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Quorum-Sensing Control of Antibiotic Synthesis in *Burkholderia thailandensis*▽

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The genome of *Burkholderia thailandensis* codes for several LuxR-LuxI quorum-sensing systems. We used *B. thailandensis* quorum-sensing deletion mutants and recombinant *Escherichia coli* to determine the nature of the signals produced by one of the systems, BtaR2-BtaI2, and to show that this system controls genes required for the synthesis of an antibiotic. BtaI2 is an acyl-homoserine lactone (acyl-HSL) synthase that produces two hydroxylated acyl-HSLs, *N*-3-hydroxy-decanoyl-HSL ($3OHC_{10}$-HSL) and *N*-3-hydroxy-octanoyl-HSL ($3OHC_8$-HSL). The *btaI2* gene is positively regulated by BtaR2 in response to either $3OHC_{10}$-HSL or $3OHC_8$-HSL. The *btaR2-btaI2* genes are located within clusters of genes with annotations that suggest they are involved in the synthesis of polyketide or peptide antibiotics. Stationary-phase cultures of wild-type *B. thailandensis*, but not a *btaR2* mutant or a strain deficient in acyl-HSL synthesis, produced an antibiotic effective against gram-positive bacteria. Two of the putative antibiotic synthesis gene clusters require BtaR2 and either $3OHC_{10}$-HSL or $3OHC_8$-HSL for activation. This represents another example where antibiotic synthesis is controlled by quorum sensing, and it has implications for the evolutionary divergence of *B. thailandensis* and its close relatives *Burkholderia pseudomallei* and *Burkholderia mallei*.

Burkholderia thailandensis, *Burkholderia pseudomallei*, *and Burkholderia mallei* are closely related betaproteobacteria. These three species show extensive genome sequence identity (37, 72). *B. mallei* is an obligate mammalian pathogen that infects solipeds (horses, mules, and donkeys) and can also cause human infections (66, 69). *B. pseudomallei* is an emerging human pathogen found in soil and stagnant water, and it infects rice farmers in Southeast Asia and people of Northern Australia (8, 70). Once thought to be a nonpathogenic saprophyte endemic to the water and soil environments of central and northeastern Thailand (4), *B. thailandensis* has recently been found in the central United States and Australia (25, 34). Sufficiently high doses of *B. thailandensis* can cause mammalian infections (4, 11, 56).

We have been interested in acyl-homoserine lactone (acyl-HSL) quorum sensing among the *B. mallei-B. pseudomallei-B. thailandensis* group because quorum sensing has been implicated in the virulence of *B. mallei* and *B. pseudomallei* and because each member of this group has multiple quorum-sensing systems (60–62). Quorum sensing involves production of and response to a self-produced extracellular signal that allows individuals to monitor the group population density (3, 21, 67). *Proteobacteria* often use acyl-HSL signals for quorum sensing. The signals are produced by members of the LuxI family of acyl-HSL synthases. The proteins that respond to acyl-HSLs are members of the LuxR family of transcription factors (22). Dozens of species possess acyl-HSL quorum-sensing systems. The genes coding for LuxR and LuxI homologs are often adjacent on a chromosome and are considered cognate pairs. The nature of the acyl side chain varies depending on the specific LuxI homolog (22, 67, 68). A LuxR homolog responds best to the acyl-HSL produced by its cognate LuxI homolog. The genes regulated by acyl-HSL quorum sensing differ depending on the species under investigation (for a review, see reference 68).

Many species of *Burkholderia* possess one or multiple acyl-HSL quorum-sensing systems (16, 64). *B. mallei*, *B. pseudomallei*, and *B. thailandensis* each possess multiple *luxR* and *luxI* homologs. These are called *bma* genes in the case of *B. mallei*, *bps* genes in the case of *B. pseudomallei*, and *bta* genes in the case of *B. thailandensis*. The three species have highly conserved *R1-I1* genes, *R3-I3* genes, and two orphan *luxR* homologs (*luxR* homologs without adjacent *luxI* homologs) called *R4* and *R5*. Both *B. pseudomallei* and *B. thailandensis*, the two species that have a known environmental lifestyle, have an additional quorum-sensing gene pair called *R2-I2*, which has been lost in *B. mallei* (45). There is a high level of interspecies amino acid sequence identity among the nonorphan R-I polypeptide pairs found in *B. mallei*, *B. pseudomallei*, and *B. thailandensis*. The cognate signal for the R1-I1 systems of each species is octanoyl-HSL (C_8-HSL) (15, 57; J. R. Chandler and E. P. Greenberg, unpublished data). The R1-I1 system in *B. pseudomallei* has

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TABLE 1. Bacterial strains used in this study

Strain	Relevant properties	Reference or source
B. thailandensis		
E264	Wild-type strain	4
JBT102	*btaI2* mutant of E264	Chandler and Greenberg, unpublished
JBT105	*btaI1*, *btaI3* double mutant of E264	Chandler and Greenberg, unpublished
JBT108	*btaR2* mutant of E264	Chandler and Greenberg, unpublished
JBT112	*btaI1*, *btaI2*, *btaI3* triple mutant of E264	Chandler and Greenberg, unpublished
BD909	BTH_II1233::Km insertion mutant of E264	This study
BD20	BTH_II1233 mutant of E264	This study
E. coli		
DH5α	ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 hsdR17*(r_k^-, m_k^-) *recA1 endA1 supE44 thi-1 gyrA relA1* λ$^-$	Invitrogen
MG4	F$^-$ λ$^-$ *ilvG rfb-50 rph-1 recA* Δ(*argF-lacIPOZYA*)*205*	48
SM10	*thi thr leu tonA lacY supE recA*::RP4–2-Tc::Mu Km λ*pir*	55
B. subtilis		
168	Wild-type strain; *trpC2*	5
3610	Wild-type strain; φ3T	9
S. pyogenes		
MGAS50055	*speA*2; cerebrospinal fluid isolate	58
S. aureus		
MN8	tstH$^+$; clinical isolate from nonmenstrual toxic shock syndrome case	51
COL	Methicillin-resistant laboratory strain	24
P. aeruginosa		
PAO1	Wild-type strain	28

been reported to control siderophore production, phospholipase C production, and the oxidative stress response (41, 57). The R3-I3 pair in *B. mallei*, called BmaR3-BmaI3, produces and responds to *N*-3-hydroxy-octanoyl-HSL (3OHC$_8$-HSL) (14), and *B. thailandensis* BtaI3 also produces 3OHC$_8$-HSL (Chandler and Greenberg, unpublished). To date, the *R2-I2* genes conserved in *B. pseudomallei* and *B. thailandensis* have received little attention.

Here, we report that the *R2-I2* gene pairs of *B. pseudomallei* and *B. thailandensis* are embedded in large clusters of genes with annotations suggesting an involvement in antibiotic synthesis. We show that in *B. thailandensis*, BtaI2 primarily produces *N*-3-hydroxy-decanoyl-HSL (3OHC$_{10}$-HSL) and lesser amounts of 3OHC$_8$-HSL. BtaR2 is a receptor that responds to the BtaI2-produced acyl-HSLs 3OHC$_{10}$-HSL and 3OHC$_8$-HSL. We also show that *B. thailandensis* produces an antibiotic that is active against gram-positive bacteria and that the BtaR2-BtaI2 quorum-sensing system is required for the production of the antibiotic.

MATERIALS AND METHODS

Bacterial culture conditions. The bacterial strains used in this study are listed in Table 1. *B. thailandensis* E264 and E264 derivatives were used throughout these investigations. Unless otherwise indicated, all bacteria except *Streptococcus pyogenes* were grown in Luria-Bertani (LB) broth. *S. pyogenes* was grown on Todd-Hewitt broth supplemented with 0.2% (wt/vol) yeast extract or on brain heart infusion medium. The following antibiotics were used at the indicated concentrations (per ml) for marker selection and for maintaining plasmids: 100 μg ampicillin, 15 μg gentamicin, 300 μg trimethoprim, and 25 μg kanamycin for *E. coli* and 100 μg trimethoprim and 150 μg kanamycin for *B. thailandensis*. To induce arabinose promoter-controlled gene expression in *E. coli*, we used 0.2% (wt/vol) L-arabinose. We used 0.2% (wt/vol) L-rhamnose to activate the rhamnose-responsive promoter in *B. thailandensis*. All bacteria were grown at 37°C.

Plasmids and recombinant DNA procedures. The plasmids used in this study are listed in Table 2. Oligonucleotides were purchased from Integrated DNA Technologies, Coralville, IA (the sequences are available upon request). *B. thailandensis* genomic DNA was purified by using a Gentra Puregene Cell Plus isolation kit (Qiagen, Valencia, CA). PCR fragments were amplified with the Failsafe PCR System and buffer J or K premix solution (Epicentre Biotechnologies, Madison, WI) or with an Expand Long Template kit (Roche Diagnostics, Pleasanton, CA). *B. thailandensis* genomic DNA was used as the template for all PCRs. Plasmid DNA was isolated by using a Qiaprep mini-spin kit, and the PCR fragments used for cloning were purified with a Qiaquick PCR gel extraction kit (Qiagen, Valencia, CA).

To create the BtaR2 expression vector pJNR2, the open reading frame of *btaR2* (BTH_II1231) from bp −18 to +705 relative to the predicted translational start site was PCR amplified. This procedure introduced PstI and SacI restriction sites at the ends of the *btaR2* fragment. The *btaR2* PCR product was ligated to PstI-SacI-digested pJN105, which carries the L-arabinose-inducible promoter (P$_{BAD}$) (44). To generate the *lacZ* transcriptional fusion constructs pI2P50 and pQF1233, *B. thailandensis* DNA upstream of *btaI2* (BTH_II1227) and BTH_II1233 was PCR amplified by using primer pairs that incorporated NcoI and HindIII restriction sites at the ends of the PCR products. These products were cloned into NcoI-HindIII-digested pQF50 (18). The *btaI2-lacZ* fusion extends from +6 to −238 in relation to the predicted translational start site. BTH_II1233-*lacZ* extends from +13 to −409 in reference to the predicted translational start site.

To regulate expression of BtaR2 in *B. thailandensis*, it was necessary to place *btaR2* under the control of an inducible promoter other than the L-arabinose promoter. This is because *B. thailandensis* is known to metabolize L-arabinose (71). We chose the rhamnose-inducible promoter because of its utility in other *Burkholderia* species (6). We constructed the BtaR2 expression vector pSCR2 by

TABLE 2. Plasmids

Plasmids	Relevant properties[a]	Reference or source
pJN105	*araC*-P_{araBAD} cloned into pBBR1MCS-5; Gm^r	44
pQF50	Broad-host-range *lacZ* fusion vector; Ap^r	18
pSCrhaB2	*rhaRS*-P_{rhaBAD}, *dhfr* cloned into pBBR1MCS; Tp^r	6
pJRC115	Mobilizable suicide vector; Tp^r	Chandler and Greenberg, unpublished
pGEM-T Easy;*amrRAB-oprA*;;*FRT*-Km	pGEM-T Easy with Δ(*amrRAB-oprA*)::*FRT*-P_{EM7}-*kam-FRT* fragment; Ap^r Km^r	Mima and Schweizer, unpublished
pI2P50	pQF50 containing a 244-bp fragment of the *btaI2* promoter extending from position +6 with respect to the translation start site to −238; Ap^r	This study
pJNR2	pJN105 containing the *btaR2* gene; Gm^r	This study
pSCR2	pSCrhaB2 containing the *btaR2* gene; Tp^r	This study
pQF1233	pQF50 containing a 422-bp fragment of the BTH_II1233 promoter extending from position +13 with respect to the translation start site to −409; Ap^r	This study
pLARA1233	pJRC115 with ΔBTH_II1233 extending from +13 with respect to the translational start site to + 060; Tp^r	This study
pKM1233	*FRT*-P_{EM7}-*kam-FRT* fragment from pGEM-T Easy;*amrRAB-oprA*;;*FRT*-Km cloned into pLARA1233; Ap^r Km^r	This study
pINF1233	pJRC115 with ΔBTH_II1233 extending from +13 with respect to the translational start site to +4313; Tp^r	This study

[a] P_{araBAD}, P_{rhaBAD}, P_{EM7}, and *dhfr* are the arabinose-inducible promoter, the rhamnose-inducible promoter, the synthetic bacterial EM7 promoter, and the dihydrofolate reductase gene.

incorporating a PCR-amplified *btaR2*-containing DNA fragment into NcoI-HindIII-digested pSCrhaB2 (6).

The suicide vector pJRC115 was used to deliver modified genes into the *B. thailandensis* genome by allelic replacement. pJRC115 and all other constructs used to create the *B. thailandensis* mutant strains JBT102, JBT105, JBT108, and JBT112 will be described elsewhere (Chandler and Greenberg, unpublished). The method used for mutant construction in *B. thailandensis* is similar to that described recently (2). To create pLARA1233, we used two-step PCR. The first step involved synthesizing DNA fragments of approximately 1 kb that were homologous to sequences flanking the intended site of deletion in BTH_II1233. These products were mixed, and a second-step PCR was done to anneal and amplify the DNA adjacent to the intended site of deletion in BTH_II1233, creating a 2-kb amplicon for subsequent site-specific recombination. Amplification created an internal XbaI restriction site in the product. The resulting PCR product was digested with XmaI and HindIII and ligated to XmaI-HindIII-digested pJRC115 to yield pLARA1233. pLARA1233 was digested with XbaI and ligated to a 1.2-kb DNA fragment from XbaI-digested pGEM-T Easy; *amrRAB-oprA*;;*FRT*-Km Δ(*amrRAB-oprA*)::*FRT*-P_{EM7}-*kam-FRT* (T. Mima and H. Schweizer, unpublished data), which contains a kanamycin resistance cassette, creating the marked suicide vector pKM1233. pINF1233 was made by ligating a 2-kb DNA fragment generated by two-step PCR, and this product was digested and ligated to XbaI-HindIII-digested pJRC115. To construct the BTH_II1233 kanamycin insertion mutant strain BD909 and the unmarked BTH_II1233 deletion strain BD20, pKM1233 and pINF1233 were mobilized from *E. coli* SM10 into *B. thailandensis*. Trimethoprim-resistant transconjugants were identified and then counterselected by a method similar to that described elsewhere (2). PCR, followed by DNA sequencing, was used to confirm the mutations. The nucleic acid sequence coordinates of insertion and replacement during the construction of BD909 and BD20 can be found in Table 2.

LC/MS/MS identification of acyl-HSLs. Acyl-HSLs were isolated from 10-ml cultures (optical density at 600 nm [OD_{600}], 4.0) of wild-type *B. thailandensis* and the indicated mutant strains. Cells were removed from the culture fluid by centrifugation, and the culture fluid was sterilized by using a 0.2-μm filter (Millipore, Billerica, MA). For liquid chromatography-electrospray ionization-tandem mass spectrometry (LC/MS/MS), 0.4 nmol of deuterated C_6-HSL (D_3-C_6-HSL) and deuterated C_{12}-HSL (D_3-C_{12}-HSL) was added to the filtered culture fluid, which was then extracted with two equal volumes of acidified ethyl acetate (0.1 ml/liter glacial acetic acid) and dried to completion under a constant stream of nitrogen gas. This was followed by a solid-phase extraction and suspension of the material in 50 μl of 5% MeOH. Ten microliters of each sample was analyzed by LC/MS/MS as described elsewhere (26). The relative abundance of acyl-HSLs in each sample was determined by integrating the area under the analyte peaks and comparing these values to those of D_3-C_6-HSL and D_3-C_{12}-HSL standard curves (26). Relative abundance is a measurement of the ratio of the areas of the analyte and internal standard peaks to the ratio of the amount of analyte and internal standard in each sample.

Acyl-HSL bioassays. To measure the BtaR2 response to acyl-HSLs, we used recombinant *E. coli* with a *btaI2-lacZ* fusion or a BTH_II1233-*lacZ* fusion. Bioassays were performed as described elsewhere (15). Briefly, an overnight culture of *E. coli* MG4 (48) containing pJNR2 and pI2P50 was used to inoculate fresh LB broth containing arabinose (starting OD_{600}, 0.05). When the OD_{600} reached 0.7, 0.5 ml of culture was added to tubes containing dried C_8-HSL, C_{10}-HSL, C_{12}-HSL, $3OHC_6$-HSL, $3OHC_8$-HSL, or $3OHC_{10}$-HSL as indicated. After 3 h with shaking at 37°C, 50 μl of chloroform was added to each tube and β-galactosidase activity was measured using a Tropix Galacto-Light Plus kit according to the manufacturer's recommendations (Applied Biosystems, Foster City, CA). *E. coli* MG4 carrying pJNR2 and pQF1233 was used to measure the regulation of the BTH_II1233 promoter by BtaR2. The protocol was identical to that described for *E. coli* MG4 containing pJNR2 and pI2P50, except that only $3OHC_8$-HSL and $3OHC_{10}$-HSL were tested as signals.

Real-time PCR. Wild-type *B. thailandensis* and the *btaR2* mutant JBT108 were grown in 25 ml of LB broth supplemented with 2% glycerol and 25 mM MOPS (morpholinepropanesulfonic acid), pH 7.0. At an OD_{600} of 2.7, 4 ml of culture was treated with RNA Protect (Qiagen, Valencia, CA), and after centrifugation, the resulting cell pellet was suspended in 200 μl of Tris-EDTA (10 mM Tris base and 1 mM EDTA, pH 8) containing 10 mg/ml lysozyme. Total RNA was purified with an RNeasy spin purification kit (Qiagen, Valencia, CA). For quantitative real-time PCR (qRT-PCR), cDNA was generated from 2 μg of RNA by using a TaqMan kit (Applied Biosystems, Foster City, CA). Primers were designed to amplify 100- to 200-bp targets for use in qRT-PCRs. The qRT-PCRs used 2× SYBR green master mix (Applied Biosystems, Foster City, CA) with 40 cycles of 15 s at 95°C followed by 60 s at 62°C. The qRT-PCR program ended with a dissociation curve that was used to verify that a single product was amplified in each reaction and that primer dimers did not form. Threshold cycle (C_T) values were obtained with a manual threshold setting of 0.2. Values were generated by the calculation $2^{(35-C_T)}$. The results are reported as the calculated transcript amount of a given gene per calculated *rpoB* transcript. The reported values show the averages and ranges of biological replicates assayed in triplicate.

Antimicrobial susceptibility testing. To assess antimicrobial activity in *B. thailandensis* culture fluid, we routinely used a diffusion disc assay (29). *B. thailandensis* cultures were grown in LB broth containing MOPS (50 mM; pH 7.0) at 37°C. Colonies grown overnight on LB agar plates were used as the starting inoculum. Unless otherwise indicated, when *B. thailandensis* cultures reached late stationary phase (OD_{600}, 9 to 10) the cells were removed by centrifugation and the culture fluid was filtered through a 0.45-μm membrane. Antibiotic assay discs (13 mm; Whatman, Florham Park, NJ) were saturated with sterile culture fluid and deposited onto LB agar plates overlaid with 100 μl of a 1:10 dilution of

TABLE 3. Relative amounts of acyl-HSLs produced by wild-type and quorum-sensing mutant strains of *B. thailandensis*

Acyl-HSL	Relative abundance[a]		
	Wild type	Δ*btaI2*	Δ*btaI1*, Δ*btaI3*
C_8-HSL	27.5 (0.2)	54.6 (0.6)	ND[b]
$3OHC_8$-HSL	11.0 (0.06)	45.4 (0.6)	12.6 (1.8)
$3OHC_{10}$-HSL	57.6 (0.5)	ND	87.4 (1.8)
$3OHC_{12}$-HSL	3.9 (0.2)	ND	ND

[a] The values are the average relative percentages of acy-HSLs from two independent cultures; the range is indicated in parentheses.
[b] ND, none detected.

an overnight culture of *B. subtilis* 168 (5) or other bacterial species as indicated. The plates were incubated at 37°C overnight.

To measure antimicrobial activity more precisely and to assess whether the activity was bactericidal or bacteriostatic, we used the following assay. Fluid from 24-h *B. thailandensis* cultures grown in LB containing 50 mM MOPS (pH 7.0) at 37°C was collected and filter sterilized (0.45-μm-pore-size membrane). Early log-phase *B. subtilis* 3610 (9) cells were inoculated into the filtered *B. thailandensis* culture fluid diluted with fresh LB-MOPS broth (the initial *B. subtilis* density was approximately 1×10^6 cells per ml). After 24 h with shaking at 37°C, the *B. subtilis* cell density was determined by plate counting on LB agar.

RESULTS

The *btaI2* gene product catalyzes the synthesis of $3OHC_8$-HSL and $3OHC_{10}$-HSL. Acyl-HSL detection often involves bioassays or bioassays coupled to thin-layer-chromatographic separation of acyl-HSLs (50, 54). These methods rely on a specific LuxR homolog for acyl-HSL detection, and they are biased for specific acyl-HSLs. It is cumbersome to conduct a comprehensive quantitative analysis of acyl-HSLs using bioassays. Thus, we employed LC/MS/MS, which detects acyl-HSLs in a complex mixture with high sensitivity irrespective of acyl side chain length or substitution and directly measures the relative abundance of acyl-HSLs (26), to analyze acyl-HSLs produced by *B. thailandensis*. We analyzed acyl-HSLs in ethyl acetate extracts of stationary-phase (OD_{600}, 4) culture fluid from wild-type *B. thailandensis* and quorum-sensing mutants lacking *btaI2* (JBT102) or both *btaI1* and *btaI3* (JBT105). Wild-type *B. thailandensis* produced C_8-HSL, $3OHC_8$-HSL, $3OHC_{10}$-HSL, and a small amount of *N*-3-hydroxy-dodecanoyl-HSL ($3OHC_{12}$-HSL) (Table 3). The *btaI2* mutant produced C_8-HSL and $3OHC_8$-HSL, but it did not produce detectable levels of $3OHC_{10}$-HSL (Table 3). This analysis suggests that BtaI2 is responsible for the synthesis of $3OHC_{10}$-HSL. The *btaI1-btaI3* double mutant produced both $3OHC_8$-HSL and $3OHC_{10}$-HSL. Taken together, the data indicate that in *B. thailandensis*, BtaI2 produces $3OHC_8$-HSL and $3OHC_{10}$-HSL. C_8-HSL appears to be produced by either BtaI1 or BtaI3. BtaI1, BtaI3, or both are also capable of producing significant amounts of $3OHC_8$-HSL. Consistent with this interpretation, the closely related *B. mallei* and *B. pseudomallei* I1 synthases produce C_8-HSL (15, 57), and the *B. mallei* BmaI3 synthesizes $3OHC_8$-HSL (14). The work of Chandler and Greenberg (unpublished data) supports the notion that BtaI1 produces C_8-HSL and BtaI3 makes $3OHC_8$-HSL in *B. thailandensis*.

BtaR2 regulates *btaI2* transcription in response to $3OHC_8$-HSL or $3OHC_{10}$-HSL. It is common for *luxI* homologs to be positively autoregulated by the acyl-HSL signal(s) produced by the proteins they encode and the transcription factor encoded by the adjacent *luxR* homolog (10, 12, 15, 23, 32, 35, 39, 52, 53). The *btaR2* and *btaI2* gene organization is unusual in that there is approximately 3.3 kb of DNA between these two genes, and this intervening DNA contains three open reading frames. We hypothesized that *btaR2* and *btaI2* may represent a cognate pair of quorum-sensing genes and that *btaI2* transcription would be activated by BtaR2 in response to $3OHC_8$-HSL, $3OHC_{10}$-HSL, or both signals. To test our hypothesis, we created a plasmid with a 244-bp DNA fragment containing the putative promoter region of *btaI2* (from −238 to +6 of the predicted translational start site) fused to a promoterless *lacZ*. This plasmid was introduced into *E. coli* with or without an arabinose-inducible *btaR2*. Activation of *btaI2-lacZ* transcription required BtaR2 and either $3OHC_8$-HSL or $3OHC_{10}$-HSL (Fig. 1A). There was also a response to *N*-decanoyl-HSL (C_{10}-HSL) or *N*-dodecanoyl-HSL (C_{12}-HSL), neither of which was detected in *B. thailandensis* culture fluid. There was no detectable response to C_8-HSL or several other acyl-HSLs tested.

To verify that *btaI2* transcription is regulated by BtaR2 in *B. thailandensis*, we measured the abundance of *btaI2* mRNA in the wild type and the *btaR2* mutant strain JBT108 by using qRT-PCR. The *btaI2* gene is the first of five genes in a putative

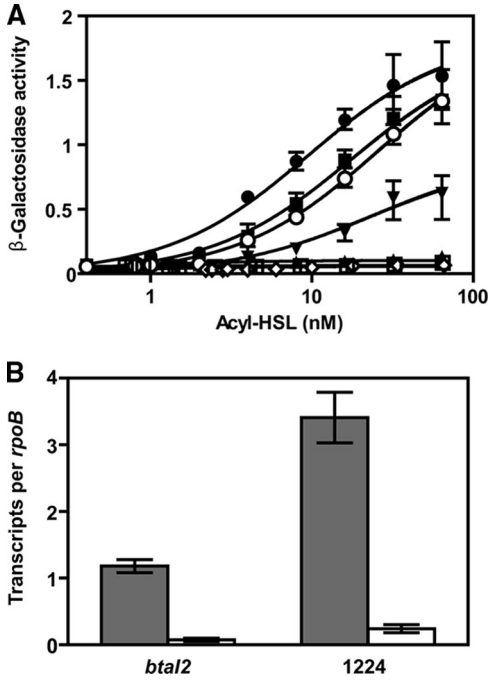

FIG. 1. Transcriptional activation of genes in the *btaI2* operon requires $3OHC_{10}$-HSL or $3OHC_8$-HSL and BtaR2. (A) Acyl-HSL dose responses of the *btaI2* promoter in *E. coli* containing a BtaR2 expression vector (pJNR2) and a *btaI2-lacZ* fusion vector (pI2P50). The following acyl-HSLs were tested: $3OHC_{10}$-HSL (■), $3OHC_8$-HSL (●), 3OHC6-HSL (□), C_{12}-HSL (▼), C_{10}-HSL (○), and C_8-HSL (▲). The open diamonds indicate the $3OHC_{10}$-HSL response in the absence of BtaR2. The error bars represent the range of three independent experiments. β-Galactosidase activity is given as millions of relative light units. (B) Relative transcript levels of *btaI2* and the downstream gene BTH_II1224 from wild-type *B. thailandensis* (gray bars) and the *btaR2* mutant strain JBT108 (white bars). The error bars represent the range of two independent experiments assayed in triplicate.

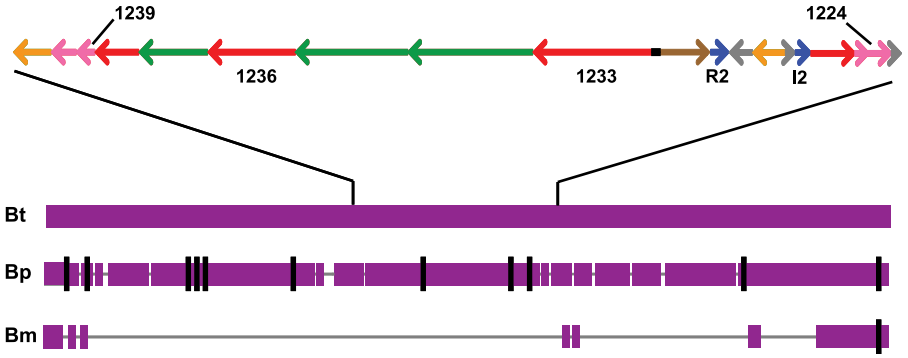

FIG. 2. Organization of the *B. thailandensis btaR2-btaI2* genomic region. Shown is a map of the genes in the immediate vicinity of *btaR2* and *btaI2*. The *btaR2* and *btaI2* genes are separated by three open reading frames encompassing about 3.3 kb of DNA. *btaI2* resides in a predicted five-gene operon that contains open reading frames annotated to function in antibiotic synthesis. *btaR2* is 3 kb upstream of a cluster containing putative antibiotic biosynthesis genes. The blue arrows labeled R2 and I2 represent *btaR2* and *btaI2*, respectively. The red arrows are annotated as NRPS genes, green arrows indicate PKS genes, pink arrows are potential accessory antibiotic synthesis genes, orange arrows are putative transport genes, the brown arrow is a metallopeptidase, and gray indicates genes of unknown function. The black lines between coding regions represent intergenic DNA. The purple bars represent the *btaR2-btaI2* genomic region and the flanking DNA, which is conserved in *B. pseudomallei* and mostly absent from the *B. mallei* chromosome. The genomic sequences were obtained from the publicly available genome sequences of *B. thailandensis* strain E264, *B. pseudomallei* strain K96243, and *B. mallei* strain ATCC 23344. The alignments were generated using the nucleotide BLAST algorithm (73). The *B. thailandensis* E264 genomic sequence was used as the reference sequence. A solid purple bar indicates congruence in nucleic acid sequence; the amino acid sequences within these regions share >90% identity. Vertical black bars represent nucleic acid sequence with dissimilarity, and gaps between purple bars are missing sequences. The arrows representing *btaR2*, *btaI2*, and their surrounding genes are drawn to scale.

operon residing on chromosome II. The qRT-PCR analysis showed that transcript levels of *btaI2* and a downstream gene (1224) were substantially higher in the wild type than they were in the *btaR2* mutant (Fig. 1B).

The *btaR2* and *btaI2* genes reside among a cluster of putative antimicrobial biosynthetic genes conserved in *B. pseudomallei* but absent in *B. mallei*. Three of the genes in the putative *btaI2* operon are predicted to code for enzymes involved in the production of secondary metabolites (Fig. 2). The second gene codes for a protein with similarity to nonribosomal peptide synthetases (NRPSs). NRPSs are involved in the production of amino acid-derived secondary metabolites (30). The third gene's product contains a domain similar to those found in NRPS enzymes, and the fourth gene codes for a protein with identity to a *Pseudomonas syringae* halogenase involved in the synthesis of the phytotoxin coronatine (59, 63). There is a cluster of genes (1233 to 1241) on chromosome II, upstream of *btaR2*, that codes for several additional NRPS polypeptides and several modular polyketide synthases (PKSs). PKSs function by incorporating fatty acids into secondary metabolites (30). Many secondary metabolites produced by NRPSs, PKSs, and hybrid NRPS-PKS enzymes are medically important antimicrobials, immunosuppressants, or anticancer molecules (19, 20). Furthermore, the cluster distal to *btaR2* also harbors genes that may play a role in the immunity to and transport of antibiotics (36, 40, 65). We hypothesize that *B. thailandensis* uses BtaR2-BtaI2 quorum sensing to control antibiotic production.

The *btaI2* and *btaR2* genes reside amid a large cluster of genes, many of which we suspect are involved in production of secondary metabolites (Fig. 2). Does *B. pseudomallei*, which possesses *btaI2* and *btaR2* homologs, have a similar cluster of genes, and does *B. mallei*, which does not have *btaI2* and *btaR2* homologs, have this cluster? The alignment in Fig. 2 shows that there is extensive identity over an approximately 120-kb *R2-I2* region of the *B. thailandensis* and *B. pseudomallei* chromosome II. This 120-kb region is completely absent in *B. mallei*. The genes flanking this region are conserved among all three species.

***B. thailandensis* produces an antibiotic.** Because *btaI2* and *btaR2* are embedded in a chromosomal region with many genes annotated as coding for functions known to be involved in antibiotic synthesis, transport, and immunity in other bacteria, we hypothesized that *B. thailandensis* produces an antibiotic and that antibiotic production may require BtaR2 and an acyl-HSL produced by BtaI2. To test the hypothesis, we first screened fluid taken from *B. thailandensis* cultures at various cell densities (Fig. 3A). To obtain the fluid, cells were removed by centrifugation and the supernatant fluid was subjected to microfiltration. The filtered culture fluid was used to saturate paper diffusion discs, which were placed on agar plates containing a growing lawn of *Bacillus subtilis.* After overnight incubation at 37°C, a zone of *B. subtilis* growth inhibition was observed around discs which had been saturated with fluid from stationary-phase cultures but not around discs saturated with fluid from logarithmic-phase cultures (Fig. 3B). Thus, *B. thailandensis* produces a secondary metabolite with antimicrobial activity.

We tested the specificity of the antibacterial component from stationary-phase culture fluid (OD_{600}, 9 to 10) by using the diffusion disc assay to assess whether the component inhibited the growth of other bacterial species. Growth of the gram-positive bacteria *Staphylococcus aureus* (including methicillin-resistant *S. aureus*) and *Streptococcus pyogenes* was inhibited by the antibiotic. Neither of the two gram-negative bacteria we tested, *E. coli* and *Pseudomonas aeruginosa*, showed growth inhibition by *B. thailandensis* culture fluid under the growth conditions we tested (Fig. 3C). The compound may

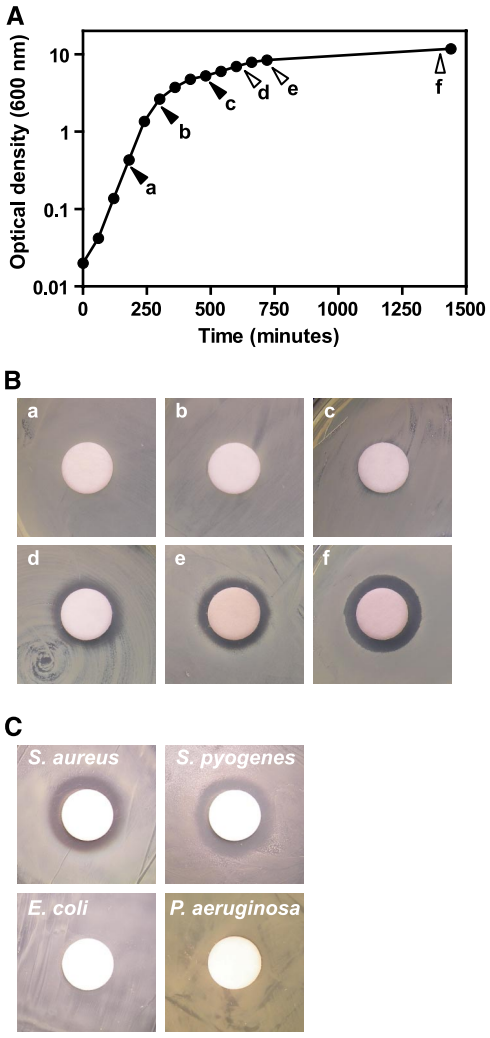

FIG. 3. Sensitivity of *B. subtilis* to a substance in *B. thailandensis* stationary-phase culture fluid. (A) Growth curve of the wild-type *B. thailandensis* strain E264. The arrowheads marked a to f indicate points where culture fluid was taken for the analysis shown in panel B. The open arrowheads indicate points in growth where antibiotic was produced. (B) Antibiotic sensitivity assays. Paper diffusion discs were saturated with fluid from a *B. thailandensis* E264 culture at the indicated points (a to f in panel A) and placed on lawns of *B. subtilis*. A zone of clearing around a diffusion disc indicates the region where *B. subtilis* growth was inhibited. (C) Antibiotic activity of *B. thailandensis* culture fluid against *S. aureus* COL, *S. pyogenes* MGAS5005, *E. coli* DH5α, and *P. aeruginosa* PAO1.

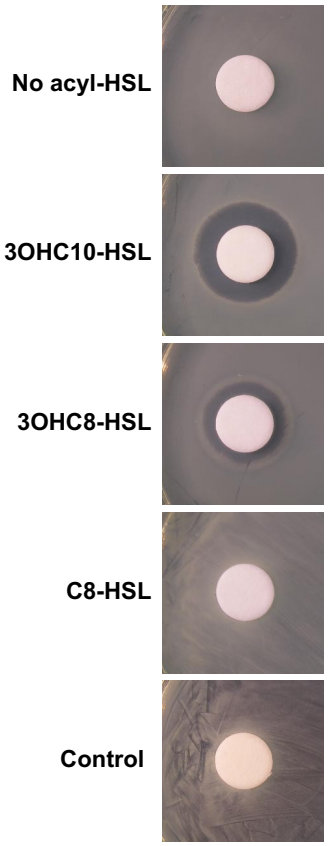

FIG. 4. A *B. thailandensis* acyl-HSL synthesis mutant requires exogenous $3OHC_8$-HSL or $3OHC_{10}$-HSL for antibiotic production. Diffusion disc assays with fluid from a stationary-phase culture of the *btaI1*, *btaI2*, *btaI3* triple mutant JBT112 grown without added signal or with 2 μM $3OHC_8$-HSL or $3OHC_{10}$-HSL, as indicated, are shown. Growth of *B. subtilis* is inhibited by culture fluid from $3OHC_{10}$-HSL- or $3OHC_8$-HSL-grown *B. thailandensis* JBT112, but not by JBT112 grown without an added acyl-HSL or in the presence of 2 μM C_8-HSL. The bottom panel shows a diffusion disc that had been soaked in sterile medium containing 2 μM $3OHC_{10}$-HSL. This control shows that $3OHC_{10}$-HSL itself is not an antimicrobial molecule.

not be active against gram-negative bacteria, or it may be that there were insufficient amounts of the antibiotic for inhibition of gram-negative bacteria in the preparations we tested.

The *btaR2-btaI2* quorum-sensing system is required for antibiotic production. To determine whether stationary-phase antibiotic production required the *btaR2-btaI2* quorum-sensing system, we first asked whether synthesis of the factor required acyl-HSL signaling as follows. We tested cell-free stationary-phase culture fluid of a *B. thailandensis* strain with mutations in all three of the acyl-HSL synthase genes, *btaI1*, *btaI2*, and *btaI3* (Chandler and Greenberg, unpublished), for anti-*B. subtilis* activity as described above (Fig. 4). Fluid from cultures of the triple acyl-HSL synthase mutant, JBT112, did not block *B. subtilis* growth unless the cultures were grown in the presence of added $3OHC_8$-HSL or $3OHC_{10}$-HSL. Addition of C_8-HSL was unable to restore antibiotic production to JBT112. As a control, we determined that $3OHC_{10}$-HSL alone does not have antibiotic activity (Fig. 4). From this experiment, we conclude that a BtaI2-produced acyl-HSL is required for production of the antibacterial factor.

To investigate whether the acyl-HSL-dependent production of antibacterial activity required BtaR2, we tested the ability of the *btaR2* mutant, JBT108, to produce an antibacterial factor. As shown in Fig. 5A, fluid from a stationary-phase JBT108 culture does not inhibit growth of *B. subtilis*, and the antibiotic synthesis phenotype was complemented by a plasmid-borne copy of *btaR2*. Another way to assess antimicrobial activity is to incubate *B. subtilis* in broth containing fluid from stationary-phase *B. thailandensis* cultures and assess bacterial growth as changes in CFU (Fig. 5B). When we incubated *B. subtilis* in

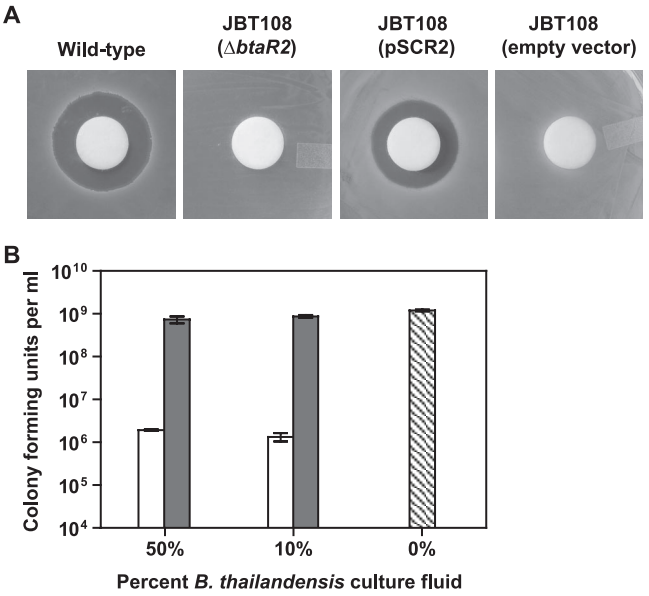

FIG. 5. *B. thailandensis* BtaR2 is required for antibiotic production. (A) A diffusion disc experiment showing antibiotic activity of fluid from a *B. thailandensis* E264 (wild-type) stationary-phase culture, a JBT108 (*btaR2* mutant) stationary-phase culture, and JBT108 complemented with the BtaR2 expression plasmid pSCR2. The control is strain JBT108 carrying the empty vector pSCrhaB2. *B. subtilis* was used as the indicator strain. (B) Influence of *B. thailandensis* culture fluid (50% or 10% [vol/vol] as indicated) on growth of *B. subtilis* in broth assessed by colony counting. The white bars represent culture fluid from the parent *B. thailandensis* E264, the gray bars indicate fluid from the *btaR2* mutant JBT108, and the hatched bar is a control *B. subtilis* culture with no added *B. thailandensis* culture fluid. The error bars indicate standard deviations.

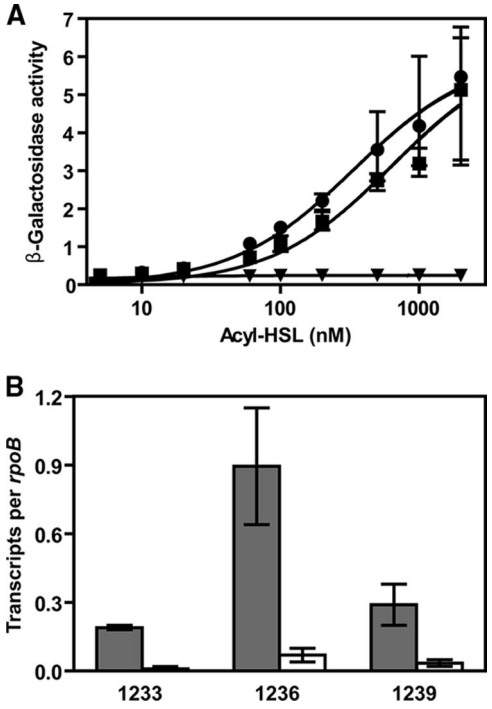

FIG. 6. Dependence of BTH_II1233, -1236, and -1239 transcription on the BtaI2-BtaR2 quorum-sensing system. (A) Acyl-HSL dose responses of the BTH_II1233-*lacZ* fusion on pQF1233 and *btaR2* on pJNR2 in *E. coli*; $3OHC_{10}$-HSL, ■; $3OHC_8$-HSL, ●. The BTH_II1233-*lacZ* response to $3OHC_{10}$-HSL in *E. coli* without a BtaR2 expression vector is also shown (▼). The error bars indicate the range of three independent experiments. β-Galactosidase activity is given as millions of relative light units. (B) Relative transcript levels of BTH_II1233 and the downstream genes BTH_II1236 and BTH_II1239 from wild-type *B. thailandensis* E264 (gray bars) and the *btaR2* mutant strain JBT102 (white bars). The values represent the range of two independent experiments assayed in triplicate.

medium containing 50% (vol/vol) fluid from a wild-type *B. thailandensis* stationary-phase culture, the *B. subtilis* cell density did not change from that of the inoculum. In the presence of 50% (vol/vol) fluid from a stationary-phase culture of the *btaR2* mutant, JBT108, the *B. subtilis* cell density increased to that seen in the absence of *B. thailandensis* culture fluid. This difference in CFU was also observed when we used 10% (vol/vol) *B. thailandensis* culture fluid from either the wild type or JBT108 (Fig. 5B). This experiment supports the conclusion that BtaR2-dependent quorum sensing is required for production of the antibacterial factor. It also suggests that the factor exerts bacteriostatic activity rather than bactericidal activity.

A putative nonribosomal peptide synthetase is required for quorum-sensing-regulated antibiotic production in *B. thailandensis*. As discussed above, the *btaI2* gene cluster and a cluster of genes located upstream of *btaR2* encode proteins annotated as NRPS and PKS enzymes, which we predict are responsible for the production of the BtaR2-BtaI2-controlled antibiotic. The cluster of genes distal to *btaR2* (1233 to 1241) is predicted to constitute an operon. We first asked whether the promoter upstream of the first gene in the cluster, 1233, was quorum sensing controlled by constructing a transcriptional fusion of −409 to +13 (with respect to the predicted translational start site) of the putative 1233 promoter to *lacZ*. We introduced a 1233 promoter-*lacZ* fusion plasmid into *E. coli* containing a BtaR2 expression vector and tested whether either $3OHC_8$-HSL or $3OHC_{10}$-HSL activated the *lacZ* reporter (Fig. 6A). Either $3OHC_8$-HSL or $3OHC_{10}$-HSL was sufficient for BtaR2-dependent induction of *lacZ*.

To obtain further data about quorum-sensing control of the 1233 to 1241 genes, we compared 1233, 1236, and 1239 transcript levels in the wild type and the *btaR2* JBT108 mutant by qRT-PCR. The wild type showed about 10-fold more transcript for these genes than the mutant (Fig. 6B). Therefore, we conclude that BtaR2 and either $3OHC_8$-HSL or $3OHC_{10}$-HSL regulate a cluster of putative secondary-metabolite genes, including 1233, 1236, and 1239.

To determine whether gene 1233 itself is required for antibiotic production, we constructed a 1233 insertion mutant strain and a 1233 in-frame deletion strain of *B. thailandensis* and tested stationary-phase culture fluid from both of these 1233 mutants for antibiotic activity. Neither strain made detectable levels of the antibiotic (Fig. 7). Thus, gene 1233 on chromosome II is required for antibiotic production. We have not tested any of the other genes in the 1233-to-1241 cluster, but we suspect that all or several are required for antibiotic production.

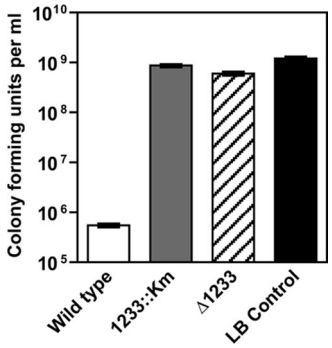

FIG. 7. BTH_II1233 mutants do not produce antibiotic. The influence of *B. thailandensis* culture fluid (10% vol/vol) on growth of *B. subtilis* in broth was assessed by colony counting. The *B. thailandensis* wild-type strain E264 is indicated by the white bar. The BTH_II1233 kanamycin insertion mutant, BD909, and the in-frame deletion mutant, BD20, are indicated by the gray and hatched bars, respectively. For reference, a control culture with no added *B. thailandensis* culture fluid is shown (black bar). The error bars indicate standard deviations.

DISCUSSION

B. thailandensis, *B. pseudomallei*, and *B. mallei* are closely related species, and it has been proposed that the limited genomic differences between these species reflect adaptations defining their specific niches. *B. mallei* is the only one of the three species that is an obligate animal pathogen (66, 69). Both *B. thailandensis* and *B. pseudomallei* exist in tropical soils (8, 70). All three species have two quorum-sensing systems in common, the so-called systems 1 and 3, which have been investigated previously (14, 15, 41, 57). The *B. thailandensis* *btaR2* and *btaI2* genes encoding quorum-sensing system 2 have conserved counterparts in *B. pseudomallei*, but not in *B. mallei*, and prior to this study, little was known about this quorum-sensing system.

The BtaR2-BtaI2 quorum-sensing system of *B. thailandensis* E264 is embedded in an approximately 120-kb DNA element, conserved in *B. thailandensis* and *B. pseudomallei* but absent in *B. mallei*. Evidence indicates that the obligate animal pathogen *B. mallei* has evolved from an ancestor common to *B. thailandensis* and *B. pseudomallei* in large part by undergoing a number of genome size reductions by deletion of regions not required for virulence (31, 45). This appears to be the case for the 120-kb *R2-I2*-containing DNA element. Other investigators have shown that both of the *B. mallei* quorum-sensing systems contribute to virulence (61). It is logical to assume that system 2 in *B. pseudomallei* is not critical for virulence. Rather, system 2 of both *B. pseudomallei* and *B. thailandensis* might be important for survival in the soil environment. System 2 is present in the two species that exhibit a saprophytic soil lifestyle but not in the species that does not occur as a soil saprophyte.

We have presented evidence that the *B. thailandensis* BtaR2-BtaI2 quorum-sensing system generates $3OHC_8$-HSL and $3OHC_{10}$-HSL and responds to either compound by regulating the synthesis of an antibiotic that is active against a variety of gram-positive bacteria. Our evidence indicates that BtaI2 is the only one of the three LuxI homologs in *B. thailandensis* that produces significant amounts of $3OHC_{10}$-HSL but that both BtaI2 and BtaI3 produce $3OHC_8$-HSL (this study and Chandler and Greenberg, unpublished). BtaR2 responds to either $3OHC_8$-HSL or $3OHC_{10}$-HSL about equally well. It is interesting that BtaR2 responds to the signals produced by its cognate acyl-HSL synthase, BtaI2, and that one of these signals is also produced by a noncognate acyl-HSL synthase, BtaI3. It is possible that $3OHC_8$-HSL production via BtaI2 may also jump-start the BtaR3-BtaI3 system. If true, this would be an aspect of network complexity that has not been observed in other bacteria with multiple acyl-HSL quorum-sensing systems. At this time, we do not know about the timing of *btaR2-btaI2* and *btaR3-btaI3* expression and whether the BtaI2-dependent production of $3OHC_8$-HSL provides an advantage to these systems or whether it is of no particular consequence.

The BtaR2-BtaI2 quorum-sensing system controls at least two gene clusters that reside on the 120-kb element that is also present in *B. pseudomallei* but absent from *B. mallei*. This 120-kb element can be considered a quorum-sensing island. We have shown that BtaR2-BtaI2 controls the production of a secondary metabolite with antibiotic activity against a variety of gram-positive bacteria. It is not a coincidence that we focused our initial assays for antimicrobial activity on *B. subtilis*, which like *B. thailandensis* is a soil bacterium. A classical view of the benefit of antibiotic production is that it can give microbes in their environment a competitive advantage over other antibiotic-sensitive microbes in the same habitat (33, 38, 49). An emerging view is that antibiotics actually serve intercellular signaling functions rather than as weapons against competitors (13, 17, 27). Our findings are consistent with the classical view and at odds with the emerging view.

B. thailandensis joins a list of bacteria that control antibiotic synthesis by an acyl-HSL quorum-sensing system. The list includes *Erwinia carotovora* (1, 43), *Pseudomonas chloroaphis* (47), and *Burkholderia vietnamiensis* (46), which is in the *B. cepacia* complex rather than the *B. mallei-B. pseudomallei-B. thailandensis* complex (7, 42). It is possible that quorum control of antibiotic synthesis in *B. thailandensis* allows microcolonies in soil environments to resist invasion by competing species and thus provides a selective advantage to the cells in the microcolony. The hypothesis that quorum-sensing control of antibiotic synthesis provides *B. thailandensis* an advantage in soil environments or in microcolonies can be tested experimentally, and such tests may shed light on the evolution of acyl-HSL quorum sensing.

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