Dual role of RsmA in the coordinated regulation of expression of virulence genes in *Pectobacterium wasabiae* strain SCC3193

Liis Andresen, Jekaterina Frolova, Lee Põllumaa and Andres Mäe

Department of Genetics, Institute of Molecular and Cell Biology, University of Tartu, Estonia

The CsrA/RsmA family of post-transcriptional regulators in bacteria is involved in regulating many cellular processes, including pathogenesis. Using a bioinformatics approach, we identified an RsmA binding motif, A(N)GGA, in the Shine–Dalgarno regions of 901 genes. Among these genes with the predicted RsmA binding motif, 358 were regulated by RsmA according to our previously published gene expression profiling analysis (WT vs *rsmA* negative mutant; Köiv *et al.*, 2013). A small subset of the predicted targets known to be important as virulence factors was selected for experimental validation. RNA footprint analyses demonstrated that RsmA binds specifically to the ANGGG motif in the 5′ UTR sequences of *celV1*, *pehA*, *pelB*, *pel2* and *prtW*. RsmA-dependent regulation of these five genes was examined in vivo using plasmid-borne translational and transcriptional fusions with a reporter *gusA* gene. They were all affected negatively by RsmA. However, we demonstrated that whereas the overall effect of RsmA on *celV1* and *prtW* was determined on both the translational and transcriptional level, expression of pectinolytic enzyme genes (*pehA*, *pel2* and *pelB*) was affected mainly on the level of transcription in tested conditions. In summary, these data indicate that RsmA controls virulence by integration of its regulatory activities at various levels.

INTRODUCTION

The RNA-binding post-transcriptional regulatory proteins CsrA/RsmA are involved in fine-tuning the control of gene expression, usually via the translation of target mRNAs (Babitzke *et al.*, 2009). From genomic data, about 200 members of the RsmA/CsrA family can be inferred in over 150 bacterial species (Bateman *et al.*, 2004), highlighting the importance of these regulatory proteins. CsrA was first identified and characterized in *Escherichia coli* (Romeo *et al.*, 1993). Although CsrA/RsmA is known to be a common virulence regulator for many animal bacterial pathogens, whole-genome sequencing revealed that CsrA/RsmA homologues are also widely distributed among plant bacterial pathogens (Feil *et al.*, 2005). The CsrA homologue RsmA was first discovered in *Erwinia carotovora* (Chatterjee *et al.*, 1995) and a few years later in *Pseudomonas fluorescens* (Blumer *et al.*, 1999). CsrA/RsmA binds to specific motifs (usually ANGGG) in single-stranded loops of target mRNAs, and when these motifs overlap the ribosome-binding site, translation is repressed (Babitzke *et al.*, 2009; Dubey *et al.*, 2005; Lapouge *et al.*, 2013; Liu *et al.*, 1997). Despite its global role in bacterial adaptation, only a few direct mRNA targets of CsrA/RsmA have been identified in *E. coli*, including *flhDC* (Yakhnin *et al.*, 2013), *moaA* (Patterson-Fortin *et al.*, 2013), *relA*, *dksA* and *rpoZ* (Edwards *et al.*, 2011); also, *hcnA* (Lapouge *et al.*, 2007) and *algU* (Martinez-Granero *et al.*, 2012) have been identified in *P. fluorescens* and *flfW* (Mukherjee *et al.*, 2011) in *B. subtilis*.

*Pectobacterium wasabiae* (*Pw*) SCC3193 (previously *Erwinia carotovora*) causes bacterial soft-rot, which is an important disease globally (Davidsson *et al.*, 2013). *Pw* is a necrotoph, and its pathogenesis relies on environmental conditions suitable for the multiplication of this opportunistic pathogen and its prolific production of plant cell wall degrading enzymes (PCWDE), which cause the typical symptoms of soft rot (Charkowski *et al.*, 2012). Among the PCWDE genes, *celV1* (Mäe *et al.*, 1995), *pehA* (Saarilahti *et al.*, 1990), *pelB* (Hei&timashimo *et al.*, 1995) and *prtW* (Marits *et al.*, 1999) have been cloned, and their secretion, regulation and role in virulence have been studied in detail. As with animal pathogens, RsmA is a central virulence regulator for *Pw* (Chatterjee *et al.*, 1995; Hyyttiäinen *et al.*, 2001; Köiv *et al.*, 2013). A non-coding small RNA (sRNA), RsmB, also participates in the RsmA regulatory circuitry of *Pw*. This sRNA contains multiple RsmA

**Abbreviations:** *GusA*, β-glucuronidase; PCWDE, plant cell wall degrading enzyme; *Pw*, *Pectobacterium wasabiae*; SD, Shine–Dalgarno; sRNA, small RNA.

Four tables are available with the online Supplementary Material.
binding sites and functions as an antagonist of CarA by sequestering it (Liu et al., 1998). RsmA represses the expression of flagella-related genes and genes encoding enzymes that degrade the cell wall (virulence factors) in plants (Chatterjee et al., 2010). Recent studies have shown these post-transcriptional regulators to have a wider range of functions in bacteria, regulating cellular processes on a scale that is underappreciated (Lapouge et al., 2008; Romeo et al., 2013; Timmermans & Van Mel-deren, 2010). We have recently demonstrated that besides being a key regulator of known virulence factors, deletion of rsmA significantly affects central metabolic and energetic pathways that are necessary for the adaptation of Pw when it colonizes the host plant. The absence of RsmA affects the mRNA levels of approximately 39% of the genes (Koiv et al., 2013).

In this study, a bioinformatics approach was used to search the Pw genomic database for genes containing potential RsmA binding sites. Potential binding sites were identified in front of 901 Rsm-affected genes, including the PCWDE genes, which are the most important virulence factors in Pw. An essential step in unravelling the role of RsmA in Pw infection is to elucidate the RsmA-mediated control pathways that are necessary for the adaptation of Pw when it colonizes the host plant. The absence of RsmA affects the mRNA levels of approximately 39% of the genes (Koiv et al., 2013).

**Methods.** *Pectobacterium wasabiae* SCC3193 (previously known as *Erwinia carotovora*) (WT), rsmA mutant (rsmA::Cm) and rsmB mutant (rsmB::Cm) have been described (Koiv et al., 2010; 2013; Nykri et al., 2013; Pirhonen et al., 1988). *E. coli* strain BL21(DE3) was used for expression of His-tagged RsmA (Novagen). Growth conditions and antibiotic concentrations were as previously used (Andresen et al., 2010; Koiv et al., 2013).

**Prediction of RsmA binding sites.** The ANGGA motifs in the genome of *Pectobacterium wasabiae* strain SCC3193 (GenBank project: PRJNA122637) (Koskimäki et al., 2012) were found using PRE-Detector software version 1.1.1 (Hiard et al., 2007). The search was restricted to nt from −16 to −8 relative to the translational start codon and no mismatches were allowed.

**Plasmids and oligonucleotides.** The plasmid-borne reporter fusions, oligonucleotides and vectors used were constructed as described in detail in Supplemental Material (Tables S2, S3, available in the online Supplementary Material). The reporter plasmid pLATS2 for transcriptional fusions was constructed from plasmid pMW119gusA (Marits et al., 1999) by PCR using the primers pKRIPT2w2 and gus_EndHindIII (Table S3). The PCR fragment contained a gusA-specific sequence together with its SD sequence and an RNaseIII-specific site. The PCR fragment was subjected to blunt-end cloning into vector pET1L.2 according to the protocol supplied by the manufacturer (Thermo Scientific). The resultant plasmid was cut with HindIII (the 1.8 kbp fragment was gel-purified) and ligated into HindIII-digested vector pMW119 (Eurogentec), generating plasmid pKRIPT2. The transcriptional reporter gene fusions pKRIPT2prtW, pKRIPT2pehA, pKRIPT2pelB, pKRIPT2celV and pKRIPT2fh were constructed by cloning PCR fragments of the regulatory regions of the respective genes (Table S2) into XbaI/EcoRI-digested vector pKRIPT2. Inserts obtained by PCR were checked for unwanted substitutions by sequencing.

The template plasmid pET24rsmA was used for the expression of histidine-tagged RsmA by cloning a NcoI/XhoI-cut PCR fragment (with primers NcoI_RsmA and XhoI_RsmA; Table S3) and cloned into the same sites of plasmid pET24(d) (Novagen).

**Purification of histidine-tagged RsmA.** An overnight culture of *E. coli* BL21(DE3) carrying plasmid pET24rsmA was diluted in fresh LB broth (1:100) and grown under aerated conditions at 37°C to OD580 0.6. Subsequently the culture was grown in the presence of 0.5 mM IPTG at 30°C for 3 h, then pelleted at 4°C. The cells were washed with 100 mM Na-phosphate buffer (pH 7), resuspended in wash buffer (100 mM Tris, pH 7.5, 1 M NaCl, 5% glycerol, 20 mM imidazole), chilled on ice for 30 min and ruptured by sonication. Histidine-tagged RsmA was purified by Ni-affinity chromatography using a histap column and an AKTA Prime Plus system (GE Healthcare Life Sciences) according to the manufacturer’s recommendations. The elution buffer contained 500 mM imidazole, 100 mM Tris pH, 7.5, 1 M NaCl and 5% glycerol. Purified RsmA-His6 was gradually dialysed against storage buffer (10 mM Tris, pH 6.8, 100 mM KCl, 10 mM MgCl2, 25% glycerol). Protein purity was confirmed on 16% Tricine-SDS-PAGE (with urea) (Schägger, 2006) and protein concentration was measured using Bradford reagent (Bio-Rad) with BSA as standard.

**Synthesis and labelling of RNA.** The pehA, pelB, pel2, prtW and celV transcripts were synthesized from the PCR products using T7 RNA polymerase. The primers used for synthesizing these fragments are shown in Table S4. The sequence of the T7 promoter was engineered into each of the 5′ primers. The PCR products were gel purified and used as templates for in vitro transcription using T7 RNA polymerase (Thermo Scientific). After RNA synthesis the reaction mixture was treated with DNase I and ethanol-precipitated. Subsequently, RNA was dephosphorylated at the 5′ end with alkaline phosphatase (FastAP, Thermo Scientific), purified by phenol/ chloroform extraction and ethanol-precipitated. Transcripts were [γ-32P]-labelled with polynucleotide kinase (Thermo Scientific) according to the instructions provided by the manufacturer. The labelled RNA was gel-purified and label incorporation was measured by liquid scintillation (Perkin Elmer).

**RNase I footprinting.** The RNA binding reaction included His-tagged RsmA at various concentrations (0–0.5 μM), labelled transcript (30 000 c.p.m.), 10 mM Tris, pH 7.5, 100 mM KCl, 10 mM MgCl2, 20 mM DTT, 1.25 μg yeast total RNA, 100 U Ribonuclease Inhibitor (Thermo Scientific) and 10% glycerol. In mixtures containing less than 0.5 μM RsmA-His6, the complementary volume of...
RsmA dialysis buffer was added. The mixtures were incubated at room temperature for 30 min and then treated with RNase I (0.00024 U µl⁻¹) for 4 min. The RNase treatment was stopped by phenol/chloroform extraction and the RNA fragments were ethanol-precipitated, resuspended in loading dye (95 % formamide, 20 mM EDTA, 0.05 % bromophenol blue, 0.05 % xylene cyanol) and loaded and separated on polyacrylamide gels using TBE as running buffer. Partial alkaline hydrolysis and RNase T1 digestion ladders (RNase V1 ladder for celV1) were run as a reference on the same gel. The resulting gels were scanned using Typhoon Phosphoimager (Amersham Biosciences).

GusA activity. The β-glucuronidase (GusA) activity of the gusA fusion constructs was measured using 4-methylumbelliferyl β-d-glucuronide (MUG; DUCHEFA Biochemie) as substrate with a method described by Andresen et al. (2010). The GusA activity of the sample was expressed as nmol substrate hydrolysed h⁻¹ ml⁻¹ per OD₆₅₀ unit, based on a standard curve of 4-methylumbelliferone. All GusA assays were performed using three technical replicates.

RESULTS

In silico analysis predicts an array of potential RsmA targets

To predict potential direct targets for RsmA we conducted a genome-wide search for the RsmA binding motif (ANGGA) near the start codon of Pw protein coding genes. CsrA binding sites in front of different genes in E. coli have been shown to lie as far as 63 nt upstream of the start codon (Mercante et al., 2009). CsrA-mediated repression often involves binding to a site overlapping the Shine–Dalgarno (SD) sequence and one or more other sites within the translation initiation region, thus preventing ribosome binding (Schubert et al., 2007). In E. coli the average distance of the ANGGA motif from the core SD sequence is 10 to 11 nt, while the distance from 8 to 16 nt is considered optimal for efficient initiation of translation (Chen et al., 1994; Ringquist et al., 1992). Therefore we limited our search to −8 to −16 nt relative to the translation start site. Our screen demonstrated that of the 901 genes with the predicted RsmA binding sites in this region, ~39 % (358) were also differently expressed in rsmA negative strain according to gene expression profiling (Fig. 1, Table S1). Specifically, expression was downregulated in 218 of these and upregulated in 140. Among the predicted targets, celV1, pehA, pelB, pel2 and prtW are known to be among the major virulence factors of Pw. In addition to the SD region we extended our search for possible RsmA binding sites to the ribosome-binding site (from −30 to +16 nt relative to the translation start site) (Hüttenhofer & Noller, 1994). The additional boundary analysis of selected genes indicated that one additional RsmA target sequence lies outside the originally characterized upstream region, −8 to −16, for two genes, celV1 and pehA (Table 1).

RsmA binds the mRNAs of virulence genes in vitro

We selected a subset of the putative RsmA-regulated targets for experimental validation. The genes celV1, pehA, pelB, pel2 and prtW were chosen because of their importance and known function in virulence. To confirm that RsmA repressed the translation of these five genes via direct RsmA-mRNA interactions, in vitro binding assays were carried out using His-tagged RsmA protein and short synthetic RNA oligonucleotides, the sequences of which were derived from the ribosome-binding site regions of the five genes (Table S4). As shown in Fig. 2 we identified one RsmA binding site in each of celV1, pehA, prtW, pel2 and pelB that overlaps the SD sequence. In addition to the binding sites predicted in silico, we identified additional RsmA binding sites upstream of the prtW and pel2 SD regions (Fig. 2). These results support the prediction that celV1, pehA, pelB, pel2 and prtW are true targets of RsmA regulation.

Effect of RsmA on efficiency of initiation of virulence gene mRNA translation

To determine whether RsmA affected the translation of the selected virulence genes in vivo, we constructed low-copy plasmid-borne translational reporter gusA fusions for pehA (pLAT2pehA), pelB (pLAT2pelB), pel2 (pLAT2pel2), prtW (pLAT2prtW) and celV1 (pLAT2celV1). In the constructs, the SD region together with the ATG start codon of gusA were replaced by a DNA fragment containing a partial sequence of the virulence gene 5’UTR including the A(N)GGA motif(s) in the SD region, start codon and small part of the reading frame cloned in-frame with the reporter gene (Fig. 3a, Table S2). All the translation constructs were transcribed from a lacZ promoter and expressed in a negative strain according to gene expression profiling.
promoter to ensure that the translational fusions are transcribed independently of the cloned sequence. To exclude the possibility that RsmA affects lacZ promoter transcription we included ffh as a negative control gene for our studies. Ffh has no RsmA recognition sequence in its SD region and it is not regulated by RsmA, according to our microarray analysis (NCBI GEO database, series accession: GSE40333).

Translational fusions with the gusA reporter were tested in three strains, the WT and both the Rsm system mutants, rsmA and rsmB. The rsmB mutant was used as a control since its phenotype resembles that of rsmA overexpression: the concentration of free RsmA in the cell is (negatively) controlled by rsmB sRNA, so rsmB inactivation increases the free RsmA level. As illustrated in Fig. 4a, the expression of the prtW-gusA translational fusion was fivefold higher in the rsmA mutant and 23 times lower in the rsmB mutant than in the WT strain. Mutation of rsmA had no effect on the expression of pehA::gusA, pelB::gusA, pel2::gusA, and modest effect on celV1::gusA in comparison to the prtW::gusA fusion (Fig. 4a). Together with the footprint results (Fig. 2), these findings suggest that RsmA had a significant negative effect on the expression of the Plac-driven translational fusion of prtW; its effect on the pehA::gusA, pelB::gusA, pel2::gusA and celV1::gusA translational fusions was less pronounced or nonexistent.

RsmA regulates the transcription of virulence genes

Data on gene expression profiling obtained by Köiv et al. (2013) were primarily derived from microarray analysis, which scarcely excluded the effects of indirect regulation by RsmA. We therefore hypothesized that RsmA also indirectly represses virulence gene expression through transcriptional regulators. To test this hypothesis we constructed low-copy plasmid-borne transcriptional reporter

Table 1. Virulence-associated PCWDE genes with RsmA binding motif in their SD region/around the ribosome-binding site (position −30 to +16 nt relative to the translation start site of the corresponding gene) and altered expression in the rsmA mutant.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Gene ID</th>
<th>FC (rsmA− WT)†</th>
<th>Distance of ANGGA from start‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>pelL</td>
<td>Pectate lyase L</td>
<td>W5S_1991</td>
<td>9.2</td>
<td>−15</td>
</tr>
<tr>
<td>celV1</td>
<td>Putative glycosyl hydrolase family 5; cellulase</td>
<td>W5S_2582</td>
<td>4.9</td>
<td>+11, −7, −15</td>
</tr>
<tr>
<td>prtW</td>
<td>Serralysin; metalloprotease</td>
<td>W5S_2894</td>
<td>4.3</td>
<td>−14</td>
</tr>
<tr>
<td>pehA</td>
<td>Polygalacturonase</td>
<td>W5S_3318</td>
<td>5.3</td>
<td>+12, −15</td>
</tr>
<tr>
<td>pelB</td>
<td>Putative pectate lyase, pel3B</td>
<td>W5S_3319</td>
<td>4.9</td>
<td>−12</td>
</tr>
<tr>
<td>pel1</td>
<td>Putative pectate lyase A</td>
<td>W5S_4192</td>
<td>4.3</td>
<td>−14</td>
</tr>
<tr>
<td>pel2</td>
<td>Pectate lyase 3</td>
<td>W5S_4193</td>
<td>6.5</td>
<td>−15</td>
</tr>
<tr>
<td>pel3</td>
<td>Putative pectate lyase A</td>
<td>W5S_4194</td>
<td>3.5</td>
<td>−15</td>
</tr>
<tr>
<td>pel4</td>
<td>Putative pectate lyase C</td>
<td>W5S_4195</td>
<td>2.5</td>
<td>−11</td>
</tr>
</tbody>
</table>

*Fold-change (FC) according to gene expression profiling (Köiv et al., 2013).
†Distance between the 5′A and the first nucleotide of Met.

Fig. 2. Footprint analysis of RsmA binding to celV1, pehA, pelB, pel2 and prtW transcripts. 5′UTR sequences of the virulence gene mRNAs were synthesized in vitro, end-labelled with [γ-32P] and subsequently used in binding reactions in the presence or absence of RsmA-His6. The positions of the RsmA footprint (asterisked) including ANGGA binding motif (marked with ellipse) and the translation initiation codon (AUG) are shown.
gusA fusions for pehA (pKRIPT2pehA), pelB (pKRIPT2pelB), prtW (pKRIPT2prtW) and celV1 (pKRIPTcelV1). Transcriptional fusion for pel2 is missing from this study as pel2 is located in a putative operon of pel genes and we were not able to construct a reliable transcriptional fusion for this gene. In the constructs reported, the transcription of the gusA reporter gene was driven by the target gene promoter. To ensure that transcriptional fusions were translated independently of the virulence gene SD, an RNase III cleavage site was introduced between the target gene and the ribosome binding sequence of the gusA reporter (Fig. 3b). Like the translational fusions, the transcriptional fusions with the gusA reporter were tested in the WT and the rsmA and rsmB mutants. The transcriptional fusions pehA :: gusA and celV1 :: gusA were strongly affected in both mutants. Expression of the pehA fusion was 2.6-fold and the celV1 fusion 4.8-fold higher in the rsmA mutant than WT. Expression of the pehA fusion was twofold and the celV1 fusion fourfold lower in the rsmB mutant than WT (Fig. 4b). Expression of the pelB :: gusA transcriptional fusion was about 2.1 times higher in the rsmA and 2.7 times lower in the rsmB mutant than WT (Fig. 4b). Thus, the total effect of RsmA could be determined by its ability to regulate the expression of pehA, celV1 and pelB, both indirectly by acting on the translation of transcriptional regulators of those genes and directly via translation of the virulence genes themselves. Finally, the transcriptional reporter activity of the prtW :: gusA fusion was about 2.4 times higher in the rsmA mutant and 2.3 times lower in the rsmB mutant than WT (Fig. 4b). The rsmA overexpression in the rsmB mutant had a stronger negative effect on the expression of the prtW :: gusA translational fusion, but had less effect on the expression of the transcriptional fusion (Fig. 4). These results suggest that RsmA controls PCWDE gene expression by integrating its regulatory activities at different levels.
DOUBLE-STRANDED RNA BINDING PROTEIN RsmA MODULATES GENE EXPRESSION IN P. AERUGINOSA

In this work, we have provided evidence that prtW, pel2, pehA, pelB, and celV1 expression remains to be determined. RsmA binding in the context of regulation implies that it probably fine-tunes the translation of these genes.

In this work, we have provided evidence that prtW, pel2, pehA, pelB, and celV1, for which the transcriptional fusions were upregulated in the rsmA mutant (Fig. 3b), were regulated by RsmA indirectly. This supports the hypothesis that negative regulation of these genes by RsmA is executed not only by binding of this protein to a specific sequence at the 5'UTR region of the mRNA, but also using other regulatory tools. Among the 358 genes of the RsmA regulon with ANGGA motifs (Fig. 1; Table S1), for which increased transcripts were recorded in an rsmA mutant, there are several with which transcriptional regulators are known or predicted (Table S1). Any of these could qualify for a candidate as RsmA-mediated indirect regulator of pehA, pel2, prtW, pelB, and celV1 expression. We assume multi-level regulation of this type will allow fine-tuning of expression of virulence genes, being dependent on RsmA level in the cell. Notably, previous studies have demonstrated that besides known functions directly regulated by RsmA/CsrA at post-transcriptional level, RsmA also indirectly affects virulence of Yersinia pseudotuberculosis by affecting expression from adhesion synthesis operon by a still-unknown mechanism (Heroven et al., 2008), via controlling translation of the transcriptional factor NahR (Pannuri et al., 2012). Additional studies established that CsrA is involved in governing the expression of several regulatory genes such as flhDC, which encodes the master regulator of motility and chemotaxis (Wei et al., 2001), relA required for the synthesis of ppGpp, the mediator of the stringent response (Edwards et al., 2011) and sdiA, the E. coli receptor of N-acetylhomoserine lactone, a mediator of quorum sensing (Yakhnin et al., 2011). The finding that the CsrA/RsmA circuitry is interconnected with other (global) regulatory networks suggests that Csr/Rsm governs cellular behaviour and physiology on a scale that is not yet fully understood.

Our finding that in P. aeruginosa the RsmA regulates the virulence genes not only directly but also indirectly reveals that its role is more complex than previously recognized. Our present and previous studies (Kõiv et al., 2013) clearly indicate that the PCWDE genes are downregulated by RsmA at both translational and transcriptional levels. We consider that fine-tuning of the RsmA activity allows bacteria to overcome a variety of environmental challenges such as surviving the host defence mechanisms by promptly modifying mRNA stability and safeguarding cells from unnecessary massive production of virulence proteins. Whether coordinated multi-step regulation of virulence traits by RsmA is a common regulatory strategy in plant-pathogenic bacteria is an important question that deserves further research.

ACKNOWLEDGEMENTS

This research was supported by Estonian Science Foundation Grant SF0180088s08 and University of Tartu Grant SLOMRARENG1.

REFERENCES

Dual role of RsmA in gene regulation


Edited by: G. Preston