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The Madagascar hissing cockroach as a novel surrogate host for *Burkholderia pseudomallei*, *B. mallei* and *B. thailandensis*

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Abstract

Background: *Burkholderia pseudomallei* and *Burkholderia mallei* are gram-negative pathogens responsible for the diseases melioidosis and glanders, respectively. Both species cause disease in humans and animals and have been designated as category B select agents by the Centers for Disease Control and Prevention (CDC). *Burkholderia thailandensis* is a closely related bacterium that is generally considered avirulent for humans. While it can cause disease in rodents, the *B. thailandensis* 50% lethal dose (LD₅₀) is typically $\geq 10^4$ -fold higher than the *B. pseudomallei* and *B. mallei* LD₅₀ in mammalian models of infection. Here we describe an alternative to mammalian hosts in the study of virulence and host-pathogen interactions of these *Burkholderia* species.

Results: Madagascar hissing cockroaches (MH cockroaches) possess a number of qualities that make them desirable for use as a surrogate host, including ease of breeding, ease of handling, a competent innate immune system, and the ability to survive at 37°C. MH cockroaches were highly susceptible to infection with *B. pseudomallei*, *B. mallei* and *B. thailandensis* and the LD₅₀ was <10 colony-forming units (cfu) for all three species. In comparison, the LD₅₀ for *Escherichia coli* in MH cockroaches was >10⁵ cfu. *B. pseudomallei*, *B. mallei*, and *B. thailandensis* cluster 1 type VI secretion system (T6SS-1) mutants were all attenuated in MH cockroaches, which is consistent with previous virulence studies conducted in rodents. *B. pseudomallei* mutants deficient in the other five T6SS gene clusters, T6SS-2 through T6SS-6, were virulent in both MH cockroaches and hamsters. Hemocytes obtained from MH cockroaches infected with *B. pseudomallei* harbored numerous intracellular bacteria, suggesting that this facultative intracellular pathogen can survive and replicate inside of MH cockroach phagocytic cells. The hemolymph extracted from these MH cockroaches also contained multinuclear giant cells (MNGCs) with intracellular *B. pseudomallei*, which indicates that infected hemocytes can fuse while flowing through the insect's open circulatory system in vivo.

Conclusions: The results demonstrate that MH cockroaches are an attractive alternative to mammals to study host-pathogen interactions and may allow the identification of new *Burkholderia* virulence determinants. The importance of T6SS-1 as a virulence factor in MH cockroaches and rodents suggests that the primary role of this secretion system is to target evasion of the innate immune system.

Keywords: Pathogenesis, Melioidosis, Glanders, Virulence, Surrogate host, Type VI secretion system

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Background

Burkholderia mallei is an obligate parasite of horses, mules and donkeys and no other natural reservoir is known [1]. The organism is a nonmotile gram-negative bacillus that is closely related to *Burkholderia pseudomallei* and *Burkholderia thailandensis*. *B. pseudomallei* is a pathogenic microbe that causes the glanders-like disease melioidosis [2] and *B. thailandensis* is a weakly pathogenic soil saprophyte [3]. While a handful of *Burkholderia* virulence determinants have been identified using rodent models of infection [4], research on the molecular mechanism(s) of pathogenesis is still a fertile area. *B. mallei*, *B. pseudomallei*, and *B. thailandensis* are able to survive and replicate inside phagocytic cells in a process that involves escape from the endocytic vacuole, replication in the cytosol, intra- and intercellular spread by actin polymerization, and fusion with uninfected cells to form multinucleated giant cells (MNGCs) [4]. Gram-negative pathogens often use secretion systems to deliver virulence factors to the cytosol of host cells, where they modulate cell physiology to favor the microbe. The exploitation of host phagocytic cells by *B. pseudomallei* involves two type III secretion systems (T3SS-1 & T3SS-3) [5–7], a type V secretion system (BimA) [8], and the cluster 1 type VI secretion system (T6SS-1) [9]. T6SS-1, occasionally referred to as *tss-5* [10], is also important for host cell interactions and virulence in *B. mallei* and *B. thailandensis* [11,12].

Small mammal models of infection have long been employed to characterize virulence factors of bacterial pathogens, but over the last decade there has been an increase in the use of surrogate hosts to study the pathogenic mechanisms of bacteria [13,14]. Several surrogate hosts have been used as alternatives to mammals to study virulence factors and host-pathogen interactions with *B. pseudomallei*, *B. mallei*, and *B. thailandensis*, including *Galleria mellonella* larvae (wax worms) [15,16], *Dictyostelium discoideum* (phagocytic amoeba) [17], *Caenorhabditis elegans* (soil nematode) [18–20], and *Solanum lycopersicum* (tomato plantlets) [21]. These alternative hosts have allowed the identification of new *Burkholderia* virulence determinants and have confirmed the importance of virulence factors previously characterized using rodent models of infection.

Insects are popular alternatives to mammalian hosts in large-scale screening studies, owing largely to the high degree of similarity between the innate immune systems of insects and mammals [22]. In both, the recognition of pathogen-associated molecular patterns (PAMPs) by Toll receptors (insects) and Toll-like receptors (mammals) results in the production of antimicrobial peptides [23]. Furthermore, insect hemocytes and mammalian neutrophils can both engulf and kill most invading microorganisms [24]. Insects are also afforded protection from microorganisms through the coagulation

and melanization of hemolymph, but they do not have an adaptive immune system.

In addition to biological similarities, several logistical issues contribute to the recent adoption of insects as alternative hosts for bacterial pathogens. Insects can be readily obtained, housed, and cared for at considerable cost savings compared to mammals. Moreover, the use of insects is not governed by animal use regulations or committees and even very large-scale experiments using insects are considered ethically acceptable. As a possible insect alternative to mammalian models of infection, we tested several *B. pseudomallei*, *B. mallei*, and *B. thailandensis* strains against juvenile Madagascar hissing cockroaches (MH cockroaches) obtained from a commercial vendor (Carolina Biological Supply Company). MH cockroaches are readily available, easily cultured, and reproduce rapidly. They are larger than wax moth larvae, slow moving compared to other species of cockroaches, and have a substantive carapace. These characteristics make them easier to manipulate and inoculate with known numbers of bacteria compared with other species of insects commonly used for similar studies. MH cockroaches thrive at 37°C, a characteristic that is essential for the analysis of mammalian pathogens.

In this study, we found the MH cockroach to be a suitable surrogate host for *B. pseudomallei*, *B. mallei*, and *B. thailandensis*. *Burkholderia* type VI secretion system mutants were attenuated in MH cockroaches, which is consistent with what is seen in rodent models of infection [9,25]. *B. pseudomallei* multiplied inside MH cockroach hemocytes and may be the primary mechanism by which this pathogen avoids elimination by the MH cockroach innate immune system. The results suggest that MH cockroaches are a good alternative to mammals for the study of *Burkholderia* species and possibly other mammalian pathogens.

Results and discussion

B. pseudomallei is virulent in the MH cockroach and T6SS-1 mutants exhibit attenuated virulence

In an attempt to determine if the MH cockroach might serve as a surrogate host for *B. pseudomallei*, we challenged juvenile MH cockroaches (Figure 1) with K96243 and T6SS mutant derivatives. T6SS-1 is a critical virulence determinant for *B. pseudomallei* in the hamster model of infection [9], while T6SS-2, T6SS-3, T6SS-4, T6SS-5, and T6SS-6 are dispensable for virulence in hamsters. Groups of eight MH cockroaches were challenged by the intra-abdominal route with 10^1 - 10^5 bacteria and deaths were recorded for 5 days at 37°C (Figure 2).

Figure 2A shows that only one MH cockroach survived for 5 days after challenge with 10^1 *B. pseudomallei* K96243 (Bp), demonstrating that the 50% lethal dose

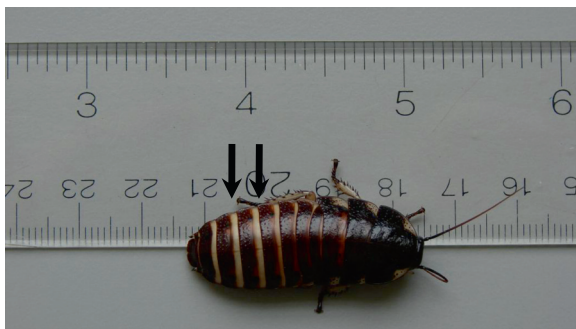


Figure 1 A representative juvenile Madagascar hissing cockroach used as a surrogate host for *B. pseudomallei*, *B. mallei*, and *B. thailandensis* infection studies. The black arrows show the locations where bacteria were inoculated into the dorsal abdominal section of the MH cockroach, between the third and the fifth terga from the posterior.

(LD₅₀) is <10 bacteria. Similarly, the LD₅₀ for K96243 in the hamster model of infection was <10 bacteria [9]. *B. pseudomallei* Δ*hcp1* is a derivative of K96243 that lacks the essential tail tube component of the T6SS-1 structural apparatus (Hcp1) and is highly attenuated in the hamster [9,26]. *B. pseudomallei* Δ*hcp1* was also attenuated in the MH cockroach (Figure 2A-E) and the LD₅₀ was ~2 × 10² bacteria on day 5, which was >20 times higher than the K96243 LD₅₀ (Table 1). In addition, a dose response was readily apparent with this strain. As the challenge dose increased from 10¹ to 10⁵ bacteria, the number and rate of MH cockroach deaths increased accordingly (Figure 2A-E). It took a challenge dose of 10⁴ Δ*hcp1* to kill all eight MH cockroaches, whereas the minimum lethal dose for K96243 was only 10² bacteria (Figure 2). The results demonstrate that *B. pseudomallei* is highly virulent in MH cockroaches and that T6SS-1 is a critical virulence factor in this insect host. Furthermore, there is a clear correlation between the virulence

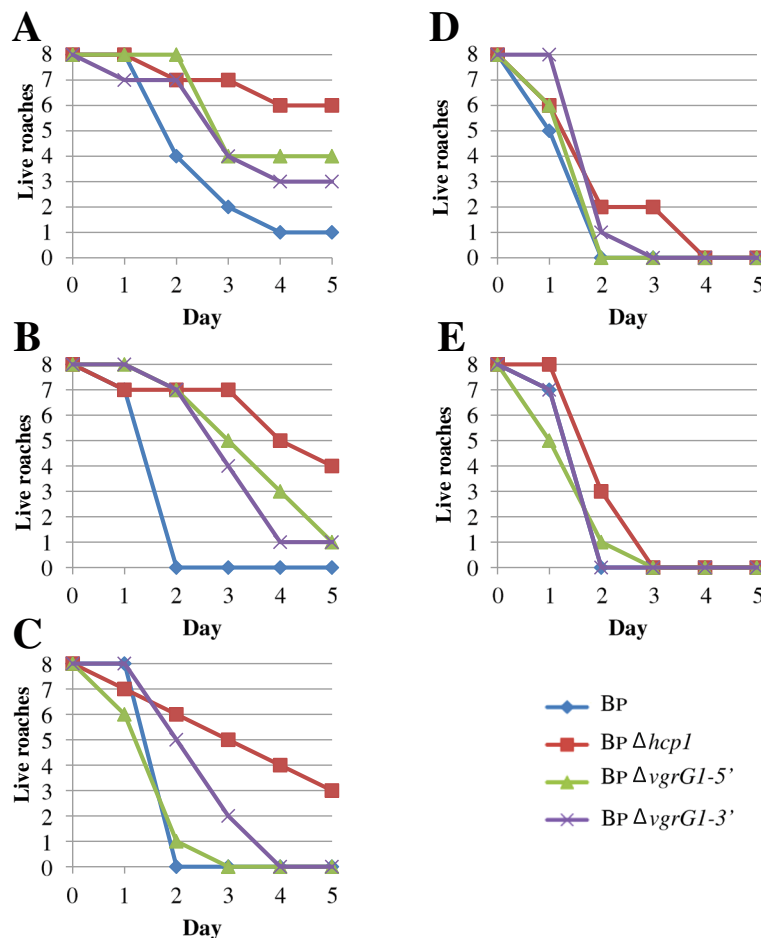


Figure 2 *B. pseudomallei* is virulent for the MH cockroach and T6SS-1 mutants are attenuated. Groups of eight MH cockroaches were challenged by the intra-abdominal route of infection and MH cockroach deaths were monitored for 5 days at 37°C. (A) 10¹ cfu. (B) 10² cfu. (C) 10³ cfu. (D) 10⁴ cfu. (E) 10⁵ cfu. Bp, K96243; Bp Δ*hcp1*, DDS1498A; Bp Δ*vgrG1-5'*, DDS1503-1A; Bp Δ*vgrG1-3'*, DDS1503-2A.

Table 1 Relative virulence of bacterial strains in Syrian hamsters and Madagascar hissing cockroaches

Bacterial strain	Syrian hamster LD ₅₀ ^a	Madagascar hissing cockroach LD ₅₀
<i>E. coli</i>		
MC4100	ND ^b	> 10 ⁵
B/r	ND	>10 ⁵
<i>B. pseudomallei</i>		
K96243	<10	<10
DDS1498A ($\Delta hcp1$)	>1000	207
DDS0518A ($\Delta hcp2$)	<10	<10
DDS2098A ($\Delta hcp3$)	<10	<10
DDS0171A ($\Delta hcp4$)	<10	<10
DDS0099A ($\Delta hcp5$)	<10	<10
DDL3105A ($\Delta hcp6$)	<10	<10
DDS1503-1A ($\Delta vgrG1-5'$)	102	<10
DDS1503-2A ($\Delta vgrG1-3'$)	>450	<10
1026b	<10	<10
MSHR305	ND	<10
<i>B. mallei</i>		
SR1	<10	<10
DDA0742 ($\Delta hcp1$)	>10 ³	>10 ³
<i>B. thailandensis</i>		
DW503	ND	<10
DDII0868 ($\Delta hcp1$)	ND	>10 ³

^a LD₅₀, 50% lethal dose [9,25,33]; ^b ND, not determined.

capacity of *B. pseudomallei* in the MH cockroach and the hamster (Table 1).

B. pseudomallei $\Delta vgrG1-5'$ and $\Delta vgrG1-3'$ are K96243 derivatives that have deletions within the gene encoding the tail spike protein (VgrG1) of the T6SS-1 structural apparatus [9,26]. These mutants were more virulent than *B. pseudomallei* $\Delta hcp1$ in the hamster model of infection [9], but were less virulent than K96243 (Table 1). There was no difference in the LD₅₀ of $\Delta vgrG1-5'$, $\Delta vgrG1-3'$, and K96243 in MH cockroaches (Table 1), however, there was a slight but not statistically significant difference in the time to death with these strains. In general, it took longer for MH cockroaches infected with $\Delta vgrG1-5'$ and $\Delta vgrG1-3'$ to die relative to K96243 (Figure 2A-C). Thus, these strains appear to have an intermediate virulence phenotype in both MH cockroaches and in hamsters (Table 1 and Figure 2).

We next examined the relative virulence of the *B. pseudomallei* $\Delta hcp2$, $\Delta hcp3$, $\Delta hcp4$, $\Delta hcp5$, and $\Delta hcp6$ mutants in MH cockroaches [9]. These mutants are each deficient in one of the other five T6SSs present in *B. pseudomallei* and all are virulent in the hamster (Table 1). Figure 3

shows that these strains are also virulent in the MH cockroach and all exhibit a clear dose response. The majority of MH cockroaches infected with a challenge dose of 10¹ bacteria were dead by day 3 (Figure 3A), but most were dead by day 1 with a challenge dose of 10⁵ bacteria (Figure 3E). Interestingly, the LD₅₀ results with these strains are remarkably similar in both MH cockroaches and hamsters (Table 1).

The virulence of two additional isolates of *B. pseudomallei* and two isolates of *Escherichia coli* were also tested in the MH cockroach. The LD₅₀s of *B. pseudomallei* 1026b and MSHR305 were <10 bacteria and the LD₅₀s for *E. coli* MC4100 and B/r were >10⁵ bacteria, the highest dose tested (Table 1). The results suggest that virulence for the MH cockroach is common among *B. pseudomallei* isolates and that not all gram-negative bacteria are pathogenic for this surrogate host (Table 1).

Taken together, the results demonstrate that *B. pseudomallei* is highly virulent in the MH cockroach and indicate that this insect might serve as a surrogate host for high throughput virulence screening assays. In addition, the MH cockroach challenge results are consistent with what is seen in the hamster model of infection and suggest that the primary function of the T6SS-1 is to evade the innate immune system.

The MH cockroach can serve as a surrogate host for *B. mallei* and *B. thailandensis*

We also evaluated the virulence of *B. mallei* and *B. thailandensis* in the MH cockroach. The LD₅₀s for *B. mallei* SR1 (Bm) and *B. thailandensis* DW503 (Bt) were <10 bacteria (Table 1) and the number and rate of deaths increased as the challenge dose increased from 10¹ to 10³ bacteria (Figure 4). Interestingly, *B. mallei* killed the MH cockroaches at a slower rate than *B. thailandensis* (and *B. pseudomallei*). It took only 2 days for *B. thailandensis* to kill 75% of the MH cockroaches with a dose of 10¹ bacteria, whereas it took *B. mallei* 5 days (Figure 4A). Similar trends were apparent with challenge doses of 10² and 10³ bacteria (Figure 4B and C). *B. mallei* does not kill rodents as quickly as *B. pseudomallei* and it is more fastidious than *B. pseudomallei* and *B. thailandensis*, so it may not be too surprising that it took longer to kill MH cockroaches [4]. These experiments demonstrate that *B. mallei* and *B. thailandensis* are both virulent in the MH cockroach and suggest that the MH cockroach might serve as a surrogate host for these bacterial species.

As mentioned above, *B. thailandensis* is considered to be avirulent in humans and exhibits a higher LD₅₀ in mammalian models of infection than *B. mallei* and *B. pseudomallei*. Mammals, unlike MH cockroaches, possess both an innate and an acquired immune system. The fact that *B. thailandensis* is highly virulent

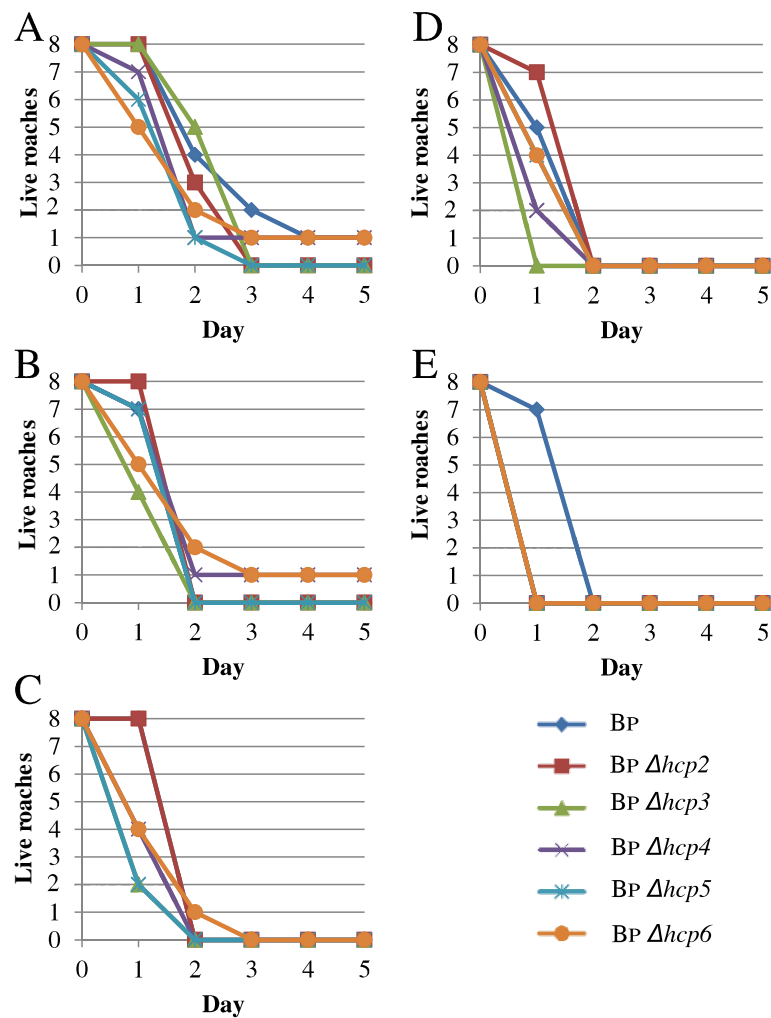


Figure 3 *B. pseudomallei* T6SS-2, T6SS-3, T6SS-4, T6SS-5, and T6SS-6 mutants are virulent in the MH cockroach. (A) 10^1 cfu. (B) 10^2 cfu. (C) 10^3 cfu. (D) 10^4 cfu. (E) 10^5 cfu. Bp, K96243; Bp $\Delta hcp2$, DDS0518A; Bp $\Delta hcp3$, DDS2098A; Bp $\Delta hcp4$, DDS0171A; Bp $\Delta hcp5$, DDS0099A; Bp $\Delta hcp6$, DDL3105A.

in the MH cockroach may suggest that the acquired immune system plays an important role in defence against *B. thailandensis*. *B. mallei* and *B. pseudomallei*, on the other hand, may have developed mechanisms to subvert the acquired immune response in mammalian species.

T6SS-1 is a critical virulence determinant for *B. mallei* in the hamster model of infection [25] and for *B. thailandensis* in the C57BL/6 mouse model of infection [27]. We challenged MH cockroaches with *B. mallei* and *B. thailandensis hcp1* mutants and found that they were highly attenuated in this surrogate host (Table 1 and Figure 4). The LD₅₀s for *B. mallei* $\Delta hcp1$ and *B. thailandensis hcp1* were $> 10^3$ bacteria on day 5, which was at least 100 times higher than their respective parental strains (Table 1 and Figure 4). The *B. mallei* results were indistinguishable from what was previously described

for SR1 and $\Delta hcp1$ using the hamster model of infection [25]. While the *B. thailandensis* strains used in this study have not been tested in hamsters, a *B. thailandensis* T6SS-1 mutant was recently shown to be avirulent in C57BL/6 mice by the aerosol route of infection [27]. Interestingly, MyD88^{-/-} mice were susceptible to the *B. thailandensis* T6SS-1 mutant, which suggests that T6SS-1 plays a role in evading the innate immune response [27]. The fact that *B. thailandensis hcp1* was attenuated in an insect host, which lacks an adaptive immune response, further supports the notion that the function of the T6SS-1 is to evade the eukaryotic innate immune system.

B. pseudomallei replicates inside MH cockroach hemocytes

Hemocytes are a key component of the MH cockroach innate immune system and we next examined if *B.*

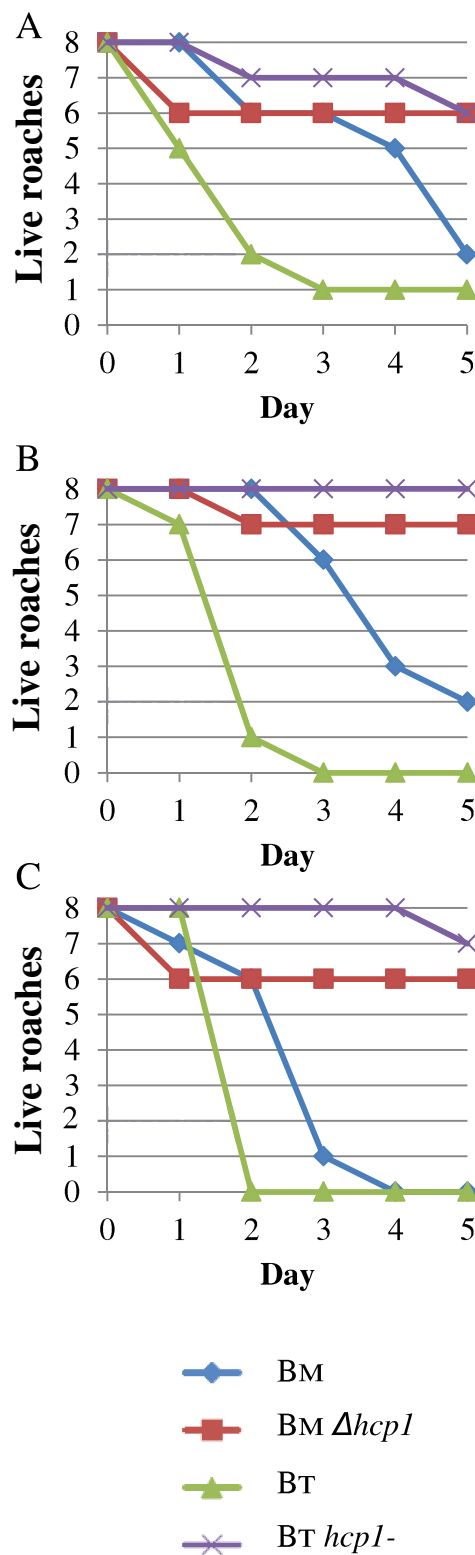


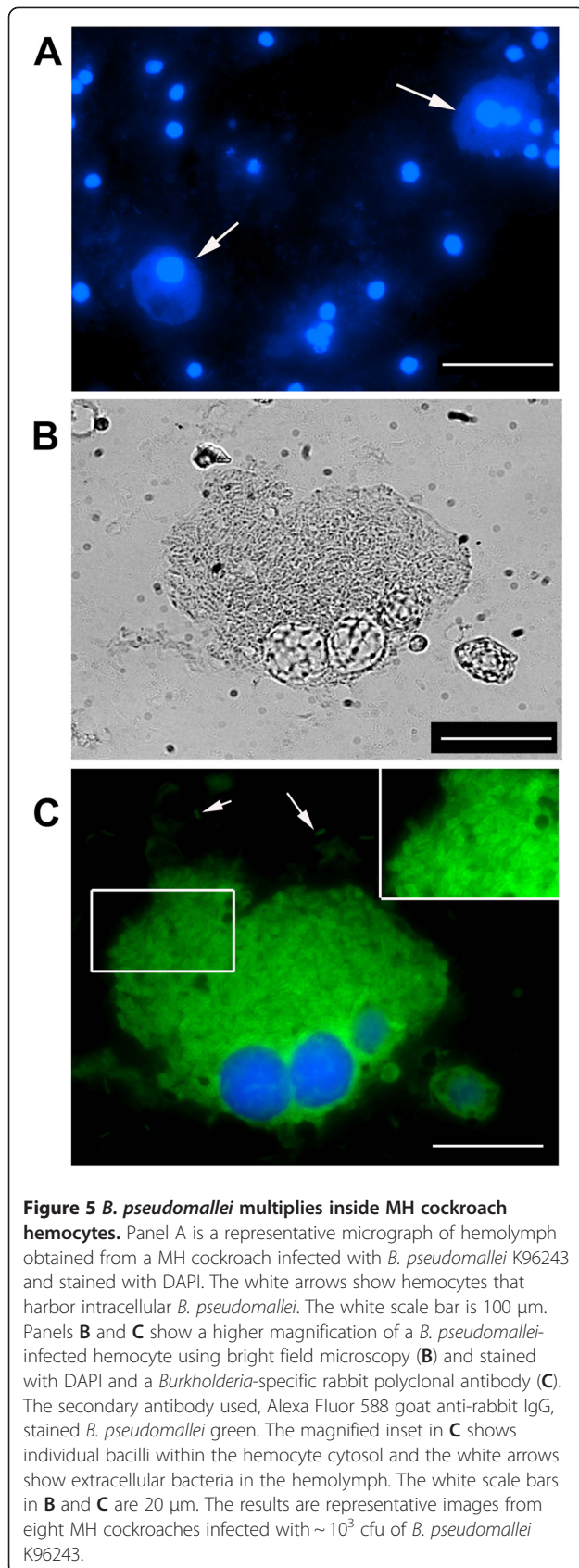
Figure 4 *B. mallei* and *B. thailandensis* are virulent for the MH cockroach and their T6SS-1 mutants are attenuated. (A) 10¹ cfu. (B) 10² cfu. (C) 10³ cfu. Bm, SR1; Bm Δhcp1, DDA0742; Bt, DW503; Bt Δhcp1, DD110868.

pseudomallei might be exploiting these phagocytic cells to gain an upper hand in the host-pathogen interaction. A group of eight MH cockroaches were infected with ~ 10³ *B. pseudomallei* K96243 and closely monitored for 48 h. MH cockroaches that appeared weak and lethargic were immediately separated from the others and hemolymph was obtained. The MH cockroach hemolymph, which contains phagocytic hemocytes, was fixed and stained with DAPI. Figure 5A shows a representative field containing the blue-staining nuclei from multiple hemocytes. As expected, the non-nuclear regions of most hemocytes could not be visualized with this fluorescent DNA stain. Interestingly, each field also contained one or two hemocytes in which the nucleus and the surrounding cytosol could be easily visualized (Figure 5A, white arrows). We speculated that these particular hemocytes might contain cytosolic *B. pseudomallei* and we stained the hemolymph with a polyclonal antibody that reacts with *B. pseudomallei*. Figure 5B and 5C show a representative micrograph of a hemocyte engorged with cytosolic *B. pseudomallei*, suggesting that the bacteria are multiplying to high numbers inside these cells. Free bacteria can also be visualized in the hemolymph outside the hemocyte, but it is unclear if these cells are alive or dead (Figure 5B and 5C). Some infected hemocytes appear to have multiple nuclei and may be multinucleated giant cells (MNGCs) (Figure 5). MNGCs have been observed in cases of human melioidosis [28] and are often formed when *B. pseudomallei* infects murine macrophage-like cell lines in vitro [9]. The formation of *B. pseudomallei*-induced MNGCs in vivo in MH cockroaches is an exciting finding and indicates that MNGCs can form in non-adherent cells freely flowing within the hemolymph.

Based on these results, we hypothesize that *B. pseudomallei* is able to survive the innate immune system of the MH cockroach by establishing an intracellular niche within the hemocyte. Infected hemocytes harboring numerous cytosolic bacteria may fuse with uninfected hemocytes to form MNGCs, which may serve as a reservoir for continued bacterial replication and protection from the antimicrobial peptides present in the surrounding hemolymph. The amplification of bacteria within phagocytic hemocytes, and their subsequent release, may eventually overwhelm the MH cockroach and lead to death.

Conclusions

Our findings indicate that the Madagascar hissing cockroach can serve as a surrogate host for the analysis of host-pathogen interactions with *B. pseudomallei*, *B. mallei*, and *B. thailandensis*. Using this system, we were able to detect virulence differences between parental strains and T6SS-1 mutants that were consistent with



what was seen in rodent models of infection. *B. pseudomallei* K96243 demonstrated the ability to multiply inside insect hemocytes and form MNGCs, which may be the primary mechanism by which it avoids killing by the MH cockroach innate immune system. The MH cockroach will probably be useful for high throughput virulence screening assays with these *Burkholderia* species as well as other bacterial pathogens.

Methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are described in Table 2. *E. coli*, *B. pseudomallei*, and *B. thailandensis* were grown at 37°C on Luria-Bertani (Lennox) agar (LB agar) or in LB broth. When appropriate, antibiotics were added at the following concentrations: 15 μ g of gentamicin (Gm), 25 μ g of streptomycin (Sm), and 25 μ g of kanamycin (Km) per ml for *E. coli* and 25 μ g of polymyxin B (Pm) and 25 μ g of Gm per ml for *B. thailandensis*. *B. mallei* was grown at 37°C on LB agar with 4% glycerol or in LB broth with 4% glycerol. All bacterial strains were grown in broth for ~ 18 h with constant agitation at 250 revolutions per minute. Phosphate-buffered saline (PBS) was used to make serial dilutions of saturated bacterial cultures and the number of cfu present in the starting culture were determined by spreading 100 μ l aliquots onto agar media and incubating for 24–48 h. A 20-mg/ml stock solution of the chromogenic indicator 5-bromo-4-chloro-3-indolyl-b-D-galactoside (X-Gal) was prepared in N,N-dimethylformamide, and 40 μ l was spread onto the surface of plate medium for blue/white screening in *E. coli* TOP10. All manipulations with *B. pseudomallei* and *B. mallei* were carried out in class II and class III microbiological safety cabinets located in designated biosafety level 3 (BSL-3) laboratories.

PCR

The two deoxyribonucleotide primers used for PCR amplification of an internal gene fragment of *B. thailandensis* BTH_II0868 (*hcp1*) were purchased from Invitrogen (Frederick, MD) and designated II0868-up (5'-AGGGCAA GATTCTCGTCCAG-3') and II0868-dn (5'-TCTCGTACGT GAACGATACG-3'). The PCR product was sized and isolated using agarose gel electrophoresis, cloned using the pCR2.1-TOPO TA Cloning Kit (Invitrogen), and transformed into chemically competent *E. coli* TOP10. PCR amplification was performed in a final reaction volume of 100 μ l containing 1X Taq PCR Master Mix (Qiagen), 1 μ M oligodeoxyribonucleotide primers, and approximately 200 ng of *B. thailandensis* DW503 genomic DNA. PCR cycling was performed using a PTC-150 MiniCycler with a Hot Bonnet accessory (MJ Research, Inc.) and heated to 97°C for 5 min. This was followed by 30 cycles

Table 2 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Source or reference
<i>E. coli</i>		
TOP10	General cloning and blue/white screening	Invitrogen
S17-1	Mobilizing strain with transfer genes of RP4 integrated on chromosome; Sm ^r , Pm ^s	[34]
MC4100	K-12 laboratory strain	[35]
B/r	B laboratory strain	[36]
<i>B. pseudomallei</i>		
K96243	Isolated in Thailand from a diabetic patient with a clinical history of short incubation, septicemic infection, and rapid progression to death	[37]
DDS1498A	K96243 derivative harboring a 162-bp in-frame deletion mutation in <i>hcp1</i> ($\Delta hcp1$)	[9]
DDS0518A	K96243 derivative harboring a 303-bp in-frame deletion mutation in <i>hcp2</i> ($\Delta hcp2$)	[9]
DDS2098A	K96243 derivative harboring a 186-bp in-frame deletion mutation in <i>hcp3</i> ($\Delta hcp3$)	[9]
DDS0171A	K96243 derivative harboring a 321-bp in-frame deletion mutation in <i>hcp4</i> ($\Delta hcp4$)	[9]
DDS0099A	K96243 derivative harboring a 192-bp in-frame deletion mutation in <i>hcp5</i> ($\Delta hcp5$)	[9]
DDL3105A	K96243 derivative harboring a 216-bp in-frame deletion mutation in <i>hcp6</i> ($\Delta hcp6$)	[9]
DDS1503-1A	K96243 derivative harboring a deletion of the 743-bp <i>StuI</i> fragment at the 5' end of <i>vgrG1</i> ($\Delta vgrG1-5'$)	[9]
DDS1503-2A	K96243 derivative harboring a deletion of the 894-bp <i>PstI</i> fragment at the 3' end of <i>vgrG1</i> ($\Delta vgrG1-3'$)	[9]
1026b	Isolated in Thailand from a human case of septicemic melioidosis with skin, soft tissue, and spleen involvement	[30]
MSHR305	Isolated from the brain of a fatal human melioidosis encephalomyelitis case in Australia	[38,39]
<i>B. mallei</i>		
SR1	ATCC 23344 sucrose-resistant derivative	[40]
DDA0742	SR1 derivative harboring a deletion of the 156 bp <i>NarI</i> - <i>SfuI</i> fragment internal to <i>hcp1</i> ; $\Delta hcp1$	[25]
<i>B. thailandensis</i>		
DW503	E264 derivative; $\Delta(amrR-oprA)$ (Gm ^s) <i>rpsL</i> (Sm ^r)	[41]
DDII0868	DW503::pGSV3-0868; Gm ^r ; <i>hcp1</i> ⁻	This study
Plasmids		
pCR2.1-TOPO	3,931-bp TA vector; pMB1 <i>oriR</i> ; Km ^r	Invitrogen
pCR2.1-0868	pCR2.1-TOPO containing 342-bp PCR product generated with II0868-up and II0868-dn	This study
pGSV3	Mobilizable Gm ^r suicide vector	[42]
pGSV3-0868	pGSV3 derivative containing <i>EcoRI</i> insert from pCR2.1-0868	This study

^a r, resistant; s, susceptible.

of a three-temperature cycling protocol (97°C for 30 s, 55°C for 30 s, and 72°C for 1 min) and one cycle at 72°C for 10 min.

DNA manipulation and plasmid conjugation

Restriction enzymes, Antarctic phosphatase, and T4 DNA ligase were purchased from Roche Molecular Biochemicals and were used according to the manufacturer's instructions. DNA fragments used in cloning procedures were excised from agarose gels and purified with a GeneClean III kit (Q · BIOgene). Bacterial genomic DNA was prepared by a previously described

protocol [29]. Plasmids were purified from overnight cultures by using Wizard Plus SV Minipreps (Promega). Plasmid pGSV3-0868 (Table 2) was electroporated into *E. coli* S17-1 (12.25 kV/cm) and conjugated with *B. thailandensis* for 8 h, as described elsewhere [30]. Pm was used to counterselect *E. coli* S17-1 (pGSV3-0868).

MH cockroach housing and manipulation

Madagascar hissing cockroaches, *Gromphadorhina laevigata*, were purchased from Carolina Biological Supply Company (Burlington, NC) as 1–2 inch nymphs (Figure 1) and were housed in the dark at

room temperature in a 7.5" w x 11.75 l x 5" h mouse cage with a filtered top (Allentown Caging Equipment Co., Inc., Allentown, NJ). The bottom of the cage was lined with cocoa mulch and a thin layer of petroleum jelly was spread around the top portion of the cage to prevent MH cockroaches from climbing up the sides. Dog food was spread on the bottom of the cage for food and the top of a petri dish was inverted and filled with water for drinking. On occasion, sliced apple wedges were placed in the cage as an additional source of food.

For bacterial infection experiments, 1.5-2 inch juvenile MH cockroaches were used (Figure 1). We also tested larger MH cockroaches (> 3 inches) and they displayed the same susceptibility as the juveniles (data not shown). Bacteria were inoculated into the dorsal abdominal section of MH cockroaches, between the third and the fifth terga (from the posterior), using a 1 ml syringe fitted with a 3/8 inch, 26-gauge needle (see Figure 1). The syringe was loaded into a Tridak STEPPER series repetitive pipette (Tridak LLC, Torrington, CT) and a 25 µl aliquot was injected into MH cockroaches. A group of eight infected MH cockroaches were placed in a 16-ounce plastic container with a few pieces of dog food and 1–2 ml of water. The containers were placed in a 37°C incubator and deaths were recorded for 5 days. Food and water levels were checked daily and replenished if needed. The LD₅₀s were calculated 5 days postinfection according to the Reed-Muench method [31].

Extraction and staining of hemolymph from infected MH cockroaches

Eight MH cockroaches were infected with ~10³ *B. pseudomallei* K96243 and monitored daily as described above. Hemolymph was extracted from MH cockroaches that were lethargic and on the verge of death. Holding the MH cockroach with its ventral side up, one hind leg was folded up towards the head to expose the membrane at the base of the leg. The membrane was punctured with a 26-gauge needle and hemolymph was immediately collected using a P200 Gilson PIPETMAN. We used a pipette tip cut with scissors approximately a 1/2 inch from the end to aid in uptake of the viscous hemolymph. The amber-colored hemolymph was transferred to a glass slide, allowed to air dry, and then fixed with methanol. The samples were initially stained with 4', 6-diamidino-2-phenylindole (DAPI) and viewed on a Nikon Eclipse TE2000-S inverted microscope equipped with a Spot-RT digital camera (Image Systems, Columbia, MD). Subsequently, the samples were incubated for 1 h with a 1:1000 dilution of rabbit polyclonal *Burkholderia* antiserum [32] and then reacted for 1 h with a 1:500 dilution of an Alexa Fluor 588 goat

anti-rabbit IgG secondary antibody (Molecular Probes) and visualized by fluorescence microscopy.

Abbreviations

MH: Cockroach; Madagascar: Hissing cockroach; T6SS-1: Ccluster 1 type VI secretion system; LD₅₀: 50% lethal dose; MNGC: Multinucleated giant cell; Cfu: Colony forming units; DAPI: 4', 6-diamidino-2-phenylindole.

Authors' contributions

NAF conceived use of the MH cockroach as a surrogate host, contributed to the experimental design, and helped draft the manuscript. WJR was involved with the extraction, staining, and fluorescence microscopy of MH cockroach hemolymph. WA participated in the study design and conducted experiments. DD designed and conducted the experiments and drafted the manuscript. All authors read and approved the final manuscript.

Authors' information

Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army or Department of Homeland Security.

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