The Rsm regulon of plant growth-promoting
*Pseudomonas fluorescens* SS101: role of small RNAs
in regulation of lipopeptide biosynthesis

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Summary

The rhizobacterium *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 con-
ducted a genome-wide search for sRNAs and per-
fomed transcriptomic analyses to identify genes
associated with the Rsm (repressor of secondary
metabolites) regulon. In silico analysis led to the iden-
tification of 16 putative sRNAs in the SS101 genome.
In frame deletion of the sRNAs *rsmY* and *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 *rsmYZ* mutant further revealed that genes associ-
ated 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
*P. fluorescens* SS101 plays a critical role in the regu-
lation of lipopeptide biosynthesis and controls the
expression of other genes involved in motility, com-
petition and survival in the plant rhizosphere.

Introduction

Computational searches of intergenic regions, promoters
and rho-independent transcription terminators (Livny
et al., 2005; 2006; Sridhar and Gunasekaran, 2013;
Wright 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 et al., 2005; Gottesman 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
*Escherichia coli* which interacts with the mRNA encoding
SodB, an iron-containing superoxide dismutase (Salvail
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 regu-
lator) act as translational repressors and their sequestra-
tion by activated sRNAs can relieve repression of the
target mRNAs.

In *Pseudomonas*, relatively few sRNAs have been
studied in detail for their functions. In *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 et al., 2002a; Valverde
et al., 2003; Kay et al., 2005; Lapouge et al., 2007; 2008).
In *P. protegens* CHA0, 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). In *Pseudomonas aeruginosa*, the two sRNAs,
RsmY and RsmZ, regulate quorum sensing and the
biosynthesis of several exoproducts (Brencic et al., 2009;
Frangipani et al., 2014). Other sRNAs described for
*P. aeruginosa* are PhrS, PrrF1 and PrrF2: PhrS is involved

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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, Δrsmyz and ΔgacA mutants of *P. aeruginosa* are both deficient in the synthesis of the quorum sensing signal N-butyl 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*, Δrsmyz and ΔgacA 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 the two sRNAs RsmY (PfISS101_4962) and RsmZ (PflSS101_1168), and the two repressor proteins RsmA (PfISS101_4138) and RsmE (PfISS101_3491), in lipopeptide biosynthesis and predicted the potential target genes of the Rsm repressor proteins. Strain SS101 was originally isolated from the rhizosphere of wheat (de Souza et al., 2003), has activity against various oomycete and fungal pathogens (de Souza et al., 2003; Tran et al., 2007; van de Mortel et al., 2009) and induces systemic resistance in tomato and Arabidopsis against several pathogens and insect pests (Tran et al., 2007; van de Mortel et al., 2012). Comparative genome analyses of multiple *Pseudomonas* species and strains (Loper et al., 2012) revealed that strain SS101 harbours 350 unique genes, which include prophage and genomic islands. Unlike many other *P. fluorescens* and *P. protegens* biocontrol strains, SS101 does not produce the typical secondary metabolites such as 2,4-diacylphloroglucinol (DAPG), phenazines, pyrroline, pyoluteorin and HCN (Loper et al., 2012). The main secondary metabolite produced by SS101 is the cyclic lipopeptide massetolide A, whose 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 *rsmY* and *rsmZ* revealed that the NRPS genes massA, massB, massC as well as the LuxR-type transcriptional regulator massAR were significantly downregulated. Via mutational 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 (PIISS101_3911) and SrpB_2 (PIISS101_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 (PIISS101_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 (PIISS101_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 (PIISS101_4081), PrrF1 (PIISS101_4589) and PrrF2 (PIISS101_3274), which are known to repress or activate the translation of target mRNAs by a base paring 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 (PIISS101_4589) and PrrF2 (PIISS101_3274) are found at different locations in the genome. We also found RgsA (PIISS101_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 (PIISS101_4962) and RsmZ (PIISS101_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 prfF1) 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 prfF1 was approximately six-fold upregulated in both gac mutants. The predicted sizes of the rsmY, rsmZ and prfF1 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 PrfF1, 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 prfF1. 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., 2009a), showed that mutations in either rsmY or rsmZ alone did not affect growth of strain SS101 (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

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

| Gene locus     | Small RNAs descriptions | Fold change in \( \Delta gacS^a \) | \( P \) value | Fold change in \( \Delta gacA^a \) | \( P \) value |
|---------------|--------------------------|---------------------------------|-------------|---------------------------------|-------------|
| PflSS101_0956 | Bacterial RNase P class A | -27.43                          | 6.46E-06    | -21.94                           | 1.11E-05    |
| PflSS101_1276 | putative t44 RNA         | -1.41                           | 0.00672     | -1.29                            | 0.0135      |
| PflSS101_1357 | RgsA RNA                 | -1.56                           | 0.0206      | -1.53                            | 0.016       |
| PflSS101_2033 | putative sRNA P15        | -1.06                           | 0.865       | -1.01                            | 0.965       |
| PflSS101_3951 | sRNA P11                 | -1.16                           | 0.615       | -1.1                             | 0.702       |
| PflSS101_3926 | srpB_2: Bacterial signal recognition particle RNA | -3.44 | 3.78E-06 | -3.22 | 5.66E-05 |
| PflSS101_4962 | RsmY RNA                 | -1.76                           | 0.0314      | 1.26                             | 0.289       |
| PflSS101_5226 | 6S SsrS RNA              | -1.92                           | 0.0112      | 2.25                             | 0.00617     |

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 \( \Delta gacS \) and \( \Delta gacA \) 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* (PIISS101_4138), *rsmE* (PIISS101_3491) and *csrA* (PIISS101_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′-Nₐ₋₉₋ₐ₋₉₋ₐ₋₉₋ₐ₋₉₋ₐ₋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: PflSS101_0554 with unknown function; *gcd* (PIISS101_1096) encoding the quinoprotein glucose dehydrogenase; *ompA* (PIISS101_1239); *aprA* (PIISS101_2560), which encodes an extracellular protease; PflSS101_2598, a gene predicted to encode a formyl-transferase domain/enoyl-CoA hydratase/isomerase family protein; and *massAR* (PIISS101_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* PA01 as references, revealed the position of the consens 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* (PIISS101_3606) and a gene predicted to encode a bacterioferritin family protein (PIISS101_0584) were significantly downregulated in the *rsmYZ* mutant. Moreover, 19 genes (PIISS101_5338–5358) homologous to the HSI-I type VI secretion system of *P. aeruginosa* (Mougou 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 (PflSS101_0205) and rebB_2 (PflSS101_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) (Schrallhammer 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 CANGGANGU/A-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 (Bencic and Lory, 2009).

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

| Gene locus     | Gene descriptions                          | Fold change ∆gacS/Wt<sup>a</sup> | P value  | Fold change ∆gacA/Wt<sup>a</sup> | P value  | Fold change ∆rsmYZ/Wt<sup>a</sup> | P value  |
|----------------|-------------------------------------------|----------------------------------|----------|----------------------------------|----------|----------------------------------|----------|
| PflSS101_0554  | conserved hypothetical protein             | −4.84                            | 0.000926 | −4.32                            | 0.00118  | −4.59                            | 0.00108  |
| PflSS101_0590  | leucine rich repeat domain protein         | 1.04                             | 0.389    | 1.12                             | 0.00821  | 1.099                            | 0.038    |
| PflSS101_1073  | conserved hypothetical protein             | 1.45                             | 0.003    | 1.26                             | 0.0125   | 1.389                            | 0.00789  |
| PflSS101_1096  | quinoprotein glucose dehydrogenase (gpd)  | −4.45                            | 0.0000343| −4.32                            | 0.0000232| −3.799                            | 0.000621 |
| PflSS101_1198  | putative pyocin R, lytic enzyme            | −1.71                            | 0.0326   | −1.69                            | 0.0332   | −1.79                            | 0.0272   |
| PflSS101_1239  | OmpA family lipoprotein                    | −22.68                           | 2.05E-06 | −16.24                           | 4.42E-06 | −11.77                           | 3.45E-07 |
| PflSS101_1789  | putative membrane protein, Pfo05661 family | −1.28                            | 0.0975   | −1.27                            | 0.0964   | −1.25                            | 0.104    |
| PflSS101_2560  | extracellular alkaline metalloprotease AprA| −44.57                           | 0.0000135| −32.98                           | 0.00109  | −51.67                           | 3.53E-07 |
| PflSS101_2598  | formyl transferase domain/                 | −37.92                           | 1.04E-06 | −32.81                           | 9.01E-06 | −35.88                           | 8.84E-07 |
| PflSS101_2598  | enoyl-CoA hydratase/isomerase family protein |                                  |          |                                  |          |                                  |          |
| PflSS101_2670  | UTP-glucose-1-phosphate uridylyltransferase | −1.06                            | 0.441    | 1.17                             | 0.0143   | 1.41                             | 0.0021   |
| PflSS101_2760  | conserved hypothetical protein             | 1.23                             | 0.183    | 1.33                             | 0.0861   | 1.15                             | 0.334    |
| PflSS101_2801  | hypothetical protein                       | −1.16                            | 0.722    | −1.01                            | 0.988    | 1.07                             | 0.87     |
| PflSS101_3147  | TonB-dependent outer membrane receptor     | −1.09                            | 0.00398  | −1.02                            | 0.721    | 1.02                             | 0.732    |
| PflSS101_3396  | transcriptional regulator, MassAR          | −43.6                            | 5.76E-07 | −36.27                           | 0.000314 | −25.96                           | 3.42E-06 |
| PflSS101_3799  | RmuC domain protein                        | 1.22                             | 0.169    | 1.17                             | 0.254    | 1.11                             | 0.469    |
| PflSS101_4067  | L-arabinose ABC transporter,               | 1.38                             | 0.0521   | 1.7                              | 0.0113   | 1.49                             | 0.0676   |
| PflSS101_5435  | conserved hypothetical protein             | 1.09                             | 0.669    | −1.1                             | 0.576    | −1.11                            | 0.562    |

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 is statistically significant (Fold change > 2; P < 0.001) in both the ∆gacS, ∆gacA and ∆rsmYZ mutant versus wild-type SS101 are shaded in grey.

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

A. Drop collapse assay with cell suspensions of wild-type SS101, Δ*gacS*, Δ*rsmAgacS*, Δ*csrAgacS*, Δ*rsmEgacS*, Δ*rsmAcsrAgacS*, Δ*rsmArsmEgacS*, Δ*csrArsmEgacS* and Δ*rsmAcsrArsmEgacS* 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, Δ*rsmAgacS*, Δ*csrAgacS*, Δ*rsmEgacS*, Δ*rsmAcsrAgacS*, Δ*rsmArsmEgacS*, Δ*csrArsmEgacS* and Δ*rsmAcsrArsmEgacS* 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 Δ*rsmAgacS* and Δ*rsmArsmEgacS* mutants are shown.

C. Growth of wild-type SS101, Δ*rsmAgacS*, Δ*csrAgacS*, Δ*rsmEgacS*, Δ*rsmAcsrAgacS*, Δ*rsmArsmEgacS*, Δ*csrArsmEgacS* and Δ*rsmAcsrArsmEgacS* 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 (PfISS101_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 (PfISS101_0896, 0898, 0899) (Fig. 5B), which function 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 of strain SS101 have also been reported previously in oxidative stress adaptation in P. aeruginosa (Polack et al., 1996; Hassett et al., 1997a,b).

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.

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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 ΔgacA and ΔrsmYZ. Additionally, we analysed genes differentially expressed in either ΔgacA/wt or in ΔrsmYZ/wt. Collectively, these analyses resulted in five genes differentially expressed in the ΔgacA mutant and 11 genes differentially expressed in the ΔrsmYZ mutant. One of the five genes (PISS101_2039) that was differentially expressed in the ΔgacA 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 ΔgacA and ΔrsmYZ 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 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, swimming 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 mutagens 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 after 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

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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-rsmE 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-rsmE constructs were transformed into the ΔgacS mutant. Electroporant 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 with 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 verify 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, glycerol 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 centrifuged (30 min, 6000 rpm, 4°C). The culture supernatant was transferred to a new tube and acidified to pH 2.0 with 9% HCl. The precipitate was obtained by centrifugation (30 min, 6000 rpm, 4°C) and washed three times with acidified dH₂O (pH 2.0). The precipitate was re-suspended in 5 ml dH₂O and the pH adjusted to 8.0 with 0.2 M NaOH; the precipitate dissolves. The solution was centrifuged (30 min, 6000 rpm, 4°C) and the supernatant transferred to a new tube and subjected to lyophilization. Analytical HPLC separations were carried out on 5 μm C18 column (Waters Symmetry column, Waters, Etten-Leur, Netherlands), a 55 min linear gradient of 0% to 100% acetonitrile + 0.1% (v/v) trifluoroacetic acid with a flow rate of 0.5 ml min⁻¹. Detection was performed with a photodiode array detector (Waters) at wavelengths from 200 to 450 nm.

**Swarming motility**

Swarming motility assays of the bacterial strains and mutants were conducted according to the method described previously (de Bruijn and Raaijmakers, 2009a). Swarming motility of wild type strain SS101 and the mutants was assessed on soft (0.6% wt/vol) standard succinate agar medium (SSM) consisting of 32.8 mM K₂HPO₄, 22 mM KH₂PO₄, 7.6 mM (NH₄)₂SO₄, 0.8 mM MgSO₄ and 34 mM succinic acid and adjusted to pH 7 with NaOH. After autoclaving, the medium was cooled down in a water bath to 55°C and kept at 55°C for 1 h. Twenty millilitres of SSM was pipetted into a 9 cm diameter petri dish, and the plates were kept for 24 h at room temperature (20°C) prior to the swarming assay. For all swarming assays, the same conditions (agar temperature and volume, time period of storage of the poured plates) were kept constant to maximize reproducibility. Overnight cultures of wild-type SS101, mutants, were washed three times with 0.9% NaCl, and 5 μl of the washed cell suspension (1 × 10⁵ cells ml⁻¹) was spot inoculated in the centre of the soft SSM agar plate and incubated for 48–72 h at 25°C.

**Transcriptional profiling**

Wild-type SS101, the ΔgacA and the ΔrsmYZ mutant were grown in King’s medium B in 24-well plates, and harvested for

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**Table 3.** Bacterial strains and mutants used in this study.

| Strain               | Relative characteristics | Reference source |
|----------------------|--------------------------|-------------------|
| *Pseudomonas fluorescens* |                          |                   |
| SS101                | Wild type, Rif'          | de Souza et al., 2003 |
| ΔgacS                | Plasposon mutant, Km'    | This study        |
| ΔgacA                | Plasposon mutant, Km'    | This study        |
| ΔrsmY                | rsmY deletion mutant     | This study        |
| ΔrsmZ                | rsmZ deletion mutant     | This study        |
| ΔrsmYZ               | rsmY rsmZ deletion mutant| This study        |
| ΔrsmgacS             | gacS deletion mutant in the ΔgacS background | This study        |
| ΔcsrAgacS            | csrA deletion mutant in the ΔgacS background | This study        |
| ΔrsmEgacS            | rsmE deletion mutant in the ΔgacS background | This study        |
| ΔrsmAcrAgacS         | rsmA csrA deletion mutant in the ΔgacS background | This study        |
| ΔrsmAcrsmEgacS       | rsmA rsmE deletion mutant in the ΔgacS background | This study        |
| ΔrsmAcrsmAcsrEgacS   | csrA rsmE deletion mutant in the ΔgacS background | This study        |
| ΔrsmAcrsmAcsrAgacS   | rsmA csrA rsmE deletion mutant in the ΔgacS background | This study        |

Rif': Rifampin resistance; Km': Kanamycin resistance.
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-ratio transformation of the raw probe-intensity data, followed by a within slide Lowess normalization. Thus, normalized sample change was estimated to declare.

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Supporting information

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