Identification of *Burkholderia mallei* and *Burkholderia pseudomallei* adhesins for human respiratory epithelial cells

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**Abstract**

**Background:** *Burkholderia pseudomallei* and *Burkholderia mallei* cause the diseases melioidosis and glanders, respectively. A well-studied aspect of pathogenesis by these closely-related bacteria is their ability to invade and multiply within eukaryotic cells. In contrast, the means by which *B. pseudomallei* and *B. mallei* adhere to cells are poorly defined. The purpose of this study was to identify adherence factors expressed by these organisms.

**Results:** Comparative sequence analyses identified a gene product in the published genome of *B. mallei* strain ATCC23344 (locus # BMAA0649) that resembles the well-characterized *Yersinia enterocolitica* autotransporter adhesin YadA. The gene encoding this *B. mallei* protein, designated boaA, was expressed in *Escherichia coli* and shown to significantly increase adherence to human epithelial cell lines, specifically HEp2 (laryngeal cells) and A549 (type II pneumocytes), as well as to cultures of normal human bronchial epithelium (NHBE). Consistent with these findings, disruption of the boaA gene in *B. mallei* ATCC23344 reduced adherence to all three cell types by ~50%. The genomes of the *B. pseudomallei* strains K96243 and DD503 were also found to contain boaA and inactivation of the gene in DD503 considerably decreased binding to monolayers of HEp2 and A549 cells and to NHBE cultures. A second YadA-like gene product highly similar to BoaA (65% identity) was identified in the published genomic sequence of *B. pseudomallei* strain K96243 (locus # BPSL1705). The gene specifying this protein, termed boaB, appears to be *B. pseudomallei*-specific. Quantitative attachment assays demonstrated that recombinant *E. coli* expressing BoaB displayed greater binding to A549 pneumocytes, HEp2 cells and NHBE cultures. Moreover, a boaB mutant of *B. pseudomallei* DD503 showed decreased adherence to these respiratory cells. Additionally, a *B. pseudomallei* strain lacking expression of both boaA and boaB was impaired in its ability to thrive inside J774A.1 murine macrophages, suggesting a possible role for these proteins in survival within professional phagocytic cells.

**Conclusions:** The boaA and boaB genes specify adhesins that mediate adherence to epithelial cells of the human respiratory tract. The boaA gene product is shared by *B. pseudomallei* and *B. mallei* whereas BoaB appears to be a *B. pseudomallei*-specific adherence factor.

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**Background**

*Burkholderia pseudomallei* is a Gram-negative bacterium readily recovered from the water and wet soils of endemic areas bordering the equator, particularly Southeast Asia and Northern Australia [1-9]. The organism is a motile, aerobic bacillus that can survive environmental extremes as well as the bactericidal activities of complement [10-12], defensins [13-15], and phagocytes [1,2,16-18]. The genome of the *B. pseudomallei* isolate K96243 has been published by the Wellcome Trust Sanger Institute and was shown to consist of two chromosomes of 4.1 and 3.2 Mbp [19]. *Burkholderia mallei* is a non-motile, host-adapted clone of *B. pseudomallei* that does not persist outside of its equine host and is endemic to certain parts of Asia, Africa, the Middle East and South America [8,9,20-25]. The genomic sequence of the *B. mallei* strain ATCC23344 has been published by...
TIGR [26] and is smaller (2 chromosomes of 3.5 and 2.3 Mbp) than that of \textit{B. pseudomallei} K96243. \textit{B. mallei} ATCC23344 was found to specify a large number of mobile DNA elements that have contributed to extensive deletions and rearrangements relative to the genome of \textit{B. pseudomallei} K96243. Despite these differences, the genes shared by the two isolates have an average identity of 99% at the nucleotide level [19,26]. The genomic sequence of several \textit{B. pseudomallei} and \textit{B. mallei} isolates are also publicly available through the NCBI genomic BLAST service (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi), which provides a wealth of resources to study these organisms.

\textit{B. pseudomallei} causes the human disease melioidosis, which is notoriously difficult to diagnose. Clinical manifestations vary greatly and may present as flu-like symptoms, benign pneumonitis, acute and chronic pneumonia, or fulminating septicemia. Infection occurs via inhalation of contaminated aerosol particles or through skin abrasions, and the risk of contracting the disease is proportional to the concentration of \textit{B. pseudomallei} in soil and water. In endemic areas, heavy rainfalls result in a shift from percutaneous inoculation to inhalation as the primary mode of infection, which leads to a more severe illness. Melioidosis commonly affects the lungs and is characterized by the spread of bacteria to various internal organs including the spleen and liver. Many patients become bacteremic and the mortality rate is high (19-51%) despite aggressive antimicrobial therapy [1-9]. \textit{B. pseudomallei} is refractory to most antibiotics and resistance mechanisms include efflux pumps and \(\beta\)-lactamases [27-36]. The recommended treatment entails the use of ceftazidime, carbapenems, TMP-SMZ, chloramphenicol and/or Augmentin for several weeks. Response to treatment is slow and eradication of \textit{B. pseudomallei} is difficult to achieve, resulting in recrudescence [1,37-39].

\textit{B. mallei} causes the zoonosis glanders, which primarily affects solipeds [8,9,20-25]. In humans, infection occurs by contact with infected animals via the cutaneous or respiratory route. The clinical manifestations of the disease include febrile pneumonia associated with necrosis of the tracheobronchial tree or putrulent skin lesions and the development of abscesses. Most patients become bacteremic and \textit{B. mallei} disseminates to the liver and spleen where it rapidly causes necrosis. Even with antibiotic treatment, the mortality rate for human glanders is 50% and the basis for this high mortality rate is not understood, though \textit{B. mallei} has been shown to be resistant to complement-mediated killing [40], macrophages [41] and antimicrobials [32,42].

One key aspect of pathogenesis by \textit{B. mallei} and \textit{B. pseudomallei} is their ability to invade and multiply within a variety of eukaryotic cells, where bacteria are shielded from the host humoral immune response and antibiotics. Once internalized, \textit{B. mallei} and \textit{B. pseudomallei} escape from endocytic vacuoles and enter the cytoplasm of infected cells where they multiply. The organisms subsequently spread to neighboring cells through a process involving the formation of actin tails and membrane protrusions that push bacteria from one cell to another. This intracellular lifestyle is crucial to virulence and has been a focus of research efforts aimed at understanding pathogenesis by \textit{B. mallei} and \textit{B. pseudomallei} [2,9,16-22,41,43-49]. Several gene products, such as BimA, type 3 secretion system effectors, and type 6 secretion proteins, have been shown to play key roles in this process. By contrast, the mechanisms used by these organisms to adhere to eukaryotic cells are poorly defined. Adherence is an essential step of pathogenesis by most infectious agents because it is necessary for colonizing a new host [50-52]. Moreover, \textit{B. pseudomallei} and \textit{B. mallei} are facultative intracellular pathogens that gain access to the interior of target cells. Though not always a prerequisite for this process, bacterial adherence is a widespread strategy that precedes and promotes invasion [50-52]. Thus far, only the \textit{B. pseudomallei} flagellum [53] and type 4 pilus [54] have been implicated in adherence and their exact roles remain to be elucidated. The present study reports the identification of \textit{B. pseudomallei} and \textit{B. mallei} gene products that mediate adherence to epithelial cells derived from the human respiratory tract, thus relevant to the aerosol route of infection by these organisms.

**Results**

**Identification of a gene shared by \textit{B. mallei} and \textit{B. pseudomallei} that encodes a potential autotransporter adhesin**

Analysis of the annotated genomic sequence of \textit{B. mallei} ATCC23344 identified the ORF locus tag number BMAA0649 as resembling members of the oligomeric coiled-coil adhesin (Oca) family of autotransporter proteins [55]. \textit{Yersinia enterocolitica} YadA [55-57] is the prototypical member of this group of adherence factors, which also includes \textit{Haemophilus influenzae} Hia [58-60] and \textit{Moraxella catarrhalis} Hag [61,62]. These Oca proteins share structural features including a C-terminal outer membrane (OM) anchor domain composed of 4 \(\beta\)-strands (also referred to as the transporter module), a surface-exposed passenger domain often containing repeated amino acid (aa) motifs, and a helical region of \(\sim40\) residues that connects the OM anchor to the surface-exposed passenger domain [55,63-65]. As illustrated in Fig 1A, BMAA0649 is predicted to possess these features. Further sequence analysis of the \textit{B. mallei} ATCC23344 gene product revealed that residues 208-362 (and 1010-1149) contain repeats with the consensus \textbf{xxxAVAIgxx[N/A]xAx} (open circles in Fig 1A), which
resemble motifs found in the N-terminus of *Y. enterocolitica* YadA (xxxSVAIGxxSxAx) [56,57] and *M. catarrhalis* Hag (GxxSIAGxx[A/S]xRx) [61]. In YadA, these AIG patterns have been shown to form a structure termed a β-roll and to specify adhesive properties. The passenger domain of BMAA0649 was also found to contain several serine-rich repeats beginning with residues SLST (colored squares in Fig 1A). Additionally, searches using the Pfam database indicated that aa 1456-1535 of BMAA0649 encode a YadA-like C-terminal domain (PF03895; expect value 3.8 e-11), which is present in most Oca molecules and is described as important for oligomerization. Taken together, these observations suggest structural and functional similarities between BMAA0649 and members of the Oca family of autotransporters. Hence, we designated this ORF of *B. mallei*
ATCC23344 boaA (Burkholderia Oca-like adhesin A). Table 1 lists characteristics of the boaA gene and its encoded product.

The published genome of B. pseudomallei K96243 was also found to specify a boaA gene product (BPSS0796, Fig 1B) that is 92.7% identical to that of B. mallei ATCC23344. Oligonucleotide primers were designed to amplify the entire boaA gene from the B. pseudomallei strain used in our laboratory, DD503, and sequence analysis of this amplicon predicted a gene product that is 94.4% and 90.6% identical to BoaA of B. mallei ATCC23344 and B. pseudomallei K96243, respectively. Database searches with the NCBI genomic BLAST service also identified boaA in several B. pseudomallei and B. mallei isolates. All nine B. mallei and 23 B. pseudomallei strains for which sequences are available through this service were found to have the gene. Characteristics of some of these ORFs are listed in Tables 1 and 2. Overall, the BoaA proteins are 82-94% identical and differ primarily in the number and/or arrangement of SLST repeats in their predicted passenger domains (data not shown). Based on these results, we conclude that BoaA is a well-conserved gene product shared by B. mallei and B. pseudomallei.

Identification of a B. pseudomallei-specific gene encoding a putative autotransporter adhesin that resembles BoaA

Further analysis of the annotated genomic sequence of B. pseudomallei K96243 identified the ORF locus tag number BPSL1705 as specifying a second Oca-like protein that is ~60% identical to BoaA. The last 776 aa of BPSL1705 and BoaA are 82.5% identical (Fig 1) and the very last 93 residues, which encompass the predicted C-terminal OM-anchoring domain and \( \alpha \)-helical region

Table 1 Characteristics of boaA and boaB genes and their encoded products

| Strain | Gene | Chromosome | Locus tag | GenBank accession # | ORF (nt) | Predicted protein (aa) | MW (Da) | Potential signal sequence cleavage site |
|--------|------|------------|-----------|---------------------|---------|------------------------|--------|----------------------------------------|
| B. mallei | boaA | 2 | BMAA0649 | YP_105401.1 | 4608 | 1535 | 140,689 | WA\(^{16}\)GV |
| NCTC10247 | boaA | 2 | BMA10247_A1776 | YP_001078959.1 | 5301 | 1766 | 162,744 | WA\(^{13}\)GV |
| B. pseudomallei | K96243 | boaA | 2 | BPSS0796 | YP_110805.1 | 4962 | 1653 | 151,565 | WA\(^{18}\)GV |
| DD503 | boaA | ND | - | EF423807 | 4680 | 1559 | 143,209 | WA\(^{18}\)AL |
| 1710b | boaA | 2 | BURPS1710b_A2381 | YP_337531.1 | 4881 | 1626 | 149,383 | WA\(^{10}\)AL |
| K96243 | boaB | 1 | BPSL1705 | YP_108306.1 | 4821 | 1606 | 148,811 | VA\(^{23}\)GT |
| DD503 | boaB | ND | - | EF423808 | 4965 | 1654 | 154,117 | VA\(^{21}\)GT |
| 1710b | boaB | 1 | BURPS1710b_2168 | YP_333563.1 | 4965 | 1654 | 154,059 | VA\(^{21}\)GT |

\(^a\)Sequence analyses were performed using Vector NTI (Invitrogen) and online tools available through the ExPASy Proteomics Server.

\(^b\)The putative signal sequence cleavage site was determined using the SignalP 3.0 server.

ND = not determined.

Table 2 Percent identity shared by boaA and boaB gene products

| BoaA (Bm ATCC23344) | BoaA (Bm NCTC10247) | BoaA (Bp K96243) | BoaA (Bp DD503) | BoaA (Bp 1710b) | BoaB (Bp K96243) | BoaB (Bp DD503) | BoaB (Bp 1710b) |
|----------------------|----------------------|------------------|-----------------|-----------------|------------------|-----------------|-----------------|
| 100                  | 86.9                 | 92.7             | 94.4            | 90.4            | 64               | 62              | 62.2            |
| BoaA (Bm ATCC23344) | BoaA (Bm NCTC10247) | BoaA (Bp K96243) | BoaA (Bp DD503) | BoaA (Bp 1710b) | BoaB (Bp K96243) | BoaB (Bp DD503) | BoaB (Bp 1710b) |

Bm = B. mallei.

Bp = B. pseudomallei.
of the molecules, were found to be particularly well-conserved (94.7% identity, Fig 1 and 2). The BPSL1705 ORF is predicted to encode a protein of 148-kDa which, as depicted in Fig 1C, possesses many of the structural features observed in BoaA including two sets of β-roll AIG motifs with the consensus xxG(S/A)(V/I)AIGxx(N/A)xAx and several SLST repeats. This high level of sequence and structural similarity between BPSL1705 and BoaA prompted us to designate this B. pseudomallei K96243 gene product BoaA.

The boaB gene was sequenced from B. pseudomallei DD503 and was predicted to encode a protein that is 96.7% identical to BoaB of B. pseudomallei K96243. Database searches using NCBI genomic BLAST revealed that the genomes of at least 10 more B. pseudomallei strains contain the gene. Overall, the BoaB proteins are highly-conserved (90-99% identity) and characteristics of the ORF from selected strains are shown in Tables 1 and 2 and Fig 2 for comparison purposes. Importantly, database searches also revealed that none of the B. mallei isolates available through the NCBI genomic BLAST database searches also revealed that none of the boaB genes were cloned from B. mallei, BoaA and BoaB proteins in E. coli

Because of their sequence and structural similarities to known bacterial adhesins, we hypothesized that BoaA and BoaB mediate adherence to human epithelial cells. To test this hypothesis, the B. mallei ATCC23344 boaA and B. pseudomallei DDS503 boaB genes were cloned into the E. coli strain EP1300. This organism does not normally adhere well to human epithelial cells [61,62,66] and therefore provides an appropriate heterologous genetic background for examining the adhesive properties of BoaA and BoaB. To verify gene expression, RNA was prepared from E. coli harboring the plasmids pCC1.3 (control), pSLboaA (specifies B. mallei ATCC23344 boaA) and pSLboaB (specifies B. pseudomallei DDS503 boaB), and analyzed by quantitative Reverse-Transcrip-

tase PCR (qRT-PCR). Fig 3A demonstrates that the boaA and boaB genes are expressed by recombinant bacteria and that the primers used in these experiments are specific for their corresponding genes. Sarkosyl-insoluble OM proteins were also extracted from E. coli cells and analyzed by western blot to ensure production of the Burkholderia proteins. Fig 3B shows that α-BoaA antibodies (Abs) react with a band of 130-kDa in the OM of E. coli expressing boaA (lane 3) whereas Abs against BoaB bind to a 140-kDa antigen in E. coli expressing boaB (lane 5). These molecular weights (MWs) are consistent with the predicted masses of the gene products (Table 1).

In addition to showing that BoaA and BoaB are associated with the OM by protein separation and western blot, we used immunofluorescent labeling of non-permeabilized E. coli cells to demonstrate their display on the bacterial surface. As depicted in Fig 3C, E. coli harboring pSLboaA and pSLboaB are labeled by the α-BoaA and α-BoaB Abs, respectively, while recombinant bacteria carrying the control plasmid pCC1.3 are not. Staining of nucleic acids with the fluorescent dye DAPI verified that comparable numbers of bacterial cells were examined (Fig 3C). Quantitative attachment assays revealed that E. coli expressing BoaA attach to HEP2 (laryngeal) and A549 (type II pneumocytes) epithelial cell lines at levels 18- and 68-fold greater than bacteria carrying pCC1.3, respectively (Fig 3D). In addition, BoaA expression was found to increase adherence to differentiated primary cultures of normal human bronchial epithelium (NHBE). Under the growth conditions used, NHBE cultures form a pseudostratified epithelium with tight junctions containing both ciliated and non-ciliated cells. This epithelium exhibits transepithelial resistance, mucus secretion, mucociliary activity, and an apical surface not submerged in tissue culture medium, thus representing an environment that is similar to the airway lumen in vivo [67-69]. Expression of the B. mallei ATCC23344 BoaA protein on the surface of E. coli also substantially increased adherence to monolayers of A549 and HEP2 cells and to NHBE cultures. Taken together, these data demonstrate that BoaA and BoaB are OM proteins mediating adherence to epithelial cells of the human respiratory tract.
**B. pseudomallei** and **B. mallei** are facultative intracellular organisms that can invade, survive and replicate in a variety of eukaryotic cells. Moreover, autotransporter adhesins often specify additional biological functions such as invasion [70], biofilm formation [71], survival within host cells [72] and intracellular motility [16]. For these reasons, we measured the ability of *E. coli* expressing *BoaA* and *BoaB* to invade epithelial cells as well as their ability to survive within murine macrophages. We also measured the ability of these recombinant strains to form biofilms on the plastic support of tissue culture plates using a crystal violet-based assay. The results of these experiments indicated that neither *BoaA* nor *BoaB* substantially increase invasion of epithelial cells, phagocytosis of recombinant bacteria by J774A.1 murine macrophages, survival inside these immune cells, or biofilm formation (data not shown).

**Construction and characterization of *Burkholderia* mutant strains**

To study the functional properties of the *boa* gene products in the native *Burkholderia* background, we constructed isogenic *boaA* mutants of *B. pseudomallei* DD503 and *B. mallei* ATCC23344 as well as an isogenic *boaB* mutant of *B. pseudomallei* DD503. A double mutant strain was also engineered in which inactivated versions of both *boaA* and *boaB* were introduced in the genome of *B. pseudomallei* DD503. Whole cell lysates and sarkosyl-insoluble OM proteins were prepared from these strains and analyzed by western blot to verify lack of expression.

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**Figure 3** Analysis of recombinant *E. coli* strains. Panel A: Total RNA was isolated from *E. coli* strains, reverse-transcribed to cDNA, and the relative levels of *boaA* or *boaB* transcript were determined by qRT-PCR. Each bar represents 4 different samples collected on 2 separate occasions. The Y-axis corresponds to the levels of *boaA* or *boaB* transcript normalized to *recA* and the error bars correspond to the standard error. Negative controls in which the reverse transcriptase enzyme was not added to reaction mixtures were included in all experiments (data not shown). Panel B: Proteins present in Sarkosyl-insoluble OM protein preparations were resolved by SDS-PAGE, transferred to PVDF membranes and analyzed by western blot with antibodies against *BoaA* (lanes 1-3) or *BoaB* (lanes 4-6). Lanes 1 & 4, *E. coli* (pCC1.3); lanes 2 & 5, *E. coli* (pSLboaB); lanes 3 & 6, *E. coli* (pSLboaA). MW markers are shown to the left in kilodaltons. Panel C: Non-permeabilized *E. coli* strains were fixed onto glass slides and fluorescently-labeled with DAPI (blue) and with α-BoaA or α-BoaB antibodies (red). Bacteria were visualized by microscopy using a Zeiss LSM 510 Meta confocal system. Representative microscopic fields are shown. Panel D: *E. coli* strains were incubated with A549 and HEp2 cells for 3-hr and with NHBE cultures for 6-hr. epithelial cells were washed to remove unbound bacteria, lysed, diluted, and spread onto agar plates to enumerate bound bacteria. The results are expressed as the mean percentage (± standard error) of inoculated bacteria adhering to epithelial cells. Asterisks indicate that the increased adherence of the indicated strains, compared to *E. coli* carrying the control plasmid pCC1.3, is statistically significant (P < 0.05). These attachment assays were performed in duplicate on at least 3 separate occasions.
of BoaA and BoaB expression in the mutants. The α-BoaA and α-BoaB Abs, however, did not react with *Burkholderia* protein preparations (data not shown). In order to determine whether the genes are expressed, total RNA was isolated from *B. pseudomallei* DD503 and *B. mallei* ATCC23344 and the relative transcript levels of boaA and boaB were assessed by qRT-PCR. Fig 4 shows that boaA and boaB are expressed by *B. pseudomallei* while *B. mallei* only expresses boaA, which is in agreement with database searches revealing that *B. mallei* isolates do not contain a boaB gene. The qRT-PCR data also demonstrate that the genes are expressed at very low levels compared to *Burkholderia recA*, which was used to normalize boaA and boaB transcript levels. These results are consistent with our inability to visualize the proteins by western blot. Other methods such as immunoprecipitation and immunofluorescence labeling also proved unsuccessful at detecting production of BoaA and BoaB by *Burkholderia* strains.

Quantitative attachment assays with recombinant bacteria indicated that BoaA or BoaB expression significantly increases the adherence of *E. coli* to monolayers of A549 and HEp2 cells and to NHBE cultures (Fig 3D). We therefore compared the ability of *Burkholderia* parent and boa mutant strains to attach to these respiratory cells. As shown in Fig 5A and 5D, inactivation of the boaA gene in *B. mallei* ATCC23344 and *B. pseudomallei* DD503 decreases adherence to A549 cells by 60 and 53%, respectively. The boaA mutation also caused a 50% reduction in the binding of *B. pseudomallei* to HEp2 monolayers (Fig 5B), and reduced adherence of *B. mallei* to these laryngeal cells by 67% (Fig 5E). Moreover, both boaA mutant strains displayed significant impairment in their abilities to attach to NHBE cultures (Fig 5C and 5F). The boaB mutation in *B. pseudomallei* DD503 decreased attachment to A549 and HEp2 cells by ~50% (Fig 5A and 5B, respectively) and caused a 62% reduction in adherence to NHBE cultures (Fig 5C). As expected, the double mutant strain DD503.boaA.boaB exhibited significantly lower attachment to epithelial cells compared to the parent strain DD503 (Fig 5A, B, and 5C). The adherence levels of the double mutant, however, did not differ significantly from that of the single mutants in any of the cell types tested. One possible explanation for this apparent lack of synergistic effect is that other adhesins expressed by the double mutant strain DD503.boaA.boaB provide a high background level of adherence. Taken together, these results demonstrate that the boaA and boaB gene products contribute to the adherence of *B. mallei* and *B. pseudomallei* to epithelial cells of the human respiratory tract.

As previously stated, autotransporter adhesins often specify additional biological functions including survival within host cells [72]. In addition, *B. pseudomallei* and *B. mallei* are facultative intracellular pathogens that are particularly proficient at replicating inside professional phagocytic cells. For these reasons, we measured the ability of our panel of *Burkholderia* mutant and parent strains to replicate within J774A.1 murine macrophages. In *B. pseudomallei* DD503, inactivation of the boa genes had no effect on phagocytosis of the organism (Fig 6A). Once inside macrophages, the boaA (DD503.boaA) and boaB (DD503.boaB) single mutants replicated at rates equivalent to that of the progenitor strain DD503 (Fig 6B). However, when both boaA and boaB genes were disrupted (DD503.boaA.boaB), intracellular growth was diminished by 60% (Fig 6B). To verify that this reduced intracellular fitness was not due to a global growth defect, we measured the growth of strains DD503 and DD503.boaA.boaB in broth as well as in tissue culture medium. We found that both strains grew at equivalent rates under both conditions (data not shown). Interestingly, the double mutant did not exhibit a growth defect in epithelial cells (data not shown). These results suggest a role for the BoaA and BoaB proteins in *B. pseudomallei*’s ability to grow inside professional phagocytes.
defect in uptake or intracellular growth was measured for the *B. mallei* ATCC23344 boaA mutant strain (data not shown). It should also be noted that none of the boa mutants showed decreased biofilm formation on the plastic support of tissue culture plates nor defects in resistance to the bactericidal activity of normal human serum (data not shown), both biological functions that are also commonly associated with Oca autotransporter adhesins [56,63,73-75].

**Discussion**

Autotransporters are involved in various biological traits of Gram-negative bacteria including invasion [70], serum resistance [56,73], phospholipolysis [76,77], cytotoxicity [78], adherence [61,79], biofilm formation [71,80], survival within eukaryotic cells [72] and intracellular motility [16]. These proteins share an N-terminal extracellular passenger domain that specifies the biological activity of the autotransporter and a C-terminus containing several β-strands, which tether the molecule to the OM. Based on the structure of this membrane-anchoring domain, autotransporters can be classified as conventional (contain 12 β-strands) or trimeric (contain 4 β-strands) [65]. One of the best characterized trimeric autotransporters is the *Y. enterocolitica* adhesin YadA. This protein, along with structurally-related adherence...
Uptake and growth of *B. pseudomallei* strains in J774A.1 murine macrophages. J774A.1 cells (duplicate wells in each of two 24-well tissue culture plates) were infected with *B. pseudomallei* strains at an MOI of 10 and incubated for 1-hr to allow phagocytosis of the organisms. Following incubation, the monolayers were incubated for 2-hr in medium containing gentamicin to kill extracellular bacteria. After gentamicin treatment (i.e. 3-hr post infection), the wells of one plate were washed, lysed, serially diluted, and spread onto agar plates to determine the number of bacteria phagocytosed by macrophages. The results of this first part of the experiments (i.e. bacterial uptake) are shown in panel A and are expressed as the percentage of bacteria (± standard error) used to infect macrophages that were phagocytosed. The wells of the other tissue culture plate inoculated with *B. pseudomallei* strains were washed once, fresh medium without antibiotics was added to wells, and the plate was incubated for an additional 5-hr. Following this incubation (i.e. 8-hr post-infection), the wells were processed as described above in order to enumerate bacterial numbers. The results of this second part of the experiments (i.e. intracellular growth of phagocytosed bacteria) are shown in panel B and are expressed as a growth/uptake ratio (± standard error) obtained by dividing the number of bacteria/well at 8-hr post infection by the number of bacteria/well at the 3-hr post infection time point. These experiments were repeated on at least 3 separate occasions. The asterisk indicates that the difference between the intracellular growth of the double mutant strain DD503 boaA boaB and that of its parent isolate DD503 is statistically significant (*P* < 0.05). Panel C shows the total number of bacteria in the inoculum (grey bars), the number of phagocytosed bacteria (open bars, 3-hr post infection) and the total number of bacteria/well at the end point of the experiment (black bars, 8-hr post infection).
proteins such as *M. catarrhalis* Hag and *H. influenzae* Hia, are often referred to as oligomeric coiled-coil adhesins (Oca) [55].

Tiyawisutsri and colleagues previously reported that the published genomic sequences of *B. pseudomallei* K96243 and *B. mallei* ATCC23344 contain several ORFs encoding putative trimeric autotransporters [81]. Of these, only BimA (i.e. *B. pseudomallei* and *B. mallei*) locus tag numbers BPSS1492 and BMAA0749, respectively) has been functionally characterized and shown to be required for actin-based motility of the organisms inside eukaryotic cells [16,17]. In the present study, we identified the boaA ORF based on similarities to the Oca proteins *Y. enterocolitica* YadA and *M. catarrhalis* Hag. Specifically, we searched the genome of *B. mallei* ATCC23344 for gene products specifying N-terminal AIG β-roll motifs, a transporter module containing 4 β-strands, and a YadA-like C-terminal domain (PF03895). We demonstrated that when expressed by *E. coli*, boaA increases adherence to the human epithelial cell lines HEp2 (laryngeal cells) and A549 (type II pneumocytes) grown as monolayers in submerged cultures. Though these cell types are relevant to the aerosol route of infection by *B. mallei* and *B. pseudomallei*, they lack important features of the airway mucosa such as cilia and mucociliary activity. The ciliated cells of the respiratory tract and other mucosal membranes keep secretions moving and contribute to preventing colonization by pathogens. For these reasons, we also measured the adherence of *E. coli* expressing BoaA to cultures of normal human bronchial epithelium (NHBE) grown in an air-liquid interface system. These cultures mimic the structure and function of the airway mucosa more accurately as they are fully differentiated, form a pseudostratified epithelium with tight junctions, contain ciliated and mucus-producing goblet cells, and exhibit mucociliary activity [67-69]. Quantitative attachment assays utilizing this culture system revealed that BoaA expression increases adherence to NHBE cultures (Fig 3D).

In addition to showing that BoaA specifies adhesive properties when expressed in the heterologous genetic background of *E. coli*, we determined that disruption of the boaA gene in the genome of *B. mallei* ATCC23344 reduces adherence of the organism to monolayers of HEp2 and A549 cells and to NHBE cultures, therefore substantiating the function of BoaA as an adhesin. Database searches using the NCBI genomic BLAST service identified boaA in several *B. pseudomallei* and *B. mallei* isolates and we demonstrated that inactivation ofboaA in the *B. pseudomallei* strain DD503 also decreases attachment to HEp2 laryngeal cells, A549 pneumocytes, and NHBE cultures. Together, our data indicate that BoaA is an adhesin common to *B. mallei* and *B. pseudomallei* and mediates adherence to host cells relevant to pathogenesis by the organisms. These findings are consistent with the recent inclusion of BoaA (i.e. *B. mallei* ATCC23344 and *B. pseudomallei* K96243 locus tag numbers BMAA0649 and BPSS0796, respectively) in the virulome of *B. mallei* and *B. pseudomallei*, which consists of a set of 650 putative virulence genes that are shared by *B. pseudomallei* and *B. mallei* but are not present in five closely-related non-pathogenic *Burkholderia* species [82].

Comparative genomic analyses revealed that several *B. pseudomallei* isolates possess a second Oca-like gene product highly similar to BoaA, which we termed BoaB. The C-terminus of BoaB is strikingly similar to that of BoaA (Fig 2) and the predicted passenger domains of the molecules contain numerous matching serine-rich SLST motifs (Fig 1). The proteins are also functionally related as they mediate adherence to the same types of host cells (Fig 3D and 5). Therefore, it is tempting to speculate that boaA and boaB are the result of gene duplication. This hypothesis would be consistent with the genomic organization of the genes. In *B. pseudomallei* strains K96243, 1710b, 1655, 576 and MSHR346, the boaB gene is located on chromosome 1 while boaA is on chromosome 2. Moreover, the boaB gene in all these isolates is preceded by two ORFs specifying an inverter and a transposase. These genes may be the remnants of mobile genetic elements possibly involved in gene duplication. Database searches also revealed that *B. mallei* isolates do not possess a boaB gene, which was likely lost during evolution of the organism into a host-adapted pathogen. Interestingly, the closely-related bacterium *Burkholderia thailandensis* has been reported by others to bind poorly to epithelial cells [83]. This organism exhibits high genomic similarities to *B. pseudomallei* and *B. mallei* and, like *B. pseudomallei*, is a natural inhabitant of the tropical soil environment. However, *B. thailandensis* is not considered pathogenic to humans or higher animals [84-87]. This difference in virulence can be attributed to the fact that *B. thailandensis* does not produce a capsule [88] and lacks the 650 genes comprising the aforementioned virulome of *B. mallei* and *B. pseudomallei*. Analysis of the published genome of the *B. thailandensis* strain E264 [89] indicated that it contains neither the boaA nor the boaB gene.

*B. pseudomallei* DD503 and *B. mallei* ATCC23344 do not produce detectable amounts of the BoaA and BoaB proteins under the conditions tested. These results are consistent with qRT-PCR experiments demonstrating that the organisms express very low levels of the boa genes relative to the *Burkholderia recA* control (Fig 4). Similar observations were made by Druar and colleagues while studying expression of the *Burkholderia* Type 3 Secretion System-3 (T3SS-3) proteins BipB and BipD [90]. These proteins were not detected in lysates of
B. pseudomallei or B. mallei grown under different conditions, even though the antibodies used in their western blot experiments recognized recombinant forms of BipB and BipD. The authors concluded that these two T3SS-3 molecules must be expressed in detectable amounts only under very specific *in vitro* conditions [90]. Using a *gfp* reporter strain, Burtnick *et al.* recently showed that the B. mallei Type 6 Secretion System-1 (T6SS-1) gene tssE is not expressed at detectable levels when bacteria are grown in LSLB or tissue culture medium, but is expressed upon phagocytosis of the organisms by murine macrophages [49]. The protein preparations tested in our studies were obtained from bacteria cultured on LSLB agar plates at 37°C, conditions which may not be optimal for expression of the BoaA and BoaB proteins. Additionally, Chantratita and colleagues reported that growth of B. pseudomallei under various conditions triggers a complex adaptive process altering the expression of surface molecules [91]. This process, termed phenotypic plasticity, was correlated with changes in the morphology of B. pseudomallei colonies grown on agar plates and appears to modulate the environmental fitness, as well as virulence, of the organism. Given their surface location and likely role in virulence (*i.e.* adherence to host cells), it is possible that BoaA and BoaB are subject to phenotypic plasticity and are expressed in detectable amounts only under very specific *in vitro* conditions. In concordance, the reduced adherence phenotype of the boaA and boaB mutant strains suggests increased level of expression of the genes when *Burkholderia* is incubated with epithelial cells. However, efforts to detect protein expression under these conditions (*i.e.* immunofluorescence, immunoprecipitation) have been unsuccessful. Of further note, studies have shown that sera from horses infected with B. mallei and sera from melioidosis patients contain antibodies reacting with BoaA (*i.e.* B. mallei ATCC23344 locus tag number BMAAA0649) [81] and with BoaB (*i.e.* B. pseudomallei K96243 locus tag number BPLS1705) [92], respectively, which indicates expression of the autotransporters *in vivo*. Determining the conditions and mechanisms that modulate expression of the Boa adhesins, and their influence on the binding of B. pseudomallei and B. mallei to host surfaces, represent key areas for future study.

Disruption of boaA and boaB in the B. pseudomallei double mutant strain DD503.boaA.boaB was found to have a significant effect on the growth of the organism within murine macrophages (Fig 6B). At present, it is not clear whether BoaA and BoaB play a direct role in intracellular replication. It is possible that the absence of both Boa proteins in the OM of DD503.boaA.boaB affects the proper surface display of another molecule involved in this phenotypic trait. One candidate is LPS, as this molecule was previously shown to play an important role in the ability of B. pseudomallei to grow inside host cells [93,94]. B. pseudomallei produces multiple T3SS and T6SS that are involved in the intracellular lifestyle of the organism. These specialized secretion apparatuses are used to inject bacterial effector proteins inside host cells where they exert cytopathic effects or manipulate signaling pathways. One important step in this process is the proper docking of bacteria to the host cell to deliver the effectors. Given their roles in adherence, it is possible that the lack of expression of the boaA and boaB gene products interferes with the delivery of T3SS and/or T6SS cell-altering effectors, which in turn reduces the intracellular fitness of the double mutant strain DD503.boaA.boaB.

The *Yersinia pestis* OM adhesin Ail was recently shown to affect delivery of Yop effector proteins to HEP2 cells and macrophages in such a manner [95]. Alternatively, the reduced intracellular growth of the double boaA boaB mutant may be due to a greater sensitivity to immune effectors produced by the macrophages. The molecular basis for this phenotype is currently being investigated.

**Conclusion**

The present study reports the identification of B. pseudomallei and B. mallei gene products mediating adherence to epithelial cells. Because of their classification as select agents, there is currently a shortage of tools for genetic studies in B. pseudomallei and B. mallei (*i.e.* paucity of acceptable antibiotic markers, lack of low copy plasmids suitable for expressing surface proteins), which precluded us from complementing mutants. Our ability to express BoaA and BoaB in *E. coli*, however, conclusively demonstrates that the proteins directly mediate binding to epithelial cells. These results, along with our analyses of the mutant strains, clearly establish that these molecules participate in adherence by *B. pseudomallei* and *B. mallei*. Adherence is an essential step in pathogenesis by most infectious agents because it is necessary for colonization and precedes invasion of host cells by intracellular pathogens. Thus, continued investigation of BoaA and BoaB will yield important information regarding the biology and virulence of these organisms.

**Methods**

**Strains, plasmids, tissue culture cell lines and growth conditions**

The strains and plasmids used in this study are described in Table 3. *B. pseudomallei* and *B. mallei* were routinely cultured at 37°C using Low Salt Luria Bertani (LSLB) agar (Teknova) supplemented with polymyxin B [PmB] (100 μg/ml for *B. pseudomallei*; 7.5 μg/ml for *B. mallei*), zeocin (100 μg/ml for *B. pseudomallei*; 7.5 μg/ml for *B. mallei*), kanamycin [Kan] (50 μg/ml for *B.
pseudomallei; 5 μg/ml for B. mallei), streptomycin [Sm] (used only for B. pseudomallei, 1000 μg/ml) and glycerol (used only for B. mallei, 5%), where indicated. Plate-grown bacteria (20-hr growth for B. pseudomallei; 40-hr growth for B. mallei) were used for extraction of Sarkosyl-insoluble outer membrane proteins, preparation of whole cell lysates, RNA isolation, as well as for adherence, invasion, bactericidal, biofilm, and macrophage assays.

E. coli was cultured using LSLB containing 15 μg/ml chloramphenicol, 50 μg/ml Kan or 50 μg/ml zeocin, where indicated. For preparation of plasmid DNA, extraction of Sarkosyl-insoluble outer membrane proteins, RNA isolation, immunofluorescence labeling, as well as for adherence, invasion and macrophage assays, recombinant E. coli strains were grown in LSLB supplemented with the EPICENTRE® Biotechnologies Copy-Control™ Induction Solution as previously reported [96].

The epithelial cell lines HEp2 (human laryngeal epithelium; ATCC CCL-23) and A549 (type II alveolar lung epithelium; ATCC CCL85) were cultured as outlined by others [97] and the murine macrophage cell line J774A.1 (ATCC TIB-67) was grown in DMEM medium (Mediatech, Inc) supplemented with 10% fetal bovine serum (Invitrogen™) at 37°C and in the presence of 7.5% CO₂. Normal human bronchial epithelium (LONZA) were expanded, cryopreserved and cultured in an air-liquid interface system as previously described [67-69]. Normal human bronchial epithelium (NHBE) were grown on Transwell permeable inserts (Corning) and their apical surfaces were exposed to air for a minimum of 3 weeks prior to use in biological assays to ensure proper cellular differentiation and the development of functional cilia.

Recombinant DNA methodology
Standard molecular biology techniques were performed as described elsewhere [98]. Genomic DNA was isolated using the Invitrogen™ Easy-DNA™ kit. Plasmid DNA was obtained with the QIAprep Spin Miniprep Kit (Qiangen). The Failsafe™ PCR System (EPICENTRE® Biotechnologies) was used to amplify the 5.5-kb boaA gene of B.

### Table 3 Strains and plasmids

| Strain   | Description                                                                 | Reference |
|----------|-----------------------------------------------------------------------------|-----------|
| **B. pseudomallei**                                  |                                                      |           |
| DD503    | Parental strain; polymyxin B<sup>R</sup> zeocin<sup>S</sup> kanamycin<sup>S</sup> streptomycin<sup>R</sup> | [107]     |
| DD503.boaA | Isogenic boaA mutant strain of DD503, polymyxin B<sup>R</sup> zeocin<sup>S</sup> kanamycin<sup>S</sup> streptomycin<sup>R</sup> | This study |
| DD503.boaB | Isogenic boaA boaB mutant strain of DD503, polymyxin B<sup>R</sup> zeocin<sup>S</sup> kanamycin<sup>S</sup> streptomycin<sup>R</sup> | This study |
| DD503.boaA. boaB | Isogenic boaA boaB double mutant strain of DD503, polymyxin B<sup>R</sup> zeocin<sup>S</sup> kanamycin<sup>S</sup> streptomycin<sup>R</sup> | This study |

| **B. mallei**                                       |                                                      |           |
| ATCC23344 | Wild-type strain; polymyxin B<sup>R</sup> zeocin<sup>S</sup> kanamycin<sup>S</sup> | [26]      |
| ATCC23344.boaA | Isogenic boaA mutant strain of ATCC23344, polymyxin B<sup>R</sup> zeocin<sup>S</sup> kanamycin<sup>S</sup> | This study |

| E. coli   | Cloning strain                                                                 | EPICENTRE* Biotechnologies                      |           |
| S17      | Strain used for conjugal transfer of suicide plasmids from E. coli to B. pseudomallei or B. mallei |           | [108]     |

| **Plasmids**                                       |                                                      |           |
| pCC1<sup>™</sup> | Cloning vector; chloramphenicol resistant (Cm<sup>R</sup>) | EPICENTRE* Biotechnologies                      |           |
| pKAS46    | Mobilizable suicide plasmid; kanamycin<sup>R</sup> and ampicillin<sup>R</sup> |           | [109]     |
| pCC1.3   | pCC1-based plasmid control, does not confer adherence; Cm<sup>R</sup> |           | [102]     |
| pSLboA   | pCC1 containing the B. mallei ATCC23344 boaA gene; Cm<sup>R</sup> |           | This study |
| pSLboAZEO | pSLboA in which a zeocin<sup>R</sup> marker was introduced near the middle of the boaA gene; Cm<sup>R</sup> and zeocin<sup>R</sup> |           | This study |
| pKASboA-ZEO | pKAS46 containing the insert from pSLboAZEO; zeocin<sup>R</sup> , ampicillin<sup>R</sup> and kanamycin<sup>R</sup> |           | This study |
| pKASboAB-ZEO | pCC1 containing the B. pseudomallei DD503 boaB gene; Cm<sup>R</sup> |           | This study |
| pSLboB-ZEO | pCC1 containing the B. pseudomallei DD503 boaB gene; Cm<sup>R</sup> and zeocin<sup>R</sup> |           | This study |
| pKASboA-ZEO | pKAS46 containing the insert from pSLboAZEO; zeocin<sup>R</sup> , ampicillin<sup>R</sup> and kanamycin<sup>R</sup> |           | This study |
| pKASboB-S Amp<sup>R</sup> | pKASboB<sup>S</sup> containing a 0.8-kb insert which corresponds to a region located within the 5' end of the B. pseudomallei DD503 boaB ORF; ampicillin<sup>R</sup> and kanamycin<sup>R</sup> |           | This study |
| pKASboB-S Amp<sup>R</sup> | pKASboB<sup>S</sup> in which the ampicillin<sup>R</sup> marker was removed; ampicillin<sup>R</sup> and kanamycin<sup>R</sup> |           | This study |
| pEM7-ZEO | Source of the zeocin<sup>R</sup> marker; ampicillin<sup>R</sup> and zeocin<sup>R</sup> | Invitrogen™ |           |
**Construction of boaA isogenic mutant strains of*B. mallei* and *B. pseudomallei***

A 0.45-kb zeocin^R_2 cassette was introduced into a unique *NheI* site located near the middle of the *boaA* ORF in pSLboaA. The resulting construct, designated pSLboaAZEO, was digested with *BamHI* and a 6.2-kb fragment corresponding to the *boaA* ORF interrupted by the zeocin^R_2 marker was excised from an agarose gel, purified with the High Pure PCR Product Purification Kit (Roche Applied Science), and treated with the EPICENTRE® Biotechnologies End-It™ DNA End Repair Kit. This blunt DNA fragment was then subcloned into the *EcoRV* site of the suicide vector pKAS46. The resulting plasmid, pKASboaAZEO, was introduced into the *E. coli* strain S17 by electroporation and subsequently transferred into*B. mallei* ATCC23344 or*B. pseudomallei* DD503 by conjugation as reported by others [99].

Upon conjugation, *B. pseudomallei* colonies were first selected for resistance to PmB (to prevent growth of *E. coli* S17) and zeocin (to select strains containing the disrupted copy of *boaA* in their genome). These putative mutants were then tested for their sensitivity to kanamycin and resistance to streptomycin, which identified strains that did not contain the suicide vector pKAS46 integrated in their genome. Lastly, these PmB^R_2 zeocin^R_2 Kan^R_2 Sm^R_2 conjugants were screened by PCR using Platinum® *Pfx* DNA Polymerase (Invitrogen™) with the primers P7 (5'-TAT CGC AAG GTT TGG AAC AAG GGC-3') and P10 (5'-ACG CCG AAT ACC CTT GAT AGC TG-3') were also used to further confirm gene replacement in the*B. pseudomallei* mutant strain. These primers amplified DNA fragments of 5-kb in the parent strain DD503 and of 5.5-kb in the isogenic *boaA* mutant. After the conjugative transfer of plasmid pKASboaAZEO into the*B. mallei* strain ATCC23344, colonies shown to be PmB^R_2, zeocin^R_2 and Kan^R_2 were screened by PCR with P7 and P8 as described above to identify the mutant strain ATCC23344 boaA. Of note, the *boaA* genes of both isogenic mutant strains DD503.boaA and ATCC23344. boaA were amplified and sequenced in their entirety to verify proper allelic exchange and successful disruption of *boaA*.

**Construction of a boaB*B. pseudomallei* isogenic mutant strain**

The plasmid pSLboaB was digested with *NheI* to remove a 162-bp fragment internal to the *boaB* ORF, treated with the End-It™ DNA End Repair Kit and ligated with the 0.45-kb zeocin^R_2 marker to yield the construct pSLboaBZEO. This plasmid was digested with *BamHI* and a 6.2-kb fragment, which corresponds to the *boaB* ORF disrupted with the zeocin^R_2 cassette, was purified from agarose gel slices, subcloned into the suicide plasmid pKAS46 and introduced into*B. pseudomallei* DD503 by conjugation as described above. Conjugants shown to be PmB^R_2 zeocin^R_2 Kan^R_2 Sm^R_2 were screened by PCR using Platinum® *Pfx* DNA Polymerase (Invitrogen™) with primers P11 (5'-AGG TGG CGA CTC AAT AGA ACC GT-3') and P12 (5'-GTT CGT GGT GTT GGC TAC GGC AAT-3') to identify the mutant strain DD503.boaB. These primers amplified a PCR product of 1.7-kb in*B. pseudomallei* DD503 and of 2.0-kb in the mutant. The primers P13 (5'-AGG TGG CGA CTC AAA TAG AAC CGT-3') and P10 were also used to further confirm gene replacement in the*B. pseudomallei* mutant strain. These primers generated amplicons of 5.2-kb and 5.5-kb in strains DD503 and DD503.boaB, respectively. Additionally, the *boaB* gene of DD503.boaB was amplified and both strands of the PCR product were sequenced to verify allelic exchange.

**Construction of a*B. pseudomallei* boaA boaB double mutant strain**

A 0.8-kb PCR product, which corresponds to a region located within the 5'-end of the*B. pseudomallei* DD503 *boaB* ORF, was amplified with Platinum® *Pfx* DNA Polymerase (Invitrogen™) using primers P14 (5'-CTC GGG CTC AAT AAC ATG GC-3') and P15 (5'-CCG AAT TCC GGT TCG TGT TGG CT-3'; EcoRI site underlined). This amplicon was digested with *EcoRI* and directionally cloned into the *EcoRV* and *EcoRI* sites of the...
suicide vector pKAS46, yielding the plasmid pKASboaB5'. This construct was digested with ApaLI to remove a 0.8-kb fragment corresponding to the ampicillin-resistance marker of pKAS46 and the resulting plasmid, pKASboaB5'AmpS was introduced into the B. pseudomallei mutant strain DD503.boaA by conjugation as described above. Conjugs shown to be PmB2 zeocinR KanR SmS were screened by PCR using the MasterAmp Extra-Long PCR kit (EPICENTRE) with primers P13 and P10 to identify the mutant strain DD503.boaA.boaB. These primers amplified PCR products of 5.2-kb in B. pseudomallei DD503 as well as in the single mutant DD503.boaA, and of 11.0-kb in the double mutant strain DD503.boaA.boaB. These results indicated that the boa gene in DD503.boaA.boaB had been disrupted by integration of the entire pKASboaB5'AmpS plasmid into the genome of B. pseudomallei.

Quantitative reverse-transcriptase PCR (qRT-PCR)

Total RNA was extracted from 10^6 bacteria with the RNeasy Kit (Qiagen). One μg of total RNA was treated with RQ1 RNase-Free DNase (Promega) and reverse transcribed with Improm II Reverse transcriptase (Promega) using random hexamers (Invitrogen) under the manufacturer’s recommended conditions. PCR quantification of specific cDNA levels was performed using a LightCycler® (Roche Applied Science) rapid fluorescence detection of specific cDNA levels was performed using a manufacturer (megag) using random hexamers (Invitrogen) with RQ1 RNAse-Free DNase (Promega) and reverse transcriptase (Promega)™

Bioinformatic Analyses

Sequence analyses were performed using Vector NTI (Invitrogen) and the various online tools available through the ExPaSy Proteomics Server (http://au.expasy.org/). Signal sequence cleavage sites were determined using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/). The B. mallei ATCC23344 boaA gene product (locus tag BMAA0649) was identified by searching the genome of the organism for the presence of a YadA-like C-terminal domain (Pfam database number PF03895) through the NCBI genomic BLAST service utilizing the tblastn and blastp programs (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). The other boaA and boaB gene products described in this study were identified using the predicted aa sequence of the B. mallei ATCC23344 BoaA protein to search the genomes of the Boa proteins (e.g. helical regions, β-strands) were identified using the PSIPRED Protein Structure Prediction Server (http://bioinf.cs.ucl.ac.uk/psipred/).

Epithelial cell adherence assays

Quantitative attachment assays were performed as previously described by our laboratory [61,67]. Monolayers of A549 and HEP2 cells and cultures of NHBE were infected with B. mallei, B. pseudomallei or recombinant E. coli strains at a MOI of 100. Duplicate assays were repeated on at least 3 occasions for each strain, and adherence is expressed as the percentage (± standard error) of bacteria attached to epithelial cells relative to the inoculum. Statistical analyses were performed using the Mann-Whitney test (GraphPad Prism.
software) and $P$ values < 0.05 are reported as statistically significant.

**Biofilm and bactericidal assays**

These experiments were performed as previously described [96,101,102]. We used 50% and 25% normal human serum in bactericidal assays with *B. pseudomallei* and *B. mallei*, respectively.

**Macrophage survival assays**

Plate-grown bacteria were suspended in 5-ml of sterile PBS supplemented with 0.15% gelatin (PBSG) to a density of $10^9$ CFU/ml. These suspensions were used to infect two identical sets of duplicate monolayers of J774A.1 cells (10$^5$ cells/well; 24-well tissue culture plate) at an MOI of 10. The inoculated tissue culture plates were centrifuged (5-min, 165 × g) and incubated for 1-hr at 37°C, time after which the medium covering the monolayers was replaced with fresh tissue culture medium containing 50 μg/ml gentamicin. After a 2-hr incubation (i.e. 3-hr post infection), the wells of one tissue culture plate were washed, J774A.1 cells were lysed with a solution containing Saponin, and serial dilutions of the well contents were spread onto agar plates to determine the number of bacteria phagocytosed by the macrophages. The wells of the other tissue culture plate were washed once, fresh medium without antibiotics was added, and the plate was incubated for an additional 5-hr. Following this incubation (i.e. 8-hr post-infection), the wells were processed as described above in order to enumerate bacteria. These experiments were repeated on at least 3 separate occasions. Statistical analyses were performed using the Mann-Whitney test (GraphPad Prism software) and $P$ values < 0.05 are reported as statistically significant.

**Epithelial cell invasion and survival assays**

These experiments were performed as described above for macrophage survival assays with some modifications. Specifically, epithelial cells were infected with an MOI of 100. The inoculated tissue culture plates were centrifuged and incubated for 3-hr at 37°C, time after which the medium covering the monolayers was replaced with fresh tissue culture medium containing 50 μg/ml gentamicin. After a 2-hr incubation (i.e. 5-hr post infection), the wells of one tissue culture plate were washed and processed to enumerate intracellular bacteria as described above. The wells of the other tissue culture plate were washed once, fresh medium without antibiotics was added to wells, and the plate was incubated for an additional 3-hr. Following this incubation (i.e. 8-hr post-infection), the wells were processed as described above. These experiments were repeated on at least 3 separate occasions. Statistical analyses were performed using the Mann-Whitney test (GraphPad Prism software) and $P$ values < 0.05 are reported as statistically significant.

**Protein preparations, western blot, and antibody production**

Sarkosyl-insoluble OM proteins were obtained as previously described by Carlone et al [103]. The methods used to prepare whole cell lysates and perform western blot experiments are described elsewhere [61,62,67,104,105]. To obtain antibodies directed against BoaA, the peptide PEPA (NYLGGFLFGPGQTSMANWGDSN) was synthesized and conjugated to maleimide-activated keyhole limpet hemocyanin (mckLH, Thermo Scientific) under the manufacturer’s recommended conditions. The sequence of PEPA corresponds to residues 78-100 of *B. pseudomallei* DD503 BoaA and encompasses aa 79-101 of *B. mallei* ATCC23344 BoaA (underlined residues in the PEPA sequence being perfectly conserved). The mckLH-PEPA conjugate was emulsified in Freund's adjuvants and used to immunize female BALB/c mice as previously reported [106]. BoaB-specific antibodies were obtained by immunizing mice with mckLH conjugated to the synthetic peptide PEBP (GWLLGTTSGTDPGPLYPGPAENN), which specifies aa 131-155 of *B. pseudomallei* DD503 BoaB. These animal studies were performed in compliance with institutional, as well as governmental, rules and regulations.

**Immunofluorescence labeling of *E. coli* and microscopy**

Plate-grown bacteria were suspended in 5-ml of sterile PBSG to a density of $10^8$ CFU/ml. Portions of these suspensions were spotted onto glass slides and dried using a warming plate. The slides were fixed with PBSG supplemented with 4% paraformaldehyde for 30-min at room temperature, washed with PBSG supplemented with 0.05% Tween 20 (PBST), and blocked overnight at 4°C using PBST supplemented with 10% goat serum (SIGMA-ALDRICH®). Next, bacteria were probed for 1-hr at room temperature with murine α-BoaA or α-BoaB antibodies diluted (1:200) in PBST supplemented with 10% goat serum. After this incubation, the slides were washed with PBST to remove unbound antibodies and incubated for 30-min at room temperature with a goat α-mouse antibody labeled with Alexa Fluor® 546 (Molecular Probes, Inc) and diluted (1:400) in PBST supplemented with 10% goat serum. Following this incubation, the slides were washed with PBST to remove unbound antibody and bacterial cells were stained using the nucleic acid dye DAPI (Molecular Probes, Inc). Slides were mounted with SlowFade® reagent (Invitrogen™) and examined by microscopy using a Zeiss LSM 510 Meta confocal system.
Abbreviations
OM: outer membrane; aa: amino acid; CRF: open reading frame; Oca: oligomeric coiled-coil-adhesin; MW: molecular weight; CFU: colony forming units; Pmb: polymyxin B; Kan: kanamycin; Sm: streptomycin; nt: nucleotide; qRT-PCR: quantitative reverse-transcriptase PCR, cDNA: complementary DNA.

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Authors’ contributions
RB helped conceive the study, participated in its design and coordination, performed most of the experiments involving live B. pseudomallei and B. mallei, and helped with redistribution of the manuscript. SL performed several of the experiments involving live B. pseudomallei and B. mallei. JL carried out the qRT-PCR experiments. WG carried out some of the macrophage survival assays with B. pseudomallei and helped with redistribution of the manuscript. RMW contributed to the qRT-PCR experiments, participated in the conception and design of the study. RH performed most of the qRT-PCR assays against B. pseudomallei and helped with the redistribution of the manuscript. SJL provided the strains B. pseudomallei DDS03, B. mallei ATCC23344, and E. coli s17, also participated in the design of the study. ERL conceived the study, participated in its design and coordination, performed experiments involving live B. pseudomallei and B. mallei, and helped with redistribution of the manuscript. All authors read and approved the final manuscript.

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