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A CsrA/RsmA translational regulator gene encoded in the replication region of a *Sinorhizobium meliloti* cryptic plasmid complements *Pseudomonas fluorescens rsmA/E* mutants

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Members of the CsrA/RsmA family are global regulatory proteins that bind to mRNAs, usually at the ribosome-binding site, to control mRNA translation and stability. Their activity is counteracted by small non-coding RNAs (sRNAs), which offer several binding sites to compete with mRNA binding. The *csrA/rsmA* genes are widespread in prokaryotic chromosomes, although certain phylogenetic groups such as Alphaproteobacteria lack this type of global regulator. Interestingly, a *csrA/rsmA*-like sequence was identified in the replication region of plasmid pMBA19a from the alphaproteobacterium *Sinorhizobium meliloti*. This *rsmA*-like allele ($rsmA_{Sm}$) is 58 % identical to *Xanthomonas axonopodis* pv. *citri* chromosomal *rsmA* and bears an unusual C-terminal extension that may fold into an extra α-helix. Homology-based modelling of $RsmA_{Sm}$ suggests that all key mRNA-binding residues are conserved and correctly positioned in the RNA-binding pocket. In fact, a 1.6 kb fragment from pMBA19a encompassing the $rsmA_{Sm}$ locus restored *rsmA/E*-dependent phenotypes of *rsmA/E gacS Pseudomonas fluorescens* mutants. The functionality of $RsmA_{Sm}$ was confirmed by the gain of control over target *aprA′–′lacZ* and *hcnA′–′lacZ* translational fusions in the same mutant background. The $RsmA_{Sm}$ activity correlated with Western blot detection of the polypeptide. Phenotype and translational fusion data from *rsmA/E P. fluorescens* mutants expressing RsmX/Y/Z RNAs indicated that $RsmA_{Sm}$ is able to bind these antagonistic sRNAs. In agreement with the latter observation, it was also found that the sRNA RsmY was stabilized by $RsmA_{Sm}$. Deletion of the C-terminal extra α-helix of $RsmA_{Sm}$ affected its cellular concentration, but increased its relative RNA-binding activity. This is believed to be the first report of the presence and characterization of a functional *csrA/rsmA* homologue in a mobile genetic element.

INTRODUCTION

Post-transcriptional regulatory mechanisms usually exert a fine-tuning control of gene expression, being in most cases dependent on RNA-binding proteins that impose translational control on target mRNAs (Kaberdin & Bläsi, 2006). This is the case for members of the CsrA/RsmA protein family. CsrA (carbon storage regulator A) was first identified and characterized in *Escherichia coli* (Romeo *et al.*, 1993), whereas RsmA (repressor of secondary metabolites A) was discovered later in *Erwinia carotovora* (Chatterjee *et al.*, 1995) and a few years later in *Pseudomonas fluorescens* (Blumer *et al.*, 1999). These are small dimeric proteins that bind to RNA sequence motifs typically (but not exclusively) present around the ribosome-binding site of target mRNAs. As a consequence of mRNA binding, CsrA/RsmA competes for ribosome access and/or influences mRNA stability, thus affecting the translation rate of the bound mRNAs (Romeo *et al.*, 2012). This regulatory mechanism is reversed by small non-coding RNA molecules (sRNAs), which bear a number of RNA motifs equivalent to those present in target mRNAs (Liu *et al.*, 1997). These RNA motifs contain conserved GGA triplets that are critical for the activity and stability of antagonistic sRNAs (Dubey *et al.*, 2005; Valverde *et al.*, 2004). Thus, the antagonistic sRNAs are able to outcompete bound mRNAs and release the translational control exerted by CsrA/RsmA proteins (Romeo *et al.*, 2012). As expected, the intracellular level of

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Abbreviations: CsrA, carbon storage regulator A; RsmA, repressor of secondary metabolites A; sRNA, small non-coding RNA.

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the antagonistic sRNAs is itself regulated by cellular and extracellular cues (Valverde & Haas, 2008). As CsrA/RsmA proteins bind to a number of mRNAs, induction of the competing mimic sRNAs results in global control of gene and operon expression (Romeo *et al.*, 2012).

Inspection of the large number of prokaryotic genome sequences available led to the conclusion that *csrA/rsmA* genes are heterogeneously present in a wide variety of eubacteria (see Table S1 available with the online version of this paper), in single or multiple copies. *E. coli* and *Bacillus subtilis*, for instance, have one *csrA* gene (Romeo & Gong, 1993; Yakhnin *et al.*, 2007), whereas *P. fluorescens* strain CHA0 encodes two homologue proteins, RsmA and RsmE (Reimmann *et al.*, 2005), and some *Pseudomonas* species, such as *Pseudomonas syringae* and *Pseudomonas putida*, have up to four CsrA/RsmA-like homologues (Lapouge *et al.*, 2008). In all cases, the *csrA/rsmA*-like genes are encoded in chromosomes. On the other hand, certain eubacterial divisions lack chromosomal CsrA/RsmA homologue genes, as is the case of sequenced Alphaproteobacteria (Table S1). Functional studies of CsrA/RsmA homologues have only been carried out in a few taxa other than Gammaproteobacteria, for example *B. subtilis*, *Helicobacter pylori* and *Borrelia burgdorferi* (Barnard *et al.*, 2004; Sze *et al.*, 2011; Yakhnin *et al.*, 2007). Interestingly, a *csrA/rsmA* homologue sequence has been detected in the replication region of the alphaproteobacterium *Sinorhizobium meliloti* cryptic plasmid pMBA19a, although it has not been further characterized (Watson & Heys, 2006). This work reports for what is believed to be the first time the functional characterization of a *csrA/rsmA* gene encoded in a mobile genetic element.

METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *P. fluorescens* strain CHA0 has been recently taxonomically reassigned to the novel species

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genetic and/or phenotypic features	Reference or source
***E. coli* strains**		
K12-BW3413	Δ*lac*U169	Romeo *et al.* (1993)
TR5-1BW3413	*csrA*::ΩKm	Romeo *et al.* (1993)
***P. aeruginosa* strains**		
PAO1	Wild-type	ATCC 15692
PAZH13	Δ*rsmA*	Heurlier *et al.* (2004)
P. fluorescens* strains		
CHA0	Wild-type	Stutz *et al.* (1986)
CHA19	Δ*gacS*	Zuber *et al.* (2003)
CHA1007	*rsmA*::ΩKm *rsmE*::ΩHg Δ*gacS aprA′–′lacZ*; Km^R Hg^R	Reimmann *et al.* (2005)
CHA1008	*rsmA*::ΩKm *rsmE*::ΩHg Δ*gacS*; Km^R Hg^R	Reimmann *et al.* (2005)
CHA1009	*rsmA*::ΩKm *rsmE*::ΩHg; Km^R Hg^R	Reimmann *et al.* (2005)
CHA1021	*rsmA*::ΩKm *rsmE*::ΩHg *aprA′–′lacZ*; Km^R Hg^R	Reimmann *et al.* (2005)
CHA1027	*rsmA*::ΩKm *rsmE*::ΩHg *hcnA′–′lacZ*; Km^R Hg^R	Reimmann *et al.* (2005)
CHA1028	*rsmA*::ΩKm *rsmE*::ΩHg Δ*gacS hcnA′–′lacZ*; Km^R Hg^R	Reimmann *et al.* (2005)
***S. meliloti* strains**		
2011	Wild-type, Sm^R	Meade & Signer (1977)
JJ1c10	Reference strain lacking cryptic plasmids	Watson & Heys (2006)
JJ1c10/pBB84	JJ1c10 bearing plasmid pBB84	Watson & Heys (2006)
MBA19	Wild-type isolate bearing cryptic plasmid pMBA19a	Watson & Heys (2006)
Plasmids		
pME6000	Cloning vector, pBBR1MCS derivative; Tc^R	Maurhofer *et al.* (1998)
pBB84	pBR328 containing a 4.5 kb *Eco*RI fragment with the *rep* region of *S. meliloti* plasmid pMBA19a; Ap^R Tc^R	Watson & Heys (2006)
pSM1	pME6000 containing the 1.6 kb *Eco*RI–*Pst*I fragment from pBB84 with ORF I encoding a CsrA/RsmA homologue; Tc^R	This work
pSM2	pME6000 containing a 0.5 kb *Eco*RI–*Pst*I fragment from pBB84 with ORF I encoding a CsrA/RsmA homologue; Tc^R	This work
pSMΔC_t	pME6000 containing a 0.45 kb *Eco*RI–*Pst*I fragment from pBB84 with an ORF I mutant encoding a CsrA/RsmA homologue bearing a C-terminal deletion (Δ62–77); Tc^R	This work

**P. fluorescens* strain CHA0 has been recently reassigned to *Pseudomonas protegens* (Ramette *et al.*, 2011).

Pseudomonas protegens (Ramette *et al.*, 2011). In this work, we keep the former taxonomic designation, which appears in all the literature related to genetics of the Gac/Rsm cascade. *Pseudomonas* spp. and *E. coli* were usually grown on nutrient agar (NA) and in nutrient yeast broth (NYB) (Valverde *et al.*, 2003), whereas *S. meliloti* was grown in agarized or liquid TY medium (Sobrero & Valverde, 2011). When required, tetracycline was added to the growth medium at 125 μg ml^{-1} for *P. fluorescens* strains and 5 μg ml^{-1} for *S. meliloti* strains. Routine incubation temperature was 28 °C. *P. fluorescens* strains were grown at 35 °C to improve their capacity to accept heterologous DNA in electrotransformation with plasmids.

DNA manipulation and cloning procedures. DNA preparations were obtained and cloning steps were carried out according to standard protocols (Sambrook *et al.*, 1989). Small-scale plasmid preparations were obtained with the one-tube cetyltrimethylammonium bromide (CTAB) method (Del Sal *et al.*, 1988) and high-quality plasmid preparations with the Jet-Quick miniprep spin kit (Genomed). PCRs were carried out as reported previously (Valverde, 2009). DNA fragments were purified from agarose gels with Qiaex II (Qiagen). All cloned PCR products were verified by sequencing from both ends by Macrogen.

Construction of plasmids. A 1.6 kb fragment from pBB84 encoding the *rsmA*$_{Sm}$ allele was subcloned as an *Eco*RI–*Pst*I insert into pME6000 to generate plasmid pSM1 (Fig. 1). Vector pSM2 was generated by PCR-amplifying the *rsmA*$_{Sm}$ locus with primers rsmAR (5′-TGTACTGCAGCATGTAATCCCGCAGCAGC-3′) and rsmAF1 (5′-TCTGAATTCTTATTCCTCGTTGGACTGG-3′), which introduced *Pst*I and *Eco*RI sites (underlined) at the borders of the amplicon. The 0.5 kb PCR product was treated with *Eco*RI/*Pst*I and cloned into pME6000 to give pSM2 (Fig. 1). Vector pSMΔC_t was generated by PCR-amplifying the *rsmA*$_{Sm}$ locus with primers rsmAR and rsmAF2 (5′-AATGAATTCTTACGGAGGAGCCGGAGGAACC-3′), thus introducing a C-terminal deletion of 48 bp (residues 62–77). The 0.45 kb PCR product was treated with *Eco*RI/*Pst*I and cloned into pME6000 to give pSMΔCt. In all three constructs the *rsmA*$_{Sm}$ gene and its own promoter region lay in the opposite orientation from that of the pME6000 vector P_{lac} promoter. Purified plasmids were transferred to *P. fluorescens* cells by electrotransformation.

Detection of RsmA-like proteins by Western blotting. Erlenmeyer flasks containing 20 ml NYB amended with 0.05 % (w/v) Triton X-100 were inoculated at 1 : 100 from overnight saturated cultures and grown with shaking at 200 r.p.m. Cells equivalent to an OD_{600} of 0.4 U ml^{-1} were centrifuged, washed with 0.9 % (w/v) NaCl, resuspended in 20 μl loading buffer [50 mM Tris/HCl, pH 6.8, 2 % (w/v) SDS, 0.1 % (w/v) bromophenol blue, 15 % (v/v) glycerol, 5 % (v/v) β-mercaptoethanol] and immediately treated at 100 °C for 10 min. Samples (15 μl) were electrophoresed in a 12 % (w/v) acrylamide-bisacrylamide gel (Laemmli, 1970) and electrotransferred onto PVDF membranes (Immobilon P; Millipore). Immunodetection of RsmA-like proteins was carried out as reported elsewhere using polyclonal antibodies raised against purified *Yersinia enterocolitica* RsmA (Reimmann *et al.*, 2005).

Modelling of RsmA$_{Sm}$ structure. Sequence alignments were generated with CLUSTAL W (Larkin *et al.*, 2007). The JPRED2 algorithm was used for secondary structure prediction (Cuff *et al.*, 1998). A model of RsmA$_{Sm}$ tertiary structure was generated by a homology-based procedure using RsmE from *P. fluorescens* CHA0 (PDB 2JPP) as a template (Schubert *et al.*, 2007). Models were obtained with the program MODELLER (Šali *et al.*, 1995), run in the TITO server (Labesse & Mornon, 1998), and structures were validated by calculating geometric (RAMPAGE – Ramachandran plot assessment) or energetic (PROSAII) parameters (Lovell *et al.*, 2003; Wiederstein & Sippl, 2007). Surface potential was calculated with APBS (Adaptative Poisson–Boltzmann Solver) (Baker *et al.*, 2001) and visualized with PyMol (http://www.pymol.org/).

Detection of HCN, exoprotease and antagonism against *Pythium ultimum* in *P. fluorescens*. For quantitative hydrogen cyanide (HCN) determinations, *P. fluorescens* cultures were grown in Parafilm-sealed 15 ml tubes containing 5 ml NYB, with shaking (200 r.p.m.). Cyanide production was quantified colorimetrically in overnight cultures (Gewitz *et al.*, 1976). Exoprotease activity was detected on skimmed milk agar plates (Sacherer *et al.*, 1994). Antagonism against *Pythium ultimum* isolate Pu-67 was assessed as hyphal growth inhibition in dual plate assays (Ongena *et al.*, 1999).

Detection of pyocyanin in *Pseudomonas aeruginosa*. Pyocyanin production was estimated from overnight cultures in King's A agar plates (Olivas *et al.*, 2012). Equal square pieces of agarized medium with bacterial lawn (approx. 2 cm^2) were excised from plates and bacterial cells were removed by repeated pipetting of 1 ml of 0.9 % (w/v) NaCl. Pyocyanin was then extracted from homogenized agar with 3 ml chloroform followed by re-extraction into 1 ml of 0.2 M HCl. Pyocyanin concentration was estimated by measuring absorbance at 520 nm. Measurements were normalized to cell density measured as the OD_{600} of cell suspensions in 0.9 % (w/v) NaCl.

Glycogen production and motility assay in *E. coli*. Glycogen content of *E. coli* strains was estimated by anthrone colorimetry, as reported previously (Valverde *et al.*, 2004). One millilitre of culture was centrifuged, and cells were washed with 0.9 % (w/v) NaCl. Then, 100 μl of 1 M NaOH was added, and the cell suspension was incubated for 30 min at 55 °C to promote cell lysis. Lysates were neutralized with 100 μl of 1 M HCl. The content of hexoses was determined by mixing an aliquot of the lysates with anthrone reagent [Sigma; 0.2 % (w/v) in 98 % sulfuric acid] and incubating in a boiling water bath for 10 min. After cooling the tubes on ice, absorption was measured at 620 nm. The standard curve was prepared with glucose (1 mg glucose equals 0.9 mg glycogen). The protein content of cell lysates was determined with the Bradford method. Glycogen content was expressed as milligrams of glycogen per milligram of protein. Spreading of *E. coli* strains by swimming motility was assessed on semi-solid agar plates [0.5 % (w/v) yeast extract, 2.5 % (w/v) nutrient broth, 0.3 % (w/v) agar]. Freshly isolated colonies were spotted onto swimming plates with a toothpick and incubated overnight in sealed plastic bags at 30 °C (Valverde *et al.*, 2004).

***β*-Galactosidase assays.** Strains were grown in 20 ml NYB (in 125 ml Erlenmeyer flasks) with shaking at 200 r.p.m. Triton X-100 was routinely added at 0.05 % (w/v) to avoid cell aggregation. β-Galactosidase activities were quantified by the Miller method (Miller, 1972), with cells permeabilized with 5 % (v/v) toluene.

RNA preparation and Northern blots. RNA preparations from *P. fluorescens* strains and Northern blotting were done as described previously (Valverde *et al.*, 2004). RsmY stability was estimated after addition of rifampicin (200 μg ml^{-1}) to near-stationary cultures and analysed by Northern blotting (Reimmann *et al.*, 2005).

RESULTS

The *rsmA*$_{Sm}$ locus in the *S. meliloti* cryptic plasmid pMBA19a

The *S. meliloti* isolate MBA19 contains the 36 kb plasmid pMBA19a, whose encoded functions are dispensable for the

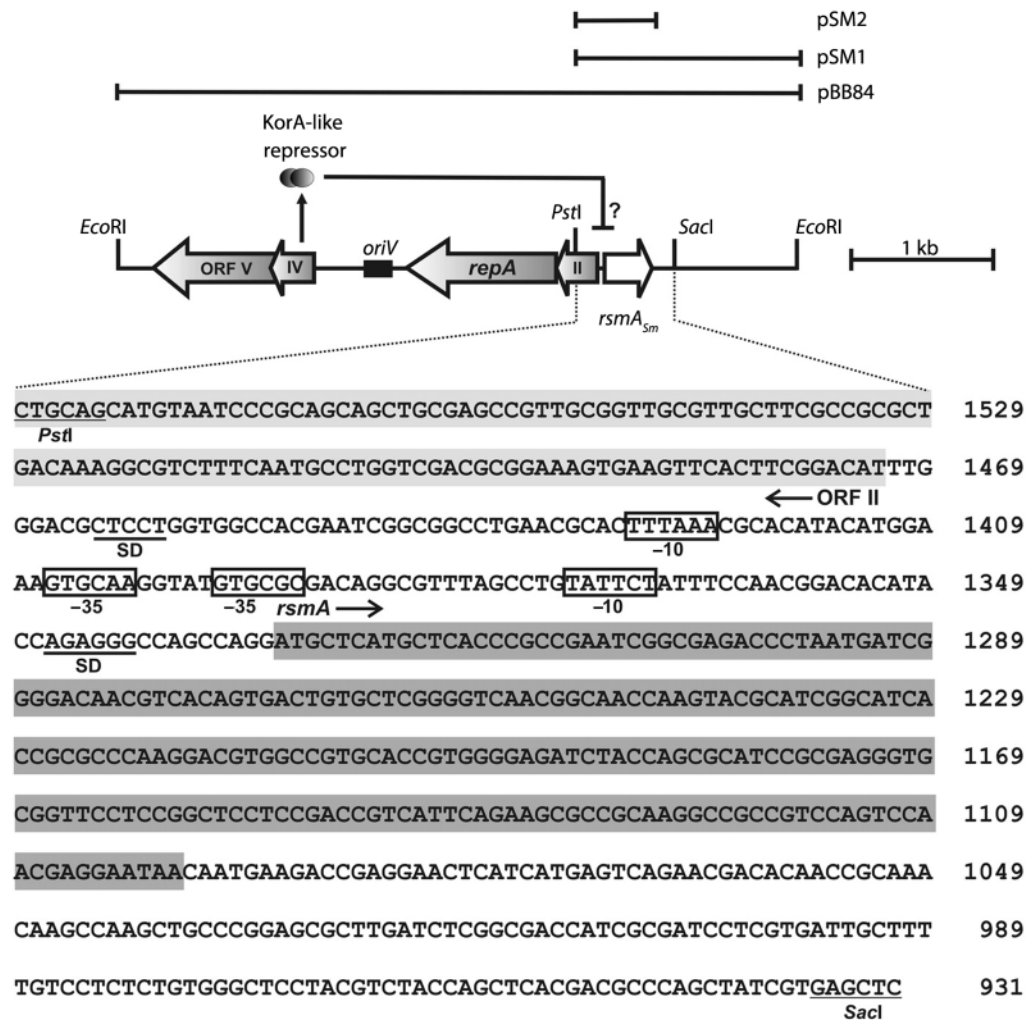

Fig. 1. A *csrA/rsmA* homologue gene (*rsmA*$_{Sm}$) in the replication region of an *S. meliloti* cryptic plasmid. Organization of the pMBA19a replication region (Watson & Heys, 2006) and sequence features of the *rsmA*$_{Sm}$ locus. The 4.5 kb replication region encodes four ORFs in addition to *rsmA*$_{Sm}$: a protein of unknown function (ORF II), the replication initiation protein RepA, a putative KorA-like transcriptional repressor possibly involved in plasmid copy number control (ORF IV), and a putative IncC-like regulatory protein which may also be implicated in plasmid maintenance and replication (ORF V). The DNA sequence of the 0.65 kb *Pst*I–*Sac*I fragment is shown below the replication region diagram. The *rsmA*$_{Sm}$ coding sequence is shaded in dark grey. The sequence of the divergently encoded ORF II gene is shaded in light grey. Putative σ^{70}-dependent promoters (boxed) were identified via the Softberry Bprom algorithm (http://linux1.softberry.com/berry.phtml). SD, putative Shine–Dalgarno motifs of *rsmA*$_{Sm}$ and ORF II transcripts. The different segments of the original pMBA19a *rep* region that were subcloned in vectors pBB84, pSM1 and pSM2 are indicated above the diagram.

host (i.e. pMBA19a is a cryptic plasmid) (Watson & Heys, 2006). In addition to genes required for plasmid replication and maintenance, the 4.5 kb replication region of pMBA19a contains a small ORF in the vicinity of the *repA* gene that is 82 % identical to the *Xanthomonas axonopodis* carbon storage regulator CsrA (NCBI reference sequence NP_642074.1) (Fig. 1). The coding sequence of this *csrA/rsmA* homologue (hereafter *rsmA*$_{Sm}$) is preceded by a typical AG-rich Shine–Dalgarno motif and by a putative σ^{70}-dependent promoter identified by the Bprom algorithm (http://linux1.softberry.com/berry.phtml), which may drive *rsmA*$_{Sm}$ expression (Fig. 1). A second divergent σ^{70}-dependent promoter was identified in the same region, which may control transcription of the ORF II–*repA* operon (Fig. 1). ORFs IV and V resemble the transcriptional repressor protein KorA and the IncC regulatory protein from the broad-host-range plasmid RK2, respectively (Fig. 1). Members of the CsrA/RsmA family, although widespread in the eubacterial domain, had not previously been found as part of mobile elements, nor identified in alphaproteobacterial species. This finding prompted us to study the functionality of this CsrA/RsmA homologue gene.

Structural features of the RsmA$_{Sm}$ polypeptide

The RsmA$_{Sm}$ homologue present in the pMBA19a *rep* region has 77 residues and a predicted molecular mass of 8.4 kDa. It is an unusual member of the CsrA/RsmA family in that the first 50 residues are strongly conserved among other bacterial species (Fig. 2a), but the last 27 residues form a C-terminal extension that resembles that of Gram-positive species (Yakhnin *et al.*, 2007) (Fig. 2b). Overall, the predicted secondary structure is similar to other CsrA/RsmA partners with a $\beta_1-\beta_2-\beta_3-\beta_4-\alpha$ arrangement, and the extra α-helix (Fig. 2b). The high level of amino acid identity found between the first 53 residues of RsmA$_{Sm}$ and those of the *P. fluorescens* RsmE homologue (Fig. 2c) served as a basis for the structural homology modelling of the RsmA$_{Sm}$ dimer excluding the C-terminal α-helix. The modelled RsmA$_{Sm}$ dimer showed almost perfect overlap with the crystal structures determined for *P. fluorescens* RsmE (Schubert *et al.*, 2007), *P. aeruginosa* RsmA (Rife *et al.*, 2005) and *Y. enterocolitica* RsmA (Heeb *et al.*, 2006) (Fig. 2d). Moreover, both the position of critical residues for CsrA/RsmA activity and a

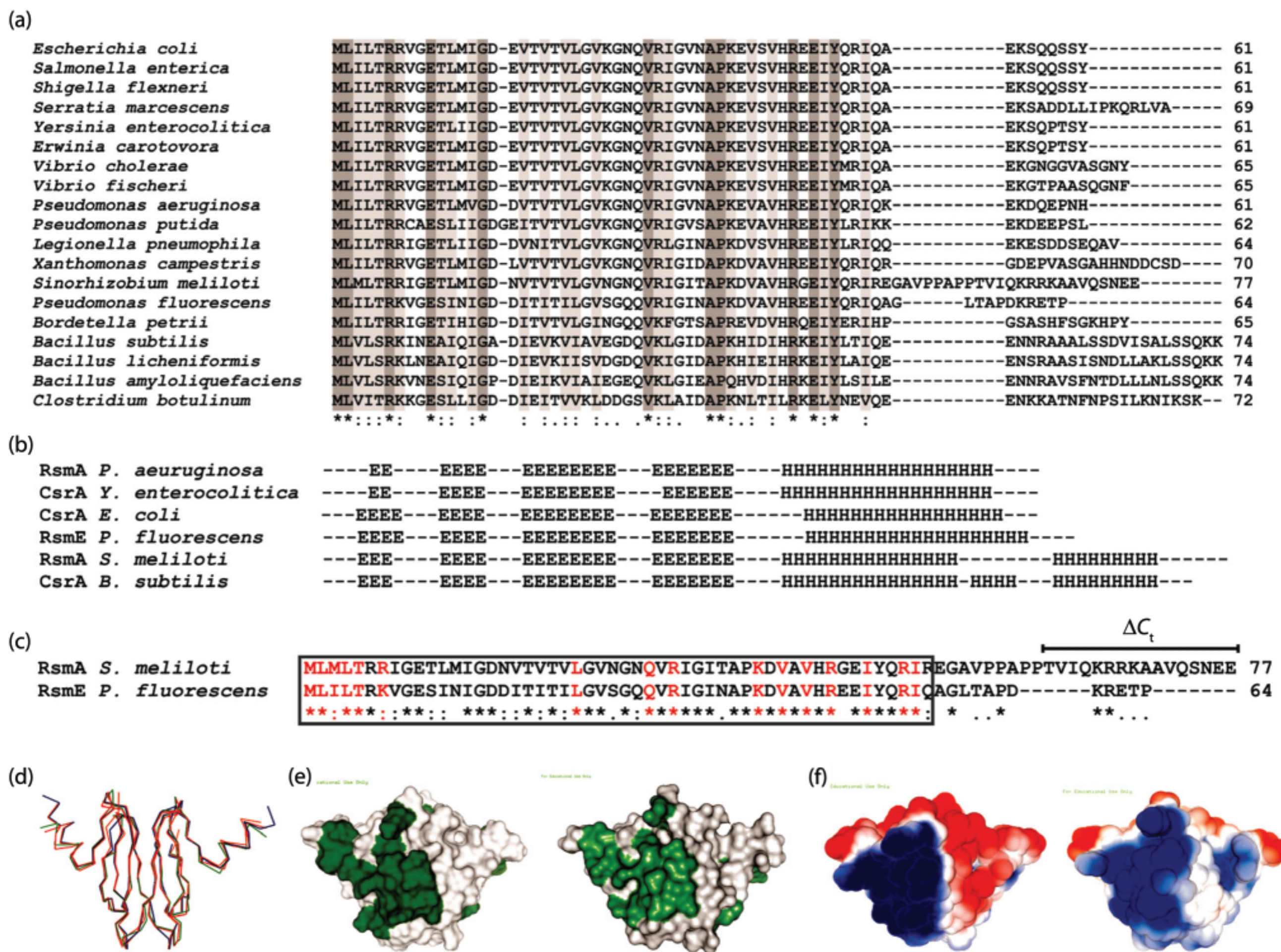

Fig. 2. Sequence features and structural modelling of RsmA$_{Sm}$ protein. (a) Sequence homology between RsmA$_{Sm}$ and selected eubacterial CsrA/RsmA proteins. (b) Comparison of the predicted secondary structure of RsmA$_{Sm}$ and selected eubacterial CsrA/RsmA proteins. H, residues predicted to fold into α-helix; E, residues predicted to fold into β-stranded regions; –, unstructured regions. (c) Sequence homology between RsmA$_{Sm}$ and *P. fluorescens* CHA0 RsmE protein. The boxed region delimits the highly conserved CsrA/RsmA core. Residues in light grey are conserved amino acids in the RNA-binding pocket. ΔC_t indicates the residues deleted in the RsmA$_{Sm}$ C-terminal region (plasmid pSMΔC_t). (d) Structural alignment of the predicted RsmA$_{Sm}$ dimer (red) and the CsrA/RsmA proteins of *P. fluorescens* (PDB 2JPP, blue), *Y. enterocolitica* (PDB 2BTI, green) and *P. aeruginosa* (PDB 1VPZ, orange). The models were validated geometrically by RAMPAGE (95.7 % of the residues are in favourable regions of the Ramachandran plot) and energetically by PROSAII with a *Z* score of −5.54, which falls within the range typically found for native proteins of similar size. (e) Location of identical and functionally conserved amino acid residues in RsmA$_{Sm}$ (left) and *P. fluorescens* RsmE (right) dimers is highlighted in green. (f) Predicted charge distribution along the surface of RsmA$_{Sm}$ (left) and *P. fluorescens* RsmE (right) dimers. Blue, positively charged residues; red, negatively charged residues.

similar surface charge distribution are conserved in the $RsmA_{Sm}$ RNA-binding pocket (Fig. 2e, f). Together, this *in silico* evidence strongly suggests that, if expressed, $RsmA_{Sm}$ may be a functional post-transcriptional repressor of the CsrA/RsmA family.

The *$rsmA_{Sm}$* gene negatively controls Gac/Rsm-dependent phenotypes in *P. fluorescens*

In *P. fluorescens* strain CHA0, the membrane-bound GacS sensor and the cytoplasmic transcriptional regulator GacA constitute a two-component system that, upon entry into early stationary phase, induces expression of *rsmX/Y/Z* genes, which encode RsmA/E-antagonistic sRNAs (Laville *et al.*, 1992; Zuber *et al.*, 2003). Thus, the simultaneous deletion of *rsmX/Y/Z* genes or of those encoding the GacS/A system allows RsmA and RsmE proteins to fully repress translation of mRNAs encoding proteins for the synthesis of several extracellular products (e.g. exoprotease AprA, HCN, antifungal compounds) (Kay *et al.*, 2005; Laville *et al.*, 1992; Zuber *et al.*, 2003). In this background, genetic inactivation of *rsmA* and *rsmE* results in maximal expression of target mRNAs (Reimmann *et al.*, 2005). Thus, *gacS rsmA rsmE* triple mutants are suitable hosts to test the functionality of heterologous members of the CsrA/RsmA family, because introduction of a foreign *csrA/rsmA*-like gene should restore translational control of target genes and reveal a negative effect on the production of extracellular metabolites. As *gacS rsmA rsmE* triple mutants are unable to express the RsmA/E-antagonistic *rsmX/Y/Z* genes (Reimmann *et al.*, 2005), introduction of a foreign *csrA/rsmA*-like gene in this background would reveal its full repressive potential towards RsmA/E mRNA targets. In turn, *P. fluorescens rsmA/E* double mutants with a wild-type and functional *gacS* gene (Reimmann *et al.*, 2005) serve to study the ability of heterologous CsrA/RsmA proteins to interact with, and to be counteracted by, the small regulatory RNAs RsmX/Y/Z.

With this in mind, the 1.6 kb *Eco*RI–*Pst*I fragment from pBB84 was subcloned into pME6000 to give vector pSM1, in which *$rsmA_{Sm}$* would be expressed from its own promoter (Fig. 1), and used to transform *P. fluorescens rsmA rsmE* mutant strains (Table 1). As the *rep* region identified for pMBA19a (Fig. 1) resembled that of the broad-host-range plasmid pVS1 (Watson & Heys, 2006), strains were also transformed with pBB84. Next, we studied the impact of *$rsmA_{Sm}$* expression on Gac/Rsm-dependent phenotypes such as antagonism of *Pythium ultimum*, AprA activity and HCN production (Fig. 3). Wild-type strain CHA0 produces the antibiotic DAPG (2,4-diacetylphloroglucinol) and inhibits growth of the oomycete *Pythium ultimum* in dual culture plates, whereas the *gacS* mutant CHA19 no longer expresses the DAPG biosynthetic genes due to repression of the *phl* operon by RsmA and RsmE proteins (Fig. 3a). Expression of *$rsmA_{Sm}$* from vector pSM1 in strain CHA1008 (*rsmA rsmE gacS*) resulted in reduced antagonism of *Pythium ultimum* (Fig. 3a), and in a drastic reduction in AprA activity (Fig. 3b) and HCN production (Fig. 3c). Surprisingly, the *$rsmA_{Sm}$* allele in pBB84 did not result in a reduction of antagonism of *Pythium ultimum*, of AprA activity or of HCN production (Fig. 3).

The *$rsmA_{Sm}$* gene acts as a repressor of Gac/Rsm-dependent genes in *P. fluorescens*

The results described above suggest strongly that $RsmA_{Sm}$ imposed a negative control on AprA activity and HCN production at the translational level. Thus, we followed the expression pattern of chromosomal *hcnA′–′lacZ* and *aprA′–′lacZ* translational fusions in *P. fluorescens rsmA rsmE gacS* mutant strains. For both reporter genes, *$rsmA_{Sm}$* in pSM1 resulted in a strong repression (Fig. 4). However, as observed for the corresponding phenotypes (Fig. 3b, c), the presence of plasmid pBB84 did not significantly affect *hcnA′–′lacZ* or *aprA′–′lacZ* expression (Fig. 4). Thus, *$rsmA_{Sm}$* is not expressed or is expressed at a very low level in *P. fluorescens* strains bearing pBB84.

$RsmA_{Sm}$ function is antagonized by *P. fluorescens* RsmX/Y/Z sRNAs

Repression of HCN, AprA and antibiotic production by $RsmA_{Sm}$ indirectly suggests that this protein is able to bind to the recognition motifs present in the vicinity of the ribosome-binding site of the corresponding mRNAs (Lapouge *et al.*, 2007). In *P. fluorescens rsmA rsmE gacS* mutants, the $RsmA_{Sm}$ repressive activity is maximal because expression of the antagonistic sRNAs RsmX/Y/Z is abolished (Kay *et al.*, 2005). We therefore wondered if the natural *P. fluorescens* Rsm sRNAs would be able to bind to and counteract $RsmA_{Sm}$. To this end, the effect of the *$rsmA_{Sm}$* gene was studied in *P. fluorescens rsmA rsmE* mutants bearing a functional *gacS* gene. As shown in Fig. 3, the strong negative effect of $RsmA_{Sm}$ on Gac/Rsm-dependent phenotypes was clearly attenuated in these *rsmA rsmE* mutants. As expected, the strong repression exerted by $RsmA_{Sm}$ on target genes was also counteracted in these strains (Fig. 4). These results suggest that $RsmA_{Sm}$ would recognize and bind to the *P. fluorescens* RsmX/Y/Z sRNAs. In *P. fluorescens* CHA0, RsmY and RsmZ sRNAs bind to RsmA and RsmE proteins to form a series of ribonucleoprotein complexes (Reimmann *et al.*, 2005). RsmA/E binding strongly stabilizes the antagonistic Rsm sRNAs (Valverde *et al.*, 2004). Thus, in the absence of RsmA/E proteins, RsmY and RsmZ sRNAs become strongly destabilized (Reimmann *et al.*, 2005). We then hypothesized that binding of $RsmA_{Sm}$ to RsmX/Y/Z sRNAs should increase sRNA half-lives in an *rsmA/E* mutant background. As shown in Fig. 5, expression of *$rsmA_{Sm}$* (from either pSM1 or pSM2 vectors; see Fig. 1) restored the wild-type stability to RsmY sRNA in the *rsmA rsmE* background. On the other hand, the low RsmY half-life displayed by the *rsmA rsmE* mutant bearing pBB84 confirmed that *$rsmA_{Sm}$* is expressed at such a low level

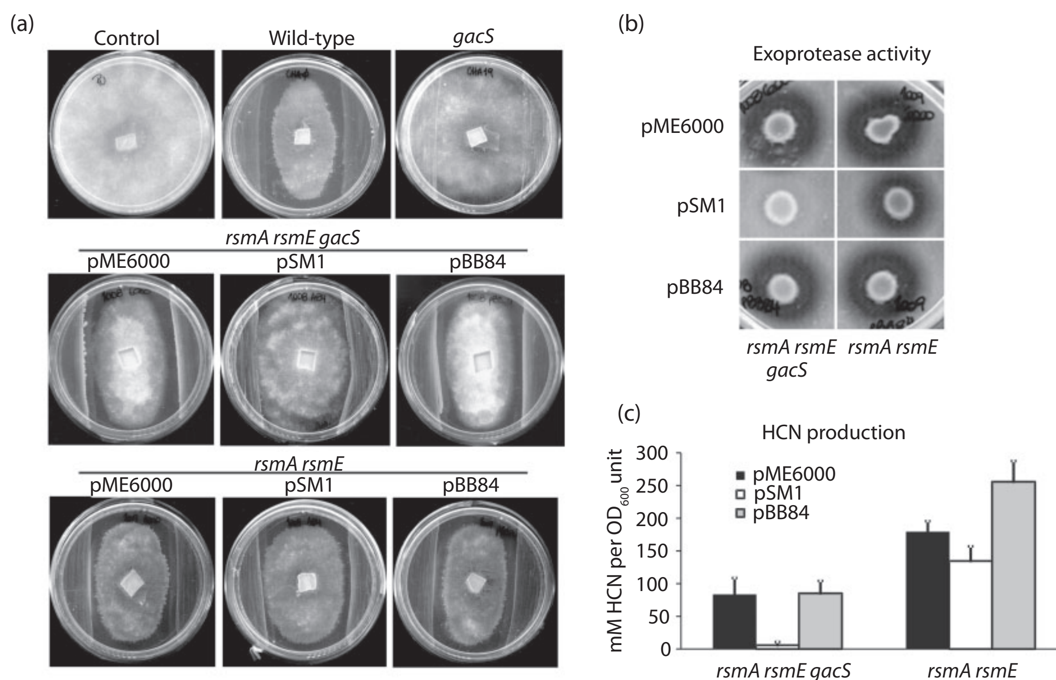

Fig. 3. Gac/Rsm-dependent phenotypes are functionally complemented by the *rsmA*$_{Sm}$ gene in *P. fluorescens*. (a) Antagonism against *Pythium ultimum* in co-culture with *P. fluorescens* cells. Malt agar plates were streaked at opposite sides with *P. fluorescens gacS rsmAE* or *rsmAE* mutant strains bearing *rsmA*$_{Sm}$ alleles, and an agar plug containing *Pythium ultimum* mycelium was seeded in the centre of the plate. Inhibition of *Pythium ultimum* growth was assessed 72 h after plate inoculation. The upper panel shows the typical antagonism displayed by wild-type strain CHA0, and the loss of antibiotic production associated with a *gacS* mutation. The control plate has not been inoculated with bacteria. (b) Exoprotease activity in skimmed milk agar plates. Strains were spotted onto plates and the degradation of casein by secreted exoprotease A was evaluated 48 h after inoculation. (c) Hydrogen cyanide (HCN) production in liquid NYB medium. Each value represents the mean of three replicate overnight cultures ± SD.

from the plasmid *rep* region that it cannot confer full protection from degradation to RsmY (Fig. 5).

The C-terminal α-helix of RsmA$_{Sm}$ is not essential for its function

Comparison of the deduced sequence of RsmA$_{Sm}$ with CsrA/RsmA proteins from other Gram-negative bacterial lineages revealed a C-terminal extension that may fold into an extra α-helix (Fig. 2b). To test if the C-terminal extension is required for RsmA$_{Sm}$ function, we removed the last 48 coding nucleotides of *rsmA*$_{Sm}$ (Fig. 2c) and cloned this allele in pME6000 to generate pSMΔC$_t$. The cellular level of the C-terminal-truncated polypeptide was about threefold lower than that of the full-length protein in Western blots (Fig. 6a). However, the RsmA$_{Sm}\Delta$C$_t$ variant was still able to strongly repress Gac/Rsm-dependent phenotypes in the heterologous test strains (Fig. S1). The results of quantitative expression of target mRNA reporters showed that the repressive activity of the RsmA$_{Sm}\Delta$C$_t$ variant was more effectively counteracted by RsmX/Y/Z sRNAs, as the expression level of both translational reporter fusions was significantly higher than in cells expressing the full-length version (Table 2). Moreover, the RsmA$_{Sm}\Delta$C$_t$ variant was able to restore wild-type RsmY sRNA stability in an *rsmA*/*E* mutant background (Fig. 5). Together, these results indicate that the extra C-terminal sequence of RsmA$_{Sm}$ is not essential for its function as a repressor of mRNA genes or for interaction with the RsmA/E-antagonistic sRNAs in the heterologous host *P. fluorescens*. Removal of the C-terminal extra α-helix is associated with lower cellular levels of the RsmA$_{Sm}$ repressor protein.

Negative control of *rsmA*$_{Sm}$ expression in the *rep* region of cryptic plasmid pMB19a

The results above indicate that the *rsmA*$_{Sm}$ allele is expressed and functional in *P. fluorescens* strains (Figs 3–5, Table 2). However, the expression level seems to depend on the genetic context of the *rsmA*$_{Sm}$ allele. For instance, *rsmA*$_{Sm}$ seems to be expressed at very low levels from vector pBB84, which contains the replication region of the rhizobial cryptic plasmid pMBA19a (Fig. 1). The expression level is so low that it was only evidenced by the slight stabilization of RsmY RNA (Fig. 5) and the slight

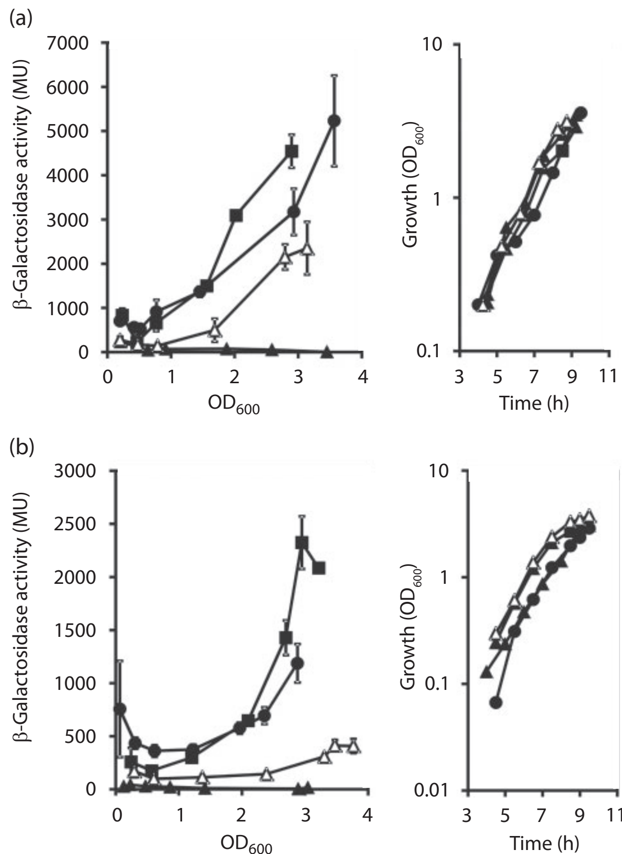

Fig. 4. Translational control of Gac/Rsm-dependent genes by RsmA$_{Sm}$ in *P. fluorescens*. β-Galactosidase activity of *P. fluorescens* strains carrying a chromosomal *hcnA′–′lacZ* (a) or an *aprA′–′lacZ* (b) fusion. Each value represents the mean of three replicate cultures ± SD. The growth curves of the tested strains are shown in the right-hand panels. Strain genetic features: *rsmA rsmE gacS*/pME6000 (●); *rsmA rsmE gacS*/pBB84 (■); *rsmA rsmE gacS*/pSM1 (▲); *rsmA rsmE*/pSM1 (△). MU, Miller units.

repression of *aprA′–′lacZ* expression (Table 2) in strains bearing pBB84; however, the same construct failed to complement RsmA/E-controlled phenotypes (Fig. 3). In contrast, *rsmA$_{Sm}$* is expressed at levels similar to those of *P. fluorescens* RsmA/E proteins from either pSM1 or pSM2 constructs (Figs 3–5, Table 2). In agreement with these observations, the RsmA$_{Sm}$ polypeptide was immunodetected in *P. fluorescens* cells bearing vectors pSM1 or pSM2, but not in cells transformed with pBB84 (Fig. 6a). That is, *rsmA$_{Sm}$* expression appears to be negatively controlled when the allele is present in the genetic context of the cryptic plasmid *rep* region (Fig. 1). This negative control seems to be relieved when the *rsmA$_{Sm}$* allele is disentangled from the plasmid copy control region (Fig. 6a).

Expression of *rsmA$_{Sm}$* is host strain-dependent

The *rsmA$_{Sm}$* allele was originally identified in a cryptic plasmid from an *S. meliloti* soil-dwelling isolate (Watson & Heys, 2006). Here, we have demonstrated that this allele is functional in the heterologous host *P. fluorescens*. We then tested if the repressor gene was expressed in the natural host *S. meliloti*. As shown in Fig. 6(b), the RsmA$_{Sm}$ polypeptide could not be detected by Western blotting in *S. meliloti* strains bearing the *rsmA$_{Sm}$* allele in the *rep* region of the cryptic plasmid pMBA19a or pBB84. The protein was not detected in strains carrying pSM1 or pSM2 constructs, in which the *rsmA$_{Sm}$* allele was disentangled from the copy number control mechanisms (Fig. 1). Strain-specific effects were discounted because the RsmA$_{Sm}$ polypeptide was not detected in two different *S. meliloti* host strains (Fig. 6b). The presence of genomic copies of KorA-like sequences in *S. meliloti* replicons was ruled out because BLASTN searches did not identify genes with significant homology to these transcriptional repressors.

Next, we studied *rsmA$_{Sm}$* expression in two other heterologous hosts for which *csrA*/*rsmA* mutants were

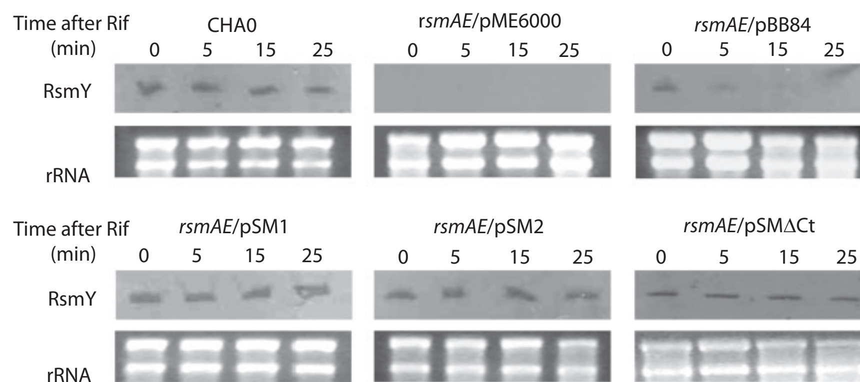

Fig. 5. RsmA$_{Sm}$ protects RsmY sRNA from degradation. RsmY transcript decay in the wild-type strain *P. fluorescens* CHA0 and in the *rsmA rsmE* double mutant bearing different vectors was determined by Northern blotting after blocking transcription with rifampicin. The amount of RNA loaded was 5 µg for the wild-type, 10 µg for the *rsmA rsmE* double mutant bearing pME6000 or pBB84, and 3 µg for the *rsmA rsmE* double mutant bearing pSM1, pSM2 or pSMΔC$_t$.

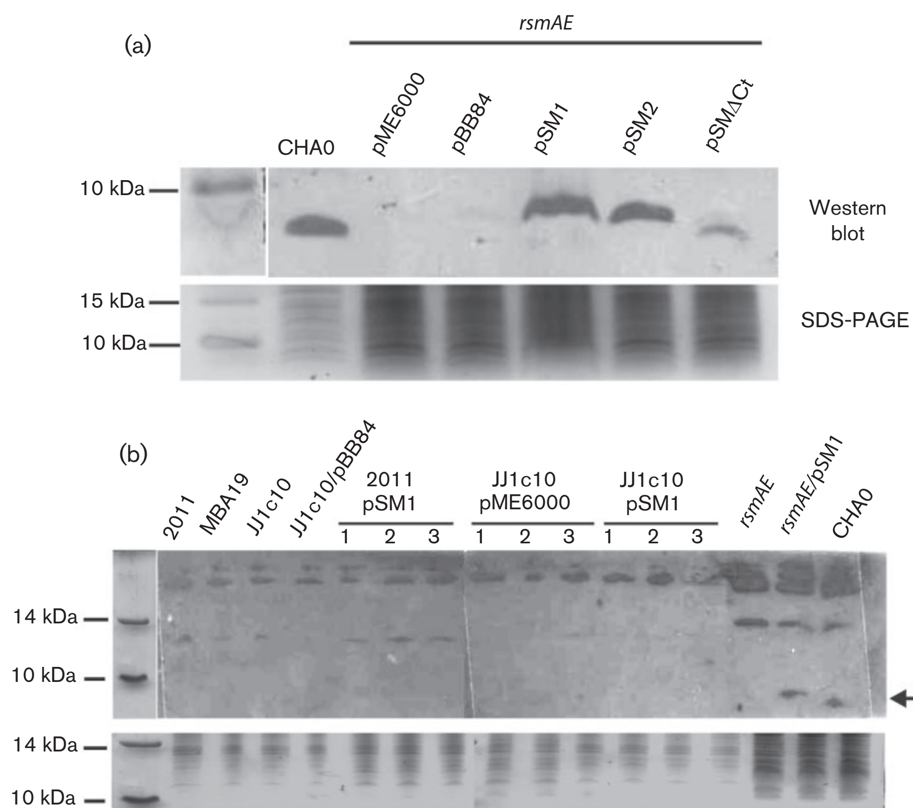

Fig. 6. Western blot detection of $RsmA_{Sm}$. Western blots of total cellular proteins from *P. fluorescens* (a) and *S. meliloti* (b) strains probed with polyclonal antibodies against *Y. enterocolitica* RsmA protein. The Coomassie blue-stained portion of the SDS-PAGE gels corresponding to the RsmA migration zone is shown under the blots to visualize protein loading. (a) Expression of $rsmA_{Sm}$ alleles in *P. fluorescens rsmAE* mutant strain CHA1009 bearing different plasmid constructs (as detailed in Table 1). Under the utilized electrophoretic conditions, RsmA and RsmE from wild-type strain *P. fluorescens* CHA0 migrated as a single band. (b) Expression of $rsmA_{Sm}$ alleles in *S. meliloti* strains bearing different plasmid constructs (as detailed in Table 1). 2011 and JJ1c10, wild-type reference strains that do not contain cryptic plasmids; MBA19, wild-type isolate bearing cryptic plasmid pMBA19a, which encodes $rsmA_{Sm}$. The arrow points to RsmA/E and $RsmA_{Sm}$ bands.

available. In the *P. aeruginosa rsmA* background, the $rsmA_{Sm}$ allele in pSM2 partially complemented pyocyanin production and the $RsmA_{Sm}$ polypeptide was detected by Western blotting (Fig. S2). In contrast, in the *E. coli* background, $rsmA_{Sm}$ in pSM2 was not able to repress glycogen production or activate the flagellar swimming

Table 2. Translational control of target mRNA reporters by the full-length $R_{Sm}A_{Sm}$ and its C-terminal-truncated variant

Reporter	Genetic background (strain)	Complementing plasmid*			
		pME6000	pBB84	pSM2	pSMΔC_t
hcnA'–' lacZ	*rsmA rsmE gacS* (CHA1028)	7209 ± 562	5461 ± 29 (1.3 ×)	134 ± 5 (53 ×)	300 ± 5 (24 ×)
	rsmA rsmE (CHA1027)	10 271 ± 1157	8944 ± 605 (1.1 ×)	1140 ± 87 (9.0 ×)	2382 ± 139 (4.3 ×)
aprA'–' lacZ	*rsmA rsmE gacS* (CHA1007)	1962 ± 159	646 ± 27 (3 ×)	10 ± 1 (196 ×)	12 ± 4 (163 ×)
	rsmA rsmE (CHA1021)	2169 ± 306	962 ± 69 (2.2 ×)	41 ± 1 (52 ×)	272 ± 18 (8 ×)

*Values shown are in Miller units. The translational repression factor exerted by the corresponding $RsmA_{Sm}$ version is indicated in parentheses. The data correspond to the mean of three replicate cultures ± SD.

motility of the *csrA* mutant (Fig. S2). Expression of $rsmA_{Sm}$ in *E. coli* could not be confirmed by Western blotting due to the strong cross-reactivity of the antibody towards other cellular proteins of similar size (data not shown). To summarize, the $rsmA_{Sm}$ allele is expressed and functional in *P. fluorescens* and *P. aeruginosa*, but it is not expressed or functional in *E. coli* and *S. meliloti*.

DISCUSSION

The eubacterial Csr/Rsm post-transcriptional regulatory circuits control diverse and unrelated cellular processes such as central carbon metabolism, motility, biofilm formation, extracellular metabolite synthesis, virulence and pathogenesis, quorum sensing and oxidative stress response (Lapouge *et al.*, 2008; Romeo *et al.*, 2012; Timmermans & Van Melderen, 2010). The circuits depend on RNA-binding proteins of the CsrA/RsmA family that control mRNA expression at the translational level, and on cognate sRNAs that titrate away CsrA/RsmA proteins to relieve bound mRNAs (Romeo *et al.*, 2012). Members of the CsrA/RsmA translational regulatory protein family can be found encoded exclusively in the chromosomes of a number of eubacterial divisions, although remarkably some lineages such as Alphaproteobacteria lack *csrA*/*rsmA* chromosomal homologues (Table S1). In this context, the identification of a *csrA*/*rsmA* gene in the replication region of a cryptic plasmid from the alphaproteobacterium *S. meliloti* (Watson & Heys, 2006) motivated us to further characterize this plasmid-encoded allele. The gene, here referred to as $rsmA_{Sm}$, is highly similar to the *rsmA* allele of *X. axonopodis* pv. *citri*, but it differs from typical CsrA/RsmA proteins of Gram-negative bacteria in that it has an extended C terminus with a predicted additional α-helix (Fig. 2). All other sequence and structural features strongly suggest that $rsmA_{Sm}$ is a translational regulator functionally related to CsrA/RsmA proteins (Fig. 2). In fact, the results presented here show that: (1) the $rsmA_{Sm}$ allele present in pSM1 is expressed and encodes a functional repressor in the heterologous host *P. fluorescens*; (2) the activity of the $RsmA_{Sm}$ protein is antagonized by the *P. fluorescens* antagonistic sRNAs RsmX/Y/Z; (3) the $RsmA_{Sm}$ protein protects RsmY from degradation; and (4) the $RsmA_{Sm}$ C-terminal extra α-helix is dispensable for the protein to function as a repressor of target mRNA genes and to be counteracted by Rsm sRNAs in *P. fluorescens*, although it is necessary to achieve a proper cellular level.

Interestingly, the $rsmA_{Sm}$ gene was poorly expressed in the heterologous host *P. fluorescens* from vector pBB84 unless the genes required for plasmid pMBA19a replication initiation and control were removed (as in pSM1 or pSM2 constructs). As the $rsmA_{Sm}$ gene is encoded divergently to the plasmid replicase operon, it may be speculated that $rsmA_{Sm}$ transcription is coincidentally subject to the negative control of replicase expression typical of IncC plasmids (Fig. 1). Although the perfect KorA-binding motif GTTTAGCTAAAC (Kostelidou & Thomas, 2002) is not present in the ORF II–$rsmA_{Sm}$ intergenic region, a less perfect repeat may serve as a negative regulatory site that directly affects $rsmA_{Sm}$ expression. This would explain the very low expression of $rsmA_{Sm}$ from the pBB84 vector in the heterologous *P. fluorescens* cells, and the gain of expression when removed from the original genetic context. As the gene was not expressed in the natural background in *S. meliloti* strains (either in pBB84 or in pSM2), we cannot speculate on the operation of the postulated negative control of $rsmA_{Sm}$ expression by the plasmid copy control mechanism in the original *S. meliloti* host.

The fact that expression of the $rsmA_{Sm}$ allele depended on the host strain (Figs 6 and S2) suggests that there might be specific transcriptional and/or translational requirements for proper $rsmA_{Sm}$ expression. Transcriptional signals and transcriptional regulation of *csrA*/*rsmA* genes have been characterized only in *E. coli* (Yakhnin *et al.*, 2011). In this enterobacterium, the promoter region spans 250 bp and *csrA* transcription is driven by five different promoters, two of which are dependent on the RpoS sigma factor (Yakhnin *et al.*, 2011). In other bacterial taxa, *csrA*/*rsmA* transcription has not yet been characterized. As the wild-type $rsmA_{Sm}$ alleles present in pSM1 and pSM2 were not expressed from the cloning vector *lac* promoter, we could delimit a minimal 250 bp region that allows expression of $rsmA_{Sm}$ in *P. fluorescens* and that may serve to further characterize the transcriptional regulation of the $rsmA_{Sm}$ gene. Sequence inspection of the 250 bp region containing the $rsmA_{Sm}$ promoter (Fig. 1) did not reveal an obvious DNA motif closely resembling the σ^{70} consensus of *E. coli* (TTGACA-N_{17}-TATAAT) (Harley & Reynolds, 1987) or that of rhizobial promoters (CTTGAC-N_{17}-CTATATc) (MacLellan *et al.*, 2006). Instead, a putative bacterial σ^{70}-dependent promoter was recognized *in silico* (GTGCGC-N_{17}-TATTCT) (Fig. 1), whose −10 and −35 motifs deviate markedly from the canonical enterobacterial, rhizobial and xanthomonad (Katzen *et al.*, 1996) consensus sequences. These observations might explain the lack of $rsmA_{Sm}$ expression in *S. meliloti* (Figs 6 and S2). In *E. coli*, we could not rule out that the protein is expressed but not able to functionally complement the *csrA* mutation (Fig. S2). It has been recently reported that the *Campylobacter jejuni* CsrA protein, expressed from an inducible *araBAD* promoter, does not repress glycogen production in *E. coli* (Fields & Thompson, 2012).

To our knowledge, this study constitutes the first functional characterization of a member of the CsrA/RsmA family that is encoded in a mobile genetic element. It could be speculated that the $rsmA_{Sm}$ gene was mobilized from the chromosome of a gammaproteobacterium to a broad-host-range plasmid that has been fortuitously detected in an *S. meliloti* soil isolate (Watson & Heys, 2006). A further transfer event from this mobile platform to the chromosome of a bacterium lacking *csrA*/*rsmA* genes, such as *S. meliloti*, might introduce unexpected global regulation and pleiotropy (Mukherjee *et al.*, 2011).

As we could not identify sequences that would encode sRNAs antagonistic to $RsmA_{Sm}$ in the 4.5 kb sequenced portion of the cryptic plasmid pMBA19a, it would be interesting to explore the whole 36 kb sequence of pMBA19a to search for putative accompanying sRNA genes that may constitute the mobilization of a complete Csr/Rsm circuit. However, the activity of CsrA/RsmA proteins may also be controlled by molecules other than sRNAs; the post-transcriptional regulatory activity of the *B. subtilis* CsrA protein is negatively controlled by direct interaction with the FliW protein (Mukherjee *et al.*, 2011). Moreover, the FliW protein is an ancestral element often encoded adjacent to *csrA* and flagellar genes in Firmicutes, thus suggesting an evolutionary and functional link to the control of flagellar motility (Mukherjee *et al.*, 2011). In this regard, the $RsmA_{Sm}$ protein is atypical in that its sequence bears features of both Gram-negative and Gram-positive CsrA/RsmA homologues: its conserved core resembles that of Alphaproteobacteria (particularly of xanthomonads) and the extended C terminus that may fold into an extra α-helix more likely resembles the CsrA/RsmA proteins of Gram-positive bacteria (Fig. 1). The evolutionary path of this apparently chimeric $rsmA_{Sm}$ gene is intriguing.

The heterogeneous phyletic distribution of *csrA*/*rsmA* genes among eubacterial divisions could be due to horizontal genetic transfer of this kind of global regulator (Table S1). A recent phylogenetic analysis of members of the CsrA/RsmA family indicates that these regulatory elements were lost from the Alphaproteobacteria and Betaproteobacteria but reappeared in the Gammaproteobacteria branch adjacent to tRNA genes, which are commonly implicated as sites of horizontal gene transfer (Mukherjee *et al.*, 2011). Thus, it would be worth extending the search for and characterization of *csrA*/*rsmA* genes in other mobile genetic elements such as phages, plasmids and genomic islands, in order to contribute to the understanding of the evolutionary history of this family of post-transcriptional regulators of mRNA expression.

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