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Dictyostelium discoideum as a Model System for Identification of *Burkholderia pseudomallei* Virulence Factors▽§

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***Burkholderia pseudomallei* is an emerging bacterial pathogen and category B biothreat. Human infections with *B. pseudomallei* (called melioidosis) present as a range of manifestations, including acute septicemia and pneumonia. Although melioidosis can be fatal, little is known about the molecular basis of *B. pseudomallei* pathogenicity, in part because of the lack of simple, genetically tractable eukaryotic models to facilitate *en masse* identification of virulence determinants or explore host-pathogen interactions. Two assays, one high-throughput and one quantitative, were developed to monitor levels of resistance of *B. pseudomallei* and the closely related nearly avirulent species *Burkholderia thailandensis* to predation by the phagocytic amoeba *Dictyostelium discoideum*. The quantitative assay showed that levels of resistance to, and survival within, amoeba by these bacteria and their known virulence mutants correlate well with their published levels of virulence in animals. Using the high-throughput assay, we screened a 1,500-member *B. thailandensis* transposon mutant library and identified 13 genes involved in resistance to predation by *D. discoideum*. Orthologs of these genes were disrupted in *B. pseudomallei*, and nearly all mutants had similarly decreased resistance to predation by *D. discoideum*. For some mutants, decreased resistance also correlated with reduced survival in and cytotoxicity toward macrophages, as well as attenuated virulence in mice. These observations suggest that some factors required by *B. pseudomallei* for resistance to environmental phagocytes also aid in resistance to phagocytic immune cells and contribute to disease in animals. Thus, *D. discoideum* provides a novel, high-throughput model system for facilitating inquiry into *B. pseudomallei* virulence.**

Burkholderia pseudomallei is a betaproteobacterium and causative agent of melioidosis in humans. *B. pseudomallei* infections are most common in Southeast Asia and Northern Australia, where melioidosis is endemic (13), and typically result from contact with contaminated soil and water after wounding or inhalation (12, 13, 33, 65). Disease onset can be rapid or sometimes after a latency of years (42, 46), with clinical manifestations ranging from localized dermal lesions and soft tissue abscesses to acute septicemia and pneumonia. Due to its prevalence in soils in areas of Thailand, high infectivity, and the fact that melioidosis can be fatal even with antibiotic therapy (13), *B. pseudomallei* is considered a biothreat agent and as such is designated a category B select agent restricted to biosafety level 3 (BSL-3) by the Centers for Disease Control and Prevention (53).

Despite its medical significance and biosecurity implications, relatively few molecular details exist regarding factors contributing to the heightened infectivity and lethality of *B. pseudomallei* relative to other bacterial pathogens. The majority of validated *B. pseudomallei* virulence factors are those which are conserved in other pathogens, e.g., exopolysaccharide capsule (3, 16, 51), lipopolysaccharide O antigen (21), type IV pili (25), and type II, III, and VI secretion systems (20, 48, 62, 71). A few additional genes have been reported to be necessary for causing melioidosis in experimental animals, including those encoding a predicted lipoprotein, an ABC transporter, a phospholipase C, a two-component regulator, and several hypothetical proteins (16, 29).

Due to various constraints when working with select agent-infected animals at BSL-3, inquiry into *B. pseudomallei* virulence mechanisms has largely focused on exploring and defining interactions between *B. pseudomallei* and mammalian phagocytes *in vitro* (31, 34). In all phagocytic cell lines examined to date, *B. pseudomallei* cells are able to escape the phagocytic vacuole and replicate freely in the cytosol, eventually causing host cells to fuse into multinucleated giant cells (MNGCs) (31, 36) through which they can migrate intracellularly or exit by actin polymerization at a single bacterial cell pole (i.e., actin-based motility [36, 64]). Despite detailed phenotypic characterization of these events and confirmation of their occurrence *in vivo* (75), genetic and molecular analyses of these phenomena have been largely limited to investigation of the role of the Inv/Mxi-Spa-like type III secretion system (T3SS), which contributes to intracellular survival in macrophages by facilitating escape from the phagocytic vacuole, promoting actin-based motility, and inducing MNGC formation (45, 64, 66, 73).

To accelerate understanding of *B. pseudomallei* virulence, it would be beneficial to have high-throughput models using eukaryotic host cells with traits mimicking those of mammalian phagocytes but lacking the cost and logistical constraints inherent with performing tissue culture infection studies in high-security BSL-3 containment. *Dictyostelium discoideum* is a unicellular protozoan that can be grown axenically in its amoebal form (67, 74); it feeds on bacteria through well-characterized phagosomal and endolysosomal mechanisms similar to those

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant properties[a]	Reference or source
Strains		
B. pseudomallei DD503	Virulent derivative of wild-type clinical isolate 1026b, Δ(*amrR-oprA*)*rpsL*, Km^s Gm^s Pm^r	43
B. pseudomallei bpsl0127k	DD503 *bpsl0127*::pKSV3, Km^r	This study
B. pseudomallei bpsl0127g	DD503 *bpsl0127*::pGSV3, Gm^r	This study
B. pseudomallei bpss0884	DD503 *bpss0884*::pKSV3, Km^r	This study
B. pseudomallei bpss1543	DD503 *bpss1543*::pKSV3, T3SS3$^-$, Km^r	This study
B. pseudomallei bpss1984	DD503 *bpss1984*::pKSV3, Km^r	This study
B. pseudomallei bpss1993	DD503 *bpss1993*::pKSV3, Km^r	This study
B. pseudomallei bpsl0127g/c+	DD503 *bpsl0127*::pGSV3 plus pSCRsl0127 Km^r Gm^r	This study
B. thailandensis DW503	Derivative of wild-type strain E264, Δ(*amrR-oprA*)*rpsL*, Km^s Gm^s Pm^r	9
E. coli S17-1	Mobilizing strain, Tra^+	57
K. pneumoniae ATCC 9621	Wild type	ATCC
P. aeruginosa PAO1 (ATCC 15692)	Wild type	ATCC
Plasmids		
pTn*Mod*-OKm′	Modular minitransposon; pMB1 ori, Km^r	18
pKSV3	Mobilizable suicide vector, Km^r	This study
pGSV3	Mobilizable suicide vector, Gm^r	22
pSCRhaB2K	Broad-host-range expression vector, Km^r	W. Nierman
pSCRsl0127	pSCRhaB2K expressing *bpsl0127*, Km^r	This study

[a] Km^r, Gm^r, and Pm^r, resistant to kanamycin, gentamicin, and polymyxin B, respectively. Km^s and Gm^s, sensitive to kanamycin and gentamicin, respectively.

of mammalian phagocytes in aspects of cytoskeletal organization, membrane trafficking, endocytic transit, and sorting events (8, 21, 37, 41, 52, 54). Indeed, studies of several bacterial pathogens, including *Pseudomonas aeruginosa*, *Legionella pneumophila*, *Vibrio cholerae*, and *Mycobacterium* spp., have determined that some genes required for resistance to predation by *D. discoideum* are also required for replication or survival in mammalian phagocytes (2, 30, 50, 58–60) and, in some cases, for causing disease in animals (1, 15, 28, 32, 47, 49, 70). However, unlike mammalian phagocytes, *D. discoideum* has a haploid genome (24) that is amenable to a diverse array of genetic manipulations (4, 17, 60), facilitating the identification of host susceptibility determinants (5, 26, 39, 58, 59) and investigation of transcriptional response to bacterial presence (27).

Here, we investigated the utility of *D. discoideum* for identification of *B. pseudomallei* virulence factors using assays designed for high-throughput macroscopic visualization of decreased resistance to predation, as well as for quantitative assessment of bacterial capacity for intra-amoebal survival and replication. We show that the closely related species *Burkholderia thailandensis* (7, 76) can be used as a surrogate in these assays because disruption of *B. pseudomallei* genes orthologous to those contributing to *B. thailandensis* resistance to predation by *D. discoideum* typically yielded an analogous phenotype. Reduced resistance to *D. discoideum* in some instances correlated with reduced survival in and cytotoxicity toward macrophage cells, as well as attenuated virulence in mice.

MATERIALS AND METHODS

Microbial strains and growth conditions. Wild-type bacteria, mutants constructed in this study, and their parental strains are listed in Table 1. Unless noted otherwise, bacteria were grown with shaking at 37°C in L broth plus 2% glycerol (LBG) or on LBG plus 1.8% agar plates with appropriate antibiotics at the following concentrations (in μg/ml): kanamycin, 50 (Km_{50}); gentamicin, 30 (Gm_{30}); and polymyxin B, 20 (Pm_{20}). Stocks of 10^7 *D. discoideum* AX3 (40) frozen in 1 ml HL-5 broth (14) plus 5% dimethyl sulfoxide were rapidly thawed at 20°C, diluted to 3 ml with HL-5, pelleted by centrifugation (500 × *g* for 2 min), resuspended in 10 ml HL-5, and aerobically propagated at 20°C to ~5×10^6 cells/ml. Cultures were maintained in log phase at 5×10^6 cells/ml ± 20% by daily dilution with HL-5 for no more than 1 week prior to use. Amoebal cell density was monitored microscopically at ×200 magnification using a hemocytometer.

Semiquantitative assay of resistance to predation by *D. discoideum*. Overnight cultures of bacteria were diluted to an optical density at 600 nm (OD_{600}) of 0.1 and grown to an OD_{600} of 1.0. After centrifugation at 7,000 × *g* for 7 min, cells were resuspended in SorC buffer (17 mM Na_2HPO_4, 33 mM KH_2PO_4, 50 μM $CaCl_2$, pH 6.0) and diluted in SorC to 5×10^7 cells/ml. Log-phase *D. discoideum* cells were pelleted by centrifugation (500 × *g* for 2 min), resuspended in SorC to 5×10^6 cells/ml, and serially diluted in 10-fold increments in SorC. *D. discoideum* dilutions were mixed 1:1 with bacteria to generate bacterium-to-amoeba ratios ranging from 10:1 to 10^6:1. Aliquots (10 μl) of each were deposited 10 mm apart on SM/5 agar (67). Plates were incubated at 20°C and visually monitored for formation of *D. discoideum* plaques in bacterial minilawns.

Quantitative gentamicin protection assay of *Burkholderia* survival within *D. discoideum*. Cultures were prepared as described above to a final concentration of 10^6 cells/ml. Equal volumes of bacteria and amoeba (multiplicity of infection [MOI] = 1) were mixed, and triplicate aliquots (0.6 ml) were placed in 24-well polystyrene plates. After incubation at 20°C for 1.5 h, Gm was added to two of the triplicate wells to 30 μg/ml. The coculture was removed from the remaining well and centrifuged at 500 × *g* for 2 min to separate amoebae from noninternalized bacteria. The pellet was resuspended in 1 ml SorC and centrifuged again, the supernatant was removed, and the amoebal pellet was lysed with 1 ml of 0.4% Triton X-100. Appropriate dilutions were spread on LBG plates to enumerate internalized bacteria. For one of the two remaining wells with Gm_{30}, the same separation and plating procedure was repeated after 20 h of incubation at 20°C. For the third well, amoebae were directly lysed after 20 h by adding 400 μl of 1% Triton X-100 and appropriate dilutions of lysate were spread on LBG plates to enumerate *Burkholderia*. Both methods gave nearly identical results, indicating that *Burkholderia* did not replicate extracellularly during the 20-h coincubation with Gm_{30}. To determine viability of *D. discoideum* in cocultures, aliquots were removed from wells and plated onto SM/5 agar harboring lawns of *Klebsiella pneumoniae*; the numbers of *D. discoideum* plaques were counted after 7 days at 20°C (5).

Transposon mutagenesis of *B. thailandensis* and screening for reduced resistance to *D. discoideum*. Three separate conjugations were performed by mixing 0.5 ml each of cultures (OD_{600}, 1.0) of *Escherichia coli* S17-1(pTn*Mod*-OKm′) (18) and *B. thailandensis* DW503. Cells were pelleted by centrifugation, resuspended in 0.1 ml of LBG, and deposited on LBG agar. After overnight incuba-

tion, cells were resuspended in 1.1 ml of LBG and 0.1- and 1-ml samples were plated on LBG $Km_{50}Pm_{20}$ plates to select for *B. thailandensis* with Tn*Mod* inserts. Colonies arising after 48 h were patched onto LBG $Km_{50}Pm_{20}$ plates. These *B. thailandensis* transposon insertion mutants were repatched at 25 per plate onto SM/5 agar that had been spread the day before with 10^7 *K. pneumoniae* cells. After overnight incubation, ~20,000 *D. discoideum* cells were deposited in the spaces between *B. thailandensis* patches and plates were incubated at 20°C for 10 days, at which time mutant patches consumed by *D. discoideum* were identified and picked from master plates for further evaluation.

Confirmation and characterization of mutants. Each of the amoeba-sensitive transposon insertion mutants obtained above was plated on M9-glucose agar (49) with Km_{50}, and colonies arising after 4 days were assayed for resistance to predation using the semiquantitative assay described above. Ten mutants which required bacterium-to-amoeba ratios >10 times higher than those required for the parental *B. thailandensis* DW503 strain to resist predation by *D. discoideum* were obtained. To exclude the possibility of secondary mutations contributing to this phenotype, transposon-inactivated alleles were individually transferred to *B. thailandensis* DW503 by transformation with purified genomic DNA (68). Each transformant exhibited the same reduction in resistance as observed for the original mutant.

To determine sites of Tn*Mod* insertion, genomic DNA from each mutant was digested with EcoRI or PstI, ligated, and transformed into *E. coli* DH5α. After being plated on LB + Km_{50}, plasmids containing Tn*Mod* with flanking *B. thailandensis* genomic DNA were isolated and subjected to outward DNA sequencing across the transposon-genome junction using Tn*Mod*-specific primers (see Table S1 in the supplemental material) to determine the genome sequence adjacent to insertion.

Construction of site-directed mutants of *B. pseudomallei*. The mobilizable suicide vectors pGSV3 (22) and pKSV3 were used for inactivation of specific *B. pseudomallei* genes; pKSV3 was constructed by removal of the transposase-containing BglII fragment of pTn*Mod*-OKm′ (18) as described before (22, 69). PCR amplicons lacking both the 5′ and 3′ ends of each gene target were generated using primers introducing SpeI and EcoRI restriction sites at the 5′ and 3′ ends, respectively (see Table S1 in the supplemental material). Amplicons were digested sequentially with each enzyme, ligated into identically digested pKSV3 or pGSV3, and transformed into *E. coli* S17-1. The resultant plasmids were conjugated into *B. pseudomallei* DD503 by mixing 0.5 ml of log-phase DD503 with 0.5 ml log-phase, plasmid-bearing *E. coli*. Cells were pelleted by centrifugation, resuspended in 0.1 ml of LB, and deposited on LBG plates. After overnight incubation, cells were resuspended in 1.1 ml of LBG and 0.1- and 1.0-ml samples were plated on LBG agar containing Pm_{20} and either Km_{50} for pKSV3 derivatives or Gm_{30} for pGSV3 derivatives to select for *B. pseudomallei* with integrated vector. *B. pseudomallei* colonies arising after 48 h were patched onto selection plates. After 48 h, cells from patches were boiled for 8 min in 1 ml of phosphate-buffered saline (PBS) and centrifuged at 10,000 × *g* for 1 min, and the supernatant was used in two PCRs: one using a primer (5′ to 3′) targeting the region immediately upstream of the predicted insertion site and a primer (3′ to 5′) targeting the rp4 region of pKSV3 or pGSV3 and a second PCR using a primer (3′ to 5′) targeting the gene immediately downstream of the predicted insertion site and a primer (5′ to 3′) targeting the antibiotic resistance determinant of pKSV3 or pGSV3 (see Table S1 in the supplemental material). The resultant amplicons were subjected to DNA sequencing to verify proper insertion.

Construction of pSCRsl0127 for complementation. The complete *bpsl0127* coding sequence (CDS) as predicted at Integrated Microbial Genomes (IMG) (img.jgi.doe.gov/cgi-bin/pub/main.cgi) plus an additional ~200 bp upstream of its ATG translation initiation codon was PCR amplified, and the resultant amplicon was ligated into pCR2.1-TOPO. After transformation into *E. coli*, resultant plasmids were purified and digested with KpnI and XbaI to release the inserts, which were then cloned into similarly digested pSCRhaB2K (11). The sequence of *bpsl0127* on the resultant plasmid (pSCRsl0127) was verified by sequencing PCR amplicons generated using vector-specific primers flanking the cloning site. pSCRsl0127 was transferred into the *bpsl0127g* mutant by conjugation as described above.

Analysis of *Burkholderia* survival and replication in macrophages. RAW 264.7 murine macrophage-like cells (ATCC accession no. TIB-71) were propagated in polystyrene flasks in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (DMEM/FBS). Twelve hours before use, fresh macrophage cells were resuspended in DMEM/FBS to a concentration of 10^6 cells/ml, and 0.6-ml aliquots were placed in 24-well polystyrene plates and grown overnight at 37°C with 5% CO_2. Overnight cultures of *Burkholderia* were diluted to an OD_{600} of 0.1 in LBG, grown to an OD_{600} of 1.0, pelleted by centrifugation, resuspended, and diluted in DMEM/FBS to a concentration of 10^7 cells/ml. Fifty-microliter samples were added to triplicate wells with macrophages (MOI, 1) and incubated at 37°C in 5% CO_2. After 1.5 h, DMEM/FBS was removed and each well was washed three times with 1 ml Hanks' balanced salt solution. Macrophage cells in one well were lysed by adding 1 ml 0.4% Triton X-100, and the lysate dilution was plated on LBG agar to enumerate internalized bacteria at 0 h. DMEM/FBS with Gm_{30} (0.6 ml) was added to the remaining two wells, and incubation was continued. After 8 h and 20 h, DMEM/FBS was removed from the wells, macrophages were lysed, and the lysate dilution was plated as described above.

Lactate dehydrogenase release assay. DMEM/FBS tissue culture medium from wells containing macrophages infected for 20 h with *B. pseudomallei* was filter sterilized, and 35 μl was added to 1 ml of 0.2 M Tris-HCl (pH 7.3)–6.6 mM NADH–30 mM sodium pyruvate. Lactate dehydrogenase activity was determined by measuring the rate of decrease of the OD_{340} over 10 min (6). No activity was detected when using fresh DMEM/FBS in the assay. Values from six independent experiments were averaged.

Microscopy. Sterile glass coverslips were placed in chambers of 24-well plates. Macrophage infections were then performed as described above. After 20 h, coverslips were washed three times with PBS, fixed with 3% paraformaldehyde for 1 h at 4°C, and then placed in chambers of a clean 24-well plate containing 3% paraformaldehyde. After overnight incubation at 4°C, coverslips were washed three times with cold PBS and stored in PBS at 4°C. Within 48 h, coverslips were imaged at ×400 on a Leica DM-IRB inverted microscope.

Virulence testing in mice. Overnight cultures were diluted to an OD_{600} of 0.2 and regrown to an OD_{600} of 1.0. An equal volume of 30% glycerol was then mixed in, and 0.6-ml aliquots frozen at −80°C until use. Aliquots were thawed and diluted to the desired concentration in sterile PBS, and 25 μl was delivered intranasally into four anesthetized female BALB/c mice. The cell concentration of each dose was determined by dilution plating. Animals were weighed and monitored daily for signs of morbidity for up to 14 days. Mice that became moribund and unresponsive to stimulation were euthanized and recorded as dead. In certain experiments, animals surviving on day 14 were sacrificed, their spleens removed and homogenized in 1 ml Trypticase soy (TS) broth, and duplicate 0.1-ml samples were plated on TS agar to enumerate bacterial content.

RESULTS

Studies with *P. aeruginosa*, *L. pneumophila*, *V. cholerae*, and *Mycobacterium* spp. have demonstrated that genes required to resist predation by, and survive intracellularly in, the soil-dwelling phagocytic amoeba *D. discoideum* likewise contribute to survival and replication in macrophage cells (2, 30, 50, 58–60) and, in some cases, to causing disease (1, 15, 28, 32, 47, 49, 70). Thus, we explored *D. discoideum* as a model for pathogenic interactions between *B. pseudomallei* and macrophages, as well as for use in a screen to identify genes potentially involved in animal pathogenesis. First we evaluated resistance of *B. pseudomallei* to predation by *D. discoideum* in reference to the previously analyzed bacteria *P. aeruginosa* (resistant) and *K. pneumoniae* (susceptible) (5, 15, 49, 50). Mixtures with various ratios of bacteria to amoebae were spotted on agar plates; within 48 h minilawns of bacteria appeared at the spotting sites. At 72 h clearing zones ("plaques") began to form in all the minilawns of the susceptible *K. pneumoniae* due to predation by *D. discoideum*. By 120 h the *K. pneumoniae* lawn was completely consumed and replaced by *D. discoideum* fruiting bodies, even at ratios as high as 10^6 *K. pneumoniae* cells per amoeba (Fig. 1A). In contrast, spots with resistant *P. aeruginosa* showed no plaques or other evidence of predation down to a ratio of 10^4 cells per amoeba (Fig. 1A); at ratios below 10^3 *P. aeruginosa* cells per amoeba, complete clearing and replacement by fruiting bodies were observed (not shown). *B. pseudomallei* showed a much higher level of resistance to predation by *D. discoideum* than *P. aeruginosa*, with plaques occurring only if the ratio of bacteria to amoebae was below 10.

Burkholderia thailandensis is very closely related to *B. pseu-*

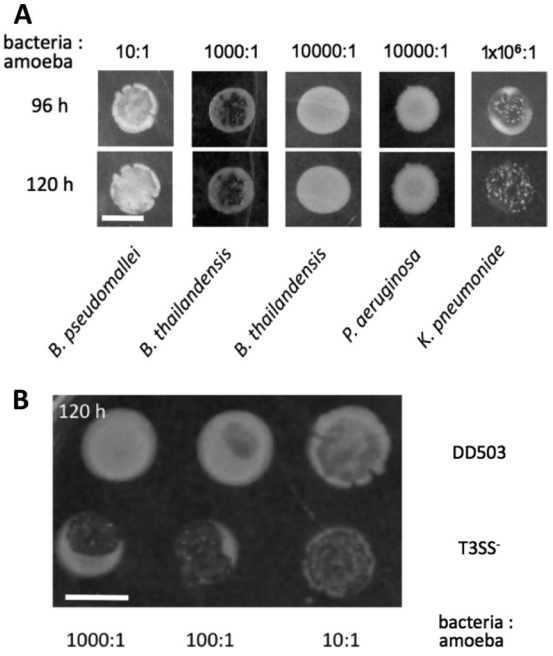

FIG. 1. Semiquantitative plaque assay for resistance to predation by *D. discoideum*. (A) Washed bacterial cells (~10^6 per ml) were mixed with washed amoebae of decreasing concentrations, and 10 μl was spotted onto SM/5 agar. After 96 h and 120 h at 20°C, plates were photographed. (B) Same conditions as for panel A except that only the 120-h time point is shown. DD503, *B. pseudomallei* DD503; T3SS$^-$, *B. pseudomallei bpss1543* mutant that is Mxi-Inv/Spa-like T3SS deficient. White scale bar, 10 mm.

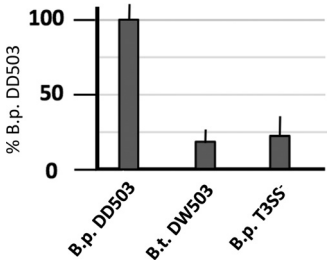

FIG. 2. Replication of *Burkholderia* inside *D. discoideum*. A gentamicin protection assay was used to enumerate intracellular numbers of *B. thailandensis* (B.t.) DW503, *B. pseudomallei* (B.p.) DD503, and *B. pseudomallei bpss1543* mutant, a T3SS-deficient mutant (T3SS$^-$) (Table 1), 20 h after internalization by *D. discoideum*. Values are averages of results of three independent experiments. Data from another three independent experiments were similar. Bars, standard errors of the means.

domallei but does not appear to cause disease in humans. However, like *B. pseudomallei*, it utilizes a T3SS for survival and replication within immune phagocytes and for causing disease in animal hosts (19, 31, 35, 44, 61, 72), albeit at a 50% lethal dose (LD_{50}) >100,000 times higher than that of *B. pseudomallei*. In the plaque assay *B. thailandensis* required an initial ratio of ~10^4 bacteria to amoeba to prevent formation of phagocytic plaques; this was ~100 times higher than the ratio required by *B. pseudomallei* (Fig. 1A).

The dramatically higher ratios of bacteria to amoebae required by the less virulent *B. thailandensis* and *P. aeruginosa* to overcome predation relative to the much more virulent *B. pseudomallei* boded well for use of the semiquantitative *D. discoideum* predation resistance plaque assay to screen for *B. pseudomallei* mutants with reduced virulence in animals. However, for this to be successful, it was essential that mutants known to have reduced virulence in animals show decreased resistance to predation by *D. discoideum*. We therefore tested *B. pseudomallei* mutants lacking a Inv/Mxi-Spa-like type III secretion system (T3SS), the loss of which results in decreased survival and altered behavior in macrophage cells, as well as in dramatically reduced virulence in murine models of disease (10, 45, 62–64, 66, 69). When this T3SS mutant (Table 1, *bpss1543* mutant) was assayed, it clearly showed dramatically decreased resistance to predation by *D. discoideum*; plaques were observed even at bacterium-to-amoeba ratios of 1,000 to 1, whereas the parent strain DD503 was fully resistant at ratios as low as 10 to 1 (Fig. 1B). It is important to note that the differences in predation resistance observed between *B. pseudomallei* DD503, the *B. pseudomallei* T3SS mutant, and *B. thailandensis* DW503 are probably not due to differences in their growth rates, since log-phase doubling times of each strain grown aerobically in SM/5 broth were within 8% (data not shown).

To further validate and characterize the semiquantitative predation assay, we determined if predation resistance measured by plaque formation correlates with survival inside the amoeba as quantified by a gentamicin protection method. Equal numbers of *Burkholderia* and amoebae were incubated together for 1 h; after the cells were washed, gentamicin was added to prevent replication of nonphagocytosed cells. At this time ~2% of the *Burkholderia* had been phagocytosed. After an additional 20 h, amoebae were lysed and released *Burkholderia* cells enumerated by plate counts. The numbers of *B. pseudomallei* DD503 released from amoebae were ~10-fold higher than counts taken after 1 h, indicating that intracellular replication had occurred; however, this had no measurable effect on amoebal viability (data not shown). Relative to the parental DD503 *B. pseudomallei* strain, the number of cells released from amoebae infected with the T3SS-deficient mutant was ~5-fold lower, indicating that only one intracellular doubling had occurred during the 20-h period (Fig. 2). The low-virulence *B. thailandensis* likewise survived phagocytosis but replicated less than once within the amoebae. Nonetheless, under these conditions *B. thailandensis* and the T3SS-deficient *B. pseudomallei* mutant were >100-fold more resistant to *D. discoideum* than *K. pneumoniae*, which exhibited a decrease in intracellular numbers of 90% over 20 h (data not shown). Growth rates of all *Burkholderia* strains in HL-5 medium lacking gentamicin or in HL-5 medium conditioned by *D. discoideum* were essentially the same, suggesting that deficiencies in intracellular replication of *B. thailandensis* and the *B. pseudomallei* T3SS mutant are not the result of *D. discoideum* toxins or metabolic by-products. Thus, quantitative reductions in intracellular replication ability of the *B. pseudomallei* T3SS mutant and the low-virulence *B. thailandensis* strain correlated well with their decreased resistance to predation as measured by the plaque assay. The lower intra-amoebal replication ability of *B. thailandensis* relative to *B. pseudomallei* implies that the latter has additional genes that enhance its resistance to *D.*

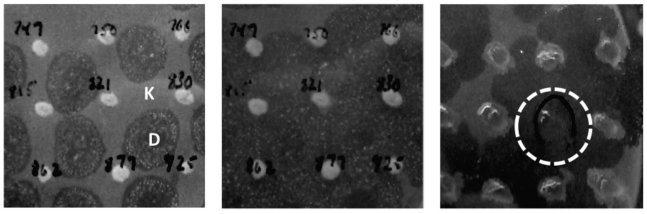

FIG. 3. Identification of *B. thailandensis* mutants with decreased resistance to predation by *D. discoideum*. *B. thailandensis* transposon insertion mutants were patched (handwritten numbers) onto lawns of *K. pneumoniae* (K). After 16 h, 10-μl suspensions of *D. discoideum* were spotted between patches. By day 2 (2 d), phagocytic plaques caused by *D. discoideum* (D) formed and increased in diameter until they merged around *B. thailandensis* patches by day 4 (4 d). Mutants with reduced resistance to predation (dashed circle) were consumed and cleared after 7 days (7 d) by advancing *D. discoideum*.

discoideum and boded well for use of *D. discoideum* to screen for mutants with reduced virulence in animals.

Identification of genes involved in predation resistance. As the results above showed that decreased resistance of *Burkholderia* to predation by *D. discoideum* correlated with published levels of virulence of these strains in animals, we investigated use of *D. discoideum* to identify new *Burkholderia* virulence genes using predation resistance as an indicator. Given the hazards and select agent restrictions associated with use of *B. pseudomallei*, we chose to first screen a transposon mutant library of *B. thailandensis* for mutants with decreased predation resistance with the belief that subsequently creating analogous mutations in orthologous genes in *B. pseudomallei* would in some cases yield a similar phenotype. By using Tn*Mod*-OKm′ (18), a library of 1,500 *B. thailandensis* mutants was generated and screened using a high-throughput version of the semiquantitative plaque assay. By 10 days, amoebae placed between patches of individual mutants consumed only a very small fraction (13 of 1,500) of the individual mutant patches, presumably due to their lowered resistance to predation caused by the transposon insertion (Fig. 3). When individually retested, two mutants did not show reduced resistance and one was auxotrophic; these were not studied further.

The 10 genes harboring insertions that reduced resistance to *D. discoideum* were determined by sequencing the 5′ and 3′ genome regions adjacent to the Tn*Mod* insertion (Table 2). Four mutants were not further pursued either because an orthologous gene is not present in *B. pseudomallei* (mutant 0312) or because the insertion was in a gene likely to have a major deleterious effect on general metabolic fitness (mutants 0001, 0937, and 1339). The reductions in resistance to predation by *D. discoideum* observed on screening plates for the remaining six mutants were reconfirmed by the plaque assay. All mutants required an initial bacterium-to-amoeba ratio of at least 10^5 to prevent the formation of *D. discoideum* plaques; these ratios were >10 times higher than that required by the parental *B. thailandensis* DW503 strain (Table 2). Differences in predation resistance of the mutants were probably not due to differences in bacterial growth rate, because doubling times of each strain in SM/5 broth were within 3% of each other (data not shown). Assessment of each mutant for survival inside *D. discoideum* using the gentamicin protection assay gave analogous results (Fig. 4A). While populations of the parental DW503 strain inside the amoeba increased 2-fold, populations of mutants 1333, 0188, and 0250 actually decreased >10-fold, similar to what was observed for *K. pneumoniae* controls. Intracellular numbers of mutants 0552, 0821, and 1205 were reduced much less (~3-fold). These reductions in intracellular survival were not the result of reduced phagocytosis by *D. discoideum*, since in parallel experiments separation of amoeba and noninternalized bacteria by centrifugation after 1 h coincubation before the addition of gentamicin showed that DW503 and all mutants were phagocytosed to the same extent (~2% of total cells added).

Resistance of *B. thailandensis* to *D. discoideum* predation correlates with survival in macrophages. Since pathways employed by *D. discoideum* for internalization and digestion of bacteria are similar to those of phagocytic mammalian immune cells (8, 23, 37, 41, 52, 54), we investigated the role of genes involved in resistance of *B. thailandensis* to predation by *D. discoideum* in resistance to killing by murine macrophages. Equal numbers of bacterial cells and macrophages were incubated together for 1.5 h, at which time the level of internalization (phagocytosis) of all strains was the same (~8% of the total added) and 4-fold higher than with *D. discoideum*. Monolayers were washed, gentamicin was added, and after 8 and 20 h of incubation intracellular numbers of bacteria were de-

TABLE 2. *B. thailandensis* transposon mutants with reduced resistance to predation by *D. discoideum*[a]

Mutant no.	Locus tag of gene with insertion	Locus tag of *B. pseudomallei* ortholog	Predicted function	Ratio required to prevent plaque formation[b]
1333	BTH_I0134	BPSL0127	Sensor kinase	10^5:1
0001	BTH_I0756	BPSL0893	Elongation factor EF-2	nt
0821	BTH_I2535	BPSL1888	Hypothetical protein	10^6:1
1205	BTH_I3341	BPSL3428	SET domain protein	10^5:1
0312	BTH_II0295	None	Drug resistance transporter	nt
0552	BTH_II0379	BPSS1993	Serine peptidase	10^5:1
0250	BTH_II0388	BPSS1984	Hypothetical protein	10^5:1
0188	BTH_II1513	BPSS0884	Hypothetical protein	10^5:1
1339	BTH_II0658	BPSS1722	Malate dehydrogenase	nt
0937	BTH_II0677	BPSS1701	tRNA pseudouridine synthase A	nt

[a] Locus tags and predicted function were derived from data at Integrated Microbial Genomes (http://img.jgi.doe.gov/).
[b] The initial ratio of bacteria to amoeba required to prevent the formation of *D. discoideum* plaques in minilawns of *B. thailandensis* when codeposited on SM/5 agar. nt, not tested.

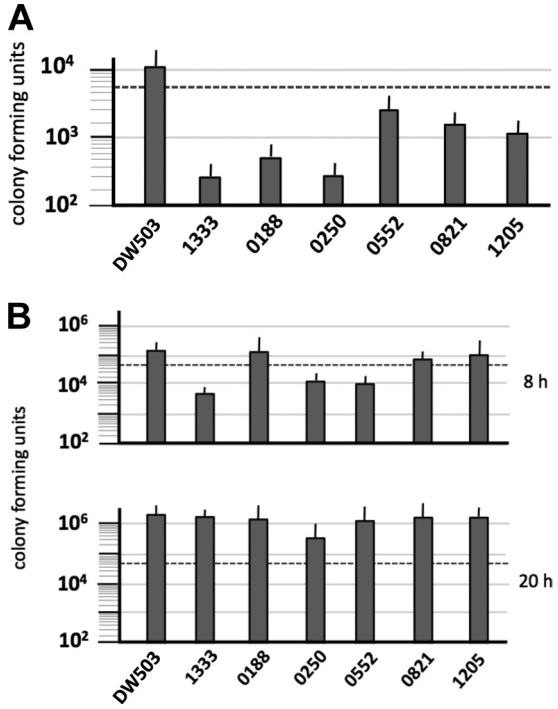

FIG. 4. Survival of *B. thailandensis* transposon mutants inside *D. discoideum* and macrophages. A gentamicin protection assay was used to measure numbers of various *B. thailandensis* transposon mutants (Table 2) in *D. discoideum* 20 h after internalization (A) or RAW 264.7 murine macrophages at 8 h and 20 h after internalization (B). Dashed line, number of cells phagocytosed prior to the addition of gentamicin (time zero [T_0]). Values are averaged results from three independent experiments. Results from another three independent experiments were similar. Error bars, standard errors of the means.

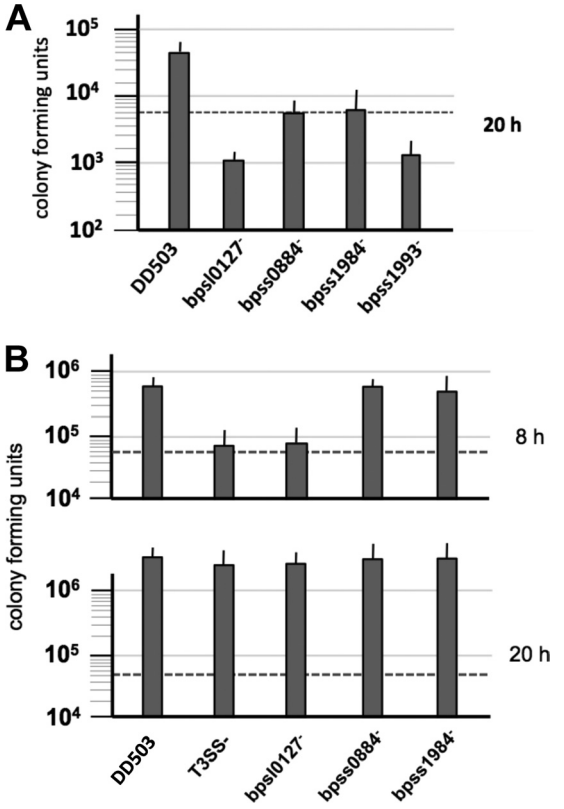

FIG. 5. Characterization of site-directed mutants of *B. pseudomallei.* Gentamicin protection assays were used to assess intracellular survival and replication of *B. pseudomallei* DD503 and various mutants derived from it (Table 1) in *D. discoideum* 20 h after internalization (A) or RAW 264.7 murine macrophages at 8 h and 20 h after internalization (B). Dashed line, number of cells phagocytosed prior to the addition of gentamicin (time zero [T_0]). Values are averaged results from three independent experiments. Results from another three independent experiments were similar. Error bars, standard errors of the means.

termined (Fig. 4B). Whereas parental *B. thailandensis* DW503 exhibited a 3-fold increase in intracellular populations by 8 h, numbers of mutant 1333, with an insertion in a two-component regulatory system, actually declined ~10-fold. Mutants 0250 and 0552 exhibited smaller decreases in intracellular populations of ~3-fold during the same initial 8-h period. However, between 8 and 20 h most mutants were able to replicate back to population levels found for the parental strain. Only mutant 0250, with a Tn*Mod* insertion in a hypothetical protein unique to *B. thailandensis* and *B. pseudomallei*, continued to exhibit reduced intracellular populations between 8 and 20 h that were ~10-fold less than that of DW503. Thus, the *B. thailandensis* mutants with dramatically reduced survival inside *D. discoideum* also showed a similar phenotype in macrophages but only at much earlier time points following phagocytosis, suggesting that *D. discoideum* may have more robust countermeasures for deterring intracellular replication by *Burkholderia* than do macrophages.

Inactivation of *bpsl0127* inhibits replication of *B. pseudomallei* in both amoeba and macrophages. We next determined whether orthologs of the *B. thailandensis* genes required for resistance to predation by *D. discoideum* functioned in a similar capacity in *B. pseudomallei*. Orthologs of genes disrupted in transposon mutants 1333, 0552, 0250, and 0188 were disrupted in *B. pseudomallei* DD503 by integration of a plasmid carrying fragments of each orthologous gene truncated at both the 5′ and 3′ ends (Table 2). Mirroring results for *B. thailandensis*, all these *B. pseudomallei* mutants required initial bacterium-to-amoeba ratios >10 times higher than those required by the parental strain to prevent the formation of *D. discoideum* plaques in the semiquantitative plaque assay; their growth rates in SM/5 were nearly identical (not shown). When assessed by the gentamicin protection assay, each mutant was reduced in its ability to replicate within *D. discoideum*, ranging from >10-fold for the *bpsl0127* and *bpss1993* mutants to ~4-fold for the *bpss0884* and *bpss1984* mutants (Fig. 5A). When these mutants were tested for survival and replication in macrophages, only the *bpsl0127* mutant and the T3SS-deficient mutant showed a phenotype; neither replicated more than once during the initial 8 h, while populations of the parental DD503 strain and of the *bpss0884* and *bpss1984* mutants increased ~10-fold (Fig. 5B). However, during the next 12 h the T3SS and *bpsl0127* mutants recovered and replicated to catch up to the parental DD503 strain by increasing their populations ~25 fold. These results for DD503 and the T3SS mutant are similar to those reported previously (10, 45, 62, 64, 66).

Some mutations affect the ability of *B. pseudomallei* to lyse macrophages. During the above-described intracellular repli-

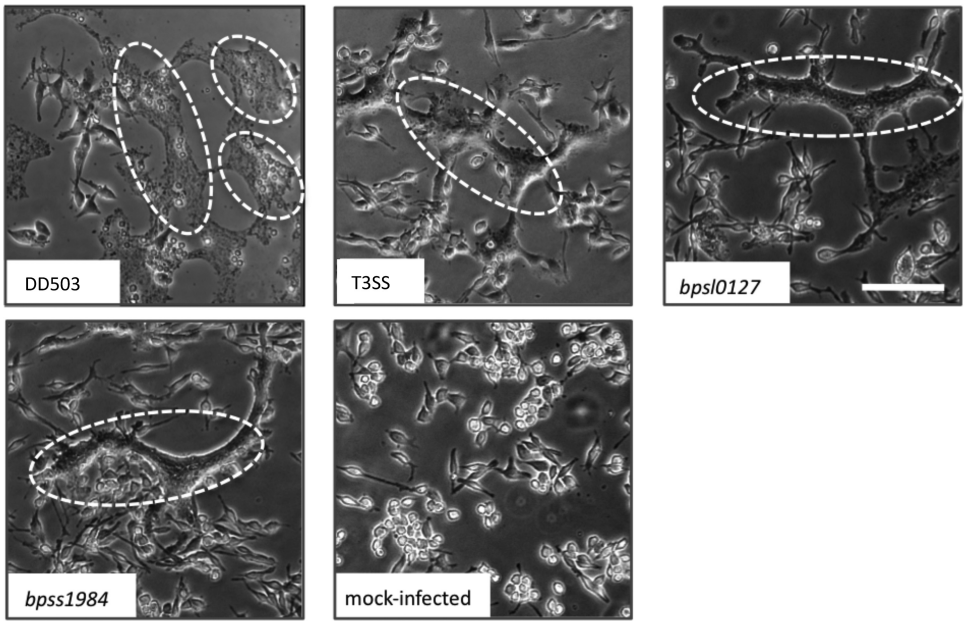

FIG. 6. Altered morphology of RAW 264.7 macrophages infected with *B. pseudomallei*. Phase-contrast microscopy of macrophage cells 20 h after infection with *B. pseudomallei* DD503 or selected site-directed mutants derived from it (Table 1) was performed as described in Materials and Methods. Dashed circles indicate multinucleated giant cells (MNGCs). Images are representative of two independent experiments. Scale bar, 100 μm.

cation assays with macrophages, we noticed that at 20 h the phenol red-containing tissue culture medium in wells with macrophages infected with the T3SS, *bpsl0127*, or *bpss1984* mutants was of lower pH (i.e., orange colored) than that in wells infected with the parental DD503 strain or the *bpss0884* and *bpss1993* mutants, which remained red. Examination of monolayers infected with DD503 by light microscopy showed a prevalence of multinucleated giant cells (MNGCs), the majority of which appeared to be lysed, leaving only a thin film of cellular debris (Fig. 6; see Fig. S1 in the supplemental material). In contrast, individual cells or MNGCs of monolayers infected with *bpsl0127*, *bpss1984*, or T3SS mutants appeared largely intact. Macrophage monolayers infected with the *bpss0884* mutant were laden with lysed multinucleated giant cells and were indistinguishable from DD503 controls (not shown). This suggests that mutations in T3SS, *bpsl0127*, or *bpss1984* reduce the ability of *B. pseudomallei* to lyse host macrophages, allowing their continued growth, which results in the lower pH.

To confirm this, quantitative determinations of macrophage lysis were performed using lactate dehydrogenase (LDH) release as an indicator of lysis (38). LDH activity in media from mock-infected wells was nearly undetectable, whereas activity in media from macrophages 20 h after infection with *B. pseudomallei* DD503 or the *bpss0884* mutant was 50-fold higher (data not shown). However, macrophages infected with mutants lacking BPSL0127, BPSS1984, or T3SS, for which lysis was much less apparent by microscopy, exhibited LDH release at levels 75% below those infected with the parental DD503 strain. Thus, although intracellular populations of the T3SS and *bpsl0127* mutants catch up from earlier deficiencies to reach those of DD503, this does not result in lysis of macrophages.

BPSL0127 is required for virulence in mice. As reduced resistance to *D. discoideum* has been shown to correlate with attenuated virulence of some human pathogens in animal models (1, 15, 48–50), we examined the lethality of the site-directed *B. pseudomallei* mutants toward BALB/c mice. Mice were inoculated intranasally with ~5,000 cells (~3 times the LD_{100}), after which they were monitored for 7 days (Fig. 7A). All mice inoculated with *B. pseudomallei* DD503 expired or had to be euthanized by the end of day 2, whereas all mice infected with a *bpsl0127* mutant or the T3SS mutant showed no signs of disease through the end of the experiment (Fig. 7A). Mutants lacking BPSS1984 or BPSS1993 were less attenuated in virulence; all mice infected with these mutants survived through day 4, twice as long as those infected with the parental strain DD503. In a second experiment, mice were infected with a *bpsl0127* mutant at doses ~50- and ~1,000-fold higher than the LD_{100} for DD503, and all but one of eight mice infected survived with no signs of morbidity for 2 weeks (Fig. 7B). The T3SS mutant gave similar results. Spleens removed from the euthanized survivors at 14 days usually showed bacterial counts of <10 CFU, suggesting that the *bpsl0127* mutants were essentially cleared by the mouse immune system rather than persisting in a latent, asymptomatic infection.

To confirm that the avirulent phenotype of the *bpsl0127* mutant was due only to inactivation of the sensor kinase, mice were infected with a *trans*-complemented *bpsl0127* mutant expressing the wild-type *bpsl0127* gene from plasmid pSCRsl0127. All mice infected with 25,000 CFU of this strain died within 4 days, whereas the uncomplemented mutant was totally avirulent at an ~40-fold-higher dose (Fig. 7B). However, at a lower dose of only 5,000 CFU, the complemented mutant was avirulent like its parent (not shown). This partial complementation may be due to instability of the plasmid

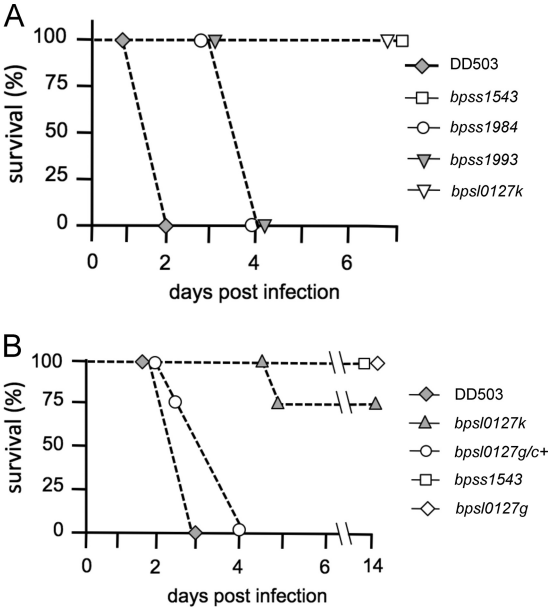

FIG. 7. Virulence of site-directed mutants of *B. pseudomallei* in mice. Groups of four BALB/c mice were intranasally infected with various doses of *B. pseudomallei* strains: (A) ~5,000-cell dose for all strains; (B) DD503, 5,000 cells; *bpsl0127k*, *bpsl0127g*, and *bpss1543* mutants, 10^6 cells; *bpsl0127g*/*c*+ (*trans*-complemented mutant), 25,000 cells. Animals were monitored daily for morbidity; survivors that became moribund and unresponsive to stimulation were euthanized and recorded as dead. Each strain was independently tested at least twice.

constitutively expressing *bpsl0127* in the absence of antibiotic selection. Alternatively, polar effects of the insertion on the cognate response regulator of *bpsl0127* immediately downstream could also affect complementation. Nonetheless, the results clearly show that the BPSL0127/BPSL0128 two-component regulatory system is absolutely required for virulence of *B. pseudomallei* in mice, possibly because it is required for activation of expression of an essential virulence factor(s).

DISCUSSION

Historically, characterization of virulence factors of intracellular bacterial pathogens has largely relied on analyses of factors required for cytotoxicity, adherence, invasion, and replication within cultured mammalian cells. However, for potentially lethal select agents requiring BSL-3 containment, such tissue culture-based systems are not facile for high-throughput identification or characterization of virulence determinants. Moreover, these procedures are labor-intensive and expensive and generate large volumes of infectious liquid waste that increase risk of exposure. To circumvent such limitations, we explored *D. discoideum* as an alternative model for identification of *Burkholderia* virulence determinants. In its amoebal form, *D. discoideum* preys upon bacteria using mechanisms that are evolutionarily conserved with those of mammalian immune phagocytes (8, 23, 37, 41, 52, 54); bacterial genes required for resistance to predation by *D. discoideum* could function similarly in macrophage cells and sometimes are required for disease in animals (1, 15, 48–50). Since the phenotype of bacterial strains with reduced resistance to predation by *D. discoideum* can be visualized macroscopically on agar plates (5, 15, 49, 50), identification of mutants altered in this process is simple, rapid, and inexpensive with minimal risk for exposure.

Using a semiquantitative plaque assay, we found that *B. pseudomallei* DD503 was resistant to predation by *D. discoideum* at bacterium-to-amoeba ratios over 100 times lower than those for an avirulent mutant lacking T3SS or its close relative *B. thailandensis*, which is only weakly pathogenic in animals (19, 44, 72). While these observations clearly support the use of the *D. discoideum* plaque assay to identify *B. pseudomallei* virulence factors, the semiquantitative plaque assay does little to illuminate the underlying mechanisms of resistance. However, using our liquid-based quantitative predation assay, we found that incubation in *Burkholderia*-conditioned medium did not affect *D. discoideum* viability, arguing against a role for toxins or metabolites in resistance to predation. Although the decreased resistance of the T3SS mutant suggests that contact-dependent secretion of effectors may be involved in killing of *D. discoideum* or blocking phagocytosis, the observations that the T3SS mutant was phagocytosed at the same level as its parental DD503 strain and that amoebal populations increased 2-fold during coculture with DD503 or mutants or in medium alone argue against this explanation.

Therefore, to gain a broader perspective of the suite of *Burkholderia* genes involved in resistance to *D. discoideum* without having to cope with the mandated select agent restrictions for construction and storage of thousands of *B. pseudomallei* mutants, we generated a *B. thailandensis* transposon mutant library and screened it for mutants with decreased resistance to predation by *D. discoideum*. Our goal was to survey genes required for resistance to *D. discoideum* and demonstrate that *B. thailandensis* is a valid surrogate for *B. pseudomallei*. Not unexpectedly, auxotrophs and other mutants altered in essential metabolic processes (e.g., elongation factor 2 and malate dehydrogenase mutants) had reduced resistance. However, additional genes, including a sensor kinase, a predicted secreted serine peptidase, and three hypothetical proteins, were also found to be important for resistance to predation and intra-amoebal survival.

The validity of the *B. thailandensis* surrogacy for *B. pseudomallei* was clearly demonstrated by the fact that disruption of *B. pseudomallei* orthologs of four genes required by *B. thailandensis* for resistance to predation by *D. discoideum* likewise resulted in reduced resistance when measured with either the plaque assay or the intra-amoebal replication assay. However, although the reduced intra-amoebal survival of the *B. thailandensis* mutants correlated with their reduced replication in macrophage cells, this correlation was generally not observed for the corresponding *B. pseudomallei* mutants, perhaps being overridden or obscured by its much higher and more aggressive virulence. Only in the case of the *bpsl0127* sensor kinase mutant did decreases in resistance to *D. discoideum* translate into reduced resistance to macrophage cells. It is tempting to speculate that *B. thailandensis* and *B. pseudomallei* as soil inhabitants share mechanisms for surviving predation by environmental phagocytes such as amoeba but that the more-virulent *B. pseudomallei* has evolved additional mechanisms to overcome destruction by mammalian immune cells.

Despite the fact that decreases in resistance to macrophages

were not apparent for all *B. pseudomallei* mutants, three mutants did exhibit attenuated virulence in mice. Most striking was the result for the *bpsl0127* sensor kinase mutant. It was avirulent in BALB/c mice even at doses nearly 1,000-fold higher than the LD_{100} of the parental DD503 *B. pseudomallei* strain, and it appeared to be completely cleared by the murine immune system rather than persisting in a latent infection, as has been reported for *B. pseudomallei* (42, 46). The molecular basis for loss of virulence is unclear, but it is plausible that this sensor kinase positively regulates critical virulence genes. The number and function of these genes are unknown, but transcriptome analyses of the *bpsl0127* mutant and strains overexpressing *bpsl0127* are in progress. However, the reason for the attenuated virulence of the *bpss1984* mutant is much less clear but could relate to the observation that, like the *bpsl0127* and T3SS mutants, disruption of *bpss1984* resulted in reduced macrophage lysis and MNGC formation. MNGCs are commonly observed in tissues of melioidosis patients (75); replication within these aggregates is hypothesized to be a means by which *B. pseudomallei* evades host immune response (36, 66). Thus, that the *bpss1984* mutant was able to replicate within macrophage cells but produced less macrophage lysis and MNGC formation deserves further inquiry. MNGC formation has not been described for bacterial pathogens outside the genus *Burkholderia*. Thus, it is noteworthy that *bpss1984* and adjacent genes (including *bpss1993*, whose inactivation also reduced predation resistance and virulence) are predicted to encode hypothetical proteins found only in pathogenic *Burkholderia* (55).

Much remains to be learned about the intracellular life cycle of *B. pseudomallei*, particularly host-pathogen interactions enabling phagosome lysis and inducing MNGC formation. While T3SS plays a role in MNGC formation (45, 66), T3SS effectors and host factors targeted by them are unknown, as is the role of other secretion systems. It is feasible that using *D. discoideum* to screen transposon mutant libraries of *Burkholderia* strains lacking T3SS or other known virulence determinants will accelerate our understanding of these processes and identify new factors contributing to *Burkholderia* pathogenesis. Also promising is the prospect of using *D. discoideum* to screen libraries of mutants *en masse* using procedures that enrich for mutants defective in phagosome lysis (56). Given that *D. discoideum* is haploid (24), easy to culture, and amenable to a diverse array of genetic manipulations and given the numerous strains with defined mutations in pathways for phagocytosis, endocytic sorting, and transit events (4, 8, 21, 26, 39, 54), this relatively simple eukaryote offers advantages over traditional cultured mammalian cells to more directly explore mechanisms utilized to subvert host defenses. Thus, based on our experimental observations and the numerous genetic advantages of *D. discoideum*, this soil-dwelling phagocyte holds promise for future *Burkholderia* research as a rapid, safe, and cost-efficient system to identify factors and dissect the mechanism of *Burkholderia* virulence.

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