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# Global Analysis of the *Burkholderia thailandensis* Quorum Sensing-Controlled Regulon

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***Burkholderia thailandensis* contains three acyl-homoserine lactone quorum sensing circuits and has two additional LuxR homologs. To identify *B. thailandensis* quorum sensing-controlled genes, we carried out transcriptome sequencing (RNA-seq) analyses of quorum sensing mutants and their parent. The analyses were grounded in the fact that we identified genes coding for factors shown previously to be regulated by quorum sensing among a larger set of quorum-controlled genes. We also found that genes coding for contact-dependent inhibition were induced by quorum sensing and confirmed that specific quorum sensing mutants had a contact-dependent inhibition defect. Additional quorum-controlled genes included those for the production of numerous secondary metabolites, an uncharacterized exopolysaccharide, and a predicted chitin-binding protein. This study provides insights into the roles of the three quorum sensing circuits in the saprophytic lifestyle of *B. thailandensis*, and it provides a foundation on which to build an understanding of the roles of quorum sensing in the biology of *B. thailandensis* and the closely related pathogenic *Burkholderia pseudomallei* and *Burkholderia mallei*.**

We have a general interest in acyl-homoserine lactone (AHL) quorum sensing (QS) and the benefit this type of cell-to-cell communication can provide to bacteria living in different environments. AHL QS, which has been identified in dozens of species of *Proteobacteria*, usually involves gene pairs coding for LuxI-family AHL signal synthases and LuxR-family AHL signal receptors, which function as transcription factors. The AHL signals are diffusible, and when they reach a critical environmental concentration, they can interact with their cognate LuxR homolog to alter global patterns of gene expression (see reference 1 for review).

AHL QS is common to many *Burkholderia* species, including *Burkholderia thailandensis*, a nonpathogenic tropical soil saprophyte. *B. thailandensis* is closely related to two pathogenic species, *Burkholderia pseudomallei* and *Burkholderia mallei* (2–4). We call these three related species the *Bptm* group (5). Members of the *Btpm* group have homologous QS systems. *B. thailandensis* and *B. pseudomallei* contain three complete QS circuits, quorum sensing circuit 1 (QS-1), QS-2, and QS-3. *B. mallei* has retained QS-1 and QS-3, but not QS-2. The *B. thailandensis* QS-1 circuit consists of the BtaI1-BtaR1 pair and the signal *N*-octanoyl homoserine lactone ($C_8$-HSL) (6, 7), QS-2 consists of BtaI2-BtaR2 and *N*-3-hydroxy-decanoyl homoserine lactone ($3OHC_{10}$-HSL) (6, 8), and QS-3 consists of BtaI3-BtaR3 and *N*-3-hydroxy-octanoyl homoserine lactone ($3OHC_8$-HSL) (6, 7). Additionally, each member of the *Bptm* group contains two orphan LuxR homologs (LuxR homologs without a cognate LuxI homolog). The *B. thailandensis* orphans are called BtaR4 and BtaR5 (6). The *B. thailandensis* QS-1 system controls aggregation, motility, and oxalic acid production, QS-2 controls synthesis of the broad-spectrum bactobolin antibiotics, and we do not know what functions are controlled by QS-3 (6–9).

We believe that *B. thailandensis* QS research will advance our understanding of several aspects of AHL signaling. First, there is not a deep understanding of why certain bacteria like *B. thailandensis* possess multiple quorum sensing systems. *B. thailandensis* can serve as a model to study what advantages multiple systems provide. Second, *B. thailandensis*, *B. pseudomallei*, and *B. mallei* are closely related species with generally conserved QS systems. *B. mallei* is a host-restricted pathogen, and *B. pseudomallei* is a soil bacterium as well as a highly infectious opportunistic pathogen. Work with either *B. pseudomallei* or *B. mallei* requires elaborate biosafety containment. Because *B. thailandensis* is not a human pathogen, it serves as a convenient model to study QS and other conserved aspects of the biology of the *Bptm* group using less-stringent non-select agent biocontainment conditions (10–13). Ultimately, we hope that comparisons of the QS regulons in *B. thailandensis*, *B. pseudomallei*, and *B. mallei* will provide insight about the evolution of AHL QS.

Little is known about the networks of genes controlled by QS in the *Bptm* group or how the multiple QS circuits might intersect. Here we describe results of a transcriptome sequencing (RNA-seq) study in which numerous QS-controlled genes are identified. This is a first step toward understanding the value of gene regulation by multiple QS circuits; it is a step toward understanding how QS benefits a saprophytic species and toward understanding how quorum sensing might benefit a saprophyte versus an opportunistic pathogen versus a host-adapted pathogen.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown

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**TABLE 1** Bacterial strains and plasmids used in this study

| Bacterial strain or plasmid | Description or relevant genotype[a] | Source or reference |
|---|---|---|
| Bacterial strains | | |
| DH10B | *E. coli* cloning vehicle | Invitrogen |
| E264 | Wild-type *B. thailandensis* | 3 |
| JBT112 | E264 Δ*btaI1* Δ*btaI2* Δ*btaI3* | 7 |
| JBT107 | E264 Δ*btaR1* | 7 |
| JBT108 | E264 Δ*btaR2* | 7 |
| JBT109 | E264 Δ*btaR3* | 7 |
| JBT110 | E264 Δ*btaR4* | 7 |
| JBT111 | E264 Δ*btaR5* | 7 |
| CM183 | E264 Δ*cdiAIB*::*tmp*; Tp$^r$ | This study |
| CM219 | E264 *glmS1 attn7*::*tmp*; Tp$^r$ | This study |
| JBT101 | E264 Δ*btaI1* | 7 |
| JBT102 | E264 Δ*btaI2* | 7 |
| JBT103 | E264 Δ*btaI3* | 7 |
| Plasmids | | |
| pTNS2 | R6K replicon TnsABC+D vector | 68 |
| pUC18T-mini-Tn7T-*tmp* | Cloning vector | 69 |
| pBD4 | pJN105 with *bmaR1*; Gm$^r$ | 16 |
| pBD5 | pQF50 with the *bmaI1* promoter; Ap$^r$ | 16 |
| pJNR2 | pJN105 with *btaR2*; Gm$^r$ | 8 |
| pI2P50 | pQF50 with the *btaI2* promoter; Ap$^r$ | 8 |

[a] Tp$^r$, trimethoprim resistant; Zeo$^r$, zeocin resistant; Gm$^r$, gentamicin resistant; Ap$^r$, ampicillin resistant.

in Luria-Bertani (LB) broth (10 g tryptone per liter, 5 g yeast extract, 5 g NaCl per liter) supplemented with 50 mM morpholinepropanesulfonic acid (MOPS) buffer (pH 7.0) when indicated. Antibiotics were added to bacteria at the following concentrations as appropriate: for *Escherichia coli*, 100 μg/ml trimethoprim (Tp), 25 μg/ml zeocin (Zeo), 100 μg/ml ampicillin (Ap), and 15 μg/ml gentamicin (Gm); for *Burkholderia thailandensis*, Tp, 100 μg/ml; and Zeo, 2 mg/ml. Where indicated, $3OHC_{10}$-HSL (2 μM), $3OHC_8$-HSL (4 μM), and $C_8$-HSL (2 μM) were added. Except where indicated, bacteria were grown at 37°C with shaking.

For RNA isolation, inocula were from 5-ml overnight LB-MOPS *B. thailandensis* cultures grown in 16-mm tubes. Fresh LB-MOPS with or without AHLs (15 ml in 125-ml flasks) was inoculated to a starting optical density at 600 nm ($OD_{600}$) of 0.05. Biological replicates were from different days.

**Mutant construction.** The contact-dependent inhibition (CDI) mutant CM183 was constructed by first generating a deletion fragment with PCR and then introducing the DNA fragment into *B. thailandensis* via natural transformation as described previously (14). To create the Δ*cdiAIB*::*tmp* deletion fragment, we first used PCR to generate three DNA molecules. The first consisted of approximately 1,000 bp of sequence upstream of the *cdi* genes and was generated with primers OCM83 and OCM85 (Table 2). This fragment contained a 3′ primer-encoded sequence complementary to the fragment carrying trimethoprim resistance. The second fragment contained the trimethoprim resistance cassette from pUC18T-mini-Tn7T-*tmp* and was made with primers OCM84 and OCM92 (Table 2). The third fragment contained approximately 1,000 bp of sequence downstream of the *cdi* genes and was made with primers OCM91 and OCM86 (Table 2). The 5′ end of this fragment contained primer-encoded sequence complementary to the 3′ end of the fragment carrying trimethoprim resistance. We next stitched the DNA molecules together by Gibson product ligation (New England BioLabs). The assembled fragment was then amplified in a final PCR with primers OCM83 and OCM86, purified, and used to transform *B. thailandensis* to yield the Tp-resistant (Tp$^r$) mutant strain CM183.

To create the Tp$^r$-marked *B. thailandensis* strain CM219, pUC18T-mini-Tn7T-*tmp* and pTNS2 were introduced into *B. thailandensis* strain E264 by electroporation. Briefly, the cells were grown to mid-exponential phase ($OD_{600}$ of 0.5), pelleted by centrifugation, and washed. First the cells were washed with an equal volume of sterile water, then with 25 ml sterile water, and then with 1 ml of 10% sterile glycerol. The pellet was resuspended in 100 μl of 10% sterile glycerol, and 1 μg of each plasmid was used for electrotransformation. The electroporation mixture was allowed a 3- to 5-h outgrowth prior to plating on selective media. Tp$^r$ colonies were selected, and we used PCR to show that the marker was inserted specifically at the *attn7* site near *glmS1*.

**Measurement of $C_8$-HSL, $3OHC_8$-HSL, and $3OHC_{10}$-HSL in *B. thailandensis* cultures.** To measure AHLs in *B. thailandensis* cultures, we twice extracted 5 ml of a culture grown to an $OD_{600}$ of 4.0 with acidified ethyl acetate. The analysis was performed as described elsewhere (15), except that high-performance liquid chromatography (HPLC) fractions containing $3OHC_{10}$-HSL were also collected. We measured the AHLs in appropriate HPLC fractions by using bioassays as described elsewhere (16). Synthetic $C_8$-L-HSL was purchased from Sigma Chemical Co. $3OHC_{10}$-L-HSL was purchased from the School of Molecular Medical Sciences at the University of Nottingham (http://www.nottingham.ac.uk/quorum/compounds.htm), and synthetic $3OHC_8$-L-HSL was synthesized as previously described (15).

**RNA isolation.** About $1 \times 10^9$ bacteria from cultures at an $OD_{600}$ of 0.5, 2.0, or 4.0 were suspended in RNAprotect bacteria reagent (Qiagen) and pelleted by centrifugation. The cell pellets were stored at −80°C. Thawed cells were suspended in 1 ml RLT buffer (Qiagen) containing 2-mercaptoethanol and lysed by bead beating. RNA was purified by using the miRNAeasy minikit (Qiagen). Contaminating DNA was removed with Turbo DNase (Ambion), and RNA was obtained by using RNeasy MinElute cleanup kit (Qiagen).

**RNA-seq library construction.** *Bptm*-specific primers were based on the sequenced genomes of *B. thailandensis* E264, *B. pseudomallei* K96243, and *B. mallei* ATCC 23344 using methods similar to the "not-so-random" primer method previously described (17). Primers predicted to anneal to the 5S, 16S, and 23S ribosomal sequences of each species were removed from a random set of hexamers. Two test RNA-seq analyses were run on RNA isolated from wild-type *B. thailandensis* cells harvested at an $OD_{600}$ of 2.0, and the remaining primers that annealed to rRNA were removed from a final *B. thailandensis*-specific hexamer pool of 949 primers. *In silico*

**TABLE 2** Primers used in this study

| Primer | Sequence[a] | Description |
|---|---|---|
| OCM83 | ATGTCCCGGGCGAAAAATGGGGATAGTGGA | Upstream *cdi* primer |
| OCM84 | ACACTCTTTGACGCTGCCTTGGATCTTGAAGTACCTATTC | Upstream forward primer for *cdi* and *tmp* junction |
| OCM85 | GAATAGGTACTTCAAGATCCAAGGCAGCGTCAAAGAGTGT | Upstream reverse primer for *cdi* and *tmp* junction |
| OCM86 | TGAACCCGGGTTTCAGGGTTGGCAAGTAGC | Downstream *cdi* primer |
| OCM91 | GCGCTTTTGAAGCTAATTCGTTGCATATGCTCGTCTGGTC | Downstream forward primer for *cdi* and *tmp* junction |
| OCM92 | GACCAGACGAGCATATGCAACGAATTAGCTTCAAAAGCGC | Downstream reverse primer for *cdi* and *tmp* junction |

[a] Restriction endonuclease sites are underlined.

analyses of the specific primers showed an average primer density (hits per gene) of 106 for *B. thailandensis*, 91 for *B. mallei*, and 87 for *B. pseudomallei*. The primer sequences are provided in Table S8 in the supplemental material.

We prepared cDNA libraries as described elsewhere (18) by using the 949-primer set and isolated RNA. Briefly, a cDNA strand was generated for the RNA. Then, the first DNA strand was converted to double-stranded DNA by RNase H-mediated nick translation. The double-stranded DNA was purified, and the ends were prepared for adapter ligation. Following adapter ligation (containing bar-coded DNA sequences), the products were PCR amplified, purified, and sequenced on an Illumina Genome Analyzer II.

**RNA-seq mapping and analysis.** Raw sequencing reads were sorted by bar-coded adapters and then aligned to the *B. thailandensis* E264 genome (the GenBank accession no. for chromosome 1 is CP000086.1 and for chromosome 2, it is CP000085.1). Aligned reads were analyzed with the Avadis NGS software package version 1.4.5 (Strand Scientific Intelligence, CA). We removed remaining reads mapping to the ribosomal sequences, determined differentially regulated genes for biological replicates using the DESeq package (with a false discovery rate [FDR] cutoff of 0.05), and proceeded with genes showing 2-fold or more regulation relative to the reference condition. Reads that partially overlapped with a gene contributed to its raw count. The Avadis NGS software package was used to compare gene expression between conditions. The data have been deposited in the NCBI sequence read archive (SRA database) (BioProject identification [ID] PRJNA233628).

**Operon, ortholog, and pseudogene analysis.** We used Regulatory Sequence Analysis Tools (RSAT) (19) to predict operons based on two criteria: genes within a predicted operon must be no more than 100 bp apart and must be transcribed in the same direction. To identify orthologs and paralogs among *B. thailandensis*, *B. pseudomallei*, and *B. mallei*, we employed a two-pronged approach. First, all genes showing QS control at an $OD_{600}$ of 2.0 were imported into the *Burkholderia* Prokaryotic Genome Analysis Tool (PGAT) (20). Orthologs and paralogs in *B. thailandensis* E264, *B. pseudomallei* 1026b, and *B. mallei* ATCC 23344 were identified by using PGAT. We used the same QS-controlled gene list to query all open reading frames in a six-frame translation of each genome in order to identify any potentially nonannotated sequence or sequences that were annotated as pseudogenes. Gene matches with 80% or more identity using basic local alignment search tool (BLAST) (21) were identified as orthologs.

The *B. thailandensis* genome and each QS-controlled gene list was further analyzed using PGAT tools to identify genes containing signal peptides and transmembrane domains and to assign each gene a functional category of gene product (COG).

**Competition experiments.** Overnight cultures of target or inhibitor *B. thailandensis* strains were back diluted into fresh LB-MOPS broth with or without AHLs to an $OD_{600}$ of 0.05 and grown to logarithmic phase ($OD_{600}$ of 0.2 to 0.6). Then, each culture was adjusted to a final $OD_{600}$ of 0.2 in phosphate buffer (pH 7.0). The partners were mixed at a 1,000:1 or 1:1 ratio of inhibitor and target. Twenty microliters of the competition mixture was then spotted onto an LB-MOPS agar plate with or without AHLs, when indicated. The plates were incubated at 30°C for 24 h. The bacteria were scraped from the agar, suspended in phosphate buffer (pH 7.0), subjected to water bath sonication for 10 min to disrupt aggregates. The bacteria were enumerated by plate counting on selective media. A competitive index (CI) was generated for each competition by dividing the final ratio of target to inhibitor cells by the starting ratio of target to inhibitor cells.

## RESULTS

**AHL-regulated genes.** We used RNA-seq transcriptomic analysis to identify *B. thailandensis* QS-controlled genes. AHL-regulated genes were identified by comparing transcripts in an AHL-negative *B. thailandensis* strain (JRC112) grown with or without exogenously added synthetic AHLs. Because the AHL-negative strain possesses wild-type copies of all five of its *luxR* homologs, it can respond to added AHLs. We identified genes regulated by the addition of the QS-1 signal ($C_8$-HSL), the QS-2 signal ($3OHC_{10}$-HSL), or the QS-3 signal ($3OHC_8$-HSL) during logarithmic growth (L growth) ($OD_{600}$ of 0.5), the transition from logarithmic growth to stationary phase (T phase) ($OD_{600}$ of 2.0), and stationary phase (S phase) ($OD_{600}$ of 4.0).

**TABLE 3** Numbers of QS-regulated genes

| AHL or transcription factor | No. of genes regulated during the following[a]: | | | | | |
|---|---|---|---|---|---|---|
| | T phase | | | S phase | | |
| | Activated | Repressed | Total | Activated | Repressed | Total |
| $C_8$-HSL | 24 | 11 | 35 | 29 | 68 | 97 |
| $3OHC_{10}$-HSL | 69 | 46 | 115 | 21 | 31 | 52 |
| $3OHC_8$-HSL | 125 | 62 | 187 | 35 | 54 | 89 |
| BtaR1 | 101 | 63 | 164 | ND[b] | ND | ND |
| BtaR2 | 20 | 0 | 20 | ND | ND | ND |
| BtaR3 | 12 | 3 | 15 | ND | ND | ND |
| BtaR4 | 0 | 0 | 0 | ND | ND | ND |
| BtaR5 | 0 | 1 | 1 | ND | ND | ND |

[a] Number of genes regulated during the transition (T) and stationary (S) phase. Regulation was determined by comparing transcripts from the AHL synthesis mutant (strain JRC112) grown with AHLs to transcripts from the mutant grown without the indicated AHL or by comparing a specific BtaR transcription factor mutant to the wild type.
[b] ND indicates that analyses were not done.

As a prelude to the transcriptomic analysis, we measured wild-type S-phase culture levels of AHLs so that we could add reasonable concentrations of these signals to the AHL synthesis mutant. We found 370 ± 100 nM $C_8$-HSL, 2.5 ± 0.5 μM $3OHC_{10}$-HSL, and 190 ± 50 nM $3OHC_8$-HSL in culture fluid. For transcriptome analysis, we used AHL concentrations similar to or exceeding the measured amounts in wild-type culture fluid (2 μM $C_8$-HSL, 2 μM $3OHC_8$-HSL, or 4 μM $3OHC_{10}$-HSL). We note that the addition of AHLs to the growth medium results in artificially high AHL levels during early growth phases. Despite the continuous presence of AHLs throughout culturing, they did not influence gene expression during L growth. However, transcript levels of dozens to hundreds of genes were affected during the T and S phases (Table 3). The greatest influence of AHLs on the transcriptome was during the T phase. Furthermore, the majority of T-phase AHL-responsive genes showed activation (65%), whereas in S phase, the majority of AHL-responsive genes were repressed (64%).Tables S1 and S2 in the supplemental material show all AHL-regulated genes. The effects of AHLs at different phases of growth are consistent with studies of other bacteria where AHL signaling is required but not sufficient for transcriptional regulation (22).

Thirty-five genes were regulated by $C_8$-HSL in T phase, and 97 were regulated in S phase, but only 4 showed a $C_8$-HSL response in both the T and S phases (see Table S3 in the supplemental material). Of the many $3OHC_{10}$-HSL-regulated genes, 32 showed a response in both T and S phases. Of the many $3OHC_8$-HSL-regulated genes, 27 showed a response in both T and S phases. Interestingly, there was considerable overlap among the QS-2 and QS-3 signal-controlled genes; 23 of the genes regulated in T and S phases by $3OHC_{10}$-HSL were also regulated by $3OHC_8$-HSL in T and S phases (Table S3).

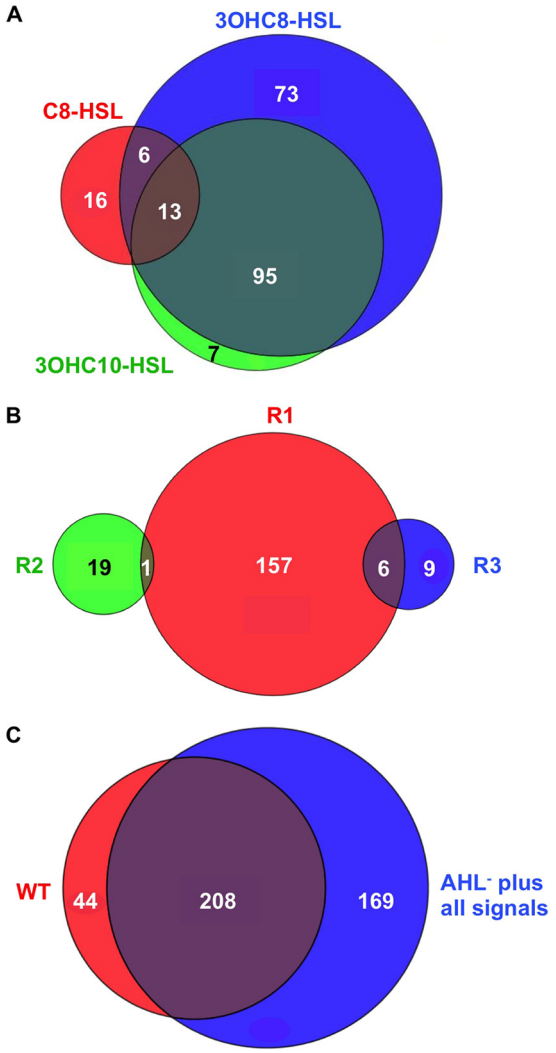


**FIG 1** Comparisons of T-phase QS-controlled genes. Venn diagrams show the relationships between QS-controlled genes in different RNA-seq experiments during T phase (the transition between exponential and stationary-phase growth). The circles show overlapping regulons under different conditions (the numbers of genes are also given). (A) The AHL synthesis mutant grown without any signals and grown with the indicated AHL. (B) The wild-type parent compared to the *btaR1* (R1), *btaR2* (R2), or *btaR3* (R3) mutant (grown in medium with all three AHLs added at the time of inoculation). (C) The wild type (WT) or the AHL synthase mutant grown with all three AHLs (AHL$^-$ plus all signals) compared to the AHL mutant grown without added AHLs.

To gain additional insight, we compared the transcriptomes at T phase of the AHL-negative mutant and the wild type, and the AHL-negative mutant grown with and without all three of the AHL signals added together (Fig. 1; see Tables S1 and S4 in the supplemental material). These comparisons address two issues. The first comparison allows a normal accumulation of each of the three signals during growth and allows us to identify QS-controlled genes that might require multiple signals. The second comparison independently addresses the issue of whether multiple signals might be required for QS control of some genes. We interpret differences in the QS-regulated genes in these two comparisons to indicate that the artificial addition of relatively high AHL concentrations from the time of inoculation results in some experimental artifacts. Regardless, our results show a large overlap in the two comparisons with 208 genes showing differential regulation in either comparison (Fig. 1C and Table S4 in the supplemental material). There were, however, 169 genes showing differential expression only in the signal-add back comparison and 44 genes showing differential expression only in the comparison of the wild type to the AHL synthesis mutant. We cannot draw conclusions about these results, but they suggest that timing of expression can be affected by exogenous additions of AHLs for some, but not all, QS-regulated genes.

**Validation of RNA-seq analysis.** Our RNA-seq analysis identified genes known to be controlled by QS or genes coding for functions known to be controlled by QS in *B. thailandensis* (see Tables S1, S2, S3, and S4 in the supplemental material). QS-1 promotes oxalate accumulation in T- and S-phase *B. thailandensis* cultures (23). RNA-seq showed that the QS-1 signal ($C_8$-HSL) induced transcription of *obc-1*, which codes for the oxalate biosynthetic enzyme. Interestingly, the other two AHLs made by *B. thailandensis*, 3OHC$_8$-HSL and 3OHC$_{10}$-HSL, also activated *obc-1*. *B. thailandensis* QS mutants are hypermotile (6, 7), and RNA-seq showed that 17 flagellar genes and 4 methyl-accepting chemotaxis protein genes were repressed by QS. QS controls aggregation in *B. thailandensis* and activates transcription of the putative exopolysaccharide (EPS) genes *bceABCDEFGHIJ* and *bceNOPRSTU* (7, 24). RNA-seq confirmed this finding. RNA-seq showed that the regulation of genes for aerobic respiration is repressed by QS. This is consistent with the finding that QS mutants have increased rates of respiration compared to the wild type (7). Finally, we know that genes required for synthesis of bactobolin antibiotics are activated by QS (8, 25). RNA-seq showed strong induction of these genes by AHLs. Thus, RNA-seq reliably identified known QS-regulated genes among a much larger overall set.

**Signal receptor specificity in the QS regulon.** The experiments described above provide information about the AHL-controlled regulons but do not provide direct information on which of the regulated genes respond to specific signal-responsive transcription factors. There are five LuxR homologs coded in the *B. thailandensis* genome, BtaR1 to BtaR5; BtaR1 to BtaR3 are cognate with BtaI1 to BtaI3, and BtaR4 and BtaR5 are orphan LuxR homologs. To gain insights into which signal receptors control what genes, we compared the transcriptome of T-phase wild-type cells to T-phase cells of strains with single null mutations in *btaR1*, *btaR2*, *btaR3*, *btaR4*, or *btaR5*. Because we were concerned that signal production by one system might depend on another system, we grew bacteria in the presence of added QS-1 to QS-3 AHLs.

BtaR1 affected transcript levels of 164 genes, BtaR2 regulated 20 genes, and BtaR3 controlled 15 genes (Table 3; see Table S5 in the supplemental material). Under the conditions of our analysis, BtaR4 did not significantly influence expression of any genes. The RNA-seq data indicated that *btaR4* was expressed at very low levels. BtaR5 affected only one transcript, a region immediately upstream of the *btaR5* open reading frame (ORF), which itself is deleted in the *btaR5* mutant we used. We presume that the upstream sequence is an untranslated *btaR5* leader. This result suggests that BtaR5 serves as a *btaR5* repressor. However, we found that exogenous AHLs activated *btaR5* transcription in the QS signal synthesis mutant. It is difficult to draw many conclusions about the orphan LuxR homologs other than to say that under the

conditions of our experiments they had very little impact on the *B. thailandensis* transcriptome.

Although there was some overlap in the genes regulated by the three different *B. thailandensis* AHLs, particularly genes regulated by $3OHC_8$-HSL and $3OHC_{10}$-HSL, there was very little overlap in the genes regulated by BtaR1, BtaR2, and BtaR3 (Fig. 1). We presume that the AHL receptors regulate generally unique sets of genes and are most sensitive to their cognate AHL. However, the AHL receptors may show some response to the noncognate signals, which we added in relative abundance to the AHL synthesis mutant. Furthermore, there may be complex regulatory networks among the QS systems that we are just beginning to understand. It is of interest that BtaR1 activated transcription of *btaR3* (see Table S5 in the supplemental material). Thus, we suggest that QS-1 induces QS-3 in a QS regulatory cascade.

We next identified genes with overlapping regulation by a signal receptor and the cognate signal. For example, we identified genes regulated by both BtaR1 and $C_8$-HSL. We believe that these genes are regulated by QS-1 specifically. Fourteen genes were activated by BtaR1 and its cognate signal $C_8$-HSL, 13 genes were coactivated by BtaR2 and $3OHC_{10}$-HSL, and 10 genes were activated and 2 were repressed by BtaR3 and $3OHC_8$-HSL (Table 4). All of the QS-2-specific genes code for bactobolin synthesis. Interestingly, almost all of the genes that appeared to be regulated by a specific signal-signal receptor system are activated rather than repressed.

**Chromosomal distribution of QS-controlled genes.** *B. thailandensis* contains two circular chromosomes (Fig. 2). Chromosome 1 is the larger of the two with 3,282 predicted ORFs, and chromosome 2 has 2,363 predicted ORFs (26). For many bacteria with two chromosomes, including members of the *Bptm* group, the larger chromosome is enriched in genes coding for essential functions, and the smaller chromosome is enriched in genes of unknown function and genes associated with adaptation (27). Interestingly, all three of the QS cognate pairs of genes (*btaI1-btaR1*, *btaI2-btaR2*, and *btaI3-btaR3*) and one of the two orphan receptor genes (*btaR4*) are found on chromosome 2. Of the 542 genes controlled by QS in the T phase, most (308) are on chromosome 2. The density of QS-controlled genes on chromosome 2 was nearly twice that of QS-controlled genes on chromosome 1 (13% of the chromosome 2 genes versus 7% of the chromosome 1 genes). Figure 2 shows the distribution of the QS-controlled genes across the *B. thailandensis* chromosomes and the pseudogene distribution. There was no enrichment for pseudogenes in the QS regulon compared to genome-wide distribution of pseudogenes.

The *B. thailandensis* genome contains 15 genomic islands (GIs), which have atypical GC content or code for bacteriophage-related genes or phage-like elements (26). The content and location of the GIs differ among strains of *B. thailandensis* and also among other *Burkholderia* species (26, 28, 29). Eighty-four QS-controlled genes mapped to 7 of the 15 genomic islands (see Table S6 in the supplemental material). Fifty-five of the 84 genes on GIs were on GI 12, which lies between BTH_II1011 and BTH_II1070. GI 12 genes are predicted to code for a bacteriophage. *B. thailandensis* is able to produce functional phages; however, it is not known which genes are precisely responsible for phage production (30, 31).

Do orthologs of *B. thailandensis* QS-controlled genes occur in *B. mallei* and *B. pseudomallei*? We identified orthologs of the *B. thailandensis* QS-controlled genes in other members of the *Bptm* group (see Table S7 in the supplemental material). We sought to gain insight into which QS-regulated factors are associated with saprophytic survival. This idea was based on the fact that the genome of *B. mallei* has experienced reductive evolution, in which it lost over 20% of its ancestral genome. Presumably, *B. mallei* lost genes of specific value for saprophytic life. Thus, the absence of *B. thailandensis* QS-controlled orthologs in the *B. mallei* genome would suggest a role in saprophytic life for those genes. We found that 77 *B. thailandensis* QS-controlled genes are absent from both *B. mallei* and *B. pseudomallei*. Forty-two of the 77 are on GIs. We identified many more orthologs of QS-regulated genes, 142, in *B. pseudomallei* but not *B. mallei*. Perhaps these genes also code for functions involved in the free-living lifestyle. Most of the *B. thailandensis* QS-regulated genes, 323, have orthologs in both *B. mallei* and *B. pseudomallei*.

**Predicted functions and locations of QS-controlled gene products.** Secreted products are overrepresented in the QS regulon of the well-studied bacterium *Pseudomonas aeruginosa* (32). An *in silico* analysis indicated that in *B. thailandensis* secreted polypeptides are not overrepresented in the large group of genes identified as QS. There was not an overrepresentation of polypeptides containing signal peptides or transmembrane domains. We also asked whether gene products predicted to code for production of secreted or excreted products might be enriched in the *B. thailandensis* QS regulon by an analysis of functional categories of gene products (COG) (see Fig. S1 in the supplemental material). Three COG groups appeared to be overrepresented among the QS-controlled regulon in comparison to genome-wide distributions: secondary metabolite biosynthesis, lipid metabolism, and those with unknown functions. The following COG groups were underrepresented: translation, transcription, intracellular trafficking and secretion, and ion transport and metabolism.

**QS controls production of predicted and characterized secondary metabolites.** QS controlled a large number of genes associated with 11 predicted and characterized secondary metabolites (Table 5). It was previously reported that QS controls the production of bactobolin (8, 25). Indeed, we observed that the genes for bactobolin production were activated by QS. We also observed that QS controls genes associated with the production of burkholdac, malleobactin, terphenyl, thailandamide, 2-alkyl-4-quinolone, rhamnolipids, and malleilactone, and genes in 3 additional uncharacterized clusters that either contain polyketide synthase (PKS) or nonribosomal peptide synthase (NRPS) genes. Interestingly, some secondary metabolite genes are QS activated, and others are QS repressed. This suggests that QS does not act as a global activator of secondary metabolite production but specifically activates and represses certain products in different conditions. For example, genes for bactobolin, burkholdac, and genes in two uncharacterized clusters (designated unknown-1 and unknown-3) showed the strongest T-phase QS activation. Alternatively, genes for malleobactin showed QS repression only during S phase. Yet other genes associated with the production of malleilactone, 2-alkyl-4-quinolone, unknown product-2, terphenyl, and thailandamide showed complex regulation with activation or repression depending on the QS signal added and the growth phase.

BtaR2 was the sole activator of the bactobolin genes and did not activate other known or predicted PKS or NRP genes. BtaR1 activated genes in multiple clusters associated with the production of terphenyl, malleilactone, and unknown-1 and unknown-3. The BtaR1-controlled genes BTH_II1209 through BTH_II1218 (un-

**TABLE 4** QS-controlled genes regulated by an AHL and its cognate receptor during T phase

| AHL and its cognate receptor and locus tag[a] | Gene | Operon[b] | Description | Fold change | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | WT[c] | QS-1[d] | QS-2[d] | QS-3[d] | All[d] | R1[e] | R2[e] | R3[e] |
| BtaR1 and $C_8$-HSL | | | | | | | | | | | |
| BTH_I1956 | | BTH_I1967-I1955 | Nonribosomal peptide synthetase, putative | 11.6+ | 3.5+ | | 3.6+ | 13.8+ | 7.8+ | | |
| BTH_I1957 | | BTH_I1967-I1955 | Hypothetical protein | 14.3+ | 5.0+ | | 4.7+ | 18.1+ | 19.9+ | | |
| BTH_I1960 | | BTH_I1967-I1955 | Hypothetical protein | 28.0+ | 6.1+ | | 5.2+ | 42.3+ | 17.1+ | | |
| BTH_I2813 | | BTH_I2814-I2813 | Hypothetical protein | | 3.2+ | | | 3.2+ | 3.9+ | | |
| BTH_II0204 | | BTH_II0206-II0204 | Peptide synthetase, putative | 5.5+ | 8.0+ | | | 9.6+ | 7.7+ | | |
| BTH_II0627 | | BTH_II0627-II0626 | Hypothetical protein | 20.3+ | 8.2+ | 10.0+ | 17.4+ | 43.3+ | 26.3+ | | |
| BTH_II1071 | *obc-1* | | Oxalate biosynthesis enzyme | 11.7+ | 8.7+ | 10.0+ | 15.4+ | 31.7+ | 15.5+ | | |
| BTH_II1161 | | | LysR family transcriptional regulator | 5.2+ | 4.7+ | | 4.3+ | 7.9+ | 4.4+ | | |
| BTH_II1210 | | BTH_II1219-II1209 | Hypothetical protein | 19.8+ | 6.9 | 4.7+ | 6.8+ | 32.8+ | 15.0+ | | |
| BTH_II1211 | | BTH_II1219-II1209 | Polyketide synthase | 10.8+ | 4.5 | | 4.2+ | 13.6+ | 12.7+ | | |
| BTH_II1339 | | | Hypothetical protein | 10.8+ | 4.0+ | 15.6+ | 30.2+ | 32.1+ | 5.3+ | | |
| BTH_II1925 | | BTH_II1925-II1923 | Chitin-binding domain-containing protein | 218.9+ | 23.4+ | 54.6+ | 71.5+ | 242.7+ | 4.4+ | | 91.7+ |
| BTH_II1997 | | | Hypothetical protein | | 5.8+ | | | | 3.8+ | | |
| BTH_II2000 | | BTH_II2001-II2000 | Hypothetical protein | 3.3+ | 6.1+ | | | 6.5+ | 5.5+ | | |
| BtaR2 and $3OHC_{10}$-HSL | | | | | | | | | | | |
| BTH_II1223 | | BTH_II1224-II1223 | Hypothetical protein | 66.8+ | | 19.8+ | 19.4+ | 46.0+ | | 12.9+ | |
| BTH_II1224 | *btaA* | BTH_II1224-II1223 | CmaB | 236.4+ | 10.5 | 130.2+ | 146.5+ | 241.2+ | | 17.2+ | |
| BTH_II1225 | *btaC* | BTH_II1228-II1225 | Phosphopantetheine-containing protein | 414.6+ | | 220.8+ | 278.0+ | 373.0+ | | 17.5+ | |
| BTH_II1226 | *btaE* | BTH_II1228-II1225 | Peptide synthetase, putative | 327.1+ | 11.5+ | 171.3+ | 206.8+ | 331.2+ | | 16.6+ | |
| BTH_II1233 | *btaK* | BTH_II1241-II1233 | Peptide synthetase, putative | 149.4+ | | 24.4+ | 19.2+ | 151.1+ | | 36.4+ | |
| BTH_II1234 | *btaL* | BTH_II1241-II1233 | JamP | 158.7+ | | 20.2+ | 16.7+ | 122.2+ | | 48.0+ | |
| BTH_II1235 | *btaM* | BTH_II1241-II1233 | JamP | 127.9+ | | 23.2+ | 23.1+ | 138.1+ | | 30.4+ | |
| BTH_II1236 | *btaN* | BTH_II1241-II1233 | Nonribosomal peptide synthetase, putative | 111.9+ | | 21.0+ | 21.5+ | 128.4+ | | 45.5+ | |
| BTH_II1237 | *btaO* | BTH_II1241-II1233 | Thiotemplate mechanism natural product synthetase | 79.8+ | | 15.8+ | 14.9+ | 96.9+ | | 33.8+ | |
| BTH_II1238 | *btaP* | BTH_II1241-II1233 | Polyketide synthase | 46.6+ | | 29.8+ | 28.1+ | 54.4+ | | 27.0+ | |
| BTH_II1240 | *btaS* | BTH_II1241-II1233 | Thioesterase II | 47.0+ | | 14.0+ | 11.8+ | 70.6+ | | 20.0+ | |
| BTH_II1241 | *btaT* | BTH_II1241-II1233 | Drug resistance transporter, Bcr/CflA family protein, putative | 75.4+ | | 15.9+ | 12.7+ | 88.2+ | | 21.6+ | |
| BTH_II1242 | *btaU* | | TauD/TfdA family dioxygenase | 220.4+ | 6.6+ | 104.3+ | 118.8 | 390.2+ | | 29.8+ | |
| BtaR3 and $3OHC_8$-HSL | | | | | | | | | | | |
| BTH_I0814 | *cysI* | BTH_I0819-I0814 | Sulfite reductase | 2.6+ | | | 4.5+ | | | | 3.8+ |
| BTH_I1020 | *kdpF* | BTH_I1023-I1020 | Potassium-transporting ATPase, KdpF subunit-related protein | 15.2+ | | 23.2+ | 30.5+ | 37.0+ | | | 28.3+ |
| BTH_I1021 | *kdpA* | BTH_I1023-I1020 | Potassium-transporting ATPase subunit A | 6.1+ | | 13.3+ | 15.1+ | 10.3+ | | | 7.5+ |
| BTH_I2299 | | BTH_I2301-I2299 | LacI family transcription regulator | 4.2+ | | 8.9+ | 11.5+ | 6.0+ | 3.4+ | | 8.4+ |
| BTH_II0022 | | | Sperm-specific protein Phi-1 | 7.3+ | | 9.2+ | 11.5+ | 7.3+ | | | 5.6+ |
| BTH_II1170 | *nirB* | BTH_II1172-II1169 | Nitrite reductase [NAD(P)H], large subunit | 8.5+ | | 6.8+ | 10.5+ | 8.3+ | 3.5+ | | 4.3+ |
| BTH_II1172 | | BTH_II1172-II1169 | Nitrate reductase | 9.2+ | | 6.0+ | 7.0+ | 8.4+ | 3.9+ | | 9.3+ |
| BTH_II1279 | | BTH_II1281-II1276 | Glyoxalase family protein family | 11.5+ | | 6.6+ | 8.6+ | 15.1+ | | | 6.5+ |
| BTH_II1307* | | | Hypothetical protein | 22.2+ | | 6.1+ | 7.4+ | 17.6+ | | | 4.5+ |
| BTH_II1720 | | | Outer membrane porin OpcP | 2.7− | | | 3.9− | 7.8− | 7.9− | | 4.2− |
| BTH_II1925 | | BTH_II1925-II1923 | Chitin-binding domain-containing protein | 218.9+ | 23.4+ | 54.6+ | 71.5+ | 242.7+ | 4.4+ | | 91.7+ |
| BTH_II2089 | *malB* | BTH_II2089-II2088 | Hypothetical protein | | | 16.6− | 18.4− | | | | 2.8− |

[a] Locus tags correspond to the *B. thailandensis* E264 genome. An asterisk after a locus tag indicates that it is a PGAT (20) predicted pseudogene.
[b] When indicated, the loci in a predicted operon are given.
[c] Fold change value and induction (+) or repression (−) in the wild-type (WT) strain compared to the AHL synthesis mutant strain JRC112 without added AHLs.
[d] Fold change value and induction (+) or repression (−) by AHLs (QS-1 for $C_8$-HSL, QS-2 for $3OHC_{10}$-HSL, QS-3 for $3OHC_8$-HSL, and All for all three AHLs) when added to strain JRC112.
[e] Fold change value and induction (+) or repression (−) in the wild-type strain compared to the indicated *btaR* mutants (*btaR1* [R1] to *btaR3* [R3]).

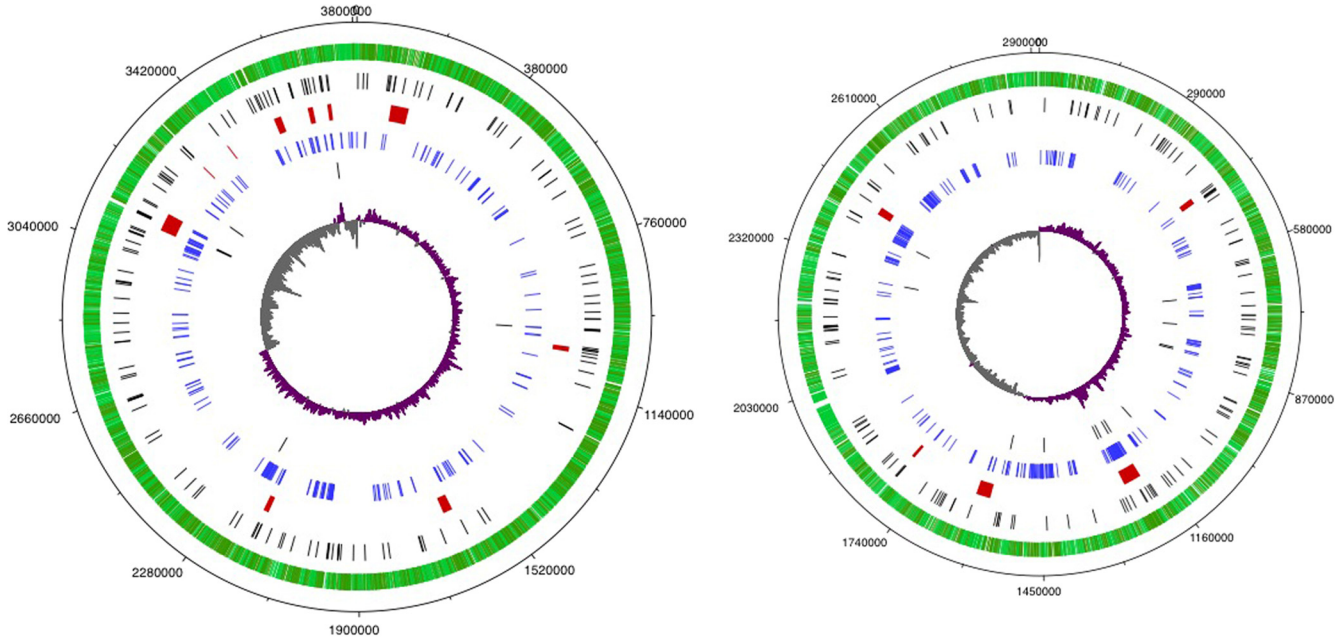


**FIG 2** Diagrams of the large and small *B. thailandensis* chromosomes. Base numbers are shown in the outermost ring. The next ring shows annotated genes in light and dark green for strand orientation. The ring of black hash marks indicates pseudogenes. The ring showing red bars illustrates genomic islands. QS-controlled genes are shown in blue in the next ring followed by pseudogenes in QS regulon (black). The innermost ring shows GC skew (positive values in purple and negative values in gray). The images were generated with DNAplotter (67).

known-3 cluster) lie immediately upstream of the QS-2 controlled bactobolin genes. BtaR3 repressed a single gene in the malleilactone gene cluster. It seems possible that the QS systems contribute independent and even opposing regulation to some secondary metabolite genes.

We do not know why QS regulation of secondary metabolites is so complex. It is tempting to speculate that QS acts to shuttle energy and precursors toward synthesis of certain products while limiting the synthesis of others. The significance of this might become clear as we learn more about the functions of the *B. thailandensis* secondary metabolites.

**QS controls contact-dependent growth inhibition.** Our analysis also revealed that 13 GI 5 genes were QS activated (see Table S6 in the supplemental material). GI 5 contains contact-dependent growth inhibition (CDI) genes. *B. thailandensis* and *B. pseudomallei* CDI systems are composed of the CdiA and CdiB two-partner secretion system and an immunity protein, CdiI. The CdiA and CdiB proteins inhibit growth of nonimmune *Burkholderia* cells (33, 34). The genes for CdiA, CdiI, CdiB, and several uncharacterized downstream genes (BTH_I2719-I2720 and BTH_I2713-I2716) were activated by the addition of the QS-2 signal ($3OHC_{10}$-HSL), the QS-3 signal ($3OHC_8$-HSL), or all three AHLs together. However, BtaR1 alone activated *cdiI*, *cdiA*, BTH_I2719, and BTH_I2716 (Table S6). These data suggest that BtaR1 activates the CDI genes but raises the possibility that BtaR1 regulation is indirect.

To test the hypothesis that QS activates CDI in *B. thailandensis*, we asked whether QS mutants were limited in their ability to inhibit growth of susceptible bacteria by using competition experiments. An AHL synthesis mutant was unable to confer wild-type levels of growth inhibition to the *cdiAIB* mutant (Fig. 3A). Additionally, wild-type levels of CDI were restored to the AHL synthesis mutant by exogenous addition of AHLs (Fig. 3A). Next, we asked which of the BtaI AHL synthases and which BtaR regulators contribute to CDI. The *btaI1*, *btaI2*, or *btaI3* signal synthase mutants were unable to confer wild-type levels of CDI; however, they were not as CDI defective as the triple *btaI123* mutant [Fig. 3A]). The *btaR1* mutant was unable to confer wild-type levels of CDI. However, the *btaR2* or *btaR3* mutant showed near wild-type CDI levels (Fig. 3B). Thus, we believe that BtaR1 is the primary QS transcriptional regulator of CDI. However, there may be substantial complexity because the QS-2 and QS-3 AHLs activated the CDI genes, and each signal synthase appears to contribute to CDI activity. We note that the competition experiments serve as another type of RNA-seq validation result.

**QS activates CPS genes.** The *B. thailandensis* genome contains multiple gene clusters that code for the production of four polysaccharides that contribute to the capsule (capsular polysaccharide [CPS]) or to EPS production: CPS I, CPS II, CPS III, and CPS IV (26, 35). Our RNA-seq findings confirm a previous observation that *B. thailandensis* QS activates two clusters of genes (the *bceI* and *bceII* genes), which are similar to the *Burkholderia cenocepacia* cepacian biosynthetic genes (7, 24). In *B. pseudomallei*, *bceI* orthologs are required for the production of CPS III (35). In T phase, select *bce* genes were activated by BtaR1, the QS-2 AHL, and the QS-3 AHL (see Tables S1 and S5 in the supplemental material). In S phase, the *bce* genes showed continued activation by the QS-2 and QS-3 AHLs but also showed activation by the QS-1 AHL (Table S2). Additionally, genes in the CPS II gene cluster (BTH_II1972-BTH_II1994) were activated as much as 30-fold by AHL additions to the AHL synthesis mutant (Table S1). These genes showed strongest QS regulation when all AHLs were present and by BtaR1 (Tables S1 and S5). We also observed that three CPS

**TABLE 5** QS control of genes for production of known or predicted secondary metabolites[a]

| Secondary metabolite and locus tag[b] | Gene | Description[c] | Fold change | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | T phase | | | | | | | | S phase | | |
| | | | WT[d] | QS-1[e] | QS-2[e] | QS-3[e] | All[e] | R1[f] | R2[f] | R3[f] | QS-1[e] | QS-2[e] | QS-3[e] |
| Unknown-1 | | | | | | | | | | | | | |
| BTH_I1950 | | AcrB/AcrD/AcrF family protein | 6.9+ | | | | 8.4+ | 5.4+ | | | | | |
| BTH_I1951 | *mexE* | Multidrug efflux RND membrane fusion protein MexE | 10.0+ | | | | 8.5+ | | | | | | |
| BTH_I1952 | | Adenylylsulfate kinase | 11.4+ | | | | 8.7+ | 5.4+ | | | | | |
| BTH_I1953 | | Peptide synthetase domain-containing protein | 14.2+ | | | | 15.6+ | 12.0+ | | | | | |
| BTH_I1954 | | Hypothetical protein | 23.0+ | | | | 28.4+ | 19.5+ | | | | | |
| BTH_I1955 | | Hypothetical protein | 14.0+ | | | | | 7.3+ | | | | | |
| BTH_I1956 | | Nonribosomal peptide synthetase, putative | 11.6+ | 3.5+ | | 3.6+ | 13.8+ | 7.8+ | | | | | |
| BTH_I1957 | | Hypothetical protein | 14.3+ | 5.0+ | | 4.7+ | 18.1+ | 19.9+ | | | | | |
| BTH_I1958 | | Dioxygenase, TauD/TfdA | 7.1+ | | | 5.1+ | 8.2+ | 13.7+ | | | | | |
| BTH_I1960 | | Hypothetical protein | 28.0+ | 6.1+ | | 5.2+ | 42.3+ | 17.1+ | | | | | |
| BTH_I1961* | | Hypothetical protein | | | | | 20.6+ | 9.5+ | | | | | |
| BTH_I1963 | | Transketolase, C-terminal subunit | | | | | 10.0+ | | | | | | |
| BTH_I1965 | | Acyltransferase family protein | 3.9+ | | | | 5.9+ | 6.2+ | | | | | |
| BTH_I1966 | *serC2* | Phosphoserine aminotransferase | | | | | 7.2+ | 9.9+ | | | | | |
| BTH_I1967 | | Glycosyltransferase, group 2 family protein | 5.3+ | | | | 8.5+ | 5.6+ | | | | | |
| BTH_I1969 | | Kinase, putative | | | | | 5.8+ | 6.1+ | | | | | |
| BTH_I1970 | | Cysteine synthase/cystathionine beta-synthase family protein | 11.8+ | | | | 27.0+ | | | | | | |
| BTH_I1971 | | Argininosuccinate lyase | 3.5+ | | | | 5.1+ | 8.0+ | | | | | |
| Burkholdac | | | | | | | | | | | | | |
| BTH_I2367 | | Dihydroaeruginoic acid synthetase | 4.0+ | | | | | | | | | | |
| Malleobactin | | | | | | | | | | | | | |
| BTH_I2415 | | TonB-dependent siderophore receptor | | | | | | | | | 3.2− | | |
| BTH_I2417 | | Nonribosomal peptide synthetase, putative | | | | | | | | | 5.1− | | |
| BTH_I2418 | | Peptide synthetase-like protein | | | | | | | | | 2.8− | | |
| Terphenyl | | | | | | | | | | | | | |
| BTH_II0204 | | Peptide synthetase, putative | 5.5+ | 8.0+ | | | 9.6+ | 7.7+ | | | | 6.4− | 4.8− |
| BTH_II0205 | | Hypothetical protein | 11.4+ | | | | 25.6+ | 12.9+ | | | | | |
| BTH_II0206 | | Hypothetical protein | | | | | 15.3+ | | | | | | |
| BTH_II0207 | | Hypothetical protein | 14.6+ | | | | 13.7+ | 10.3+ | | | | | |
| Unknown-2 | | | | | | | | | | | | | |
| BTH_II0562 | | BarD | | | | | | | | | 5.3− | 8.6− | 10.2− |
| BTH_II0563 | | Peptide synthetase, putative | | | | | | | | | 4.6− | 9.8− | 11.4− |
| BTH_II0564 | | BarB2 | 4.1+ | | | | 11.5+ | | | | | | |
| BTH_II0566 | | Demethylmenaquinone methyltransferase | | | | | | | | | 4.8− | 9.3− | 9.2− |
| BTH_II0567 | | Branched-chain amino acid aminotransferase | | | | | | | | | 3.8− | 7.2− | 6.3− |
| BTH_II0569 | *mhpF* | Acetaldehyde dehydrogenase | | | | | | | | | 4.1− | 6.6− | 6.2− |
| BTH_II0570 | *mhpE* | 4-Hydroxy-2-ketovalerate aldolase | | | | | | | | | 5.1− | 10.0− | 9.6− |
| BTH_II0571 | | Pectin degradation protein KdgF | | | | | | | | | 4.2− | | 6.3− |
| Unknown-3 | | | | | | | | | | | | | |
| BTH_II1209 | | Hypothetical protein | 18.6+ | | 4.6+ | 8.3+ | 54.6+ | 32.8+ | | | | | |
| BTH_II1210 | | Hypothetical protein | 19.8+ | 6.9+ | 4.7+ | 6.8+ | 32.8+ | 15.0+ | | | | | |
| BTH_II1211 | | Polyketide synthase | 10.8+ | 4.5+ | | 4.2+ | 13.6+ | 12.7+ | | | | | |
| BTH_II1212 | | Syringomycin biosynthesis enzyme, putative | | | | | 17.0+ | | | | | | |
| BTH_II1213 | | Peptide synthetase-like protein | 10.7+ | | | | 18.0+ | 8.2+ | | | | | |
| BTH_II1214 | | Peptide synthetase, putative | 11.1+ | | | 4.7+ | 18.2+ | 6.6+ | | | | | |
| BTH_II1216 | | D-Cysteine desulfhydrase, putative | 10.5+ | | | | | | | | | | |
| BTH_II1218* | | AMP-binding domain-containing protein | 27.7+ | | 5.3+ | 6.4+ | 28.4+ | 12.5+ | | | | | |
| Bactobolin | | | | | | | | | | | | | |
| BTH_II1222 | | 4-Hydroxyphenylpyruvate dioxygenase | 12.8+ | | | | 11.5+ | | 9.7+ | | | | |
| BTH_II1223 | | Hypothetical protein | 66.8+ | | 19.8+ | 19.4+ | 46.0+ | | 12.9+ | | | | 13.5+ |
| BTH_II1224 | *btaA* | CmaB | 236.4+ | 10.5+ | 130.2+ | 146.5+ | 241.2+ | | 17.2+ | | 2.8+ | 35.0+ | 18.4+ |
| BTH_II1225 | *btaC* | Phosphopantetheine-containing protein | 414.6+ | | 220.8+ | 278.0+ | 373.0+ | | 17.5+ | | | | |
| BTH_II1226 | *btaE* | Peptide synthetase, putative | 327.1+ | 11.5+ | 171.3+ | 206.8+ | 331.2+ | | 16.6+ | | | 69.9+ | |
| BTH_II1227 | *btaI2* | *N*-acyl homoserine lactone synthase | 1563+ | | | | | | 17.6+ | | | | |
| BTH_II1228 | *btaF* | Hypothetical protein | 8.5+ | | | | | | | | | | |
| BTH_II1229 | *btaG* | Sodium/hydrogen exchanger | 24.3+ | | | 11.0+ | 23.4+ | | 30.7+ | | | | |
| BTH_II1230 | *btaH* | Hypothetical protein | 5.2+ | | 6.2+ | | 4.9+ | | | | | | |
| BTH_II1231 | *btaR2* | ATP-dependent transcription regulator LuxR | | | | 3.6+ | | | 26.2+ | | | | |
| BTH_II1232 | *btaJ* | Oligopeptidase A | 11.3+ | | 7.5+ | 8.3+ | 13.7+ | | | | | | |
| BTH_II1233 | *btaK* | Peptide synthetase, putative | 149.4+ | | 24.4+ | 19.2+ | 151.1+ | | 36.4+ | | | | |

(Continued on following page)

**TABLE 5** (Continued)

| Secondary metabolite and locus tag[b] | Gene | Description[c] | Fold change | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | T phase | | | | | | | S phase | | |
| | | | WT[d] | QS-1[e] | QS-2[e] | QS-3[e] | All[e] | R1[f] | R2[f] | R3[f] | QS-1[e] | QS-2[e] | QS-3[e] |
| BTH_II1234 | *btaL* | JamP | 158.7+ | | 20.2+ | 16.7+ | 122.2+ | | 48.0+ | | | | |
| BTH_II1235 | *btaM* | JamP | 127.9+ | | 23.2+ | 23.1+ | 138.1+ | | 30.4+ | | | | |
| BTH_II1236 | *btaN* | Nonribosomal peptide synthetase, putative | 111.9+ | | 21.0+ | 21.5+ | 128.4+ | | 45.5+ | | | | |
| BTH_II1237 | *btaO* | Thiotemplate mechanism natural product synthetase | 79.8+ | | 15.8+ | 14.9+ | 96.9+ | | 33.8+ | | | | |
| BTH_II1238 | *btaP* | Polyketide synthase | 46.6+ | | 29.8+ | 28.1+ | 54.4+ | 27.0+ | | | | | |
| BTH_II1239 | *btaQ* | Acetyltransferase | 56.7+ | | | | 75.5+ | 30.0+ | | | | | |
| BTH_II1240 | *btaS* | Thioesterase II | 47.0+ | | 14.0+ | 11.8+ | 70.6+ | | 20.0+ | | | | |
| BTH_II1241 | *btaT* | Drug resistance transporter, Bcr/CflA family protein, putative | 75.4+ | | 15.9+ | 12.7+ | 88.2+ | | 21.6+ | | | | |
| BTH_II1242 | *btaU* | TauD/TfdA family dioxygenase | 220.4+ | 6.6+ | 104.3+ | 118.8+ | 390.2+ | | 29.8+ | | 4.0+ | | |
| Thailandamide | | | | | | | | | | | | | |
| BTH_II1674 | | Polyketide synthase | 3.5+ | | | | | | | | | | |
| BTH_II1677 | | Phenolpthiocerol synthesis type i polyketide synthase PpsA | | | | | | | | | 4.1− | | 6.0− |
| Rhamnolipid | | | | | | | | | | | | | |
| BTH_II1075 | *rhlA1* | Rhamnosyltransferase 1, subunit A | 3.0+ | | | | | | | | | | |
| BTH_II1076* | *rhlB1* | Rhamnosyltransferase I, subunit B | 5.4+ | | | | | | | | | | |
| BTH_II1077 | | EmrB/QacA family drug resistance transporter | 5.2+ | | | | | | | | | | |
| BTH_II1081 | | Multidrug resistance protein | 3.5+ | | | | | | | | | | |
| BTH_II1879 | | EmrB/QacA family drug resistance transporter | 3.7+ | | | | | | | | | | |
| BTH_II1880* | *rhlB2* | Rhamnosyltransferase I, subunit B | 5.7+ | | | | | | | | | | |
| 2-Alkyl-4-quinolone | | | | | | | | | | | | | |
| BTH_II1929 | *hmqG* | Hypothetical protein | | | 3.7− | | | | | | 3.1− | 6.3− | 5.9− |
| BTH_II1930 | *hmqF* | AMP-binding domain-containing protein | 10.8+ | | 3.7− | 4.2− | | | | | | 5.7− | 4.6− |
| BTH_II1931 | *hhqE* | Metallo-beta-lactamase domain-containing protein | | | | 5.3− | | | | | | | |
| BTH_II1932 | *hhqD* | 3-Oxoacyl-(acyl carrier protein) synthase III | | | 5.4− | 5.2− | | | | | 3.2− | 7.7− | 8.5− |
| BTH_II1933 | *hhqC* | Hypothetical protein | | | 5.6− | 4.3− | | | | | | | |
| BTH_II1935 | *hhqA* | Acetyl-CoA synthetase, putative | | | 5.2− | 4.4− | | | | | | 4.9− | 4.3− |
| Malleilactone | | | | | | | | | | | | | |
| BTH_II2088 | *malA* | Thiotemplate mechanism natural product synthetase | | | 12.6− | 16.6− | | | | | 5.0− | 11.8− | 13.5− |
| BTH_II2089 | *malB* | Hypothetical protein | | | 16.6− | 18.4− | | | | 2.8− | 4.4− | 10.9− | 15.7− |
| BTH_II2090 | *malC* | Syringomycin synthesis regulator SyrP, putative | | | 10.1− | 10.8− | | | | | 5.3− | 9.5− | 10.6− |
| BTH_II2091 | *malD* | Hypothetical protein | 8.6+ | | 9.9− | 9.6− | 8.0+ | | | | 4.4− | 7.9− | 12.2− |
| BTH_II2092 | *malE* | Gamma-aminobutyraldehyde dehydrogenase | | | 5.4- | 4.4- | | | | | 4.4- | 26.4- | 7.5- |
| BTH_II2093 | *malF* | Polyketide synthase, putative | | | 7.0− | 7.0− | 6.2+ | | | | 3.8− | 8.1− | 8.8− |
| BTH_II2094 | *malG* | Ketol-acid reductoisomerase | | | | | | | | | 3.5− | 6.2− | 6.6− |
| BTH_II2095 | *malI* | Diaminopimelate decarboxylase, putative | 5.6+ | | 5.7− | 6.7− | | | | | 4.1− | 8.5− | 8.5− |
| BTH_II2096 | *malJ* | Long-chain-fatty-acid–CoA ligase, putative | 4.9+ | | 5.3− | 5.6− | 3.6+ | | | | 3.8− | 8.4− | 8.7− |
| BTH_II2097 | *malK* | Putative lipoprotein | | | 4.9− | | | | | | | | |
| BTH_II2098 | *malL* | Malonyl CoA-acyl carrier protein transacylase | | | | | | | | | 4.5− | 7.3− | 9.3− |
| BTH_II2099 | *malM* | AMP-binding domain-containing protein | 9.8+ | | 5.6− | 5.1− | 11.0+ | 4.0+ | | | 4.1− | 7.9− | 8.5− |

[a] QS-controlled genes during T or S phase predicted or shown to code for the production of secondary metabolites.
[b] Locus tags correspond to the *B. thailandensis* E264 genome. An asterisk after a locus tag indicates that it is a pseudogene predicted by PGAT (20) analysis.
[c] RND, resistance-nodulation-cell division; CoA, coenzyme A.
[d] Fold change value and induction (+) or repression (−) in the wild-type strain compared to the AHL synthesis mutant strain JRC112 without added AHLs.
[e] Fold change value and induction (+) or repression (−) by AHLs (QS-1 for $C_8$-HSL, QS-2 for $3OHC_{10}$-HSL, QS-3 for $3OHC_8$-HSL, and All for all three AHLs) when added to strain JRC112.
[f] Fold change value and induction (+) or repression (−) in the wild-type strain compared to the indicated *btaR* mutants (*btaR1* [R1] to *btaR3* [R3]).

I genes (BTH_I1330, BTH_I1331, and BTH_I1341) showed modest QS activation in T phase (Table S1).

## DISCUSSION

We have generated a deep transcriptomics data set to begin to learn about the activities controlled by QS in *B. thailandensis*. The data set includes transcriptomes for an AHL synthesis mutant at three different points in growth with or without each of the three *B. thailandensis* AHLs added individually and at one point in growth with all three AHLs added together. We also generated transcriptome data for the wild type and for strains with single deletions of *btaR1*, *btaR2*, *btaR3*, *btaR4*, and *btaR5*. These data allow us to paint a general picture of genes controlled by QS, of which there are many. However, even with this large data set, we

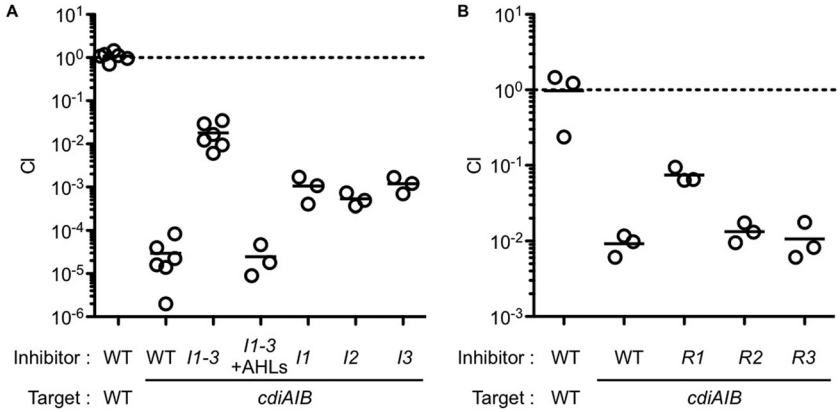


**FIG 3** QS controls contact-dependent growth inhibition. Unmarked inhibitor cells of either wild-type *B. thailandensis* E264 (WT), the *btaI1-3* mutant (*I1-3*), or individual *btaR* (*R1*, *R2*, or *R3*) or *btaI* (*I1*, *I2*, or *I3*) mutants were cocultured with $Tp^r$-marked target cells of either the WT (CM219) or a *cdiAIB* mutant (CM183) at a starting inhibitor-to-target ratio of 1,000:1 (A) or 1:1 (B). AHLs ($C_8$-HSL, $3OHC_8$-HSL, and $3OHC_{10}$-HSL were added where indicated. The competitive index (CI) is the final ratio of target to inhibitor cells divided by the starting ratio of target to inhibitor cells. A CI of 1 (indicated by the broken line) shows equal fitness. Each symbol represents the value for a biological replicate, and the short black bars show the mean values.

do not have a complete picture of the QS-controlled regulon. The data reveal that different genes are QS induced or repressed at different points during culture growth, that genes can be regulated by noncognate signals and receptors, and that some regulated genes likely respond to multiple signals and receptors. These features are not unexpected. Studies with *P. aeruginosa* have shown that many QS-controlled genes are coregulated by other factors (see reference 32 for an overview). Accumulation of AHL signals is necessary but not sufficient for activation of many genes (control is via AND logic gates). We also know that *P. aeruginosa lasB* transcription responds to both LasR and RhlR (36, 37). Our data show that based on transcriptomics alone, it is difficult to determine which genes are directly regulated by a specific QS transcription factor, which QS circuits might be controlled by others, and whether there is a hierarchy of QS circuits. Yet we can get a sense of the breadth of the QS-controlled regulon and although we cannot derive a comprehensive list of genes that are influenced by QS under any condition and at any stage of growth, we can obtain a picture of the types of activities influenced by QS. It is clear that *B. thailandensis* QS has a large global influence on gene expression.

Many bacteria contain multiple AHL QS systems, and numerous efforts have been directed toward understanding the independent and combined roles of each signaling circuit in a given species. For example, *B. cenocepacia* (38), *P. aeruginosa* (22, 32, 39), *Pseudomonas chloroaphis* (40), and several *Yersinia* species (41–43) each contain two or more complete QS systems, which act synergistically, independently, and even in opposition to regulate QS-controlled factors. By examining both the AHL signal- and AHL signal receptor-regulated transcripts in *B. thailandensis*, we learned that there is considerable overlap among the AHL-regulated factors and more divergence among the receptor-regulated genes. For example, there were 108 genes regulated by both the QS-2 or QS-3 signals ($3OHC_{10}$-HSL and $3OHC_8$-HSL). However, there were no genes regulated by both BtaR2 and BtaR3. The overlapping regulation by the QS-2 and QS-3 AHLs can in part be explained by the fact that BtaR2 can respond to both $3OHC_{10}$-HSL and $3OHC_8$-HSL (8). We assume that BtaR2 and BtaR3 regulate unique gene sets but that particularly at high concentrations the QS-3 signal substitutes for the QS-2 signal and vice versa.

There was also overlap in the genes regulated by BtaR1 and BtaR3. Because our *btaR1* null mutant showed reduced *btaR3* expression compared to the wild type, we believe the overlap might result in part from a hierarchical QS network with BtaR1 controlling the BtaR3 system.

What types of *B. thailandensis* activities are regulated by QS? We found that many genes involved in production of secondary metabolites were QS controlled. Secondary metabolites are often excreted or secreted during stationary phase, and they are not catabolic end products. Microbial secondary metabolites can function as antibiotics, surfactants, siderophores, pigments, immunosuppressants, signaling molecules, and virulence factors. It is not uncommon for QS to control production of secondary metabolites. Examples include carbapenem production by *Erwinia carotovora* (44–46), phenazine production by *Pseudomonas chloroaphis* (47), violacein by *Chromobacterium violaceum* (48), bactobolin by *B. thailandensis* (8, 25), mupirocin by *Pseudomonas fluorescens* (49), and rhamnolipids, pyocyanin, hydrogen cyanide, and pyoverdin by *P. aeruginosa* (50–53). Control of secondary metabolite genes by QS in *B. thailandensis* is complex. Examples of *B. thailandensis* QS-controlled secondary metabolite genes include those coding for functions involved in the synthesis of malleobactin, malleilactone, terphenyl, thailandamide, quinolones, three compounds of unknown function, rhamnolipids, and bactobolin. Two gene clusters for metabolites of unknown function (designated unknown-1 and unknown-3) were strongly activated by multiple AHLs. They also showed regulation by BtaR1, but not BtaR2 or BtaR3. In contrast, bactobolin production was activated by BtaR2, but not BtaR1 or BtaR3.

The QS control of quinolone synthesis genes, malleilactone synthesis genes (*malABCDEFGIJKLM*), and unknown product-2 was particularly complex in that depending on conditions, these genes can either be induced or repressed by QS. For example, the addition of $C_8$-HSL, $3OHC_{10}$-HSL, or $3OHC_8$-HSL individually to the AHL synthase mutant repressed many *mal* genes during both T and S phases. However, when all three AHLs were added together or when we compared the wild-type strain to the $AHL^-$ mutant, QS activated a subset of *mal* genes in T phase. Additionally, we observed that BtaR1 activated *malM* and BtaR3 repressed

*malB*. Previous work showed that malleilactone had iron-binding activity and mild antibacterial activity against Gram-positive bacteria (54). It is of interest that the *mal* genes are adjacent to the orphan QS receptor gene, *btaR4*. There is some evidence that BtaR4 might activate the *mal* genes under certain conditions (54). Although little or no malleilactone was produced during laboratory growth, *B. thailandensis malF* and *btaR4* mutants were attenuated for virulence of the worm *Caenorhabditis elegans* and the slime mold *Dictyostelium discoideum* (54). Presumably BtaR4 activates *mal* gene expression during infection (54). Our RNA-seq analysis of the *btaR4* mutant is consistent with this idea. We did not find any BtaR4-dependent gene expression in laboratory-grown T-phase cells.

Our analysis revealed themes regarding roles of each QS system. QS-1 regulates factors that are likely involved in cell aggregation or biofilm formation. Specifically, BtaR1 or $C_8$-HSL activates three separate gene clusters important for CPS or EPS production, the *bceI* and *bceII* genes for CPS III, and the genes for CPS II. The role of CPS III in *B. pseudomallei* is controversial; one publication reports that it is a virulence factor (55), and another indicates it is involved in the saprophytic lifestyle of this species and is not a virulence factor (35). The other QS-controlled CPS, CPS II, remains largely uncharacterized. Like CPS III, the genes for CPS II are present in *B. thailandensis* and *B. pseudomallei*, but not in *B. mallei*.

Additionally, we discovered that BtaR1 activates the CDI operon. CDI mediates intraspecies growth inhibition or killing and also promotes biofilm growth in *B. thailandensis* (33, 34, 56). Finally, QS-1 represses many motility genes. Repression of motility genes often correlates with biofilm formation. Thus, the coregulation genes for CPS or EPS, CDI, and motility by BtaR1 or $C_8$-HSL suggest that QS-1 may promote an aggregate or biofilm lifestyle.

One additional *B. thailandensis* QS-1-controlled trait is oxalate production (23). QS-1-controlled oxalate production can spare *B. thailandensis* from catastrophic high-pH-induced stationary-phase cell death when growing on amino acids as the primary carbon source (23). The RNA-seq analysis showed that the oxalate biosynthetic gene, *obc-1*, is activated by QS. Furthermore, another gene important for oxalate production, *qsmR*, was controlled by QS. However, *qsmR* was repressed by QS in *B. thailandensis* (see Table S1 in the supplemental material) but is required for oxalate production in *B. thailandensis* (23). This may represent a situation where *obc-1* activation leads to oxalate production, and this is followed by QsmR repression of oxalate synthesis such that an extracellular pH homeostasis is achieved.

What is the primary role of QS-2? The QS-2 and QS-3 signals are similar ($3OHC_{10}$-HSL and $3OHC_8$-HSL), and there is overlap in the genes they regulate, possibly because BtaR2 and BtaR3 can respond to either signal. Therefore, to assess which genes are activated by QS-2, we can sort for those regulated by both $3OHC_{10}$-HSL in the signal synthase mutant and BtaR2 (wild type versus a *btaR2* null mutant). This list encompasses 13 bactobolin synthesis genes. There were five additional bactobolin genes regulated by BtaR2 that did not appear in the list of genes regulated by $3OHC_{10}$-HSL in the AHL synthase mutant. Perhaps this is related to differences in timing of gene expression. This is consistent with previous investigations showing that some bactobolin genes are activated by QS-2 and that bactobolin production itself depends on QS-2 (8). The bactobolin genes are found in *B. thailandensis* and *B pseudomallei*, but not *B. mallei* (8). Our results suggest that the primary role of QS-2 is control of bactobolin synthesis. It appears that the bactobolin genes serve a role in saprophyte growth.

The QS-3 circuit strongly activates BTH_II1925, which codes for a predicted chitin-binding domain-containing protein (CBP). BMAA1785, the *B. mallei* ortholog of BTH_II1925 (>95% amino acid sequence identity) contributes to virulence in an insect infection model (57). This gene is also conserved in *B. pseudomallei*. Chitin-binding proteins, as well as chitinases, have been identified in the QS regulons of multiple species, including *P. aeruginosa* (18, 58), *B. cenocepacia* (38, 59), and *C. violaceum* (60). A gene annotated as *kdpF* is also strongly activated by BtaR3 (28-fold), and is adjacent to and presumably cotranscribed with *kdpA* (8-fold). These genes, along with three other genes (*kdpB*, *kpdC*, and *kdpD*), are predicted to code for a P-type ATPase high-affinity potassium ion transporter complex that functions in adaptation to osmotic stress in several bacteria (61). Interestingly, *kdpB* did not show BtaR3 activation, but it was activated by the addition of AHL to the AHL synthesis mutant.

Generally, AHL QS has been considered in the context of a transition between a free-living and host-associated lifestyle (5). *B. thailandensis* is considered to be a soil saprophyte. Our analysis indicates that genes coding for production of secondary metabolites are heavily represented in the QS regulon. A number of the secondary metabolites have antimicrobial properties and may provide groups of *B. thailandensis* a competitive advantage in multispecies soil habitats.

Of the *B. thailandensis* genes we identified as QS controlled, 40% are absent from the genome of the host-restricted pathogen *B. mallei*. The *B. mallei* genome has experienced massive reductive evolution, presumably losing genes for saprophytic survival and maintaining those for host colonization and persistence (62). Thus, we believe many of the 40% of *B. thailandensis* QS-controlled genes absent in the *B. mallei* genome are for adaptation to variations in saprophytic habitats. This includes many, but not all, of the genes for characterized and predicted antimicrobial factors. It also includes certain CPS and EPS genes. Might specific exopolysaccharides promote survival and group activities in the soil, while others play roles in host association?

This deep transcriptomics analysis of QS-controlled genes in *B. thailandensis* lays the groundwork for comparative transcriptome studies in *B. pseudomallei* and *B. mallei*. A global survey of QS-controlled factors in these pathogens has not yet been done despite the link between QS and virulence in both species (15, 63–66). Conserved elements may provide insights about the role of QS during infections and how QS can be adapted from a system providing benefit in a saprophyte to one providing benefit in a pathogen.

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