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Complex autoregulation of the post-transcriptional regulator RsmA in *Pseudomonas aeruginosa*

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RsmA is a post-transcriptional RNA-binding protein that acts as a pleiotropic global regulator of mRNAs in the opportunistic pathogen *Pseudomonas aeruginosa*. Upon binding to its target, RsmA impedes the translation of the mRNA by the ribosome. The RsmA regulon affects over 500 genes, many of which have been identified as important in the pathogenicity of *P. aeruginosa*. Whilst the regulatory function of RsmA is relatively well characterized, the genetic regulation of *rsmA* itself at the transcriptional and translational levels remains poorly understood. Here, we show that RsmA is capable of self-regulation through an unorthodox mechanism. This regulation occurs via direct interaction of the protein with an RsmA-binding site located in the early portion of its coding sequence. To the best of our knowledge this is the first report of such an unusual regulation in pseudomonads.

INTRODUCTION

Rapid adaptation of bacteria requires the detection of and response to diverse environmental cues. Post-transcriptional regulation is used by bacteria to quickly adapt to changing conditions. The well-characterized CsrA/RsmA family of post-transcriptional regulators is widespread among Gram-negative bacteria and can globally affect gene expression (Romeo *et al.*, 2013). This family consists of small dimeric RNA-binding proteins that have the capacity to recognize a GGA trinucleotide present in the loop portion of a stem–loop located in the 5′ untranslated region (UTR) of a mRNA (Lapouge *et al.*, 2008). The GGA motif can be present in multiple copies in the 5′ UTR of a target mRNA. However, one GGA trinucleotide is almost always positioned close to the Shine–Dalgarno region, thus hindering the attachment to the ribosome and blocking the translation of the target mRNA (Baker *et al.*, 2002, 2007; Dubey *et al.*, 2005). Accordingly, CsrA/RsmA proteins typically act as negative regulators of mRNA translation. However, in some cases their RNA-binding activity can act as a positive translational regulator by stabilizing the mRNA (Romeo *et al.*, 2013).

In the Gram-negative opportunistic pathogen *Pseudomonas aeruginosa*, RsmA is a pleiotropic post-transcriptional regulator that modulates the expression of >500 genes (Brencic & Lory, 2009; Burrowes *et al.*, 2006). RsmA indirectly activates the expression of genes associated with the establishment of acute infections whilst repressing those implicated in the development of chronic infections (Brencic & Lory, 2009). This post-transcriptional regulator is under the control of the GacA/GacS two-component system, which is exclusively responsible for the transcription of the small RNAs (sRNAs) RsmY and RsmZ (Brencic *et al.*, 2009). These sRNAs possess numerous RsmA-binding sites and thus act as 'baits' titrating free RsmA proteins in the cell (Sonnleitner & Haas, 2011). Furthermore, many other systems can affect the activity of the Gac system, thus modulating the levels of these RsmA-repressing sRNAs (Goodman *et al.*, 2004, 2009; Ventre *et al.*, 2006). The importance of post-transcriptional regulation in *P. aeruginosa* is more complex than initially thought, as a new post-transcriptional regulator (RsmN), which shares little structural homology with RsmA, but possesses a similar mechanism of action, was reported recently (Marden *et al.*, 2013; Morris *et al.*, 2013).

Whilst the regulatory function of RsmA is relatively well characterized, the genetic regulation of *rsmA* itself at the transcriptional and translational levels remains poorly understood. The genetic regulation of *csrA*, coding for the RsmA homologue in *Escherichia coli*, is complex and dependent on the presence of multiple promoters that are activated at different stages during cell growth. Interestingly, CsrA negatively controls its translation by directly binding to its own mRNA (Yakhnin *et al.*, 2011b).

In the present study, we demonstrate that RsmA is capable of binding its own mRNA, promoting a negative feedback regulatory loop. Two RNA attachment sites are implicated in this RsmA–*rsmA* interaction. We identified an RsmA-binding motif in the 5′ portion of its coding sequence, revealing an important difference from the conventional CsrA/RsmA regulation mechanism.

Abbreviations: sRNA, small RNA; UTR, untranslated region

One supplementary table and four supplementary figures are available with the online Supplementary Material.

METHODS

Strains, plasmids and growth conditions. The bacterial strains used in this study are listed in Table 1. *P. aeruginosa* and *E. coli* strains were cultivated in Tryptic Soy Broth (TSB) medium at 37 °C with shaking (240 r.p.m.) in a TC-7 roller drum (New Brunswick) or on TSB agar plates. Antibiotics used for selection were 125 μg tetracycline ml^{-1} and 25 μg triclosan ml^{-1}.

β-Galactosidase assays. Activity of *lacZ* fusion reporters was tested for β-galactosidase activity with ONPG (Thermo Fisher Scientific) as substrate (Miller, 1972). Each experiment was performed in triplicate, at least twice. Overnight TSB cultures were diluted at a starting OD_{600} 0.05 in TSB and incubated as above. Results were obtained for five sampling points during bacterial growth over 8 h.

Purification of His_6-tagged RsmA. *E. coli* BL21(DE3) cells containing the pET29a(+)-RsmA-H6 plasmid were grown overnight in TSB with 30 μg kanamycin ml^{-1}. In the morning, the culture was diluted 1 : 100 in 100 ml pre-warmed LB. Cells were grown to mid exponential phase (OD_{600} 0.7) and IPTG was added to the culture at a final concentration of 1 mM for protein expression induction. Cells were harvested after 4 h. Cell pellets were resuspended in 20 ml 0.5 M NaCl, 20 mM NaH_2PO_4 and Tris/HCl (pH 7.65), and ruptured by sonication. Lysed cells were pelleted by centrifugation at 15 000 ***g*** for 45 min at 4 °C. Prior to purification, the supernatant was filtered on a 0.22 μM nitrocellulose filter. RsmA-His_6 was purified by using a HisTrap FF crude 5 ml column (GE Healthcare) with an Äkta FPLC system (GE Healthcare) following the manufacturer's recommendations. The protein-containing fractions were concentrated and the protein stored in a Tris/HCl (pH 7.63)/33 % glycerol conservation buffer. Purity and identity of the protein was assessed by SDS-PAGE followed by MS analysis. Concentration was estimated using a Bradford assay (Bio-Rad) with BSA as standard. The concentrated protein was stored at −20 °C until use.

Table 1. Strains/plasmids used in this study

Strain/plasmid	ED no.	Phenotype/genotype	Reference
E. coli			
SM10 (λpir)	222	*thi thr leu tonA lacY supE recA* : : RP4-2-Tc : : Mu Km *λpir*	Simon *et al.* (1983)
BL21(DE3)	778	*F–, ompT gal dcm lon hsdSB*(r_B^-, m_B^-)*λ*(*DE3* [*lacI lacUV5-T7 gene1 ind1sam7 nin5*])	Studier & Moffatt (1986)
DH5α	78	*fhuA2*Δ(*argF–lacZ*)*U169 phoA glnV44 Φ80*Δ(*lacZ*)*M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Woodcock *et al.* (1989)
P. aeruginosa			
UCBPP-PA14	14	WT strain	Rahme *et al.* (1995)
PA14 *rsmA*$^-$	282	MaR2xT7 transposon mutant	Liberati *et al.* (2006)
Plasmids			
mini-CTX-*lacZ*		Self-proficient integration vector with *lacZ* reporter	Hoang *et al.* (2000)
pET29a(+)-RsmA-H6		His_6-tagged RsmA expression vector	Brencic & Lory (2009)
pGEM-T Easy		Linearized vector with 3′ T-overhangs	Promega
pFJP1		*rsmA* WT pGEM integration Cb^R	
pFJP2		*rsmA* WT −40 5′ UTR pGEM integration Cb^R	This study
pFJP3		*rsmA* with 5′ UTR GGA→GAA inserted in pGEM Cb^R	This study
pFJP4		*rsmA* with coding sequence GGA→GAA inserted in pGEM integration Cb^R	This study
pFJP5		*rsmA* with double GGA→GAA inserted in pGEM integration Cb^R	This study
pFJP6		*rsmA* with hairpin structure disruption inserted in pGEM Cb^R	This study
pFJP7		*rsmA* with compensatory hairpin inserted in pGEM Cb^R	This study
pFJP8		*rsmA* Shine–Dalgarno point mutation inserted in pGEM Cb^R	This study
pFJP9		*rsmA* mini-CTX-*lacZ* WT reporter	This study
pFJP10		*rsmA* mini-CTX-*lacZ* −40 5′ UTR WT reporter	This study
pFJP11		*rsmA* mini-CTX-*lacZ* BS1 GGA→GAA reporter	This study
pFJP12		*rsmA* mini-CTX-*lacZ* BS2 GGA→GAA reporter	This study
pFJP13		*rsmA* mini-CTX-*lacZ* BS1/2 GGA→GAA reporter	This study
pFJP14		*rsmA* mini-CTX-*lacZ* BS2 no hairpin reporter	This study
pFJP15		*rsmA* mini-CTX-*lacZ* BS2 hairpin compensatory mutation reporter	This study
pFJP16		*rsmA* mini-CTX-*lacZ* Shine–Dalgarno GGA→GAA reporter	This study

RNA electrophoretic mobility shift assay. Fragments of *rsmA* mRNA were synthesized from PCR products using a T7 RNA polymerase. The primer sequences used are listed in Table S1 (available in the online Supplementary material). The T7 RNA polymerase promoter sequence was added to the 5′ portion of the primers. The obtained PCR fragments were purified using a BioBasic (Canada) purification kit following the manufacturer's recommendations. *In vitro* transcription was carried out in 20 µl reactions containing 20 pmol purified PCR fragment, 2 mM rNTPs, 10 µg pyrophosphatase ml^{-1} (Roche), 80 mM HEPES/KOH (pH 7.5), 24 mM $MgCl_2$, 50 mM DTT, 2.5 mM spermidine, 1 U T7 polymerase, 1 mg competitor tRNA ml^{-1} and completed with RNase-free distilled H_2O, and incubated at 37 °C for a total of 3 h. Once the transcription was completed, remaining DNA fragments were removed using 1 U RNase-free DNase I (Promega). RNA fragments were gel-purified by 12 % 8 M urea denaturing PAGE and dephosphorylated using 2.5 pmol RNA, 2 µl 10 × Antarctic phosphatase buffer, 1 U Antarctic phosphatase enzyme $µl^{-1}$ and completing to 20 µl with RNase-free distilled H_2O. The 5′ ends of the RNA fragments were radiolabelled by phosphorylation using 1 U T4 polynucleotide kinase $µl^{-1}$ and 1.85×10^5 Bq [γ-^{32}P]ATP. Labelled RNA fragments were gel-purified as described, resuspended in RNase-free distilled H_2O and stored at −20 °C until use. The RNA-binding reaction consisted of the recombinant RsmA-His_6 dimer at various concentrations, radiolabelled RNA transcript (0.6 pM), 10 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl, 50 mM KCl and 5 mM DTT, and the final mixture was adjusted to 20 µl with RNase-free distilled H_2O. The RNA-binding reaction was incubated at room temperature for 30 min, mixed with 3 µl loading dye (40 % sucrose, 0.05 % xylene cyanol and 0.05 % bromophenol blue) and loaded on a 10 % (29 : 1) native polyacrylamide gel using Tris/borate/EDTA as the running buffer. A Typhoon PhosphorImager FLA9500 (GE Healthcare) and ImageQuant software were used for gel scanning and analysis.

Translational *rsmA′–′lacZ* fusion constructions. Specific point mutations in the *rsmA* upstream intergenic region and/or coding sequence, the entire upstream intergenic region of *rsmA* and the first 36 nt of the ORF were synthesized by GenScript. The resulting plasmids were cloned into *E. coli* DH5α. Plasmid extractions were carried out using a Miniprep kit (BioBasic) and purity was assessed by gel electrophoresis. The various *rsmA* mutated alleles and the *lacZ* genes were synthesized by PCR directly from plasmids using different sets of primers (Table S1). A fusion PCR between the synthesized *rsmA* transcripts and the first 668 bp of the *lacZ* gene was performed, and the resulting fragment was purified from a 1 % agarose gel. The *rsmA′–′lacZ* fragments were ligated in pGEM-T Easy (Promega), the plasmids transformed in $CaCl_2$ thermocompetent *E. coli* DH5α cells and clones selected on TSB agar plates supplemented with 100 µg carbenicillin ml^{-1} after overnight incubation at 37 °C. Positive clones were identified by digesting Miniprep products with *Eco*RI for the presence of the insert. Overnight double digestion at 37 °C of the pGEM-T Easy plasmids containing the inserts and the destination vector mini-CTX-*lacZ* was performed using *Pst*I (Fermentas) and *Aat*II (NEB) restriction enzymes. The released inserts were gel-purified before ligation in the destination vector. Overnight ligation between the digested inserts and vector was performed using Feldan T4 ligase following the manufacturer's guidelines. Selection of the clones was done on TSB agar containing 15 µg tetracycline ml^{-1}. Positive clones were identified by double digestion of Miniprep products. The mini-CTX-*lacZ* plasmids containing the different constructions were conjugated into WT *P. aeruginosa* strain PA14 for integration in the unique *attB* site (Hoang *et al.*, 1998), giving stable, chromosomal translational reporters.

RESULTS

RsmA represses its own expression

Several recent reports (and unpublished data from our laboratory) have shown that the major *rsmA* transcriptional start site during planktonic cell growth starts at position −40 before the ATG codon (Dötsch *et al.*, 2012; Marden *et al.*, 2013; Wurtzel *et al.*, 2012). Analysis of the primary sequence of the *rsmA* 5′ UTR showed that many GGA trinucleotides are present in that region (Fig. 1a). Knowing that proteins of the CsrA/RsmA family associate with a GGA trinucleotide exposed in the loop portion of a stem–loop generally located close to the ribosome-binding site on their target mRNAs that blocks their translation (Dubey *et al.*, 2005), we hypothesized that a possible autoregulation of RsmA on its own mRNA is possible in *P. aeruginosa*. Thus, to investigate if such a regulation either at the transcriptional or translational level exists,

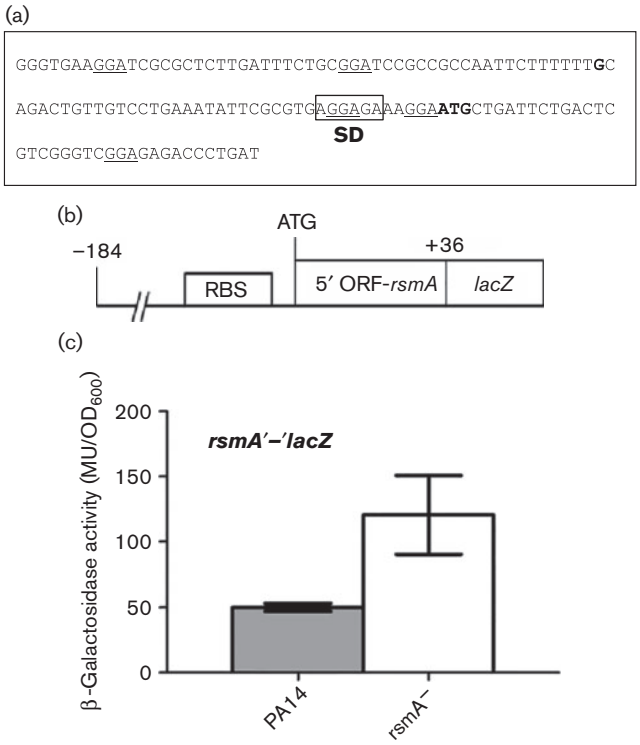

Fig. 1. Expression of the *rsmA′–′lacZ* reporter in different backgrounds. (a) Primary sequence analysis of *rsmA*. Underlined, GGA trinucleotides; bold nucleotide, −40 transcriptional start site; boxed, Shine–Dalgarno (SD) sequence; bold ATG codon, translational start site. (b) Fusion of the entire *rsmA* intergenic region and the first seven codons of its coding sequence with the 5′ end of the *lacZ* gene. RBS, ribosome-binding site; ATG, +1. (c) Expression of *rsmA* during the early growth stage using a WT reporter in various genetic backgrounds. Reporter gene activity is shown as a ratio of Miller units (MU)/OD_{600}. Data represent the mean ± SD of triplicate cultures.

we monitored the expression of *rsmA* in various backgrounds using a chromosomal *rsmA′–′lacZ* reporter containing the whole *rsmA* intergenic region and the first 12 codons of its coding sequence (Fig. 1b). As shown in Fig. 1(c), the expression of *rsmA* was increased in a Δ*rsmA* mutant background when compared with the WT, suggesting that RsmA is, in some way, responsible for negatively affecting its own expression either at the transcriptional or translational level.

RsmA binds to its own coding sequence

To further clarify how RsmA negatively affects its own expression, RNA mobility shift assays were carried out to determine whether or not RsmA can directly bind its own mRNA, thus promoting negative regulation specifically on its own translation. Upon testing the RNA fragments including the presence of possible RsmA-binding sites, we found that all the tested RNA molecules were bound by RsmA, thus blocking its own translation (Fig. 2). Unexpectedly, we observed that the coding sequence of *rsmA* was by itself sufficient for a protein–RNA interaction (lane ATG). Regulation of target mRNAs by proteins of the CsrA/RsmA family is usually exerted on the 5′ UTR of a transcript. To understand this result, we used *in silico* RNA folding of the full *rsmA* coding sequence to identify if a probable RsmA-binding site in the ORF is present that might be responsible for the observed effect. Indeed, upon analysis, a putative RsmA-binding site at position +25 after the translation start codon was identified (Fig. 3). Given that the major *rsmA* transcriptional start site is located at position −40 before the AUG codon, we investigated a portion of the *rsmA* transcript spanning from −40 to +38 around the start codon, allowing for the formation of two possible RsmA-binding sites (BS1 and BS2), to test whether or not the observed *in vitro* interaction was due to these two predicted RsmA-binding sites (Fig. 3). Interestingly, the GGA trinucleotide present in the Shine–Dalgarno sequence was never found to be exposed in a loop portion of a stem–loop during *in silico* analyses. Thus, we decided not to focus on that portion of the mRNA. Indeed, upon testing by RNA mobility shift assays, we observed that RsmA bound the WT −40 to +38 transcript (Fig. 4a, WT). Next, to decipher which of BS1 or BS2 was responsible for this RsmA–*rsmA* interaction promoting negative translational regulation, we inserted point mutations only affecting the primary nucleotide sequence at various positions in the WT −40 to +38 transcript. The presence of RsmA-binding sites was confirmed by the abolition of protein–RNA interactions when GGA trinucleotides were mutated into GAA in BS1, BS2 or both (Fig. 3), when compared with the WT transcript (Fig. 4a). Looking at the effect of an alteration in the secondary RNA structure on RsmA–*rsmA* regulation, we disrupted the formation of the predicted hairpin structure of BS2 in the early coding sequence of *rsmA* (Fig. 3, GG→CC). The introduction of such a mutation induced a loss of interaction between *rsmA* and RsmA (Fig. 4b, M4). Confirming BS2, the further introduction of a compensatory mutation that reinstated the

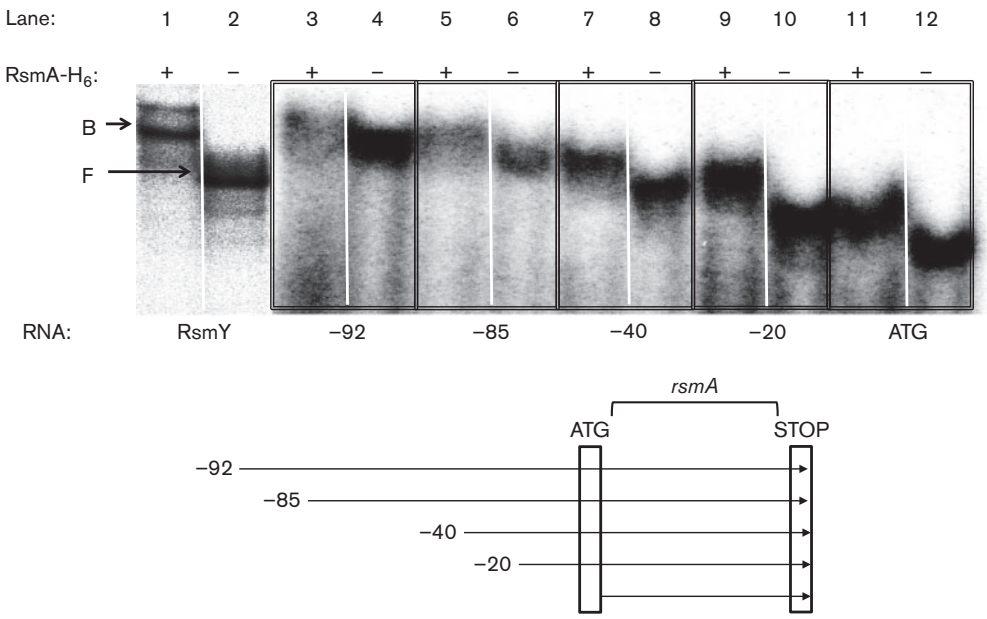

Fig. 2. RNA mobility shift assay with purified RsmA. Determination of RsmA–*rsmA* interaction using radiolabelled RNA fragments and purified RsmA. Odd lanes, RNA with protein; even lanes, RNA fragment only. The −92, −85, −40, −20 and ATG sites represent the 5′ end of the RNA molecule relative to the ATG codon. The 3′ end of each fragment is the TGA (STOP) codon of *rsmA*. RsmY sRNA was used as positive control in the experiment. H_6, hexahistidine; B, bound RNA; F, free RNA.

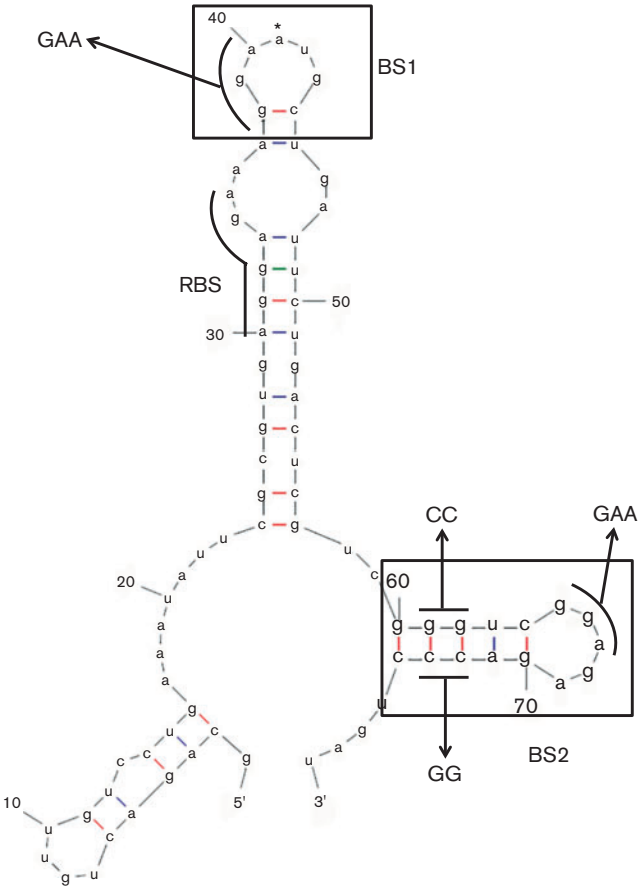

Fig. 3. The −40 to +38 *rsmA* transcript secondary RNA structure. RNA folding prediction of the *rsmA* −40 to +38 sequence using M-Fold. Boxed, putative RsmA-binding sites; BS1, binding site 1; BS2, binding site 2; curved lines, GGA trinucleotides; RBS, ribosome-binding site; arrows, inserted nucleotide polymorphisms. *AUG (START) codon of *rsmA*.

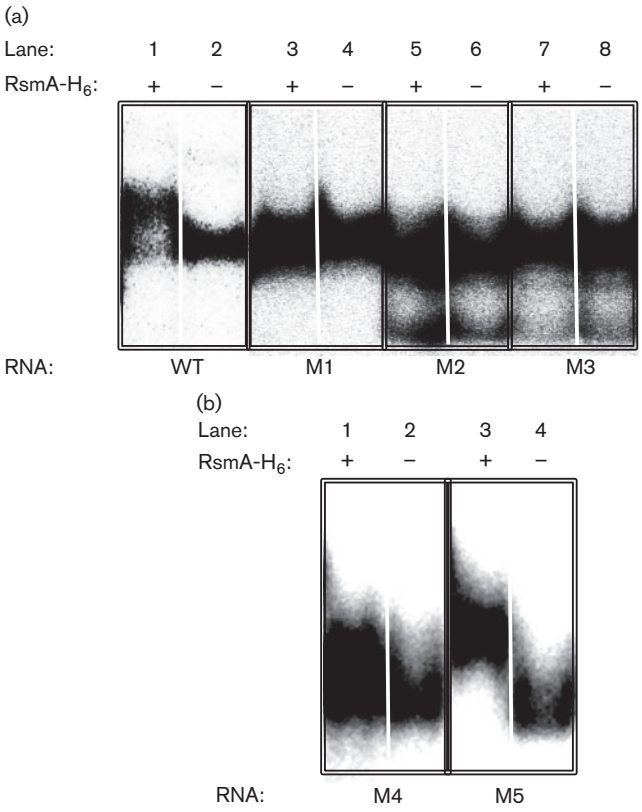

Fig. 4. RNA mobility shift assay with purified RsmA. (a) Effect of GGA→GAA trinucleotide mutations on the −40 to +38 radiolabelled RNA fragments for RsmA binding. WT, WT −40 to +38 *rsmA* fragment; M1, GGA→GAA in BS1; M2, GGA→GAA in BS2; M3, GGA→GAA in BS1 and BS2. (b) Effect of a destabilizing hairpin mutation on RsmA binding. M4, *rsmA* fragment with no hairpin in BS2 (GG→CC); M5, *rsmA* fragment with compensatory hairpin mutation in BS2 (CC→GG). Odd lanes, RNA with protein; even lanes, RNA fragment only. WT *rsmA* fragment was used as positive control in the experiment. H_6, hexahistidine.

formation of the hairpin structure to a WT conformation in M4 (Fig. 3, CC→GG) restored the affinity for RsmA towards its own transcript (Fig. 4b, M5). These results demonstrated that not only the primary nucleotide sequence is important for protein–RNA interaction, but also the presence of secondary RNA structures is essential for RsmA–*rsmA* interaction.

In vivo rsmA self-regulation is driven by multiple binding sites

To investigate the relevance of each of the identified RsmA-binding sites (BS1 and BS2), the same point mutations used in our *in vitro* gel-shift assays were introduced in an *in vivo* setting by constructing various translational *rsmA′–′lacZ* reporters (Fig. S1). Compared with the WT (Fig. 5a), the substitution of a GGA→GAA trinucleotide located in BS1 did not affect the capacity of RsmA to bind to its own transcript as the translational expression of *rsmA′–′lacZ* was still upregulated in a Δ*rsmA* background (Fig. 5b). However, the introduction of the same mutation within BS2 located in the coding sequence resulted in a complete loss of translational regulation by RsmA on itself (Fig. 5c). Surprisingly, the abolishment of both potential binding sites in BS1 and BS2 restored a WT expression pattern of *rsmA′–′lacZ* where inhibition of *rsmA* was observed (Fig. 5d). As the RsmA-binding site located within BS2 seems to be more important for the RsmA–*rsmA* interaction in an *in vivo* setting, we tested the effect of a destabilizing hairpin mutation on the translation of *rsmA*. We observed that the deletion of the stem–loop structure abolished the capacity of RsmA to repress its own translation as there was no significant difference in *rsmA* translation between the Δ*rsmA* strain and the WT (Fig. 5e). Conversely, the introduction of a compensatory mutation that restored the hairpin structure

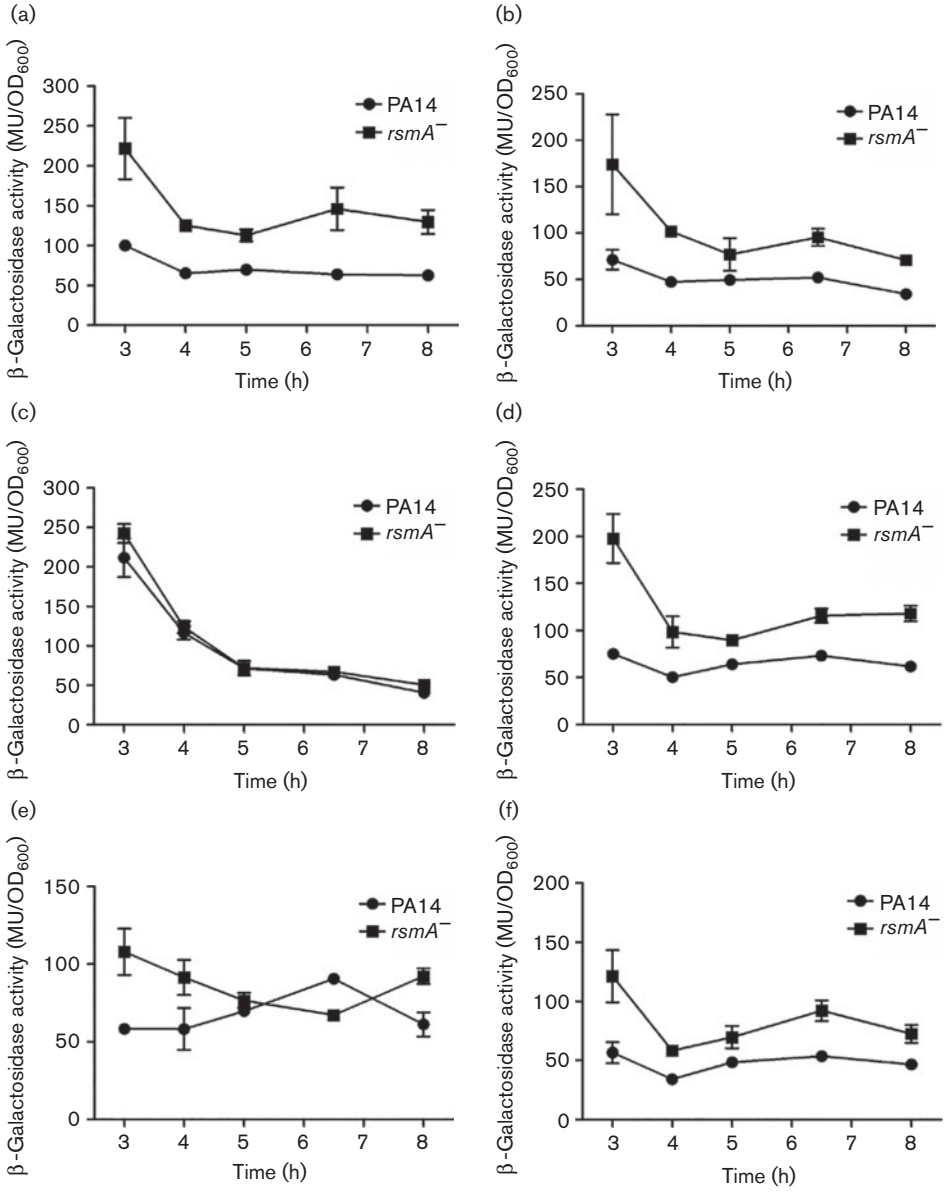

Fig. 5. Time-course of expression of *rsmA* determined by using a translational *rsmA′–′lacZ* reporter. (a) Expression of WT −40 *rsmA′–′lacZ* reporter. (b) Expression of *rsmA* containing GGA→GAA in BS1. (c) Expression of *rsmA* with GGA→GAA in BS2. (d) Expression of *rsmA* with GGA→GAA in BS1 and BS2. (e) Expression of *rsmA* with a destabilized hairpin (GG→CC) in BS2. (f) Expression of *rsmA* with a compensatory hairpin mutation (CC→GG) in BS2. Reporter gene activity is shown as a ratio of Miller units (MU)/OD_{600}. Data represent the mean ± SD of triplicate cultures.

allowed for the capacity of RsmA to interact with its transcript and inhibit the translation (Fig. 5f).

DISCUSSION

Translational regulation of target genes by the CsrA/RsmA protein family generally occurs by binding of the protein to a GGA motif exposed in a loop of a stem–loop located in the Shine–Dalgarno region of the 5′ UTR of a mRNA, thus hindering the formation of the ribosomal complex (Dubey *et al.*, 2005). In our study, the identification of several GGA trinucleotides in the primary sequence of the 5′ UTR of *rsmA* initially suggested that an autoregulation of RsmA was possible, maybe similar to what was observed for CsrA in *E. coli* (Yakhnin *et al.*, 2011b). Using a translational *rsmA′–′lacZ* reporter, we indeed observed that *rsmA* translation was de-repressed in a Δ*rsmA* background. However, we could not decipher at this point if the observed effect was due to direct regulation

implicating RsmA or via another regulator at the transcriptional or translational level.

To understand how RsmA affects its own expression, we used both *in vitro* and *in vivo* approaches to unravel the mechanisms implicated in this regulation. Our initial shift assays unexpectedly indicated that the coding sequence of *rsmA* alone was sufficient for protein–RNA interaction, which is atypical for regulators of the CsrA/RsmA family. RNA folding predictions of the full-length *rsmA* ORF revealed the presence of a potential RsmA-binding site at position +25 after the start codon. Using the *rsmA* major transcriptional start site (position −40) that was identified in three independent studies (Dötsch *et al.*, 2012; Marden *et al.*, 2013; Wurtzel *et al.*, 2012) and corroborated in our laboratory (data not shown), we performed shift assays with a shorter *rsmA* RNA molecule spanning positions −40 to +38 relative to the AUG codon, including our structure of interest. We observed that RsmA was indeed capable of binding its own mRNA, thus directly regulating its own translation negatively. However, a second possible, more conventional, binding site located in the 5′ UTR of the same RNA molecule that could be implicated in protein–RNA interaction was also identified. To investigate these BS1 and BS2 binding sites, we introduced point mutations, potentially affecting the capacity of RsmA to bind to the mRNA, in strategically located positions in the transcript by (i) modifying the GGA trinucleotide exposed in the loop portion of a stem–loop and (ii) destabilizing the predicted hairpin structure, both elements known to be important for RsmA binding activity, or (iii) restoring the formation of the latter. As expected, disrupting elements required for RsmA-binding activity abolished the capacity of RsmA to bind its own mRNA. Furthermore, the insertion of a compensatory stem–loop mutation restored a WT interaction, supporting the importance of the presence of a stem–loop structure for RsmA–*rsmA* interaction *in vitro* (Fig. S2). To confirm our results *in vivo*, we constructed various translational *rsmA′–′lacZ* reporters containing the same point mutations used in our RNA shift assays (Fig. S1) and containing both BS1 and BS2 identified to be important for RsmA–*rsmA* interaction. The determination of the *rsmA′–′lacZ* activity using a construct containing a GGA→GAA mutation in BS1 resulted in no difference in translational regulation when compared with the WT *rsmA′–′lacZ* reporter. This suggested that BS1 is not a critical regulatory binding site for RsmA self-regulation, leaving BS2 as the major RsmA target site. Indeed, when we inserted a similar mutation within the GGA trinucleotide exposed in BS2, we completely abolished the capacity of RsmA to repress its own translation, suggesting that this binding site is the major element responsible of RsmA-mediated autoregulation. However, the introduction of a GGA→GAA mutation in both BS1 and BS2 resulted in WT *rsmA* regulation. If BS2 is indeed the dominant regulatory switch, the absence of these two binding sites should be somewhat similar to what was observed for *rsmA′–′lacZ* activity for the mutation in BS2. Still, the importance of BS2 *in vivo* was further supported by the loss of regulation by RsmA on itself when a destabilizing mutation of the hairpin structure was inserted. Indeed, during early cellular growth, a negative translational regulation was noticeable, but was lost during the other growth stages. Furthermore, the introduction of a compensatory mutation allowing the formation of the hairpin structure re-established a WT regulation of RsmA on itself.

Overall, our results indicate that RsmA is a major regulatory factor affecting *rsmA* at the translational level, but that another, still unidentified, regulatory element is likely involved. RsmN, a novel RNA-binding protein different from the CsrA/RsmA protein family, could have been the mediator in such translational control. However, it has already been established that RsmN cannot directly affect RsmA translation (Marden *et al.*, 2013). To exclude the possibility of RsmA blocking its own translation through a GGA located in the Shine–Dalgarno sequence typically important for CsrA/RsmA-mediated regulation of mRNAs, we introduced a GGA→GAA nucleotide substitution in that region (Fig. S1). The insertion of such a mutation did not affect the capacity of RsmA to bind to its own transcript *in vitro* nor did it have an effect on *rsmA* translation (Figs S3 and S4). Therefore, in *P. aeruginosa*, and conversely to what has previously been reported for proteins of the CsrA/RsmA family, the presence of an RsmA-binding site in the early portion of its coding region supersedes the need for the presence of a GGA in the region of the Shine–Dalgarno for *rsmA* self-regulation itself. A recent study characterizing RsmN also noticed that RsmA can bind its own mRNA *in vitro* (Marden *et al.*, 2013), but did not investigate the RsmA-binding site.

Our data strongly indicate that RsmA self-regulation in *P. aeruginosa* diverges from the conventional protein–RNA regulation by proteins of the CsrA/RsmA family as the main binding site is located in the *rsmA* coding sequence. Furthermore, this regulation is more complex than initially thought and might implicate additional unknown regulatory elements acting at the translational level. This strengthens the importance of the control over *rsmA* through complex multiple alternative regulatory mechanisms as it is a global regulator. To the best of our knowledge, this is the first report of such a phenomenon in pseudomonads. The presence of a similar unorthodox regulation mechanism has been reported in a study investigating the regulation of CsrA on the *sdiA* mRNA in *E. coli* (Yakhnin *et al.*, 2011a). These authors demonstrated that CsrA could directly bind to two sites in the early coding sequence of that target gene, thus preventing translation by the ribosome. Thus, such an unusual regulatory mechanism exists in proteobacteria, but has yet to be further elucidated. Regulatory RNA elements are typically not sought in coding sequences as they are considered to be mostly limited to UTRs. However, our results indicate otherwise and suggest that unexplained results previously reported in studies investigating the targets of RsmA (Brencic & Lory, 2009) could be explained by other instances of the new mechanism identified here.

Lastly, sequence alignment of multiple *rsmA* coding sequences from other *P. aeruginosa* strains (PAO1, PA7)

shows that the RsmA-binding site identified in this study is also present, indicating that this new mechanism is likely a feature of this species. In contrast, the RsmA-binding site in the early coding sequence is absent from other *Pseudomonas* species (*Pseudomonas stutzeri*, *Pseudomonas fluorescens* and *Pseudomonas entomophila*), suggesting different mechanisms for environmental adaptation to different ecological niches as RsmA is a pleiotropic gene regulator.

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