Two rsaM homologues encode central regulatory elements modulating quorum sensing expression in *Burkholderia thailandensis*

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Running title: RsaM-like proteins in *Burkholderia thailandensis*
Abstract

The bacterium *Burkholderia thailandensis* possesses three conserved *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 (*3OHC₁₀-HSL*), as well as *N*-3-hydroxy-octanoyl-homoserine lactone (*3OHC₈-HSL*), which are produced by the LuxI-type synthase BtaI1, BtaI2, and BtaI3, and modulated by the LuxR-type transcriptional regulators BtaR1, BtaR2, and BtaR3. Both *btaR₁/btaI₁* and *btaR₂/btaI₂* gene clusters contain an additional gene that is conserved in the *Burkholderia* genus, homologous to a gene coding for the negative AHL biosynthesis modulatory protein RsaM originally identified in the phytopathogen *Pseudomonas fuscovaginae*, and hence designated *rsaM₁* and *rsaM₂*. We have characterized the function of these two *rsaM* homologues and demonstrated their involvement in the regulation of AHLs biosynthesis in *B. thailandensis* strain E264. We measured the production of *C*₈-HSL, *3OHC₁₀-HSL*, and *3OHC₈-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 monitored the transcription of *btaI₁*, *btaI₂*, and *btaI₃* using chromosomal mini-CTX-*lux* transcriptional reporters. The expression of *btaR₁*, *btaR₂*, and *btaR₃* was also measured by quantitative reverse-transcription PCR (qRT-PCR). We demonstrate that the QS-1 system is repressed by RsaM1, whereas RsaM2 principally represses the QS-2 system. We also found that both *rsaM₁* and *rsaM₂* are QS-controlled, as well as negatively auto-regulated. We conclude that RsaM1 and RsaM2 are an integral part of the QS modulatory 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.
Quorum sensing (QS) is a global regulatory mechanism of genes expression depending on bacterial density. QS is commonly involved in the coordination of genes expression associated with the establishment of host-pathogen interactions and acclimatization to the environment. We present the functional characterization of the two rsaM homologues designated rsaM1 and rsaM2 in the regulation of the multiple QS systems coexisting in the non-pathogenic bacterium Burkholderia thailandensis, widely used as a model system for the study of the pathogen Burkholderia pseudomallei. We found that inactivation of these rsaM homologues, which are clustered with the other QS genes, profoundly affects the QS regulatory circuity of B. thailandensis. It is proposed that these genes code for QS repressors and we conclude that they constitute essential regulatory components of the QS modulatory network of B. thailandensis, and provide additional layers of regulation to modulate the expression of QS-controlled genes, including those encoding virulence/survival factors and linked to environmental adaptation in B. pseudomallei.
Introduction

Quorum sensing (QS) is a widespread bacterial intercellular communication system that coordinates expression of specific genes in a cell 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 accumulate in the environment as bacterial growth progresses until a threshold concentration is reached allowing the coordination of the expression of specific genes. Gram-negative bacteria typically 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 include a luxI homologue encoding the AHL synthase, resulting in a typical self-inducing loop of AHLs (3).

The Burkholderia genus encompasses heterogeneously species colonizing diverse ecological niches, such as soil, water, plants, as well as animals, including humans (4). The Burkholderia cepacia complex (Bcc), for instance comprises notable opportunistic human pathogens deleterious in both cystic fibrosis (CF) individuals and immunocompromised patients (5). Bcc members carry luxI and luxR homologues referred as cepI and cepR genes, respectively. These genes are constitutive of the AHL-based QS system designated CepI/CepR (6). The LuxI-type synthase CepI is responsible for N-octanoyl-homoserine lactone (C8-HSL) biosynthesis, which generally constitutes the predominant AHL found in the Burkholderia genus (6). The LuxR-type transcriptional regulator CepR modulates the expression of QS target genes in conjunction with C8-HSL, including the cepI gene creating the QS typical auto-regulation loop (6). The genetic organization of cepI and cepR is conserved among Burkholderia spp. (7). These genes are generally separated by a gene encoding an RsaM-like protein that was originally identified in the plant pathogen Pseudomonas fuscovaginae and shown to be a major negative regulator of both AHLs biosynthesis and expression of the AHL synthase-coding genes (8). It was also recently reported to act as a global regulator mediating the expression of numerous genes through and out of the QS regulon in P. fuscovaginae (9). The function of RsaM-like proteins has been investigated in the Burkholderia genus, and could be important for balancing and fine-tuning of QS-dependant regulation (10). RsaM-like proteins do not possess 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 (11). Their precise underlying regulatory mechanism is currently unknown.

The non-pathogenic tropical soil saprophyte \textit{Burkholderia thailandensis}, as well as the closely-related human pathogen \textit{Burkholderia pseudomallei}, both encode two conserved RsaM-like proteins of uncharacterized function (7). The genome of \textit{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 \textit{B. pseudomallei}, and were reported to be involved in the regulation of several virulence genes and to be essential to its pathogenicity (12). The QS-1, QS-2, and QS-3 systems were recently reported to be hierarchically and homeostatically organized, and integrated into an intricate modulatory network, including transcriptional and post-transcriptional interactions between each QS circuits (13). The QS-1 AHL synthase BtaI1 produces C8-HSL (14) which associates with the BtaR1 transcriptional regulator to activate the expression of the \textit{btaI1} gene (13, 15). The QS-2 system is responsible for the biosynthesis of both N-3-hydroxy-decanoyl-homoserine lactone (3OHC\textsubscript{10}-HSL) and N-3-hydroxy-octanoyl-homoserine lactone (3OHC\textsubscript{8}-HSL) (16). The \textit{btaI2} gene, which encodes the BtaI2 synthase, is positively controlled by the BtaR2 transcriptional regulator in association with 3OHC\textsubscript{10}-HSL and 3OHC\textsubscript{8}-HSL (13, 16). The QS-3 system is composed of the BtaR3 transcriptional regulator and the BtaI3 synthase responsible for 3OHC\textsubscript{8}-HSL production as well (14). The \textit{btaI3} gene encoding BtaI3 is activated by BtaR3 (13). While both the QS-1 and QS-2 gene clusters include an \textit{rsaM} homologue, no \textit{rsaM} gene is present in the vicinity of \textit{btaI3/btaR3} (7).

The central aim of this study was to further explore the QS modulatory circuitry of \textit{B. thailandensis} E264 to identify additional transcriptional and/or post-transcriptional regulators of the QS-1, QS-2, and QS-3 systems. We functionally characterized the role of \textit{rsaM1} and \textit{rsaM2} in the regulation of the \textit{B. thailandensis} E264 AHL-based QS circuits. We established that they negatively modulate AHLs biosynthesis and that they are finely integrated into the complex QS circuitry of \textit{B. thailandensis} E264. This study provides new insights on the intricate interplay existing between the QS systems of \textit{B. thailandensis}, and is essential in unraveling the regulatory mechanism underlying QS-dependent genes expression, including those encoding virulence/survival factors and linked to environmental adaptation in \textit{B. pseudomallei}.
Materials and methods

Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table S1. Unless otherwise stated, all bacteria were cultured at 37°C in Tryptic Soy Broth (TSB; BD Difco™, Mississauga, ON, Canada), with shaking (240 rpm) in a TC-7 roller drum (New Brunswick, Canada), or in 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, while Tc was used at 15 µg/mL for *Escherichia coli* DH5α. All measurements of the optical density (OD$_{600}$) were acquired with a Thermo Fisher Scientific NanoDrop® ND-1000 Spectrophotometer.

Plasmids construction

All plasmids used in this study are described in Table S2. Amplification of *btaR2* was conducted from genomic DNA of *B. thailandensis* E264 using appropriate primers (Table S3). The amplified products were digested with the FastDigest restriction enzymes BamHI and HindIII (Thermo Fisher Scientific) and ligated using T4 DNA ligase (Bio Basic, Inc., Markham, ON, Canada) within the corresponding restriction sites in the pME6000 plasmid (17), generating the constitutive expression vector pMCG21. All primers were purchased from Alpha DNA (Montreal, QC, Canada).

Recombinant strains construction for BtaR2 expression

The pME6000 and pMCG21 constitutive expression vectors were introduced in *B. thailandensis* E264 strains by electroporation. Briefly, bacterial cultures were grown to an OD$_{600}$ = 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 electroporator 2510 (Eppendorf Scientific, Westbury, NY, USA). Cells were outgrown for 1 hr in 1 mL Lysis Broth (LB) (Alpha Biosciences, Inc., Baltimore, MD, USA) at 37°C and plated on Tc selective media.
Reporter strains construction

The mini-CTX-\textit{btaI}-1-lux, mini-CTX-\textit{btaI}-2-lux, and mini-CTX-\textit{btaI}-3-lux transcriptional reporters were integrated into the chromosome of \textit{B. thailandensis} E264 strains through conjugation with \textit{E. coli} \chi7213 followed by selection with Tc. Successful chromosomal insertion of the \textit{btaI}-1-lux, \textit{btaI}-2-lux, and \textit{btaI}-3-lux plasmids was confirmed by PCR using appropriate primers.

LC-MS/MS quantification of AHLs

The concentration of AHLs was determined from cultures of \textit{B. thailandensis} E264 at different times during bacterial growth by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The samples were prepared and analyzed as described previously (18). 5,6,7,8-tetradeutero-4-hydroxy-2-heptylquinoline (HHQ-d4) was used as an internal standard. All experiments were performed in triplicate and carried out at least twice independently.

Measurement of the \textit{btaI}-1-lux, \textit{btaI}-2-lux, and \textit{btaI}-3-lux reporters’ activity

Expressions from the promoter regions of \textit{btaI}-1, \textit{btaI}-2, or \textit{btaI}-3 were quantified by measuring the luminescence of \textit{B. thailandensis} E264 cultures carrying the corresponding chromosomal reporters as formerly described (13). Overnight bacterial cultures were diluted in TSB to an initial OD\textsubscript{600} = 0.1 and incubated as indicated above. The luminescence was regularly determined from culture samples using a multi-mode microplate reader (Cytation\textsuperscript{TM} 3, BioTek Instruments, Inc., Winooski, VT, USA) and expressed in relative light units per culture optical density (RLU/OD\textsubscript{600}). All experiments were performed with three biological replicates and repeated at least twice.

Quantitative reverse-transcription PCR experiments

Total RNA of \textit{B. thailandensis} E264 cultures at an OD\textsubscript{600} = 4.0 was extracted with the PureZOL RNA Isolation Reagent (Bio-Rad Laboratories, Mississauga, ON, Canada) and treated twice with the TURBO DNA-free\textsuperscript{TM} Kit (Ambion Life Technologies, Inc., Burlington,
ON, 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* (19). The
*ndh* gene displayed a stable expression under the different genetic contexts tested. All primers
used for cDNA amplification are presented in Table S4. Genes expression differences
between *Burkholderia thailandensis* E264 strains were calculated using the $2^{(-\Delta\Delta(CT))}$ formula
(20). A threshold of 0.5 was chosen as significant. For experiments with additions of AHLs,
cultures were supplemented or not with 10 µM C₈-HSL, 3OHC₁₀-HSL, and 3OHC₆-HSL
(Sigma-Aldrich Co., Oakville, ON, Canada) from stocks prepared in HPLC-grade acetonitrile.
Acetonitrile only was added in controls. All experiments were performed in triplicate and
carried out at least twice independently.

**Data analysis**

Unless otherwise stated, data are reported as mean +/- standard deviation (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). Probability values less than 0.05 were considered
significant.

**Results**

The QS-1 and QS-2 gene clusters of *B. thailandensis* each carry an *rsaM* homologue

The *btaI* (*BTH_I1512*) and *btaR* (*BTH_I1510*) genes, respectively encoding the BtaI1
AHL synthase and the BtaR1 transcriptional regulator of the *B. thailandensis* E264 QS-1
system, are separated by the *BTH_I1511* gene that codes for an hypothetical protein
conserved in the *Burkholderia* genus (7, 10, 11, 21-23). This hypothetical protein of 147
amino acids is similar to RsaM-like proteins and displays 35.8% identity with the negative
AHL biosynthesis modulatory protein RsaM of the phytopathogen *P. fuscovaginae* UPB0736
Interestingly, another rsaM homologue, encoding a hypothetical protein of uncharacterized function, is present on the genome of *B. thailandensis* E264 between the QS-system *btaI*2 (*BTH_I1227*) and *btaR*2 (*BTH_I1231*) genes that code for the LuxI-type synthase *BtaI*2 and the LuxR-type transcriptional regulator *BtaR*2, respectively. This hypothetical protein of 135 amino acids encoded by the *BTH_I1228* gene is 32.4% identical to RsaM (Fig. S1A).

Since the *BTH_I1511* and *BTH_I11228* genes are directly adjacent to *btaI*1 and *btaI*2 in the genome of *B. thailandensis* E264, respectively, and transcribed in the same direction (Fig. S1B), we wondered whether they could be co-transcribed. *BTH_I1228* is indeed predicted to be arranged in operon with *btaI*2 (www.burkholderia.com). According to our transcriptomic analyses (S. Le Guillouzer, M.-C. Groleau, F. Mauffrey, R. Vilmur, and E. Déziel, unpublished data), neither *BTH_I1511*, nor *BTH_I11228* are co-transcribed with *btaI*1 and *btaI*2, respectively (Fig. S1B), as confirmed by reverse-transcription PCR (RT-PCR) experiments (data not shown).

The function of the putative proteins encoded by the *BTH_I1511* and *BTH_I11228* genes is unknown. While *BTH_I11228* is located within a cluster responsible for bactobolin biosynthesis (14, 16, 24), its involvement was actually not demonstrated. To determine whether *BTH_I11511* and *BTH_I11228* are functionally similar to the RsaM-encoding gene of *P. fuscovaginae* UPB0736, which was described as an important repressor of AHLs biosynthesis (8), we investigated the impact of these genes on the production of the predominant AHLs detected in *B. thailandensis* E264. *B. thailandensis* E264 produces 3OHC10-HSL and, to lesser extents, C8-HSL and 3OHC8-HSL (13-16). The total concentration of these AHLs was measured at various time intervals of the bacterial growth by LC-MS/MS in the *B. thailandensis* E264 wild-type and in the null mutants *BTH_I1511*- and *BTH_I11228*-. The mutants both overproduce AHLs when compared to the wild-type strain (Fig. 1). Interestingly, the impact of *BTH_I11511* on total AHL biosynthesis was more pronounced than the effect of *BTH_I11228* (Fig. 1). Of note, the *BTH_I11511*- mutant displays an initially delayed growth phenotype (Fig. 1). These observations indicate that the hypothetical proteins encoded by the *BTH_I11511* and *BTH_I11228* genes constitute, as for RsaM in *P. fuscovaginae* UPB0736, negative regulators of AHL biosynthesis and were thus designated RsaM1 and RsaM2, respectively.
RsaM1 and RsaM2 are repressors of, respectively, QS-1 and QS-2 systems

To determine the effect of RsaM1 and RsaM2 on the QS-1, QS-2, and QS-3 systems, we measured the respective production of C8-HSL, 3OHC10-HSL, and 3OHC8-HSL in the wild-type strain E264 and in the rsaM1- and rsaM2- mutants throughout the bacterial growth phases. To gain additional insights, we also monitored expression of the AHL synthase-coding genes btaI1, btaI2, and btaI3 in the same backgrounds using chromosomal transcriptional fusion reporters.

We observe a dramatic overproduction of C8-HSL in the rsaM1- mutant when compared to the wild-type strain, indicating that RsaM1 negatively affects the biosynthesis of this AHL (Fig. 2A). Expression of the btaI1 gene, (14) was accordingly enhanced in the absence of RsaM1, suggesting that it is repressed by RsaM1 (Fig. 2C). Strikingly, the impact of RsaM1 on C8-HSL biosynthesis (approximately 200 fold) was more important than its effect on btaI1 expression (approximately 2-fold), implying that RsaM1 might also intervene at post-transcriptional levels. Additionally, C8-HSL concentrations also augmented in the rsaM2- mutant, in comparison with the wild-type strain, from the stationary phase (OD600 ≈ 8.0) (Fig. 2B). However, no discernible difference in btaI1 transcription was detected in the absence of RsaM2 (Fig. 2C). Altogether, these data suggest that RsaM1 represses the production of C8-HSL by controlling btaI1 transcription, including putative post-transcriptional modulations as well, whereas the negative impact of RsaM2 on C8-HSL biosynthesis appears to not result from btaI1 regulation.

The levels of 3OHC10-HSL, as well as expression of the btaI2 gene, were unaffected in the absence of RsaM1 (Fig. 3). Thus, neither the production of 3OHC10-HSL, nor btaI2 transcription is under RsaM1 control. Interestingly, 3OHC10-HSL concentrations were strongly increased in the rsaM2- mutant throughout both the exponential and stationary phases, indicating that RsaM2 negatively affects 3OHC10-HSL biosynthesis (Fig. 3A). btaI2 transcription was similarly upregulated in the absence of RsaM2 (Fig. 3B), suggesting that RsaM2 represses 3OHC10-HSL biosynthesis by modulating the expression of btaI2.

The levels of 3OHC8-HSL in cultures of the rsaM1- mutant (14) were also higher compared to the wild-type strain (Fig. 4A). Unexpectedly, expression of the btaI3 gene was not increased in the absence of RsaM1, suggesting that the negative impact of RsaM1 on 3OHC8-HSL production does not result from btaI3 regulation (Fig. 4C). 3OHC8-HSL concentrations were also increased in the rsaM2- mutant in comparison with the wild-type strain during the
stationary phase (Fig. 4B), showing that the production of 3OHC₈-HSL is repressed by RsaM2 as well. Nevertheless, no visible change in expression of btaI₃ was noticed in the absence of RsaM2, revealing that the RsaM2-dependent regulation of 3OHC₈-HSL biosynthesis might also not be linked to btaI₃ (Fig. 4C).

While the concentrations of both C₈-HSL and 3OHC₈-HSL were enhanced in the rsaM₁-mutant, the impact on the former was more important (Figs. S2A and S2B). Additionally, C₈-HSL, 3OHC₁₀-HSL, and 3OHC₈-HSL levels were all increased in the absence of RsaM2, however, 3OHC₁₀-HSL levels were more affected (Figs. S2A and S2C). Collectively, these findings indicate that the QS-1 system is mainly repressed by RsaM1, whereas RsaM2 principally represses the QS-2 system.

RsaM₁ negatively regulates transcription of btaR₁ but expression of the btaR₂ gene is not modulated by RsaM2

In order to determine whether the impact of RsaM1 and RsaM2 on AHL biosynthesis also involves the BtaR1, BtaR2, and BtaR3 transcriptional regulators, (13, 15, 16), we monitored expressions of their respective encoding genes by quantitative reverse-transcription PCR (qRT-PCR) in the wild-type strain and in the rsaM₁- and rsaM₂- mutants during the exponential phase. Interestingly, we observed an increase in btaR₁ transcription in the absence of RsaM1 (Fig. 5A), which correlates with the expression profile of btaI₁ in this background (Fig. 5B). However, no variation was observed in the rsaM₂- mutant when compared to the wild-type strain (Fig. 5). Thus, while expression of both btaR₁ and btaI₁ are negatively regulated by RsaM1, RsaM2 does not impact any of the QS-1 system regulatory genes. Taken together, these results suggest that the negative impact of RsaM1 on the QS-1 system also implies regulation of btaR₁, whereas RsaM2 might exclusively act at post-transcriptional levels. Furthermore, no discernible difference was detected in the btaR₂ gene transcription in the rsaM₁- mutant strain in comparison with the wild-type strain, and its expression was also unchanged in the absence of RsaM2, showing that RsaM1 nor RsaM2 modulate expression of btaR₂ (data not shown). Consequently, while RsaM1 seems to have no effect on the QS-2 system, RsaM₂-dependent regulation of the QS-2 system is apparently not linked to btaR₂ control but might rather go through regulation of btaI₂ transcription. Moreover, neither RsaM₁ nor RsaM₂ modulate the transcription of btaR₃ (data not shown).

Collectively, these observations indicate that both RsaM1 and RsaM2 do not regulate 3OHC₈-
HSL biosynthesis through the QS-3 system genes \textit{btaR}3 and \textit{btaI}3, which encodes the BtaI3 synthase mainly responsible for 3OHC₈-HSL production.

**The \textit{rsaM1} and \textit{rsaM2} genes are QS-controlled**

Our transcriptomic sequencing analyses indicate that QS positively regulates the expression of \textit{rsaM1} and \textit{rsaM2} (S. Le Guillouzer, M. C. Groleau, F. Mauffrey, R. Villemur, and E. Déziel, unpublished data). Majerczyk \textit{et al.} (15) also reported that the \textit{rsaM2} gene is QS-controlled, but not \textit{rsaM1}.

In order to ascertain that \textit{rsaM1} is under QS control, we monitored \textit{rsaM1} expression by qRT-PCR in the \textit{B. thailandensis} E264 wild-type strain and in the AHL-null \textit{ΔbtaI}1\textit{ΔbtaI}2\textit{ΔbtaI}3 mutant supplemented or not with exogenous AHLs during the exponential phase. We observed that expression of \textit{rsaM1} is reduced in the absence of AHLs (Fig. 6A), confirming that QS positively modulates \textit{rsaM1} transcription. Furthermore, expression of \textit{rsaM1} was restored to wild-type levels in the culture of the AHL-null mutant strain supplemented with either \textit{C₈}-HSL or 3OHC₈-HSL (Fig. 6A). Moreover, adding 3OHC₁₀-HSL to the culture of the \textit{ΔbtaI}1\textit{ΔbtaI}2\textit{ΔbtaI}3 mutant background did not significantly enhance \textit{rsaM1} transcription (Fig. 6A). To gain insights into the QS-dependent regulation of \textit{rsaM1}, we also measured expression of \textit{rsaM1} in the \textit{ΔbtaR}1, \textit{ΔbtaR}2, and \textit{ΔbtaR}3 mutants vs. the \textit{B. thailandensis} E264 wild-type strain during the exponential phase. While no obvious change in \textit{rsaM1} transcription was visible in the absence of neither BtaR2, nor BtaR3 under our conditions, expression of \textit{rsaM1} was decreased in the \textit{ΔbtaR}1 mutant when compared to the wild-type strain (Fig. 6B). Taken together, these data indicate that expression of \textit{rsaM1} is positively regulated by the QS-1 system and might be activated by BtaR1 in association with \textit{C₈}-HSL or 3OHC₈-HSL, whereas BtaR2 and BtaR3 are not directly involved in the modulation of \textit{rsaM2} transcription.

Expression of \textit{rsaM2} was lowered in the absence of AHLs, confirming that the \textit{rsaM2} gene is activated by QS (Fig. 7A). Furthermore, \textit{rsaM2} transcription was restored to wild-type levels in cultures of the \textit{ΔbtaI}1\textit{ΔbtaI}2\textit{ΔbtaI}3 mutant strain supplemented with either 3OHC₁₀-HSL or 3OHC₈-HSL (Fig. 7A). Moreover, adding \textit{C₈}-HSL did not significantly increase \textit{rsaM2} transcription, revealing that the \textit{rsaM2} gene is not activated by \textit{C₈}-HSL (Fig. 7A). Interestingly, we observed that expression of \textit{rsaM2} was also downregulated in the \textit{ΔbtaR}2 mutant strain.
mutant in comparison with the wild-type strain, meaning that the *rsaM*2 gene is positively controlled by BtaR2, whereas no discernible difference in *rsaM*2 transcription was detected in the absence of neither BtaR1, nor BtaR3 under the conditions of our experiments (Fig. 7B). Altogether, these results indicate that expression of *rsaM*2 is positively regulated by the QS-2 system and might be activated by BtaR2 in response to 3OHC<sub>10</sub>-HSL or 3OHC<sub>8</sub>-HSL, whereas BtaR1 and BtaR3 do not intervene in *rsaM*2 regulation.

Collectively, these observations highlight that expression of *rsaM*1 is activated by the QS-1 system, which is negatively controlled by RsaM1, whereas *rsaM*2 transcription is positively regulated by the QS-2 system, which is repressed by RsaM2, showing that these RsaM-like proteins are integrated into the QS modulatory web of *B. thailandensis* E264.

**Expression of RsaM1 and RsaM2 are negatively auto-regulated**

To further explore the RsaM1 and RsaM2 molecular mechanisms of action, *rsaM*1 and *rsaM*2 expressions were assessed by qRT-PCR in the wild-type strain of *B. thailandensis* E264 and in the *rsaM*1- and *rsaM*2- mutants during the exponential phase. Expression of *rsaM*1 was increased in the *rsaM*1- mutant when compared to the wild-type strain, and the same was observed for *rsaM*2 expression in the *rsaM*2- mutant (Figs. S3A and S3B). However, the absence of RsaM2 had no impact on *rsaM*1 transcription (Fig. S3A) and *rsaM*2 transcription was unchanged in the *rsaM*1- mutant in comparison with the wild-type strain (Fig. S3B). Altogether, these results indicate that RsaM1 and RsaM2 repress their own expression, but are not transcriptionally hierarchically organized.

**Discussion**

*B. thailandensis* E264 can synthesize C<sub>8</sub>-HSL, 3OHC<sub>10</sub>-HSL, and 3OHC<sub>8</sub>-HSL (13-16), with 3OHC<sub>10</sub>-HSL being the most abundant AHL detected during the different stages of growth. This reveals that the production of C<sub>8</sub>-HSL and 3OHC<sub>8</sub>-HSL might be under stringent control. These signaling molecules mediate the activity of three BtaR/BtaI QS systems. Here, we initiated the study of two uncharacterized genes present in the QS-1 and QS-2 gene clusters. RsaM1 and RsaM2 were shown to dramatically restrict the production of AHLs, highlighting...
their deep involvement in the complex organization of the multiple AHL-based QS circuits of 
*B. thailandensis* E264, as summarized in *Fig. 8*.

A gene conserved in the *Burkholderia* genus within the QS-1 system (7, 10, 11, 21-23) is 
divergently transcribed from *btaR1* and oriented in the same direction as *btaI1* (*Fig. 8* and 
*Fig. S1B*). This gene encodes an hypothetical protein, homologous to the negative AHL 
biosynthesis modulatory protein RsaM originally identified in the plant pathogen *P. fuscovaginae* UBP0736 (8, 9), hence designated RsaM1. In *B. cenocepacia* H111, the 
homologue *BcRsaM* was described as an important repressor of the CepI/CepR QS system, 
and proposed to inhibit the production of C8-HSL by regulating 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 (10, 11). While the transcription of the QS 
*cepI*, *cepR*, and *cepR2* genes were shown to be lowered in the *rsaM-* mutant of *B. 
cenocepacia* H111 (10), *btaI1* and *btaR1* expressions were both increased in our RsaM1 of *B. 
thailandensis* E264, correlating with the accumulation of C8-HSL observed in this 
background. Consequently, RsaM1 could repress the transcription of *btaI1* and *btaR1*, 
suggesting that its mode of action in *B. thailandensis* differs from that of *BcRsaM*. Still, the 
impact of RsaM1 on C8-HSL biosynthesis was dramatically higher than its effect on *btaI1* 
expression (*Figs. 2A and 2C*), which hints that RsaM1 might also act at post-transcriptional 
and/or post-translational levels, as proposed for *BcRsaM*. Thus, RsaM1 could repress as well 
the expression of *btaI1* and *btaR1* directly or indirectly, for instance by modulating the 
synthase activity and/or stability of BtaI1 (14), or by controlling the functionality of BtaR1. 
Clearly, while BtaR1 is considered the principal regulator of *btaI1* expression, RsaM1 plays a 
major role in modulating the production of C8-HSL.

We recently determined that expression of *btaI1* is positively controlled by BtaR1, as well as 
activated by the presence of C8-HSL. The accumulation of this AHL detected in the Δ*btaR1* 
mutant background is not directly induced by the impact of BtaR1 on *btaI1* expression, but 
could involve additional transcriptional and/or post-transcriptional regulators under its control 
(13). Interestingly, we observed that *rsaM1* expression is positively controlled by BtaR1/C8- 
HSL (*Fig. 6*), suggesting that the overproduction of C8-HSL detected in the Δ*btaR1* mutant 
strain is indirectly induced by BtaR1 through RsaM1. This also reveals that the QS-1 system 
is negatively auto-regulated, maybe counteracting the positive feedback loop mediated by 
BtaR1/C8-HSL for C8-HSL biosynthesis, and thus be necessary to maintain the production of 
this AHL within optimal and adequate levels according to specific environmental conditions,
as previously suggested for the RsaL repressor in *P. aeruginosa* PAO1 (23, 25). In agreement with the finding that *rsaM* is positively and directly regulated by CepR in *B. cenocepacia* J2315 (10, 26), we found a putative *lux*-box sequence in the promoter region of the *rsaM* gene ([Fig. S1C](#)), suggesting that *rsaM* could also be directly under BtaR1 control in *B. thailandensis* E264. Nevertheless, *rsaM* transcription was not completely abolished in the absence of BtaR1, indicating that the QS-dependent regulation of the *rsaM* gene is more complex and requires further investigation.

Strikingly, the absence of RsaM1 was associated with a growth delay of the mutant ([Fig. 1](#)), correlating with aggregation of cells. This could be linked to the high levels of C8-HSL produced in this background and thus over-activation of phenotypes controlled by the QS-1 system such as auto-aggregation and oxalate production (14, 15, 27, 28). Thus, RsaM1 could be necessary for the regulation of phenotypic traits under BtaR1/C8-HSL control, requiring substantial concentrations of C8-HSL under specific environmental conditions, as suggested for the RsaL repressor in *B. kururiensis* M130 (29).

Interestingly, RsaM1 also represses the production of 3OHC8-HSL ([Fig. 4A](#)), an AHL synthesized by both BtaI2 and BtaI3 (14, 16). Because no overproduction of 3OHC10-HSL, the main AHL produced by BtaI2 (16), was observed in the *rsaM*1-mutant background ([Fig. 3A](#)), we assume that the QS-2 system is not influenced by RsaM1. Thus, while RsaM1 seems to act on 3OHC8-HSL by modulating the QS-3 system, although no impact on expression of *btaI3* and *btaR3* was observed, we propose that its negative impact is going through an effect on the activity and/or stability of the BtaI3 synthase. This would add another regulatory layer linking the QS-1 and QS-3 systems in *B. thailandensis* E264, already shown to be hierarchically organized at the transcriptional level (13, 15). Additionally, it is conceivable that concentrations of 3OHC8-HSL produced by BtaI1 in the wild-type strain are below our detection limit and that these levels become detectable in the QS-1-boosted *rsaM*1-mutant background. Indeed, we previously reported production of both C8-HSL and traces amounts of 3OHC8-HSL by the same synthase in a *Burkholderia* strain from the *cepacia* complex (30), and the *B. pseudomallei* KHW BpsI and *B. mallei* ATCC 23344 BmaI1 synthases were both shown to produce low levels of 3OHC8-HSL in addition to C8-HSL, (31, 32). Additionally, while we demonstrated that *rsaM*1 expression is controlled by BtaR1 in response to C8-HSL, activation of this gene by 3OHC8-HSL might also involve BtaR1 ([Fig. 6](#)). This is further supported by the fact that the *B. pseudomallei* KHW BpsR and *B. mallei* ATCC 23344 BmaR1 transcriptional regulators were reported to specifically respond to both C8-HSL and
3OHC₈-HSL, with C₈-HSL eliciting the strongest response (31, 32). This is in agreement with
the finding that the BtaR1-controlled genes identified in transcriptomic analyses were
generally affected by both C₈-HSL and 3OHC₈-HSL (15). This would also explain why
3OHC₈-HSL levels increased in the absence of BtaR1, following the same kinetic as C₈-HSL
(13), indicating that RsaM1 could play a part in the accumulation of this AHL detected in the
ΔbtaR1 mutant background as proposed for C₈-HSL. Nevertheless, additional experiments
will be necessary to confirm the production of 3OHC₈-HSL by BtaI1.

Importantly, the fact that the production of both C₈-HSL and 3OHC₈-HSL, but not 3OHC₁₀-
HSL, is stringently repressed by RsaM1, is consistent with the recent finding that C₈-HSL and
3OHC₈-HSL are synthesized at low levels throughout the different stages of growth (13), and
could reasonably justify the predominance of 3OHC₁₀-HSL in B. thailandensis E264.

Interestingly, an rsaM homologue encoding a hypothetical protein designated RsaM2 is also
found directly adjacent to btai2 and transcribed in the same direction (Fig. 8 and Fig. S1B).
We recently corroborated that btai2 transcription is principally activated by BtaR2 in
association with 3OHC₁₀-HSL (13). While BtaI2 is mainly responsible for the production of
this AHL (16), and we observed that 3OHC₁₀-HSL biosynthesis is almost completely
abolished in the Δbtai2 mutant (Fig. S4B), we demonstrated that the production of 3OHC₁₀-
HSL is positively regulated by BtaR2 (13). Furthermore, our results show that RsaM2
represses the biosynthesis of 3OHC₁₀-HSL, as well as the expression of btai2, indicating that
RsaM2-dependent regulation of the QS-2 system is apparently not linked to btaiR2 control but
might rather go through regulation of btai2 transcription. Since 3OHC₁₀-HSL biosynthesis
and expression of btai2 were similarly affected by RsaM2 (Fig. 3), the activity and/or
stability of BtaI2 might not be altered by RsaM2. However, we do not exclude that RsaM2
represses 3OHC₁₀-HSL biosynthesis by controlling the functionality of BtaR2. Moreover, we
observed that expression of rsaM2 is activated by the QS-2 system (Fig. 7). Consequently,
while expression of btai2 is directly activated by BtaR2, we demonstrated that BtaR2 also
represses btai2 transcription indirectly through RsaM2 control. This homeostatic control of
the QS-2 system appears similar to what proposed for the RsaL repressor in P. aeruginosa
PAO1 (23, 25).

We also highlighted that the production of 3OHC₈-HSL is negatively regulated by RsaM2
(Fig. 4B), and because we determined that expressions of neither btai3, nor btaiR3 are under
RsaM2 control, we must conclude that RsaM2 does not influence this AHL by modulating the
QS-3 system. Interestingly, the production of 3OHC$_8$-HSL is repressed by RsaM1 as well (Fig. 4A). This could also explain why inactivating either rsaM1 or rsaM2 does not result in btaI3 activation; investigating the expression of this gene in a double rsaM1-rsaM2- mutant would be necessary to determine the precise regulatory mechanism directing 3OHC$_8$-HSL biosynthesis through these RsaM-like proteins. Nevertheless, since BtaI2 produces this AHL in addition to 3OHC$_{10}$-HSL, albeit to a lesser extent (16), we suppose that the negative impact of RsaM2 on the production of 3OHC$_8$-HSL, as the RsaM2-dependent regulation of 3OHC$_{10}$-HSL biosynthesis, is linked to btaI2 regulation. Remarkably, we noticed that the production of 3OHC$_{10}$-HSL is repressed by RsaM2 from the exponential phase, whereas 3OHC$_8$-HSL biosynthesis is repressed by RsaM2 from the stationary phase. This is consistent with our hypothesis that 3OHC$_8$-HSL is produced by BtaI2 at the expense of 3OHC$_{10}$-HSL (13), meaning that BtaI2 mainly synthesizes 3OHC$_{10}$-HSL during the logarithmic growth, whereas it principally synthesizes 3OHC$_8$-HSL during the stationary phase, further confirmed by 3OHC$_8$-HSL being produced in the stationary phase, but almost not in the logarithmic growth when BtaI3 expression is very low (Fig. S4C).

We confirmed that the production of C$_8$-HSL is completely abolished in the absence of BtaI1, indicating that this AHL is exclusively produced by this synthase (Fig. S4A); indeed, no other synthase than the homologs BpsI and BmaI1 have been associated with the production of this AHL in B. pseudomallei KHW and B. mallei ATCC 23344, respectively (31, 32). Consequently, 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 (Figs. 2B and 2C). The QS-1 and QS-2 systems were recently found to be sequentially organized (13), and we indeed confirmed that BtaR2 negatively modulates the production of C$_8$-HSL by repressing the expression of btaI1 (Figs. S5 and S6). We propose that the negative impact of RsaM2 on the production of C$_8$-HSL might involve post-transcriptional regulation, underscoring an additional regulatory layer connecting the QS-1 and QS-2 systems in B. thailandensis E264.

We also demonstrated that RsaM1 and RsaM2 repress their own transcription (Fig. S3). Negative auto-regulation of these RsaM-like proteins could be necessary to maintain AHLs at appropriate levels depending on particular environmental conditions, and might further contribute to the correct timing of the QS-1, QS-2, and QS-3 systems response. Since RsaM1 and RsaM2 had no impact on rsaM2 and rsaM1 expression, respectively, we hypothesize that these RsaM homologues act on the biosynthesis of common AHLs independently of each other, thus ensuring the specificity of the regulation of the QS-1, QS-2, and QS-3 systems.
Figure 8 shows the proposed interactions between the QS-1, QS-2, and QS-3 systems and the RsaM homologues RsaM1 and RsaM2. We previously showed that the first system activated in B. thailandensis E264 is QS-2 with 3OHC10-HSL production starting earlier and at higher levels than the other AHLs (13). BtaR2 activates rsaM2 expression. RsaM2 has a negative impact on 3OHC10-HSL. We also proposed that the QS-2 system has a negative impact on the QS-1 system (13) and we believe this effect goes through RsaM1. However, there is no effect on rsaM1 expression in a ΔbtaR2 mutant, which adds to our previous hypothesis that BtaR2 acts on the transcription of btaI. Our qRT-PCR experiments indicate that BtaR1 activates expression of rsaM1, which controls negatively the biosynthesis of C8-HSL. Since our previous results show that a ΔbtaR1 mutant produces higher levels of C8-HSL than the wild-type with no matching overexpression of btaI1, it is possible in the inactivation of btaR1 affects rsaM1 expression and thus BtaI1 activity. The effect of the QS-1 system on the QS-3 system was also formerly detailed (13).

Conclusion

We reported that the QS-1, QS-2, and QS-3 systems are hierarchically and homeostatically arranged in B. thailandensis E264 and we also observed that these QS systems are integrated into an intricate network, including additional unspecified transcriptional and/or post-transcriptional regulators (13). The present study uncovers the crucial role of the two newly identified RsaM homologues designated RsaM1 and RsaM2 in the modulation of AHL signaling (Fig. 8). We demonstrated that the QS-1 system is mainly repressed by RsaM1, whereas RsaM2 principally represses the QS-2 system. Additionally, these AHL biosynthesis regulatory proteins were shown to be an integral part of the QS modulatory circuitry, contributing to the temporal expression of the multiple AHL-based QS circuits of B. thailandensis E264. The precise underlying molecular mechanism of action of RsaM-like proteins remains 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 (7-11, 21-23).

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References


Figure legends

Figure 1. The biosynthesis of AHLs is increased in the rsaM1 and rsaM2 knockout mutants. Total AHLs (3OHC\textsubscript{10}-HSL + C\textsubscript{8}-HSL + 3OHC\textsubscript{8}-HSL) production (bars) was monitored by LC-MS/MS at various times during growth (lines) in cultures of the \textit{B. thailandensis} E264 wild-type strain and its rsaM1- and rsaM2- mutants. The error bars represent the standard deviation of the average for three replicates.

Figure 2. C\textsubscript{8}-HSL biosynthesis and expression from the btaI\textsubscript{1} promoter in the wild-type and the rsaM1- and rsaM2- mutant strains of \textit{B. thailandensis} E264. The production of C\textsubscript{8}-HSL was quantified using LC-MS/MS at various times during growth in cultures of the wild-type and of (A) the rsaM1- and (B) rsaM2- mutant strains of \textit{B. thailandensis} E264. The error bars represent the standard deviation of the average for three replicates. (C) The luminescence of the chromosomal \textit{btaI}\textsubscript{1}-lux transcriptional fusion was monitored in cultures of the \textit{B. thailandensis} E264 wild-type strain and of the rsaM1- and rsaM2- mutants. The luminescence is expressed in relative light units per culture optical density (RLU/OD\textsubscript{600}).

Figure 3. 3OHC\textsubscript{10}-HSL biosynthesis and expression from the btaI\textsubscript{2} promoter in the wild-type and the rsaM1- and rsaM2- mutant strains of \textit{B. thailandensis} E264. (A) The production of 3OHC\textsubscript{10}-HSL was quantified using LC-MS/MS at various times during growth in cultures of the wild-type and of the rsaM1- and rsaM2- mutant strains of \textit{B. thailandensis} E264. The error bars represent the standard deviation of the average for three replicates. (B) The luminescence of the chromosomal \textit{btaI}\textsubscript{2}-lux transcriptional fusion was monitored in cultures of the \textit{B. thailandensis} E264 wild-type strain and of the rsaM1- and rsaM2- mutants. The luminescence is expressed in relative light units per culture optical density (RLU/OD\textsubscript{600}).

Figure 4. 3OHC\textsubscript{8}-HSL biosynthesis and expression from the btaI\textsubscript{3} promoter in the wild-type and the rsaM1- and rsaM2- mutant strains of \textit{B. thailandensis} E264. The production of 3OHC\textsubscript{8}-HSL was quantified using LC-MS/MS at various times during growth in cultures of the wild-type and of (A) the rsaM1- and (B) rsaM2- mutant strains of \textit{B. thailandensis} E264. The error bars represent the standard deviation of the average for three replicates. (C) The luminescence of the chromosomal \textit{btaI}\textsubscript{3}-lux transcriptional fusion was monitored in cultures of the \textit{B. thailandensis} E264 wild-type strain and of the rsaM1- and rsaM2- mutants. The luminescence is expressed in relative light units per culture optical density (RLU/OD\textsubscript{600}).
Figure 5. Expressions of btaR1 and btaI1 are both repressed by RsaM1. The relative transcript levels of (A) btaR1 and (B) btaI1 were assessed by qRT-PCR experiments in cultures of the wild-type and of the rsaM1- and rsaM2- mutant strains of B. thailandensis E264. The results are presented as relative quantification of genes transcription compared to the wild-type normalized to 100%. The error bars represent the standard deviation of the average for three replicates. **, p < 0.01; ns, non-significant.

Figure 6. QS positively regulates rsaM1 transcription. (A) The relative transcript levels of rsaM1 from the B. thailandensis E264 wild-type and its ΔbtaI1ΔbtaI2ΔbtaI3 mutant strain were estimated by qRT-PCR experiments. Cultures were supplemented with 10 µM C8-HSL, 3OHC10-HSL, or 3OHC8-HSL. Acetonitrile only was added in controls. The results are presented as relative quantification of genes transcription compared to the wild-type normalized to 100%. The error bars represent the standard deviation of the average for three replicates. (B) The relative transcript levels of rsaM1 were assessed by qRT-PCR experiments in cultures of the wild-type and of the ΔbtaR1, ΔbtaR2, and ΔbtaR3 mutant strains of B. thailandensis E264. **, p < 0.01; *, p < 0.05; ns, non-significant.

Figure 7. Expression of rsaM2 is activated by QS. (A) The relative transcript levels of rsaM2 from the B. thailandensis E264 wild-type and its ΔbtaI1ΔbtaI2ΔbtaI3 mutant strain were monitored by qRT-PCR experiments. Cultures were supplemented with 10 µM C8-HSL, 3OHC10-HSL, or 3OHC8-HSL. Acetonitrile only was added in controls. The results are presented as relative quantification of genes transcription compared to the wild-type normalized to 100%. The error bars represent the standard deviation of the average for three replicates. (B) The relative transcript levels of rsaM2 were quantified by qRT-PCR experiments in cultures of the wild-type and of the ΔbtaR1, ΔbtaR2, and ΔbtaR3 mutant strains of B. thailandensis E264. **, p < 0.01; *, p < 0.05; ns, non-significant.

Figure 8. Proposed model of the QS regulatory network of B. thailandensis E264.
Legends for supplemental material

Figure S1. **B. thailandensis** possesses two conserved RsaM-like proteins designated RsaM1 and RsaM2. (A) Sequence alignment of the RsaM1 and RsaM2 proteins of **B. thailandensis** E264 with the **P. fuscovaginae** UPB0736 RsaM homologue. (B) Genetic arrangement of rsaM1 and rsaM2 with btaI1 and btaI2, respectively. (C) A putative lux-box is present in the promoter region of the rsaM1 gene, which is homologous to characterized lux-box sequences in Proteobacteria.

Figure S2. **AHLs production profiles in the wild-type and the rsaM1- and rsaM2- mutant strains of B. thailandensis** E264. The biosynthesis of AHLs (bars) was monitored by LC-MS/MS at various times during growth (lines) in cultures of the (A) wild-type and the (B) rsaM1- and (C) rsaM2- mutant strains of **B. thailandensis** E264. The error bars represent the standard deviation of the average for three replicates.

Figure S3. **Expression of rsaM1 and rsaM2 is negatively auto-regulated.** The relative transcript levels of (A) rsaM1 and (B) rsaM2 from the **B. thailandensis** E264 wild-type and its rsaM1- and rsaM2- mutant strains were estimated by qRT-PCR experiments. The results are presented as relative quantification of genes transcription compared to the wild-type normalized to 100%. The error bars represent the standard deviation of the average for three replicates. ***, p < 0.001; ns, non-significant.

Figure S4. **AHL biosynthesis in the wild-type and the ΔbtaI1, ΔbtaI2, and ΔbtaI3 mutant strains of B. thailandensis** E264. The production of (A) C8-HSL, (B) 3OHC10-HSL, and (C) 3OHC8-HSL was quantified using LC-MS/MS during the exponential and stationary phases in cultures of the wild-type and of the ΔbtaI1, ΔbtaI2, and ΔbtaI3 mutant strains of **B. thailandensis** E264, respectively. The error bars represent the standard deviation of the average for three replicates.

Figure S5. **Complementation of C8-HSL production by BtaR2.** The biosynthesis of C8-HSL was quantified using LC-MS/MS during the logarithmic growth in cultures of the wild-type and of the ΔbtaR2 mutant strains of **B. thailandensis** E264. The error bars represent the standard deviation of the average for three replicates.

Figure S6. **Expression of btaI1 is positively modulated by BtaR2.** The relative transcript levels of (A) btaI1 and (B) btaR1 from the **B. thailandensis** E264 wild-type and its ΔbtaR2 mutant strains were estimated by qRT-PCR experiments during the logarithmic growth.
results are presented as relative quantification of genes transcription compared to the wild-type normalized to 100%. The error bars represent the standard deviation of the average for three replicates.
Table S1. Bacterial strains used in this study.

Table S2. Plasmids used in this study.

Table S3. Primers used for PCR.

Table S4. Primers used for qRT-PCR.
### Table S1. Bacterial strains used in this study.

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Table S2. Plasmids used in this study.

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Table S3. Primers used for PCR.

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### Table S4. Primers used for qRT-PCR.

<table>
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Fig. 1. The biosynthesis of AHLs is increased in the rsaM1 and rsaM2 knockout mutants. Total AHLs (3OHC_{10}-HSL + C_{8}-HSL + 3OHC_{8}-HSL) production (bars) was monitored by LC-MS/MS at various times during growth (lines) in cultures of the B. thailandensis E264 wild-type strain and its rsaM1- and rsaM2-mutants. The error bars represent the standard deviation of the average for three replicates.
Fig. 2. C₈-HSL biosynthesis and expression from the btaI promoter in the wild-type and the rsaM₁- and rsaM₂- mutant strains of B. thailandensis E264. The production of C₈-HSL was quantified using LC-MS/MS at various times during growth in cultures of the wild-type and of (A) the rsaM₁- and (B) rsaM₂- mutant strains of B. thailandensis E264. The error bars represent the standard deviation of the average for three replicates. (C) The luminescence of the chromosomal btaI-lux transcriptional fusion was monitored in cultures of the B. thailandensis E264 wild-type strain and of the rsaM₁- and rsaM₂- mutants. The luminescence is expressed in relative light units per culture optical density (RLU/OD₆₀₀).
Fig. 3. 3OHC₁₀-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 3OHC₁₀-HSL was quantified using LC-MS/MS at various times during growth in cultures of the wild-type and of the rsaM₁- and rsaM₂- mutant strains of B. thailandensis E264. The error bars represent the standard deviation of the average 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 of the rsaM₁- and rsaM₂-mutants. The luminescence is expressed in relative light units per culture optical density (RLU/OD₆₀₀).
Fig. 4. 3OH C<sub>8</sub>-HSL biosynthesis and expression from the btaI<sub>3</sub> promoter in the wild-type and the rsaM<sub>1</sub>- and rsaM<sub>2</sub>- mutant strains of B. thailandensis E264. The production of 3OH C<sub>8</sub>-HSL was quantified using LC-MS/MS at various times during growth in cultures of the wild-type and of (A) the rsaM<sub>1</sub>- and (B) rsaM<sub>2</sub>- mutant strains of B. thailandensis E264. The error bars represent the standard deviation of the average for three replicates. (C) The luminescence of the chromosomal btaI<sub>3</sub>-lux transcriptional fusion was monitored in cultures of the B. thailandensis E264 wild-type strain and of the rsaM<sub>1</sub>- and rsaM<sub>2</sub>- mutants. The luminescence is expressed in relative light units per culture optical density (RLU/OD<sub>600</sub>).
Fig. 5. Expressions of \textit{btaR}1 and \textit{btaI}1 are both repressed by RsaM1. The relative transcript levels of (A) \textit{btaR}1 and (B) \textit{btaI}1 were assessed by qRT-PCR experiments in cultures of the wild-type and of the \textit{rsaM}1- and \textit{rsaM}2- mutant strains of \textit{B. thailandensis} E264. The results are presented as relative quantification of genes transcription compared to the wild-type normalized to 100%. The error bars represent the standard deviation of the average for three replicates. **, p < 0.01; ns, non-significant.
Fig. 6. QS positively regulates $rsaM_1$ transcription. (A) The relative transcript levels of $rsaM_1$ from the $B. thailandensis$ E264 wild-type and its $\Delta btaI_1\Delta btaI_2\Delta btaI_3$ mutant strain were estimated by qRT-PCR experiments. Cultures were supplemented with 10 µM C8-HSL, 3OHC$_{10}$-HSL, or 3OHC$_8$-HSL. Acetonitrile only was added in controls. The results are presented as relative quantification of genes transcription compared to the wild-type normalized to 100%. The error bars represent the standard deviation of the average for three replicates. (B) The relative transcript levels of $rsaM_1$ were assessed by qRT-PCR experiments in cultures of the wild-type and of the $\Delta btaR_1$, $\Delta btaR_2$, and $\Delta btaR_3$ mutant strains of $B. thailandensis$ E264. **, $p < 0.01$; *, $p < 0.05$; ns, non-significant.
Fig. 7. Expression of rsaM2 is activated by QS. (A) The relative transcript levels of rsaM2 from the B. thailandensis E264 wild-type and its ΔbtaI1ΔbtaI2ΔbtaI3 mutant strain were monitored by qRT-PCR experiments. Cultures were supplemented with 10 µM C8-HSL, 3OHC10-HSL, or 3OHC8-HSL. Acetonitrile only was added in controls. The results are presented as relative quantification of genes transcription compared to the wild-type normalized to 100%. The error bars represent the standard deviation of the average for three replicates. (B) The relative transcript levels of rsaM2 were quantified by qRT-PCR experiments in cultures of the wild-type and of the ΔbtaR1, ΔbtaR2, and ΔbtaR3 mutant strains of B. thailandensis E264. **, p < 0.01; *, p < 0.05; ns, non-significant.
Fig. 8. Schematic model of the QS regulatory network of B. thailandensis E264.
Fig. S1. *B. thailandensis* possesses two conserved RsaM-like proteins designated RsaM1 and RsaM2. (A) Sequence alignment of the RsaM1 and RsaM2 proteins of *B. thailandensis* E264 with the *P. fuscovaginae* UPB0736 RsaM homologue. (B) Genetic arrangement of the RsaM1- and RsaM2-encoding genes with *btaI*1 and *btaI*2, respectively. (C) The rsaM1 gene encoding RsaM1 possesses in its promoter region a putative lux-box, which is homologous to characterized lux-box sequences in Proteobacteria.
Fig. S2. AHLs production profiles in the wild-type and the rsaM1- and rsaM2- mutant strains of B. thailandensis E264. The biosynthesis of AHLs (bars) was monitored by LC-MS/MS at various times during growth (lines) in cultures of the (A) wild-type and the (B) rsaM1- and (C) rsaM2- mutant strains of B. thailandensis E264. The error bars represent the standard deviation of the average for three replicates.
Fig. S3. Expressions of rsaM1 and rsaM2 are negatively auto-regulated. The relative transcript levels of (A) rsaM1 and (B) rsaM2 from the B. thailandensis E264 wild-type and its rsaM1- and rsaM2- mutant strains were estimated by qRT-PCR experiments. The results are presented as relative quantification of genes transcription compared to the wild-type normalized to 100%. The error bars represent the standard deviation of the average for three replicates. ***, p < 0.001; ns, non-significant.
Fig. S4. AHLs biosynthesis in the wild-type and the ΔbtaI1, ΔbtaI2, and ΔbtaI3 mutant strains of B. thailandensis E264. The production of (A) C8-HSL, (B) 3OHC10-HSL, and (C) 3OHC8-HSL was quantified using LC-MS/MS during the exponential and stationary phases in cultures of the wild-type and of the ΔbtaI1, ΔbtaI2, and ΔbtaI3 mutant strains of B. thailandensis E264, respectively. The error bars represent the standard deviation of the average for three replicates.
The biosynthesis of C₈-HSL was quantified using LC-MS/MS during the logarithmic growth in cultures of the wild-type and of the ΔbtaR₂ mutant strains of B. thailandensis E264. The error bars represent the standard deviation of the average for three replicates.

**Fig. S5. Complementation of C₈-HSL production by BtaR2.**

The biosynthesis of C₈-HSL was quantified using LC-MS/MS during the logarithmic growth in cultures of the wild-type and of the ΔbtaR₂ mutant strains of B. thailandensis E264. The error bars represent the standard deviation of the average for three replicates.
Fig. S6. Expression of *btaI*1 is positively modulated by BtaR2. The relative transcript levels of (A) *btaI*1 and (B) *btaR*1 from the *B. thailandensis* E264 wild-type and its ΔbtaR2 mutant strains were estimated by qRT-PCR experiments during the logarithmic growth. The results are presented as relative quantification of genes transcription compared to the wild-type normalized to 100%. The error bars represent the standard deviation of the average for three replicates.