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MEETING PRESENTATION

Two *rsaM* Homologues Encode Central Regulatory Elements Modulating Quorum Sensing in *Burkholderia thailandensis*

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ABSTRACT The bacterium *Burkholderia thailandensis* possesses three *N*-acyl-L-homoserine lactone (AHL) quorum sensing (QS) systems designated BtaI1/BtaR1 (QS-1), BtaI2/BtaR2 (QS-2), and BtaI3/BtaR3 (QS-3). These QS systems are associated with the biosynthesis of *N*-octanoyl-homoserine lactone (C_8-HSL), *N*-3-hydroxy-decanoyl-homoserine lactone ($3OHC_{10}$-HSL), and *N*-3-hydroxy-octanoyl-homoserine lactone ($3OHC_8$-HSL), which are produced by the LuxI-type synthases BtaI1, BtaI2, and BtaI3 and modulated by the LuxR-type transcriptional regulators BtaR1, BtaR2, and BtaR3. The *btaR1-btaI1* and *btaR2-btaI2* gene clusters each carry an additional gene encoding a homologue of the QS repressor RsaM originally identified in the phytopathogen *Pseudomonas fuscovaginae* and thus here named *rsaM1* and *rsaM2*, respectively. We have characterized the functions of these two conserved *rsaM* homologues and demonstrated their involvement in the regulation of AHL biosynthesis in *B. thailandensis* strain E264. We quantified the production of C_8-HSL, $3OHC_{10}$-HSL, and $3OHC_8$-HSL by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) in the wild-type strain and in the *rsaM1* and *rsaM2* mutants, and we monitored *btaI1*, *btaI2*, and *btaI3* expression using chromosomal mini-CTX-*lux* transcriptional reporters. The transcription of *btaR1*, *btaR2*, and *btaR3* was also measured by quantitative reverse transcription-PCR (qRT-PCR). We observed that RsaM1 mainly represses the QS-1 system, whereas RsaM2 principally represses the QS-2 system. We also found that both *rsaM1* and *rsaM2* are QS controlled and negatively autoregulated. We conclude that RsaM1 and RsaM2 are an integral part of the QS circuitry of *B. thailandensis* and play a major role in the hierarchical and homeostatic organization of the QS-1, QS-2, and QS-3 systems.

IMPORTANCE Quorum sensing (QS) is commonly involved in the coordination of gene transcription associated with the establishment of host-pathogen interactions and acclimatization to the environment. We present the functional characterization of two *rsaM* homologues in the regulation of the multiple QS systems coexisting in the nonpathogenic bacterium *Burkholderia thailandensis*, which is widely used as a model system for the study of the human pathogen *Burkholderia pseudomallei*. We found that inactivation of these *rsaM* homologues, which are clustered with the other QS genes, profoundly affects the QS circuitry of *B. thailandensis*. We conclude that they constitute essential regulatory components of the QS modulatory network and provide additional layers of regulation to modulate the transcription of QS-controlled genes, particularly those linked to environmental adaptation.

KEYWORDS *Burkholderia pseudomallei*, acyl-homoserine lactone, gene regulation, repressor

Quorum sensing (QS) is a widespread cell-cell communication system that coordinates the expression of specific genes in a bacterial population density-dependent manner (1). QS is mediated by diffusible signaling molecules called autoinducers, which are synthesized and secreted in response to fluctuations in cell density. They accumu-

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late in the environment as bacterial growth progresses until a threshold concentration is reached that allows bacteria to synchronize their activities and to function as multicellular communities. Gram-negative bacteria commonly possess homologues of the LuxI/LuxR system initially characterized in the bioluminescent marine bacterium *Vibrio fischeri* (2). The signaling molecules *N*-acyl-L-homoserine lactones (AHLs) are produced by the LuxI-type synthases. These AHLs activate the LuxR-type transcriptional regulators that modulate the expression of QS target genes, which usually contain a *lux* box sequence in their promoter region. These genes frequently include a *luxI* homologue encoding the AHL synthase, resulting in a typical self-inducing loop of AHLs (3).

The *Burkholderia* genus encompasses heterogeneous species colonizing diverse ecological niches, such as soil, water, plants, and animals, including humans (4, 5). The *Burkholderia cepacia* complex (Bcc), for instance, comprises notable opportunistic human pathogens deleterious to both cystic fibrosis (CF) patients and immunocompromised individuals (6). Bcc members carry *luxI* and *luxR* homologues, namely, *cepI* and *cepR*, respectively, coding for the AHL-based QS system CepI/CepR (7). CepI is a LuxI-type synthase responsible for *N*-octanoyl-homoserine lactone (C_8-HSL) biosynthesis, which generally is the predominant AHL found in members of the *Burkholderia* genus (7). The LuxR-type transcriptional regulator CepR modulates the expression of QS target genes in conjunction with C_8-HSL, including the *cepI* gene itself, creating the typical QS autoregulation loop (7). The genetic organization of *cepI* and *cepR* is conserved among *Burkholderia* spp. (8). Interestingly, they are generally separated by a gene encoding an RsaM-like protein originally identified in the plant pathogen *Pseudomonas fuscovaginae* (9, 10), which was shown to be a major negative regulator of both AHL biosynthesis and expression of AHL synthase-coding genes (9). RsaM actually acts as a global regulator mediating the transcription of numerous genes through and out of the QS regulon in *P. fuscovaginae* (10). The function of RsaM-like proteins could therefore be important for balancing and fine-tuning QS-dependent regulation in members of the *Burkholderia* genus (11). These proteins do not present any sequence similarity with biochemically or structurally characterized proteins, such as DNA-binding motifs, and constitute single-domain proteins with unique topology presenting a novel fold (12). Their precise underlying regulatory mechanism thus remains unknown.

The nonpathogenic soil saprophyte *Burkholderia thailandensis* and the closely related human pathogen *Burkholderia pseudomallei* (13) both encode two conserved RsaM-like proteins of uncharacterized function (8). The genome of *B. thailandensis* contains three LuxI/LuxR-type QS systems designated BtaI1/BtaR1 (QS-1), BtaI2/BtaR2 (QS-2), and BtaI3/BtaR3 (QS-3). These QS systems are also found in *B. pseudomallei* and were reported to be involved in the regulation of several virulence genes and to be essential to its pathogenicity (14, 15). We recently thoroughly dissected the QS circuitry of *B. thailandensis* and found that the QS-1, QS-2, and QS-3 systems are hierarchically and homeostatically organized, and they are integrated into an intricate modulatory network, including transcriptional and posttranscriptional interactions (16). The QS-1 system is responsible for C_8-HSL production (17). The BtaR1 transcriptional regulator activates the expression of the *btaI1* gene encoding the BtaI1 synthase (16, 18). The QS-2 system is responsible for the biosynthesis of both *N*-3-hydroxy-decanoyl-homoserine lactone ($3OHC_{10}$-HSL) and *N*-3-hydroxy-octanoyl-homoserine lactone ($3OHC_8$-HSL) (19). The *btaI2* gene, which codes for the BtaI2 synthase, is positively and directly controlled by the BtaR2 transcriptional regulator in association with $3OHC_{10}$-HSL and $3OHC_8$-HSL (16, 19). The QS-3 system is composed of the BtaR3 transcriptional regulator and the BtaI3 synthase responsible for $3OHC_8$-HSL production (17). The *btaI3* gene is activated by BtaR3 (16). While both the QS-1 and QS-2 gene clusters include an *rsaM* homologue (8), here named *rsaM1* and *rsaM2*, respectively, no homologue of *rsaM* is present in the vicinity of *btaR3* or *btaI3* (8).

The central aim of this study was to further elucidate the QS modulatory network of *B. thailandensis* E264 by characterizing the roles of RsaM1 and RsaM2 in the regulation of its components. We established that they negatively affect the biosynthesis of AHLs

and that they are central to the homeostasis of the QS circuitry of *B. thailandensis* E264. This study provides new insights on the intricate interplay existing between the various elements of *B. thailandensis* QS systems and is essential in unraveling the regulatory mechanism underlying QS-dependent gene expression in this bacterium.

RESULTS

The QS-1 and QS-2 gene clusters of *B. thailandensis* each carry an *rsaM* homologue. The *B. thailandensis* E264 QS-1 system *btaI1* (*BTH_II1512*) and *btaR1* (*BTH_II1510*) genes, encoding the BtaI1 synthase and the BtaR1 transcriptional regulator, respectively, are separated by the *BTH_II1511* gene that codes for a hypothetical protein conserved in members of the *Burkholderia* genus (8, 11, 12, 20–22). This hypothetical protein of 147 amino acids is similar to RsaM-like proteins and displays 35.8% identity with the QS repressor RsaM of the phytopathogen *P. fuscovaginae* UPB0736 (http://www.uniprot.org/uniprot/Q2T542) (see Fig. S1A in the supplemental material). Interestingly, another *rsaM* homologue, encoding a hypothetical protein of uncharacterized function, is present on the genome of *B. thailandensis* E264 between the QS-2 system *btaI2* (*BTH_II1227*) and *btaR2* (*BTH_II1231*) genes that code for the BtaI2 synthase and the BtaR2 transcriptional regulator, respectively. This hypothetical protein of 135 amino acids encoded by the *BTH_II1228* gene is 32.4% identical to *P. fuscovaginae* UPB0736 RsaM (http://www.uniprot.org/uniprot/Q2T5X5) (Fig. S1A). Therefore, the putative proteins encoded by the *BTH_II1511* and *BTH_II1228* genes were designated RsaM1 and RsaM2, respectively.

Since the *rsaM1* and *rsaM2* genes are directly adjacent to *btaI1* and *btaI2* on the genome of *B. thailandensis* E264, respectively, and are transcribed in the same direction (Fig. S1B), we asked whether they could be cotranscribed. *rsaM2* is indeed predicted to be arranged in a operon with *btaI2* (http://www.burkholderia.com/). According to our transcriptomic analyses obtained by RNA-sequencing (RNA-seq) (S. Le Guillouzer, M. C. Groleau, F. Mauffrey, R. Villemur, and E. Déziel, unpublished data), neither *rsaM1* nor *rsaM2* is cotranscribed with the *btaI1* or *btaI2* gene, respectively (Fig. S1B), as confirmed by reverse transcription-PCR (RT-PCR) experiments (Fig. S2).

The functions of the *rsaM1* and *rsaM2* genes are unknown. While *rsaM2* is located within a cluster responsible for bactobolin biosynthesis (19, 23, 24), its involvement was actually not demonstrated. To determine whether *rsaM1* and *rsaM2* are functionally similar to the RsaM-encoding gene of *P. fuscovaginae* UPB0736, which was described as an important repressor of AHL production (9), we investigated the impact of these genes on the biosynthesis of the following predominant AHLs produced by *B. thailandensis* E264: $3OHC_{10}$-HSL and, to lesser extents, C_8-HSL and $3OHC_8$-HSL (16–19). Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was used to measure the total concentrations of these AHLs at various time intervals of the bacterial growth in the *B. thailandensis* E264 wild-type strain and in *rsaM1* and *rsaM2* null mutants. These mutants both overproduced AHLs compared to the wild-type strain (Fig. 1). Interestingly, the impact of RsaM1 on total AHL concentrations was more pronounced than the effect of RsaM2 (Fig. 1). Of note, the *rsaM1* mutant displayed a delayed growth phenotype (Fig. 1), and more cell aggregation was observed in this background (Fig. S3). Altogether, these observations indicate that the RsaM1 and RsaM2 proteins of *B. thailandensis* E264 constitute negative regulators of QS, as previously reported for *P. fuscovaginae* UPB0736 RsaM (9).

RsaM1 mainly represses the QS-1 system, and RsaM2 principally represses the QS-2 system. Since we confirmed the involvement of the BtaI1, BtaI2, and BtaI3 synthases in C_8-HSL, $3OHC_{10}$-HSL, and $3OHC_8$-HSL biosynthesis, respectively (Fig. 2), we determined the effects of RsaM1 and RsaM2 on the QS-1, QS-2, and QS-3 systems by measuring the respective production of C_8-HSL, $3OHC_{10}$-HSL, and $3OHC_8$-HSL in the wild-type strain and in the *rsaM1* and *rsaM2* mutants of *B. thailandensis* E264 throughout the bacterial growth phases. To gain additional insights, we also monitored the expression of the AHL synthase-coding genes *btaI1*, *btaI2*, and *btaI3* in the same

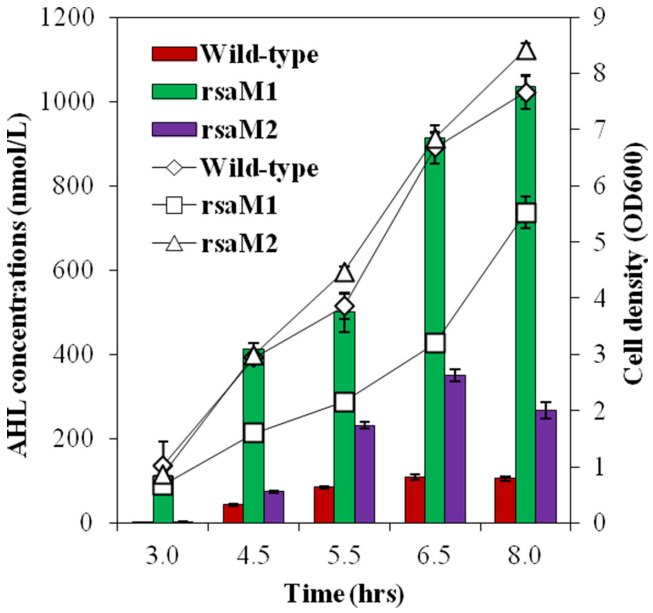

FIG 1 AHLs are overproduced by *rsaM1* and *rsaM2* mutants. Total concentrations of AHLs ($3OHC_{10}$-HSL, C_8-HSL, and $3OHC_8$-HSL) (bars) were monitored by LC-MS/MS at various times during growth (lines) in cultures of the *B. thailandensis* E264 wild-type strain and isogenic *rsaM1* and *rsaM2* mutants. The error bars represent the standard deviations of the averages for three replicates.

backgrounds using the chromosomal *btaI1-lux*, *btaI2-lux*, and *btaI3-lux* transcriptional reporters, respectively.

We observed a dramatic overproduction of C_8-HSL in the *rsaM1* mutant compared to the wild-type strain during the early exponential (optical density at 600 nm [OD_{600}], ≈3.0) and late-exponential (OD_{600}, ≈5.0) phases, indicating that RsaM1 represses the biosynthesis of C_8-HSL (Fig. 3A). The transcription of the *btaI1* gene was accordingly enhanced in the absence of RsaM1, suggesting that RsaM1 intervenes in the modulation of C_8-HSL production by regulating the transcription of *btaI1* (Fig. 3C). Interestingly, the impact of RsaM1 on C_8-HSL biosynthesis (approximately 200-fold) was larger than its effect on *btaI1* transcription (approximately 2-fold) (Fig. 3). We also detected a small, but reproducible, augmentation of C_8-HSL concentrations from the stationary phase (OD_{600}, ≈6.0) in the *rsaM2* mutant compared to the wild-type strain, highlighting that the production of C_8-HSL is negatively modulated by RsaM2 as well (Fig. 3B). However, no discernible difference in the transcription of *btaI1* was detected in the absence of RsaM2 (Fig. 3C). Thus, the negative impact of RsaM2 on C_8-HSL production might not result from regulation of *btaI1* transcription.

While $3OHC_{10}$-HSL production, as well as the transcription of *btaI2*, was unaffected in the absence of RsaM1 (Fig. 4), the concentrations of $3OHC_{10}$-HSL were strongly increased in the *rsaM2* mutant compared with the wild-type strain throughout both the late-exponential and stationary phases (Fig. 4A), and *btaI2* transcription was similarly upregulated (Fig. 4B). These data suggest that RsaM2 represses $3OHC_{10}$-HSL biosynthesis by modulating the transcription of *btaI2*.

The levels of $3OHC_8$-HSL were also higher from the logarithmic growth in the *rsaM1* mutant than in the wild-type strain (Fig. 5A). Unexpectedly, the transcription of the *btaI3* gene was not increased, suggesting that the negative impact of RsaM1 on $3OHC_8$-HSL production does not involve the regulation of *btaI3* transcription (Fig. 5C). Additionally, $3OHC_8$-HSL concentrations were augmented during the stationary phase in the *rsaM2* mutant in comparison with the wild-type strain, showing that the production of $3OHC_8$-HSL is repressed by RsaM2 as well (Fig. 5B). Nevertheless, no visible change in the transcription of *btaI3* was noticed in the absence of RsaM2, revealing that the RsaM2-dependent control on $3OHC_8$-HSL biosynthesis might not be linked to modulation of *btaI3* transcription (Fig. 5C).

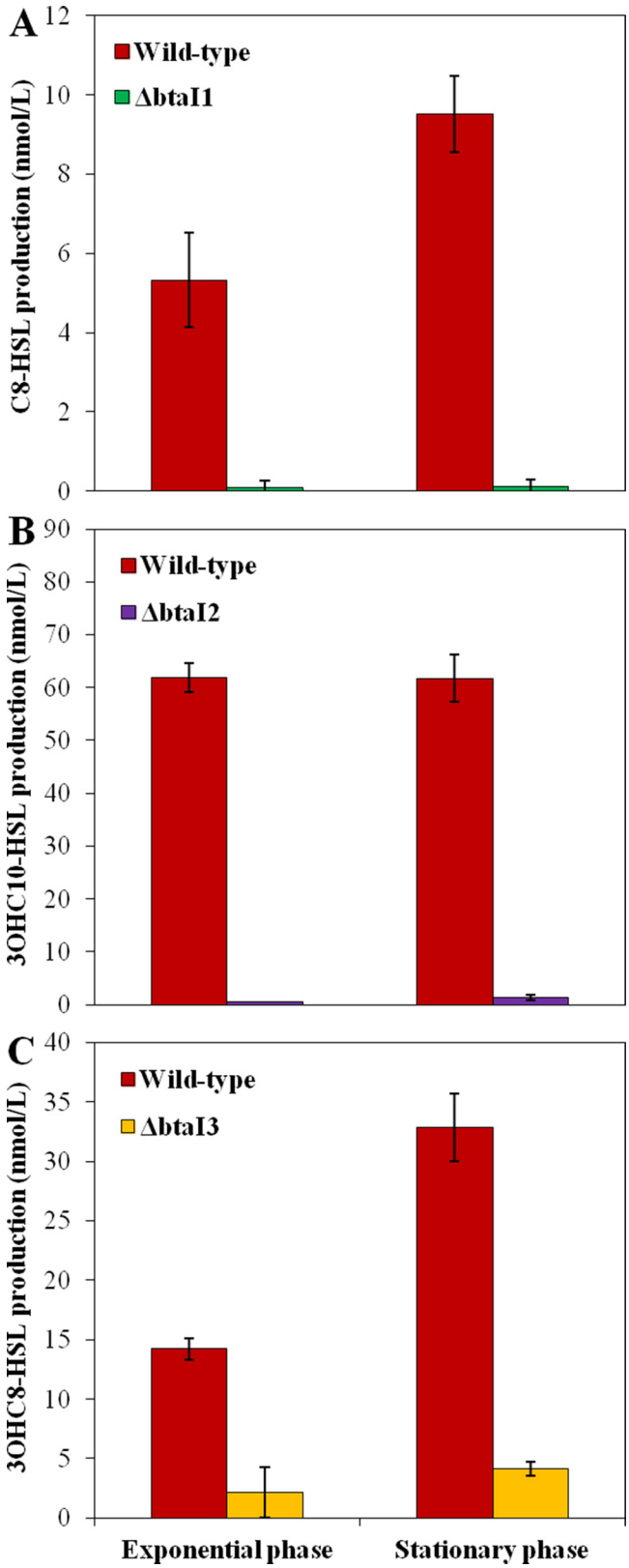

FIG 2 AHL biosynthesis in the wild-type strain of *B. thailandensis* E264 and the Δ*btaI1*, Δ*btaI2*, and Δ*btaI3* mutants. The production of C_8-HSL (A), $3OHC_{10}$-HSL (B), and $3OHC_8$-HSL (C) was quantified using LC-MS/MS during the exponential and stationary phases in cultures of the wild-type strain of *B. thailandensis* E264 and the Δ*btaI1* (A), Δ*btaI2* (B), and Δ*btaI3* (C) mutants, respectively. The error bars represent the standard deviations of the averages for three replicates.

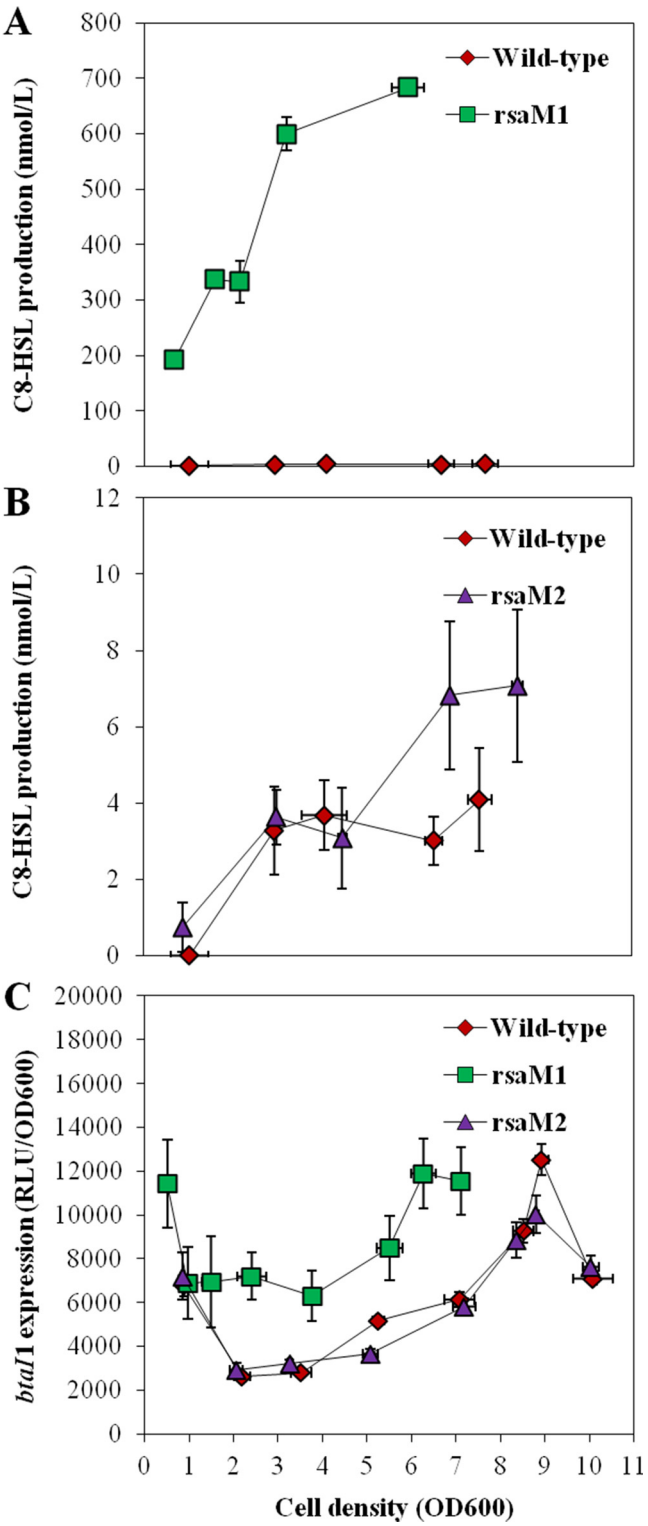

FIG 3 C_8-HSL biosynthesis and expression from the *btaI1* promoter in the wild-type and the *rsaM1* and *rsaM2* mutant strains of *B. thailandensis* E264. (A and B) The production of C_8-HSL was quantified using LC-MS/MS at various times during growth in cultures of the wild-type strain and of the *rsaM1* (A) and *rsaM2* (B) mutant strains of *B. thailandensis* E264. The error bars represent the standard deviations of the averages for three replicates. (C) The luminescence of the chromosomal *btaI1-lux* transcriptional fusion was monitored in cultures of the *B. thailandensis* E264 wild-type strain and the *rsaM1* and *rsaM2* mutants. The luminescence is expressed in relative light units per optical density of the culture at 600 nm (RLU/OD_{600}).

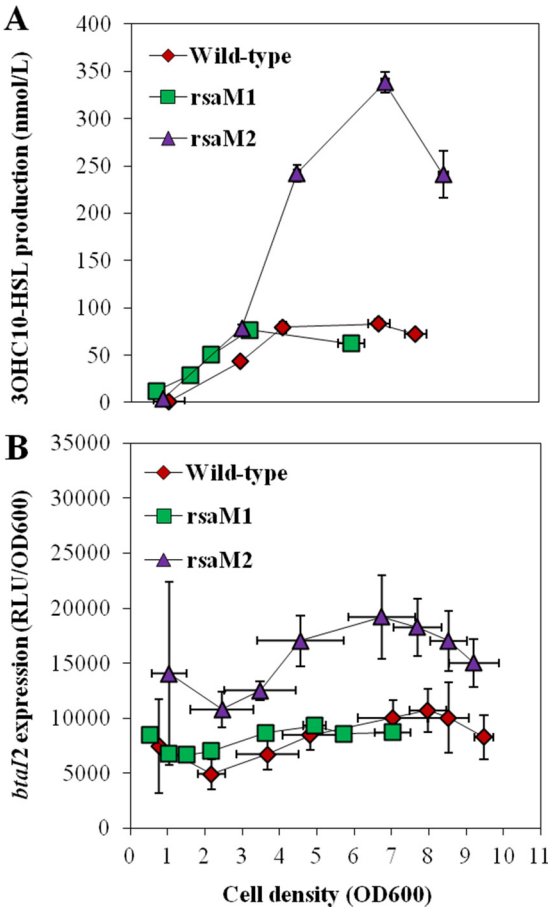

FIG 4 3OHC$_{10}$-HSL biosynthesis and expression from the *btaI2* promoter in the wild-type and the *rsaM1* and *rsaM2* mutant strains of *B. thailandensis* E264. (A) The production of 3OHC$_{10}$-HSL was quantified using LC-MS/MS at various times during growth in cultures of the wild-type and the *rsaM1* and *rsaM2* mutant strains of *B. thailandensis* E264. The error bars represent the standard deviations of the averages for three replicates. (B) The luminescence of the chromosomal *btaI2-lux* transcriptional fusion was monitored in cultures of the *B. thailandensis* E264 wild-type strain and the *rsaM1* and *rsaM2* mutants. The luminescence is expressed in relative light units per optical density of the culture at 600 nm (RLU/OD$_{600}$).

While the concentrations of both C$_8$-HSL and 3OHC$_8$-HSL were enhanced in the *rsaM1* mutant background, the impact on C$_8$-HSL biosynthesis was more pronounced than the effect on 3OHC$_8$-HSL production (Fig. S4A and B). Additionally, the amounts of C$_8$-HSL, 3OHC$_{10}$-HSL, and 3OHC$_8$-HSL were all increased in the *rsaM2* mutant background; however, 3OHC$_{10}$-HSL levels were the most affected (Fig. S4A and C). Collectively, these findings indicate that RsaM1 mainly represses the QS-1 system, whereas RsaM2 principally represses the QS-2 system.

RsaM1 negatively regulates *btaR1* gene transcription, but transcription of the *btaR2* gene is not modulated by RsaM2. In order to determine whether the impact of RsaM1 and RsaM2 on AHL biosynthesis also implicates the BtaR transcriptional regulators, we monitored the levels of transcription of *btaR1*, *btaR2*, and *btaR3* by quantitative reverse transcription-PCR (qRT-PCR) in the wild-type strain and in the *rsaM1* and *rsaM2* mutants of *B. thailandensis* E264 during the exponential phase. We observed an increase in *btaR1* transcription in the absence of RsaM1 (Fig. 6A), but no significant variation was noticed in the *rsaM2* mutant compared to the wild-type strain (Fig. 6A), correlating with the transcription profiles of *btaI1* in these backgrounds (Fig. S5). Thus, the transcription of both *btaR1* and *btaI1* is negatively regulated by RsaM1, suggesting that the negative impact of RsaM1 on the production of C$_8$-HSL involves the regulation of *btaR1* and *btaI1* transcription, whereas RsaM2 does not apparently impact the QS-1 system genes transcription to repress

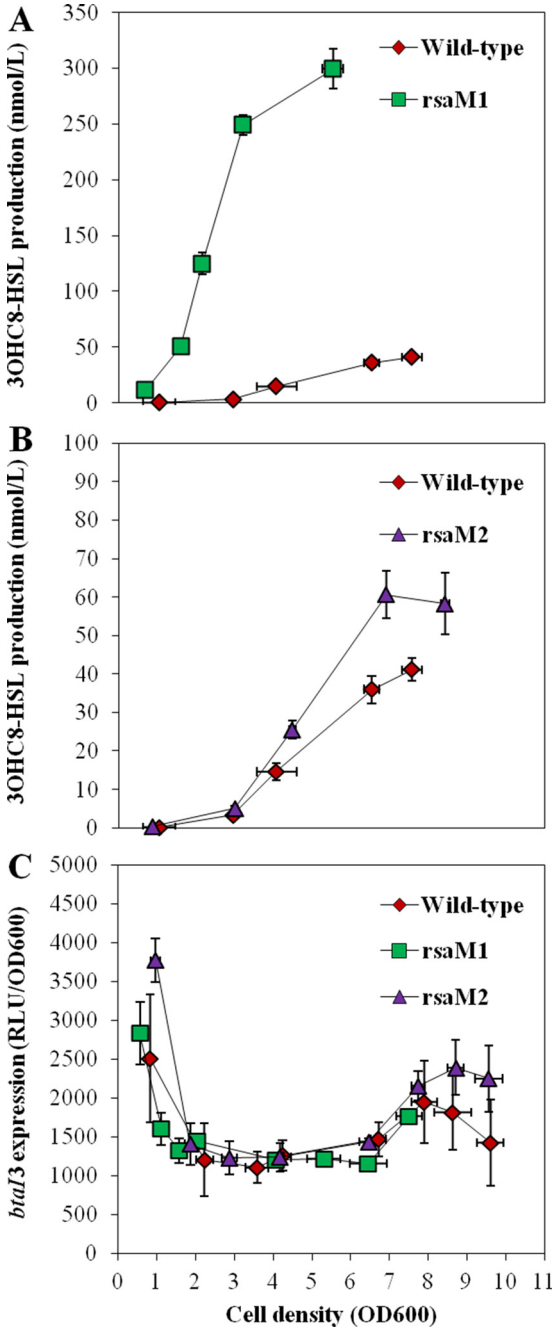

FIG 5 3OHC$_8$-HSL biosynthesis and expression from the *btaI3* promoter in the wild-type and the *rsaM1* and *rsaM2* mutant strains of *B. thailandensis* E264. (A and B) The production of 3OHC$_8$-HSL was quantified using LC-MS/MS at various times during growth in cultures of the wild-type strain and of the *rsaM1* (A) and *rsaM2* (B) mutant strains of *B. thailandensis* E264. The error bars represent the standard deviations of the averages for three replicates. (C) The luminescence of the chromosomal *btaI3-lux* transcriptional fusion was monitored in cultures of the *B. thailandensis* E264 wild-type strain and the *rsaM1* and *rsaM2* mutants. The luminescence is expressed in relative light units per optical density of the culture at 600 nm (RLU/OD$_{600}$).

C$_8$-HSL biosynthesis. Furthermore, no significant difference was detected in *btaR2* transcription in both the *rsaM1* and *rsaM2* mutant strains compared to the wild-type strain, showing that neither RsaM1 nor RsaM2 modulates the transcription of *btaR2* (Fig. 6B). Consequently, while RsaM1 seems to have no effect on the QS-2 system, the RsaM2-dependent control on 3OHC$_{10}$-HSL biosynthesis is not likely linked to the regulation of *btaR2* transcription and therefore appears to solely result from

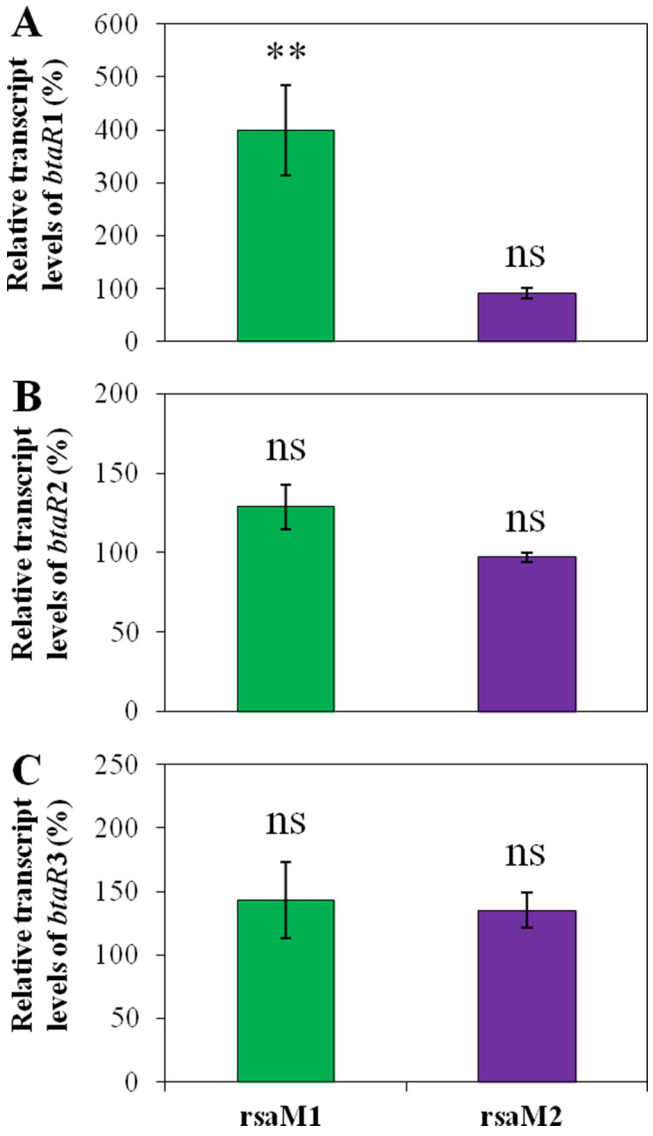

FIG 6 RsaM1 negatively regulates the transcription of *btaR1*, but *btaR2* transcription is not modulated by RsaM2. The relative transcript levels of *btaR1* (A), *btaR2* (B), and *btaR3* (C) were assessed by qRT-PCR in cultures of the wild-type and the *rsaM1* and *rsaM2* mutant strains of *B. thailandensis* E264. The results are presented as relative quantification of transcription of the gene compared to the wild-type strain, which was set at 100%. The values are means ± standard deviations (error bars) for three replicates. Values that are significantly different are indicated by asterisks as follows: **, $P < 0.01$. Values that are not significantly different (ns) are also indicated.

modulation of *btaI2* transcription. Moreover, neither RsaM1 nor RsaM2 had a significant impact on the transcription of *btaR3* (Fig. 6C). These observations indicate that the production of $3OHC_8$-HSL is not controlled by RsaM1 and RsaM2 through modulation of the transcription of the QS-3 system genes.

The *rsaM1* and *rsaM2* genes are QS controlled. The transcription of the *rsaM2* gene, but not *rsaM1* gene transcription, was reported to be activated by QS (18). Our transcriptomic analyses indicate that QS indeed stimulates *rsaM2* transcription, as well as the transcription of *rsaM1* (Le Guillouzeret al., unpublished).

In order to ascertain that the transcription of *rsaM1* is positively controlled by QS, we monitored *rsaM1* transcription by qRT-PCR in the *B. thailandensis* E264 wild-type strain and in the AHL-defective Δ*btaI1* Δ*btaI2* Δ*btaI3* mutant supplemented with exogenous AHLs or not supplemented with AHLs during the exponential phase. We observed that the transcription of *rsaM1* was reduced in the absence of AHLs, confirming that QS

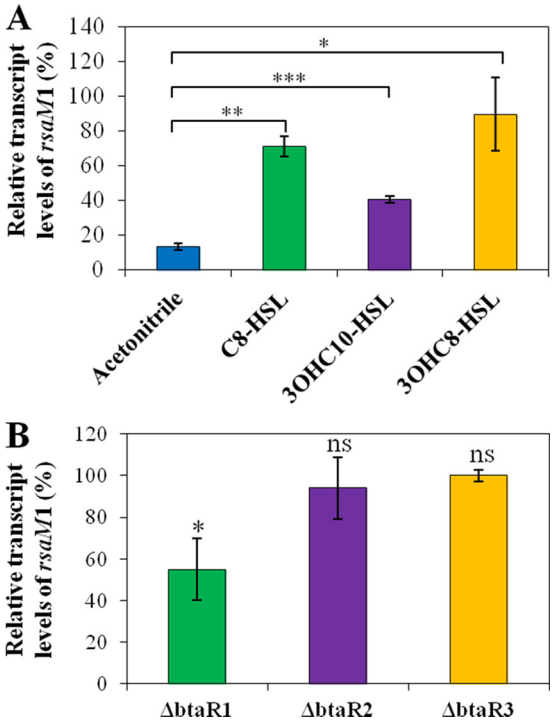

FIG 7 QS positively regulates *rsaM1* transcription. (A) The relative transcript levels of *rsaM1* from the *B. thailandensis* E264 wild-type strain and its Δ*btaI1* Δ*btaI2* Δ*btaI3* mutant strain were estimated by qRT-PCR. Cultures were supplemented with 10 μM C_8-HSL, $3OHC_{10}$-HSL, or $3OHC_8$-HSL. Acetonitrile only was added to the controls. The results are presented as relative quantification of transcription of the gene compared to the wild-type strain, which was set at 100%. The error bars represent the standard deviations of the averages for three replicates. (B) The relative transcript levels of *rsaM1* were assessed by qRT-PCR in cultures of the wild-type and the Δ*btaR1*, Δ*btaR2*, and Δ*btaR3* mutant strains of *B. thailandensis* E264. ***, $P < 0.0001$; **, $P < 0.01$; *, $P < 0.05$; ns, nonsignificant.

activates *rsaM1* transcription (Fig. 7A). Furthermore, the transcription of *rsaM1* was significantly enhanced in cultures of the Δ*btaI1* Δ*btaI2* Δ*btaI3* triple-mutant strain supplemented with C_8-HSL, $3OHC_{10}$-HSL, or $3OHC_8$-HSL (Fig. 7A). To gain insights into the QS-dependent regulation of *rsaM1*, we also measured the transcription of *rsaM1* in the Δ*btaR1*, Δ*btaR2*, and Δ*btaR3* mutants versus the *B. thailandensis* E264 wild-type strain during the exponential phase. While no obvious change in *rsaM1* transcription was visible in the absence of neither BtaR2 nor BtaR3, the transcription of *rsaM1* was decreased in the Δ*btaR1* mutant compared to the wild-type strain (Fig. 7B). Taken together, these data indicate that the transcription of *rsaM1* is positively regulated by the QS-1 system, whereas the QS-2 and QS-3 systems are likely not involved in the modulation of *rsaM1* transcription.

The transcription of *rsaM2* was lowered in the absence of AHLs, confirming that the *rsaM2* gene is activated by QS as well (Fig. 8A). Moreover, *rsaM2* transcription was significantly enhanced in cultures of the Δ*btaI1* Δ*btaI2* Δ*btaI3* triple-mutant strain supplemented with $3OHC_{10}$-HSL or $3OHC_8$-HSL (Fig. 8A). Interestingly, we observed that the transcription of *rsaM2* was also downregulated in the Δ*btaR2* mutant compared to the wild-type strain, meaning that the *rsaM2* gene is positively controlled by BtaR2, whereas no discernible difference in *rsaM2* transcription was detected in the absence of BtaR1 or BtaR3 (Fig. 8B). Altogether, our results indicate that the transcription of *rsaM2* is positively modulated by the QS-2 system, whereas the QS-1 and QS-3 systems apparently do not intervene in the regulation of *rsaM2* transcription.

Collectively, these observations highlight that the transcription of *rsaM1* is activated by the QS-1 system, which is negatively regulated by RsaM1, whereas *rsaM2* transcription is stimulated by the QS-2 system, which is negatively regulated by RsaM2, showing

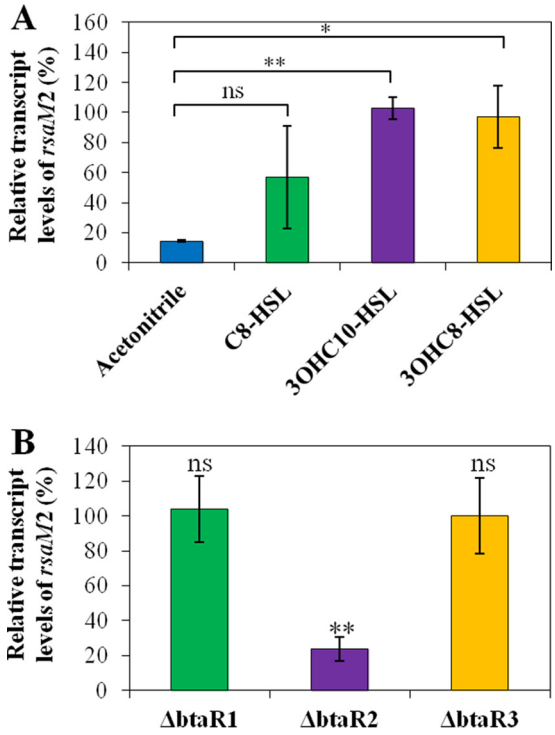

FIG 8 The transcription of *rsaM2* is activated by QS. (A) The relative transcript levels of *rsaM2* from the *B. thailandensis* E264 wild-type strain and its Δ*btaI1* Δ*btaI2* Δ*btaI3* mutant strain were monitored by qRT-PCR. Cultures were supplemented with 10 μM C_8-HSL, $3OHC_{10}$-HSL, or $3OHC_8$-HSL. Acetonitrile only was added to the controls. The results are presented as relative quantification of transcription of the gene compared to the wild-type strain, which was set at 100%. The error bars represent the standard deviations of the averages for three replicates. (B) The relative transcript levels of *rsaM2* were quantified by qRT-PCR in cultures of the wild-type and the Δ*btaR1*, Δ*btaR2*, and Δ*btaR3* mutant strains of *B. thailandensis* E264. **, $P < 0.01$; *, $P < 0.05$; ns, nonsignificant.

that these repressors are deeply integrated into the QS modulatory network of *B. thailandensis* E264.

***rsaM1* and *rsaM2* are negatively autoregulated.** To further explore the RsaM1 and RsaM2 molecular mechanisms of action, the levels of transcription of *rsaM1* and *rsaM2* were assessed by qRT-PCR in the *B. thailandensis* E264 wild-type strain and in the *rsaM1* and *rsaM2* mutants during the exponential phase. The transcription of *rsaM1* was strongly increased in the absence of RsaM1 (Fig. 9A), and the same was observed for *rsaM2* transcription in the *rsaM2* mutant compared to the wild-type strain (Fig. 9B). However, the absence of RsaM2 had no impact on *rsaM1* transcription (Fig. 9A), and the transcription of *rsaM2* was unchanged in the *rsaM1* mutant in comparison with the wild-type strain (Fig. 9B). Altogether, our results indicate that RsaM1 and RsaM2 repress their own transcription but do not influence each other.

DISCUSSION

While the function of RsaM-like proteins was previously investigated in a few *Burkholderia* species (11, 12, 21), their involvement in the complex organization of the multiple QS circuitries found in the closely related species of the *Burkholderia pseudomallei-B. thailandensis-B. mallei* group had not been addressed. Here, we initiated a study of the two *rsaM* homologues present on the genome of *B. thailandensis* E264.

The *rsaM1* gene, which is divergently transcribed from *btaR1* and oriented in the same direction as *btaI1*, encodes an RsaM-like protein initially characterized in the plant pathogen *P. fuscovaginae* (8, 11, 12, 20–22) (see Fig. S1A and B in the supplemental material). The RsaM protein of *P. fuscovaginae* UPB0736 was reported to negatively control the AHL-based QS systems PfsI/PfsR and PfvI/PfvR (9, 22). It is hypothesized to

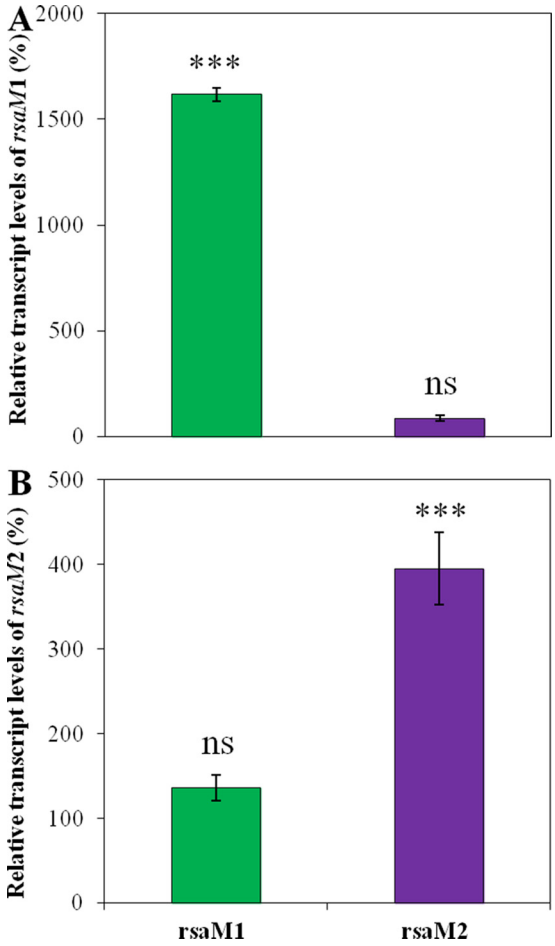

FIG 9 The *rsaM1* and *rsaM2* genes are negatively autoregulated. The relative transcript levels of *rsaM1* (A) and *rsaM2* (B) from the wild-type *B. thailandensis* E264 strain and its *rsaM1* and *rsaM2* mutant strains were estimated by qRT-PCR. The results are presented as relative quantification of transcription of the gene compared to the wild-type strain, which was set at 100%. The error bars represent the standard deviations of the averages for three replicates. ***, $P < 0.001$; ns, nonsignificant.

directly repress the transcription of the LuxI-type synthase PfsI- and PfvI-encoding genes. However, it could also act indirectly, for instance, by inhibiting the functionality of the LuxR-type transcriptional regulators PfsR and PfvR, which are required for activation of *pfsI* and *pfvI* gene transcription, respectively. In the Bcc member *Burkholderia cenocepacia*, an RsaM-like protein homologue, namely, *Bc*RsaM, was described as an important repressor of the production of C_8-HSL affiliated with the CepI/CepR QS system and proposed to regulate the activity and/or stability of the LuxI-type synthase CepI and the LuxR-type transcriptional regulator CepR, as well as the orphan LuxR-type transcriptional regulator CepR2 (11, 12). The transcription of the *cepI*, *cepR*, and *cepR2* genes of *B. cenocepacia* H111 was seen to be lowered in an *rsaM* mutant in comparison with the wild-type strain (11). However, in *B. thailandensis* E264, we found that the transcription of both *btaI1* and *btaR1* was increased in the absence of RsaM1 (Fig. 3C and 6A), correlating with the accumulation of C_8-HSL in this background (Fig. 3A). Consequently, RsaM1 could repress the transcription of *btaI1* and *btaR1*, suggesting that its mode of action in *B. thailandensis* E264 differs from that of *Bc*RsaM. However, we noticed that the impact of RsaM1 on C_8-HSL biosynthesis was dramatically greater than its effect on *btaI1* transcription, which hints that RsaM1 could also act at post-transcriptional levels, as proposed for *Bc*RsaM. Thus, RsaM1 could directly repress the transcription of the *btaI1* and *btaR1* genes or indirectly do so, for instance, by modulating the activity and/or stability of BtaI1 or by controlling the functionality of BtaR1.

This demonstrates that while BtaR1 is considered the principal regulator of the QS-1 system, RsaM1 plays a major role in modulating the production of C_8-HSL (Fig. 10). Strikingly, the absence of RsaM1 was associated with a growth defect in tryptic soy broth (TSB) medium (Fig. 1) and leads to an aggregative growth phenotype in modified M9 medium (see Fig. S3 in the supplemental material). This could be linked to the prominent levels of C_8-HSL produced in the *rsaM1* mutant compared to the wild-type strain, and thus overactivation of phenotypes controlled by the QS-1 system, such as autoaggregation, biofilm development, and oxalate production (17, 18, 25–27).

We recently reported that the transcription of *btaI1* is activated by BtaR1/C_8-HSL, meaning that the QS-1 system is positively autoregulated (16) (Fig. 10). We indeed confirmed that *btaI1* transcription is downregulated in the Δ*btaR1* mutant compared to in the wild-type strain (16, 18). However, we observed an accumulation of C_8-HSL in the absence of BtaR1 (16). We thus hypothesized that additional regulatory elements are involved in the modulation of C_8-HSL production (16). The finding that BtaR1 and C_8-HSL activate the transcription of *rsaM1* might explain why more C_8-HSL is detected in the absence of BtaR1 (Fig. 7). In fact, it is possible that the mutation in *btaR1*, which appears to affect *rsaM1* transcription, results indirectly in C_8-HSL overproduction. Moreover, it reveals that the QS-1 system is also negatively autoregulated through RsaM1, presumably counteracting with the positive-feedback loop mediated by BtaR1/C_8-HSL for the biosynthesis of C_8-HSL. This could be necessary to modulate the QS response depending on specific environmental conditions, as previously suggested for other negative regulators of QS. For instance, the QteE and RsaL repressors in the human opportunistic pathogen *Pseudomonas aeruginosa* modulate the timing and extent of the QS response and likely increase *P. aeruginosa* phenotypic plasticity and population fitness, ultimately facilitating the colonization of challenging environments, including higher organisms (22, 28–33). The RsaL protein is also found ubiquitously in the group of nonpathogenic plant-associated nitrogen-fixing *Burkholderia* spp., such as *Burkholderia kururiensis*, and its role is hypothesized to be a switch to turn on/off the AHL signaling system under various environmental conditions (22, 34). We found a putative *lux* box sequence in the promoter region of *rsaM1* that might be specifically recognized by BtaR1/C_8-HSL to stimulate *rsaM1* transcription (Fig. S1C). Consistently, the CepR transcriptional regulator of *B. cenocepacia* K56-2 was shown to positively and directly control the transcription of the *rsaM* gene in association with C_8-HSL (35, 36). Nevertheless, *rsaM1* displayed different transcriptional profiles in the Δ*btaR1* mutant and in the Δ*btaI1* Δ*btaI2* Δ*btaI3* mutant backgrounds (Fig. 7), indicating that the QS-dependent regulation of *rsaM1* transcription might be more complex and will need further investigation.

We suppose that RsaM1 does not control the QS-2 system, since neither the biosynthesis of 3OHC$_{10}$-HSL (Fig. 4A), which we confirmed constitutes the main AHL produced by BtaI2 (19) (Fig. 2B), nor the transcription of *btaI2* and *btaR2* is affected in the *rsaM1* mutant compared to the wild-type strain (Fig. 4B and 6B). Therefore, we must deduce that the effect of RsaM1 on 3OHC$_8$-HSL production (Fig. 5A), which is also synthesized by BtaI2 (19), rather involves modulation of the QS-1 and/or QS-3 systems. We indeed confirmed that BtaI3 principally synthesizes 3OHC$_8$-HSL (17) (Fig. 2C). However, RsaM1 seems to have no impact on *btaI3* and *btaR3* transcription (Fig. 5C and 6C). An explanation could be that RsaM1 indirectly modulates the QS-3 system through the control of other regulatory elements that would affect the production of 3OHC$_8$-HSL, thus further connecting the QS-1 and QS-3 systems in *B. thailandensis* E264 that were shown to be transcriptionally linked (16, 18) (Fig. 10). Interestingly, the QS repressor RsaM of *P. fuscovaginae* UPB0736 was reported to control several genes encoding transcriptional factors and could consequently intervene directly in the modulation of gene expression, as well as indirectly via auxiliary regulators (10, 22). In order to further understand the molecular mechanism of action of RsaM1, we propose to define the RsaM1 regulon, for instance, by performing RNA-seq analyses and/or chromatin immunoprecipitation sequencing (ChIP-Seq) analyses. Still, it is also conceivable that RsaM1 affects 3OHC$_8$-HSL biosynthesis by directly regulating the QS-1 system.

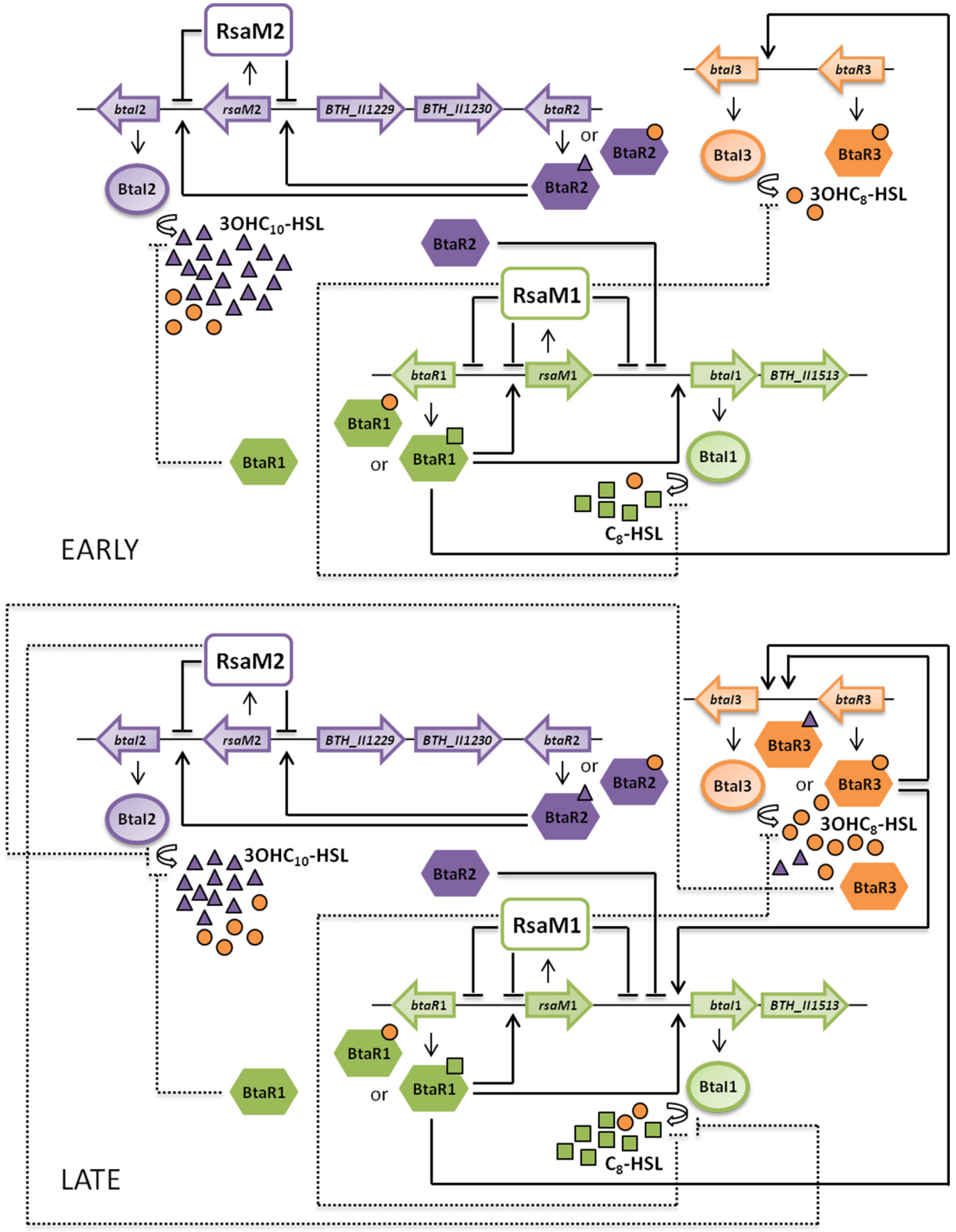

FIG 10 Proposed involvement of RsaM1 and RsaM2 in the QS circuitry of *B. thailandensis* E264. The QS-1 system is composed of the BtaI1 synthase, which is principally responsible for C_8-HSL biosynthesis (17) and is hypothesized to produce $3OHC_8$-HSL, as well as the BtaR1 transcriptional regulator that stimulates the transcription of *btaI1* in association with C_8-HSL (16, 18). The BtaI2 synthase, which synthesizes both $3OHC_{10}$-HSL and $3OHC_8$-HSL (19), as well as the BtaR2 transcriptional regulator that activates *btaI2* transcription in conjunction with these AHL signaling molecules (16, 18, 19), constitute the QS-2 system. Furthermore, the QS-1 and QS-2 systems contain *rsaM* homologues designated *rsaM1* and *rsaM2*, respectively. The RsaM1 protein mainly represses the production of C_8-HSL. It could act directly by repressing the transcription of *btaI1*

(Continued on next page)

In fact, $3OHC_8$-HSL could be produced via BtaI1 in the wild-type strain in concentrations under our detection limit, and then those levels become detectable in the QS-1 system-boosted *rsaM1* mutant. We previously reported that besides C_8-HSL, the homologue of this AHL synthase can produce trace amounts of $3OHC_8$-HSL in the Bcc member *Burkholderia ambifaria* (37). Additionally, the *B. pseudomallei* KHW BpsI and *Burkholderia mallei* ATCC 23344 BmaI1 synthases, which are homologous to BtaI1 (38–40), were both shown to produce $3OHC_8$-HSL in addition to C_8-HSL, albeit at lower concentrations (41, 42), and the *B. pseudomallei* KHW BpsR and *B. mallei* ATCC 23344 BmaR1 transcriptional regulators, which are homologous to BtaR1 (38–40), were reported to specifically respond to both C_8-HSL and $3OHC_8$-HSL (41, 42). Accordingly, the BtaR1-controlled genes identified in transcriptomic analyses were generally affected by both C_8-HSL and $3OHC_8$-HSL (18). This then explains why these AHLs exhibit similar production profiles (16). Additional experiments, however, will be necessary to confirm the possible production of $3OHC_8$-HSL by BtaI1.

The *rsaM2* gene, which is found directly adjacent to *btaI2* and is transcribed in the same direction, encodes an additional RsaM-like protein (Fig. S1A and B). The transcription of *btaI2* and $3OHC_{10}$-HSL production are activated by BtaR2, which constitutes the main regulator of the QS-2 system (16, 18, 19). Additionally, we demonstrated that the QS-2 system is negatively modulated by RsaM2 (Fig. 4), whereas the transcription of *rsaM2* is stimulated by the QS-2 system (Fig. 8). Consequently, while *btaI2* transcription is directly activated by BtaR2, it seems that BtaR2 also represses the transcription of *btaI2* indirectly through RsaM2 control (Fig. 10). We assume that the negative regulation exerted by RsaM2 restrains the QS-2 system response by limiting the self-inducing loop that leads to the accumulation of $3OHC_{10}$-HSL, showing again an important homeostatic modulation of AHL production in *B. thailandensis* E264. The negative impact of RsaM2 on the production of $3OHC_8$-HSL, as for the RsaM2-dependent regulation of $3OHC_{10}$-HSL biosynthesis, might result from modulation of *btaI2* transcription (Fig. 10). Remarkably, we noticed that the production of $3OHC_{10}$-HSL is repressed by RsaM2 from the exponential phase (Fig. 4A), whereas $3OHC_8$-HSL biosynthesis is repressed by RsaM2 from the stationary phase (Fig. 5B). This is consistent with our proposal that $3OHC_8$-HSL is produced by BtaI2 at the expense of $3OHC_{10}$-HSL in the stationary phase (16). Since the transcription of neither *btaI3* nor *btaR3* seems to be under RsaM2 control, we conclude that RsaM2 does not influence $3OHC_8$-HSL biosynthesis by modulating the QS-3 system gene transcription.

It is not clear how C_8-HSL biosynthesis is repressed by RsaM2 when no matching overexpression of *btaI1* is observed in the absence of RsaM2 (Fig. 3B and C), as we confirmed the loss of C_8-HSL production in the Δ*btaI1* mutant in comparison with the wild-type strain, indicating that this AHL is exclusively synthesized by BtaI1 (17) (Fig. 2A). We recently reported that the QS-1 and QS-2 systems are transcriptionally linked (16) and indeed determined that C_8-HSL biosynthesis and the transcription of *btaI1*, but not *btaR1*, are repressed by BtaR2 (Fig. S6). Therefore, while the QS-2 system appears to directly repress the production of C_8-HSL by modulating *btaI1* transcription, it is

FIG 10 Legend (Continued)
and *btaR1* or indirectly, for instance, by modulating the activity and/or stability of BtaI1 or by controlling the functionality of BtaR1. The RsaM2 protein principally represses the production of $3OHC_{10}$-HSL, as well as *btaI2* transcription but not the transcription of *btaR2*. The *rsaM1* and *rsaM2* genes are negatively autoregulated and activated by the QS-1 and QS-2 systems, respectively, showing an important homeostatic modulation of AHL biosynthesis. Moreover, an interdependence between the QS-1 and QS-2 systems was observed. The production of C_8-HSL, as well as *btaI1* transcription, but not the transcription of *btaR1*, is indeed negatively controlled by BtaR2 (16). Since RsaM2 seems to have no impact on the transcription of *btaI1* and *btaR1*, the negative modulation of C_8-HSL biosynthesis by RsaM2 might involve other regulatory elements, underscoring an additional modulatory layer connecting the QS-1 and QS-2 systems. While neither the transcription of *btaI2* nor *btaR2* transcription are under BtaR1 control, BtaR1 appears to repress the production of $3OHC_{10}$-HSL (16, 18). Similarly, $3OHC_{10}$-HSL biosynthesis was shown to be negatively controlled by the QS-3 system (16, 18), which is composed of the BtaI3 synthase and the BtaR3 transcriptional regulator. BtaI3 is mainly responsible for $3OHC_8$-HSL biosynthesis (17) and is hypothesized to produce $3OHC_{10}$-HSL (16), whereas the transcription of *btaI3* is stimulated by BtaR3 in association with $3OHC_8$-HSL and $3OHC_{10}$-HSL (16). An interdependence between the QS-1 and QS-3 systems was observed as well, since *btaI3* transcription is likely activated by BtaR1/C_8-HSL from the exponential phase, and BtaR3, in conjunction with $3OHC_8$-HSL and $3OHC_{10}$-HSL, was suggested to positively modulate the transcription of *btaI1* from the stationary phase (16). Additionally, RsaM1 could repress the production of $3OHC_8$-HSL by targeting the QS-1 and/or QS-3 systems, thus further connecting these QS circuitries.

possible that C_8-HSL biosynthesis is also negatively and indirectly controlled by the QS-2 system, underscoring an additional modulatory layer connecting the QS-1 and QS-2 systems in *B. thailandensis* E264 (Fig. 10). In fact, the negative impact of RsaM2 on the production of C_8-HSL could involve additional transcriptional and/or posttranscriptional regulators. More experiments will thus be necessary to determine the precise underlying molecular mechanism of action of RsaM2.

We demonstrated that RsaM1 and RsaM2 repress their own transcription (Fig. 9). Negative autoregulation of these repressors could be necessary to maintain AHLs at appropriate levels depending on particular environmental conditions, likely contributing further to the hierarchical and homeostatic expression of the QS-1, QS-2, and QS-3 systems (Fig. 10).

Conclusion. We recently reported the complex organization of the QS-1, QS-2, and QS-3 systems in *B. thailandensis* E264 and we observed that these QS systems are integrated into an intricate modulatory network, including the required involvement of additional regulators (16). The study described here uncovers the central role of RsaM1 and RsaM2 in the modulation of AHL signaling in this bacterium (Fig. 10). We demonstrated that RsaM1 mainly represses the QS-1 system, whereas RsaM2 principally represses the QS-2 system. Additionally, RsaM1 and RsaM2 were shown to be an integral part of the QS circuitry in *B. thailandensis*, contributing to the temporal activation of its multiple QS systems by modulating the production of AHLs. The precise underlying molecular mechanism of action of these proteins is, however, currently unknown and has to be further investigated in the future given their importance in the regulation of QS-controlled genes in the *Burkholderia* genus and other proteobacteria (8–12, 20–22).

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table S1 in the supplemental material. Unless stated otherwise, all bacteria were cultured at 37°C in tryptic soy broth (TSB; BD Difco, Mississauga, Ontario, Canada), with shaking (240 rpm) in a TC-7 roller drum (New Brunswick, Canada), or on petri dishes containing TSB solidified with 1.5% agar. When required, antibiotics were used at the following concentrations: 200 μg/ml tetracycline (Tc) and 100 μg/ml trimethoprim (Tp) for *B. thailandensis* E264, and 15 μg/ml Tc for *Escherichia coli* DH5α. All measurements of optical density at 600 nm (OD_{600}) were acquired with a Thermo Fisher Scientific NanoDrop ND-1000 spectrophotometer.

Construction of plasmids. All plasmids used in this study are described in Table S2. Amplification of *btaR2* was performed from genomic DNA from *B. thailandensis* E264 using the appropriate primers (Table S3). The amplified product was digested with the FastDigest restriction enzymes BamHI and HindIII (Thermo Fisher Scientific) and inserted by T4 DNA ligase (Bio Basic, Inc., Markham, Ontario, Canada) within the corresponding restriction sites in the pME6000 plasmid (43), generating the constitutive expression vector pMCG21. All primers were purchased from Alpha DNA (Montreal, Quebec, Canada).

Construction of recombinant strains. The pME6000 and pM6000-*btaR2* constitutive expression vectors were introduced in *B. thailandensis* E264 strains by electroporation. Briefly, bacterial cultures were grown to an OD_{600} of 1.0, pelleted by centrifugation, and washed several times with 1 ml of sterile water. The pellets were concentrated 100-fold in 100 μl of sterile water and electroporated using a 1-mm-gap disposable electroporation cuvette at 1.8 kV with an Eppendorf Electroporator 2510 (Eppendorf Scientific, Inc., Westbury, NY). Cells were grown for 1 h in 1 ml lysogeny broth (LB) (Alpha Biosciences, Inc., Baltimore, MD) at 37°C then plated on Tc-selective medium.

Construction of reporter strains. Chromosomal integration of the mini-CTX-*btaI1-lux*, mini-CTX-*btaI2-lux*, and mini-CTX-*btaI3-lux* transcriptional reporters at the *attB* locus in *B. thailandensis* E264 strains was performed through conjugation with the auxotrophic *E. coli* χ7213. Overnight bacterial cultures of *B. thailandensis* E264 strains were diluted in 1.5 ml TSB to an initial OD_{600} of 0.1 and incubated as described above. Overnight bacterial cultures of *E. coli* χ7213 carrying the corresponding chromosomal reporters were diluted in 1.5 ml TSB supplemented with 62.5 μg/ml diaminopimelic acid (DAP) to an initial OD_{600} of 0.1 and statically grown at 37°C. When the cultures reached an OD_{600} of 0.5, they were pelleted by centrifugation. The pellets were resuspended together in 100 μl TSB and then spotted onto TSB agar plates containing DAP and incubated overnight at 37°C. The bacterial strains were suspended in 1 ml TSB and then plated on Tc-selective medium. Successful chromosomal insertion of the *btaI1-lux*, *btaI2-lux*, and *btaI3-lux* plasmids was confirmed by PCR using appropriate primers.

LC-MS/MS quantification of AHLs. The concentration of AHLs was determined from samples of *B. thailandensis* E264 cultures obtained at different time points during bacterial growth, using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The samples were prepared and analyzed as described previously (37). 5,6,7,8-Tetradeutero-4-hydroxy-2-heptylquinoline (HHQ-d4) was used as an internal standard. All experiments were performed in triplicate and conducted at least twice independently.

Measurement of the activities of *btal1-lux*, *btal2-lux*, and *btal3-lux* reporters. The levels of transcription from the promoter regions of *btal1*, *btal2*, or *btal3* were quantified by measuring the luminescence of *B. thailandensis* E264 cultures carrying the corresponding chromosomal reporters, as described previously (16). Overnight bacterial cultures were diluted in TSB to an initial OD_{600} of 0.1 and incubated as indicated above. The luminescence was regularly determined from culture samples using a multimode microplate reader (Cytation 3; BioTek Instruments, Inc., Winooski, VT) and expressed in relative light units per optical density of the culture at 600 nm (RLU/OD_{600}). All experiments were performed with three biological replicates and repeated at least twice.

Quantitative reverse transcription-PCR experiments. Total RNA of *B. thailandensis* E264 cultures at an OD_{600} of 4.0 was extracted with the PureZOL RNA isolation reagent (Bio-Rad Laboratories, Mississauga, Ontario, Canada) and treated twice with the Turbo DNA-free kit (Ambion Life Technologies, Inc., Burlington, Ontario, Canada), according to the manufacturer's instructions. Extractions were done on three different bacterial cultures. Quality and purity controls were confirmed by agarose gel electrophoresis and UV spectrophotometric analysis, respectively. cDNA synthesis was performed using the iScript reverse transcription supermix (Bio-Rad Laboratories), and amplification was accomplished on a Corbett Life Science Rotor-Gene 6000 thermal cycler using the SsoAdvanced universal SYBR green supermix (Bio-Rad Laboratories), according to the manufacturer's protocol. The reference gene was *ndh* (44). The *ndh* gene displayed stable transcription under the different genetic contexts tested. All primers used for cDNA amplification are presented in Table S4. Differences in gene transcription between *B. thailandensis* E264 strains were calculated using the $2^{-\Delta\Delta CT}$ formula (45). A threshold of 0.5 was chosen as significant. For experiments with AHL additions, cultures were supplemented with 10 μM C_8-HSL, $3OHC_{10}$-HSL, and $3OHC_8$-HSL (Sigma-Aldrich Co., Oakville, Ontario, Canada) or not supplemented with AHLs from stocks prepared in high-performance liquid chromatography (HPLC)-grade acetonitrile. Acetonitrile only was added to the controls. All experiments were performed in triplicate and conducted at least twice independently.

Data analysis. Unless stated otherwise, data are reported as means ± standard deviations (SD). Statistical analyses were performed with the R software version 3.3.3 (http://www.R-project.org/) using one-way analysis of variance (ANOVA) or a *t* test. Probability values of less than 0.05 were considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00727-17.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

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