

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% chlorophenylalanine (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 *flhC2* (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 OD₆₀₀ 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% CO₂. 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 × 10⁶ 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 MgSO₄ 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% NaHCO₃, 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 piperacillin/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.

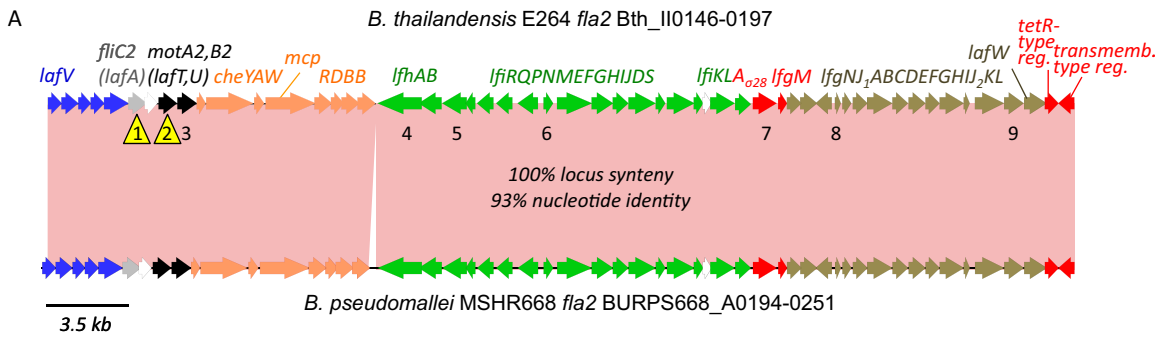
Photothermal delivery was performed essentially as described (7). The photothermal nanoblade uses a laser pulse to induce ultrafast bubble cavitation at the tip of a titanium (Ti)-coated micropipette for efficient cell membrane opening and large cargo delivery. HEK293 cells were cultured in multiwell plates or in LabTek glass bottom dishes. Approximately 5 × 10⁸ *Bt* cells were washed three times in Hank's Balanced Salts and resuspended at 10⁸–10⁹ 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 × 10⁸ 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/cm². 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 MgCl₂ 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



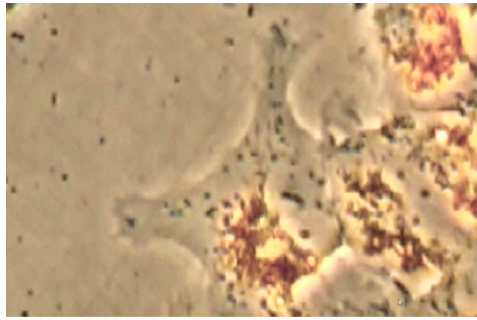
B

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

E

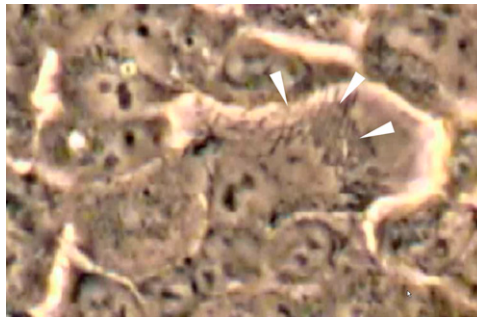
Fig. S3. The *fla2* loci of *B. thailandensis* and *B. pseudomallei* are highly conserved and are predicted to encode lateral flagella. (A) Map of the *B. thailandensis* E264 and *B. pseudomallei* MSHR668 *fla2* chromosome 2 flagellar and chemotaxis loci. As described by Tuanyok et al. (1, 2), *fla2* 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.



Movie S1. *B. thailandensis* E264 exhibits rapid intracellular motility. Real-time video is shown of HEK293 infected at an MOI of 1 with BtE264 $\Delta bimA \Delta motA1$ 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. $\Delta c/pV1$ T65S mutants exhibit rapid flagellar motility and replicate extensively inside cells. HEK293 cells were infected using an MOI of 1 with BtE264 $\Delta c/pV1$ mutant bacteria. Live infected cells were visualized by phase contrast videomicroscopy 8 h after infection. Despite their capability for rapid intracellular motility, $\Delta c/pV1$ 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 $\Delta bimA$ 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](#)