

Octanoyl-Homoserine Lactone Is the Cognate Signal for *Burkholderia mallei* BmaR1-BmaI1 Quorum Sensing[▽]

Breck A. Duerkop,¹ Ricky L. Ulrich,² and E. Peter Greenberg^{1*}

Department of Microbiology, University of Washington School of Medicine, Seattle, Washington 98195-7242,¹ and
Bacteriology Division, United States Army Medical Research Institute of
Infectious Diseases, Fort Detrick, Maryland 21704²

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Acyl-homoserine lactones (HSLs) serve as quorum-sensing signals for many *Proteobacteria*. Members of the LuxI family of signal generators catalyze the production of acyl-HSLs, which bind to a cognate receptor in the LuxR family of transcription factors. The obligate animal pathogen *Burkholderia mallei* produces several acyl-HSLs, and the *B. mallei* genome has four *luxR* and two *luxI* homologs, each of which has been established as a virulence factor. To begin to delineate the relevant acyl-HSL signals for *B. mallei* LuxR homologs, we analyzed the BmaR1-BmaI1 system. A comparison of acyl-HSL profiles from *B. mallei* ATCC 23344 and a *B. mallei* *bmaI1* mutant indicates that octanoyl-HSL synthesis is BmaI1 dependent. Furthermore, octanoyl-HSL is the predominant acyl-HSL produced by BmaI1 in recombinant *Escherichia coli*. The synthesis of soluble BmaR1 in recombinant *E. coli* requires octanoyl-HSL or decanoyl-HSL. Insoluble aggregates of BmaR1 are produced in the presence of other acyl-HSLs and in the absence of acyl-HSLs. The *bmaI1* promoter is activated by BmaR1 and octanoyl-HSL, and a 20-bp inverted repeat in the *bmaI1* promoter is required for *bmaI1* activation. Purified BmaR1 binds to this promoter region. These findings implicate octanoyl-HSL as the signal for BmaR1-BmaI1 quorum sensing and show that octanoyl-HSL and BmaR1 activate *bmaI1* transcription.

Burkholderia mallei is a rod-shaped proteobacterium that exists as an obligate animal pathogen. Members of the equine family (including horses and mules) serve as the natural hosts for *B. mallei*, which causes a disease called glanders (9). *B. mallei* can infect other animals including mice, hamsters, guinea pigs, monkeys, and humans (27). Human glanders, if untreated, is often fatal. *B. mallei* can be transmitted by aerosol, and because the infectious dose is very low, it is considered to be a biowarfare threat. Little is known about *B. mallei* pathogenesis, and there is no effective *B. mallei* vaccine. To identify suitable glanders therapies, it is important to begin to understand *B. mallei* virulence genes. This obligate animal pathogen has several putative acyl-homoserine lactone (HSL) quorum-sensing signal generators and receptors that are critical for virulence in animal models (39).

Quorum sensing allows bacteria to monitor their population density and affect gene transcription at critical population levels (17, 41). Many host-associated *Proteobacteria* utilize small amphipathic acyl-HSL signals for quorum sensing. These signals can diffuse out of and into cells and, upon reaching a threshold concentration, bind transcriptional regulators that control the expression of specific sets of genes. Acyl-HSL signaling was first identified in the luminescent marine bacterium *Vibrio fischeri*, which produces blue light at high cell densities (28). The *V. fischeri* quorum-sensing circuit depends on two proteins, LuxI and LuxR (11). The LuxI protein is an *N*-3-(oxohexanoyl)-HSL (3OC₆-HSL) synthase, and LuxR is a 3OC₆-HSL-responsive luminescence gene transcriptional acti-

vator. The luminescence of *V. fischeri* is used for its mutualistic symbiosis in the light organs of marine animal hosts (16, 34). Quorum sensing allows *V. fischeri* to discriminate between a high population density inside the animal host and a low population density in the seawater environment.

Many other *Proteobacteria* that associate with animal or plant hosts in a nonobligate fashion possess systems homologous to the *V. fischeri* quorum-sensing system. These systems often control virulence or symbiosis functions and are thought to allow the discrimination between host and nonhost environments (for reviews, see references 16 and 41). Acyl-HSL quorum sensing involves paired LuxI-LuxR homologs. The LuxI homologs produce acyl-HSLs with different side chain lengths, different substitutions on the third carbon in the side chain (fully protonated, carbonyl, and hydroxyl), and sometimes a single carbon-carbon double bond in the center of the side chain. A LuxR homolog shows the greatest sensitivity to the signal produced by its cognate LuxI homolog. Genomes often contain additional unpaired LuxR homologs, and the functions of these homologs are just beginning to be understood (6, 32).

The genomes of numerous obligate animal pathogens have been sequenced, and sequence analyses indicate that they do not have acyl-HSL quorum-sensing systems, yet *B. mallei* possesses several such systems, and these systems are critical for virulence (39). The reasons why *B. mallei* acyl-HSLs are involved in virulence are unknown. We hope that by studying *B. mallei* quorum sensing, we can learn about the general significance of acyl-HSL signaling in pathogenic bacteria.

There are two other *Burkholderia* species that are very closely related to *B. mallei*, *Burkholderia thailandensis*, which is a soil bacterium with relatively low animal virulence, and *Burkholderia pseudomallei*, an opportunistic pathogen that is the causative agent of an emerging disease called melioidosis (3, 4,

* Corresponding author. Mailing address: Department of Microbiology, University of Washington School of Medicine, 1959 N. E. Pacific Street, Box 357242, Seattle, WA 98195-7242. Phone: (206) 616-2881. Fax: (206) 616-2968. E-mail: epgreen@u.washington.edu.

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42). The *B. mallei* genome appears to be a degenerate form of the *B. pseudomallei* genome (30). For example, the genome of *B. pseudomallei* has three *luxI-luxR* homolog pairs and two additional *luxR* homologs. The *B. mallei* genome is missing one of the *luxI-luxR* pairs. Two recent studies of quorum sensing in *B. pseudomallei* focused on a cognate acyl-HSL synthase and receptor pair called BpsR and BpsI (24, 38). These studies indicate that the relevant signal for this system is octanoyl-HSL (C_8 -HSL) and that BpsR activates siderophore synthesis and the synthesis of a nonspecific DNA binding protein called DpsA. Quorum sensing has been studied in more depth in a different complex of *Burkholderia* species, the *Burkholderia cepacia* complex. In *Burkholderia cenocepacia*, there are two *luxI-luxR* homolog pairs. The primary product of CeiI is C_8 -HSL, and the primary product of CciI is hexanoyl-HSL (C_6 -HSL) (22, 26). Another member of the complex, *Burkholderia vietnamiensis*, appears to have a similar set of systems (8). All known members of the *B. cepacia* complex are soil bacteria. Some species cause significant plant diseases, and some also infect humans with underlying health issues (25).

We are interested in identifying which acyl-HSL synthases of *B. mallei* are responsible for the production of specific acyl-HSLs and which LuxR homologs respond to these acyl-HSLs. There are two fundamental reasons to address these issues: First, there are robust animal models for *B. mallei* infections (15, 19), and identifying signals and receptors is a first step in developing *B. mallei* quorum-sensing inhibitors. The efficacy of such virulence inhibitors in preventing or resolving infections can be tested in precise ways with animal models. Second, *B. mallei* represents an unusual case in that quorum sensing is often important for infection by nonobligate but not by obligate bacterial pathogens. We hope that a systematic molecular study of *B. mallei* quorum sensing will reveal why it is important for this obligate animal pathogen to maintain acyl-HSL quorum-sensing systems.

MATERIALS AND METHODS

Bacterial culture conditions. Unless otherwise specified, all *Escherichia coli* strains were grown in Luria-Bertani (LB) broth. For growth of *B. mallei*, we used LB broth plus glycerol (4%, vol/vol). The acyl-HSL bioassay strain *Agrobacterium tumefaciens* KYC55 was grown in tryptone-yeast extract broth (2). Antibiotics were added to growth media at the following concentrations (per ml): 100 μ g ampicillin, 34 μ g chloramphenicol, and 15 μ g gentamicin for *E. coli*; 5 μ g gentamicin for *B. mallei*; and 100 μ g spectinomycin, 25 μ g gentamicin, and 5 μ g tetracycline for *A. tumefaciens*. For the induction of the arabinose promoter, we used 0.4% L-arabinose. For the induction of *lac* promoters, we used 0.4 mM isopropyl- β -D-thiogalactoside (IPTG). All *E. coli* and *B. mallei* cultures were incubated at 37°C with shaking, and *A. tumefaciens* was incubated at 30°C with shaking.

Strain and plasmid construction. The bacterial strains and plasmids used are described in Table 1. We used standard cloning protocols. Oligonucleotides (Table 1) were purchased from Integrated DNA Technologies (Coralville, IA). Genomic DNA was used as a template for the PCR amplification of all *B. mallei* quorum-sensing genes. To create the BmaI expression plasmid pBD2, the open reading frame of the *bmaI* gene (BMA_A1347) from bp +1 to +612 relative to the predicted translational start site was PCR amplified by using primers *bmaI*B5 and *bmaI*B6 (for this and all other sequence information, refer to The Institute for Genomic Research *Burkholderia mallei* Genome website at <http://cmr.tigr.org/tigr-scripts/CMR/GenomePage.cgi?org=gbm>). This procedure introduced EcoRI and XbaI restriction sites at the ends of the *bmaI* fragment. The *bmaI* PCR product was ligated into EcoRI-XbaI-digested pBAD24, which carries an L-arabinose-inducible promoter (P_{BAD}) (18). To generate pBD1, we used PCR by using primers *bmaI*B1 and *bmaI*B2 to create a *bmaI* (BMA_A1345)-containing DNA fragment (bp +1 to +720 with respect to the start of *bmaI* translation). This PCR fragment was ligated into NdeI-XhoI-digested pET17b (Novagen, Madison, WI). To construct *bmaI::lacZ* expression vector pBD5, we

used PCR to amplify a fragment of *B. mallei* genomic DNA extending from -395 to -1 bp relative to the predicted translational start site of *bmaI* by using primers *PbmaI*B7 and *PbmaI*B8. The fragment was cloned into NcoI-HindIII-digested pQF50 (13). To obtain a *bmaI* expression plasmid compatible with pBD5, we constructed pBD4 by PCR amplification of a *bmaI*-containing fragment of *B. mallei* genomic DNA (bp +1 to +720 in relation to the start of *bmaI* translation) using primers *bmaI*B3 and *bmaI*B4. The fragment contained EcoRI and SacI restriction sites and was subsequently cloned into EcoRI-SacI-digested pJN105 (29), putting *bmaI* under the control of the P_{BAD} promoter. The *lux* box mutant plasmid pBD5a was created by overlap extension PCR with primers *PbmaI*I(Δ*lux*1) and *PbmaI*I(Δ*lux*2). This yielded a DNA fragment with a deletion from nucleotides -80 to -71 (with respect to the predicted translational start codon) of the *bmaI* promoter (1). We performed a second PCR with primers *PbmaI*B7 and *PbmaI*B8 to amplify this mutant promoter for cloning into NcoI-HindIII-digested pQF50. To construct N-terminal histidine fusion vector pQF5016b.*bmaI*, we cloned the *bmaI* DNA fragment at bp +1 to +720 into NdeI-BamHI-digested pJLQhis (21).

Detection of 14 C-labeled acyl-HSLs. A radiotracer assay similar to that described previously by Singh et al. (37) was used to monitor acyl-HSLs produced by recombinant *E. coli*. Briefly, a 50-ml culture of *E. coli* DH5 α containing pBD2 was grown to an optical density at 600 nm (OD_{600}) of 0.3, at which point we added L-arabinose. When the OD_{600} reached 0.7, the culture was centrifuged at $2,750 \times g$ for 20 min, and the cell pellet was suspended in 2 ml of phosphate-buffered saline containing 10 mM glucose. After 10 min at 37°C with shaking, we added 5 μ Ci of L-[1- 14 C]methionine (American Radiolabeled Chemicals, St. Louis, MO). After an additional 3 h of incubation, the cell suspension was extracted with two equal volumes of acidified ethyl acetate (glacial acetic acid, 0.1 ml/liter). The ethyl acetate fraction was evaporated to dryness under a stream of N_2 gas. The residue was suspended in 50% methanol, and the entire extract was separated by C_{18} reverse-phase high-performance liquid chromatography (HPLC). Each HPLC fraction was mixed with 4 ml of Complete Counting Cocktail (catalog no. 3a70B; Research Products International, Mt. Prospect, IL), and radioactivity was detected by using a Beckman LS 1800 liquid scintillation counter.

Acyl-HSL bioassays. To compare the acyl-HSL profiles from wild-type *B. mallei* ATCC 23344 and *bmaI* mutant strain RJ16, we used the *A. tumefaciens* bioreporter strain KYC55, which carries a *traI-lacZ* fusion and a $P_{17-*traR*}$ overexpression plasmid. The *traI-lacZ* fusion in this strain is induced by an array of acyl-HSLs with side chain lengths ranging from C_6 to C_{10} . The third carbon of the acyl group can either be fully reduced, contain a carbonyl, or contain a hydroxyl moiety (44) (data not shown). Ten-milliliter cultures of *B. mallei* ATCC 23344 and *bmaI* mutant strain RJ16 were grown to an OD_{600} of 1.8, after which the cells were removed by centrifugation, and the culture fluid was extracted twice with acidified ethyl acetate and dried under N_2 gas. The dried material was reconstituted in 50% methanol, and the entire extract was separated by HPLC. A 40- μ l sample of each HPLC fraction was added to 0.5 ml of the *A. tumefaciens* bioreporter. The cells were incubated with shaking at 30°C for 16 h. We then added 50 μ l of chloroform to each culture tube. The samples were incubated at room temperature for 10 min, and β -galactosidase activity was monitored using a Tropix Galacto-Light Plus kit according to the manufacturer's protocol (Applied Biosystems, Foster City, CA).

To measure the BmaR1 response to acyl-HSLs directly, we used recombinant *E. coli* with a *bmaI-lacZ* fusion and *bmaI* on compatible plasmids as follows. A culture of *E. coli* MG4 containing pBD4 and pBD5 grown overnight was used as the inoculum (starting OD_{600} of 0.05). When the OD_{600} reached 0.5, we added L-arabinose to induce BmaR1 expression. This culture was added to tubes containing dried C_8 -HSL, C_{10} -HSL, dodecanoyl-HSL (C_{12} -HSL), or 3-hydroxy-octanoyl-HSL (3OHC $_8$ -HSL) as indicated. The volume of culture in each tube was 0.5 ml. After 2 h at 37°C, the β -galactosidase activity was measured as described above. To assess the necessity of the *lux*-box-like element for the transcriptional activation of the *bmaI-lacZ* fusion, we tested a *bmaI* promoter lacking the distal half-site of the *lux*-box-like element. One-milliliter cultures of *E. coli* MG4(pBD4, pBD5a) were grown to an OD_{600} of 0.5, induced with arabinose, and transferred into tubes containing either 20 nM C_8 -HSL or no C_8 -HSL. Growth was continued for 2 h with shaking, and β -galactosidase activity was measured as described above.

Assessing the solubility of BmaR1. A culture of *E. coli* BL21(DE3)(pLysS) containing pBD1 was used to inoculate flasks containing 25 ml of LB broth plus 50 mM MOPS (morpholinepropanesulfonic acid) buffer (pH 7.0) and 5 μ M C_8 -HSL, 5 μ M C_{10} -HSL, or no acyl-HSL as indicated. When the culture reached an optical density of 0.7, we added IPTG to induce BmaR1 expression. After 17 h with shaking at 16°C, cells were harvested by centrifugation at $2,750 \times g$ for 20 min. Cell pellets were frozen, thawed at room temperature, suspended in 1 ml of purification buffer (25 mM Tris-HCl [pH 7.8], 150 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 0.05% Tween 20) (35), and sonicated on ice. The cell lysates were clarified by ultracentrifugation at $163,000 \times g$ for 30 min at 4°C. The soluble and insoluble

TABLE 1. Bacterial strains, plasmids, and primers used in this study

Bacterial strain, plasmid, or primer	Genotype, phenotype, or primer sequence ^a (5'-3')	Reference, source, or description
Strains		
<i>B. mallei</i>		
ATCC 23344	Wild-type strain	30
RJ16	<i>bmaI1</i> mutant of ATCC 23344; <i>bmaI1</i> ::pRUI1 Gm ^r	39
<i>E. coli</i>		
DH5 α	F ⁻ ϕ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>hsdR17</i> (r _K ⁻ m _K ⁺) <i>recA1</i> <i>endA1</i> <i>phoA</i> <i>supE44</i> <i>thi-1</i> <i>gyrA96</i> <i>relA1</i> λ ⁻	Invitrogen
MG4	F ⁻ λ ⁻ <i>ilvG</i> <i>rfb-50</i> <i>rph-1</i> <i>recA</i> Δ (<i>argF-lacIPOZYA</i>)205	33
BL21(DE3)(pLysS)	F ⁻ <i>ompT</i> <i>gal</i> <i>dcm</i> <i>lon</i> <i>hsdS_B</i> (r _B ⁻ m _B ⁻) λ (T7 RNAP) pLysS Cm ^r	Novagen
<i>A. tumefaciens</i> KYC55 bioreporter	Derivative of R10 carrying pJZ384, pJZ410, and pJZ372; Gm ^r Sp ^r Tc ^r	44
Plasmids		
pBAD24	<i>araC</i> -P _{BAD} cloned into pDHB60, optimized SD; Ap ^r	18
pET17b	T7 expression vector; Ap ^r	Novagen
pJN105	<i>araC</i> -P _{BAD} cloned into pBBR1MCS-5; Gm ^r	29
pQF50	Broad-host-range <i>lacZ</i> fusion vector; Ap ^r	13
pUC19	Commonly used plasmid cloning vector; Ap ^r	Invitrogen
pBD1	pET17b containing the <i>bmaR1</i> gene; Ap ^r	This study
pBD2	pBAD24 containing the <i>bmaI1</i> gene; Ap ^r	This study
pBD4	pJN105 containing the <i>bmaR1</i> gene; Gm ^r	This study
pBD5	pQF50 containing a 395-bp fragment of the <i>bmaI1</i> promoter extending from position -1 with respect to the translation start site to position -395; Ap ^r	This study
pBD5a	pBD5 with a deletion of the first 10 bp of the <i>bmaI1</i> promoter <i>lux</i> box; Ap ^r	This study
pQF5016b. <i>bmaR1</i>	N-terminal histidine fusion vector pJLQhis containing the <i>bmaR1</i> gene; Ap ^r	This study
pJLQhis	N-terminal histidine fusion vector containing the <i>qscR</i> gene; Ap ^r	21
Primers		
<i>bmaR1</i> B1	NNNNNNCATATGGAAGTTCGCTGGCAAGACG	<i>bmaR1</i> forward primer
<i>bmaR1</i> B2	NCTCGAGTTACGGCGCGTCGATGA	<i>bmaR1</i> reverse primer
<i>bmaR1</i> B3	NGAATTCATGGAAGTTCGCTGGCAAGACG	<i>bmaR1</i> forward primer
<i>bmaR1</i> B4	NNGAGCTCTTACGGCGCGTCGATGA	<i>bmaR1</i> reverse primer
<i>bmaI1</i> B5	AAGAATTCAAAATGCGAAGTTCGTTTCATGGCG	<i>bmaI1</i> forward primer
<i>bmaI1</i> B6	TTTCTAGATCAGGCGGCTTCGGCGGG	<i>bmaI1</i> reverse primer
<i>PbmaI1</i> B7	NNCCATGGGACGTGCGCAGCAACGTGAT	<i>bmaI1</i> promoter forward primer
<i>PbmaI1</i> B8	NNNAAGCTTGTCTCTCTCGTGCCTGTGTC	<i>bmaI1</i> promoter reverse primer
<i>PbmaI1</i> (Δ <i>lux1</i>)	CAGCCCCGCTTTCCCGAGTTAACAGTTACCTGTCC	Forward primer for overlap extension PCR
<i>PbmaI1</i> (Δ <i>lux2</i>)	GGACAGGTAAGTGTAACTCGGGAAAGCGGGGCTG	Reverse primer for overlap extension PCR
1p99a	AACTGAAGTGGCCGTCCGC	Specific probe forward primer
1p99b	GCCCGTCGCCATGAACG	Specific probe reverse primer
M13F	TGTAAAACGACGGCCAGT	Nonspecific probe forward primer
M13R	GTGTGGAATTGTGAGCGG	Nonspecific probe reverse primer

^a SD, Shine-Dalgarno sequence. Endonuclease restriction sites are underlined.

protein fractions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels (12% polyacrylamide) were stained with Coomassie brilliant blue dye. Protein concentrations were estimated by using a Bradford assay (Bio-Rad, Hercules, CA), and bovine serum albumin (New England Biolabs, Beverly, MA) was used as a standard.

BmaR1 purification and in vitro DNA binding assay. BmaR1 was purified from recombinant *E. coli* as an N-terminal histidine fusion protein by using nickel affinity chromatography according to a previously published protocol for the purification of His-tagged QscR (21). Briefly, pQF5016b.*bmaR1* was used to transform *E. coli* BL21(DE3)(pLysS). His-tagged BmaR1 was expressed in a 400-ml culture of BL21(DE3)(pLysS, pQF5016b.*bmaR1*) according to the pro-

col described above for the expression of native BmaR1 from pBD1. The clarified cell extract was separated by nickel column chromatography (QIAGEN, Valencia, CA), proteins were eluted in a step gradient of imidazole, and the fractions containing His-tagged BmaR1 (200 mM to 300 mM imidazole) were pooled and dialyzed in 1 liter of purification buffer for 12 h followed by dialysis in fresh purification buffer for an additional 12 h. All purification and dialysis steps were performed at 4°C. The concentration of the purified His-tagged BmaR1 preparation was determined as described above.

To measure DNA binding activity of purified His-tagged BmaR1, we used a gel shift assay similar to those described elsewhere previously (35, 40). A 332-bp target DNA was generated by PCR with primers 1p99a and 1p99b and end labeled with

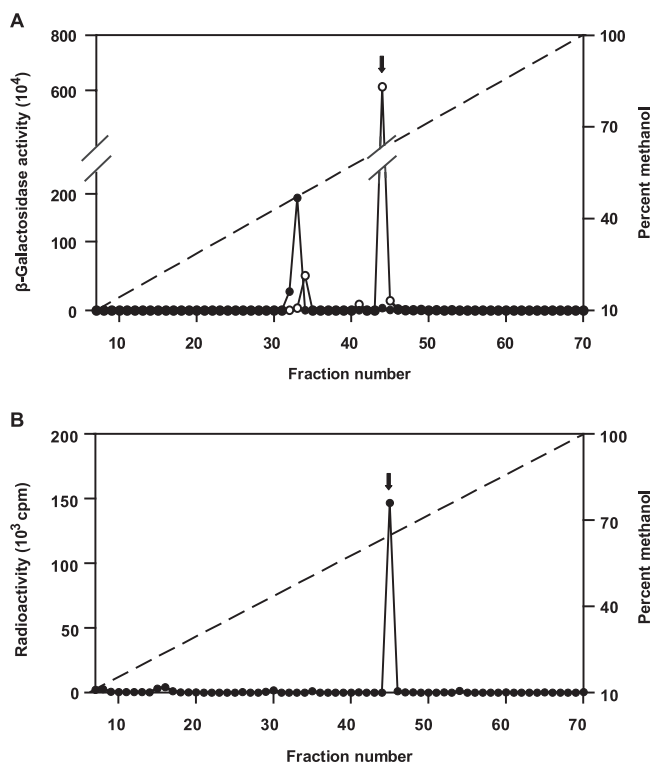


FIG. 1. Methanol gradient HPLC separation of acyl-HSLs produced by *B. mallei* and recombinant *E. coli* containing *bmaI1*. (A) HPLC profile of ethyl acetate-extracted culture fluid from *B. mallei* (○) and *bmaI1* mutant strain RJ16 (●). Acyl-HSL-containing HPLC fractions were identified by using a broad-specificity *A. tumefaciens* bioreporter strain. Peak sizes in this assay do not correlate with relative abundances, and although it has a broad specificity, the reporter does not respond to all acyl-HSLs. β -Galactosidase activity is reported in relative light units. (B) HPLC profile of ethyl acetate-extracted *E. coli* containing *bmaI1* vector pBD2. This analysis involved a radiotracer assay (see Materials and Methods). The radiotracer assay provides a measure of the relative abundances of acyl-HSLs present and serves to identify any acyl-HSL that is sufficiently hydrophobic to move into the ethyl acetate phase during extraction. This method will identify all acyl-HSLs reported to date. Short-chain acyl-HSLs are less efficiently extracted than long-chain acyl-HSLs, and their concentrations could be underestimated by up to 25%. The percent methanol is indicated as the dashed line. The arrow indicates the fraction in which synthetic C_8 -HSL is eluted.

γ - ^{32}P as described previously (35). An end-labeled nonspecific 120-bp molecule generated by PCR amplification with pUC19 as the template and M13F and M13R as primers was included in reaction mixtures as a control. The DNA binding reaction mixtures contained 4 fmol each of target and nonspecific DNA in a final volume of 20 μ l of 20 mM Tris $\cdot$ HCl (pH 7.5), 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 100 μ g per ml bovine serum albumin, and 5% glycerol. Purified BmaR1 was added to the DNA binding reactions at the indicated concentrations, and after 20 min at room temperature, the DNA molecules were resolved on a 5% Tris-glycine-EDTA polyacrylamide gel. Radioactivity was detected with a Storm PhosphorImager (GE Healthcare, Piscataway, NJ).

RESULTS

BmaI1 functions as a C_8 -HSL synthase. Fluid from *B. mallei* cultures contains several acyl-HSLs in unknown abundances (39). To better understand which acyl-HSL signal is produced by BmaI1, we fractionated stationary-phase culture extracts from either wild-type *B. mallei* or a *bmaI1* mutant (RJ16) by HPLC and

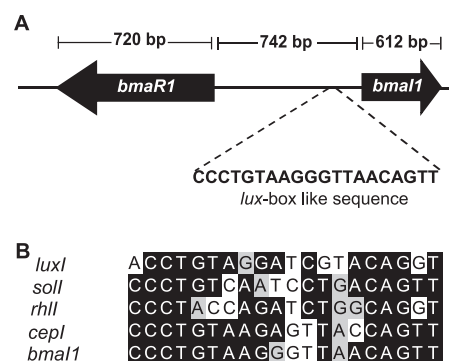


FIG. 2. The *bmaR1-bmaI1* *B. mallei* genomic region. (A) Map showing the divergently transcribed *bmaR1-bmaI1* region and the sequence of a lux-box-like element centered 70.5 bp upstream of the *bmaI1* translation start site. (B) Alignment of the lux box element shown in A with the lux boxes of *V. fischeri luxI* (GenBank accession no. Y00509) (12), *R. solanacearum soli* (accession no. AF021840) (14), *Pseudomonas aeruginosa rhlI* (accession no. U40458) (5), and *B. cepacia cepI* (accession no. AF019654) (22).

compared the acyl-HSL elution profiles by monitoring bioactivity. There were two acyl-HSLs evident in the wild-type extract, and the extract from the *bmaI1* mutant showed only one of these acyl-HSLs (Fig. 1A). This suggests that the acyl-HSL missing in the *bmaI1* mutant is synthesized by BmaI1. This acyl-HSL coelutes with C_8 -HSL. We calculated that the concentration of C_8 -HSL in the *B. mallei* culture fluid was 50 nM. The acyl-HSL that was eluted in the 50% methanol fraction for both extracts was identified as being 3OHC $_8$ -HSL (Fig. 1A). We calculated that this molecule was 1 to 2 nM in the wild-type culture fluid. To establish that BmaI1 is a C_8 -HSL synthase and to determine whether it produces other acyl-HSLs, we expressed this protein in *E. coli* and monitored acyl-HSL production using a [^{14}C]methionine incorporation assay (37). The radiotracer assay will identify acyl-HSLs regardless of whether they are detected by the bioassay, which has a limited range, and because one ^{14}C is incorporated into each molecule of acyl-HSL, the radiotracer assay provides information on the relative abundances of acyl-HSL products. As shown by this assay, fluid from a recombinant *E. coli* culture contained one major fraction of radioactivity that coeluted with C_8 -HSL (Fig. 1B). A low level of 3OHC $_8$ -HSL was also detected. The level of 3OHC $_8$ -HSL was about 1% of the C_8 -HSL level.

C_8 -HSL serves as the cognate acyl-HSL for BmaR1. The synthesis of many LuxR homologs in a soluble, active form requires the presence of their cognate acyl-HSL or a closely related acyl-HSL during bacterial growth. In the absence of an appropriate acyl-HSL, these polypeptides form as inactive aggregates (35, 40, 45). As a test of the hypothesis that C_8 -HSL is the BmaI1-generated signal, we examined the solubility of BmaR1, which is encoded by a gene adjacent to *bmaI1* (Fig. 2A), in cell extracts of *E. coli* containing a BmaR1 expression vector grown in the presence of several different acyl-HSLs (Fig. 3). In the presence of C_8 -HSL (5 μ M), a large proportion of the overexpressed polypeptide was in the soluble fraction. There was less soluble BmaR1 when cells were grown in the presence of C_{10} -HSL (5 μ M) than there was when the cells were grown in the presence of C_8 -HSL. However, there was little or no soluble BmaR1 obtained from cells grown without

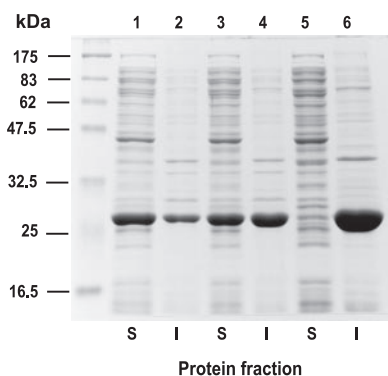


FIG. 3. Solubility of BmaR1 in extracts of recombinant *E. coli* grown in the presence of acyl-HSLs. An SDS-PAGE analysis of soluble (S) and insoluble (I) polypeptides from *E. coli* BL21(DE3)(pLysS) containing the BmaR1 expression vector pBD1. Cells were grown in the presence of C₈-HSL (lanes 1 and 2) or C₁₀-HSL (lanes 3 and 4) or with no acyl-HSL (lanes 5 and 6). The predicted molecular weight of BmaR1 is 26,600. A prestained protein ladder is shown in the left lane, and the molecular masses of the markers are shown in kDa.

added acyl-HSL or cells grown in the presence of the following acyl-HSLs: butanoyl-HSL (C₄-HSL), 3OC₆-HSL, or C₁₂-HSL (data not shown). This experiment supports the conclusion that BmaI1 is a C₈-HSL synthase that generates a signal for its cognate receptor, BmaR1.

The *bmaI1* promoter is activated by BmaR1 and C₈-HSL. Many *luxI* homologs are autoregulated by the acyl-HSL generated by the enzymes that they encode together with their LuxR homolog (5, 10, 23, 36). The promoters of these autoregulated *luxI* homologs usually contain a recognizable *lux*-box-like sequence, an 18- to 20-bp element resembling the 20-bp *lux* box to which LuxR binds. There is a *lux*-box-like sequence centered 70.5 bp upstream of the *bmaI1* translational start site (Fig. 2B). Thus, we hypothesized that the *bmaI1* gene is positively autoregulated by BmaR1 and C₈-HSL. To test our hypothesis, a 395-bp DNA fragment containing the presumed *bmaI1* promoter was fused to a promoterless *lacZ* (from positions -1 with respect to the translation start codon to -395). Expression of the *bmaI1::lacZ* reporter required BmaR1 and C₈-HSL (Fig. 4A). The concentration of C₈-HSL required for half-maximal *bmaI1* promoter activation was 16 nM. This value is within the range of responses for other LuxR homologs to their cognate signals (20, 31, 43). We also measured BmaR1-dependent *bmaI1* activation by C₁₀-HSL, C₁₂-HSL, and 3OHC₈-HSL. All of these acyl-HSLs served as poor substitutes for C₈-HSL (Fig. 4A). This finding supports the view that C₈-HSL is the cognate acyl-HSL for the BmaR1-BmaI1 system.

To test the hypothesis that the *lux*-box-like sequence in the *bmaI1* promoter region is required for activation by BmaR1, we constructed a plasmid with a deletion of the distal 10 bp of the *lux*-box-like element. In *E. coli* containing this construct, the basal level of *lacZ* expression was similar to that in *E. coli* containing the full-length *bmaI1-lacZ* fusion, but *lacZ* expression from the *lux*-box-like sequence deletion plasmid was not activated by BmaR1 and C₈-HSL (Fig. 4B). These findings support the view that in the presence of C₈-HSL, BmaR1 binds

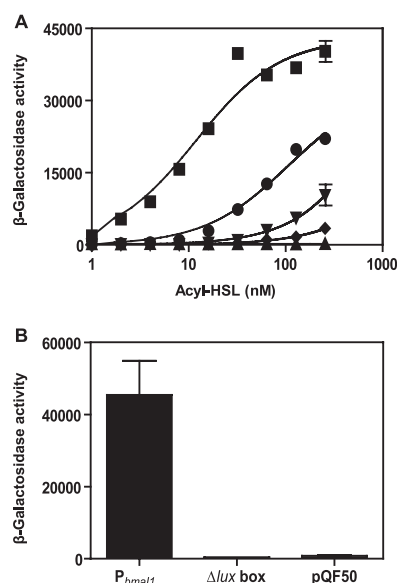


FIG. 4. Dependence of *bmaI1* transcription on C₈-HSL, BmaR1, and the *lux*-box-like sequence embedded in the *bmaI1* promoter region. (A) Acyl-HSL dose response of the *bmaI1* promoter in *E. coli* containing a BmaR1 expression vector (pBD4) and a *bmaI1-lacZ* reporter (pBD5). The following acyl-HSLs were added at the concentrations indicated: C₈-HSL (■), C₁₀-HSL (●), C₁₂-HSL (▼), or 3OHC₈-HSL (◆). ▲, control showing the C₈-HSL response of *E. coli* with the reporter plasmid but no BmaR1 expression plasmid. (B) Expression of the *bmaI1-lacZ* reporter in the full-length promoter construct (P_{*bmaI1*}) and in a construct with a deletion of the distal 10 bp of the *lux*-box-like element (Δ *lux* box). The empty vector (pQF50) is shown as a control. We added 20 nM C₈-HSL to all cultures. Values are means $\pm$ standard deviations for three independent experiments.

to the *lux*-box-like element that and this binding leads to transcriptional activation.

Purified BmaR1 binds to the *bmaI1* regulatory element. To study the interaction of BmaR1 with the *bmaI1* promoter, we purified BmaR1 as an N-terminal histidine fusion protein from recombinant *E. coli*. The presence of C₈-HSL during culture growth was required in order to obtain soluble His-tagged BmaR1. Furthermore, all attempts to remove C₈-HSL during purification resulted in the formation of insoluble aggregates of BmaR1 (data not shown). After nickel affinity chromatography of the soluble polypeptides (in the presence of C₈-HSL), we obtained a highly purified BmaR1 fraction for use in gel shift experiments (Fig. 5A). When incubated with 5 μ M C₈-HSL, BmaR1 bound specifically to a 332-bp promoter DNA fragment extending from positions -304 to +28 with respect to the *bmaI1* translation start site (Fig. 5B). Without C₈-HSL in the binding reaction, we did not observe a band shift (data not shown). We presume that BmaR1 did not remain in a soluble form without C₈-HSL. Thus, in vitro data support the conclusion that BmaR1 regulates the transcription of *bmaI1* by interacting with *bmaI1* regulatory DNA directly.

DISCUSSION

The genome of the obligate animal pathogen *B. mallei* has four *luxR* homologs and two *luxI* homologs (39). Two of the

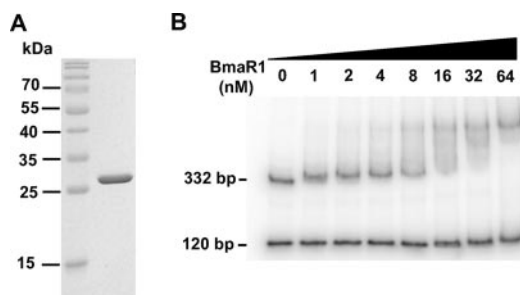


FIG. 5. Binding of purified BmaR1 to *bmaI1* promoter DNA. (A) SDS-PAGE of His-tagged BmaR1 after nickel column affinity chromatography. The left lane shows the molecular mass markers, and the right lane shows column-purified protein. (B) DNA mobility shift assay. Each lane contained approximately 4 fmol of the 332-bp *bmaI1* target DNA and a 120-bp nonspecific probe. The molar amount of BmaR1 in each binding reaction is indicated. All binding reactions contained 5 μ M C_8 -HSL.

luxR homologs (*bmaR1* and *bmaR3*) are linked to adjacent *luxI* homologs (*bmaI1* and *bmaI3*, respectively). The two other *luxR* homologs are examples of what have been called orphan quorum-sensing receptors (6, 32). Such orphans are common in bacteria that have cognate pairs, and in the cases where signal binding to an orphan has been studied, the orphans are capable of responding to acyl-HSL signals for one of the cognate pairs (21, 32). It is unusual for an obligate pathogen to possess an acyl-HSL quorum-sensing system, much less multiple systems, and previous studies have implicated all of the quorum-sensing signal generators and receptors in the virulence of *B. mallei* (39). We have chosen to begin a systematic study of acyl-HSL production and responses to specific acyl-HSLs in *B. mallei* by determining the nature of the signal involved in BmaR1-BmaI1 quorum sensing. The *bmaR1-bmaI1* region shares a great degree of sequence identity with the *bpsR-bpsI* gene pair in the closely related bacterial species *B. pseudomallei*. The predominant acyl-HSL synthesized by BpsI is C_8 -HSL (24, 38). We have shown that *B. mallei* produces C_8 -HSL, whereas a *bmaI1* mutant does not (Fig. 1A). Furthermore, C_8 -HSL is the predominant acyl-HSL produced by recombinant *E. coli* expressing BmaI1 (Fig. 1B). Thus, we conclude that BmaI1 is a C_8 -HSL synthase. This appears to be true for the closely related BpsI from *B. pseudomallei* and the less closely related CepI from *B. cenocepacia* (22). Unlike *B. mallei*, neither *B. pseudomallei* nor *B. cenocepacia* is an obligate animal pathogen (7).

Generally, *luxR* homologs linked to *luxI* homologs code for proteins that sense the acyl-HSL coded by the linked *luxI* homolog (5, 10, 23, 36). We have two lines of evidence that support the hypothesis that this is true for BmaR1 and that further support the conclusion that BmaI1 is a C_8 -HSL synthase. First, we know that many LuxR homologs require the presence of their cognate acyl-HSL in order to fold into a soluble active form. In fact, in cell lysates of recombinant *E. coli* grown in the absence of added acyl-HSLs, little or no BmaR1 is found in the soluble protein fraction. When C_8 -HSL is added to the growth medium, there is an abundance of BmaR1 in the soluble protein fraction (Fig. 3). Growth of bacteria in the presence of some but not all other acyl-HSL can also lead to the production of soluble active BmaR1, but C_8 -

HSL serves this purpose more effectively than any of the other acyl-HSLs that we tested (Fig. 3 and data not shown). Second, we asked whether the *bmaI1* promoter was activated by BmaR1, as is the case for many other *luxI* homologs and their cognate LuxR homologs. Expression of a *bmaI1-lacZ* fusion is dependent on BmaR1 in recombinant *E. coli*, and it is also dependent upon the presence of an acyl-HSL with the greatest sensitivity to C_8 -HSL (Fig. 4). Notably, C_{10} -HSL showed activation of the *bmaI1* promoter, but C_{10} -HSL activation was weaker than the C_8 -HSL response. BmaR1 is one of four LuxR homologs in *B. mallei*, and at present, we cannot conclude whether any of these other proteins might activate the *bmaI1* gene. Analysis of a *bmaI1* deletion construct showed that the activation of the *bmaI1* promoter required the presence of an inverted repeat with sequence similarity to binding sites for LuxR family members from other bacteria. Thus, we believe that BmaI1 is a C_8 -HSL synthase and that the cognate BmaR1 responds to C_8 -HSL. The specific product of BmaI3 remains to be determined, as do the signals to which BmaR3 and the two *B. mallei* orphan receptors respond.

We also purified BmaR1 as a His-tagged protein to confirm that it could bind to *bmaI1* promoter DNA directly (Fig. 5). In a fashion similar to that of many LuxR homologs, the synthesis of soluble BmaR1 in bacteria required the presence of the cognate acyl-HSL, in this case, C_8 -HSL. The continuous presence of C_8 -HSL during purification was required to keep BmaR1 in a soluble form. There is one other similar example of which we are aware, which is LuxR itself (40). The interactions of LuxR homologs and their cognate acyl-HSLs have been divided into three classes: (i) those which require a signal to fold into a soluble, active protein and bind the signal irreversibly, (ii) those which require a signal for folding but bind the signal reversibly, and (iii) those which do not require a signal to fold into an active conformation and bind signals reversibly (M. Schuster and E. P. Greenberg, unpublished data). We believe that BmaR1 is an example of the second type of protein. If C_8 -HSL bound irreversibly, the solubility characteristics of BmaR1 would not be expected to change upon the removal of unbound C_8 -HSL. Regardless, our DNA binding experiments with purified BmaR1 show that it can interact with *bmaI1* promoter DNA directly. Based on the fact that the activation of a *bmaI1-lacZ* reporter in recombinant *E. coli* depends on BmaR1, C_8 -HSL, and a 20-bp inverted repeat similar in sequence to binding sites for LuxR homologs of other bacteria, this demonstration of direct binding comes as no surprise.

This study of *B. mallei* BmaR1 and BmaI1 function provides important basic information about quorum sensing in *B. mallei*, tools for identification of the genes controlled by this quorum-sensing circuit, and discovery of inhibitors of this system. We believe that this work represents a useful systematic way to determine the cognate acyl-HSL for a given LuxR-LuxI pair of genes by capitalizing on our general knowledge of acyl-HSL signaling systems and employing an indiscriminant radiotracer assay to determine relative abundances of acyl-HSLs produced by any given LuxI homolog. This approach avoids artifacts that can result from the vastly different relative sensitivities of bioassays to different acyl-HSLs.

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