The Rsm regulon of plant growth-promoting \textit{Pseudomonas fluorescens} SS101: role of small RNAs in regulation of lipopeptide biosynthesis

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Summary

The rhizobacterium \textit{Pseudomonas fluorescens} SS101 inhibits growth of oomycete and fungal pathogens, and induces resistance in plants against pathogens and insects. To unravel regulatory pathways of secondary metabolite production in SS101, we conducted a genome-wide search for sRNAs and performed transcriptomic analyses to identify genes associated with the Rsm (repressor of secondary metabolites) regulon. \textit{In silico} analysis led to the identification of 16 putative sRNAs in the SS101 genome. In frame deletion of the sRNAs \textit{rsmY} and \textit{rsmZ} showed that the Rsm system regulates the biosynthesis of the lipopeptide massetolide A and involves the two repressor proteins RsmA and RsmE, with the LuxR-type transcriptional regulator MassAR as their most likely target. Transcriptome analyses of the \textit{rsmYZ} mutant further revealed that genes associated with iron acquisition, motility and chemotaxis were significantly upregulated, whereas genes of the type VI secretion system were downregulated. Comparative transcriptomic analyses showed that most, but not all, of the genes controlled by RsmY/RsmZ are also controlled by the GacS/GacA two-component system. We conclude that the Rsm regulon of \textit{P. fluorescens} SS101 plays a critical role in the regulation of lipopeptide biosynthesis and controls the expression of other genes involved in motility, competition and survival in the plant rhizosphere.

Introduction

Computational searches of intergenic regions, promoters and rho-independent transcription terminators (Livny \textit{et al}., 2005; 2006; Sridhar and Gunasekaran, 2013; Wright \textit{et al}., 2013) combined with experimental approaches (Sharma and Vogel, 2009) have revealed the presence of several small RNAs (sRNAs) in bacterial genomes. In general, two types of regulatory sRNAs have been described (Majdalani \textit{et al}., 2005; Gottesman \textit{et al}., 2006; Pichon and Felden, 2007; Gottesman and Storz, 2011). The first targets specific messenger RNAs (mRNAs) by base pairing. An example is RyhB in \textit{Escherichia coli} which interacts with the mRNA encoding SodB, an iron-containing superoxide dismutase (Salvail \textit{et al}., 2010). The second type interacts with RNA-binding proteins of the RsmA/CsrA family. RsmA (regulator of secondary metabolism) and CsrA (carbon storage regulator) act as translational repressors and their sequestration by activated sRNAs can relieve repression of the target mRNAs.

In \textit{Pseudomonas}, relatively few sRNAs have been studied in detail for their functions. In \textit{Pseudomonas protegens} strain CHA0, the sRNAs RsmX, RsmY and RsmZ are under the control of the GacS/GacA two-component system and regulate the production of a range of secondary metabolites (Heeb \textit{et al}., 2002a; Valverde \textit{et al}., 2003; Kay \textit{et al}., 2005; Lapouge \textit{et al}., 2007; 2008). In \textit{P. protegens} CHA0, Gac/Rsm-mediated regulation of secondary metabolites involves sequestration of the repressor proteins RsmA and RsmE that act posttranslationally by binding to the target mRNA (Blumer \textit{et al}., 1999; Reimmann \textit{et al}., 2005; Lapouge \textit{et al}., 2008). In \textit{Pseudomonas aeruginosa}, the two sRNAs, RsmY and RsmZ, regulate quorum sensing and the biosynthesis of several exoproducts (Brencic \textit{et al}., 2009; Frangipani \textit{et al}., 2014). Other sRNAs described for \textit{P. aeruginosa} are PhrS, PrrF1 and PrrF2: PhrS is involved

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\textit{Microbial Biotechnology} (2015) 8(2), 296–310
doi:10.1111/1751-7915.12190

\textbf{Funding Information} We are very grateful to the Dutch Graduate School of Experimental Plant Sciences (EPS) for financing this project.

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in the regulation of quinolone biosynthesis (Sonleitner and Haas, 2011; Sonleitner et al., 2011), and PrrF1 and PrrF2 contribute to iron acquisition (Wilderman et al., 2004; Sonleitner and Haas, 2011).

Most of the known sRNAs in Pseudomonas and other Gram-negative bacterial genera are under the control of the Gac/Rsm signal transduction pathway. Based on the proposed model, the phosphorylated regulator GacA binds to a conserved element upstream of the sRNA promoter, referred to as the GacA box, to activate their expression (Lapouge et al., 2008). In many cases, mutations or deletions of the sRNAs result in phenotypes similar to that of GacS/GacA mutants. For example, \( \Delta \text{gac}YZ \) and \( \Delta \text{gac}A \) mutants of P. aeruginosa are both deficient in the synthesis of the quorum sensing signal N-butanoyl-homoserine lactone, hydrogen cyanide (HCN), pyocyanin, elastase and chitinase as well as in biofilm formation (Kay et al., 2006; Brencic et al., 2009). In Pseudomonas entomophila, \( \Delta \text{gac}YZ \) and \( \Delta \text{gac}A \) mutants were both deficient in the production of entolysin (Vallet-Gely et al., 2010). Similarities in phenotypes of rsm and gac mutants have also been described for Pectobacterium carotovorum (Liu et al., 1998), E. coli (Weibacher et al., 2003), Salmonella enterica (Fortune et al., 2006) and Legionella pneumophila (Sahr et al., 2009).

In this study, we conducted a genome-wide search for sRNAs in Pseudomonas fluorescens strain SS101 and performed transcriptomic analyses to identify genes associated with the Rsm regulon and with the Gac regulon. We addressed the function of the Rsm regulon, involving sRNAs in P. fluorescens, \( \Delta \text{rsmYZ} \) and \( \Delta \text{gac}A \) mutants which are both deficient in the synthesis of the quorum sensing signal. Massetolide A is a cyclic lipopeptide massetolide A, which biosynthesis is governed by the non-ribosomal peptide synthetase (NRPS) genes massABC and regulated by the GacS/GacA system (de Bruijn and Raaijmakers, 2009a). Massetolide A contributes to biofilm formation, swarming motility, antimicrobial activity and defense against protozoan predators (Mazzola et al., 2009; Raaijmakers et al., 2010). Here, genome-wide transcriptional analysis of mutants with deletions in \( \text{rsm}Y \) and \( \text{rsm}Z \) revealed that the NRPS genes massA, massB, massC as well as the LuxR-type transcriptional regulator massAR were significantly downregulated. Via mutualistic and phenotypic analyses, we show that the Rsm system regulates massetolide biosynthesis as well as several other genes and traits in the rhizobacterium P. fluorescens SS101.

**Results and discussion**

**Small RNAs in P. fluorescens SS101**

A total of 68 tRNAs and 19 rRNAs were found in the SS101 genome (Table S1). Genome-wide analyses revealed 16 predicted sRNAs including homologues of the two signal recognition particle RNAs SrpB_1 (PfISS101_3911) and SrpB_2 (PfISS101_3926) (Table 1). Signal recognition particle (Srp) is a ribonucleoprotein complex that participates in multiple protein targeting pathways in bacteria (Koch et al., 1999) and is primarily involved in the incorporation of proteins in the inner membrane (Rosenblad et al., 2009). Furthermore, we also found a 6S SsrS RNA (PfISS101_5226) in the SS101 genome. In E. coli, 6S RNA is encoded by the srrS gene which regulates transcription during late exponential and stationary growth (Wassarman, 2007). Bacterial Ribonuclease P (PfISS101_0956) was found in the SS101 genome and represents a ribonucleoprotein complex comprised of a single RNA (~ 400 nt) and a single small protein subunit (~ 14 kDa) with the RNA as the catalytic subunit of the enzyme involved in the maturation of tRNA transcripts (Ellis and Brown, 2009). We also found homologues of PhrS (PfISS101_4081), PrrF1 (PfISS101_4589) and PrrF2 (PfISS101_3274), which are known to repress or activate the translation of target mRNAs by a base pairing mechanism. In P. aeruginosa, the two prrF sRNA genes are found in tandem. Homologous genes in other Pseudomonas species are located considerably distant from each other on the chromosome (Wilderman et al., 2004). Also in SS101, PrrF1 (PfISS101_4589) and PrrF2 (PfISS101_3274) are found at different locations in the genome. We also found RgsA (PfISS101_1357) in the SS101 genome, which is an sRNA probably regulated indirectly by GacA and directly by the stress sigma factor RpoS (Gonzalez et al., 2008).

Two other sRNAs found in the SS101 genome were RsmY (PfISS101_4962) and RsmZ (PfISS101_1168) (Table 1). In P. protegens and P. aeruginosa, RsmY and RsmZ regulate secondary metabolite production by...
sequestering RNA-binding proteins (e.g. CsrA, RsmA) that act as translational repressors (Kay et al., 2005; Gottesman and Storz, 2011). In P. aeruginosa, the expression of all Gac-regulated genes was shown to be RsmY/Z dependent (Brencic et al., 2009). For the other sRNAs detected in the SS101 genome (Table 1), the functions are poorly understood or not known from other Pseudomonas species. Here, we will specifically focus on the sRNAs in strain SS101 that are regulated by the GacS/GacA two-component system.

**Small RNAs in P. fluorescens SS101 regulated by the GacS/A system**

Transcriptomic analyses of both gacS and gacA mutants of P. fluorescens SS101 (Tables S2, S3) revealed that the expression of three sRNAs (rsmY, rsmZ and prrF1) was significantly (> 2-fold, \( P < 0.001 \)) altered (Table 1). Expression of rsmY and rsmZ was significantly downregulated in both gacS and gacA mutants, whereas expression of prrF1 was approximately six-fold upregulated in both gac mutants. The predicted sizes of the rsmY, rsmZ and prrF1 transcripts were 118 bp, 133 bp and 112 bp respectively. Subsequent prediction of their secondary structures revealed eight GGA motifs in both RsmY and RsmZ, with three in predicted loop regions respectively (Fig. 1A). In contrast, only one GGA motif was found in PrrF1, which is localized to a predicted stem (Fig. 1A). Repeated GGA motifs in loop regions of the secondary structure, as predicted for RsmY and RsmZ, are an essential characteristic of sRNAs for sequestration of RsmA and homologous repressor proteins (Lapouge et al., 2008). Previous work also showed that the regions upstream of these sRNAs contain a conserved 18 bp sequence which corresponds to the GacA-binding site for activation of these sRNAs (Heeb et al., 2002b; Kay et al., 2005). For SS101, we indeed found this typical GacA-binding box upstream of rsmY and rsmZ (Fig. 1B and C), but not for prrF1. Therefore, our subsequent functional analyses focused on rsmY and rsmZ.

**Role of RsmY and RsmZ in lipopeptide biosynthesis in P. fluorescens SS101**

The location of rsmY and rsmZ in the genomes appears to be conserved, at least to some extent, for the different Pseudomonas species and strains (Fig. 1B and C). In frame deletion, mutants were generated to investigate the role of rsmY and rsmZ in the regulation of massetolide A biosynthesis. The drop collapse assay, a reliable proxy for detection of massetolide A and other lipopeptide surfactants (de Bruijn et al., 2008; de Bruijn and Raaijmakers, 2009a), showed that mutations in either rsmY or rsmZ alone did not affect massetolide A production (Fig. 2A). However, mutations in both rsmY and rsmZ resulted in loss of massetolide A production which was confirmed by reversed phase-high-performance liquid chromatography (RP-HPLC) (Fig. 2B). Also swarming motility of SS101, a phenotype that depends on massetolide production (de Bruijn et al., 2008), was abolished in the rsmYZ double mutant (Fig. 2C). Mutations in rsmY or rsmZ alone did not affect growth of strain SS101 (Fig. 2D). However, mutations in both rsmY and rsmZ slightly enhanced growth in the early exponential phase but had an adverse effect on growth during the late exponential and stationary phase; similar changes in growth

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**Table 1. Small non-coding RNAs in P. fluorescens SS101.**

<table>
<thead>
<tr>
<th>Gene locus</th>
<th>Small RNAs descriptions</th>
<th>Fold change in ΔgacS(^a)</th>
<th>( P ) value</th>
<th>Fold change in ΔgacA(^a)</th>
<th>( P ) value</th>
</tr>
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<tr>
<td>PflSS101-1056</td>
<td>Bacterial RNase P class A 1.46</td>
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<td>1.37</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>PflSS101-1168</td>
<td>RsmZ RNA -27.43</td>
<td>6.4E-06</td>
<td>-21.94</td>
<td>1.11E-05</td>
<td></td>
</tr>
<tr>
<td>PflSS101-1276</td>
<td>putative t44 RNA -1.41</td>
<td>0.00672</td>
<td>-1.29</td>
<td>0.0135</td>
<td></td>
</tr>
<tr>
<td>PflSS101-1357</td>
<td>RgsA RNA -1.56</td>
<td>0.0206</td>
<td>-1.53</td>
<td>0.016</td>
<td></td>
</tr>
<tr>
<td>PflSS101-2033</td>
<td>putative sRNA P15 -1.06</td>
<td>0.865</td>
<td>-1.01</td>
<td>0.965</td>
<td></td>
</tr>
<tr>
<td>PflSS101-3274</td>
<td>PrrF2 RNA 1.14</td>
<td>0.772</td>
<td>-1.02</td>
<td>0.965</td>
<td></td>
</tr>
<tr>
<td>PflSS101-3926</td>
<td>sRNA P11 1.32</td>
<td>0.257</td>
<td>1.29</td>
<td>0.319</td>
<td></td>
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<tr>
<td>PflSS101-3951</td>
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<td>-1.1</td>
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<td>PflSS101-5226</td>
<td>6S SsrS RNA 1.23</td>
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<td>PrrF1 RNA 6.05</td>
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<td>PflSS101-4738</td>
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<td>sRNA P26 -1.17</td>
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<td>RsmY RNA -3.44</td>
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<td>-3.22</td>
<td>5.56E-05</td>
<td></td>
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<tr>
<td>PflSS101-5194</td>
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<td>0.0314</td>
<td>1.26</td>
<td>0.289</td>
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<td>PflSS101-5226</td>
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<td>0.0112</td>
<td>2.25</td>
<td>0.00617</td>
<td></td>
</tr>
</tbody>
</table>

All predicted small non-coding RNAs in P. fluorescens SS101 are indicated.

\( a. \) Positive values correspond to higher expression, negative values to lower expression (compared with the wild type). The sRNAs for which the expression is statistically significant (fold change \( >2; \ P < 0.001 \)) in both the ΔgacS and ΔgacA mutant versus wild-type SS101 are shaded in grey.
dynamics were observed for the gacS and gacA mutants of strain SS101 (Fig. 2D). These changes in growth dynamics are most likely not related to a lack of massetolide production, because growth of the site-directed massA biosynthesis mutant of SS101 was similar to that of the wild type (de Bruijn and Raaijmakers, 2009a). In summary, these results indicated that both RsmY and RsmZ are an integral component of the GacS/

GacA signal transduction cascade and regulate massetolide biosynthesis in P. fluorescens SS101.

Deletion of repressor proteins restores massetolide production

Previous studies with P. protegens CHA0 have shown that Gac/Rsm-mediated regulation of secondary metabolites involves sequestration of the repressor proteins RsmA and RsmE that act post-transcriptionally by binding to the target mRNA (Blumer et al., 1999; Reimmann et al., 2005; Lapouge et al., 2008). Hence, the next step was to determine if these repressor proteins are present in SS101 and if they play a role in Gac/Rsm-mediated regulation of massetolide biosynthesis. In silico analysis of the SS101 genome led to the identification of rsmA (PfISS101_4138), rsmE (PfISS101_3491) and csrA (PfISS101_3653). Phylogenetic analyses showed that they clustered closely with their homologues in other P. fluorescens strains and Pseudomonas species at both DNA and protein levels (Fig. S1). To decipher their role in regulation of massetolide biosynthesis, deletion mutants were made for each of these three repressors in the gacS mutant background of strain SS101. The gacS mutant does not produce massetolide, but according to the regulatory model, a mutation of the repressor proteins would alleviate translational repression and restore production. The results of the drop collapse assay and RP-HPLC analyses showed that a deletion of either rsmA or csrA in the gacS mutant did not restore massetolide production (Fig. 3A and B). Based on the drop collapse assay, a mutation in the rsmE gene partially affected the surface tension (Fig. 3A), but massetolide production was not detectable by RP-HPLC analysis (Fig. 3B). A double mutation in rsmE and rsmA fully restored massetolide production (Fig. 3A and B). A single deletion of either one of the repressor genes did not affect growth as compared with that of the gacS mutant, whereas stacked deletions of rsmA and rsmE in the gacS mutant changed the growth dynamics back to that of the wild type (Fig. 3C). We conclude that Gac/Rsm-mediated regulation of massetolide biosynthesis via rsmY and rsmZ implicates the two small RNA binding proteins RsmA and RsmE, whereas CsrA is not involved.

Potential targets of the RsmA/RsmE repressor proteins in P. fluorescens SS101

To determine the potential targets of the RsmA and RsmE repressor proteins, we conducted a whole genome search for putative Rsm binding sites at or near the 5′ untranslated leader mRNA by using the conserved motif 5′-N17−20 CANGGANG15′-3′ (N is any nucleotide) (Lapouge et al., 2008). A total of 17 genes were found with this conserved motif located in the ribosome binding site (RBS) (Table 2). For six of these 17 genes, transcription was significantly downregulated in the gacS/gacA mutants and also in the rsmYZ double mutant (Table 2). These six genes included: PfISS101_0554 with unknown function; gcd (PfISS101_1096) encoding the quinoprotein glucose dehydrogenase; ompA (PfISS101_1239); aprA (PfISS101_2560), which encodes an extracellular protease; PfISS101_2598, a gene predicted to encode a formyl-transferase domain/enoyl-CoA hydratase/isomerase family protein; and massAR (PfISS101_3396), the LuxR-type transcriptional regulatory gene located upstream of the massA biosynthesis gene and essential for massetolide biosynthesis (de Bruijn and Raaijmakers, 2009a,b). There was no GacA box sequence upstream of massA, massBC or massBCR (LuxR type regulator downstream of massBC). Alignment of the 5′ untranslated leader regions of these six putative target genes, with hcnA and aprA of P. protegens CHA0 and P. aeruginosa PAO1 as references, revealed the position of the consensus motif close to the RBS (Fig. 4A). When the alignment for massAR was performed with genes of several closely related LuxR-type transcriptional regulator genes flanking other lipopeptide biosynthesis genes in different Pseudomonas species and strains, similar consensus motifs were found (Fig. 4B). Based on these findings, we postulate that (i) the LuxR-type transcriptional regulator MassAR is the most likely target of the RsmA and RsmE repressor proteins in Gac/Rsm-mediated regulation of massetolide biosynthesis in P. fluorescens SS101; and (ii) lipopeptide biosynthesis in other Pseudomonas species is most likely regulated in a similar manner.

Other genes of the Rsm regulon in P. fluorescens SS101

To explore the potential roles of rsmY and rsmZ in global gene regulation in strain SS101, we conducted a genome-wide microarray analysis on the rsmYZ double mutant and the wild-type strain, both sampled in the mid-exponential growth phase (OD600 ∼ 0.6). In rsmYZ, the expression of rsmY and rsmZ was reduced 89 and 82-fold, respectively, due to the deletion of the corresponding genes. Various other significant changes in gene expression were observed with 121 and 272 genes significantly (fold change > 2.0; P < 0.001) up- and downregulated respectively (Table S4; Table S5). Next to the genes involved in massetolide biosynthesis, the chitinase encoding gene chiC (PfISS101_3606) and a gene predicted to encode a bacterioferritin family protein (PfISS101_0584) were significantly downregulated in the rsmYZ mutant. Moreover, 19 genes (PfISS101_5338–5358) homologous to the HSI-I type VI secretion system of P. aeruginosa (Mougous et al., 2006) were downregulated (Fig. 5A). Another type
VI secretion system HSI-II was not differentially regulated in the rsmYZ mutant. The putative functions of these type VI secretion systems in SS101, including a role in antibacterial activity or in plant-growth promotion (Decoin et al., 2014), are yet unknown.

Transcriptomic analysis also revealed that rebB_1 (PfSS101_0205) and rebB_2 (PfSS101_0206) were downregulated more than 44-fold and 93-fold, respectively, in the rsmYZ mutant (Table S3). For certain endosymbionts, such as Caedibacter in Paramecium, these genes have been reported to encode insoluble proteins referred to as refractile bodies (R bodies) (Schralhammer et al., 2012). It has been noted that R bodies unwind under certain conditions and are associated with toxicity, i.e. the ability to kill symbiont-free competitors. For free-living bacteria, including P. fluorescens SS101, the functions of these R bodies are not known yet. Given that not all downregulated genes in rsmYZ double mutant harbour the conserved motif 5′-A/U CANGGANG12/-3′ in the ribosome-binding site (data not shown), we postulate that the altered expression of these genes might be due to indirect regulation by the Rsm regulon as was reported for P. aeruginosa (Brench and Lory, 2009).

Table 2. Predicted target genes of the RsmA and RsmE repressor proteins in P. fluorescens SS101.

<table>
<thead>
<tr>
<th>Gene locus</th>
<th>Gene descriptions</th>
<th>Fold change</th>
<th>P value</th>
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<td>PfSS101_0554</td>
<td>conserved hypothetical protein</td>
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<td>0.000926</td>
<td>-4.32</td>
<td>0.00118</td>
<td>-4.59</td>
<td>0.00108</td>
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<td>leucine rich repeat domain protein</td>
<td>1.04</td>
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<td>1.12</td>
<td>0.00821</td>
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<td>PfSS101_1073</td>
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<td>0.0125</td>
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<td>PfSS101_2560</td>
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<td>PfSS101_2670</td>
<td>UTP–glucose-1-phosphate uridylyltransferase</td>
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All predicted target genes of Gac/Rsm cascade in P. fluorescens SS101 are indicated.

a. Positive values correspond to higher expression, negative values to lower expression (compared with the wild type). The target genes for which the expression was statistically significant (Fold change >=2; P < 0.001) in both the ΔgacS, ΔgacA and ΔrsmYZ mutant versus wild-type SS101 are shaded in grey.

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Fig. 3. Phenotypic and chemical analyses of *P. fluorescens* strain SS101, ΔgacS mutant and single, double or triple mutants disrupted in *rsmA*, *rsmE* and *csrA* in the ΔgacS background.

A. Drop collapse assay with cell suspensions of wild-type SS101, ΔgacS, ΔrsmAgacS, ΔcsrAgacS, ΔrsmEgacS, ΔrsmAcsrAgacS, ΔrsmArsmEgacS, ΔcsrArsmEgacS and ΔrsmAcsrArsmEgacS mutants. Bacterial cultures grown for 2 days at 25°C on KB agar plates were suspended in sterile water to a final density of 1 × 10^10 cells ml^-1, and 10-μl droplets were spotted on parafilm, and crystal violet was added to the droplets to facilitate visual assessment. A flat droplet is a highly reliable proxy for the production of the surface-active lipopeptide massetolide A.

B. Reversed phase-high-performance liquid chromatography chromatograms of cell-free culture extracts of wild-type SS101, ΔrsmAgacS, ΔcsrAgacS, ΔrsmEgacS, ΔrsmAcsrAgacS, ΔrsmArsmEgacS, ΔcsrArsmEgacS and ΔrsmAcsrArsmEgacS mutants as described in A. The wild-type strain SS101 produces massetolide A (retention time of approximately 18–21 min) and various other derivatives of massetolide A (minor peaks with retention times ranging from 12 to 18 min) which differ from massetolide A in the amino acid composition of the peptide moiety. AU stands for absorbance unit. Representative chromatograms of ΔrsmAgacS and ΔrsmArsmEgacS mutants are shown.

C. Growth of wild-type SS101, ΔrsmAgacS, ΔcsrAgacS, ΔrsmEgacS, ΔrsmAcsrAgacS, ΔrsmArsmEgacS, ΔcsrArsmEgacS and ΔrsmAcsrArsmEgacS mutants in liquid broth at 25°C. At different time points, the optical density of the cell cultures was measured spectrophotometrically (600 nm). Mean values for four biological replicates are given; the error bars represent the standard errors of the mean.

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Genes upregulated in the rsmYZ mutant represent genes involved in iron acquisition, chemotaxis and cell motility (Fig. 5B). Also, gabT (PflSS101_0208), which is involved in γ-aminobutyric acid utilization, was upregulated in the rsmYZ mutant. Upregulation was also found for three genes of the fagA-fumC-orfX-sodA operon (PflSS101_0896, 0898, 0899) (Fig. 5B), which functions in oxidative stress adaptation in P. aeruginosa (Polack et al., 1996; Hassett et al., 1997a,b).

Comparison of the Rsm regulon and the Gac regulon of P. fluorescens SS101

Many of the genes differentially regulated in the rsmYZ mutant have also been reported previously in oxidative stress adaptation in P. aeruginosa (Polack et al., 1996; Hassett et al., 1997a,b).

Fig. 4. A. Alignment of the upstream regions of five putative target genes of the RsmA and RsmE repressor proteins of P. fluorescens SS101. The aprA and hcnA genes of P. protegens CHA0 and P. aeruginosa PA01 were used as references. The translation initiation ATG codon is shown at the 3’ end. B. Alignment of the regions upstream of LuxR-type transcriptional regulatory genes that flank different lipopeptide biosynthesis gene clusters in Pseudomonas fluorescens SS101, Pseudomonas sp. MIS38, P. fluorescens P90-1, P. protegens Pf-5, P. fluorescens SBW25, P. syxantha BG33R, P. putida PCL1445, P. entomophila L48 and Pseudomonas syringae pv. tomato DC3000. The translation initiation ATG codon is shown at the 3’ end.

Fig. 5. Whole genome transcriptome analysis of P. fluorescens SS101 and the ΔrsmYZ mutant. Heat maps showing significant log2-fold changes (P < 0.001) in the expression of genes in the ΔrsmYZ versus wild-type cells. Wild-type SS101 and the ΔrsmYZ mutant were grown in liquid KB at 25°C to an optical cell density of OD600 = 0.6. The fold changes shown here represent averages of three biological replicates. A represents known genes that were downregulated in the ΔrsmYZ mutant, whereas B represents known genes upregulated in the ΔrsmYZ mutant versus wild-type SS101. For a list of all genes differentially regulated in the ΔrsmYZ mutant versus wild-type SS101, we refer to Tables S2 and S3.
ously to be differentially expressed in Gac mutants of other *Pseudomonas* species and strains (Brencic et al., 2009; Hassan et al., 2010; Cheng et al., 2013; Wang et al., 2013). In *P. aeruginosa*, the GacS/GacA transduction system acts exclusively through its control over the transcription of *rsmY* and *rsmZ* (Brencic et al., 2009). However, the possibility that the system directly regulates other genes cannot be excluded for other *Pseudomonas* species and strains. For instance, in *L. pneumophila*, LetA (orthologue of GacS) regulates expression of flagellar genes by a mechanism that appears to be independent of RsmY and RsmZ (Sahr et al., 2009). In our study, comparative analyses of the Gac regulon and Rsm regulon of *P. fluorescens* SS101 were conducted according to Sahr and colleagues (2009). Briefly, we made a direct comparison (fold change > 2.0, *P* value < 0.05) of the gene expression pattern of Δ*gacA* and Δ*rsmYZ*. Additionally, we analysed genes differentially expressed in either Δ*gacA*/*wt* or in Δ*rsmYZ/*wt*. Collectively, these analyses resulted in five genes differentially expressed in the Δ*gacA* mutant and 11 genes differentially expressed in the Δ*rsmYZ* mutant. One of the five genes (PflSS101_2039) that was differentially expressed in the Δ*gacA* mutant is located directly downstream of *gacA*. Hence, its differential expression is most likely due to a polar effect of the *gac* mutation. Therefore, this gene was excluded from the comparison. In summary, the expression of four and 11 genes varied in Δ*gacA* and Δ*rsmYZ* mutants respectively. One of these four genes is related to iron uptake, one is involved in amino acid transport and metabolism, and two genes are predicted to encode a hypothetical protein. The 11 genes uniquely expressed in the Δ*rsmYZ* mutant (Table S6) were all significantly upregulated. One gene, encoding a secondary thiamine-phosphate synthase enzyme, showed the most increased expression (nine-fold change), but its function in strain SS101 is not known yet.

In summary, this analysis suggests that most, not all, of the genes controlled by GacS/GacA two-component system are controlled via RsmY/RsmZ.

**Conclusions**

Through *in silico* analyses of the genome of the rhizobacterium *P. fluorescens* SS101, 16 small RNAs were identified. Subsequent experiments revealed, for the first time, that the Rsm signal transduction pathway plays a critical role in the regulation of massetolide biosynthesis, a cyclic lipopeptide important for biofilm formation, swarming motility, antimicrobial activity and induction of systemic resistance in plants. We showed that the effects of the two sRNAs RsmY and RsmZ are channeled through the RsmA and RsmE repressor proteins, and we predicted that the LuxR-type transcriptional regulator MassAR is one of the targets of these repressor proteins in strain SS101. To date, most information on the Rsm regulon in *Pseudomonas* species comes from studies on *P. aeruginosa* and *P. protegens*. Here, new information is provided that the Rsm system regulates lipopeptide biosynthesis in *P. fluorescens* SS101 and possibly other *Pseudomonas* species. Our study also provided, for the first time, a whole genome comparison of the Rsm and Gac regulons in a *Pseudomonas* species other than *P. aeruginosa*. The results of these analyses revealed that most but not all of the genes controlled by RsmY/RsmZ are also controlled by the GacS/GacA two-component system, whereas in *P. aeruginosa*, the Gac regulon controls downstream genes exclusively through the sRNAs RsmY and RsmZ.

**Experimental procedures**

Bioinformatic prediction of sRNAs in *P. fluorescens* SS101 genome

sRNA searches were performed by BLAST and YASS (Noe and Kucherov, 2005) against the Rfam database (http://rfam.janelia.org/), as well as by erpIn (Gautheret and Lambert, 2001), INFERNAL (Nawrocki et al., 2009) and DARN (Zytnicki et al., 2008), which are included in the RNAspace package (Cros et al., 2011).

Bacterial strains and cultural conditions

Bacterial strains used in this study are listed in Table 3. *Pseudomonas fluorescens* strains were cultured in liquid King’s medium B (KB) (King et al., 1954) at 25°C. The gacS and gacA plasmid mutants were obtained with plasmid pTnModOKm (Dennis and Zylstra, 1998). *Escherichia coli* strain DH5α was used as a host for the plasmids used for site-directed mutagenesis. *Escherichia coli* strains were grown on Luria–Bertani (LB) plates or in LB broth (Bertani, 1951) amended with the appropriate antibiotics.

Bacterial mutagenesis

Site-directed mutagenesis of the two small RNAs and three repressor protein genes was performed with the pEX18Tc suicide vector as described by de Bruijn and colleagues (de Bruijn et al., 2008). The primers used are listed in Table S7. For each mutant construct, two fragments were amplified: Up and down fragments. In the first-round polymerase chain reaction (PCR), the up and down fragments were amplified respectively. The first round PCR was performed with Pfu polymerase (Promega). The program used for the PCR consisting 1 min denaturation at 95°C, followed by 30 cycles of 95°C 1 min, Tm 30 s and 72°C 2 min. The last step of the PCR was 72°C for 7 min. All fragments were separated on a 1% (wt/vol) agarose gel and purified with an Illustra GFX PCR DNA and Gel Band Purification Kit. The second round PCR was performed by mixing equimolar amounts of the up and down fragments as templates, up forward and down reverse primers were added in the Pfu PCR reaction system. All fragments were separated on a 1% agarose gel, and bands
of the right size were purified with a Qiagen kit. The fragments were digested with EcoRI and HindIII and cloned into pEX18Tc. *Escherichia coli* DH5α was transformed with pEX18TC-rsmY, pEX18TC-rsmZ, pEX18TC-rsmA, pEX18TC-csrA or pEX18TC-csrE plasmids by heat shock transformation according to method of Inoue and colleagues (Inoue et al., 1990), and transformed colonies were selected on LB supplemented with 25 μg ml⁻¹ tetracycline (Sigma). Integration of the inserts was verified by restriction analysis of the plasmids. The plasmid inserts were verified by sequencing (Macrogen, Amsterdam, the Netherlands). The correct pEX18Tc-rsmY and pEX18Tc-rsmZ constructs were subsequently electroporated into *P. fluorescens* SS101; pEX18Tc-rsmA, pEX18Tc-csrA and pEX18Tc-csrE constructs were transformed into the ΔgacS mutant. Electrocompetent cells were obtained according to the method of Choi and colleagues (2006), and electroporation occurred at 2.4 kV and 200 μF. After incubation in SOC medium [2% Bacto tryptone (Difco), 0.5% Bacto yeast extract (Difco), 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose (pH 7)] for 2 h at 25°C, the cells were plated on KB supplemented with tetracycline (25 μg ml⁻¹) and rifampin (50 μg ml⁻¹). The single crossover colonies obtained were grown in LB overnight at 25°C and plated on LB supplemented 5% sucrose to accomplish the double crossover. The plates were incubated at 25°C for at least 48 h, and colonies were re-streaked on LB supplemented with tetracycline (25 μg ml⁻¹) and on LB supplemented with 5% sucrose. Colonies that grew on LB with sucrose, but not on LB with tetracycline, were selected and subjected to colony PCR to confirm the deletion of the genes.

### Lipopeptide extraction and RP-HPLC separation

Massetolide extractions and RP-HPLC analysis were conducted according to the methods described previously (de Bruijn *et al.*, 2008; de Bruijn and Raaijmakers, 2009a). Briefly, *Pseudomonas* strains were grown on *Pseudomonas* agar plates (*Pseudomonas* agar 38 g l⁻¹, glucose 10 g l⁻¹) for 48 h at 25°C. The cells were suspended in sterile de-mineralized water (~40 ml per plate), transferred to 50 ml tubes, shaken vigorously for 2 min and then centrifu-
RNA isolation at the mid-exponential growth stage (OD600 = 0.6). Cells of these strains were collected in triplicates. Total RNA was extracted with Trizol reagent (Invitrogen) and further purified with the NucleoSpin RNA kit (Macherey-Nagel). A tiling microarray for P. fluorescens SS101 was developed in the MicroArray Department (MAD), University of Amsterdam (UvA), Amsterdam, the Netherlands. In total, 134,276 probes (60 mer) were designed with, in general, a gap of 32 nucleotides between adjacent probes on the same strand and an overlap by 14 nucleotides when regarding both strands. In addition, 5000 custom negative control probes were hybridized, and used as an internal control to validate the designed probes in a comparative genomic hybridization experiment of four arrays. Probes were annotated and assembled into probe sets for known genes based on location information retrieved from the Pathosystems Resource Integration Center (http://patricbrc.org). Probes outside of known genes were labelled as Intergenic Region. Complementary DNA (cDNA) labelling was conducted as described previously (52). Briefly, cDNA was synthesized in presence of Cy3-dUTP (Cy3) for the test samples and with Cy5-dUTP (Cy5) for the common reference. The common reference was made by an equimolar pool of the test samples (3 μg per sample). Five micrograms of total RNA per reaction was used and yielded 1.5–2.5 μg cDNA for each sample with more than 16 pmol of Cy3 or Cy5 dye per microgram. Hybridizations were performed according to Pennings and colleagues (Pennings et al., 2011). Slides were washed according to the procedures described in the Nimblegen Arrays User’s Guide – Gene Expression Arrays Version 5.0 and scanned in an ozone-free room with a Agilent DNA microarray scanner G2565CA (Agilent Technologies). Feature extraction was performed with NIMBLESCAN v2.5 (Roche Nimblegen). Data pre-processing consisted of log2 transformation of the raw probe-intensity data, followed by a change to a LOWESS normalization. Thus, normalized sample transformations of the raw probe-intensity data, followed by a Multi-Array Analysis algorithm (Irizarry, et al., 2003). All results described were found to be significant using a false discovery rate of less than 5%. The ARAYSTAR 12 software (DNASTAR, Madison, Wisconsin, USA) was used for analysing the pre-normalized array data. Statistical analyses were carried out with the normalized data using a moderated t-test to determine differential transcript abundance. Genes with a fold change > 2 and P-value < 0.05 were considered to be differentially regulated.

Acknowledgements
We thank the MAD lab: Dutch Genomics Service & Support Provider for conducting the microarray analysis. This publication is No.5680 of the Netherlands Institute of Ecology (NIOO-KNAW).

Conflict of interest
The authors of this manuscript have no conflicts of interest to declare.

References


Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Fig. S1.** Phylogenetic analyses of RsmA/CsrA-like proteins in different *Pseudomonas* species and strains. The phylogenetic tree is based on amino acid sequences of RsmA, RsmE and CsrA from 23 bacterial genomes, and was generated by neighbor-joining (NJ) (Saitou and Nei, 1987) in MEGA 6 (Tamura et al., 2013). The evolutionary distances were computed using Jones–Taylor–Thornton (JTT) model. The variation rate among sites was modelled with a gamma distribution. Bootstrap values (1000 repetitions) are shown on branches. Rsm proteins from *P. fluorescens* strain SS101 are indicated in bold.

**Table S1.** RNA and rRNA in SS101.

**Table S2.** Whole genome transcriptome analysis of ΔgacS/Δagr.
Table S3. Whole genome transcriptome analysis of ΔgacA/wt.
Table S4. Whole genome transcriptome analysis of ΔrsmYZ/wt, up-regulated genes with \( P < 0.001 \), fold change > 2.
Table S5. Whole genome transcriptome analysis of ΔrsmYZ/wt, down-regulated genes with \( P < 0.001 \), fold change > 2.

Table S6. Unique expression genes in ΔgacA and ΔrsmYZ mutants.
Table S7. Primers used for in frame deletion mutagenesis of the small RNAs and repressor proteins.
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