A *Burkholderia thailandensis* Acyl-Homoserine Lactone-Independent Orphan LuxR Homolog That Activates Production of the Cytotoxin Malleilactone

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ABSTRACT

Burkholderia thailandensis has three acyl-homoserine lactone (AHL) LuxR-LuxI quorum-sensing circuits and two orphan LuxR homologs. Orphans are LuxR-type transcription factors that do not have cognate LuxI-type AHL synthases. One of the orphans, MalR, is genetically linked to the *mal* gene cluster, which encodes enzymes required for production of the cytotoxic polyketide malleilactone. Under normal laboratory conditions the *mal* gene cluster is silent; however, antibiotics like trimethoprim induce *mal* transcription. We show that trimethoprim-dependent induction of the *mal* genes requires MalR. MalR has all of the conserved amino acid residues characteristic of AHL-responsive LuxR homologs, but in *B. thailandensis*, MalR activation of malleilactone synthesis genes is not responsive to AHLs. MalR can activate transcription from the *mal* promoter in *E. coli* without addition of AHLs or trimethoprim. Expression of *malR* in *B. thailandensis* is induced by trimethoprim. Our data indicate that MalR binds to a *lux* box-like element in the *mal* promoter and activates transcription of the *mal* genes in an AHL-independent manner. Antibiotics like trimethoprim appear to activate *mal* gene expression indirectly by somehow activating *malR* expression. MalR activation of the *mal* genes represents an example of a LuxR homolog that is not a receptor for an AHL quorum-sensing signal. Our evidence is consistent with the idea that *mal* gene activation depends solely on sufficient transcription of the *malR* gene.

IMPORTANCE

LuxR proteins are transcription factors that are typically activated by acyl-homoserine lactone (AHL) signals. We demonstrate that a conserved LuxR family protein, MalR, activates genes independently of AHLs. MalR is required for transcription of genes coding for synthesis of the cytotoxic polyketide malleilactone. These genes are not expressed when cells are grown under normal laboratory conditions. In laboratory culture, MalR induction of malleilactone requires certain antibiotics, such as trimethoprim, which increase *malR* expression by an unknown mechanism. At sufficient levels of *malR* expression, MalR functions independently of any external signal. Our findings show that MalR is an activator of the silent malleilactone biosynthesis genes and that MalR functions independently of AHLs.

Acyl-homoserine lactone (AHL) quorum-sensing systems are widespread among the *Proteobacteria*. AHLs are generated by members of the LuxI family of AHL synthases. Members of the LuxR protein family serve as cognate AHL-dependent transcription factors. Often, the genes targeted by LuxR family members have an 18- to 20-base inverted repeat in their promoter regions that shows conservation across species. These repeat elements are called *lux* box-like sequences and serve as binding sites for the transcription factors. Sequence identity in pairwise comparisons of LuxR polypeptides is rather limited (18 to 35%), but there is a conserved N-terminal AHL binding region and a conserved C-terminal DNA binding region where sequence identity is higher and where there are highly conserved residues (see references 1 and 2 for reviews).

Burkholderia thailandensis has three pairs of *luxI-luxR*-type genes called *btaI1-btaR1*, *btaI2-btaR2*, and *btaI3-btaR3*. Each of the *luxR* homologs is linked to its cognate *luxI* homolog, as is the case for *luxI-luxR*-type gene pairs in many other bacteria. This species also has two additional *luxR* homologs, *btaR4* (now called *malR* [see below]) and *btaR5* (3). LuxR family members like MalR and BtaR5 are called orphans (4) or solos (5), because the genes encoding these polypeptides are not associated with a *luxI* ho-

molog. Relatively few orphans have been characterized. Some orphans, like *Pseudomonas aeruginosa* QscR or *Salmonella enterica* serovar Typhimurium SdiA, show complete conservation of all of the invariant residues in the signal binding region of AHL-responsive LuxR family members and in fact respond to AHLs that are self-produced (6) or exogenously supplied (7, 8). Other LuxR family members, like *Xanthomonas oryzae* OryR and *Photorhab-*

dus asymbiotica PauR, show conservation in the AHL binding region but vary with respect to at least one of the conserved residues. OryR and PauR do not respond to AHLs. Rather, they respond to host-derived factors or an endogenously produced secondary metabolite, respectively (9, 10).

We are interested in the *B. thailandensis* MalR protein for several reasons. First, it shows complete identity in all of the residues conserved among AHL-responsive LuxR family members. Second, it is adjacent to and divergently transcribed from the *mal* gene cluster, which is required for the production of the *B. thailandensis* cytotoxic polyketide malleilactone. The genes for MalR and malleilactone biosynthesis are silent when *B. thailandensis* is grown in standard laboratory media but expressed in cells grown with certain antibiotics, such as trimethoprim (11). Third, *malR* and the *mal* genes are required for *Caenorhabditis elegans* infections (12). There is a *lux* box-like sequence in the promoter region of the *mal* operon. One might imagine that MalR is an AHL-responsive activator of the *mal* operon. However, the *mal* operon is silent under laboratory conditions where AHLs are produced. Perhaps MalR binds to a signal other than an AHL. This would be unusual, but perhaps not unique (13), for a LuxR family member showing complete identity with the conserved residues in the AHL binding and DNA binding regions. We seek to understand how the *mal* operon is regulated because its product, malleilactone, appears to be a virulence factor and because it may be regulated by MalR in an unusual manner.

Here, we show MalR is required for trimethoprim activation of the malleilactone genes and *mal* gene transcription is stimulated by several, but not all, antibiotics. Activation of the *mal* genes does not involve any of the AHLs tested in *B. thailandensis* or in recombinant *Escherichia coli*. We also show that induction is not the consequence of a general antibiotic stress response, nor does it involve a direct interaction of MalR with a malleilactone-inducing antibiotic. Our results are consistent with a model whereby certain antibiotics activate expression of the silent *malR* gene and the *malR* gene product in turn activates transcription of genes for malleilactone synthesis.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and reagents. We used *B. thailandensis* strain E264 (14) and *E. coli* strain DH5α or MG4 for genetic manipulations and recombinant DNA expression, respectively (see Table S1 in the supplemental material). Our *B. thailandensis malA-lacZ* chromosomal insertion mutant (BT01447) was from a sequence-defined transposon mutant library (15). This mutant has a transposon insertion in the *malA* coding sequence after bp 5704 (out of 8,379) relative to the predicted *malA* translational start site. The other strains are listed in Table S1 in the supplemental material.

All *E. coli* growth was in Luria-Bertani (LB) broth at 37°C with shaking, and *B. thailandensis* was grown in morpholinepropanesulfonic acid (MOPS)-buffered LB broth (LB-MOPS) at 30°C with shaking. When appropriate, the following antibiotics were used (per milliliter): 100 μg trimethoprim (*E. coli* and *B. thailandensis*) and 15 μg gentamicin and 100 μg ampicillin (*E. coli*). We added IPTG (isopropyl-β-D-thiogalactopyranoside) as indicated.

We measured β-galactosidase activity with a Tropix Galacto-Light Plus chemiluminescence kit according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). β-Galactosidase activity in *B. thailandensis* 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 DNeasy blood and tissue kit, PCR, plasmid pu-

rification kit, or gel extraction kit (Qiagen) according to the manufacturer's protocol.

Genetic manipulations. We used standard procedures for DNA manipulations (16). To assess *malA-lacZ* expression in a *malR*-null background, an unmarked, in-frame *malR* deletion was introduced into the *malA-lacZ* reporter strain BT01447 by using the deletion construct pJRC115 *btaR4*, which we now call *malR*, and methods described previously (17). To assess *malA-lacZ* activity in an AHL synthase-deficient strain, we prepared genomic DNA from BT01447 and introduced it into the *btaI1 btaI2 btaI3* triple mutant JBT112 (17) by using natural transformation as described previously (18). The pUC18-mini-Tn7T derivatives were introduced into *B. thailandensis* strains with the helper plasmid pTNS2 by electroporation, as previously described (19). We used PCR to verify insertion into the *attn7* site near *glmS1* as previously described (15). Where indicated, we removed the trimethoprim resistance marker *dhfrIIb* from pUC18-mini-Tn7T derivatives by using plasmid pFLPe2, as described previously (20).

The plasmids are listed in Table S1 in the supplemental material. To generate pJN105.malR for recombinant *malR* expression in *E. coli*, *malR* was amplified from genomic DNA isolated from *B. thailandensis* E264 by using primers that incorporated restriction sites (NheI and SpeI) into the product. The amplicon was cut with NheI and SpeI and ligated into NheI-SpeI-digested pJN105 to make pJN105.malR. To generate the *E. coli malA-lacZ* expression vector pQF50.malA, the region upstream of *malA* extending from positions −1 to −500 with respect to the translational start site was amplified by PCR by using primers that incorporated restriction sites (NcoI and HindIII) into the product. The amplicon was digested with NcoI and HindIII and ligated to NcoI-HindIII-digested pQF50 to make pQF50.PmalA. To make the *malA-lacZ* expression vector with mutations in the putative *lux* box, we used a synthetic DNA fragment (IDT gBlock) identical to the PCR amplicon used to make pQF50.PmalA, except with the base substitutions T4C and G5A in the putative *lux* box. This gBlock was digested with NcoI and HindIII and ligated to NcoI-HindIII-digested pQF50 to make pQF50.mutPmalA. To make the p*lac-malR* expression cassette, we first constructed the IPTG-inducible expression vector pUC18-mini-Tn7T-LAC-Tp by digesting the fragment containing the *lac* promoter and *lacI* repressor gene from pUC18-mini-Tn7T-LAC with NsiI and SpeI and ligating it to NsiI-SpeI-digested pUC18-mini-Tn7T-Tp (21). We then cloned *malR* into this vector by cutting *malR* from pJN105.malR using the NheI and SpeI sites and ligating it to SpeI-cut pUC18-mini-Tn7T-LAC-Tp. We verified the orientation of *malR* with respect to the *lac* promoter in the resulting construct, pUC18-mini-Tn7T-LAC-Tp.malR, by PCR.

Transcription reporter assays. To assess MalR activation of *malA* expression in recombinant *E. coli*, we used *E. coli* MG4 with arabinose-inducible *malR* (pJN105.malR) and either pQF50.PmalA with *lacZ* fused to the wild-type *malA* promoter or pQF50.mutPmalA with *lacZ* fused to the T4C and G5A mutant *malA* promoter. Overnight cultures were used as starters by diluting them to an OD$_{600}$ of 0.05. When experimental cultures reached an OD$_{600}$ of 0.5, L-arabinose was added at the concentrations indicated to induce MalR expression. These cultures were added to sterile 16-mm test tubes or test tubes containing dried AHLs, as indicated. The volume of culture in each tube was 0.5 ml. After 2 h at 37°C with shaking, β-galactosidase activity was measured as described above.

To assess MalR activation of *malA* in *B. thailandensis*, we used a *B. thailandensis malA-lacZ* reporter strain (BT01447) (15). Logarithmic-phase cultures were diluted to an OD$_{600}$ of 0.05 in growth medium supplemented with antibiotics or IPTG at the concentrations indicated. When used, AHLs were added to the culture tube and dried prior to adding inoculated LB broth. The final AHL concentrations were 5 μM. After 24 h at 30°C with shaking, β-galactosidase activity was measured as described above.

Purification of malleilactone. Malleilactone was purified from *B. thailandensis* as follows. A stationary-phase culture of *B. thailandensis* E264 was used as the inoculum for 8 640-ml cultures in 4-liter Erlenmeyer
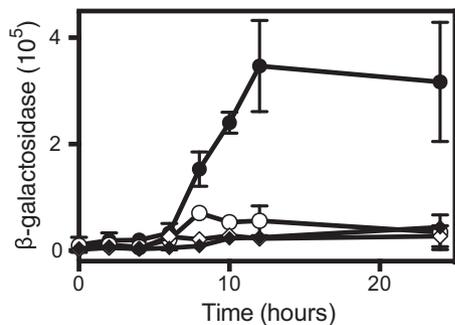
FIG 1 Trimethoprim-dependent expression of *malA* requires MalR. Shown is growth-adjusted activity of a *malA-lacZ* chromosomal reporter in the *malR* mutant (TT04) (diamonds) or its isogenic parent (BT01447) (circles) grown with trimethoprim (solid symbols) or without trimethoprim (open symbols). The data are the means ± ranges from the results of two independent experiments.

flasks. The growth medium was LB-MOPS plus 9 μg/ml trimethoprim. Cells were inoculated to an OD_{600} of 0.05. After 32 h at 30°C with shaking (200 rpm), the cultures were extracted with 1 volume of ethyl acetate, and the extract was dried over Na_2SO_4 and evaporated completely in a vacuum. The remaining residue was resolved through a Kupchan liquid extraction scheme. Briefly, the dried extract was dissolved in 200 ml of methanol (MeOH)-H_2O (9:1) and extracted four times with 200 ml hexanes. The remaining methanolic extract was diluted in 800 ml of MeOH-H_2O (6:4), which was extracted four times with an equal volume of CH_2Cl_2. High-performance liquid chromatography–mass spectrometry (HPLC-MS) analysis of the three resulting fractions (MeOH-H_2O, CH_2Cl_2, and hexanes) showed that malleilactone was in the CH_2Cl_2 fraction. This fraction was dried, suspended in a small volume (2 to 3 ml) of CH_2Cl_2, and resolved on a silica gel column (16 g; diameter, 1.25 cm; length, 20 cm). The column was equilibrated in CH_2Cl_2, loaded with the malleilactone-containing mixture, and eluted with 3 column volumes (CV) of CH_2Cl_2, followed by 3 CV (each) of 2.5%, 5%, 7.5%, and 10% MeOH in CH_2Cl_2. Fractions containing malleilactone, as judged by thin-layer chromatography (TLC) with 5% MeOH in CH_2Cl_2 as a solvent, were pooled; the pooled fractions consisted of a number of malleilactone analogs. Pure malleilactone A was obtained by a final HPLC step consisting of a preparative Eclipse XCB-C_8 column (Agilent; 7 μm; 21.2 by 250 mm) operating at 12 ml/min with an elution program that contained an isocratic step (6 min; 28% acetonitrile [MeCN] in H_2O) followed by a gradient from 28 to 100% MeCN over 36 min. Both MeCN and H_2O contained 0.1% NH_4OH, which helped stabilize the malleilactone. This procedure yielded 4 mg of pure malleilactone, which was verified by UV-visible (λ_{max} = 370 nm), ^1H nuclear magnetic resonance (NMR), and high-resolution electrospray ionization (ESI)-MS ([M+H]$^+$ observed, 307.1907; [M+H]$^+$ calculated, 307.1909).

RESULTS

MalR is required for trimethoprim induction of *mal* biosynthesis genes. MalR is a LuxR orphan with all of the LuxR family conserved amino acids and 35% amino acid sequence identity to the *P. aeruginosa* AHL-responsive orphan QscR. MalR is genetically linked to a 13-gene cluster, the malleilactone (*mal*)-biosynthetic genes. The *mal* genes are transcriptionally activated by trimethoprim (11). To address the question of whether MalR is required for trimethoprim to induce expression of the *mal* genes, we used a *B. thailandensis* strain with a chromosomal *lacZ* fusion to *malA*, the first gene in the *mal* cluster (15). We compared *malA-lacZ* activation in a *malR* mutant to that in wild-type *malR*. The *lacZ* reporter was activated by trimethoprim in the wild type, as previously reported (11), but not in the *malR* mutant (Fig. 1).

These results show that MalR is required for trimethoprim activation of the *mal* biosynthesis genes.

Influence of antibiotics on *mal* gene expression and the *B. thailandensis* growth rate. Trimethoprim is a dihydrofolate reductase inhibitor. In addition to trimethoprim, several other antibiotics activate the *mal* operon. They include fluoroquinolone DNA gyrase inhibitors and the cell wall biosynthesis inhibitors piperacillin, ceftazidime, and cefotaxime (11). The fact that these antibiotics are structurally distinct and target different activities suggests that they might not interact with MalR directly. An alternative explanation is that they affect the growth rate of *B. thailandensis* at the sublethal concentrations tested, and this might correlate with activation of the *mal* genes. We first tested the influence of trimethoprim, the strongest *mal* gene inducer (11), on growth and *mal* gene induction by using our *B. thailandensis malA-lacZ* reporter. At concentrations that induced *mal* expression, trimethoprim also inhibited bacterial growth (Table 1 and Fig. 2), and in fact, the level of *mal* gene expression was inversely correlated with growth over a range of trimethoprim concentrations (Fig. 2). We also tested other antibiotics at sub-MIC but growth-slowing concentrations. Not all of the antibiotics we tested served to activate *malA-lacZ*. Kanamycin, for example, did not activate *lacZ* expression, but it did inhibit growth (Table 1 and Fig. 2). Thus, slow growth is not sufficient for activation of the *mal* genes, but it may be necessary.

Our *malA-lacZ* reporter disrupts *malA*, which is a predicted polyketide synthase thought to be critical for malleilactone synthesis (12). Thus, malleilactone itself is likely not required for trimethoprim and other antibiotics to activate the *mal* genes. Nevertheless, malleilactone might influence *mal* gene expression. To address this possibility, we purified malleilactone from cultures of trimethoprim-treated *B. thailandensis* cells, where it was estimated to be at a concentration of 0.8 μg/ml (see Materials and Methods). At this concentration, our purified malleilactone had a minimal effect on growth or *mal* gene expression in the *B. thailandensis* reporter strain (Fig. 2). However, at a higher concentration (15 μg/ml), malleilactone reduced growth by 70 to 80% and showed potency as a *malA* activator comparable to that of trimethoprim (Fig. 2).

Transcription from the *malA* promoter in recombinant *E. coli* requires MalR and the *lux* box-like sequence. We engi-

TABLE 1 Abilities of antibiotics to activate *malA-lacZ* in *B. thailandensis*

Antibiotic (μg/ml)[a]	Antibiotic class	Relative *malA-lacZ* activity[b]
None		1
Trimethoprim (15)[c]	THF pathway inhibitor[d]	9.7 (0.4)
Kanamycin (115)	Ribosomal inhibitor	0.5 (0.1)
Zeocin (1,200)	DNA intercalator	1.5 (0)
Mitomycin C (10)	DNA cross-linker	4.3 (0.1)
Sulfamethoxazole (3,000)	THF pathway inhibitor	10 (0.7)
Malleilactone (15)	Unknown	12.3 (1.5)

[a] The concentration of antibiotic used is indicated in parentheses, and in each case, the concentration resulted in a 70 to 80% reduction in the growth yield, as determined by the OD_{600} taken at the time of β-galactosidase measurement.
[b] Growth-adjusted β-galactosidase activity relative to that of the untreated control. The β-galactosidase activity and OD_{600} were determined after 24 h. The values are the means of the results of two independent experiments, with the range indicated in parentheses.
[c] Trimethoprim was previously shown to activate *malA-lacZ* (11).
[d] THF pathway, tetrahydrofolate reductase pathway.

FIG 3 Expression of *malA* requires MalR and an intact *lux* box-like sequence in recombinant *E. coli*. (A) Activation of the *malA* promoter in *E. coli* containing an arabinose-inducible *malR* expression vector (pJN105.malR) and a *lacZ* reporter with either the wild-type *malA* promoter (pQF50.PmalA) (circles) or the *malA* promoter with the T4C and G5A base substitutions (pQF50.mutPmalA) (triangles). The values are the means ± ranges of the results of two independent experiments. Asterisks, *E. coli* containing pJN105.malR and pQF50.PmalA grown with 0.4 or 0.004% arabinose and 5 μM (each) C$_8$-HSL, 3OHC$_8$-HSL, and 3OHC$_{10}$-HSL (the *B. thailandensis* AHLs). Similar results were obtained with 5 μM C$_4$-, C$_6$-, C$_{10}$-, C$_{12}$-, C$_{14}$-, 3OHC$_6$-, and *p*-coumaroyl-HSL. (B) *malR-malA B. thailandensis* genomic region. The putative *lux* box-like element is centered 63.5 bp upstream of the *malA* translation start site. The red letters indicate the T4C and G5A base substitutions in pQF50.PmalA. (C) Alignment of the *malA lux* box-like sequence with the *lux* boxes from *Vibrio fischeri luxI*, *P. aeruginosa rhlI*, and *B. mallei bmaI1*.
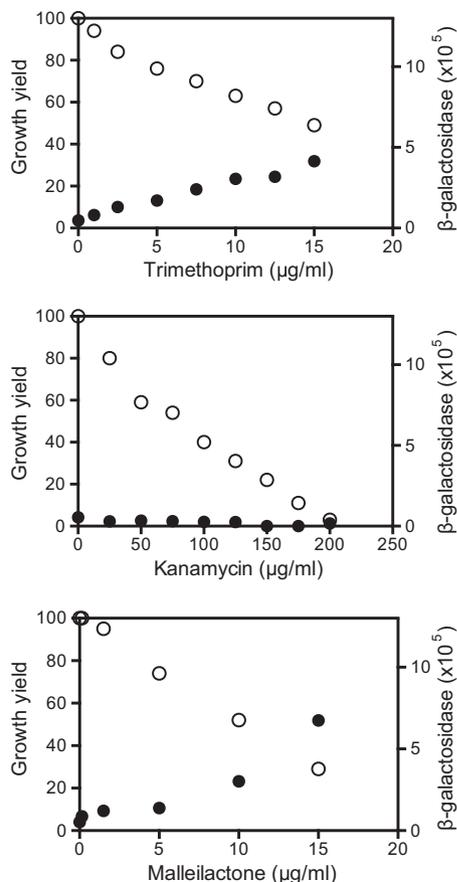
FIG 2 Effect of antibiotics on growth (open circles) and activation of a chromosomal *malA-lacZ* reporter (solid circles). The data are from the *B. thailandensis malA-lacZ* reporter BT01447 grown 24 h with antibiotics as indicated. The final growth yield is a percentage of that without added antibiotic measured as the OD$_{600}$. β-Galactosidase activity is reported as relative light units normalized to the OD$_{600}$.

neered a plasmid with a 500-bp fragment containing the presumed *malA* promoter fused to a promoterless *lacZ* (from positions −1 to −500 with respect to the *malA* translation start codon) and a plasmid with an arabinose-inducible *malR*. In *E. coli*, *lacZ* expression was dependent on the presence of both plasmids and on arabinose (Fig. 3A).

The *lux* box-like sequence is centered 63.5 bp upstream of the *malA* translational start site (Fig. 3B and C). We tested the hypothesis that MalR requires this *lux* box-like sequence to activate *malA* by introducing the base substitutions T4C and G5A (Fig. 3B), which are critical for transcription factor binding in LuxR-LuxI-type systems (22). In *E. coli* containing this construct, the basal level of *lacZ* expression was similar to that in *E. coli* containing the full-length *malA-lacZ* fusion; however, the T4C and G5A substitutions abolished MalR-dependent activation of *malA-lacZ* (Fig. 3A). Our results are consistent with the idea that MalR binds to the *lux* box-like element upstream of *malA* and that this binding leads to transcriptional activation of *malA*. Activation of reporter transcription in *E. coli* did not require AHLs or antibiotics.

MalR-dependent activation of the *mal* genes is not influenced by AHLs. Although activation of the *malA-lacZ* reporter in *E. coli* does not require an AHL, it might nevertheless be influ-
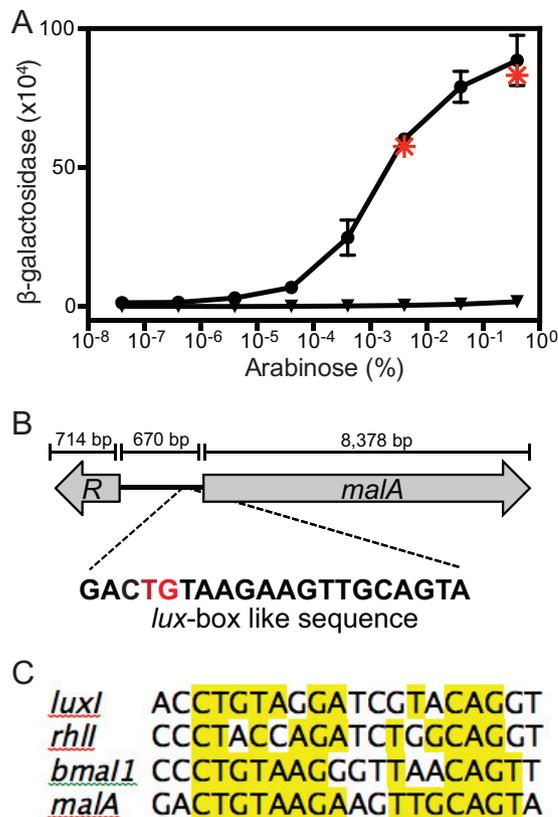
enced by AHLs either positively or negatively. Thus, we measured *lacZ* expression in our *E. coli* reporter containing both the *malA-lacZ* expression vector and the arabinose-dependent *malR* expression vector as follows. We grew the *E. coli* reporter strain in the presence of arabinose at a concentration that elicits maximal or half-maximal MalR-dependent *malA-lacZ* activation (0.4 or 0.004% arabinose, respectively) plus the following AHLs (5 μM each): octanoyl-homoserine lactone (HSL), 3-hydroxy-octanoyl-HSL, and 3-hydroxy-decanoyl-HSL (the *B. thailandensis* AHLs); butyryl-HSL, hexanoyl-HSL, decanoyl-HSL, dodecanoyl-HSL, tetradecanoyl-HSL, and 3-hydroxy-hexanoyl-HSL (C$_4$-, C$_6$-, C$_{10}$-, C$_{12}$-, C$_{14}$-, and 3OHC$_6$-HSL); and *p*-coumaroyl-HSL. Addition of any of the above-listed AHLs to the growth medium did not positively or negatively affect *lacZ* transcription (Fig. 3A and data not shown).

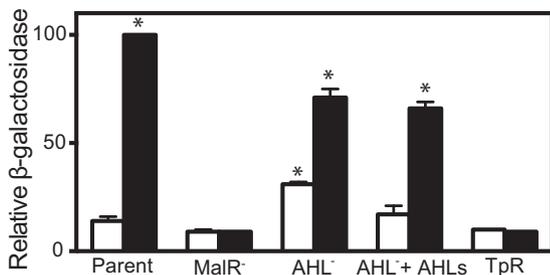It is possible that AHLs do not affect MalR or transcription

FIG 4 Transcriptional activation of a *malA-lacZ* chromosomal fusion in *B. thailandensis*. Growth-adjusted β-galactosidase activity is shown as a percentage of wild-type control values. The open bars indicate activity in the absence of trimethoprim, and the solid bars indicate activity in the presence of 15 μg/ml trimethoprim. Parent, *B. thailandensis* with the *malA-lacZ* chromosomal reporter (BT01447); MalR⁻, BT01447 with a *malR* deletion (TT04); AHL⁻, BT01447 with deletions of all three of the AHL synthase genes (Δ*btaI1-3*) (TT10); AHLs, 5 μM (each) C_8-HSL, $3OHC_8$-HSL, and $3OHC_{10}$-HSL; TpR, BT01447 with the *dhfrII* gene conferring trimethoprim resistance inserted in the neutral chromosomal *attn7* site (TT05). The values are the means and ranges of the results from two independent experiments. Statistical analysis was done with a *t* test compared to the untreated parent; *, $P \leq 0.05$.

FIG 5 IPTG-dependent activation of *malA-lacZ* by a *lac* promoter-controlled *malR* in *B. thailandensis*. Cells were grown with 15 μg/ml trimethoprim (solid symbols) or without trimethoprim (open symbols) and IPTG as indicated. Shown is the growth-adjusted activity of a *malA-lacZ* chromosomal reporter in the native *malR* deletion with a *lac* promoter-controlled *malR* strain (TT08) (circles) and an isogenic strain with the *lac* promoter but no *malR* (TT07) (squares). The values are the means ± ranges from the results of two independent experiments. Statistical analysis was done by *t* test of the trimethoprim-treated strain compared to the identical strain grown without trimethoprim; *, $P \leq 0.05$.

from the *malA* promoter in recombinant *E. coli* but that they do have an influence on *malA* expression in *B. thailandensis*. To test this possibility, we measured *lacZ* expression from the *malA-lacZ* reporter in the parent *B. thailandensis* strain, a *malR* mutant, and a mutant incapable of producing any AHLs. Cells were grown both with and without added AHLs (Fig. 4). As previously shown (Fig. 1), trimethoprim induced *lacZ* expression in the *malR* wild type but not in the *malR* mutant. Trimethoprim also induced *malA-lacZ* activity in the AHL synthesis mutant (Fig. 4). In the absence of trimethoprim, self-produced or exogenously added AHLs modestly repressed *malA-lacZ* reporter activity by about 2-fold (Fig. 4). Because *malR* is not expressed under these conditions (11), we posit that this may be due to cross-regulation by one of the other LuxR homologs encoded in the *B. thailandensis* chromosome. Our results with both recombinant *E. coli* and *B. thailandensis* are consistent with our conclusion that trimethoprim activates *malA-lacZ* expression in a MalR-dependent fashion and show that the AHLs we tested, including the *B. thailandensis* AHLs, are not required for MalR-mediated activation of the *mal* operon.

MalR-dependent activation of *malA* transcription correlates with *malR* transcription. Our results with recombinant *E. coli* (Fig. 3) suggest the possibility that MalR does not require a ligand to activate *mal* gene expression. It may be that a sufficient level of MalR is the only requirement for activation of *mal* gene expression and that trimethoprim and certain other antibiotics somehow activate *malR* transcription. In fact, trimethoprim, piperacillin, and malleilactone all induce *malR* expression in *B. thailandensis* (11) (see Fig. S1 in the supplemental material). Furthermore, we constructed a strain with p*lac*-*malR* in a neutral site (*glmS1*) on the chromosome of a *B. thailandensis malR* mutant containing the *malA-lacZ* reporter. We monitored *malA* expression in this strain, grown with or without trimethoprim and increasing concentrations of IPTG to induce p*lac*-*malR* expression. We found that activation of *malA-lacZ* required IPTG but not trimethoprim (Fig. 5).

Although trimethoprim was not required for *malA-lacZ* activation, it had a modest stimulatory influence on IPTG-dependent
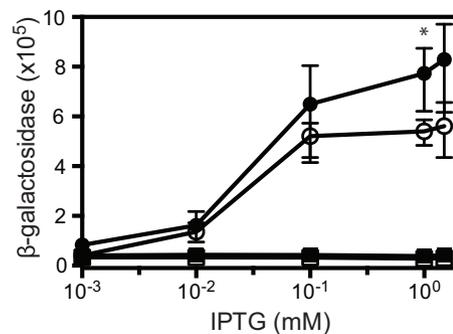
activation of *malA-lacZ*. This raised the possibility that trimethoprim does interact with MalR directly to affect its ability to activate *mal* gene expression. We addressed the possibility that trimethoprim is a ligand for MalR by introducing a gene coding for a trimethoprim-resistant dihydrofolate reductase (*dhfrIIb*) into our reporter strain. We found that *dhfrIIb* completely abolished trimethoprim-dependent activation of *malA-lacZ* (Fig. 5). Because the *dhfrIIB* product changes the target of trimethoprim and does not affect trimethoprim itself, we conclude that although trimethoprim activation of the *mal* operon is MalR dependent, the trimethoprim effect does not result from a direct interaction of trimethoprim and MalR. This is consistent with the hypothesis that trimethoprim serves to activate *malR* transcription and that elevated levels of MalR are sufficient to activate the otherwise silent *mal* genes in laboratory cultures of *B. thailandensis*.

DISCUSSION

Here, we describe an unusual LuxR family protein, MalR, which retains all of the conserved amino acid residues in the AHL binding domains of LuxR family members involved in AHL quorum sensing but does not require an AHL for activity. Generally, LuxR family members that do not respond to AHLs have one or more substitutions in the conserved residues of the AHL binding domain. For example, *X. oryzae* OryR responds to a plant-derived signal (9), *P. asymbiotica* PauR responds to dialkylresorcinols (9, 10), and *Serratia* sp. CarR appears to be like MalR in that it does not seem to require an AHL or any exogenously added ligand for activity (23). CarR activity may also correspond to its level of transcription (24, 25). Although CarR has a change in one of the conserved AHL binding residues, genetic evidence supports the idea that the conversion to AHL independence occurred through mutations in the C terminus (23). The C-terminal mutations in CarR (23) are in a region that is not present in MalR.

The *B. thailandensis mal* gene cluster is silent in laboratory-grown cultures, but *mal* gene expression can be activated by certain antibiotics. We have shown that antibiotic activation of the *mal* genes requires MalR. Our results also indicate that antibiotics activate *mal* gene expression indirectly by driving *malR* expres-

sion. This conclusion is based on our results with *B. thailandensis* (Fig. 1 and 4) and a *B. thailandensis* AHL signal synthesis mutant and a trimethoprim-resistant mutant (Fig. 4), as well as with *E. coli* (Fig. 3), showing that sufficient levels of *malR* expression are all that is needed to activate *mal* gene expression. In our experiments, MalR does not appear to require an AHL or a ligand added to the culture medium to influence *mal* gene expression. It is possible that a common metabolite in *B. thailandensis* and *E. coli* serves as a ligand. Recently, the *E. coli* LuxR orphan SdiA was shown to bind a conserved metabolite (1-octanoyl-*rac*-glycerol) in the absence of its cognate AHL (13). SdiA has some activity in the absence of AHLs, as do a few other AHL-responsive LuxR homologs (e.g., *Burkholderia cenocepacia* CepR2) (26, 27). It is not known if any other LuxR proteins bind common metabolites, like SdiA. In ways other than the AHL requirement, MalR is similar to LuxR quorum-sensing signal receptors. For example, MalR requires an intact *lux* box-like sequence to activate the promoter of the malleilactone-biosynthetic genes.

Transcription of *malR* is activated by several, but not all, of the antibiotics we tested. In every case, antibiotics that activated *mal* gene expression also slowed the growth of *B. thailandensis*, with growth and *mal* activation inversely correlated. However, the influence of an antibiotic on growth was not in itself sufficient for *mal* gene activation. Some antibiotics slowed growth but did not affect *mal* gene activation. We imagine that the presence of certain antibiotics activates a specific cellular response pathway, which in turn activates expression of *malR*. Malleilactone may activate the *mal* genes through a similar pathway, although the cellular target of malleilactone is unknown. We find it interesting that antibiotic modulation of AHL-responsive LuxR family members has been reported elsewhere (28, 29). In fact, several classes of antibiotics activate transcription of the *P. aeruginosa* orphan QscR (29). MalR might be a degenerate AHL quorum-sensing signal receptor. There is increasing evidence that AHL receptors play an important role in interspecies competition (30, 31). Antibiotics and cytotoxic factors are often activated by AHL quorum sensing (31–33), and as discussed above, MalR (11) and QscR (29) are antibiotic activated. The *mal* genes code for synthesis of malleilactone, which is a cytotoxin that also has antibiotic activity against several Gram-positive bacteria (12), and as we show here (Fig. 2), malleilactone even slows the growth of *B. thailandensis* at high concentrations. Activation of the *mal* genes by low, nonlethal concentrations of antibiotics might be an alarm response whereby *B. thailandensis* reacts to danger by mounting an attack against competitors.

B. thailandensis is closely related to two human pathogens, *Burkholderia pseudomallei* and *Burkholderia mallei*. The malleilactone-biosynthetic gene clusters are conserved (>80% identity at the amino acid sequence level), and MalR shows 100% identity across these three species. *B. pseudomallei* is the causative agent of melioidosis, an often fatal emerging infectious disease that is prevalent in northeast Thailand (34). One of the difficulties in treatment is the high level of antibiotic resistance in *B. pseudomallei*. Trimethoprim and sulfamethoxazole are two of the few clinically useful antibiotics (35). Both of these antibiotics activate malleilactone production in *B. thailandensis* (Table 1). These findings, together with the finding that malleilactone is important for *B. thailandensis* virulence in *C. elegans* (12), suggest the need to evaluate how antibiotics regulate virulence and the gene homologs for malleilactone and MalR in *B. pseudomallei*. Finally, it is interesting

that at sufficiently high concentrations (15 μg/ml) malleilactone itself inhibits the growth of *B. thailandensis*. Members of the genus *Burkholderia* are quite insensitive to most antibiotics, and this creates clinical difficulties (36). It is not unreasonable to think that malleilactone might provide a scaffold for anti-*Burkholderia* agents or that discovery of the malleilactone target might be helpful in drug discovery.

REFERENCES

1. **Fuqua C, Parsek MR, Greenberg EP.** 2001. Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. Annu Rev Genet **35**:439–468. http://dx.doi.org/10.1146/annurev.genet.35.102401.090913.
2. **Whitehead NA, Barnard AM, Slater H, Simpson NJ, Salmond GP.** 2001. Quorum-sensing in Gram-negative bacteria. FEMS Microbiol Rev **25**:365–404. http://dx.doi.org/10.1111/j.1574-6976.2001.tb00583.x.
3. **Ulrich RL, Hines HB, Parthasarathy N, Jeddeloh JA.** 2004. Mutational analysis and biochemical characterization of the *Burkholderia thailandensis* DW503 quorum-sensing network. J Bacteriol **186**:4350–4360. http://dx.doi.org/10.1128/JB.186.13.4350-4360.2004.
4. **Fuqua C.** 2006. The QscR quorum-sensing regulon of *Pseudomonas aeruginosa*: an orphan claims its identity. J Bacteriol **188**:3169–3171. http://dx.doi.org/10.1128/JB.188.9.3169-3171.2006.
5. **Subramoni S, Venturi V.** 2009. PpoR is a conserved unpaired LuxR solo of *Pseudomonas putida* which binds N-acyl homoserine lactones. BMC Microbiol **9**:125. http://dx.doi.org/10.1186/1471-2180-9-125.
6. **Chugani SA, Whiteley M, Lee KM, D'Argenio D, Manoil C, Greenberg EP.** 2001. QscR, a modulator of quorum-sensing signal synthesis and virulence in *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A **98**:2752–2757. http://dx.doi.org/10.1073/pnas.051624298.
7. **Michael B, Smith JN, Swift S, Heffron F, Ahmer BM.** 2001. SdiA of *Salmonella enterica* is a LuxR homolog that detects mixed microbial communities. J Bacteriol **183**:5733–5742. http://dx.doi.org/10.1128/JB.183.19.5733-5742.2001.
8. **Smith JN, Ahmer BM.** 2003. Detection of other microbial species by *Salmonella*: expression of the SdiA regulon. J Bacteriol **185**:1357–1366. http://dx.doi.org/10.1128/JB.185.4.1357-1366.2003.
9. **Ferluga S, Bigirimana J, Hofte M, Venturi V.** 2007. A LuxR homologue of *Xanthomonas oryzae* pv. *oryzae* is required for optimal rice virulence. Mol Plant Pathol **8**:529–538. http://dx.doi.org/10.1111/j.1364-3703.2007.00415.x.
10. **Brachmann AO, Brameyer S, Kresovic D, Hitkova I, Kopp Y, Manske C, Schubert K, Bode HB, Heermann R.** 2013. Pyrones as bacterial signaling molecules. Nat Chem Biol **9**:573–578. http://dx.doi.org/10.1038/nchembio.1295.
11. **Seyedsayamdost MR.** 2014. High-throughput platform for the discovery of elicitors of silent bacterial gene clusters. Proc Natl Acad Sci U S A **111**:7266–7271. http://dx.doi.org/10.1073/pnas.1400091111.
12. **Biggins JB, Ternei MA, Brady SF.** 2012. Malleilactone, a polyketide synthase-derived virulence factor encoded by the cryptic secondary metabolome of *Burkholderia pseudomallei* group pathogens. J Am Chem Soc **134**:13192–13195. http://dx.doi.org/10.1021/ja3052156.
13. **Nguyen Y, Nguyen NX, Rogers JL, Liao J, MacMillan JB, Jiang Y, Sperandio V.** 2015. Structural and mechanistic roles of novel chemical ligands on the SdiA quorum-sensing transcription regulator. mBio **6**:e02429–14. http://dx.doi.org/10.1128/mBio.02429-14.
14. **Brett PJ, DeShazer D, Woods DE.** 1998. *Burkholderia thailandensis* sp. nov., a *Burkholderia pseudomallei*-like species. Int J Syst Bacteriol **48**:317–320. http://dx.doi.org/10.1099/00207713-48-1-317.
15. **Gallagher LA, Ramage E, Patrapuvich R, Weiss E, Brittnacher M,**

Manoil C. 2013. Sequence-defined transposon mutant library of *Burkholderia thailandensis*. mBio **4**:e00604–13. http://dx.doi.org/10.1128/mBio.00604-13.

16. **Sambrook J, Russell D.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

17. **Chandler JR, Duerkop BA, Hinz A, West TE, Herman JP, Churchill ME, Skerrett SJ, Greenberg EP.** 2009. Mutational analysis of *Burkholderia thailandensis* quorum sensing and self-aggregation. J Bacteriol **191**:5901–5909. http://dx.doi.org/10.1128/JB.00591-09.

18. **Thongdee M, Gallagher LA, Schell M, Dharakul T, Songsivilai S, Manoil C.** 2008. Targeted mutagenesis of *Burkholderia thailandensis* and *Burkholderia pseudomallei* through natural transformation of PCR fragments. Appl Environ Microbiol **74**:2985–2989. http://dx.doi.org/10.1128/AEM.00030-08.

19. **Majerczyk C, Brittnacher M, Jacobs M, Armour CD, Radey M, Schneider E, Phattarasokul S, Bunt R, Greenberg EP.** 2014. Global analysis of the *Burkholderia thailandensis* quorum sensing-controlled regulon. J Bacteriol **196**:1412–1424. http://dx.doi.org/10.1128/JB.01405-13.

20. **Choi KH, Mima T, Casart Y, Rholl D, Kumar A, Beacham IR, Schweizer HP.** 2008. Genetic tools for select-agent-compliant manipulation of *Burkholderia pseudomallei*. Appl Environ Microbiol **74**:1064–1075. http://dx.doi.org/10.1128/AEM.02430-07.

21. **Choi KH, Schweizer HP.** 2006. Mini-Tn7 insertion in bacteria with single attTn7 sites: example *Pseudomonas aeruginosa*. Nat Protoc **1**:153–161. http://dx.doi.org/10.1038/nprot.2006.24.

22. **Antunes LC, Ferreira RB, Lostroh CP, Greenberg EP.** 2008. A mutational analysis defines *Vibrio fischeri* LuxR binding sites. J Bacteriol **190**:4392–4397. http://dx.doi.org/10.1128/JB.01443-07.

23. **Poulter S, Carlton TM, Spring DR, Salmond GP.** 2011. The *Serratia* LuxR family regulator CarR 39006 activates transcription independently of cognate quorum sensing signals. Mol Microbiol **80**:1120–1131. http://dx.doi.org/10.1111/j.1365-2958.2011.07634.x.

24. **Slater H, Crow M, Everson L, Salmond GP.** 2003. Phosphate availability regulates biosynthesis of two antibiotics, prodigiosin and carbapenem, in *Serratia* via both quorum-sensing-dependent and -independent pathways. Mol Microbiol **47**:303–320. http://dx.doi.org/10.1046/j.1365-2958.2003.03295.x.

25. **Fineran PC, Slater H, Everson L, Hughes K, Salmond GP.** 2005. Biosynthesis of tripyrrole and beta-lactam secondary metabolites in *Serratia*: integration of quorum sensing with multiple new regulatory components in the control of prodigiosin and carbapenem antibiotic production. Mol Microbiol **56**:1495–1517. http://dx.doi.org/10.1111/j.1365-2958.2005.04660.x.

26. **Ryan GT, Wei Y, Winans SC.** 2013. A LuxR-type repressor of *Burkholderia cenocepacia* inhibits transcription via antiactivation and is inactivated by its cognate acylhomoserine lactone. Mol Microbiol **87**:94–111. http://dx.doi.org/10.1111/mmi.12085.

27. **Malott RJ, O'Grady EP, Toller J, Inhulsen S, Eberl L, Sokol PA.** 2009. A *Burkholderia cenocepacia* orphan LuxR homolog is involved in quorum-sensing regulation. J Bacteriol **191**:2447–2460. http://dx.doi.org/10.1128/JB.01746-08.

28. **Liu Z, Wang W, Zhu Y, Gong Q, Yu W, Lu X.** 2013. Antibiotics at subinhibitory concentrations improve the quorum sensing behavior of *Chromobacterium violaceum*. FEMS Microbiol Lett **341**:37–44. http://dx.doi.org/10.1111/1574-6968.12086.

29. **Yeom DH, Im SJ, Kim SK, Lee JH.** 2014. Activation of multiple transcriptional regulators by growth restriction in *Pseudomonas aeruginosa*. Mol Cells **37**:480–486. http://dx.doi.org/10.14348/molcells.2014.0105.

30. **Chandler JR, Heilmann S, Mittler JE, Greenberg EP.** 2012. Acyl-homoserine lactone-dependent eavesdropping promotes competition in a laboratory co-culture model. ISME J **6**:2219–2228. http://dx.doi.org/10.1038/ismej.2012.69.

31. **An D, Danhorn T, Fuqua C, Parsek MR.** 2006. Quorum sensing and motility mediate interactions between *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens* in biofilm cocultures. Proc Natl Acad Sci U S A **103**:3828–3833. http://dx.doi.org/10.1073/pnas.0511323103.

32. **Duerkop BA, Varga J, Chandler JR, Peterson SB, Herman JP, Churchill ME, Parsek MR, Nierman WC, Greenberg EP.** 2009. Quorum-sensing control of antibiotic synthesis in *Burkholderia thailandensis*. J Bacteriol **191**:3909–3918. http://dx.doi.org/10.1128/JB.00200-09.

33. **Bainton NJ, Stead P, Chhabra SR, Bycroft BW, Salmond GP, Stewart GS, Williams P.** 1992. N-(3-oxohexanoyl)-L-homoserine lactone regulates carbapenem antibiotic production in *Erwinia carotovora*. Biochem J **288**:997–1004. http://dx.doi.org/10.1042/bj2880997.

34. **Limmathurotsakul D, Wongratanacheewin S, Teerawattanasook N, Wongsuvan G, Chaisuksant S, Chetchotisakd P, Chaowagul W, Day NP, Peacock SJ.** 2010. Increasing incidence of human melioidosis in Northeast Thailand. Am J Trop Med Hyg **82**:1113–1117. http://dx.doi.org/10.4269/ajtmh.2010.10-0038.

35. **Dance D.** 2014. Treatment and prophylaxis of melioidosis. Int J Antimicrob Agents **43**:310–318. http://dx.doi.org/10.1016/j.ijantimicag.2014.01.005.

36. **Bazzini S, Buroni S, Udine C, Pasca MR, Riccardi G.** 2014. Molecular basis for antibiotic resistance in the genus *Burkholderia*, p 183–196. *In* Coenye T (ed), *Burkholderia*: from genomes to function. Caister Acadmic Press, Norfolk, United Kingdom.