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# Cross-Species Comparison of the *Burkholderia pseudomallei*, *Burkholderia thailandensis*, and *Burkholderia mallei* Quorum-Sensing Regulons

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***Burkholderia pseudomallei*, *Burkholderia thailandensis*, and *Burkholderia mallei* (the *Bptm* group) are close relatives with very different lifestyles: *B. pseudomallei* is an opportunistic pathogen, *B. thailandensis* is a nonpathogenic saprophyte, and *B. mallei* is a host-restricted pathogen. The acyl-homoserine lactone quorum-sensing (QS) systems of these three species show a high level of conservation. We used transcriptome sequencing (RNA-seq) to define the quorum-sensing regulon in each species, and we performed a cross-species analysis of the QS-controlled orthologs. Our analysis revealed a core set of QS-regulated genes in all three species, as well as QS-controlled factors shared by only two species or unique to a given species. This global survey of the QS regulons of *B. pseudomallei*, *B. thailandensis*, and *B. mallei* serves as a platform for predicting which QS-controlled processes might be important in different bacterial niches and contribute to the pathogenesis of *B. pseudomallei* and *B. mallei*.**

Our interest in *Burkholderia thailandensis*, *Burkholderia pseudomallei*, and *Burkholderia mallei*, which we call the *Bptm* group (1), stems from the fact that this triad shares a high degree of genetic similarity but the species have very divergent lifestyles. *B. thailandensis* is a soil saprophyte common to tropical and subtropical regions and is not a human pathogen (2, 3). *B. pseudomallei* is found in environments similar to those for *B. thailandensis*, but it is also an opportunistic pathogen that causes the emerging infectious disease melioidosis (4). *B. mallei* is the causative agent of a zoonotic disease that most commonly causes glanders in equines (5). Unlike *B. pseudomallei* and *B. thailandensis*, *B. mallei* is a host-restricted pathogen and does not have a saprophytic reservoir.

*B. thailandensis* and *B. pseudomallei* diverged from a common ancestor about 47 million years ago and have close 16S rRNA sequence similarities (6). More than 85% of their genes are conserved and their genomes are highly syntenic, with only four large-scale inversions (6). Genomic islands provide a major source of species-specific genes in *B. thailandensis* and *B. pseudomallei* (6, 7). The third member of the *Bptm* group, *B. mallei*, is believed to have evolved from an ancestral *B. pseudomallei* isolate following an animal infection. The *B. mallei* genome (5.8 Mb) is 20% smaller than the *B. pseudomallei* genome (7.2 Mb) yet retains high nucleotide sequence identity (99%) (8, 9). The expansion of genomic insertion sequences (ISs) facilitated numerous deletion events that resulted in reductive evolution of the *B. mallei* genome (8). Presumably, many genes needed for environmental survival were lost from *B. mallei*, while those important for host survival were maintained (8, 10). *B. mallei* has few species-specific genes; a multi-isolate query of *B. mallei* variable genes showed that all have *B. pseudomallei* orthologs (10). Despite this, the *B. mallei* genome is highly plastic due to the large number of IS elements and simple sequence repeats that facilitate homologous recombination (8–10).

Many *Proteobacteria*, including *Burkholderia* species, use acyl-homoserine lactone (AHL) quorum-sensing (QS) cell-to-cell signaling systems to differentiate between a low-population-density and a high-population-density state. AHL signals are made by members of the LuxI family of signal synthases and can diffuse in and out of cells. Once a critical AHL concentration is reached, the AHL binds to and influences the activity of a LuxR family receptor that is also a transcription factor. The active LuxR then initiates changes in transcription. In this way, bacteria can sense their population density (AHL concentration) and coordinate their behavior (see reference 11 for a review).

Our group is interested in AHL signaling and its role in different bacterial lifestyles. Frequently, AHL QS allows opportunistic pathogens and symbionts to sense and respond to lifestyle shifts that occur as a bacterium cycles between a free-living (low-population-density) state and a host-associated (high-population-density) state (12–15). In fact, QS is important for the virulence of many species (16). There is also mounting evidence that QS is important in environmental reservoirs where it can allow bacteria to mount interspecies attacks and compete for limited resources or facilitate other survival strategies (17, 18). Though many genes are controlled by QS, genes coding for secreted products such as virulence factors, toxins, biofilm components, and antimicrobials are frequently activated by AHL signaling.

Members of the *Bptm* group share homologous AHL QS systems (19–21). *B. thailandensis* and *B. pseudomallei* each contain three complete AHL QS circuits, QS-1 through QS-3 (which consist of the cognate pairs BtaI1-BtaR1, BtaI2-BtaR2, and BtaI3-

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BtaR3 for *B. thailandensis* and BpsI1-BpsR1, BpsI2-BpsR2, and BpsI3-BpsR3 for *B. pseudomallei*). During *B. mallei*'s reductive evolution, it lost a large genomic region containing the QS-2 genes and thus only contains the QS-1 and QS-3 systems (BmaI1-BmaR1 and BmaI3-BmaR3). Additionally, each member of the *Bptm* group contains two orphan or solo LuxRs (receptors that do not have a cognate LuxI signal synthase and may or may not respond to an AHL). These are designated for each species (*B. thailandensis*, *B. pseudomallei*, or *B. mallei*) as R4 and R5. The QS systems are highly conserved across the *Bptm* group; orthologous LuxI and LuxR proteins show between 95 and 100% amino acid identity. Furthermore, the AHLs that each homologous circuit produces and responds to are identical. The QS-1 signal is *N*-octanoyl homoserine lactone ($C_8$-HSL) (22–26), the QS-2 signal is *N*-3-hydroxy-decanoyl homoserine lactone ($3OHC_{10}$-HSL) (24, 27), and the QS-3 signal is *N*-3-hydroxy-octanoyl homoserine lactone ($3OHC_8$-HSL) (22, 24, 28).

There is limited information on the global roles of the QS circuits in the *Bptm* group. Of particular interest, we do not know if the QS-controlled factors among these species are conserved, as their LuxR and LuxI homologs are, or if they are divergent. Here, we describe a global analysis and comparison of the QS regulons of each member of the *Bptm* group, identify a core group of QS-controlled genes shared by all members of the group, and highlight similarities and differences between the three species. We believe this information will provide a foundation on which to generate ideas about how QS is used in different bacterial lifestyles and how signaling systems might change with niche adaptation. Additionally, QS has been associated with the virulence of both *B. pseudomallei* and *B. mallei*. *B. pseudomallei* mutants are attenuated in multiple infection models and show aberrant intracellular replication (21, 26, 29, 30). These studies imply a requirement for QS in melioidosis, yet it is not known which QS-controlled factor or factors are used in the host. A current hypothesis is that QS regulates the acute-to-chronic disease shift in *B. mallei* (31). As is the case for *B. pseudomallei*, the QS-controlled factors utilized by *B. mallei* in the host are unknown. Characterization of the QS-controlled factors in these pathogens should lead to a deeper understanding of meliodosis and glanders.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The strains and plasmids used are listed in Table S1 in the supplemental material. Bacteria were grown in Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract, 5 g NaCl per liter) supplemented with 50 mM morpholinepropanesulfonic acid (MOPS) buffer (pH 7.0) where indicated. For *B. pseudomallei*, 1.6 mM adenine sulfate and 0.005% thiamine-HCl were added to the growth medium. Antibiotics were used at the following concentrations: for *Escherichia coli*, 35 μg/ml kanamycin (Kan) and 25 μg/ml zeocin (Zeo); for *B. pseudomallei*, 2 mg/ml Zeo and 1 mg/ml Kan. Where stated, synthetic $3OHC_{10}$-HSL (4 μM; University of Nottingham [http://www.nottingham.ac.uk/quorum/compounds.htm]), $3OHC_8$-HSL (2 μM; synthesized as previously described [31]) and $C_8$-HSL (2 μM; Sigma Chemical Co.) were added. Except where indicated, bacteria were grown at 37°C with shaking. All experiments with *B. mallei* were performed in a class II biological safety cabinet housed in a biological safety level 3 (BSL-3) enhanced laboratory. All experiments with *B. pseudomallei* employed strain Bp82, which is exempt from the select agent list, and were performed in a BSL-2 laboratory.

For RNA isolation from *B. mallei* and *B. pseudomallei* cells, inocula were from 5-ml overnight cultures. Fresh medium with or without AHLs (20 ml in 125-ml flasks) were inoculated to a starting optical density at 600 nm ($OD_{600}$) of 0.05. All RNA samples were from cells at the transition from exponential growth to stationary phase (T phase; $OD_{600}$, 2.0).

**Measurements of $C_8$-HSL, $3OHC_8$-HSL, and $3OHC_{10}$-HSL in *B. pseudomallei* cultures.** To measure AHLs in *B. pseudomallei* cultures, we twice extracted 5 ml of a culture grown to an $OD_{600}$ of 4.0 with acidified ethyl acetate. The extract was then dried under $N_2$ gas, dissolved in 50 μl 50% methanol, and subjected to $C_{18}$ reverse-phase high-performance liquid chromatography, and fractions containing $C_8$-HSL, $3OHC_8$-HSL, and $3OHC_{10}$-HSL were individually collected. We used previously described bioassays to measure the AHLs: $C_8$-HSL was measured using the bioreporter *E. coli*(pBD4, pBD5) (23), and $3OHC_8$-HSL and $3OHC_{10}$-HSL were measured using the bioreporter *E. coli*(pJNR2, pI2P50) (27). Standard curves were generated by using synthetic $C_8$-L-HSL, $3OHC_{10}$-L-HSL, and $3OHC_8$-L-HSL.

**Mutant construction.** The *bpsI2* (BP1026B_II1251) and *bpsI3* (BP1026B_II1673) deletions were constructed by using the dual-plasmid method of Lopez et al. (32) and standard molecular biology procedures with *E. coli* DH10B as a cloning vehicle. To create gene deletion vectors, we used overlap extension PCR to generate approximately 1,000 bp of DNA flanking each gene with genomic DNA from *B. pseudomallei* Bp82 as a template. The flanking sequences were joined together and PCR amplified to generate each deletion construct. Primers used in cloning are listed in Table S1 in the supplemental material and included two sets of four primers each: OCM53-OCM56 for *bpsI2* and OCM64-65 and OCM70-71 for *bpsI3*. The *bpsI2* deletion construct and pEXKm5 were digested with SmaI and ligated to yield pCM139. The *bpsI3* deletion construct and pEXKm5 were digested with XhoI and ExoRI and ligated to yield pCM134.

To create the *B. pseudomallei* Δ*bpsI1* Δ*bpsI2* Δ*bpsI3* triple mutant, the unmarked successive deletions were generated in strain CM135 (*B. pseudomallei* Bp82 Δ*bpsI1*) with pCM139, pCM134, and pBADSce, as previously described (33), to yield strain CM153 (*B. pseudomallei* Bp82 Δ*bpsI1* Δ*bpsI2* Δ*bpsI3*).

**RNA isolation, RNA-seq library construction, and RNA-seq analysis.** RNA isolation, library construction, and transcriptome sequencing (RNA-seq) analysis were done as described previously using a primer set we developed to limit rRNA amplification in the *Bptm* group (34). Sequencing reads were aligned to the *B. pseudomallei* 1026b genome or to the *B. mallei* ATCC 23344 genome and analyzed by using Avadis NGS software. Differentially regulated genes were determined for biological replicates by using differential expression sequence analysis (DESeq; with a false discovery rate [FDR] cutoff of 0.05) and showed 2-fold or more regulation relative to the reference condition. The data for biological replicates were deposited in the NCBI sequence read archive (SRA) database.

**Ortholog and pseudogene analysis.** We used the *Burkholderia* Prokaryotic Genome Analysis tool (35) to identify orthologs and pseudogenes among the QS-controlled genes for each *B. pseudomallei* 1026b, *B. mallei* ATCC 23344, and *B. thailandensis* E264 list. We compared these lists with each other to identify areas of overlap and divergence. For orthologs that had more than one paralog in another species, each paralog was also considered.

**Microarray data accession number.** The data from the RNA-seq analysis have been deposited in the NCBI SRA database under BioProject ID PRJNA241448. The tables are organized by locus tag.

## RESULTS

**Approach to identify QS-controlled genes in the *Bptm* group.** We sought to identify and compare QS-controlled factors in *B. pseudomallei*, *B. thailandensis*, and *B. mallei* by using an RNA-seq method we recently employed for *B. thailandensis* (34). For each species, we compared transcripts from the wild-type (QS-proficient parent) strain, *B. thailandensis* E264, *B. pseudomallei* Bp82, or *B. mallei* GB8 and the corresponding AHL-negative mutant, *B. thailandensis* JBT112 (22), *B. pseudomallei* CM153, or *B. mallei* CM38 (31). Next, we compared transcripts from each AHL-negative mutant grown with and without added QS signals, either

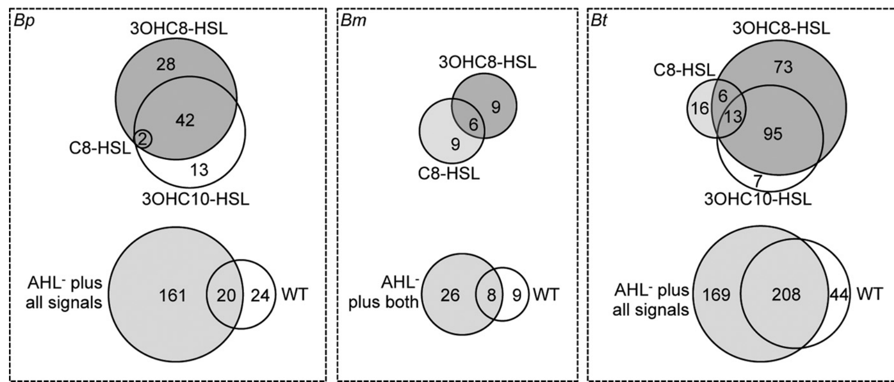


**FIG 1** Venn diagrams showing the relationship between QS-controlled genes in *B. pseudomallei* (*Bp*), using strain Bp82 as the wild type (WT) and strain CM153 as the AHL-negative mutant, *B. mallei* (*Bm*), using strains GB8 as WT and CM38 as the AHL-negative mutant, and *B. thailandensis* (*Bt*), using strains E264 as WT and JBT1122 as the AHL-negative mutant. The circles show overlapping regulons under different conditions (the numbers of genes are also given). For each species, the top diagrams show QS-controlled genes identified when the AHL-negative mutant was grown without any signals, compared to growth with the indicated AHL. The bottom diagrams show QS-controlled genes when the WT or the AHL-negative mutant grown with multiple AHLs ($AHL^-$ plus all signals or $AHL^-$ plus both) was compared to the AHL-negative mutant grown without added AHLs.

individually or added together. The AHL-negative mutants contained wild-type copies of the LuxR family regulators and as such could respond to added AHL. Each RNA sample was isolated from cells transitioning from exponential growth to stationary phase (T phase; $OD_{600}$ of 2). The *B. thailandensis* data have been published previously (34). The comparison of wild types to quorum-sensing signal synthesis mutants should reveal responses to all of the signals as they accumulate normally during the growth cycle, but this approach is limited in that we cannot derive any information about which signals might be responsible for any given response. A second issue with this approach is that, unavoidably, we are comparing two different strains with each other. Although the mutants are derived from the wild-type strains and one would expect them to be isogenic, the *Bptm* group is known to be genetically plastic, and there might be genomic changes other than those of which we are aware. The experiments in which a specific signal or all signals are added back to the signal synthesis mutant is not subject to either of the issues discussed above, but in these experiments signal concentrations are artificially high early in growth (see below).

We sought to add an excess and consistent amount of each AHL during RNA-seq sampling for all three species, to ensure analogous sampling conditions and facilitate cross-species comparisons. We previously reported the AHL abundances in stationary-phase cultures of *B. thailandensis* E264 (34) and *B. mallei* GB8 (31). For *B. pseudomallei* Bp82, we found that stationary-phase cultures contained 98 $\pm$ 10 nM (mean $\pm$ standard deviation) $C_8$-HSL, 28 $\pm$ 2 nM $3OHC_8$-HSL, and 648 $\pm$ 144 nM $3OHC_{10}$-HSL. Although each species accumulated different concentrations of AHLs, we added equal amounts of synthetic AHLs in our experiments (2 $\mu$M $C_8$-HSL, 2 $\mu$M $3OHC_8$-HSL, and 4 $\mu$M $3OHC_{10}$-HSL). We acknowledge that these concentrations exceed the physiological concentrations measured for each species, but we chose high concentrations in an effort to saturate the QS systems.

For *B. thailandensis* and *B. pseudomallei*, we identified genes activated and repressed by addition of $C_8$-HSL, $3OHC_{10}$-HSL, $3OHC_8$-HSL, or all three AHLs together. For *B. mallei*, which only contains the QS-1 and QS-3 systems, we identified genes controlled by $C_8$-HSL, $3OHC_8$-HSL, or both AHLs together. The single addition of an AHL to an AHL-negative strain allows us to evaluate the contribution of an individual AHL to gene regulation, which cannot be achieved by looking at single AHL synthesis mutants, as such strains would contain the other AHL synthases and data interpretation would be complex. A complete list of QS-controlled genes under all conditions can be found in the supplemental material. Figure 1 (and also Table S2 in the supplemental material) shows the QS-controlled genes of *B. pseudomallei*. Figure 1 (and also Table S3 in the supplemental material) shows those of *B. mallei*. The QS regulon of *B. thailandensis* has been described previously (34), but it is shown in Fig. 1 (and has been reformatted in Table S4 in the supplemental material) for the purposes of this report.

Table 1 summarizes the number of QS-activated and QS-repressed genes in each species under each condition tested. *B. thailandensis* showed the highest number of QS-controlled genes, followed by *B. pseudomallei* and then *B. mallei*. The majority of the QS-controlled genes under each condition were QS activated.

**Cross-species comparison of QS-controlled orthologs in the *Bptm* group.** We predicted that the identification of conserved and divergent factors across the *Bptm* QS regulons could provide functional clues as to why certain bacterial processes are QS controlled in different niches. For example, QS-controlled factors common to only the saprophytic species (*B. thailandensis* and *B. pseudomallei*) may be most useful in saprophytic life. QS-controlled factors shared by the pathogenic species (*B. pseudomallei* and *B. mallei*) might be important in host association. Factors common to all members of the *Bptm* group may serve a conserved function important for *Burkholderia* physiology in several habitats.

To compare the QS-controlled orthologs in the *Bptm* group, we first determined if the genes in each regulon had orthologs in the other species of the group. A total of 517 genes showed QS control in *B. thailandensis*. Of these, 449 have *B. pseudomallei* orthologs and 310 have *B. mallei* orthologs. A total of 216 genes were QS controlled in *B. pseudomallei*. Of these, 143 have *B. mallei* orthologs and 175 have *B. thailandensis* orthologs. A total of 43 genes showed QS control in *B. mallei*. Of these, all have *B. pseu-*

**TABLE 1** Summary of QS-controlled genes identified in the *Bptm* group

| | No. of QS-controlled genes induced (+) or repressed (−) in the presence of: | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | WT[a] | | $C_8$-HSL[b] | | $3OHC_{10}$-HSL[b] | | $3OHC_8$-HSL[b] | | All AHLs[c] | | $C_8$-HSL and $3OHC_8$-HSL[c] | |
| Species | + | − | + | − | + | − | + | − | + | − | + | − |
| *B. pseudomallei* | 40 | 1 | 2 | 0 | 53 | 4 | 65 | 7 | 120 | 55 | ND[d] | ND |
| *B. thailandensis* | 161 | 88 | 24 | 11 | 69 | 46 | 125 | 62 | 271 | 106 | ND | ND |
| *B. mallei* | 14 | 2 | 14 | 0 | ND | ND | 14 | 0 | ND | ND | 32 | 1 |

[a] Numbers of QS-controlled genes that were induced or repressed in the WT compared to the corresponding AHL-negative mutant.
[b] Numbers of QS-controlled genes that were induced or repressed when the indicated AHL was added to the medium for the corresponding AHL-negative mutant.
[c] Numbers of QS-controlled genes that were induced or repressed when either all three AHLs or both $C_8$-HSL and $3OHC_8$-HSL were added simultaneously to the medium for the corresponding AHL-negative mutant of the species.
[d] ND, not done.

*domallei* orthologs and 35 have *B. thailandensis* orthologs. We next compared the orthologs from the QS regulon of one species to the QS regulon of the other species. Systematically, we determined the number of shared orthologs among the QS regulons of the *Bptm* group.

Seven conserved orthologs are present in the QS regulons of *B. pseudomallei*, *B. thailandensis*, and *B. mallei* and represent a core group of QS-controlled factors. Seventy-one orthologs are shared by the QS regulons of *B. thailandensis* and *B. pseudomallei*. Two QS-controlled orthologs are shared by only *B. mallei* and *B. pseudomallei*, and nine orthologs are shared by the QS regulons of *B. mallei* and *B. thailandensis*. The Venn diagram in Fig. 2 shows a comparison of all QS-controlled orthologs across the species in the *Bptm* group.

**The core *Bptm* QS-controlled factors.** We identified seven orthologs that showed QS regulation in all three *Burkholderia* species. These orthologs code for a predicted chitin-binding protein (CBP), two products involved with malleilactone synthesis, the Obc1 enzyme for oxalate biosynthesis, and three hypothetical proteins of unknown function (Table 2). Figure 3 shows synteny maps of the orthologs in the core regulon. Nearly all of the core QS-controlled genes showed QS activation.

**QS-controlled genes common to *B. pseudomallei* and *B. thailandensis*.** Comparison of the *Bptm* QS regulons showed that there are 71 orthologs uniquely QS controlled in *B. pseudomallei* and *B. thailandensis* (Fig. 2; see also Table S5 in the supplemental material). The majority of these, 39 (55%), do not have orthologs in the *B. mallei* ATCC 23344 genome. This majority exceeds the genome-wide distributions in *B. pseudomallei* and *B. thailandensis*; 20.7% of the genes in *B. pseudomallei* 1026b and 30.5% of the genes in *B. thailandensis* E264 do not have a *B. mallei* ATCC 23344 ortholog. Thus, the QS regulon uniquely shared by the saprophytic species is enriched for genes no longer present in *B. mallei*.

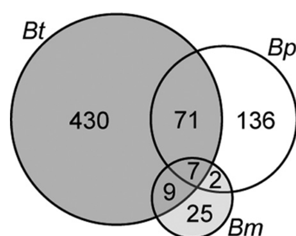


**FIG 2** Comparison of QS-controlled orthologs in the *Bptm* group. A Venn diagram shows the quantity of shared and unique QS-controlled orthologs in *B. thailandensis* (*Bt*), *B. pseudomallei* (*Bp*), and *B. mallei* (*Bm*).

**QS-controlled genes unique to *B. pseudomallei* and *B. mallei*.** We identified two orthologs unique to the *B. pseudomallei* and *B. mallei* QS regulons, BP1026B_I1678/BMA1121 (Fig. 4B) and BP1026B_I1564/BMA1011. Neither of these orthologs is found in the genome of *B. thailandensis*. The BP1026B_I1564/BMA1011 ortholog codes for a hypothetical protein with no conserved domains. Genes neighboring BP1026B_I1564 are QS controlled in *B. pseudomallei* (BP1026B_I1563 to -I1566), but the orthologous region is not in *B. mallei*. BP1026B_I1678/BMA1011 is discussed in greater detail below.

**The *B. pseudomallei* QS regulon.** We identified a total of 216 QS-controlled genes in *B. pseudomallei* (Table 1). Two genes (BP1026B_II1267 and BP1026B_II1268) were activated by $C_8$-HSL. $3OHC_{10}$-HSL regulated 57 genes and $3OHC_8$-HSL regulated 72 genes, of which 44 are the same. Comparison of the AHL-negative mutant to the Bp82 parent or to itself with all three AHLs added together showed that 41 and 175 genes were differentially regulated, respectively. Twenty of these were differentially regulated in both comparisons (see Fig. 1 for an overview).

**The *B. mallei* QS regulon.** We identified a total of 43 QS-controlled genes in *B. mallei*. This is considerably fewer than the hundreds regulated by QS in *B. pseudomallei* and *B. thailandensis*. The vast majority of the QS-controlled genes in *B. mallei* showed QS activation (Table 1). The QS-1 signal $C_8$-HSL positively regulated 14 genes. The QS-3 signal $3OHC_8$-HSL induced five of those activated by $C_8$-HSL, as well as an additional nine genes (Fig. 1). When both AHLs were added together ($C_8$-HSL and $3OHC_8$-HSL), 32 genes were activated and 1 was repressed. *B. mallei* does not possess a QS-2 system, and therefore we did not use $3OHC_{10}$-HSL in our analyses. When we compared wild-type *B. mallei* to the AHL-negative strain, we observed that 16 genes were differentially regulated. Fourteen genes showed higher activity in the wild-type strain, indicative of QS activation, and two were QS repressed. Seven genes identified as QS controlled by comparing the wild type to the AHL-negative strain were also QS controlled by the addition of both $C_8$-HSL and $3OHC_8$-HSL together (Fig. 1).

## DISCUSSION

The *Bptm* group is an interesting triad. Each species has a different lifestyle: *B. thailandensis* is a saprophyte, *B. pseudomallei* is an opportunistic pathogen, and *B. mallei* is a host-restricted pathogen. Yet, these bacteria share a high degree of genetic similarity.

**TABLE 2** The *Bptm* core of QS-controlled genes[a]

| Locus tag number in[b]: | | | | Fold change | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *B. pseudomallei* | | | | | *B. mallei* | | | | *B. thailandensis* | | | | |
| *B. pseudomallei* | *B. mallei* | *B. thailandensis* | Gene, description | WT[c] | QS-1[d] | QS-2[d] | QS-3[d] | All AHLs[d] | WT[c] | QS-1[d] | QS-3[d] | Both[d] | WT[c] | QS-1[d] | QS-2[d] | QS-3[d] | All AHLs[d] |
| I2932 | 0021 | I0515 | Hypothetical protein | 8.1+ | | | | | | | 3.2+ | 4.3+ | 8.8+ | | | 3.8+ | 12.8+ |
| II0335 | A1451.1 | II2093 | *malF*, polyketide nonribosomal peptide synthase | | | | | 5.5+ | | 5.2+ | | 7.1+ | | | 7.0− | 7.0− | 6.2+ |
| II0339 | A1447 | II2089 | *malB*, hypothetical protein | | | | | 7.4+ | | 6.7+ | 6.6+ | 10.0+ | | | 16.6− | 18.4− | |
| II0548 | A1785 | II1925 | Putative chitin-binding protein | | | 46.2+ | 74.5+ | 185.9+ | | | 20.3+ | 24.5+ | 218.9+ | 23.4+ | 54.6+ | 71.5+ | 242.7+ |
| II0841 | A0609 | II1638 | Hypothetical protein | 19.5+ | | 17.1+ | 20.7+ | | | | | 3.7+ | | | 18.1+ | 33.1+ | 6.4+ |
| II0970 | A1348 | II1513 | Hypothetical protein | | | | | 7.4+ | 7.7+ | 7.2+ | | 9.5+ | | | | | 7.9+ |
| II1441 | A0912 | II1071 | *obc1*, oxalate biosynthesis | 23.3+ | | | | | | 2.2+ | | 3.3+ | 11.7+ | 8.7+ | 10.0+ | 15.4+ | 31.7+ |

[a] Orthologs present in the QS regulons of *B. pseudomallei*, *B. mallei*, and *B. thailandensis*.
[b] Locus tags correspond to the *B. pseudomallei* 1026b genome (BP1026B_[locus tag number]), the *B. mallei* ATCC 23344 genome (BMA_[locus tag number]), or the *B. thailandensis* E264 genome (BTH_[locus tag number]).
[c] The fold change due to induction (+) or repression (−) in the *B. pseudomallei* Bp82, *B. mallei GB8*, or *B. thailandensis* E264 WT parent compared to the AHL-negative mutant CM153, CM38, or JRC112, without added AHLs.
[d] The fold change due to induction (+) or repression (−) by AHLs (QS-1 for $C_8$-HSL, QS-2 for $3OHC_{10}$-HSL, QS-3 for $3OHC_8$-HSL, both for $C_8$-HSL and $3OHC_8$-HSL, or for all three AHLs) when added to the AHL-negative mutant CM153, CM38, or JRC112.

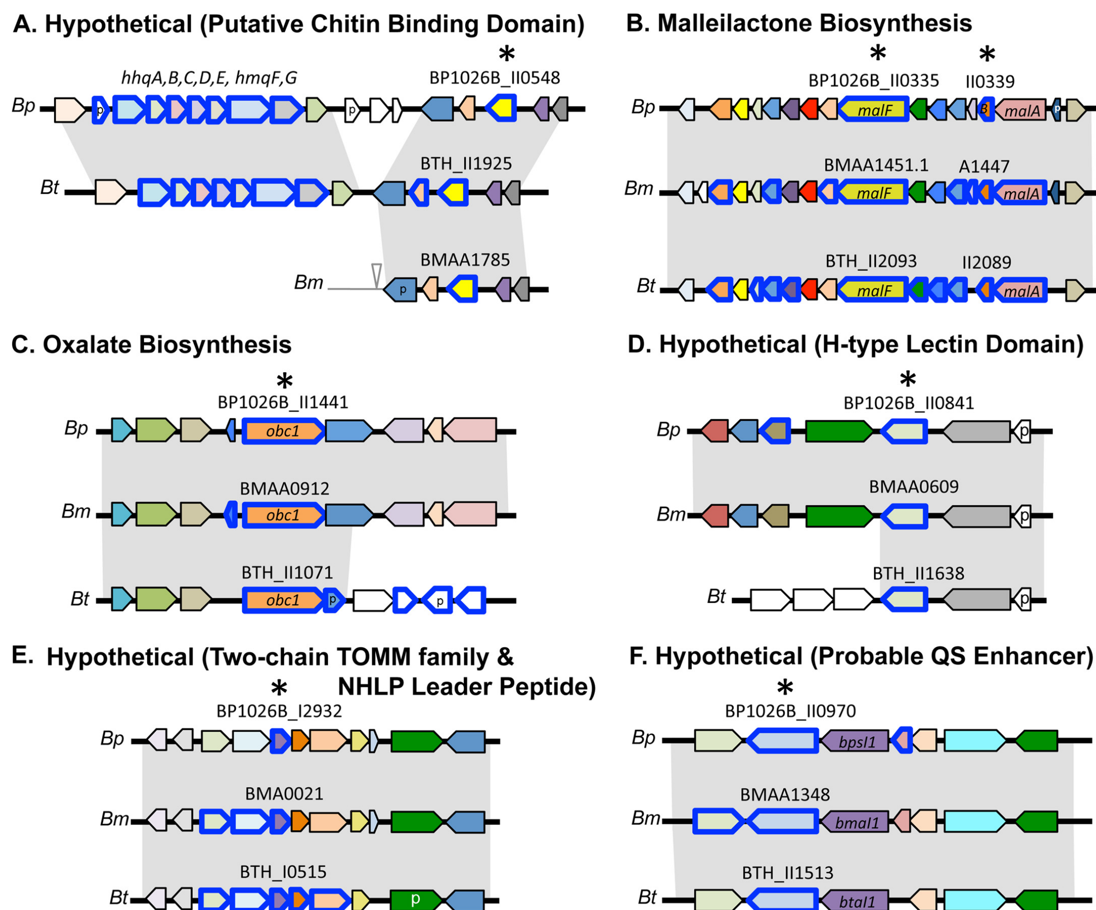


**FIG 3** Synteny maps of chromosome regions in *B. pseudomallei* 1026b (*Bp*), *B. mallei* ATCC 23344 (*Bm*), and *B. thailandensis* E264 (*Bt*), showing orthologs of the core QS regulon. Relevant genes are indicated by the locus tag or gene name. Genes are shown as arrows, and orthologs are color coded across species. An asterisk above a gene indicates that it is part of the core. Orthologs outlined in blue show QS control. An open triangle indicates an insertion sequence, and pseudogenes are labeled with a p. (A) Genes surrounding a hypothetical protein with a predicted chitin-binding domain. (B) Mallielactone biosynthesis region. (C) Region containing the oxalate biosynthetic gene, *obc1*. (D) Region containing a hypothetical protein with an H-type lectin domain. (E) Region containing a hypothetical protein with a two-chain TOMM family domain and an NHLP leader peptide. (F) Region containing a hypothetical protein that has a reductase (HMGR) domain and likely acts as a QS-1 enhancer.

The *Bptm* group affords us an opportunity to explore how homologous genetic regulatory circuits may be used in different ways. We are interested in QS, and we identified QS-controlled genes in each species during the transition from exponential growth to stationary-phase growth (T phase) in LB broth. Recently, we published a comprehensive survey of QS-controlled factors in *B. thailandensis* and found that T-phase cells exhibited the largest number of QS-controlled genes, with 65% showing QS activation (34). For this reason and because QS is generally thought to regulate important factors as bacteria transition through high-population density growth to stationary phase, we focused our *B. pseudomallei* and *B. mallei* QS analyses on T-phase cells. However, we acknowledge that our analysis was limited, and additional QS-controlled factors would likely be identified under different growth conditions.

The largest set of QS-controlled genes was in the saprophyte *B. thailandensis* (>500 genes). The QS regulon of the opportunistic pathogen *B. pseudomallei* included between 200 and 300 genes, and that of the host-adapted *B. mallei* contained only about 40 genes. With this information, we asked which genes had QS-controlled orthologs in all three species. Is there a core set of QS-controlled genes in the *Bptm* group? Seven orthologs showed QS control in all members of the *Bptm* group. We refer to the set of seven as the core QS regulon. These orthologs code for a predicted CBP, two products involved with malleilactone synthesis, the Obc1 enzyme for oxalate biosynthesis, and three hypothetical proteins of unknown function (Table 2 and Fig. 3).

The predicted CBP orthologs (Fig. 3A) contain a chitin-binding 3 family domain (which is often associated with cellulose and chitin binding) and are predicted to be membrane associated and located primarily on the outside of the cell. The *B. mallei* putative CBP ortholog BMAA1785 is a virulence factor in a wax moth larva infection model (36). Malleilactone is a polyketide synthase-derived product that shows iron-binding activity and acts as a virulence factor for *B. thailandensis* in a *Caenorhabditis elegans* infection model (37). We recently reported that AHLs differentially regulate the *mal* genes in *B. thailandensis* (34). In *B. pseudomallei*, *malB* and *malF* showed QS activation when all three AHLs were added to an AHL-negative mutant; in *B. mallei*, many *mal* genes (*malA* to *malM*) were QS activated by single or combined additions of AHLs to an AHL-negative mutant. The *obc1* genes code for an oxalate biosynthetic enzyme. Oxalate is an anion made by

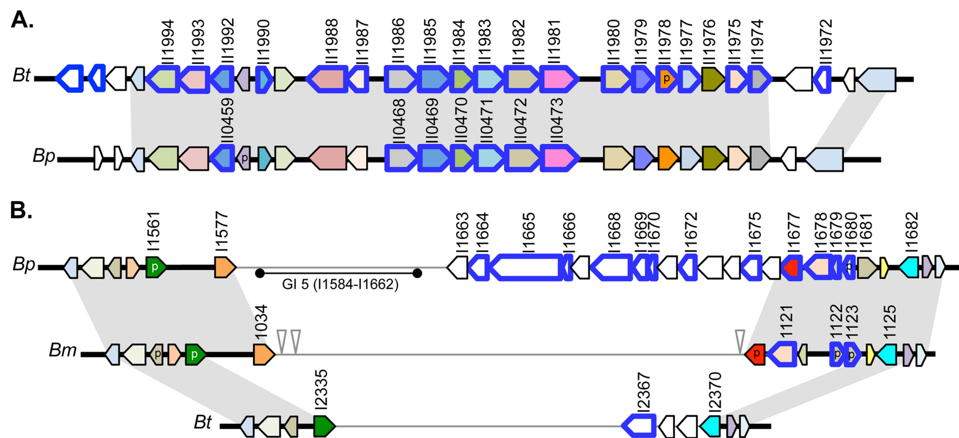


**FIG 4** Synteny maps of the chromosome regions in *B. pseudomallei* 1026b (*Bp*), *B. mallei* ATCC 23344 (*Bm*), and *B. thailandensis* E264 (*Bt*), showing QS-controlled genes. Relevant genes are indicated by locus tags that correspond to the *Bp* genome (BP1026B_[locus tag number]), *Bm* genome (BMA_[locus tag number]), or the *Bt* genome (BTH_[locus tag number]). Genes are shown as arrows, and orthologs are color coded across species. Species-specific genes are white. Orthologs outlined in blue show QS control. An open triangle indicates an insertion sequence, pseudogenes are indicated with a p, and genomic islands (GI) are indicated with a bulleted black horizontal line. (A) The CPS II genes. (B) Genes for a *B. pseudomallei*-specific secondary metabolite and orthologs unique to the *B. mallei* and *B. pseudomallei* QS regulons.

many species in several domains of life. This anion serves diverse functions; it is a virulence factor for some pathogenic fungi, it can act as a chelator for certain metals, it is an end product of metabolism in many animal and plant tissues, and it can serve as a carbon source for some bacteria (38–40). Oxalate production is QS dependent and serves an important role in pH homeostasis of *B. thailandensis* and *B. pseudomallei* (33, 41). These bacteria activate *obc1* via the QS-1 system, and the consequent oxalate production serves to counter ammonia-induced base toxicity and prevent cell death in stationary phase (33). The observation that *obc1* is QS activated in *B. mallei* raises the possibility that *B. mallei* also uses oxalate to prevent base-induced toxicity.

The remaining three QS-controlled core orthologs code for uncharacterized hypothetical proteins. One set (BP1026B_II0841, BTH_II1638, and BMAA0609) is found only in closely related *Burkholderia* species, including *B. oklahomensis*, and codes for a protein with an H-type lectin domain (Table 2 and Fig. 3D). H-type lectin domains are frequently involved with carbohydrate binding and cell recognition or adhesion (42). Another of the core orthologs is encoded by BP1026B_I2932, BTH_I0515, and BMA0021 (Table 2 and Fig. 3E). This ortholog is predicted to code for a polypeptide with a domain characteristic of thiazole/oxazole-modified microcins (TOMMs). TOMMs are ribosomally produced peptides that contain posttranslationally installed heterocycles. TOMMs have diverse activities (antibacterial, antitumor, or morphogenic) (43). Finally, BP1026B_II0970, BTH_II1513, and BMAA1348 are members of the QS-controlled core. These genes code for proteins with a hydroxymethylglutaryl-coenzyme A reductase (HMGR) domain (Table 2 and Fig. 3F). HMGR enzymes are involved in mevalonate and ultimately isoprenoid synthesis. These products are used in signal transduction networks or lipid synthesis (44). In all species, the gene for this hypothetical HMGR protein lies immediately downstream of the QS-1 *luxI* homolog (*bpsI1*, *btaI1*, or *bmaI1*) (Fig. 3F). Though *B. thailandensis* is considered nonpathogenic, it is able to replicate in cultured mammalian cells and is able to resist predation by amoeba (45, 46). *B. thailandensis* and *B. pseudomallei* mutants in this ortholog (BP1026B_II0970/BTH_II1513) showed reduced survival in *Dictyostelium discoideum* amoeba but were able to replicate to wild type-levels during intracellular infection assays in RAW 264.7 cells (46). In *Burkholderia cenocepacia*, an orthologous gene acts as an enhancer of AHL-mediated phenotypes (47). The organization of the QS-1 system and the QS-enhancer genes shows a high degree of synteny among *B. cenocepacia* and the *Bptm* group, raising the possibility that this gene has a broad role in the QS-controlled phenotypes of related *Burkholderia*.

Are there QS-controlled genes common to the two species that can live as saprophytes? We found a large group of QS-controlled factors (71 orthologs) unique to the QS regulons of *B. pseudomallei* and *B. thailandensis* (see Table S5 in the supplemental material). A striking trend is that many of these genes are absent from the *B. mallei* genome. They include the gene clusters for capsule polysaccharide synthesis II (CPS II) (Fig. 4A), several secondary metabolites (including the PQS-like signal 2-alkyl-4-quinolone [Fig. 3A]), bactobolin, and an additional uncharacterized product made by genes upstream of the bactobolin cluster (Table 3).

We also observed instances where gene clusters conserved in the saprophytic QS regulons were present but showed degeneration in the *B. mallei* genome. In *B. pseudomallei* and *B. thailandensis*, QS controls genes for an uncharacterized secondary metabolite (BP1026B_I1157 and -I1158/BTH_I1950 to -I1970) (Table 3). Genes in the orthologous *B. mallei* cluster are not QS controlled. Interestingly, one of these orthologs in the *B. mallei* cluster (BMA1365) is a predicted nonfunctional pseudogene. Another example is an operon that encodes predicted histidine transport functions (BP1026B_I0929 to BP1026B_I0932 [BP1026B_I0929-I0932]/BTH_I1772-I1774). This operon was QS repressed in *B. pseudomallei* and *B. thailandensis* (see Table S5 in the supplemental material). The orthologous region in *B. mallei* is not QS controlled, and the first gene in the operon is a pseudogene. A final example involves a small uncharacterized operon (BP1026B_II1878-II1880/BTH_II0626-BTH_II0627) that shows QS activation in both *B. pseudomallei* and *B. thailandensis* but not *B. mallei*. The BP1026B_II1880/BTH_II0626 ortholog codes for an acetyl-

**TABLE 3** QS-controlled genes associated with secondary metabolite production

| Secondary metabolite | Locus tag(s) for production of the metabolite by[a]: | | |
|---|---|---|---|
| | *B. pseudomallei* | *B. thailandensis* | *B. mallei* |
| 2-Alkyl-4-quinolone | BP1026B_II0535-II0541 | *BTH_II1929-II1935* | |
| Bactobolin | BP1026B_II1232-II1254 | BTH_II1223-II1242 | |
| Burkholdac | | BTH_I2357-I2369 | |
| Isonitrile | BP1026B_II0180-II0185 | BTH_II0229-I2357 | BMAA1919-A1924 |
| Maleobactin | BP1026B_I1731-I1736 | BTH_I2414-I2419 | BMA1177-1183 |
| Malleilactone | BP1026B_II0328-II0340 | *BTH_II2088-II2099* | BMAA1446-A1459 |
| Pyochelin | BP1026B_II0641-II0648 | BTH_II1826-II1833 | |
| Rhamnolipid 1 | BP1026B_II0593-II0598 | BTH_II1075-II1081 | BMAA0459-A0464 |
| Rhamnolipid 2 | BP1026B_II1432-II1437 | BTH_II1875-II1881 | BMAA0919-A0925 |
| Terphenyl | BP1026B_II0147 | BTH_II0204 | |
| Thailandamide | | *BTH_II1662-II1681* | |
| Unknown | BP1026B_I1157-I1176 | BTH_I1952-I1971 | BMA1620-1639 |
| Unknown | BP1026B_II1935-II1945 | BTH_II0562-II0572 | |
| Unknown | BP1026B_II1250-II1267 | BTH_II1209-II1218 | |
| Unknown | BP1026B_II2504-II2509 | BTH_II2344-II2349 | BMAA2085-A2090 |
| Unknown | BP1026B_I1663-I1681 | | BMA1122*-1038 |
| Unknown | BP1026B_II1103-II1108 | | BMAA1200-A1206* |
| Syrbactin | BP1026B_II1345-II1353 | | BMAA1016-A1021*; BMAA1117-A1119* |
| Malleipeptin | BP1026B_II1742-II1746 | | BMAA1642-A1647 |

[a] Genetic determinants for predicted and characterized secondary metabolites are indicated by locus tags for *B. pseudomallei* 1026b, *B. thailandensis* E264, and *B. mallei* ATCC 23344. Orthologous regions are shown for each metabolite across species columns. Underlined text corresponds to loci that showed QS activation. Italics correspond to loci that showed both positive and negative regulation by QS under different conditions. An asterisk indicates that a *B. mallei* cluster is interrupted by an insertion sequence, compared to the orthologous *B. pseudomallei* cluster.

transferase family protein with 10 transmembrane domains. The *B. mallei* ortholog (BMAA0415) is a pseudogene. We suggest that perhaps functional degeneration as well as gene loss have driven a reduction in the QS-controlled genes in *B. mallei*.

Finally, there are genes that are QS controlled in only *B. pseudomallei* and *B. thailandensis* yet have *B. mallei* orthologs. We cannot exclude the possibility that they are QS controlled in *B. mallei* under conditions that we did not test or that they are not QS controlled in *B. mallei* for another reason. *B. mallei* has lost over 20% of its ancestral genome, and this may have pleotropic regulatory impacts.

Our cross-species analysis also identified two orthologs unique to the genomes and QS regulons of *B. pseudomallei* and *B. mallei*, BP1026B_I1678/BMA1121 and BP1026B_I1564/BMA1011. BP1026B_I1678/BMA1121 each code for JmjC domain-containing polypeptides. JmjC domains are found in members of the cupin metalloenzyme superfamily. The function of BP1026B_I1678/BMA1121 remains to be determined. In eukaryotes, Jumonji (jmj) family proteins are involved in histone modification by methylation. Jmj domains are present in bacterial proteins but remain uncharacterized (48). In *B. pseudomallei*, BP1026B_I1678 is flanked by numerous QS-controlled genes, including a gene cluster for a predicted secondary metabolite found only in this species (Fig. 4B). Examination of the orthologous region in *B. mallei* showed that a large IS-mediated deletion event likely occurred near BMA1121 and that two neighboring pseudogenes showed QS activation (Fig. 4B). The second ortholog that is uniquely QS controlled by *B. pseudomallei* and *B. mallei* is BP1026B_I1564/BMA1011. Genes neighboring BP1026B_I1564 are also QS activated in *B. pseudomallei* and code for uncharacterized hypothetical proteins, one of which contains an LpqC (poly-3-hydroxybutyrate depolymerase) domain and a signal peptide, suggesting a role as a secreted hydrolase.

We identified a group of nine orthologs controlled by QS in *B. mallei* and *B. thailandensis* but not *B. pseudomallei*. All nine factors have orthologs in the *B. pseudomallei* genome. A close look at these factors showed that the majority of them actually group to genes associated with the core *Bptm* QS regulon. Four of the nine are malleilactone biosynthesis genes (Fig. 3B), and two map to a TOMM gene cluster (Fig. 3E).

QS contributes to *B. pseudomallei* virulence (21, 26, 29), yet it is unknown which QS-controlled factors are important in the host. The most strongly QS-controlled *B. pseudomallei* genes code for CPS II (also QS controlled in *B. thailandensis*), a predicted CBP (which is part of the core regulon), and the production of secondary metabolites. The observation that QS strongly activates the CPS II genes (BP106B_II0468 to -II0473) is consistent with the observation that QS promotes biofilm formation in *B. pseudomallei* (24). However, *B. pseudomallei* produces four CPS or exopolysaccharide clusters, and we do not know which of these contribute to cell aggregation or surface adherence.

As is true for *B. thailandensis* (34), QS controls many *B. pseudomallei* genes involved in secondary metabolite production. These *B. pseudomallei* secondary metabolite genes include those coding for bactobolin, malleilactone, 2-alkyl-4-quinolone, and two uncharacterized products (Table 3), all of which are also regulated by QS in *B. thailandensis*. The *B. pseudomallei* QS regulon also contains a predicted secondary metabolite gene cluster unique to this species (Table 3 and Fig. 4B). Genes involved in production of several factors previously associated with *B. pseudomallei* virulence showed complex QS regulation. The type III secretion system effector genes, *bopE*, *bipD*, and *bsaM*, were repressed by QS, while the genes for *Burkholderia* lethal factor 1 (BP1026B_1486) and *wbiD* for lipopolysaccharide biosynthesis were QS activated.

How do our results compare to those from other studies of QS

gene regulation in the *Bptm* group? The best-studied member of the group is *B. pseudomallei*. Thus, we compared our *B. pseudomallei* findings to previous reports on QS-controlled factors in *B. pseudomallei* (see Table S6 in the supplemental material). Two groups identified QS-1-controlled factors in different *B. pseudomallei* isolates (Bp008 and PP844) (49, 50), and a third group used microarrays to study yet another *B. pseudomallei* strain (K96243) (unpublished data, available at http://www.melioidosis.info/about.aspx). Nearly half, 97, of the 216 *B. pseudomallei* QS-controlled genes we identified were also identified in other studies. This overlap provides a validation of the results reported by the different groups, and QS-controlled genes uniquely found by the different groups may be due to strain-to-strain variations, sampling differences, or other variations in methodology and analysis.

The relatively small *B. mallei* QS regulon included genes for the predicted CBP discussed above, malleilactone biosynthesis, and the JmjC domain-containing protein, also discussed above. Additionally, the *benABC* operon was QS activated. It is unclear where *B. mallei*, which is a host-restricted bacterium, might come into contact with benzoate. However, the BenB ortholog in *B. pseudomallei* was identified as an antigen in human sera, suggesting that this factor is produced *in vivo* during melioidosis (51). We note that we did not see the *ben* operon in our *B. pseudomallei* QS regulon.

Comparison of the QS regulons of the *Bptm* group affords the opportunity to begin to address how a QS regulon might evolve. There is suggestive evidence that some QS-controlled factors in *B. mallei* are actively being maintained and some are not. For example, the region coding for the predicted CBP (Fig. 3A) shows conservation and divergence among the QS regulons and genomes of the *Bptm* group; the predicted CBP genes in each species are orthologous, but the neighboring genes in *B. mallei* are divergent. The genes for the predicted CBP orthologs are among the most highly QS-activated genes in each species. In *B. mallei*, the CBP is a virulence factor in an insect infection model (36). This highlights the fact that this protein is functional in *B. mallei*. However, the role of this protein in an insect (which has a high composition of chitin) might be very different than its role in a mammal or in the environment. The region coding for the putative CBP ortholog also codes for a PQS-like cluster found in *B. thailandensis* and *B. pseudomallei* but not *B. mallei*. The PQS cluster in *B. thailandensis* and in *B. pseudomallei* is QS regulated (Fig. 3A). Presumably, the PQS cluster was eliminated from the *B. mallei* genome by IS element-mediated gene loss, a driving force for *B. mallei* genome erosion (8). It seems significant that the QS-activated CBP gene is retained in *B. mallei*.

There also appear to be regions of the *B. mallei* genome and QS regulon that are decaying remnants of the ancestral *B. pseudomallei* isolate from which *B. mallei* evolved. The synteny map of such a region (Fig. 4B) has been discussed previously, as it contains *B. pseudomallei* sequence for a predicted *B. pseudomallei*-unique secondary metabolite. In this region, there is considerable divergence among the three *Burkholderia* species. The *B. pseudomallei* region shows extensive regulation by QS. Many of the QS-controlled orthologs are absent from the *B. mallei* genome (there is a large deletion and a number of pseudogenes), but some that remain show QS control. It seems unlikely that this region is functional in *B. mallei*. As discussed above, there are multiple examples of QS-controlled orthologs in *B. pseudomallei* and *B. thailandensis* that are pseudogenes in *B. mallei* and also not regulated by QS. Such observations represent other instances of functional degeneration in the *B. mallei* genome that are associated with loss of QS control.

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