

Virulence of *Burkholderia mallei* Quorum-Sensing Mutants

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Many *Proteobacteria* use acyl-homoserine lactone-mediated quorum-sensing (QS) to activate specific sets of genes as a function of cell density. QS often controls the virulence of pathogenic species, and in fact a previous study indicated that QS was important for *Burkholderia mallei* mouse lung infections. To gain in-depth information on the role of QS in *B. mallei* virulence, we constructed and characterized a mutant of *B. mallei* strain GB8 that was unable to make acyl-homoserine lactones. The QS mutant showed virulence equal to that of its wild-type parent in an aerosol mouse infection model, and growth in macrophages was indistinguishable from that of the parent strain. Furthermore, we assessed the role of QS in *B. mallei* ATCC 23344 by constructing and characterizing a mutant strain producing AiiA, a lactonase enzyme that degrades acyl-homoserine lactones. Although acyl-homoserine lactone levels in cultures of this strain are very low, it showed full virulence. Contrary to the previous report, we conclude that QS is not required for acute *B. mallei* infections of mice. QS may be involved in some stage of chronic infections in the natural host of horses, or the QS genes may be remnants of the QS network in *B. pseudomallei* from which this host-adapted pathogen evolved.

Burkholderia mallei, the causative agent of the equine disease glanders, is a member of the class *Betaproteobacteria*. It infrequently infects humans and other mammals in areas where equine disease is endemic (including regions in the Middle East and Asia) (1–3). *B. mallei* is classified as a category B priority pathogen by the Centers for Disease Control and Prevention because human disease is severe, the species is highly infectious by aerosol delivery, and options for disease prevention and treatment are limited (<http://www.bt.cdc.gov/agent/agentlist-category.asp>). Furthermore, *B. mallei* was deployed as a bioweapon during World Wars I and II, was weaponized by multiple countries until 1972 (4, 5), and remains a potential agent of bioterrorism (4). Despite the documented virulence of *B. mallei*, little is known about the mechanisms of bacterial colonization and pathogenesis that lead to disease progression. *B. mallei* is a host-restricted pathogen that is closely related to the nonpathogenic saprophyte *Burkholderia thailandensis* and the opportunistic human pathogen *Burkholderia pseudomallei*, which causes the emerging infectious disease of melioidosis (6). *B. thailandensis* and *B. pseudomallei* have been isolated from soils and waters in regions of endemicity in Southeast Asia and Northern Australia (7–10). *B. mallei*, however, has only been isolated from infected animals. These three species represent a unique situation where closely related bacteria occupy diverse and divergent niches.

Our particular focus is on the role of acyl-homoserine lactone (AHL) quorum sensing (QS) in *Proteobacteria* during microbial adaptations to changing environments. AHL QS allows cells of a bacterial species to assess population density and execute group activities when diffusible AHL signals reach a critical concentration (11). AHL QS was first described as a system for transcriptional activation of luminescence genes in the marine bacterium *Vibrio fischeri*. This luminescent bacterium possesses *luxI*, which codes for an enzyme that synthesizes 3-oxo-C₆-homoserine lactone (3OC₆-HSL), as well as *luxR*, which codes for a 3OC₆-HSL-dependent activator of luminescence gene transcription (12). *V. fischeri* has a dual lifestyle; it lives freely in seawater at low densities and can also colonize light organs of certain marine animals at high population densities. QS allows *V. fischeri* to discriminate

between its low-population-density seawater habitat and the high-population-density, light-organ habitat. AHL quorum sensing is most commonly viewed as a means to allow bacteria to discern a low-density, free-living lifestyle from a higher-density, host-associated lifestyle (12, 13).

We are particularly interested in *B. mallei* because it is a rare example of a host-restricted pathogen with a functional AHL QS system (1, 14, 15). There is no apparent free-living lifestyle. The genome of *B. mallei* contains two sets of linked *luxI-luxR* homologs, *bmaI1-bmaR1* and *bmaI3-bmaR3*, and two additional *luxR*-like genes called *bmaR4* and *bmaR5* (15). The *bmaI1* gene product is an octanoyl-homoserine lactone (C₈-HSL) synthase, and *bmaI3* codes for a 3-hydroxy-octanoyl-HSL (3OHC₈-HSL) synthase (14–16). Previously, it was reported that a number of *B. mallei* AHL QS mutants showed a strong virulence attenuation phenotype in mouse and hamster models of acute infection (15). This publication encouraged us to think it might be possible to develop anti-QS therapeutic approaches for *B. mallei* infections and to better understand the role of QS in an acute infection. Here, we present several lines of evidence that QS is not required for acute *B. mallei* disease in mice, and we discuss possible explanations for maintenance of a functional quorum-sensing circuit in light of these findings.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacteria and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown in low-salt Luria-Bertani (LB) broth (10 g of tryptone, 5 g of yeast extract, 5

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TABLE 1 Bacterial strains and plasmids and PCR primers

| Strain, plasmid, or primer | Relevant characteristics or sequence ^a | Source or reference |
|----------------------------|--|---------------------|
| <i>E. coli</i> strains | | |
| DH10B | | Invitrogen |
| RH03 | SM10(λ pir) Δ asd::FRT Δ aphA::FRT | 17 |
| <i>B. mallei</i> strains | | |
| GB8 | Derived by passage of ATCC 23344 in horse | D. Woods |
| ATCC 23344 | Human isolate | 6 |
| CM8 | GB8 Δ bmaI1 | This study |
| CM9 | GB8 Δ bmaI3 | This study |
| CM38 | GB8 Δ bmaI1 Δ bmaI3 | This study |
| CM3 | GB8 with MCS-luxCDABE fusion-ble; Zeo ^r | This study |
| CM4 | GB8 with PbmaI1-luxCDABE fusion-ble; Zeo ^r | This study |
| CM11 | GB8 with PbmaI3-luxCDABE fusion-ble; Zeo ^r | This study |
| CM31 | ATCC 23344 with S12 ribosomal protein promoter- <i>aiiA</i> fusion construct; Zeo ^r | This study |
| Plasmids | | |
| pBADScce | Zeo ^r | 17 |
| pEXKm5 | <i>sacB</i> plasmid; Km ^r | 17 |
| pTNS2 | R6K replicon TnsABC+D expression vector | 18 |
| pUC18-mini-Tn7T-Gm-lux | Gm ^r on mini-Tn7T; luxCDABE transcriptional fusion vector; Ap ^r , Gm ^r | 18 |
| pCM53 | pUC18-mini-Tn7T-luxCDABE-ble; luxCDABE transcriptional fusion vector; Zeo ^r , Ap ^r | This study |
| pBD4 | pJN105 derivative with <i>bmaR1</i> ; Gm ^r | 14 |
| pBD5 | pQF50 with <i>bmaI1</i> promoter; Ap ^r | 14 |
| pBD7 | pQF50 with <i>bmaI3</i> promoter; Ap ^r | B. Duerkop |
| pCM56 | pUC18-mini-Tn7T-pbmaI1-luxCDABE-ble; Zeo ^r | This study |
| pCM66 | pUC18-mini-Tn7T-pbmaI3-luxCDABE-ble; Zeo ^r | This study |
| pUC18T-mini-Tn7T-Zeo-FRT | Zeo ^r on mini-Tn7T; Zeo ^r , Ap ^r | 19 |
| pCM112 | pUC18T-mini-Tn7T-ble-pS12; Zeo ^r , Ap ^r | This study |
| pAD123::aiiA | pAD123 containing <i>aiiA</i> from <i>B. cereus</i> ATCC 14579 | S. B. Peterson |
| pCM117 | pUC18T-mini-Tn7T-ble-pS12- <i>aiiA</i> ; Zeo ^r , Ap ^r | This study |
| pCM02 | <i>bmaI1</i> deletion plasmid for making unmarked <i>bmaI1</i> deletions; Km ^r | This study |
| pCM03 | <i>bmaI3</i> deletion plasmid for making unmarked <i>bmaI3</i> deletions; Km ^r | This study |
| pJNR2 | pJN105 with <i>btaR2</i> ; Gm ^r | 20 |
| pI2P50 | pQF50 containing the <i>btaI2</i> promoter; Ap ^r | 20 |
| Primers | | |
| bmaI1-A | ATCACCCGGGCGATCATGTTTCGAGCTGAGCGC | This study |
| bmaI1-B | CAGCAGCGGGTTCGAGATCGAGGAAAGTTCGCATGTTCGTCCTCGTG | This study |
| bmaI1-C | AGGACGACATGCGAACTTTCCTCGATCTCGACCCGCTGCTGTGC | This study |
| bmaI1-D | TGATGCGGCCGAGGAGAACGAGCGGTATATCCGCA | This study |
| bmaI3-A | ATCACCCGGGCTCAACGCGAATATCGAACAG | This study |
| bmaI3-B | AAACGACAAGACGCGTGTCTGCTTGGTTCTCCGATG | This study |
| bmaI3-C | ATCCATCGGAGAACCAAGCAGACACGCGTCTTGTCG | This study |
| bmaI3-D | AATCTAGAATTCATCACGTCGAGCGGATTCTTC | This study |
| S12 prom-A | GATCGAGCTGTTGACTCGCTTGGGATTTTCGGAATATCATGCCGGGTGTGCA | This study |
| S12 prom-B | CACCCGGCATGATATTCGGAATAATCCCAAGCGAGTCAACAGCTC | This study |

^a Endonuclease restriction sites are underlined.

g of NaCl per liter), and *B. mallei* was grown in low-salt LB broth supplemented with 4% glycerol and 50 mM morpholinepropanesulfonic acid (MOPS), pH 7.0. Antibiotics were used where appropriate at the following concentrations for *E. coli*: gentamicin (Gm), 20 μ g/ml; kanamycin (Km), 35 μ g/ml; zeocin (Zeo) 25 μ g/ml. For *B. mallei*, we used Km (5 μ g/ml) and Zeo (25 μ g/ml). Diaminopimelic acid (DAP; Sigma) was added (400 μ g/ml) when needed. All experiments with live *B. mallei* bacteria were performed in a class II biological safety cabinet housed in a biological safety level 3 (BSL-3)-enhanced laboratory.

Construction of *B. mallei* mutants. We used standard molecular biology procedures with *E. coli* DH10B as a cloning vehicle. The *bmaI1* (BMAA1347) and *bmaI3* (BMAA1577) deletions were constructed by using a strategy based on the dual-plasmid method of Lopez et al. for *B.*

pseudomallei (17). To create the gene deletion vectors, we used overlap extension PCR to generate approximately 1,000 bp of DNA flanking each gene with genomic DNA (gDNA) from *B. mallei* ATCC 23344 as the template. The flanking sequences were annealed together and PCR amplified to generate each deletion construct. Primers used in cloning are listed in Table 1 and include two sets of four primers for the *bmaI1* and *bmaI3* deletion constructs, bmaI1-A-D and bmaI3-A-D, respectively. Primer-encoded restriction sites were introduced into the ends of the PCR products during amplification. The *bmaI1* deletion construct and pEXKm5 were digested with NotI and SmaI to yield pCM02 following ligation. The *bmaI3* deletion construct and pEXKm5 were digested with SmaI and EcoRI and ligated to yield pCM03 following ligation.

The *bmaI1* or *bmaI3* deletion vector pCM02 or pCM03 was used to

transform *E. coli* RHO3, which was used for mating with *B. mallei* to create the unmarked *B. mallei* *bmaI1* and *bmaI3* mutants. For matings, we mixed equal volumes of overnight cultures of *B. mallei* GB8 and *E. coli* RHO3 with either pCM02 or pCM03 and plated the mixtures on low-salt LB agar with DAP. After 10 to 16 h at 37°C, cells were scraped from plates and suspended in 1 ml of low-salt LB broth. The suspended cells were pelleted by centrifugation, washed in 1 ml of low-salt LB broth, and suspended in 300 μ l of low-salt LB broth. Cells from the 300- μ l suspension were serially diluted and plated on low-salt LB agar containing Km. After 24 h at 37°C, single colonies were picked as candidate merodiploids. Candidate merodiploids were grown in low-salt LB broth for 10 to 16 h at 37°C and then transformed with pBADScE as follows. Nine milliliters of culture was pelleted by centrifugation, washed with 9 ml of 300 mM sucrose, pelleted, washed with an additional 6 ml of 300 mM sucrose, and centrifuged, and the pellet was suspended in 100 μ l of 300 mM sucrose. The sucrose-suspended cells were then electrotransformed with 0.5 to 1 μ g of pBADScE. The transformation mixture was suspended in 1 ml of low-salt LB broth, grown for 1 to 3 h at 30°C, and then plated on low-salt LB agar plus Zeo. Plates were incubated for 5 days at 30°C, after which colonies were picked and patched on low-salt LB agar and low-salt LB agar plus Km. We screened for Km-sensitive *B. mallei* isolates and confirmed unmarked gene deletions by DNA sequencing. We cured the mutants of pBADScE by growth at 37°C. As a confirmation of plasmid curing, we showed that the mutants were Zeo sensitive. The double *bmaI1 bmaI3* mutant was generated by introducing the *bmaI1* mutation into a confirmed *bmaI3* mutant *B. mallei* strain, as described above.

The chromosomal *bmaI1* promoter-*lux* and *bmaI3* promoter-*lux* fusions were generated by exploiting a mini-Tn7 tool previously used with *B. mallei* (21). To generate fusion plasmids, the Gm resistance gene was removed from pUC18-mini-Tn7T-Gm-*lux* by digestion with EagI and SpeI. The vector was then Klenow treated to create blunt ends and self-ligated. The resulting plasmid was digested with KpnI, blunt ended with the Klenow fragment, and ligated with the *ble* gene (*Zeo*^r) fragment from pBADScE that had been treated with PmII, Klenow, and NotI. The resulting plasmid, pCM53, is essentially pUC18 with a mini-Tn7T-MCS-*luxCDABE-ble* insert (where MCS is multiple-cloning site). We found this gene organization necessary to achieve low-background luminescence. For construction of the *bmaI1* promoter-*luxCDABE* fusion vector (pCM56), we isolated a *bmaI1* promoter from pBD5 following treatment with EcoRI, Klenow, and then BglII. The vector, pCM53, was cut with PstI, Klenow treated, and then digested with BamHI. The *bmaI1* promoter and the cut vector were ligated to yield pCM56. The plasmids were used to transform *E. coli* DH10B for maintenance and purification. To create the *bmaI3* promoter fusion construct, the *bmaI3* promoter from pBD7 was isolated following treatment with HindIII, Klenow, and then BglII. The vector, pCM53, was prepared as described above. The *bmaI3* promoter fragment and cut vector were then ligated together to generate pCM66. For each fusion, DNA sequencing confirmed that the promoter was driving *luxCDABE* expression.

The *bmaI1* promoter-*lux* and *bmaI3* promoter-*lux* fusions on pCM56 and pCM66 were inserted in the *B. mallei* GB8 chromosomal Tn7 attachment site (*attTn7*) site downstream of *glmS1* by electroporation together with pTNS2 essentially as described above to yield *B. mallei* strains CM4 and CM11, respectively. Transformants were selected as *Zeo*^r colonies. The resulting colonies were screened for insertion in either *glmS1* or *glmS2* by PCR. Isolates with a single insertion downstream of *glmS1* were used for our subsequent studies. The promoterless fusion construct on pCM53 was also introduced into the chromosome in this way to yield *B. mallei* strain CM3.

The AHL-lactonase producing *B. mallei* ATCC 23344 derivative CM31 was constructed by introducing the S12 ribosomal protein promoter-driven *B. cereus* *aiiA* gene into the *attTn7* site by coelectrotransformation with pCM117 and pTNS2, as described above. To construct pCM117, the S12 ribosomal protein promoter was introduced into pUC18T-mini-Tn7T-Zeo-FRT (where FRT is Flp recognition target). The S12 ribosomal pro-

tein promoter was prepared by using previously described methods (22). Briefly, the oligonucleotide S12 prom-A was annealed with a complementary oligonucleotide, S12 prom-B. The annealed oligonucleotides comprise a short double-stranded fragment encoding the S12 ribosomal protein promoter flanked by BamHI and PstI sticky ends. This promoter fragment was then treated with T4 polynucleotide kinase and was ligated to BamHI- and PstI-cut pUC18T-mini-Tn7T-Zeo-FRT to create pCM112. Then, the *Bacillus cereus* ATCC 14579 AHL lactonase gene was inserted immediately downstream of the S12 ribosomal protein promoter to generate pCM117. Briefly, a lactonase gene (*aiiA*) fragment was purified from *aiiA*::pAD123 following digestion with XbaI and EcoRI. The *aiiA* fragment was then treated with Klenow for blunt-end ligation with pCM112, which had been cut with EcoRI and also Klenow treated. DNA sequencing confirmed the orientation of the *aiiA* gene and the S12 promoter. The ligation product was pCM117.

In vitro luminescence measurements. *B. mallei* colonies from a low-salt LB agar plate were used to inoculate a 3-ml culture of low-salt LB medium. This culture and four additional 3-ml cultures with successive 100-fold dilutions of the initial inoculum were incubated overnight with shaking at 37°C. The next day, flasks were selected that contained cultures with an optical density at 600 nm (*OD*₆₀₀) below 0.5 and were used to inoculate a fresh low-salt LB culture at the desired optical density at *OD*₆₀₀, maintaining a flask-to-volume ratio of 1:10. The *OD*₆₀₀ and luminescence were measured throughout growth with aeration.

To assess activity of promoters fused to the *Photobacterium luminescens* *luxCDABE* in *B. mallei*, we diluted cultures into fresh low-salt LB broth in wells of a 96-well plate, aerated the cells, and measured luminescence by using a SpectraMax M3 reader. We normalized the luminescence values as relative light units (RLU) per 100 μ l of culture.

Measurements of C₈-HSL and 3OHC₈-HSL in B. mallei cultures. To measure AHLs in stationary *B. mallei* cultures, we twice extracted 20 ml of an overnight culture with acidified ethyl acetate and dried the extract under a stream of N₂ gas. The extract was dissolved in 1 ml of acidified ethyl acetate. We used a previously described bioassay to measure C₈-HSL in the ethyl acetate concentrate. The reporter in this assay was *E. coli* (pBD4, pBD5) (14). We generated a standard curve by using synthetic C₈-L-HSL (Sigma Chemical Co.). We measured 3OHC₈-HSL as follows. We dried 500 μ l of the ethyl acetate concentrates and dissolved the dried material in 50 μ l 50% methanol. The methanol solution was separated by C₁₈ reverse-phase high-performance liquid chromatography (HPLC), and fractions in which 3OHC₈-HSL eluted were collected and pooled. We measured 3OHC₈-HSL in the pooled fractions by a previously described bioassay that makes use of *E. coli* (pJNR, pI2P50) (20). We determined the 3OHC₈-HSL HPLC elution profile and prepared standard curves for the bioassay by using 3OHC₈-L-HSL synthesized as described below.

Synthesis of 3OHC₈-HSL. We prepared 3OHC₈-L-HSL by coupling (*R/S*)-3-hydroxyoctanoic acid with L-homoserine lactone hydrobromide according to published protocols (23). (*R/S*)-3-hydroxyoctanoic acid was prepared by alkaline hydrolysis of the product from the Reformatsky coupling of methyl bromoacetate and hexanal (24).

Cell culture and mouse infection assays. Murine macrophage-like RAW 264.7 cells were grown in Dulbecco's modified Eagle's medium (DMEM; HyClone) containing 4.5 mg/ml glucose, 0.1 mg/ml sodium pyruvate, 4 mM L-glutamine, and 10% fetal bovine serum (FBS; Sigma) and incubated at 37°C with 5% CO₂. For infection studies, cells were seeded at 2.5 \times 10⁵ cells/cm² in 24-well tissue culture plates 12 to 24 h prior to infection. The monolayers were infected at a multiplicity of infection (MOI) of 0.1. Immediately after bacteria were added, the plates were centrifuged for 5 min at 500 \times g, and this was followed by incubation for 1 h at 37°C. The RAW 264.7 cells were then washed with Dulbecco's phosphate-buffered saline (DPBS; HyClone) to remove extracellular bacteria. To measure invasion, the monolayer cells were lysed by treatment with 0.2% Triton X-100 in DPBS for 5 min. Bacterial cell counts in the lysis solution were determined by plating and are reported as numbers of CFU. To measure bacterial replication, RAW 264.7 cells were infected as

described above except that we added DMEM containing Km (250 µg/ml) to the monolayers following the DPBS wash. The infected cell layers were incubated for 20 h prior to lysis and bacterial cell counting. As a control to confirm the absence of extracellular bacterial cells, we plated 50 to 100 µl of DMEM over the cell layers at 20 h of incubation and found no CFU.

The *B. mallei* inocula for RAW 264.7 cell infections were prepared as follows. Starter cultures were inoculated from growth on a fresh agar plate into low-salt LB broth containing 4% glycerol and 50 mM MOPS, pH 7.0. After 6 to 8 h at 37°C, starter cultures were used to inoculate low-salt LB broth containing 4% glycerol and 50 mM MOPS, pH 7.0, so that the optical density at 600 nm at the time of inoculation was 0.01. Cultures were grown for 10 to 16 h, diluted in DPBS, and used to infect RAW 264.7 cells as described above.

Animal infection experiments were with 6- to 8-week-old female BALB/c mice (Charles River Laboratories, Wilmington, MA). Mice were housed under specific-pathogen-free conditions. All mouse experiments were approved by the Institutional Animal Care and Use Committee of the University of Washington. The AeroMP (Biaera Technologies, Frederickburg, MD) aerosol management platform was used to deliver *B. mallei* to mice. All components of the AeroMP, except air pump, vacuum, desiccator, electronics interface box, and computer, were housed inside a class II biological safety cabinet. Mice were exposed to aerosols generated from 10-ml 10 mM phosphate-buffered (pH 7.0) *B. mallei* suspensions by using a Hi-Flo MiniHeart nebulizer (WestMed, Inc., Tucson, AZ) running at 15 liters/min (generator, 5.4 liters/min; dilution, 9.6 liters/min) and delivered to mice in a whole-body exposure chamber at ambient temperature and pressure. To measure deposition, one mouse per infection group was sacrificed, and the lungs were removed and homogenized in 2 ml of 10 mM phosphate buffer (pH 7.0) in gentle MACS C tubes (Mitenyi Biotech, Inc.). The homogenate was serially diluted, and CFU numbers were determined by plate counting. Following exposure, mice were observed for the development of symptoms of lethal glanders disease including weight loss, hypothermia, piloerection, forced ventilation, ocular discharge, impaired mobility, and altered physical behaviors. Mice that manifested symptoms of imminent death were euthanized with pentobarbital (intraperitoneal injection, 200 mg/kg).

Bacterial suspensions for mouse infections were prepared as follows. Bacteria from a fresh agar plate were used to inoculate 25 ml of low-salt LB broth containing 4% glycerol and 50 mM MOPS, pH 7.0, in a 125-ml baffled flask. After growth for 12 to 16 h at 37°C with shaking at 225 rpm, cells were pelleted by centrifugation, suspended in 3 ml of low-salt LB broth containing 15% glycerol, and frozen in 0.75-ml aliquots at -80°C. On the day of an animal infection, an aliquot was thawed and used to prepare aerosol suspensions.

In vivo luminescent images were acquired following mouse infection with luminescent bacteria, essentially as described above. Following exposure to bacteria, mice were imaged by an IVIS Spectrum *in vivo* imaging system and analyzed with the Living Image software program (PerkinElmer, CA) once every other day. For luminescent imaging, the system acquires a photograph and a luminescence image simultaneously and automatically generates an overlay image with a scale bar. Before *in vivo* imaging, mice were anesthetized with 3% isoflurane in an oxygen-filled chamber. Once anesthetized, mice were transferred to a sealable optically clear visualization chamber (PerkinElmer, CA); the chamber was then connected to a continuous supply of isoflurane, and the mice were imaged.

RESULTS

Characterization of *B. mallei* GB8 quorum-sensing signal synthesis mutants. We are interested in understanding the role of QS in the biology of the host-adapted pathogen *B. mallei*. The mutants used in the previous study of QS and virulence of *B. mallei* (15) no longer exist. Several years after the initial description of these mutants (15), the antibiotic marker used to disrupt the QS genes was disallowed for use. U.S. government authorities asked

TABLE 2 AHL levels in stationary phase *B. mallei* cultures

| Strain | Mean concn (nM [range]) ^a | |
|--|--------------------------------------|-------------------------|
| | C ₈ -HSL | 3OH C ₈ -HSL |
| GB8 (wild type) | 158 (±89) | 5.2 (±0.4) |
| CM8 (<i>BmaI1</i> ⁻) | ≤1 | ≤1 |
| CM9 (<i>BmaI3</i> ⁻) | 207 (±25) | ≤1 |
| CM38 (<i>BmaI1</i> ⁻ <i>BmaI3</i> ⁻) | ≤1 | ≤1 |
| ATCC 23344 (wild type) | 294 (±170) | 3.5 (±0.4) |
| CM31 (ATCC 23344 <i>aiiA</i>) | 6 (±4) | ≤1 |

^a Values are the means from at least two independent biological replicates.

the authors of the previous publication to destroy their constructs, and they complied. We constructed QS mutant strains containing unmarked deletions of the AHL synthase genes *bmaI1*, *bmaI3*, or both *bmaI1* and *bmaI3* by adapting a method recently developed by Lopez et al. (17) for generating mutations in the closely related *B. pseudomallei*, as described in Materials and Methods. We used *B. mallei* GB8, which was isolated from a horse infected with the sequenced strain ATCC 23344 (Don Woods, personal communication).

We measured AHLs in stationary-phase cultures of the parent and the three QS mutants (Table 2). The wild-type *B. mallei* GB8 produced much more of the BmaI1 signal (C₈-HSL) than the BmaI3 signal (3OHC₈-HSL). The *bmaI1* mutant (CM8) did not produce detectable levels of either AHL. The *bmaI3* mutant (CM9) produced wild-type levels of C₈-HSL, but it did not produce detectable levels of 3OHC₈-HSL. These data are consistent with the idea that BmaI1 is required for C₈-HSL and that C₈-HSL is required for activation of *bmaI3*. BmaI3 is required for 3OHC₈-HSL production, but this does not influence C₈-HSL production. Finally, as expected, the double *bmaI1 bmaI3* mutant (CM38) did not produce detectable levels of either AHL.

Expression of *bmaI1* and *bmaI3* during *B. mallei* growth. We sought to better understand the relationship between culture density, *bmaI1* and *bmaI3* expression, and AHL accumulation. To do this, we employed strains CM4 and CM11 (Table 1), which contain a *lux* operon fusion to either the *bmaI1* or *bmaI3* promoter at a neutral site in the *B. mallei* chromosome. Growth, promoter activity, and AHL accumulation of each strain were monitored (Fig. 1). Both *bmaI1* promoter activity and the product of BmaI1 (C₈-HSL) showed a rapid rise starting in late logarithmic phase. We could not detect *bmaI3* promoter activity, and we were able to detect the product of BmaI3 (3OHC₈-HSL) only when the culture was in late stationary phase following overnight growth (Fig. 1 and data not shown). At least under the conditions of our experiments, both the BmaI1 and BmaI3 proteins are functional, but *bmaI1* is expressed at much higher levels than *bmaI3*. Furthermore, the BmaI3-BmaR3 system appears to depend on the BmaI1-BmaR1 system (Table 2).

***B. mallei* GB8 QS mutants are virulent.** It was previously reported that virulence of *B. mallei* QS mutants is severely attenuated in an aerosol mouse infection (15). We asked whether virulence was attenuated in our unmarked QS deletion mutants. *B. mallei* is a facultative intracellular pathogen (25). As is the case for several intracellular pathogens, the ability to replicate inside host cells has been linked to virulence. For this reason, tissue culture infection in macrophages can serve as a proxy to identify virulence

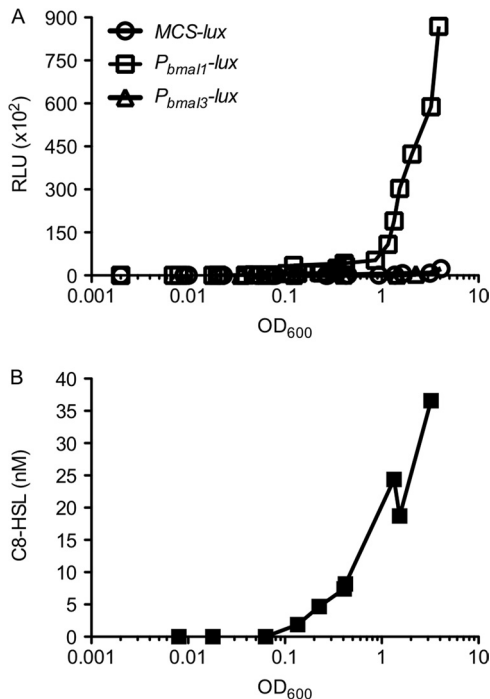


FIG 1 *B. mallei* *bmaI1* and *bmaI3* promoter activity and AHL production. (A) Luminescence (RLU, relative light units) was monitored during growth of *B. mallei* cells containing a *bmaI1* promoter-*lux* fusion in a neutral site on the chromosome (strain CM4; squares) or a *bmaI3* promoter-*lux* fusion at a neutral site (strain CM11; triangles). A strain containing a promoterless *lux* cassette at the same neutral site (CM3; circles) was used as a control. (B) C₈-HSL concentrations during growth of strain CM4. Cell density was measured as optical density at 600 nm (OD₆₀₀). We did not detect 3OHC₈-HSL in strain CM11 during exponential and early-stationary-phase growth (data not shown). The data for each strain are from three separate cultures, which were started at different densities (OD₆₀₀ of 0.01, 0.05, or 0.1). The BSL-3 requirement necessitated this approach to capture entire growth curves (stationary phase commences at a density of >2) within 10 h.

factors. Thus, we began by following the growth of *B. mallei* GB8 and its unmarked QS deletion mutants in macrophage-like RAW 264.7 cells (Fig. 2). To our surprise, intracellular replication levels of wild-type *B. mallei* GB8 and the QS mutants were indistinguishable.

It could be that the connection between QS and virulence reported previously involved a step that was independent of macrophage growth or that we were unable to uncover the QS mutant phenotype during this *in vitro* infection assay for other reasons. Therefore, we also used a mouse aerosol infection model similar to that described in the previous publication on QS and virulence in *B. mallei* (15). With this whole-body aerosol infection model, we estimated the 50% lethal dose (LD₅₀) of wild-type GB8 to be between 5,000 to 10,000 CFU (Fig. 3B). The LD₅₀ of wild-type GB8 is modestly higher than the reported LD₅₀ of wild-type ATCC 23344 (1,000 CFU) by aerosol delivery (26). When we tested wild-type GB8 or its QS null strain (CM38) at a dose slightly above the determined LD₅₀ (20,000 CFU), all mice succumbed to infection within 1 week (Fig. 3A). Similarly, we observed comparable survival kinetics of wild-type GB8 and the single *bmaI1* or *bmaI3* mutant (CM8 or CM9) at doses near the LD₅₀ (Fig. 3B). In some instances, mice infected with either the single *bmaI1* or *bmaI3* mutant

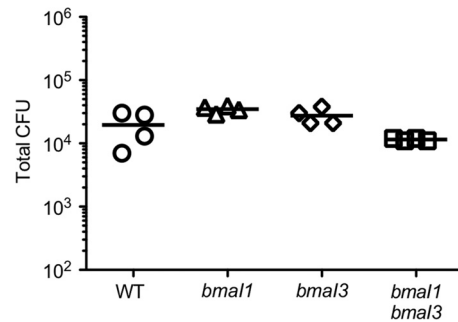


FIG 2 Replication of wild-type *B. mallei* and *B. mallei* AHL synthesis mutants in RAW 264.7 macrophage-like cells. Monolayers were infected with the wild-type (WT) GB8, the *bmaI1* mutant (CM8), the *bmaI3* mutant (CM9), or the double *bmaI1 bmaI3* mutant (CM38) at an MOI of 0.1. The AHL synthesis mutations did not influence initial attachment (data not shown). The amount of intracellular bacteria harvested from RAW 264.7 cells after 20 h is shown as total CFU count.

showed decreased survival relative to mice infected with the isogenic wild-type strain (Fig. 3B). These results suggest that the QS mutants might be slightly more virulent than the wild type. Alternatively, the small differences may represent inherent variability that occurs at doses around the LD₅₀. It is clear from these experiments that all QS mutants of strain GB8 are virulent, indicating that in our horse-passaged derivative of *B. mallei* ATCC 23344, quorum sensing is not critical for virulence during acute disease in mouse aerosol infections.

Perhaps the discrepancy between our experiments and the prior finding that quorum sensing is required for virulence of *B. mallei* ATCC 23344 reflects bacterial strain differences. Although the original study used strain ATCC 23344, we utilized a derivative of that strain, which was passed through a horse (GB8). The *B. mallei* genome is unstable and plastic, and it has been shown that passage of *B. mallei* through animals can result in genetic changes (27). Therefore, we sought to study strain ATCC 23344 itself. For unknown reasons, we found it difficult to construct QS mutants of strain ATCC 23344. Thus, we developed a different strategy to create an AHL-negative ATCC 23344 strain. We constructed a *B. mallei* ATCC 23344 derivative, which carries the *B. cereus* AHL lactonase gene *aiiA* driven by a strong promoter (CM31). We measured AHLs in stationary-phase cultures of strain CM31 and found that C₈-HSL was about 2% of the level seen in the wild-type ATCC 23344. We could not detect any 3OHC₈-HSL (Table 2). We found that virulence of the AHL lactonase-expressing strain CM31 was comparable to its parent ATCC 23344 in the murine infection model (Fig. 3C).

Is QS active during infection? Apparently, quorum sensing is not required for acute *B. mallei* murine aerosol infections. Our evidence indicates that during *in vitro* growth of *B. mallei*, *bmaI1* is expressed in late logarithmic phase, and *bmaI3* is expressed in stationary phase and then only poorly. These genes may or may not be expressed during an acute infection. We sought to evaluate if the strongly QS-activated *bmaI1* promoter was induced in the mouse during infection. To assess *bmaI1* expression, we infected mice with the luminescent *bmaI1* promoter fusion strain CM4. We followed *bmaI1* expression by monitoring luminescence in whole animals (Fig. 4). Strong luminescence appeared in the lungs of mice infected with strain CM4 at the time that an individual showed marked signs of

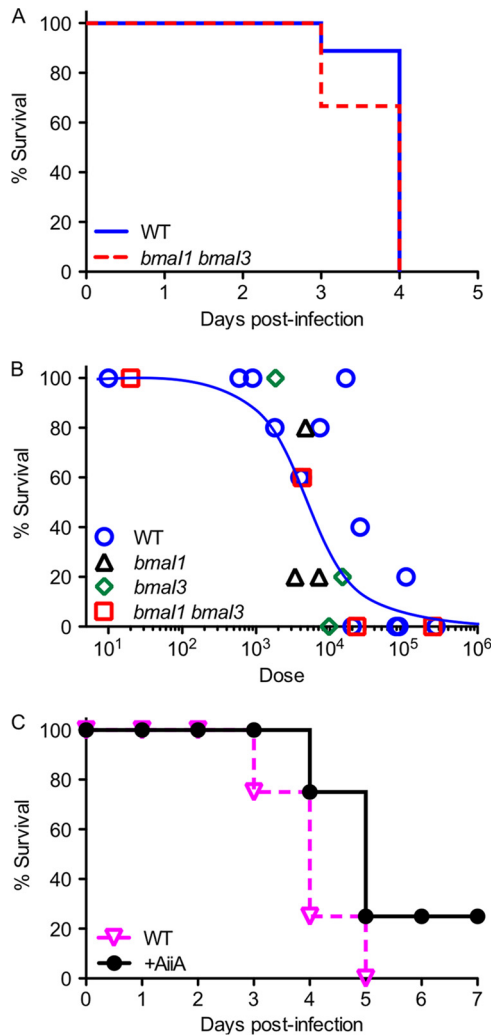


FIG 3 Mouse survival after *B. mallei* infection. (A) Mice were infected by aerosol delivery of wild-type (WT) GB8 or the double *bmal1 bmal3* mutant CM38. Results are from two independent experiments. The data were plotted using GraphPad Prism software, which generated survival fraction calculations (Kaplan-Meier limit method) and analyzed the data (log rank Mantel-Cox test). By this analysis the wild-type and mutant results are not statistically different. The initial doses for each replicate of WT GB8 were 20,000 CFU and 79,000 CFU, and the doses of CM38 were 24,800 CFU and 20,000 CFU. (B) Mouse survival was monitored for 1 week following aerosol infection with increasing doses of wild-type GB8 *B. mallei* (WT), the *bmal1* mutant CM8, the *bmal3* mutant CM9, or the double *bmal1 bmal3* mutant CM38. Each data point represents one exposure group from independent experiments consisting of at least four mice. (C) Mice were infected with wild-type *B. mallei* ATCC 23344 (WT) or ATCC 23344 producing the AHL lactonase AiiA CM31 (+AiiA). Initial doses were 2,184 CFU and 1,416 CFU for ATCC 23344 and CM31, respectively. The results shown are from one experiment. In a second similar experiment, all infected mice experienced comparable signs of acute disease through day 4 postinfection, after which the mice were euthanized. For most experiments each infection group consisted of five mice. Some of the groups in Fig. 3B consisted of four mice. Initial doses were measured as deposition (see Materials and Methods).

acute disease (most notably respiratory distress, reduced mobility, ocular discharge, and decreased body temperature). Thus, though *bmal1* is not required for *B. mallei* disease in this animal model, the BmaI1-BmaR1 system is active at least after the disease has progressed.

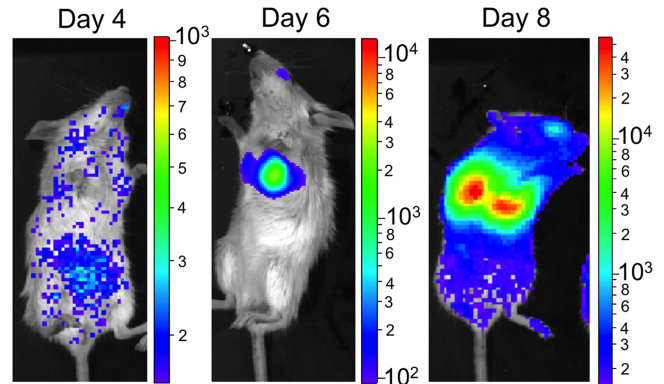


FIG 4 *In vivo* imaging of *bmal1* activity during acute murine gladders. Mice were infected with 3,900 CFU of *B. mallei* strain CM4 with a *bmal1* promoter-*lux* fusion. Infected mice were imaged using an *in vivo* imaging system (see Materials and Methods). Luminescence is shown from a single CM4-infected mouse on days 4, 6, and 8 following infection. On day 8, the mouse was moribund and was euthanized following imaging. Note that the luminescence scale bar for each image is different.

DISCUSSION

To probe the role of QS in *B. mallei* pathogenesis, we constructed mutants of the horse-passaged strain GB8 with clean, unmarked deletions in the two AHL QS signal synthase genes. As expected, a double *bmal1 bmal3* mutant does not produce AHLs. The *bmal3* mutant does not produce the BmaI3 signal (3OHC₈-HSL), but it does produce the BmaI1 signal (C₈-HSL). The *bmal1* mutant does not produce detectable levels of either AHL: it behaves like the double mutant. We interpret this result to mean that the BmaI1-BmaR1 quorum-sensing circuit is required for activation of *bmal3* expression. This sort of hierarchical quorum-sensing circuitry has been well established in the case of *Pseudomonas aeruginosa*, where the LasR-LasI QS circuit is required to activate a battery of genes including a second QS circuit, RhlR-RhlI (28, 29).

Substantial amounts of the BmaI1 signal C₈-HSL are produced by both of the wild-type *B. mallei* strains we examined, GB8 and ATCC 23344. Much lower levels of the BmaI3 product 3OHC₈-HSL are produced by the wild-type strains, and 3OHC₈-HSL is not detected until cultures reach late stationary phase (Fig. 1 and Table 2). We note that measured levels of 3OHC₈-HSL are low, in the range of 5 nM compared to levels of 150 to 300 nM of C₈-HSL. Two recently discovered QS systems are fully functional at signal levels even lower than those we measured for 3OHC₈-HSL. In certain species of *Bradyrhizobium*, signal concentrations in cultures reach about 5 to 10 nM, and these concentrations are in considerable excess with responses saturating at signal concentrations on the order of 10 pM (30, 31).

We assessed the impact of all three of our *B. mallei* quorum-sensing mutants on growth in macrophage-like cells and on virulence in an acute murine infection (Fig. 2 and 3). QS did not appear to have any significant influence on *B. mallei* pathogenesis in either of these infection models. This result was surprising for three reasons. First and foremost, a previous report provided evidence that both the BmaI1-BmaR1 and BmaI3-BmaR3 systems were key virulence determinants (15). We used infection parameters nearly identical to those previously employed, including a similar infectious dose and aerosol delivery method. Second, QS has been previously linked to virulence in the related opportunist-

tic pathogen *B. pseudomallei*; three independent studies examined the role of C₈-HSL QS in a total of five infection models using three different parent and mutant strains (32–34). In each case, the mutants were attenuated. In one of these studies, mutants defective in the 3OHC₈-HSL QS system were also attenuated (34). Thus, it seems that QS in the opportunistic pathogen *B. pseudomallei* is relevant in acute disease. We were surprised when the same did not appear to be true for *B. mallei*. Third, evidence indicates that *B. mallei* evolved from a clone of *B. pseudomallei* and that it has undergone expansive genetic loss and pseudogene acquisition, with a reduction of about 20% of its ancestral genome (approximately 1.4 Mb of DNA). Genome erosion is one factor that defines *B. mallei* as a host-restricted pathogen equipped for life and persistence in an animal reservoir. It is believed that genes likely important for saprophytic survival and unwanted immunogenicity were inactivated or have been eliminated from the *B. mallei* genome (5, 35). In fact *B. pseudomallei* has three AHL quorum-sensing gene pairs, *bpsI1-bpsR1*, *bpsI2-bpsR2*, and *bpsI3-bpsR3*. A large *bpsI2-bpsR2*-containing *B. pseudomallei* chromosomal region has been eliminated from the *B. mallei* genome. Implicit in the fact that *bmaI1-bmaR1* and *bmaI3-bmaR3* have been conserved in *B. mallei* is the idea that they are important for the success of this pathogen in an animal host.

What are possible explanations for the discrepancy between our finding and the previous finding that QS is a key virulence determinant in *B. mallei*? Unfortunately, the mutants used in the prior study no longer exist, and we cannot do comparative analysis. As discussed above, the genome of *B. mallei* is plastic (6). Laboratory passage of this species can result in the accumulation of spontaneous mutations or insertions/deletions (indels), which can attenuate virulence (27, 36). Ulrich et al. (15) had more limited genetic tools available to study *B. mallei*, and the mutants they examined had antibiotic resistance markers on plasmids inserted in the quorum-sensing genes. It is possible that their mutant construction selected for attenuation. It is also possible that the differences reflect the fact that we used a horse-passaged derivative of the *B. mallei* strain studied previously. To address this possibility, we constructed an AHL lactonase-producing derivative of the strain used in the original study. During *in vitro* growth, this strain made barely detectable levels of AHLs. Yet it remained virulent in mice (Table 2 and Fig. 3). Thus, we do not believe that the explanation for the discordant results resides in differences in parental strains (with the caveat that we did not measure AHLs in infected mice). Regardless of the explanation, our results showing that QS is not required for acute *B. mallei* disease following aerosol infection of mice reduces enthusiasm for development of anti-quorum-sensing therapeutics for *B. mallei* infections.

Several key factors for acute *B. mallei* infections in mice have been identified. These factors include capsular polysaccharide (36), a type III secretion system, and a type VI secretion system (37, 38). We infer that QS must not be required for activation of genes coding for these known virulence factors.

We do not know why the *bmaI1-bmaR1* and *bmaI3-bmaR3* genes have been retained in the *B. mallei* genome. We do know, however, that the AHL synthase genes produce functional, active proteins. Perhaps the genes are simply silent in the host, and the cost of their maintenance is low enough that there has been little or no selection against them. However, we showed that the *bmaI1* promoter is active during a mouse infection by using luminescence imaging of mice infected with *B. mallei* carrying a *bmaI1*

promoter-driven *lux* gene cassette (Fig. 4). Glanders is a complicated disease in that it appears *B. mallei* has specifically evolved for persistence in the horse host. For example, glanders in horses most commonly presents in chronic and subacute forms (2, 3, 39). However, in other mammals, including humans, glanders is more commonly a life-threatening acute disease (2, 40, 41). It may be that QS is important during acute infection in the horse. However, we believe it more likely that QS is involved in establishment or maintenance of a chronic phase of infection. Whether or not this is the case will require further experimentation to reveal the genes controlled by QS in *B. mallei*, and it will require studies of chronic infections. It is interesting that *bmaI1* promoter activity in mouse lungs coincided with the onset of overt disease symptoms. Perhaps QS could serve in the development of a chronic state in horses, whereas mice do not limit the acute infection to the same extent.

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