Quorum sensing and RsaM regulons of the rice pathogen *Pseudomonas fuscovaginae*

Gordana Uzelac, † Hitendra Kumar Patel, † Giulia Devescovi, † Danilo Licastro and Vittorio Venturi

**Abstract**

*Pseudomonas fuscovaginae* (Pfv) is an emerging plant pathogen causing sheath brown rot in rice, as well as diseases in other gramineae food crops including maize, sorghum and wheat. *Pfv* possesses two conserved *N*-acyl homoserine lactone (AHL) quorum sensing (QS) systems called PfvI/R and PfsI/R, which are repressed by RsaL and RsaM, respectively. The two systems are not hierarchically organized and are involved in plant virulence. In this study the AHL QS PfsI/R, PfvI/R and RsaM regulons were determined by transcriptomic analysis. The PfsI/R system regulates 98 genes, whereas 26 genes are regulated by the PfvI/R AHL QS system; only two genes are regulated by both systems. RsaM, on the other hand, regulates over 400 genes: 206 are negatively regulated and 260 are positively regulated. More than half of the genes controlled by the PfsI/R system and 65% by the PfvI/R system are also part of the RsaM regulon; this is due to RsaM being involved in the regulation of both systems. It is concluded that the two QS systems regulate a unique set of genes and that RsaM is a global regulator mediating the expression of different genes through the two QS systems as well as genes independently of QS.

**INTRODUCTION**

*Pseudomonas fuscovaginae* (Pfv) is a Gram-negative fluorescent pseudomonad belonging to the subclass *Gammaproteobacteria* that currently comprises 144 species [1]. *Pfv* is one of the 18 described plant pathogenic *Pseudomonas* species [2] causing sheath brown rot in rice (*Oryza sativa*), as well as being pathogenic to other gramineae food crops including maize (*Zea mays*) [3], sorghum (*Sorghum bicolor*) and wheat (*Triticum aestivum*) [4]. Recently, *Pfv* has also been reported as being able to colonize rice plants as an endophyte [5]. When rice infection by *Pfv* occurs at a later stage, the lower part of the sheath becomes brown and, later on, the whole sheath becomes necrotic [6]. Biochemical studies have shown that *Pfv* produces different types of phytotoxins, namely syringotoxin, fuscopeptin A (FP-A) and fuscopeptin B (FP-B) [7, 8], which are believed to generate the disease symptoms. It was previously established that quorum sensing (QS) gene regulation and phytotoxin production, type IV pili biosynthesis, type VI secretion system, arginine biosynthesis and sulfur metabolism are involved in *Pfv* pathogenicity on rice [9, 10]. QS has been shown to synchronize virulence-associated gene expression in many plant pathogenic bacteria [11, 12].

QS is a bacterial cell–cell communication process that involves the production, detection and response to extracellular signalling molecules also called autoinducers [13, 14]. At low cell density, QS signals are present at a concentration below the threshold, whereas at high cell density the cumulative production of signals leads to a local high signal concentration, allowing detection and response [15]. Gram-negative bacteria most commonly communicate using *N*-acyl homoserine lactones (AHLs) as signals and the LuxI-family proteins are the major class of enzymes responsible for AHL synthesis. At a critical cell density, AHLs reach quorum concentrations and interact directly with LuxR-type proteins, and the resulting LuxR/AHL complex then binds specific promoter sequences, called lux boxes, thereby affecting the expression of target genes [16]. QS regulates many community phenotypes in bacteria such as biofilm formation, swimming and swarming, conjugation, bioluminescence, production of extracellular enzymes, virulence factor secretion, and pigment production [12, 17–20].

We previously reported that 11 *Pfv* strains isolated from diseased rice grown in different parts of the world possess two conserved AHL QS systems involved in virulence. These two AHL QS systems, namely PfsI/R and PfvI/R, are not
hierarchically organized and are both negatively regulated by repressor proteins encoded by genes located intergenically between the AHL synthase encoding genes and the luxR response regulator (Fig. 1). The PfsI/R system is regulated by repressor RsaM, while the PfvI/R system is regulated by RsaL repressor and also weakly by RsaM [9]. The RsaL repressor regulates the PfvI/R system via the transcriptional regulation of pfvI. RsaM is a novel regulator (protein of 167 aa) displaying homology to other putative QS regulators of *Burkholderia* sp., which are also intergenically located between the AHL QS systems genes; the mode of action of RsaM is currently unknown [21]. Both QS systems have been shown to be involved in plant pathogenicity in rice [9].

In this study, the AHL QS PfsI/R, the PfvI/R and the RsaM regulons were established by transcriptomic analysis. Interestingly, RsaM regulates a large set of genes; 206 genes are downregulated and 260 genes are upregulated. Differently from RsaM, the PfsI/R system modulates 98 genes whereas 26 genes are controlled by the PfvI/R AHL QS system. The majority of genes positively regulated by RsaM are involved in carbon transport and metabolism, lipid transport and metabolism, amino acid transport, transcription and cell motility. Likewise, the PfvI/R system modulates the expression of several genes involved in transport. On the other hand, the PfsI/R system upregulates 72% of the genes of its regulon, of which 37% are involved in translation, ribosomal structure and biogenesis. 53% of the genes controlled by the PfsI/R and 65% by the PfvI/R are also part of the RsaM regulon. Interestingly, only two genes are regulated by both AHL QS systems. The present study highlights the unique regulons of the PfvI/R, the PfsI/R and the RsaM that is a global regulator that modulates gene expression in the two QS systems as well as independently of the QS.

**METHODS**

**Bacterial strains, plasmids, media and recombinant DNA techniques**

The bacterial strains and plasmids used in this study are listed in Table 1. *Pfv* strains were grown at 30°C in Luria–Bertani (LB) medium [22] and M8 medium [23] was used for swarming assays. *Escherichia coli* DH5α and *E. coli* S17-1 were grown at 37°C in LB medium. Bacterial biosensor strains used for AHL detection *Chromobacterium violaceum* CVO26 and VRR07 were grown at 30°C in LB medium. When required, antibiotics were added at the following concentrations: ampicillin 100 µg ml⁻¹ (*Pfv*, *E. coli*); gentamicin 50 µg ml⁻¹ (*Pfv*) and 15 µg ml⁻¹ (*E. coli*); kanamycin 100 µg ml⁻¹ (*Pfv*) and 50 µg ml⁻¹ (*E. coli*); tetracycline 40 µg ml⁻¹ (*Pfv*) and 20 µg ml⁻¹ (*E. coli*). pGEM-T Easy vector (Promega Corp., Madison, WI, USA) was used for cloning. When necessary, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) was added at a final concentration of 80 µg ml⁻¹. Routine DNA manipulation steps such as digestion with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, ligation with T4 DNA ligase and transformation of *E. coli* were performed as described previously [24]. Plasmids were purified by using EuroGold columns (EuroClone, Pero, Milan, Italy); total DNA from *Pfv* was isolated by sarkosyl-pronase lysis, as described previously [25]. Digestion with restriction enzymes was conducted according to the supplier’s recommendations (New England BioLabs, USA). DNA was ligated with T4 DNA ligase (New England BioLabs, USA) according to the manufacturer’s recommendations. The sets of specific primers (Sigma-Aldrich) used in this study are listed in Table 1.

**Total RNA extraction, RNA-Seq experiment and analysis**

Isolation of total RNA was carried out from three independent biological replicate cultures of *Pfv* UB0736 (*Pfv* WT), *Pfv* 0736IDM (0736IDM), *Pfv* 0736IDM (pBBRPsfl), 0736IDM (pBBRPsfl), *Pfv* 0736IDM (pBBRPsfl) and *Pfv* 0736RSAM (0736RSAM). The cultures were grown at 30°C and 180 r.p.m. until the onset of stationary phase. RNA was purified from 2 × 10⁶ cells using the RiboPure bacterial RNA isolation kit (Ambion Inc., Austin, TX, USA) and following the manufacturer’s instructions. Total RNA was treated with RNase-free DNase (Ambion, Life Technologies, USA) and the purity of RNA was assessed by PCR on total RNA with GoTaq polymerase (Promega) using RecA Fw and RecAR ev primers. RNA-Seq experiments were performed by IGA Technology Services (Udine, Italy), and Illumina HiSeq 2000 (Illumina) was used for sequencing. The resulting sequences were mapped against RefSeq assembly accession: GCF_000251185.1 (cite PMID: 22535942 PMCID) using BWA software (cite PMID: 20080505). Finally, Bioconductor libraries GenomicFeatures and DESeq2 (cite PMID 23950696 and 25516281) were used to calculate gene expression levels and fold changes between samples. The cut-off FDR-adjusted P value was 0.01, with a minimum twofold change. The RNA-Seq data has been submitted to the Sequence Read Archive (SRA); submission number SUB2084775 and SRA number SRP093692.

**Purification and detection of AHLs**

Five *Pfv* strain (*Pfv* WT, 0736IDM, 0736IDMPsfl, 0736IDMPsfl and 0736RSAM) cultures that were used for RNA extraction were also used to perform AHL extraction and detection. Spent supernatant (20 ml) was extracted with an equal volume of ethyl acetate with 0.1% acetic acid. The mixture was centrifuged (5000 r.p.m., 5 min) and the ethyl acetate phase collected. After drying, the extract was resuspended in 20 µl ethyl acetate and was run on C18 reverse-phase chromatography plates besides synthetic AHLs used as standards (Fluka-Sigma-Aldrich), using 60% (v/v) methanol in water as the mobile phase. The plate was then overlaid with a layer of LB agar containing *Agrobacterium tumefaciens* NTL4/pZLR4 as an AHL biosensor as previously described [26] (Fig. S1, available in the online Supplementary Material).

**Real-time quantitative PCR (RT-qPCR)**

Transcript expression of selected genes was quantified by real-time PCR using the CFX96 Touch qPCR system (Bio-Rad). DNase-treated RNA (1 µg) was used as a starting
Table 1. Strains, plasmids and primers used in this study

<table>
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<tr>
<th>Strains or mutants</th>
<th>Relevant characteristics</th>
<th>Reference</th>
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<td>[61]</td>
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<tr>
<td>0736IDM (0736IDM)</td>
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<td>[9]</td>
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<td>0736RSAM (0736RSAM)</td>
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<td><em>P. fuscovaginae</em></td>
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<td>0736IDMpBBRPfsI (0736IDMPfsI)</td>
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material to generate cDNA following the manufacturer’s protocol. For all the RT-qPCR experiments, three independent biological replicates were performed. cDNA was generated by using Reverse Transcription system kit (Promega) with random hexamers. The synthesized cDNA samples were diluted to 25 ng µl⁻¹, and 2 µl cDNA was mixed with GoTaq qPCR Master Mix kit (Promega), containing SYBR green, and specific primers in a final volume of 12 µl. Gene-specific primers (Table 1) were designed using Primer 3 software [27], and recA was used as a reference gene. Fold change in gene expression was quantified by using the comparative D cycle threshold (CT) method [28]. The relative transcription levels were normalized to the reference gene and compared with expression levels of RNA-Seq.

**Gene promoter studies**

Transcriptional gene promoter activity studies of six promoters were performed on Pfv WT and derivatives 0736IDM and 0736RSAM. All constructs were initially made in the promoter probe vector pBBRGFPGm; however it was later established that the 0736RSAM was resistant to high concentrations of gentamicin. The reason for this phenotype is currently unknown. Promoters were then cloned in the promoter probe vector pMPGF. This is an IncPI-based plasmid which carries a promoterless GFP gene and harbours the gene for tetracycline resistance. The promoters of the six genes were: gene 4954 coding for a hypothetical protein, gene 1770 coding for a hypothetical protein, gene 3905 coding for trehalose permease IIC subunit, gene 5554 coding for urocanase, gene 5160 coding for formate dehydrogenase subunit gamma and gene 1166 coding for dihydrodipicolinate synthase. PCR-amplified promoter regions (genomic DNA of Pfv WT was used as a template) were transcriptionally fused to a promoterless gfp gene in vectors pBBRGFP and pMPGF. These gfp-based reporter plasmids were constructed by amplifying the gfp gene, deprived of its promoter, from plasmid pBBR2-GFP [29] using the primers GFPEF and GFPPPR. The amplified gfp was first cloned in the pMOS vector (Blunt-ended PCR Cloning Kit, American Biociences), sequenced and then cloned as an EcoRI/PstI fragment in pMP220 vector, generating pMPGF or as an EcoRI/Sall fragment in pBBRMCS5 generating pBBRGFP. The gene promoter constructs pBBR–promoters–GFP were then electroporated [30] into Pfv WT and 0736IDM while pMP–promoters–GFP were electroporated into Pfv WT and 0736RSAM. Gene promoter activity was determined as the amount of GFP fluorescence at 510 nm measured in overnight cultures on a microplate reader (Perkin Elmer EnVision 2104).

**RNA-Seq regulon overlapping comparisons**

The rationale of the experimental set-up for the determination of the PfsI/R, Pfvl/R and RsaM regulon is described in the Results section. We compared RNA-Seq data of Pfv WT and 0736IDM in order to locate genes regulated by QS systems that were repressed by RsaM in Pfv WT. Comparison between 0736IDM and 0736RSAM revealed genes regulated by RsaM in the absence of both QS systems. Genes regulated by the PfsI/R system were obtained by comparing RNA-Seq profile between 0736IDM and 0736DMPsvl. Comparison of RNA-Seq profiles between 0736IDM and 0736DMPsvl revealed genes regulated by the Pfvl/R system. The Pfvl/R, PfsI/R and RsaM regulons were then compared and overlapped in order to reveal which genes are co-regulated. The genes differentially expressed more than twofold were selected.

**Phenotypic assays, swarming motility and quorum quenching assay**

For bacterial motility assays, Pfv WT, 0736IDM and 0736RSAM were grown in LB medium for 24 h at 30 °C and 150 r.p.m., and adjusted to an optimal density of 1.0. Adjusted culture (2 µl) was spotted onto the 0.5 % M8 agar
plates. In order to analyse motility in the presence of polyamines, M8 plates were supplemented with putrescine (Sigma-Aldrich) (400 µM) [31]. The plates were incubated at 26, 30 and 37 °C. The diameter of swarming zones were measured after 24 h, and mean value was calculated. All experiments were performed in triplicate.

For detection of quorum quenching (QQ) activity, agar well diffusion assays were performed as described previously by Lozo et al. [32]. A spot-on lawn was used for semiquantitative measurement of QQ activity through the QQ zone (considered as a zone of absence of the pigment violacein around the wells made in C. violaceum lawns), which contains 50 µl overnight culture of the Pfv strains to be tested.

**Mutant construction and expression of penicillin acylase pgaA gene**

The vector pBBR1MCS5 [33] has been used for the expression of the Pfv pgaA gene; the cloning ORF of the pgaA gene was carried out in two steps, using two pairs of primers. Firstly, primers Pen1Fw and Pen1Rev were used to amplify the first part of the gene, and primers Pen2Fw and Pen2Rev were used to amplify the second part of the gene. Amplified DNA fragments were cloned in pGEM-T Easy vector, and then sub-cloned as the entire gene into pBBR1MCS5 vector. The pgaA gene was expressed in Pfv WT. Chromosomal knockout of the same gene was constructed using conjugative suicide vector pKNOCK-Km. The pgaA gene was inactivated in Pfv WT by amplifying via PCR an internal fragment of this gene using primers PenMutFw and PenMutRev, cloning it in pKNOCK-Km and then using it as a suicide plasmid via conjugation into Pfv WT and selecting for insertion into the chromosome via homologous recombination. Km-resistant colonies were selected and the mutation of the targeted gene was confirmed by PCR.

**RESULTS**

**Rationale and set-up for RNA transcriptional profiling in Pfv to determine the Pfvl/R, PfsI/R and RsaM regulons**

The Pfvl/R and the PfsI/R systems of Pfv are controlled by repressors; RsaL regulates the expression of pfvl, whereas RsaM regulates pfsI and in part pfvl [9]. In order to identify the Pfvl/R, PfsI/R and RsaM regulons, an RNA-Seq experiment was performed using five different Pfv genetic backgrounds: (i) in the WT; (ii) in the double pfvl-pfsI AHL synthases mutant (0736IDM); (iii) in the double synthase mutant 0736IDM harbouring the plasmid pBBRpfvl, which results in the expression and production of Pfvl-AHLs; (iv) in the double synthase mutant 0736IDM harbouring the plasmid pBBRpfsI, which results in the expression and production of PfsI-AHLs; and (v) in the repressor rsaM mutant 0736RSAM, which results in the hyperproduction of PfsI-AHLs and an increase in Pfvl-AHLs. The Pfv WT used here produced very small quantities of AHLs since both QS systems are strongly repressed. Therefore we set up the RNA-seq in the genetic background as described earlier in order to ensure that the PfsI/R and Pfvl/R systems were active. The double mutant (0736IDM) did not produce any AHLS and when harbouring the pfsI or pfvl AHL synthase in a plasmid, it resulted in the biosynthesis of AHLS in considerably larger amounts when compared to the WT strain. Similarly, the rsaM mutant overexpressed AHLS as originally reported [9]. This experimental set-up and evidence of the AHLS produced by the cultures used for the RNA purifica- tion in these five genetic backgrounds is provided in Fig. S1.

**QS in the Pfv WT strain is not active**

We have previously established that Pfv UPB0736, as well as other 11 tested Pfv strains, produces very small quantities of AHLS under laboratory conditions due to the two AHL QS systems being strongly negatively regulated. Transcriptomics studies via RNA-Seq performed here did not show any gene with a statistically significant difference in gene expression when the WT Pfv UPB0736 results were compared to the ones from 0736IDM. It was therefore concluded that, under the conditions of growth used here, PfsI/R and Pfvl/R AHL QS systems were not active to regulate gene expression. It was concluded that this is most probably due to the repression caused by RsaL and RsaM.

**PfsI/R and Pfvl/R regulons**

It was established that 26 genes were regulated by Pfvl/R and 98 genes by PfsI/R by at least twofold (Tables S1 and S4, respectively). PfsI/R positively regulated 70% of its regulon genes, mostly classified as genes involved in translation, ribosomal structure and biogenesis (Fig. 2a, and Table S5). Eleven 30S ribosomal and nine 50S ribosomal proteins were positively regulated by PfsI/R at a similar level of two-threefold (Table S5). Furthermore, we found that the PfsI/R QS system upregulates the 16S rRNA-processing protein RimM by 3.5-fold. This system had a also positive impact on translation through the regulation of genes such as tyrosyl-tRNA synthetase, valyl-tRNA synthetase and phenylalanyl-tRNA synthetase (Table S5), as well as in trafficking secretion by regulating the three genes coding the preprotein translocase subunits SecD, SecY and YajC. The PfsI/R system was also involved in the negative regulation of genes encoding for different chaperones such as CbpM, Hsp90, DnaK, GroEL and protein disaggregation chaperone. PfsI/R also had a positive influence on the transport and metabolism of different amino acids such as glycine, histidine and serine (Table S5). Using a threefold cut-off, beside the AHL synthase gene, we found that the PfsI/R system upregulates five other genes mostly involved in process of translation and ribosomal structure as well as in amino acid transport and metabolism (Table S6).

Similarly, about half of the genes regulated by the Pfvl/R AHL QS system encode for transporters, mostly involved in histidine and arginine amino acid transport, or involved in histidine degradation. Inorganic ion transport and metabolism were also positively regulated by the presence of PfvI-AHLs (Table S2 and Fig. S2). Using a cut-off of threefold
change, beside the AHL synthase gene, only two genes were scored as negatively regulated (Table S3). Together, both AHL QS systems were involved in the regulation of 126 genes as summarized in Tables S1 and S4 based on the order of fold change, and Tables S2 and S5 based on the functional classification.

**RsaM regulon**

It was of interest to determine the regulon of RsaM since it regulates PfsI/R stringently and, in part, PfVl/R [9]. Transcriptional profiling resulted in the identification of a surprisingly large number of genes being differentially expressed in 0736RSAM when compared to the 0736IDM (Table S7). By using a cut-off of twofold change ratio, 466 genes were found to be regulated by RsaM; 56 % (260 genes) were positively regulated and 44 % (206 genes) were negatively regulated. As expected, a significant proportion of these genes (approximately 16 %) were also QS-controlled by either PfsI/R and/or PfVl/R since RsaM regulates PfsI/R stringently and, in part, the PfVl/R QS system too [9]. This meant that 53 % of PfsI/R and 65 % of the PfVl/R genes were also regulated via RsaM (highlighted Tables S1 and S4).

Interestingly, 13.4 % of RsaM-regulated genes displayed a considerable differential expression of over eightfold and 23.7 % between four- and eightfold. The genetic loci regulated by RsaM encode for proteins that can be grouped into 20 classes (Fig. 2b). RsaM was involved in the regulation of a high number of genes encoding for proteins involved in processes of energy production and conversion (8.6 % of total genes), and genes encoding for proteins involved in translation, ribosomal structure and biogenesis (10.3 %), where most of them are overexpressed in 0736IDM compared with 0736RSAM. Of the total genes regulated by RsaM, 32 % were transporters, 8.58 % of total genes were involved in transport of different carbohydrates, 6.43 % in lipid transport, 14.4 % in amino acid transport, 3 % in nucleotide transport and 2.3 % of total genes were involved in inorganic ion transport. On the other hand, 6.86 % of total genes were involved in cell wall and membrane envelope processing and 6 % encoded for transcriptional regulators belonging to several different families including AraC, Fis, GntR, LacI, LuxR and LysR. From these, one LuxR (pfsR) regulator was the only transcriptional regulator that was downregulated by RsaM, with all of the others being positively regulated. Moreover, 3.4 % of the genes regulated by RsaM were involved in motility, all flagellar and almost all chemotaxis-related genes being positively regulated by this regulator. All genes classified in other categories are presented in Table S8.

Some of the proteins encoded by genes downregulated by RsaM can be putatively labelled as virulence-associated factors, such as the LPS biosynthesis proteins, type VI secretion proteins, fusaric acid resistance protein (the rsaM mutant is affected in resistance to fusaric acid, see below and Fig. 4b) and penicillin acylase. Similarly, several positively regulated genes can also be considered as virulence-associated; for example, the five genes encoding spermidine-putrescine transporters are positively regulated by RsaM. It has been shown that putrescine can behave as a signalling molecule and a QS system that responds to this molecule is able to control different community behaviours such as swarming in *Proteus mirabilis* [34] and biofilm production in *Yersinia pestis* [35]. The integration host factors (IHF) gene was positively regulated by RsaM, since it was approximately seven times less expressed in the 0736RSAM when compared with the 0736IDM. It has been reported that IHF is involved in the regulation of bioluminescence in *Vibrio harveyi*, as well as virulence factor elastase in *Vibrio vulnificus*, which are both regulated by QS [36, 37]. Furthermore, RsaM strongly repressed the expression of the cluster of six genes (putative desaturase, four hypothetical proteins and penicillin acylase) located downstream of *pfsI* (Fig. 1). All genes were strongly overexpressed in 0736RSAM compared with PfVl WT or 0736IDM and are most likely organized as an operon (Fig. 2b, d and Table S7). Upstream of *pfsI*, gene 4949 was strongly regulated by RsaM; the encoded protein was annotated as an isomerase displaying 67 % similarity with the phenazine biosynthesis protein (PhzF) of *P. aeruginosa* and 68 % with PhzF in *Pseudomonas fluorescens*. This gene was overexpressed more than 30 times in 0736RSAM compared with PfVl WT and it was also positively regulated by the PfVl/R QS system (see below). All the genes regulated by RsaM are shown in Table S7 based on the order of fold change and Table S8 based on the functional classification of loci.

Using a threefold cut-off, 166 genes were regulated by RsaM, 25.9 % were downregulated and 74.1 % upregulated. The genes most likely organized in an operon structure with the *pfsI* gene (Fig. 1), were up to 212-fold overexpressed in 0736RSAM compared with 0736IDM. RsaM strongly downregulated the copper chaperone CopZ, Fe/S-binding proteins, cytochrome proteins, several proteins involved in transcription and several others as summarized in Table S9. Strongly positively regulated genes involved in lipid, carbohydrate and amino acid transport, and metabolism were overexpressed in 0736IDM up to 30-fold compared with 0736RSAM (Table S9).

**Overlap of the RsaM regulon with PfsI/R and PfVl/R regulons**

As RsaM negatively regulates *pfsI* and in part also *pfvI*, it was of interest to determine the overlap with the PfsI/R and PfVl/R regulons established in this study. Both *pfsI* and *pfvI* were negatively regulated by RsaM as expected. The *pfsI* gene was strongly repressed by RsaM since it was 53 times more expressed in 0736RSAM than 0736IDM. Interestingly, *pfvI* was also negatively regulated by RsaM since it was 7.7 times more expressed in 0736RSAM compared with 0736IDM. Interestingly, the gene located upstream of *pfsI*, annotated as coding for an isomerase, is a putative phenazine biosynthesis protein and was also strongly regulated by RsaM through the PfsI/R system (Fig. 1). Overall, 53 % of the PfsI/R regulon determined in this study was also part of the RsaM regulon. Eleven of the 17 ribosomal protein-encoding genes regulated by RsaM were also regulated by PfsI/R. Two genes encoding for RNA helicases were negatively regulated by RsaM with a...
difference of almost sixfold and were positively regulated by the PfsI/R system. The genes encoding for the preprotein translocase subunit YajC and SecD were also regulated by RsaM through the PfsI/R system. The copper chaperone was the only chaperone regulated by RsaM and one of the chaperones regulated by the PfsI/R system. In summary, there was considerable overlap in the RsaM and PfsI/R regulon (Fig. 2a–c, Table S4), which indirectly also validated the RNA-Seq data as well as indicating the important role of RsaM in the QS response via regulation of the PfsI/R AHL QS system.

Interestingly, 65% of the genetic loci of the Pfvl/R regulon were also controlled by RsaM (Figs 2c and S2, Table S1), where most of the genes were involved in the transport of arginine and histidine. A putative six-gene operon involved in the transport and metabolism of histidine was strongly upregulated by RsaM; four of these genes were also regulated by the Pfvl/R system. Two genes encoding for methylmalonate-semialdehyde dehydrogenase and beta-alanine-pyruvate transaminase were positively regulated by RsaM, and repressed by Pfvl-AHL. Both genes are involved in valine metabolism and are putatively organized in an operon. Pfvl-AHL had a positive effect on the expression of the genes involved in inorganic ion transport and metabolism, such as two Ton-B-dependent receptor and multidrug DMT transporter permease, which were downregulated by RsaM.

Only two genes were part of the regulons of both AHL QS systems and both were also part of the RsaM regulon.

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**Fig. 1.** Gene map of the two QS loci of Pfv UPB0736. (a) Map of the 9.98 kb region of the pfsI/R QS system and surrounding genes strongly regulated by PfsI/R and RsaM. (b) Map of the 1.6 kb region of the pfvl/R QS system.

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(Fig. 2c); gene 5561 putatively encodes for a permease and gene 5562 for an imidazolonepropionase.

In conclusion, most of the genes in the two PfvI/R and PfsI/R regulons were also part of the RsaM regulon, indicating that regulation of AHL QS by RsaM plays a fundamental role in monitoring gene expression in response to cell density in Pfv.

**RT-qPCR, gene promoter studies and phenotypic analysis of some genes of the regulons**

It was of interest to perform a set of studies in order to further validate the RNA-Seq data. Seven genes were randomly selected and RT-qPCR was carried out with gene-specific primers (Table 1). The RNA samples extracted from three biological replicates were used as templates for both
transcriptomic analysis (RNA-Seq) and RT-qPCR. Through analysis of the expression of the seven genes plus a reference one (recA) from Pfv WT, 0736IDM and 0736RSAM, it was established that the expression patterns determined by RNA-Seq were in line with the expression levels obtained by qPCR (Fig. 3).

We also studied the expression of six randomly selected genes by cloning their gene promoters in a promoter probe vector in order to generate transcriptional fusions (as described in the Methods section). RNA-Seq analysis showed that all gene promoters except gene 4954 were positively regulated by RsaM. Three gene promoters (of gene 4954, gene 5554 and gene 5160) showed rather low promoter activity. However, comparison of promoter activity between Pfv WT and the double synthase mutant 0736IDM showed that there was no significant difference for all six promoters; all the differences are less than 0.5 (log2 fold change), and these trends of expression patterns were in correlation with RNA-Seq data (Fig. S3). Analysis of the activity of the promoters in the rsaM mutant 0736RSAM revealed that five of the six promoters showed correlation with RNA-Seq data, while promoter prom4954, of which the expression was highly dependent on RsaM, surprisingly displayed promoter activity opposite to that determined in the RNA-Seq experiment (Fig. S3). The reason for this is opposite behaviour is currently unknown.

In conclusion, the RT-qPCR and promoter studies were in line with the results of the RNA-Seq data. In addition, the significant overlap between the RsaM regulon and the regulons of PfsI/R and PfvI/R (see above) is a strong indication that the statistically significant RNA-Seq data resulting from three biological replicates is consistent.

The RsaM regulon identified here is rather large and contains many genes that are organized in operons. Several genes have been implicated in surface motility, such as the seven genes involved in flagellar biosynthesis and regulation (flgA, flhF, flgL, flgE, flgC, flhA, flgB) that were shown to be upregulated by RsaM. Phenotypic analysis confirmed that 0736RSAM was able to swarm less well when compared to Pfv WT or 0736IDM at 26°C and 30°C; no strain tested was able to swarm at 37°C (Fig. 4a).

The transcriptomic data revealed that the penicillin acylase-encoding gene (pgaA) as well as neighbouring genes, which are putatively organized in an operon (see above and Fig. 1), were overexpressed in 0736RSAM when compared to the WT. Previous studies showed that besides catalysis of deacylation of natural penicillins, this enzyme can act as a QQ molecule for AHLs by degrading the acyl-side chain [38, 39]. In order to determine whether the pgaA gene of Pfv was involved in QQ, we tested whether it altered AHL levels. We noted a significant difference in the QQ activity of the 0736RSAM when compared to the Pfv WT or 0736IDM when using middle chain AHLs and no difference using longer chain AHLs. Surprisingly, the penicillin acylase mutant 0736PGA behaved just like the Pfv WT, creating a similar QQ zones. One possible explanation for this effect is that other protein(s) responsible for the observed increase in QQ activity in the rsaM mutant. Analysing the whole genome of Pfv we found two other genes encoding putative QQ proteins; however these were not regulated by RsaM. It is therefore currently unknown which genes regulated by RsaM are involved in the RsaM-dependent QQ activity observed here.

**DISCUSSION**

In this study we performed transcriptome studies to unravel the PfsI/R, PfvI/R regulons and RsaM targets resulting in a global picture of the role played by AHL QS and the RsaM regulator in Pfv. The PfsI/R and PfvI/R QS systems together regulate 124 genes; the PfsI/R system regulates 98 and the PfvI/R 26 genes. Only two loci (involved in histidine metabolism) are co-regulated by the two systems, indicating that
Acinetobacter baumannii which harbour an AHL-QS system, including LasR. This repressor binds a region that overlaps the –10/–35 promoter sequence for σ70 of its regulated genes [40, 41].

RsaM is involved in the regulation of approximately 450 genes, where 56% are positively regulated while 44% are negatively regulated, indicating that RsaM is a major/global regulator of gene expression in Pfv. The RsaM regulon confirmed previous experimental data that RsaM strongly represses the pfsI/R AHL QS system; pfsI and pfsR are regulated by RsaM by a factor of 53- and 7.7-fold, respectively. A similar considerable difference of over 50-fold in gene expression has also been observed in the six genes located downstream of pfsI, which are most likely organized as an operon. Four of these genes encode for hypothetical proteins, whereas one encodes a putative penicillin acylase (pgA). Penicillin acylases have been previously reported to be QQs [38, 39, 42]. We observed that Pfv has QQ activity which is significantly affected by RsaM. PgaA was however not linked to the phenotypically detected QQ. The pgaA shows similarity with penicillin acylase (KcPGA) from Kluvera citrophila, which has been reported to be able to cleave AHFs [39]. We have reported here that, in Pfv, both AHL QS and QQ activity are controlled by RsaM.

Many other genes and putative operons are regulated by RsaM, including 21 transcriptional regulators. This indicates that many of the 466 genes of the RsaM regulon might be controlled indirectly. All regulators are positively regulated with the exception of pfsR (Table S8). It is therefore likely that many members of the RsaM regulon will be regulated indirectly via these transcriptional regulators. It is also currently unknown whether RsaM regulates pfsR directly or indirectly and whether this is connected to the stringent negative regulation of pfsI. Our previous study clearly demonstrated that RsaM stringently regulates PfsI-based AHL production [9]. In view of the RsaM regulon data reported here, this could be due (at least in part) by its regulation of PfsR. RsaL-P. aeruginosa directly represses the lasI AHL synthase gene interfering in the binding of the RNA polymerase at the promoter region. The RsaL-P. aeruginosa regulon contains over 200 genes, half of which are also QS-regulated [43]. RsaL controls 130 genes independently from its effect on 3OC12-HSL levels via regulation of lasI, some of which being important for pathogenicity. RsaM and RsaL are therefore two examples of small repressor genes that are intergenically located in AHL QS systems and play major roles in the regulation of these systems as well as regulating many other genes independently of QS. RsaM negatively regulates 45 genes involved in the different steps of translation, ribosomal structure and biogenesis; some are regulated via PfsI/R and others independently of QS (Table S8). RsaM also negatively regulates several proteins involved in replication and transcription and cell-cycle control, including the

the two AHL QS systems are acting independently of each other. They are, however, linked via RsaM since this repressor regulates the PfsI/R system stringently and also in part Pfv/R. It is therefore of importance to understand the mode of regulation by RsaM and, in particular, to find what stimulus (or stimuli) it responds to. The importance of this RsaM response is highlighted by the result reported here that the WT strain and the double pcvI–pfsI mutant result in a similar RNA-Seq profiles, thus not having statistically significant differences in any of the loci. This indicates that AHL QS in Pfv is very much under the control of RsaM and RsaM, which are likely to play a role in switching them on/off, thus adding a further control in addition to cell density.

RsaL homologs are present in many different bacterial species belonging to β-, γ- and δ-divisions, whereas RsaM is present in few species limited to β- and γ-proteobacteria which harbour an AHL-QS system, including Burkholderia sp., Actinobacter baumannii, Acidithiobacillus ferrooxidans, and Halothiobacillus neapolitanus [21]. So far, Pfv represents the only example in which RsaL and RsaM homologs are found in the same genome. RsaL has been studied in some detail in P. aeruginosa and in Psuedomonas putida, where it has been shown to act as a repressor of the AHL synthase-encoding genes lasI and ppuI, respectively. This repressor binds a region that overlaps the –10/–35 promoter sequence for σ70 of its regulated genes [40, 41].

![Fig. 4. Phenotypic analysis. (a) Comparison of surface motility between Pfv WT, 0736IDM and 0736RSAM incubated at 26 °C, 30 °C and 37 °C for 24 h. Means denoted by different letters are significantly different at P<0.01. (b) Difference in resistance to fusaric acid between Pfv WT and 0736RSAM in correlation with RNA-Seq data.](image-url)
housekeeping RpoD sigma factor (Table S8). Global regulators have been shown to modulate QS-dependent genes and vice versa. Schuster et al. [44] and Schuster and Greenberg [45] reported that the stationary phase RpoS sigma regulates approximately 40% of the genes that are part of the AHL QS regulon. In addition, Wagner et al. [46] determined that AHL QS in P. aeruginosa regulates the expression of many genes involved in basic cellular process, such as DNA replication, RNA transcription and translation, cell division and amino acid biosynthesis.

RsaM regulates many genes involved in carbohydrate transport and metabolism with the highest impact on several MFS family transporters, C4 dicarboxylate ABC transporters, sugar ABC transporters and man-PTS transport system of glucose, mannose and trehalose. MFS family transporters and sugar transporter permeases are also part of QS regulons in P. aeruginosa and Burkholderia thailandensis [47, 48]. Lipid transport and metabolism is positively regulated by RsaM and also not via the AHL QS systems. Amino acid transport and metabolism genes, on the other hand, are regulated by RsaM via PfvI/R or PfsI/R QS. Genes involved in histidine transport and metabolism are dependent on PfvI/R and PfsI/R and these are the only genes regulated by both AHL QS systems in Pfv. Histidine metabolism is also regulated by the AHL QS system in Burkholderia sp., but not in P. aeruginosa [47, 48].

Cell motility is positively regulated by RsaM and not by QS, as also shown phenotypically. Flagellar genes and chemotaxis proteins have been reported to be negatively regulated by QS in B. thailandensis, thus QS mutants were hypermotile [48]. Interestingly, Jang et al. [49] reported that in the rice pathogen Burkholderia glumae flagellar biosynthesis and motility are not QS-regulated at 28 °C, but are dependent on the QS system at 37 °C although expression levels are low. In Pfv, motility is also temperature-dependent, regulation is independent of QS but it is regulated by RsaM. There was no difference in motility between Pfv WT and mutants in the presence of putrescine, even though RsaM positively regulates five genes involved in putrescine-spermidine transport. Interestingly, RsaL_P. aeruginosa positively regulates genes encoding for putrescine-spermidine transporters [50]. Expression of the genes encoding for these transporters is also dependent on QS in B. thailandensis [44, 46, 48].

A possible virulence-associated factor regulated by RsaM is the IHF. IHF can bind to specific DNA sequences with high affinity and has been linked with the regulation of a number of cellular process including transcription [51], DNA replication [52] and recombination [53, 54]. Recently, it has been reported that IHF behaves as a key co-activator of the luxCDABE bioluminescence genes since it is involved in the regulation of LuxR, ensuring a timely expression of bioluminescence in V. harveyi [36]. IHF regulates a high number of other LuxR-regulated promoters, suggesting that IHF plays an important role in transcriptional activation during V. harveyi QS. Since both IHF and PfsR are regulated by RsaM in Pfv, it cannot be excluded that IHF also plays a major role in the regulation of genes regulated by QS.

RsaM is involved in controlling antibiotic resistance independently of AHL QS, as shown phenotypically since the rsaM mutant is more resistant to fusaric acid (Fig. 4) and gentamicin. The genes responsible for these resistance phenotypes observed in the rsaM mutant are currently unknown. Gentamicin is a bactericidal antibiotic that induces cell death, unlike bacteriostatic antibiotics that inhibit cell growth. The cellular response to a bactericidal antibiotic leads to overflow metabolism and the formation of reactive oxygen species (ROS) [55–57]. The two classes of antibiotics act differently on cellular respiration; growth inhibition from bacteriostatic antibiotics is associated with suppressing cellular respiration, while cell death from bactericidal antibiotics is associated with accelerated respiration [58]. The RNA-Seq data show that RsaM is involved in perturbation of cellular respiration through regulation of 40 genes coding for proteins involved in energy production and conversion. We found that formate dehydrogenase subunits alpha, beta and gamma, as well as the ferredoxin gene, are overexpressed in Pfv WT compared with 0736RSAM. The regulation of these loci could be responsible for the observed resistances to the antibiotics; the relationship between one gene/operon and drug resistance is not always clear since multiple differences in genes expression are often required to acquire high levels of resistance to a specific antibiotic(s) [59].

The mechanism of repression by the RsaM regulator is currently unknown. RsaM is a novel regulator without any functional domains or significant homology with functionally characterized proteins (max. 25% homology on protein level encoded by a putative gene intergenically located between the AHL QS system gene in Burkholderia sp.). The characterization of RsaM in Burkholderia cenocepacia J2315 (BcRsaM) revealed that this regulator has no known DNA binding motifs and direct binding of BcRsaM to the cepI AHL synthease promoter was not detected in in vitro assays. Michalska et al. [60] propose that the modulatory action of BcRsaM might result from interaction with other components of the QS machinery rather than from direct binding to the DNA promoter [60]. It must be noted that unlike what occurs in Burkholderia spp., the RsaM of Pfv strongly represses AHL QS thus playing a switch role in the QS response as mentioned above.

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Conflicts of interest
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