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MEETING PRESENTATION

Malleilactone Is a *Burkholderia pseudomallei* Virulence Factor Regulated by Antibiotics and Quorum Sensing

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ABSTRACT *Burkholderia pseudomallei*, the causative agent of melioidosis, encodes almost a dozen predicted polyketide (PK) biosynthetic gene clusters. Many of these are regulated by LuxR-I-type acyl-homoserine (AHL) quorum-sensing systems. One of the PK gene clusters, the *mal* gene cluster, is conserved in the close relative *Burkholderia thailandensis*. The *B. thailandensis mal* genes code for the cytotoxin malleilactone and are regulated by a genetically linked LuxR-type transcription factor, MalR. Although AHLs typically interact with LuxR-type proteins to modulate gene transcription, the *B. thailandensis* MalR does not appear to be an AHL receptor. Here, we characterize the *mal* genes and MalR in *B. pseudomallei*. We use chemical analyses to demonstrate that the *B. pseudomallei mal* genes code for malleilactone. Our results show that MalR and the *mal* genes contribute to the ability of *B. pseudomallei* to kill *Caenorhabditis elegans*. In *B. thailandensis*, antibiotics like trimethoprim can activate MalR by driving transcription of the *mal* genes, and we demonstrate that some of the same antibiotics induce expression of *B. pseudomallei malR*. We also demonstrate that *B. pseudomallei* MalR does not respond directly to AHLs. Our results suggest that MalR is indirectly repressed by AHLs, possibly through a repressor, ScmR. We further show that malleilactone is a *B. pseudomallei* virulence factor and provide the foundation for understanding how malleilactone contributes to the pathology of melioidosis infections.

IMPORTANCE Many bacterially produced polyketides are cytotoxic to mammalian cells and are potentially important contributors to pathogenesis during infection. We are interested in the polyketide gene clusters present in *Burkholderia pseudomallei*, which causes the often-fatal human disease melioidosis. Using knowledge gained by studies in the close relative *Burkholderia thailandensis*, we show that one of the *B. pseudomallei* polyketide biosynthetic clusters produces a cytotoxic polyketide, malleilactone. Malleilactone contributes to *B. pseudomallei* virulence in a *Caenorhabditis elegans* infection model and is regulated by an orphan LuxR family quorum-sensing transcription factor, MalR. Our studies demonstrate that malleilactone biosynthesis or MalR could be new targets for developing therapeutics to treat melioidosis.

KEYWORDS *Burkholderia*, quorum sensing, polyketide

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Burkholderia pseudomallei is the causative agent of melioidosis, an often fatal and difficult to treat condition estimated to be responsible for ~90,000 deaths worldwide per year (about 40% of total infections) (1). Despite the severity and worldwide incidence of melioidosis, the basic biology and pathogenesis mechanisms of this species remain relatively poorly understood, in part because of the difficulty of handling it in the laboratory. Because *B. pseudomallei* poses a high risk to national security and public health, it is considered a tier 1 select agent and, as such, requires biosafety level 3 (BSL3) safety conditions for handling. Due to this restriction, much knowledge of *B. pseudomallei* stems from studies conducted on a close relative, *Burkholderia thailandensis*, which is safe for handling in BSL2 laboratory conditions. *B. thailandensis* and *B. pseudomallei* have a high degree of genetic relatedness and share about 85% of their genes. Species are differentiated by some metabolic capabilities, species-specific genes (including virulence genes in *B. pseudomallei*), and four large-scale (>10 kb) genomic inversions (2, 3). In addition to *B. thailandensis*, two *B. pseudomallei* attenuated strains were recently excluded from select agent regulations (4, 5) and thus can be used in BSL2 laboratory conditions. These strains provide new opportunities to study *B. pseudomallei* directly.

We are interested in understanding the pathogenic mechanisms of *B. pseudomallei*, particularly those mediated by small-molecule toxins, which are emerging as an important contributor to *B. pseudomallei* virulence in mammalian infections (6, 7). A substantial portion of the *B. pseudomallei* genome is dedicated to polyketide (PK) and nonribosomal peptide (NRP) secondary metabolism, with almost a dozen predicted PK/NRP gene clusters (6). Some of these gene clusters are also present in *B. thailandensis*, which has been used as a surrogate in some cases (8–10). Some of the clusters have been studied directly in *B. pseudomallei*, particularly those that are not shared in *B. thailandensis* (6, 7, 11–13). In many cases, no polyketide products, or very low concentrations, are synthesized in standard laboratory conditions, making them difficult to identify and study. In some cases, the products can be generated by using a genetic approach of synthetically inducing transcription of the biosynthetic genes (6, 8, 14). In others, specific laboratory conditions can trigger production (9, 15).

Many predicted secondary metabolite gene clusters in *B. pseudomallei* are regulated by acyl-homoserine lactone (AHL) quorum sensing (16, 17). These systems typically involve *luxI-luxR* gene pairs that code for signal synthase and receptor proteins, respectively. In some cases, *luxR* genes are not linked to any *luxI* genes, and these are called orphans or solos (18, 19). Many LuxR orphans are AHL responsive; however, at least some appear to be signal independent (20, 21). In *B. pseudomallei*, there are three LuxR-LuxI signaling systems, BpsR1-I1, BpsR2-I2, and BpsR3-I3. There are also two orphans, MalR (also called BpsR4) and BpsR5. MalR in *B. thailandensis* has been shown to control production of malleilactone (20), the product of a PK/NRP gene cluster and a cytotoxin that contributes to *B. thailandensis* virulence in nematodes (8). At least in *B. thailandensis*, MalR appears to be an unusual LuxR orphan, in that it is not a receptor for AHLs (20).

In this study, we focus on the malleilactone (*mal*) biosynthetic genes in *B. pseudomallei*, and specifically on the products and regulation of the *mal* cluster. The protein products of the *mal* biosynthetic genes and *malR* share ~80% identity in *B. thailandensis* and *B. pseudomallei*. The *mal* genes are silent in *B. thailandensis* under standard laboratory conditions. Expression of the *mal* cluster seems to be induced by certain antibiotics, including several used to treat melioidosis infections. Induction is through increased expression of *malR*. Here, we use knowledge gained from studies of the *B. thailandensis mal* genes to guide studies in *B. pseudomallei*. We demonstrate that the product of the *B. pseudomallei* genes is also malleilactone and that malleilactone contributes to *B. pseudomallei* virulence, as it does in *B. thailandensis*. The results of this work provide new information on small-molecule biosynthesis and regulation in *B. pseudomallei* and the biology of this understudied pathogen.

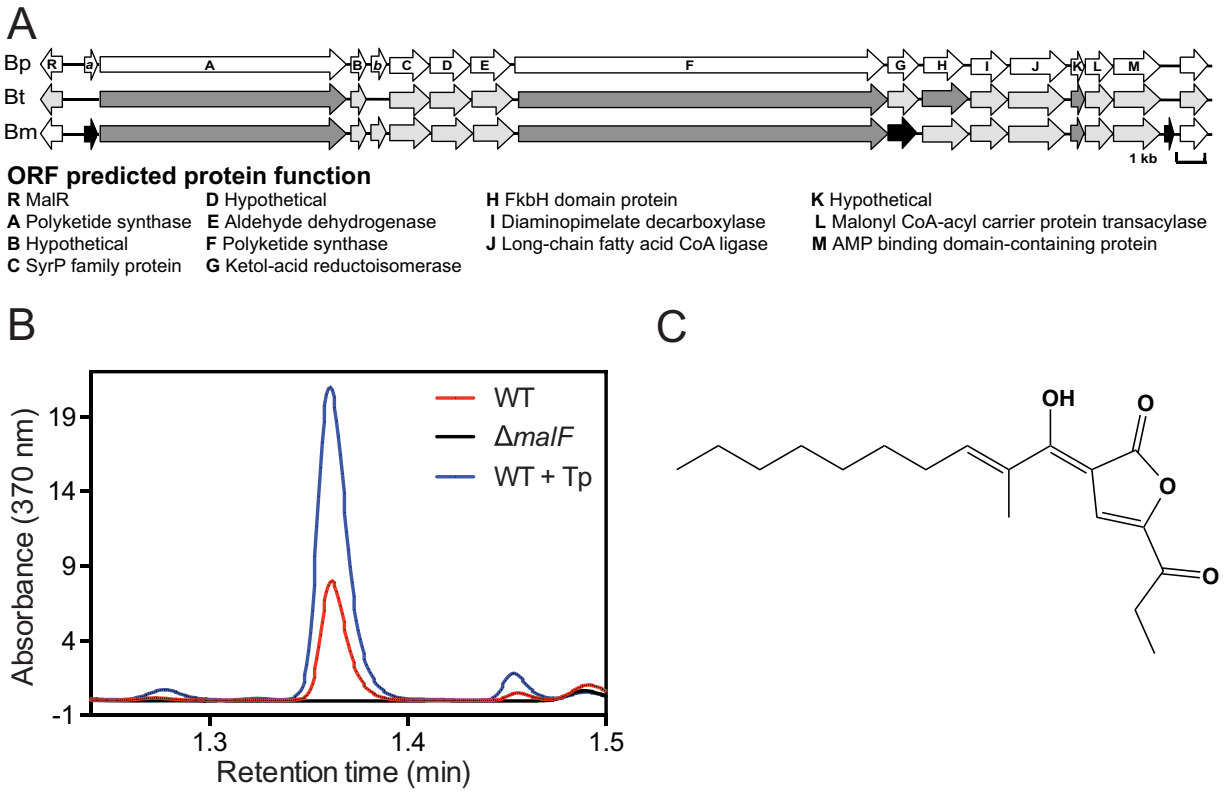

FIG 1 *B. pseudomallei mal* genes code for production of malleilactone. (A) The *mal* gene clusters from *B. pseudomallei* (Bp), *B. thailandensis* (Bt), and *B. mallei* (Bm), with predicted function of each encoded protein. Shading indicates percent identity to the *B. pseudomallei* protein sequences. White, 100%; light gray, 90 to 99%; dark gray, 80 to 89%; black, <80%. (B) Production of a malleilactone-like product in *B. pseudomallei* cultures. Levels of malleilactone production in the wild type (WT), Δ*malF* strain, and WT grown with 4.5 μg/ml trimethoprim (Tp) are shown. (C) Proposed *B. pseudomallei* malleilactone structure consistent with liquid chromatography-mass spectrometry approach (LC-MS)/MS and nuclear magnetic resonance (NMR) results.

RESULTS

The *B. pseudomallei mal* genes code for malleilactone biosynthesis. The *B. thailandensis* malleilactone (*mal*) biosynthetic genes are in an ~35-kb region that is also present in *B. pseudomallei* and *B. mallei* (Fig. 1A). The predicted proteins encoded by this cluster share ~80 to 90% amino acid identity in all three species. The genomic organization of this region is also well conserved, with the exception of two genes coding for hypothetical proteins ($MalB_a$ and $MalB_b$) which are found in *B. pseudomallei* and *B. mallei* but not in *B. thailandensis*. The product of the *mal* genes in *B. thailandensis* is malleilactone. Based on the sequence conservation among these three species, we predicted that the *B. pseudomallei mal* gene product might be similar or identical to malleilactone. To test this prediction, we deleted the *B. pseudomallei malF* gene that is required for *B. thailandensis* malleilactone production (8). We compared the secondary metabolic profile of the *B. pseudomallei malF* mutant with that of the parental wild-type strain (Bp82) using a high-resolution liquid chromatography-mass spectrometry approach (HR-LC-MS). In the *B. pseudomallei* Bp82 culture, we observed a relatively small peak corresponding to the molecular weight of *B. thailandensis* malleilactone, which was absent in *malF* mutant cultures (Fig. 1B).

Under standard conditions, *B. pseudomallei* produced limited quantities of the presumed malleilactone product, which precluded further studies to characterize the structure of this compound. In *B. thailandensis*, malleilactone production is triggered by subinhibitory concentrations (below the MIC) of the antibiotic trimethoprim (15, 20). Thus, we grew *B. pseudomallei* cells with 4.5 μg/ml trimethoprim, a concentration that slowed growth by about 25%. Cells grown with this trimethoprim concentration produced ~5-fold-higher titers of the presumed malleilactone compound than did

TABLE 1 Ability of antibiotics to activate P*malA-lacZ* in *B. pseudomallei*

Growth inhibitor (μg/ml)[a]	Antibiotic mechanism	Relative P*malA-lacZ* activity[b]
None		1
Trimethoprim (15)	THF pathway inhibitor[c]	10.7 ($\pm$0.4)
Sulfamethoxazole (500)	THF pathway inhibitor	10.5 ($\pm$3.1)
Ceftazidime (1.75)	Cell wall synthesis inhibitor	4.9 ($\pm$0.7)
Piperacillin (10)	Cell wall synthesis inhibitor	4.7 ($\pm$1.1)
Ciprofloxacin (1)	DNA gyrase inhibitor	4.1 ($\pm$0.8)
Imipenem (1.7)	Cell wall synthesis inhibitor	1.8 ($\pm$0.2)
Polymyxin B (600–700)	Membrane permeabilization	1.4 ($\pm$0.1)
Kanamycin (150)	30S ribosome inhibitor	0.9 ($\pm$0.1)
Paraquat (500–700 nM)[d]	Reactive oxygen stress	1.3 ($\pm$0.1)

[a]The concentration of antibiotic used is indicated in parentheses, and in each case this concentration resulted in about 50 to 70% reduction of growth yield as determined by the optical density at 600 nm (OD_{600}) measured at the time of β-galactosidase measurement, compared with that of an identically grown untreated culture.

[b]Growth-adjusted β-galactosidase activity is given relative to that of the untreated control. β-Galactosidase activity and optical density at 600 nm (OD_{600}) were determined after 24 h. Values are the mean of at least three independent experiments, with the statistical range of the median indicated in parentheses.

[c]THF, tetrahydrofolate reductase.

[d]Paraquat generates oxidative stress through superoxide production.

untreated cells (Fig. 1B). Thus, we used trimethoprim as an inducer to generate sufficient quantities for further characterization. We initially characterized the product using a tandem HR-MS (HR-MS/MS) approach. The HR-MS/MS fragmentation pattern of the *B. pseudomallei* malleilactone-like product was consistent with that of *B. thailandensis* malleilactone (Fig. 1C; see also Fig. S1 in the supplemental material). We also purified the product and conducted structural analysis by nuclear magnetic resonance spectroscopy (NMR). We used a purification procedure similar to that reported previously for *B. thailandensis* (20), with an important modification. We found that compound stability during and after purification was significantly improved by using a neutral pH in the mobile-phase buffer during secondary purification (pH 6.5) (see Materials and Methods). Our NMR data agreed well with those published for *B. thailandensis*-produced malleilactone (8, 14) (see Fig. S2 and Table S2 in the supplemental material). Together, our results demonstrate that the product of the *B. pseudomallei mal* genes is malleilactone, and that malleilactone production is enhanced during growth with trimethoprim.

Antibiotic activation of the *B. pseudomallei mal* genes. Trimethoprim is an inhibitor of the tetrahydrofolate biosynthesis pathway. Several antibiotics with other cellular targets also induce *B. thailandensis mal* gene expression, including fluoroquinolone DNA gyrase inhibitors and the cell wall biosynthesis inhibitors piperacillin and ceftazidime (15). These antibiotics activate *mal* gene expression at concentrations that slow growth. However, slowed growth is not sufficient to induce the *mal* cluster, as some antibiotics such as the ribosome inhibitor kanamycin do not induce *B. thailandensis mal* gene expression. We were interested in identifying which antibiotics can induce expression of the *mal* genes in *B. pseudomallei*. To identify *mal*-inducing antibiotics, we constructed a reporter strain by fusing a promoterless *lacZ* gene to the promoter of *malA*, the first gene in the *mal* gene cluster, and introducing this reporter into the *glmS3* site in the *B. pseudomallei* genome. We tested a variety of antibiotics at sublethal but growth-slowing concentrations. Our results show that trimethoprim induces the P*malA-lacZ* reporter (Table 1), consistent with our mass spectrometry results (Fig. 1B). *B. pseudomallei mal* genes were also induced by antibiotics other than trimethoprim, such as sulfamethoxazole (which inhibits the tetrahydrofolate biosynthetic pathway, similarly to trimethoprim), and, to a lesser extent, ciprofloxacin (a fluoroquinolone), ceftazidime, and piperacillin. It is of note that trimethoprim, sulfamethoxazole, and ceftazidime are used clinically to treat melioidosis, although it is unclear whether this finding has any importance *in vivo*. Three antibiotics, namely, kanamycin, polymyxin B (an outer membrane-integrity disruptor), and imipenem (a β-lactam), did not activate expression of the *mal* genes.

Because some antibiotics trigger induction of oxidative stress response pathways through production of reactive oxygen species (22), we wondered if induction of oxidative stress could lead to activation of *mal* gene expression. Thus, we tested whether Paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride), which induces oxidative stress through the production of superoxide, can induce *mal* gene expression. We tested Paraquat at concentrations that slow growth, and our results showed that Paraquat did not induce the *mal* gene cluster, consistent with the idea that *mal* gene expression is not induced by oxidative stress. Instead, our results suggest that a variety of antibiotics with different cellular targets can induce expression of the *B. pseudomallei mal* biosynthetic genes. Some of the same antibiotics also induce the *B. thailandensis mal* genes, suggesting a similar mechanism of antibiotic-dependent *mal* gene regulation in both species.

Malleilactone cytotoxicity and *B. pseudomallei* virulence. Previously, malleilactone was shown to be cytotoxic to several bacteria and to mammalian cells (8). Thus, we assessed the toxicity of our malleilactone preparation. We tested the concentration of malleilactone required to cause a 50% decrease in the viability of cultured human A549 bronchial epithelial cells and human Jurkat T lymphocyte cells after 48 h in culture (IC_{50}). The IC_{50} of malleilactone was found to be 7.4 μg/ml (24 μM) for the A549 cell line and 2.5 μg/ml (8 μM) for the Jurkat cell line (Fig. S3). This IC_{50} is comparable with those previously reported for malleilactone against other mammalian cells (8). We also determined the MIC of malleilactone against several bacterial species. Malleilactone was cytotoxic to some Gram-positive bacterial species, including *Staphylococcus aureus*, at concentrations ranging from 5 to 20 μg/ml (18 to 70 μM) (see Table S1 in the supplemental material).

The cytotoxicity of malleilactone suggests that it might function as a virulence factor in *B. pseudomallei*. We used a *Caenorhabditis elegans* infection model to test whether malleilactone contributes to *B. pseudomallei* virulence. Previous studies indicate that *B. pseudomallei* rapidly kills *C. elegans* through production of several secreted toxins, although only one has been identified (11, 23–26). Toxins also appear to be a significant contributor to *B. pseudomallei* pathogenicity in mammalian infections (6, 7). We tested the virulence of our *malF* biosynthesis mutant and a strain with a deletion in *malR*. MalR is an orphan LuxR and is encoded adjacent to the *mal* biosynthesis genes. The *B. thailandensis* MalR is important for *C. elegans* infections (8) and has been shown to be a *mal* gene regulator (20) (Fig. 1A). For our virulence assays, we placed *C. elegans* worms on a lawn of *B. pseudomallei* cells on nematode growth medium supplemented with adenine and thiamine and monitored worm survival daily. Under these conditions, the attenuated *B. pseudomallei* Bp82 strain was able to kill *C. elegans* worms within about 3 days (Fig. 2). *C. elegans* worms on Bp82 lawns also showed slowed movement and were reproduction deficient, as they produced fewer eggs than identically treated nematodes that were fed nonpathogenic *Escherichia coli*. Thus, similar to findings for virulent *B. pseudomallei* strains (23, 25, 26), our results demonstrate that Bp82 was able to kill *C. elegans* in the presence of adenine and thiamine in the growth medium. Compared with Bp82, the *malF* and *malR* mutants were slower to kill *C. elegans* under identical growth conditions (Fig. 2). These results support the notion that malleilactone production and the putative regulator MalR are important for *B. pseudomallei* virulence.

Induction of the *mal* genes relies on MalR and sufficient *malR* expression. MalR appears to be a key *mal* gene regulator in *B. thailandensis*, and our *C. elegans* infection results indicate that MalR is also important in *B. pseudomallei*. To test whether *B. pseudomallei* MalR regulates the *mal* genes, we introduced a short-half-life P*malA-luxCDABE* reporter into the neutral *glmS3* site in the genome of our *malR* mutant and the Bp82 parent and monitored reporter induction in each strain grown with or without trimethoprim. Not surprisingly, we observed little to no P*malA-luxCDABE* reporter induction in either strain grown without trimethoprim (Fig. 3) At 15 μg/ml (a concentration that reduced the growth yield by 50% at 24 h), trimethoprim activated the Bp82 reporter by about 6-fold, with maximal induction at 8 to 10 h (Fig. 3). However, there

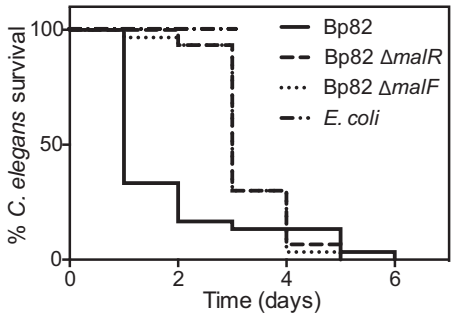

FIG 2 Malleilactone contributes to *B. pseudomallei* killing of *C. elegans*. Death of *C. elegans* over time when placed on a lawn of *B. pseudomallei* Bp82 WT, Bp82 Δ*malF*, or Bp82 Δ*malR* or *E. coli* OP50 cells. In the case of *E. coli*, generation of new offspring prevented survival counts after day 3. Results are from one experiment with 30 worms on each strain, but they are representative of similar experiments done on separate days. 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 median survival of each mutant is statistically different from that of the Bp82 parent (median survival is 3 days for Δ*malF* and Δ*malR* strains and 1 day for WT; $P < 0.0001$).

was no trimethoprim-dependent reporter induction in *malR* mutant cells, showing that MalR is important for trimethoprim activation of *mal* gene transcription. In *B. thailandensis*, MalR is not activated by trimethoprim directly, but rather the antibiotic increases MalR activity by driving *malR* transcription. We tested whether trimethoprim similarly increases *B. pseudomallei malR* transcription by using quantitative droplet digital PCR. Our results show that trimethoprim increases *B. pseudomallei malR* transcription 4-fold (see Fig. S4 in the supplemental material), consistent with the idea that trimethoprim activates *B. pseudomallei* MalR indirectly by driving *malR* transcription.

Our results suggest that MalR activity correlates with the level of *malR* expression. We sought to more directly test this hypothesis. We constructed an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *malR* expression cassette by fusing *malR* to the P*lac* promoter and moved this cassette into the neutral *glmS3* site in the genome of our *malR* mutant. We measured malleilactone production in this P*lac-malR*-containing strain and also in the *malR* mutant and Bp82 parent strains containing the empty P*lac* cassette (Fig. 4). Consistent with our previous results (Fig. 1B and 2), malleilactone was relatively low in the P*lac*-labeled Bp82 and even lower in the *malR* mutant. However, the *malR* mutant containing P*lac-malR* had malleilactone levels that exceeded those produced by Bp82 by more than 7-fold ($P < 0.015$). Thus, *malR* expression is sufficient to induce malleilactone production to high levels, even in the absence of trimethoprim.

Malleilactone production is repressed by *N*-3-hydroxy-octanoyl homoserine lactone and *N*-3-hydroxy-decanoyl homoserine lactone. Results with *B. thailandensis* indicate that MalR is not a typical LuxR family protein, in that it is not an AHL receptor.

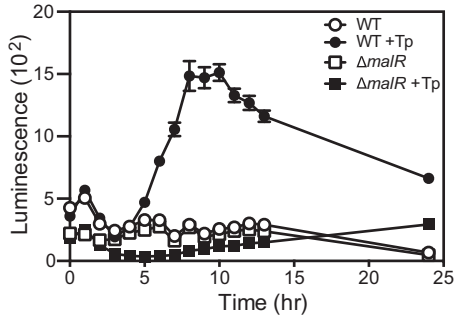

FIG 3 MalR is required for trimethoprim-dependent expression of *malA* in *B. pseudomallei*. Shown is the growth-adjusted activity of a P*malA-luxBCABE* chromosomal reporter in the *malR* mutant or its isogenic parent, Bp82 WT, grown with 15 μg/ml trimethoprim (Tp) or without trimethoprim. Results are the means and standard errors from three independent experiments.

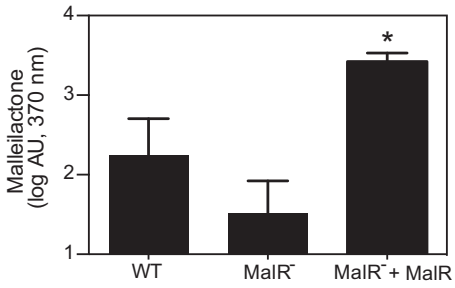

FIG 4 Expression of *lac* promoter-controlled *malR* is sufficient for malleilactone production in *B. pseudomallei*. Cells were grown with isopropyl-β-D-thiogalactopyranoside (IPTG) to induce *malR* expression, and cultures were collected at stationary phase (24 h), extracted with ethyl acetate, and assessed for relative malleilactone, as described in Materials and Methods. Data shown are the growth-adjusted peak areas corresponding with malleilactone at 370 nm (the malleilactone λ_{max}). WT, Bp82 with the P*lac* cassette; MalR⁻, Bp82 Δ*malR* with the P*lac* cassette; MalR⁻ + MalR, Bp82 Δ*malR* with the P*lac-malR* cassette. All cultures were grown with 1 mM IPTG. Results are the means and standard errors from three independent experiments. *, statistical significance by *t* test ($P < 0.015$), compared with both WT and MalR⁻.

Previous studies with recombinant *E. coli* showed that *B. thailandensis* MalR activation of the *malA* promoter is not influenced, either positively or negatively, by AHLs (20). MalR proteins in *B. pseudomallei* and *B. thailandensis* differ by only 5 amino acids. Some of these changes could possibly affect the ability of MalR to respond to AHLs. Thus, we engineered plasmids to test *B. pseudomallei* MalR activity in recombinant *E. coli*. We used a plasmid with an arabinose-inducible *B. pseudomallei malR* and another plasmid containing a 1,000-bp fragment containing the *B. pseudomallei malA* promoter (from positions −1 to −1000 with respect to the predicted MalA translational start site) fused to a promoterless *lacZ* gene. In *E. coli*, *lacZ* activation required arabinose induction of *B. pseudomallei* MalR and was not influenced by any of the AHLs tested (see Fig. S5 in the supplemental material), including the *B. pseudomallei* AHLs *N*-octanoyl homoserine lactone (C8-HSL), *N*-3-hydroxy-octanoyl homoserine lactone (3OHC8-HSL) and *N*-3-hydroxy-decanoyl homoserine lactone (3OHC10-HSL).

Our results indicate that *B. pseudomallei* MalR does not interact with AHLs directly. However, activation of the *mal* genes might be regulated by AHLs through other indirect mechanisms. To test this possibility, we used a *B. pseudomallei* mutant with deletions in all three of the AHL synthase genes (*bpsI1*, *bpsI2*, and *bpsI3*). We compared malleilactone produced by this strain with that of the wild type. In our experiments, the AHL mutant showed ~10-fold higher malleilactone than the Bp82 parent (Fig. 5A). Malleilactone levels were restored to wild-type levels by exogenously adding all three of the *B. pseudomallei*-produced AHLs to the growth medium (5 μM each). When added individually, 3OHC8-HSL, and to a lesser extent 3OHC10-HSL, repressed malleilactone production but C8-HSL did not.

How does quorum sensing repress malleilactone production? Based on our previous results, MalR is a likely candidate for indirect regulation by AHLs. In support of this idea, introducing a *malR* deletion to the AHL synthase mutant reduced malleilactone to almost undetectable levels (Fig. 5A). We hypothesized that AHLs regulate MalR indirectly by repressing *malR* expression. To test this hypothesis, we assessed *malR* expression in the triple AHL mutant and wild-type parent throughout growth. We also assessed *malR* expression in the AHL mutant with 3OHC8-HSL added to the culture medium. We sampled cells at three culture optical densities, namely, 2 (the transition between logarithmic and stationary phase), 4 (stationary phrase), and 6 (stationary phase). For all three strains, *malR* expression was relatively low until the culture reached an optical density of 6. At this density, *malR* levels were almost 2-fold higher in the AHL mutant compared with those of the wild type, and more than 3-fold higher in the AHL mutant compared with those of the mutant grown with AHLs ($P < 0.05$) (Fig. 5B). We also assessed *malA* expression in these cells. Our results showed that the pattern of

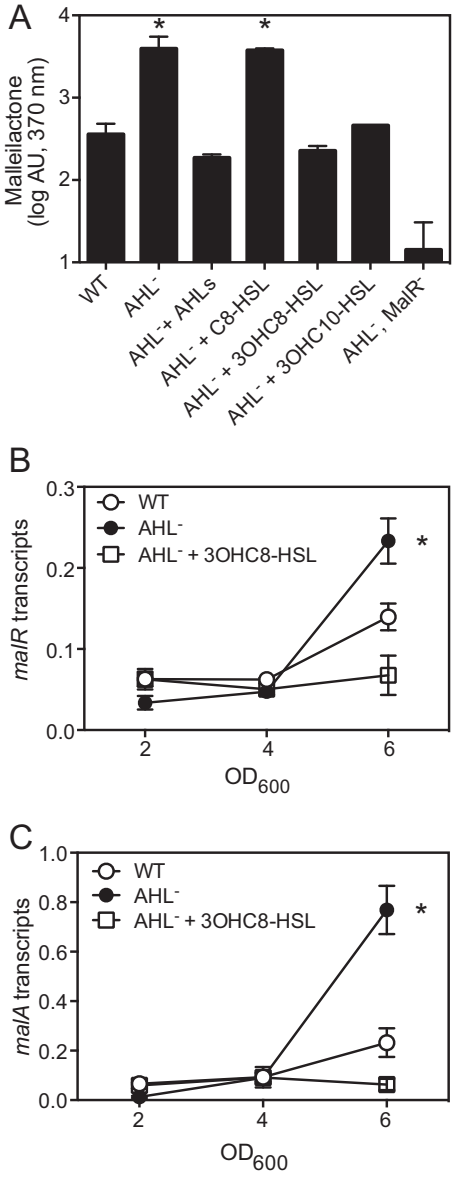

FIG 5 AHL-dependent regulation of malleilactone biosynthesis. (A) Cultures were collected during stationary phase (24 h), extracted with ethyl acetate, and assessed for relative malleilactone, as described in Materials and Methods. Data shown are the growth-adjusted peak areas corresponding with malleilactone at 370 nm (the malleilactone λ_{max}). WT, Bp82; AHL$^-$, Bp82 with deletions in all three of the AHL synthase genes (*bpsI1* to *-3*) (CM139); AHLs, 5 μM (each or individually, as indicated) C8-HSL, 3OHC8-HSL, and 3OHC10-HSL; AHL$^-$ MalR$^-$, Bp82 with deletions of *bpsI1* to *-3* and *malR*. (B and C) AHL-dependent regulation of *malR* (B) and *malA* (C) transcription in Bp82 WT, AHL$^-$ cells, and AHL$^-$ cells with 5 μM 3OHC8-HSL added to the culture medium. Transcript abundance was normalized to the *rpoD* RNA polymerase sigma factor gene transcripts. In all cases, results are the means and standard errors from three independent experiments. *, statistical significance by *t* test compared with the wild type ($P \leq 0.05$).

malA expression was similar to that of *malR*, with maximal induction at a culture optical density of 6 (Fig. 5C). These results are consistent with the idea that *malR* transcription is induced in the AHL mutant, causing subsequent induction of the malleilactone biosynthesis genes. Our results suggest that *malR* expression is repressed by AHLs very late in growth. It is possible that other mechanisms repress *malR* at lower culture densities.

Malleilactone production is repressed by a quorum sensing-activated regulator, ScmR. The *B. pseudomallei* AHL-responsive regulators BpsR1, BpsR2, and BpsR3

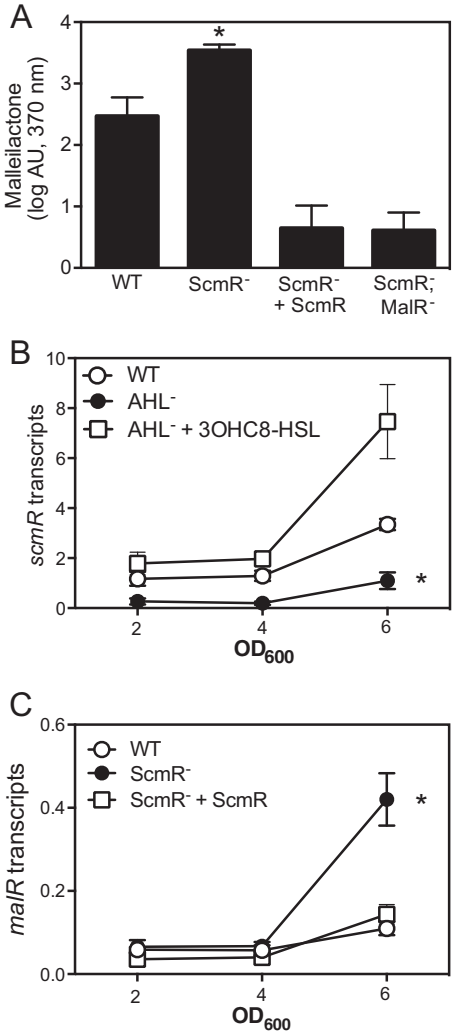

FIG 6 Regulation and activity of *B. pseudomallei* ScmR. (A) Cultures were collected during stationary phase (24 h), extracted with ethyl acetate, and assessed for relative malleilactone, as described in Materials and Methods. Data shown are the growth-adjusted peak areas corresponding with malleilactone at 370 nm (the malleilactone λ_{max}). WT, Bp82 with the P*lac* cassette inserted into the *glmS3* site in the genome; ScmR$^-$, Bp82 *glmS3*::P*lac* with a deletion in *scmR*; ScmR$^-$ + ScmR, Bp82 with P*lac-scmR* inserted into *glmS3*; ScmR,$^-$ MalR$^-$, Bp82 *glmS3*::P*lac* with deletions in *scmR* and *malR*. (B) AHL-dependent regulation of *scmR* transcription. WT, Bp82; AHL$^-$, Bp82 with deletions in all three of the AHL synthase genes (*bpsI1* to *-3*) (CM139); AHL$^-$ + 3OHC8-HSL, CM139 with 5 μM 3OHC8-HSL added to the culture medium. (C) ScmR-dependent regulation of *malR* transcription. Strains are described in the panel A legend. Transcript abundance (B and C) was normalized to the *rpoD* RNA polymerase sigma factor gene transcripts. Strains in panels A and C were grown with 1 mM IPTG. In all cases, results are the means and standard errors from three independent experiments. *, statistical significance by *t* test ($P \leq 0.05$).

typically function as gene promoter activators (27–30); thus, we hypothesized that quorum sensing represses *malR* indirectly through another regulator. A likely candidate is the putative transcriptional regulator ScmR. In *B. thailandensis*, ScmR is a quorum sensing-activated repressor of malleilactone production and other secondary metabolites (31). The *B. pseudomallei* and *B. thailandensis* ScmR proteins are 99% identical. To test the role of *B. pseudomallei* ScmR in malleilactone production, we deleted *scmR* in Bp82 and compared malleilactone produced by the *scmR* mutant to that of the parent. Our results showed that deleting *scmR* increased malleilactone production almost 4-fold (Fig. 6A) ($P < 0.01$). We were able to restore malleilactone production in the *scmR* mutant to that of the wild type by expressing *scmR* from the IPTG-inducible P*lac* promoter (Fig. 6A). To test whether *B. pseudomallei* quorum sensing activates *scmR* transcription, we measured *scmR* transcripts in our triple AHL mutant, the wild-type

parent, and the AHL mutant supplemented with 3OHC8-HSL. We found that *scmR* transcripts were 3-fold lower in the AHL mutant than in wild-type cells ($P < 0.01$) (Fig. 6B), suggesting that *B. pseudomallei scmR* transcription is controlled by quorum sensing. We also assessed the importance of MalR in ScmR-dependent regulation of malleilactone production. MalR is required for induction of malleilactone in the *scmR* mutant, because deleting *malR* in the *scmR* mutant nearly abolished malleilactone production (Fig. 6A). We hypothesized that ScmR might repress malleilactone production by decreasing *malR* transcription. To test this hypothesis, we measured *malR* transcripts in an *scmR* mutant and compared these with those of the wild type at culture optical densities of 2, 4, and 6. In support of our hypothesis, *malR* transcripts were 2-fold higher in the *scmR* mutant compared with those of identically grown wild-type cells during later growth ($P < 0.05$) (Fig. 6C), a pattern consistent with that of *malR* expression in the AHL synthase mutant (Fig. 5B). Together, our results support the idea that malleilactone production is controlled by quorum sensing indirectly through ScmR, and that ScmR acts by repressing transcription of *malR*, thereby explaining why little to no malleilactone is produced under standard laboratory conditions.

DISCUSSION

The *mal* gene cluster is well conserved in *B. thailandensis*, *B. pseudomallei*, and also *B. mallei* (~80% identity). These three species also share a high degree of genetic similarity. Yet, they each have a distinctly different lifestyle. *B. thailandensis* is a saprophyte, *B. pseudomallei* is an opportunistic pathogen, and *B. mallei* is a host-restricted pathogen that causes a disease called glanders. We are interested in studying this group because it provides a unique opportunity to understand how homologous systems adapt to different lifestyles. The finding that the malleilactone biosynthesis cluster is conserved in all three species suggests that it is important for diverse lifestyles. In a saprophytic lifestyle, malleilactone might be important for competition with other strains or species of bacteria. Malleilactone is also important for virulence in an animal host (Fig. 2) (8). There is evidence from recent transposon sequencing (Tn-seq) studies that malleilactone contributes to pathogenesis in mouse models of melioidosis (32, 33). These studies use pools of *B. pseudomallei* transposon mutant populations to infect the lungs of mice, and in these studies, insertions in *malR*, *malA*, and some of the other *mal* biosynthetic genes reduced fitness in the mouse lung and spleen (e.g., *malA* mutants were 3-fold and 39-fold reduced in mouse lungs and spleen, respectively) (32, 33). The exact role of malleilactone in *B. pseudomallei* fitness during infections *in vivo* remains to be determined.

The regulatory circuits controlling malleilactone production appear to be well conserved in *B. pseudomallei* and *B. thailandensis*. The *B. pseudomallei mal* genes are induced by at least some of the same antibiotics as the *B. thailandensis mal* genes, such as trimethoprim, piperacillin, ceftazidime, and ciprofloxacin (15, 20) (Table 1). Furthermore, the *mal* genes in both species are not induced by kanamycin (15, 20) (Table 1). These results suggest that the pathway of antibiotic-dependent regulation of the *mal* genes might be conserved in these two species. Quorum-sensing repression of *mal* gene expression is also similar in *B. thailandensis* and *B. pseudomallei*. In *B. pseudomallei*, malleilactone production is repressed by 3OHC8-HSL and 3OHC10-HSL (Fig. 5A). These AHLs are specific to the BpsR2-I2 system (27). This system is conserved in *B. thailandensis*, where it also represses malleilactone production (31). However, this system is missing from the *B. mallei* genome. Instead, in *B. mallei* the *mal* genes appear to be controlled by the C8-HSL-responsive system BmaR1-I1 (homologous to BpsR1-I1) (17). Despite the potential variation in which system controls malleilactone production, the conservation of quorum control of malleilactone production across these three species suggests an important association with cell density that is broadly beneficial in this group.

Our results show that ScmR represses malleilactone production in *B. pseudomallei*. They support a model whereby BpsR2-I2 activates production of the ScmR repressor

during later stages of growth, and ScmR decreases malleilactone production by repressing transcription of *malR* (Fig. 6). ScmR also represses malleilactone production in *B. thailandensis*; however, in *B. thailandensis* ScmR was not shown to repress *malR* during late log phase (31). It is possible that *malR* that repression also occurs in *B. thailandensis* but only in late stationary phase, as it does in *B. pseudomallei* (Fig. 6). Alternatively, this result might represent a difference in the regulatory circuits of *B. thailandensis* and *B. pseudomallei*. The finding that ScmR represses malleilactone in *B. pseudomallei* suggests that ScmR might function as a global repressor of *B. pseudomallei* secondary metabolism, as it does in *B. thailandensis*. ScmR is also conserved in *B. mallei*, the causative agent of the disease glanders, and might be a useful target for eliciting production of other secondary metabolites in the *B. pseudomallei*-group pathogens. As some ScmR-dependent secondary metabolites are implicated in virulence (31), ScmR might also serve as a target for development of novel antivirulence therapeutics to treat melioidosis and glanders.

Our results show that *B. pseudomallei* malleilactone is structurally identical to that of *B. thailandensis* and corroborate previous studies showing that malleilactone is cytotoxic to both cultured mammalian cells and several kinds of Gram-positive bacteria. In our experiments, *B. thailandensis* and *B. pseudomallei* were relatively insensitive to malleilactone (MIC $>$ 200 μg/ml) (Table S1). Previously, we showed that *B. thailandensis* growth is slowed by about 50% when 10 to 15 μg/ml of malleilactone is added to culture flasks (20). These results suggest that *B. thailandensis*, and possibly other bacterial species, is sensitive to malleilactone under certain conditions. In addition to cytotoxicity, malleilactone could also have another role as an iron-chelating molecule that binds or possibly imports iron. The butyrolactone core of malleilactone has the potential to coordinate metal ions, and malleilactone has been shown to weakly interact with iron (8). Furthermore, the *mal* genes are transcriptionally induced during growth in low iron (34), suggesting a possible link to extracellular iron levels (35).

MATERIALS AND METHODS

Bacterial strains, culture conditions, and reagents. All bacterial strains, plasmids, and primers used in this study are listed in Tables S3 to S5 in the supplemental material. We used *B. pseudomallei* strain Bp82 (4), a select agent-excluded Δ*purM* derivative of the fully virulent strain 1026b. Bp82 is an adenine and thiamine auxotroph. We refer to Bp82 as wild-type *B. pseudomallei*. We used *E. coli* strains DH5α and DH10B (Invitrogen) for genetic manipulations and strain BW27783 (36), which constitutively expresses the arabinose transporter, for recombinant *E. coli* experiments. Bacteria were grown in Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter) supplemented with 50 mM morpholinepropanesulfonic acid (MOPS) where indicated, or in M9 minimal medium supplemented with 0.4% glucose and 10 mM *para*-chloro-phenylalanine (*p*-Cl-Phe; Sigma) for *B. pseudomallei* counterselection during mutant construction. All *B. pseudomallei* growth media were supplemented with 1.6 mM adenine hemisulfate and 0.005% thiamine HCl, with the exception of LB agar plates, which were not supplemented. All growth was at 37°C with shaking at 250 rpm, with the exception of *C. elegans* infection assays, which were at 20°C (see below). Acyl-homoserine lactones were purchased from Sigma-Aldrich and Caymen Chemicals, stored in ethyl acetate acidified with 0.1 ml/liter glacial acetic acid, and added to empty culture tubes and dried down prior to addition of bacterial cultures. When appropriate, the following antibiotics were used (per ml): 15 μg (*E. coli*) and 100 μg (*B. pseudomallei*) gentamicin, 100 μg ampicillin (*E. coli*), 50 μg (*E. coli*) and 1 mg (*B. pseudomallei*) kanamycin, and 25 μg (*E. coli*) and 2 mg (*B. pseudomallei*) zeocin. We added 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) as indicated.

We assayed β-galactosidase activity by using a Tropix Galacto-Light Plus chemiluminescence kit according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). In all cases, β-galactosidase activity is reported as light units relative to the optical density at 600 nm (OD_{600}). Genomic DNA, PCR and DNA fragments, and plasmid DNA were purified by using a Puregene Core A kit, plasmid purification miniprep kit, or PCR cleanup/gel extraction kits (Qiagen or IBI-MidSci) according to the manufacturer's protocol.

Genetic manipulations. Unmarked, in-frame deletions of *malR*, *malF*, and *scmR* were constructed using previously described methods (37). Briefly, DNA fragments containing ~500 to 1,000 bp of DNA flanking each gene were generated by PCR or DNA synthesis (Genscript, New Jersey) and fused together to create an unmarked, nonpolar deletion of each gene with incorporated HindIII and XbaI sites. These fragments were digested with HindIII and XbaI and cloned into HindIII- and XbaI-digested pEX18Km-*pheS*, which was transformed into Bp82 by electroporation. Merodiploids were selected on LB agar containing kanamycin, and deletion mutants were counterselected on M9 agar with *p*-Cl-Phe. Mutant strains were verified by testing for kanamycin sensitivity and by PCR-amplifying the deletion region and sequencing the PCR product. The pUC18miniTn7T derivatives were introduced by electroporation into *B.*

pseudomallei strains with the helper plasmid pTNS3, as previously described (38). We used PCR to verify insertion into the *attn7* site near *glmS3*, as previously described (38).

For recombinant *malR* expression in *E. coli*, *malR* was amplified from genomic DNA isolated from *B. pseudomallei* Bp82 by using primers that incorporated restriction sites (EcoRI and XmaI) into the product. The amplicon was cut with EcoRI and XmaI and ligated to EcoRI- and XmaI-digested pJN105 to make pJN105 P_{BAD}-*malR*. To generate a *lacZ* fusion to the *malA* promoter for recombinant *E. coli* experiments, the region upstream of *B. pseudomallei malA* extending from positions −1 to −476 with respect to the translational start site was synthesized (Genscript, New Jersey), with restriction sites (NcoI and HindIII) incorporated into the product. The G block was digested with NcoI and HindIII and ligated to NcoI- and HindIII-digested pQF50 to make pQF50 P*malA-lacZ*. To make the P*lac-malR* expression cassette, we first constructed the IPTG-inducible expression vector pUC18-mini-Tn7T-Km-P*lac* by digesting the kanamycin (Km) resistance gene from pUC18-mini-Tn7T-Km-FRT (38) with SalI and NsiI and using this fragment to replace the trimethoprim (Tp) resistance gene in SalI-NsiI-digested pUC18-min-Tn7T-LAC-Tp (20) to make pUC18-mini-Tn7T-LAC-Km. We then cloned *malR* into this vector by PCR-amplifying *malR* from the Bp82 genome using primers that incorporated restriction sites (SacI and HindIII) into the product. The amplicon was cut with SacI and HindIII and ligated into SacI- and HindIII-digested pUC18-mini-Tn7T-Km-P*lac* to generate pUC18-mini-Tn7T-Km-P*lac-malR*. The P*lac-scmR* expression cassette was made by introducing an EcoRI-HindIII-cut *scmR* PCR-generated amplicon to the EcoRI-HindIII-cut pUC18-mini-Tn7T-Km P*lac-malR*. To make the pUC18-mini-Tn7T-Zeo-P*malA-lux* reporter plasmid, the *B. pseudomallei malA* promoter region from bp −1 to −1,244 with respect to the translational start site was amplified from Bp82 genomic DNA using primers that incorporated BamHI and PstI. The fragment was digested with BamHI and PstI and then ligated into BamHI- and PstI-digested plasmid pCM53 (promoterless *lux* reporter plasmid) (39). To make the pUC18-mini-Tn7T-Zeo-P*malA-lacZ* reporter plasmid, we first constructed a pUC18-mini-Tn7T-Zeo-P*malA*-GFP intermediate plasmid. This was done by PCR-amplifying the GFP gene from pUC18-mini-Tn7T-Kan-GFP (40) and incorporating restriction sites PstI and SpeI, PCR-amplifying the promoter and open reading frame of the zeocin resistance gene (*ble*) from pUC18-mini-Tn7T-Zeo-P*malA-lux* and incorporating restriction sites SpeI and StuI, and ligating both of those fragments into PstI- and StuI-digested pUC18-mini-Tn7T-Zeo-P*malA-lux* (releasing the *luxCDABE* cassette and the *ble* gene). Then, the *lacZ* gene was subcloned from the pUC18-mini-Tn7T-Gm-*lacZ* plasmid (41) by digesting with AseI, treating with Klenow (New England BioLabs) to blunt-end the fragment, and then digestion with PstI. The *lacZ* was ligated to pUC18-mini-Tn7T-Zeo-P*malA*-GFP plasmid digested with SpeI, Klenow-treated, and then digested with PstI (to remove the GFP gene) to generate pUC18-mini-Tn7T-Zeo-P*malA-lacZ*. In all cases, constructs generated using PCR were verified by sequencing.

HR-LC-MS measurements. For measuring malleilactone in *B. pseudomallei* cultures, samples were prepared by diluting stationary-phase *B. pseudomallei* cultures to an OD_{600} of 0.05 into 50 ml of LB-MOPS in a 250-ml flask, and growing cultures for 24 h with 250 rpm shaking at 37°C. *B. pseudomallei* growth media was supplemented with adenine and thiamine, and, where indicated, cultures were also supplemented with trimethoprim at 4.5 μg/ml, IPTG at 1 mM, or synthetic AHLs at 5 μM each. Each culture was extracted with an equivalent volume of ethyl acetate, and the organic phase was collected using a separatory funnel. The organic phase was then dried down under nitrogen air and suspended in 300 μl dimethyl sulfoxide (DMSO) plus 1,300 μl acetonitrile. Subsequent dilutions in acetonitrile were then performed as needed. Injections of 2 μl each were made on nm Acquity ultraperformance liquid chromatography (UPLC) system (Waters) with a wash of 500 μl acetonitrile and 800 μl of water/methanol (1:1) between each injection. The UPLC was coupled to a photodiode array detector and an LCT Premier TOF mass spectrometer (Waters), and the system was fitted with an Acquity C_{18} ethylene bridged hybrid (BEH) column (2.1 × 50 mm, 1.7 μm; Waters). The chromatography was developed using an aqueous mobile phase with NH_4OH at pH 9.8 and an organic phase of acetonitrile increased by a gradient of 5% to 99% over 2.7 min and then held at 99% acetonitrile for 0.4 min; the flow rate was 0.6 ml/min. Relative malleilactone was reported as the growth-adjusted total area under the 370 nm peak corresponding with malleilactone (the λ_{max} of malleilactone is 373 nm). In all cases, fragments were monitored using mass spectrometry to validate the molecular weight of malleilactone in the expected peak.

Mass spectral data were collected by negative ion electrospray from 100 to 1,100 *m/z*, with capillary set to 2,500 V, desolvation gas set to 300°C, and cone set to 35 V. Relative malleilactone was reported using the peak area at 370 nm, the λ_{max} of malleilactone. For LC-MS/MS experiments, negative ion electrospray spectra were acquired on a Waters Qtof Premier (Waters/Micromass, Manchester, UK) mass spectrometer operated in v mode at a resolution of 10,000. Spectra were acquired over the mass range 50 to 1,000 u, accumulating data for 3 s per cycle. LC-MS/MS spectra were simultaneously collected using collision-induced dissociation (CID), using an MS1 quadrupole set to transmit the precursor mass (*m/z* 305.1), with a selection window of 4 u. The collision energy was ramped from 15 to 45 eV per acquisition cycle to obtain a distribution of fragments from low to high mass. Product ions in the CID spectra ranged from the same mass to 2 mmu and the relative abundances of CID fragments ranged from the same to better than 5%.

***B. pseudomallei* malleilactone purification and analysis.** Samples for malleilactone purification were grown and ethyl acetate extracted as described for mass spectrometry analysis, except growth was in 650 ml of media with 4.5 μg/ml trimethoprim in a 1.6-liter flask, and the dried ethyl acetate extract was reconstituted in 10 ml acetonitrile. For comparison, *B. thailandensis* cultures were grown similarly except with 15 μg/ml trimethoprim and no adenine or thiamine added to the growth media. For malleilactone purification, two separate rounds of chromatography were performed on a Waters autopurification system equipped with a Waters 996 photodiode array (PDA), a 2525 binary pump, a 2767

sample manager, a Waters ZQ single quad mass spectrometer, and a Waters XBridge C_{18} column (5 μm, 19 by 150 mm). For the first round of chromatography, injections of 1,200 μl each were made using an aqueous mobile phase with NH_4OH at pH 9.8 and an organic phase of acetonitrile which increased by a gradient from 25% to 45% over 4 min, followed by a hold at 100% for 2 min; the flow rate was 20 μl/min. The fractions were pooled and evaporated using a Genevac instrument at 35°C, and the residue was reconstituted in 10 ml of acetonitrile. For the second round of chromatography, injections of 1,200 μl each were made using an aqueous mobile phase with 20 mM NH_4HCOO at pH 7.0 and an organic mobile phase of acetonitrile with a gradient of 35% to 55% over 4 min, followed by a hold at 100% for 2 min. Fractions were triggered by UV at 370 nm and mass at 305 *m/z* with a mass spectrometer set to negative ionization, with the capillary at 2.9 kV, the source at 100°C, and the cone at 50 V. Final fractions were pooled and evaporated under a stream of N_2 gas and stored dry at −20°C. For NMR characterization, purified malleilactone was dissolved in $CDCl_3$ (Cambridge Isotopes) and transferred to a 5-mm tube (Wilmad-LabGlass). All NMR spectra were acquired on a 500 MHz AVIII spectrometer (Bruker) equipped with a cryogenically cooled broadband observe probe. Data were processed and visualized using Topspin (Bruker) and MestReNova (Mestrelab) software.

Malleilactone susceptibility experiments. The MICs of malleilactone against bacterial pathogens were determined using a protocol adapted from the 2003 guidelines of the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS), using the microtiter MIC method. Inocula for each test organism were prepared by suspending a colony from an LB agar plate into tryptic soy broth and growing for 3 to 5 h at 35°C with shaking, then adjusting the culture turbidity in phosphate-buffered saline (PBS) to the equivalent of a 1.0 McFarland standard (3×10^8 CFU/ml). These cell suspensions were used as inocula for microtiter MIC assays. Inocula (2.5 μl), which each corresponded to 1×10^6 cells, was added to 100 μl wells, each containing malleilactone diluted in cation-adjusted Mueller-Hinton II broth, and these were incubated with shaking for 24 h at 37°C. The MIC was defined as the lowest concentration of malleilactone (in micrograms per milliliter) in which bacterial growth in the well was not measurable by determining the turbidity at 600 nm (A_{600}) on a BioTek Synergy 2 plate reader.

Malleilactone cytotoxicity experiments. The minimum concentration causing 50% loss of viability (IC_{50}) of malleilactone against cultured A549 and Jurkat cells was measured using propidium iodide staining and flow cytometry (42). Briefly, A549 cells (ATCC CCL-185) were cultured in Dulbecco's modified Eagle medium (DMEM) (D6429; Sigma). Jurkat cells (ATCC TIB-152) were cultured in RPMI 1640 medium (R8758; Sigma). Media was supplemented with fetal bovine serum (FBS; 10%), penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were maintained in a humidified 5% CO_2 incubator at 37°C. A 96-well microtiter plate was seeded with 8,000 cells in 200 μl medium per well and grown at 37°C for 16 h. Cells were then treated with fresh medium containing concentrations of malleilactone ranging from 10^{-12} to 10^{-4} M. After incubation at 37 °C for 48 h, adherent A549 cells were washed with phosphate-buffered saline (pH 7.4) and suspended by treatment with trypsin-EDTA solution (50 μl) for 10 min at 37°C, followed by addition of propidium iodide (3 μM final concentration) in complete medium (100 μl). For Jurkat cells in suspension, propidium iodide (2 μl, 3 μM final concentration) was added directly to the medium. Viable cells were quantified by light scattering and exclusion of propidium iodide using a CytoFLEX flow cytometer. The data from 3 replicates were used to generate a dose-responsive curve. These curves were fitted by nonlinear regression with an inhibitor versus response variable-slope 4-parameter model (GraphPad Prism 7) to determine the concentration that causes 50% loss of viability (IC_{50}) values.

***C. elegans* infections.** To assess the contributions of MalR and malleilactone genes in *B. pseudomallei* virulence, we used a *Caenorhabditis elegans* infection model using the N2 Bristol wild-type *C. elegans* strain. *C. elegans* were cultured at 20°C on solid nematode growth medium (NGM; 3 g NaCl, 2.5 g Bacto peptone, 17 g Bacto agar, 5 μg cholesterol, 1 mM [each] $CaCl_2$ and $MgSO_4$, and 1 mM KPO_4 buffer [pH 6.0] per liter), and the *E. coli* strain OP50 was used as the *C. elegans* food source. When *C. elegans* worms cultured on *B. pseudomallei* strain cultures, 3.2 mM adenine and 0.010% thiamine were added to the NGM plates. For infection assays, overnight cultures of *B. pseudomallei* or the *E. coli* OP50 food source were diluted to an OD_{600} of 0.1, 30 to 40 μl were spread onto the center of the NGM plates, and the plates were incubated overnight at 37°C. Plates were acclimated to room temperature for ~1 h prior to seeding the plates with *C. elegans* (10 late larval [L4]-staged worms per plate). Survival of the worms was monitored every 24 h until all worms were dead or until generation of new offspring prevented survival counts (in the case of *E. coli*). Of note, in every case, few to no deaths of OP50-fed worms were observed, and very few offspring were ever generated by worms that were fed *B. pseudomallei*.

Droplet digital PCR. RNA was harvested from *B. pseudomallei* cells at the indicated durations or optical densities, using methods described previously (43). Droplet digital PCR was performed on QX200 droplet digital PCR (ddPCR) (Bio-Rad) system using EvaGreen Supermix. Each reaction mixture contained 0.25 to 0.5 ng of cDNA template, 900 nM each primer, and 10 ul EvaGreen Supermix in a 20-μl final volume. After generating 40 μl of oil droplets, 40 rounds of PCR were conducted using the following cycling conditions: 95°C for 30 s, 58°C for 30 s, and 68°C for 30 s. Absolute transcript levels were determined using QuantaSoft software (Bio-Rad). In all cases, a no-template control reaction was performed, which contained no detectable transcripts. The *rpoD* RNA polymerase sigma factor gene was used as a reference gene unless otherwise noted, and the results are reported as the calculated transcript amount of a given gene per calculated *rpoD* transcript.

Transcription reporter assays. To assess MalR activation of *malA* in *B. pseudomallei*, we used a *B. pseudomallei* strain with either a P*malA-lacZ* or a P*malA-luxCDABE* fusion cassette introduced to the neutral *glmS3* site in the chromosome (see Table S3 in the supplemental material). Logarithmic-phase cultures were diluted to an OD_{600} of 0.05 in LB broth supplemented with antibiotics at the concentrations

indicated. Reporter activity was determined by measuring β-galactosidase activity as described above or by direct luminescence measurements on a BioTek Synergy 2 plate reader.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00008-18.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

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