at the tip of a titanium (Ti)-coated micropipette for efficient cell membrane opening and large cargo delivery. HEK293 cells were cultured in multiwell plates or in LabTek glass bottom dishes. Approximately 5×10^8 *Bt* cells were washed three times in Hank's Balanced Salts and resuspended at 10^8–10^9 cfu/mL. Ti-coated pulled glass capillary pipettes were fabricated by pulling (P-97; Sutter Instrument) a 1-mm diameter borosilicate glass capillary tube, followed by Ti thin film deposition onto the tapered ends. Pipettes were loaded with 1–2 μL of 5×10^8 cfu/mL bacterial suspension. The laser pulse system was a Q-switched, frequency-doubled Nd:YAG laser (Minilite I; Continuum) operated at 532 nm wavelength and 6 ns pulsewidth. The laser beam was sent into the fluorescence port of an inverted microscope (AxioObserver; Zeiss) and then through the objective lens (40×, 0.6 NA), to generate a 260-μm–wide laser spot on the sample plane. The optimized laser fluence used for cargo delivery was 180 mJ/cm^2. The excitation laser pulse was synchronized with a liquid delivery system (FemtoJet; Eppendorf), using a switch. A pressure of 100–350 hPa was used to deliver 1–2 pL of a bacterial suspension per pulse. Approximately one bacterium was successfully delivered into a cell every two pulses. Following delivery, cells were washed and treated with antibiotics as described above.

Microscopy. HEK293 cells were grown as described above on glass coverslips or in glass-bottom dishes treated with dilute Matrigel liquid. Following infection or nanoblade delivery of bacteria, cells were washed with Hank's and fixed with ambient temperature 4% paraformaldehyde in PBS containing 3 mM $MgCl_2$ and 10 mM EGTA for 15 min. Cells were permeabilized using PBS containing 0.2% Triton X-100. Nonspecific binding sites were blocked in PBS containing 1% BSA, 10% calf serum, and 2% goat serum for 1 h at room temperature. Antibody incubations were carried out for 1 h at room temperature in blocking buffer, using the following dilutions: rabbit *Bt* antiserum (8), 1:1,000 (for *Bt*) and 1:50 (*Bp*); mouse α-LAMP-1 monoclonal antibody (Santa Cruz), 1:40; and Alexa-Fluor 488- or 555-labeled phalloidin and secondary antibodies, 1:150 (Molecular Probes). Permanent mounts of specimens were created using ProLong Gold (Invitrogen). Glass-bottom dishes and slides were analyzed using a Leica SP5-II AOBS confocal microscope setup or an Olympus BX51 upright fluorescent microscope. Live cell bright-field and fluorescence imaging and video recording were performed using a Zeiss Axiovert 40CFL inverted fluorescence microscope with a Canon digital camera. Image processing was performed with Leica's LAS-AF software suite or Adobe Photoshop CS2.

Image and Data Analysis. Figures, tables, and graphs were prepared using Microsoft PowerPoint and Excel. Statistical analysis was performed with Student's *t* test, using Excel and GraphPad.

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

2. Mima T, Schweizer HP (2010) The BpeAB-OprB efflux pump of Burkholderia pseudomallei 1026b does not play a role in quorum sensing, virulence factor

production, or extrusion of aminoglycosides but is a broad-spectrum drug efflux system. *Antimicrob Agents Chemother* 54:3113–3120.
3. Barrett AR, et al. (2008) Genetic tools for allelic replacement in Burkholderia species. *Appl Environ Microbiol* 74:4498–4508.
4. Kovach ME, et al. (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* 166:175–176.
5. Dull T, et al. (1998) A third-generation lentivirus vector with a conditional packaging system. *J Virol* 72:8463–8471.
6. French CT, et al. (2009) The Bordetella type III secretion system effector BteA contains a conserved N-terminal motif that guides bacterial virulence factors to lipid rafts. *Cell Microbiol* 11:1735–1749.
7. Wu TH, et al. (2011) Photothermal nanoblade for large cargo delivery into mammalian cells. *Anal Chem* 83:1321–1327.
8. Burtnick MN, et al. (2008) Burkholderia pseudomallei type III secretion system mutants exhibit delayed vacuolar escape phenotypes in RAW 264.7 murine macrophages. *Infect Immun* 76:2991–3000.

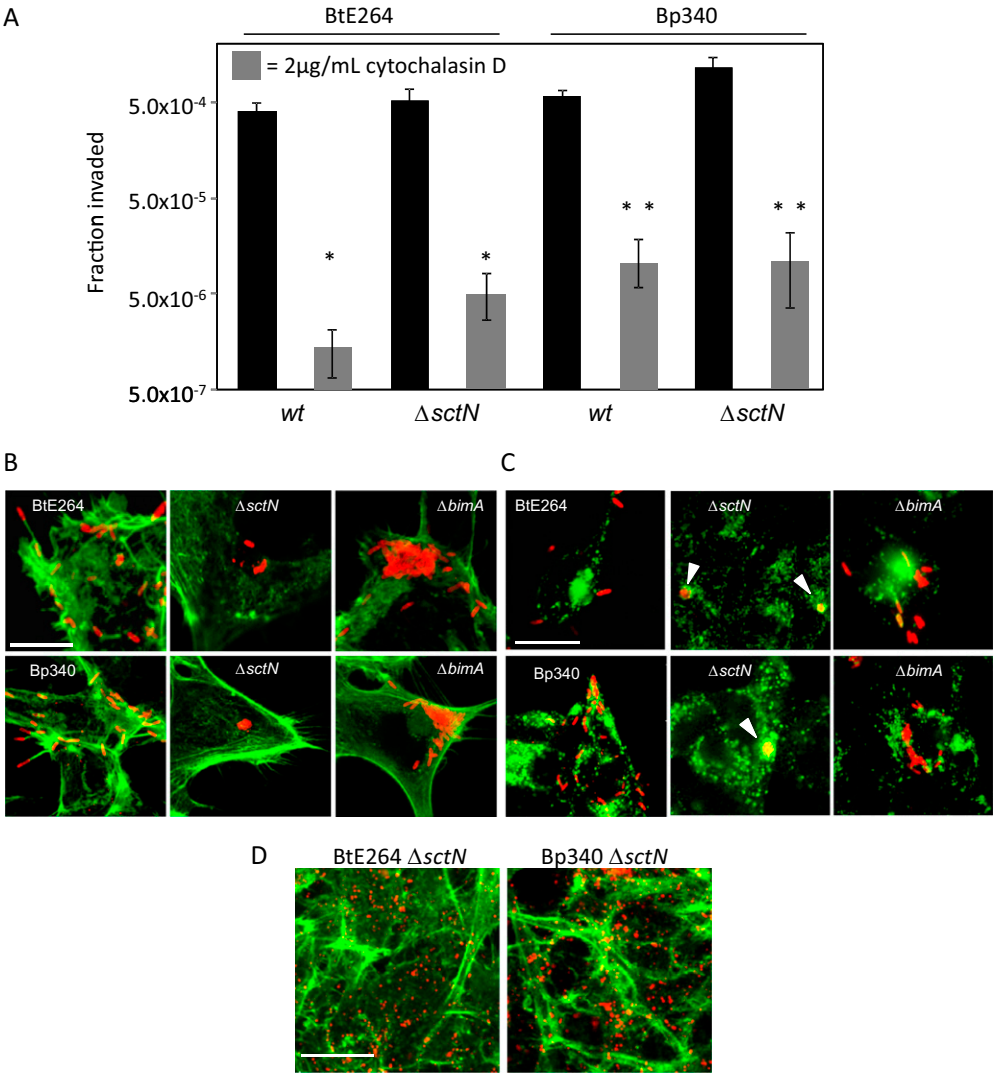


Fig. S1. T3SS$_{Bsa}$ does not facilitate invasion in HeLa epithelial cells. (*A*) Invasion efficiency of *B. thailandensis* E264 and *B. pseudomallei* Bp340 and Δ*sctN* mutants 2 h after infection of HeLa cells. Cells were untreated (black bars) or treated in parallel with 2 μg/mL cytochalasin D (gray bars), which blocks rearrangement of cortical actin, 30 min before infection with bacteria using an MOI of 10. Values shown are averages of three independent experiments performed in triplicate. Error bars are ±SEM. *$P < 0.001$; **$P < 0.05$. (*B*) Fluorescence confocal microscopy of HEK293 cells 8 h postinfection with nonmutant BtE264 (*Upper*) or Bp340 (*Lower*) and Δ*sctN* and Δ*bimA* derivatives. Green, actin; red, bacteria. (Scale bar, 20 μm.) (*C*) HEK293 cells infected with BtE264 (*Upper*) or Bp340 (*Lower*) and indicated mutants were stained for LAMP1 (green) and bacteria (red) 8 h after infection. Arrowheads (*Center*) indicate LAMP1-positive endosomal vesicles containing bacteria. (Scale bar, 20 μm.) (*D*) Bt and Bp *sctN* mutants eventually lose viability and are degraded as a result of endosomal entrapment. HEK293 cells were infected with BtE264 or Bp340 Δ*sctN* mutants and stained for actin (green) and bacteria (red). Bacterial debris remains 16 h after infection with Δ*sctN* mutants. Images are single 0.3-μm Z-sections and are representative of four experiments. (Scale bar, 10 μm.)

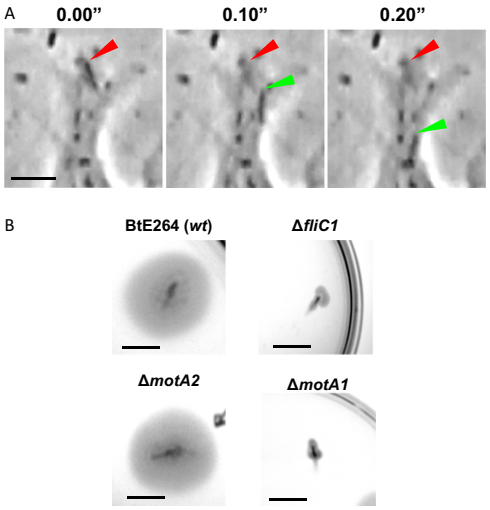
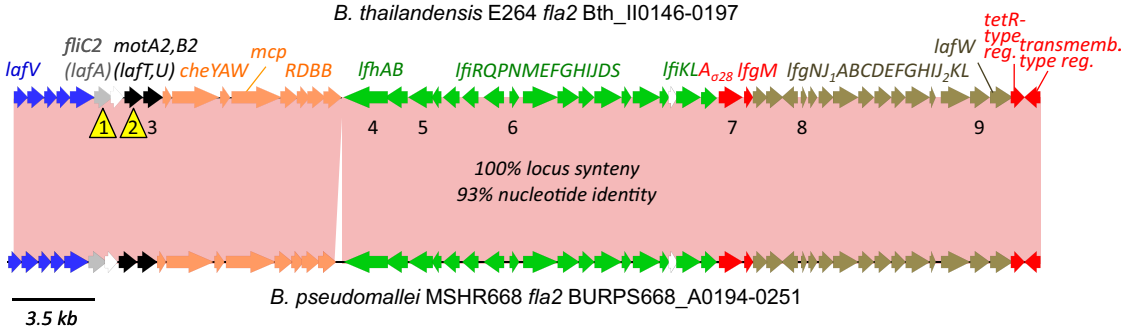


Fig. S2. The *fla2* locus of *B. thailandensis* is required for rapid intracellular motility but does not facilitate swarming in soft agar. (*A*) HEK293 cells were infected with BtE264 Δ*bimA*Δ*motA1* (MOI = 1) and examined 8 h later by live phase-contrast microscopy. Shown is a 200-ms time-lapse image series of two, motile bacterial cells joined end to end (~6 μm in total length) inside a HEK293 cell. The red pointer indicates the lagging pole of the rear-most bacterium, and green pointers denote the distance traveled. From this information, the movement rate of the bacteria is estimated to be 25 μm/s. (*B*) Soft agar swarming assays (1). BtE264 *WT*, Δ*fliC1*, Δ*motA1* (*fla1*) mutants or a Δ*motA2* (*fla2)* mutant were inoculated into 0.4% L-agar and incubated at 37 °C for 18 h. Swarming motility is indicated by bacterial growth (dark gray) radiating from the central site of inoculation. Images are representative of multiple independent experiments. (Scale bar, 1 cm.)

1. Gardel CL, Mekalanos JJ (1996) Alterations in Vibrio cholerae motility phenotypes correlate with changes in virulence factor expression. *Infect Immun* 64:2246–2255.

A



B

	Bt, Bp locus tags	product name	homolog	species	E
1	BTH_II0151, BURPS668_A0199	flagellin FliC2	LafA	*Citrobacter rodentium, Vibrio parahaemolyticus*	9e-58, 4e-55
2	BTH_II0153, BURPS668_A0201	flagellar motor protein MotA2	LafU	*E. coli, Aeromonas samonicida*	2e-58, 5e-79
3	BTH_II0154, BURPS668_A0202	flagellar motor protein MotB2	LafT	*E. coli, V. parahaemolyticus*	2e-44, 8e-43
4	BTH_II0163, BURPS668_A0211	flagellar biosynthesis protein FlhA	LfhA	*E. coli, A. salmonicida*	0.0, 0.0
5	BTH_II0165, BURPS668_A0214	flagellar biosynthetic protein FliR	LfiR	*E. coli, A. salmonicida*	6e-40, 2e-35
6	BTH_II0170, BURPS668_A0222	flagellar hook-basal body component FliE	LfiE	*C. rodentium, A. salmonicida*	3e-15, 2e-10
7	BTH_II0180, BURPS668_A0233	RNA pol. σ factor 28 for flagellar operon FliA	LfiA	*Chromobacterium violaceum, C. rodentium*	4e-58, 7e-53
8	BTH_II0184, BURPS668_A0237	flagellar basal body protein FlgB	LfgB	*E. coli, A. salmonicida*	2e-27, 2e-26
9	BTH_II0194, BURPS668_A0247	flagellar hook associated protein FlgL	LfgL	*E. coli, C. rodentium*	2e-24, 3e-22

Fig. S3. The *fla2* loci of *B. thailandensis* and *B. pseudomallei* are highly conserved and are predicted to encode lateral flagella. (*A*) Map of the *B. thailandensis* E264 and *B. pseudomallei* MSHR668 *fla2* chromosome 2 flagellar and chemotaxis loci. As described by Tuanyok et al. (1, 2), *fla2* loci are highly conserved and syntenic between *B. thailandensis* and Australian *B. pseudomallei* species (red shading) but are not present in southeast Asian isolates of *B. pseudomallei* or in *B. mallei*. Color coding: blue, putative flagellar-accessory/modification genes; orange, *che* chemotaxis; green, *lfi* basal body/export/assembly; brown, *lfg* flagellar hook/rod/ring components; black, *mot/laf* motor components; gray, *fliC/lafA* flagellin; red, putative regulatory loci; and white, hypothetical protein/unknown function. The position of Δ*motA2* and Δ*fliC2* mutations used in this study is indicated by the position of the yellow arrowhead. Map is to scale. (Scale bar, 3.5 kb.) The predicted *fla2* gene products from *Bt* E264 and *Bp* MSHR668 genomic sequence data were analyzed by protein BLAST (BLAST-P). Numbers below the locus map in *A* indicate a representative subset of gene loci for which the analysis is shown in *B*. Listed are the *Bt* and *Bp* locus tag designations, predicted gene products, and highly similar homologs in other species that are known or indicated to possess lateral flagella.

1. Tuanyok A, et al. (2008) Genomic islands from five strains of Burkholderia pseudomallei. *BMC Genomics* 9:566.
2. Tuanyok A, et al. (2007) A horizontal gene transfer event defines two distinct groups within Burkholderia pseudomallei that have dissimilar geographic distributions. *J Bacteriol* 189: 9044–9049.

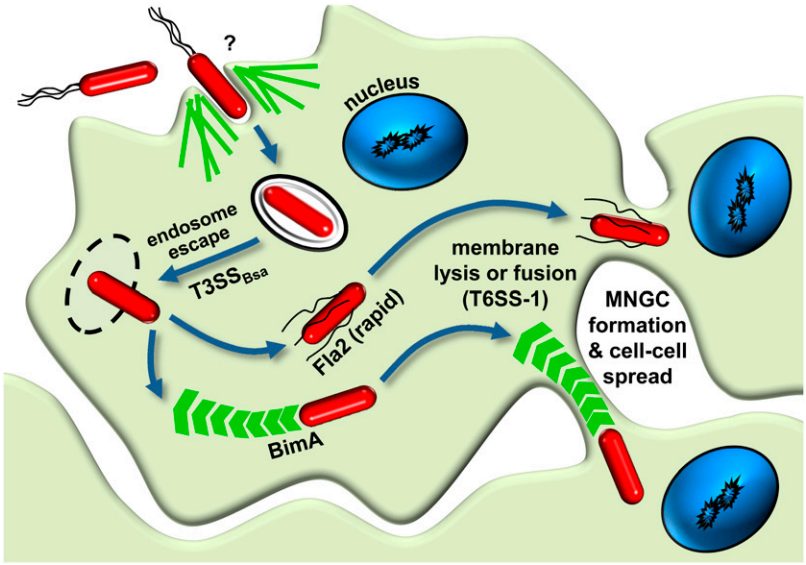
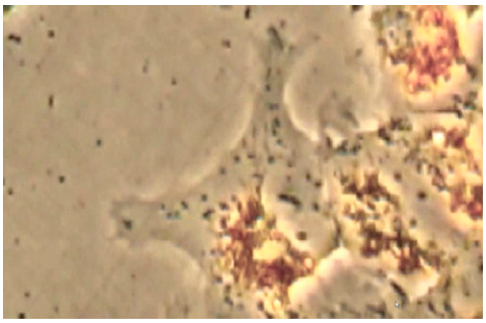


Fig. S4. Model of the intracellular life cycle. T3SS$_{Bsa}$-independent invasins, which have yet to be identified, facilitate actin-dependent internalization of *B. thailandensis* and *B. pseudomallei* into single-membrane primary endosomes. The activity of T3SS$_{Bsa}$ is required for escape from the primary endosome and entry into the cytoplasm. Once the primary endosome has been breached, T3SS$_{Bsa}$ is dispensable for all subsequent events in the intracellular life cycle, including intracellular replication, motility, cell–cell spread, and MNGC formation. Either one of two independent motility systems, flagellar (Fla2) or actin based (BimA), is required for efficient cell–cell spread. T6SS-1 facilitates intercellular spread and multinucleate giant cell (MNGC) formation, possibly through the fusion of host cell membranes. Membrane interaction facilitated by motility and T6SS-1 can also lead to cell death. The life-cycle model for *B. thailandensis* and *B. pseudomallei* contrasts with that of *Listeria* and *Shigella* because it does not require engulfment of membrane protrusions with bacteria at their tips, obviating the need for lysis of double-membrane vesicles for cell–cell spread.

Table S1. Summary of intracellular life-cycle dissection

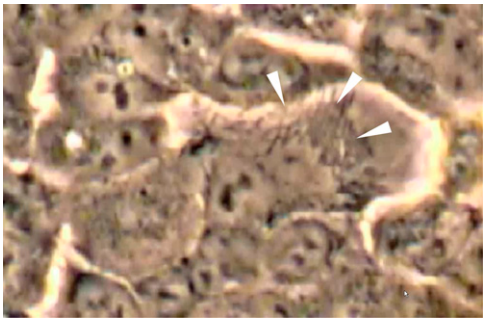
	Infection					Nanoblade delivery			
	Endosome escape	Actin motility	Flagellar motility	MNGC formation	Plaque formation	Actin motility	Flagellar motility	MNGC formation	Plaque formation
*Bt*E264 (*WT*)	+	+	+	+	+	+	+	+	+
Δ*sctN*	–	–	–	–	–	+	+	+	+
Δ*bimA*	+	–	+	+	+	–	+	+	+
Δ*sctN*Δ*bimA*	–	–	–	–	–	–	+	+	+
Δ*clpV1*	+	+	+	–	±	+	+	–	±
Δ*sctN*Δ*clpV1*	–	–	–	–	–	+	ND	–	±
Δ*bimA*Δ*clpV1*	+	–	+	–	±	–	ND	–	±
Δ*sctN*Δ*bimA*Δ*clpV1*	–	–	–	–	–	–	ND	–	±
Δ*motA1*	+	+	+	+	+	ND	ND	ND	ND
Δ*motA2*	+	+	–	+	+	ND	ND	ND	ND
Δ*bimA*Δ*motA2*	+	–	–	–	±	ND	ND	ND	ND
Δ*fliC2*	+	+	–	+	+	ND	ND	ND	ND

Following infection or photothermal nanoblade delivery of BtE264 *WT* and indicated mutants in HEK293 cells, phenotypes were scored for endosome escape, actin motility, flagellar motility, multinucleate giant cell (MNGC) formation, and plaque formation. +, observed; ±, near limit of detection; –, not observed; ND, not done. Endosome escape (infection only) was assessed by fluorescence microscopy of cells fixed and stained for LAMP1 endosome marker and bacteria 8 h after infection. + means at least 50% of intracellular bacteria were observed free in the cytoplasm or not colocalized with LAMP1. Actin motility: + denotes that at least 10% of bacteria in cells were associated with actin tails 8 h after infection or nanoblade delivery, respectively. Flagellar motility: + denotes rapid intracellular motility exhibited by bacteria 8 h after infection or nanoblade delivery in a significant portion (>10%) of infected cells. Multinucleate giant cell (MNGC) formation: + indicates that MNGCs were observed in the red–green mixed-cell assay for cell fusion 12 h after infection or bacterial delivery with the photothermal nanoblade. Plaque formation: 24 h following infection or nanoblade delivery, + indicates average plaque size >250 μm in diameter, ± indicates plaques <250 μm, and – indicates that plaques were not observed. Data are representative of at least three independent experiments.



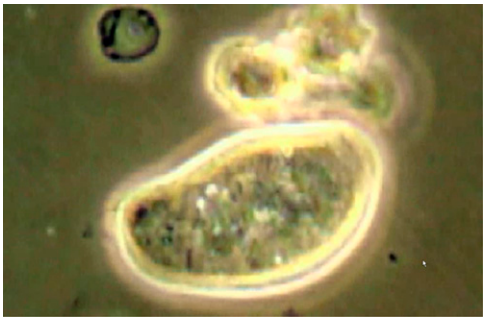
Movie S1. *B. thailandensis* E264 exhibits rapid intracellular motility. Real-time video is shown of HEK293 infected at an MOI of 1 with BtE264 Δ*bimA*Δ*motA1* double-mutant bacteria. Rapid motility is independent of *bimA* (actin polymerization) and *motA1* (motor subunit locus in the *fla1* flagellar gene cluster), but is dependent on *motA2* (motor subunit in the *fla2* gene cluster). Cells were observed by phase-contrast videomicroscopy 8 h after infection.

Movie S1



Movie S2. Δ*clpV1* T6SS mutants exhibit rapid flagellar motility and replicate extensively inside cells. HEK293 cells were infected using an MOI of 1 with BtE264 Δ*clpV1* mutant bacteria. Live infected cells were visualized by phase contrast videomicroscopy 8 h after infection. Despite their capability for rapid intracellular motility, Δ*clpV1* mutants exhibited cell–cell spread defects and accumulated to high numbers inside cells. A large group of intracellular bacteria can be observed (arrowheads in still photo) along with numerous motile individual bacteria throughout the viewfield.

Movie S2



Movie S3. Rapid motility in unattached HEK293 cells confirms intracellular localization of bacteria. Motile intracellular bacteria can be observed inside a live, floating HEK293 cell. Cells were infected (MOI = 1) with BtE264 Δ*bimA* mutant bacteria and detached from the growth surface by treatment with 0.25% trypsin-EDTA 8 h later. Cells were examined using live phase-contrast videomicroscopy.

Movie S3