Homologues of the RNA binding protein RsmA in *Pseudomonas syringae* pv. *tomato* DC3000 exhibit distinct binding affinities with non-coding small RNAs and have distinct roles in virulence

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**SUMMARY**

*Pseudomonas syringae* pv. *tomato* DC3000 (PstDC3000) contains five RsmA protein homologues. In this study, four were functionally characterized, with a focus on RsmA2, RsmA3 and RsmA4. RNA electrophoretic mobility shift assays demonstrated that RsmA1 and RsmA4 exhibited similar low binding affinities to non-coding small RNAs (ncsRNAs), whereas RsmA2 and RsmA3 exhibited similar, but much higher, binding affinities to ncsRNAs. Our results showed that both RsmA2 and RsmA3 were required for disease symptom development and bacterial growth *in planta* by significantly affecting virulence gene expression. All four RsmA proteins, especially RsmA2 and RsmA3, influenced γ-amino butyric acid utilization and pyoverdine production to some degree, whereas RsmA2, RsmA3 and RsmA4 influenced protease activities. A single RsmA, RsmA3, played a dominant role in regulating motility. Furthermore, reverse transcription quantitative real-time PCR and western blot results showed that RsmA proteins, especially RsmA2 and RsmA3, regulated target genes and possibly other RsmA proteins at both transcriptional and translational levels. These results indicate that RsmA proteins in PstDC3000 exhibit distinct binding affinities to ncsRNAs and have distinct roles in virulence. Our results also suggest that RsmA proteins in PstDC3000 interact with each other, where RsmA2 and RsmA3 play a major role in regulating various functions in a complex manner.

**Keywords:** CsrA, post-transcriptional regulation, *Pseudomonas syringae*, RsmA, type III secretion, virulence.

**INTRODUCTION**

The Gac/Rsm signal transduction system has been elaborately studied in many bacterial species (Babitzke and Romeo, 2007; Lapouge et al., 2008). It has been widely reported that the GacS/GacA two-component system (TCS) regulates pleotropic phenotypes, including virulence, stress responses, biofilm formation, production of extracellular enzymes, secondary metabolites and quorum sensing (Heeb and Haas, 2001; Lapouge et al., 2008). The GacA homologues specifically initialize the transcription of non-coding regulatory small RNAs (ncsRNAs), such as *csrB* and *csrC* in *Escherichia coli* (Jonas and Melefors, 2005; Vakulskas et al., 2009; Martínez et al., 2014), and *rsmW, rsmY, rsmZ* and *rsmV* in *Pseudomonas aeruginosa* (Janssen et al., 2018). These ncsRNAs contain numerous GGA motifs and bind and sequester the function of the RNA binding protein CsrA (carbon storage regulator) or its homologues RsmA and RsmE (repressor of secondary metabolites) (Reimmann et al., 2005; Vakulskas et al., 2015). As important post-transcriptional regulators, the RsmA/CsrA family proteins inhibit translation or stability of transcripts of target genes by binding specific GGA motifs within apical loops of the RNA secondary structures in the 5′ untranslated regions (UTR), one of which overlaps or is close to the Shine–Dalgarno (SD) sequence or ribosome binding sites (RBSSs) of target mRNAs, thus blocking ribosome access (Blumer et al., 1999; Vakulskas et al., 2015). On the other hand, the RsmA/CsrA family proteins can also positively regulate the expression of target genes. CsrA protects *flhDC* mRNA by inhibiting the 5′ end-dependent RNase E cleavage pathway in *E. coli* (Yakhnin et al., 2013). RsmA activates the expression of *hag* gene by directly binding to the 5′ UTR and stabilizing its mRNA in *Xanthomonas citri* (Andrade et al., 2014).

The CsrA protein was first reported in *E. coli* by affecting glycogen biosynthesis and gluconeogenesis (Romeo et al., 1993). Later, CsrA was deemed to be a global regulator, which regulates multiple important pathways in many bacteria (Timmermans and Van Melderson 2010). RsmA and RsmE are highly conserved CsrA homologues and play a major role in regulation of virulence in diverse pathogenic bacteria. In *X. citri* subsp. *citri* and *X. campestris* pv. *campestris*, an *rsmA*
mutant caused significantly reduced virulence in the host plant, and delayed or completely abolished hypersensitive response (HR) in the non-host plant tobacco (Andrade et al., 2014; Chao et al., 2008). In Erwinia amylovora, a csrA mutant did not induce HR on tobacco or cause disease on immature pear fruits. It was compromised in motility and had reduced exopolysaccharide (EPS) amylovoran and expression of type III secretion system (T3SS) genes. In addition, CsrA in E. amylovora may indirectly affected uptakes of antibiotics through the Rcs system (Ancona et al., 2016; Ge et al., 2018; Lee et al., 2018). In Pectobacterium carotovorum, overexpression of rsmA inhibited motility, biofilm formation, EPS and secondary metabolite, antibiotics and pigment productions (Mukherjee et al., 1996). Absence of rsmA resulted in less tissue-macerating (soft-rotting) in plant hosts through affecting quorum sensing required for extracellular lytic enzymes (Chatterjee et al., 1995, 2003; Cui et al., 2001). RsmA influenced expression of adhesion synthesis operon and indirectly affected virulence of Yersinia pseudotuberculosis (Heroven et al., 2008). Loss of csrA greatly down-regulated SPI-1 intestinal epithelial cell invasion and other virulence gene expression in S. typhimurium (Lawhon et al., 2003). In Pseudomonas protegens, RsmA and RsmE controlled metabolism and antibiotic biosynthesis (Reimann et al., 2005; Wang et al., 2017). In Pseudomonas putida, RsmA, RsmE and RsmI negatively affected c-di-GMP pools and biofilm formation (Huertas-Rosales et al., 2016, 2017).

Pseudomonas syringae pv. tomato DC3000 (PstDC3000) causes bacterial speck disease on tomato and Arabidopsis thaliana by secreting effectors through the T3SS and producing the phytotoxin coronatine (Zhao et al., 2003). Extracellular protease, pyoverdine siderophore and alginate EPS also contribute to its virulence (Swingle et al., 2008; Vargas et al., 2013). Earlier bioinformatic studies showed that P. syringae pv. tomato possesses seven small RNAs, i.e. rsmX homologues (rsmX1-5) as well as rsmY and rsmZ (Moll et al., 2010). However, the molecular mechanism and virulence-related target genes of the CsrA/RsmA proteins in PstDC3000 remain elusive. A recent study identified five alleles of CsrA (RsmA) proteins present in PstDC3000 and characterized three single mutants (csrA1 to csrA3) (Ferreiro et al., 2018). They showed that CsrA2 (RsmA2) is the most conserved member among 250 prokaryotic genomes examined, and CsrA3 (RsmA3) is nearly identical to CsrA2, but is present only in the Pseudomonas fluorescens group (RsmE). They further reported that CsrA3 and CsrA2 were found to play roles in motility, syringafactin and alginate production, and promote growth in planta, but not symptom development (Ferreiro et al., 2018). Here, we labelled these CsrA proteins in PstDC3000 as RsmA proteins because deduced amino acids of RsmA proteins in PstDC3000 shared relatively higher identities and similarities to RsmA proteins from other Pseudomonas strains than to CsrA proteins from E. coli and Erwinia species (Fig. S1; Table S1). In our study, our major goal was to determine the roles of the rsmA genes in virulence and other virulent-related phenotypes. We also explored the interactions between different RsmA proteins in PstDC3000 and determined the binding affinities of these RsmA proteins to ncRNAs.

RESULTS

**In vitro characterization of the rsmA mutants**

We characterized four rsmA genes in PstDC3000 by generating four overexpression strains (DC3000(pRsmA1, pRsmA2, pRsmA3, pRsmA4)), four single mutants (rsmA1, rsmA2, rsmA3, rsmA4), three double mutants (rsmA2/rsmA3, rsmA2/rsmA4, rsmA3/rsmA4), one triple mutant (rsmA2/rsmA3/rsmA4) and one quadruple mutant (rsmA1/rsmA2/rsmA3/rsmA4). Our results using overexpression strains indicate that overexpression of RsmA2, RsmA3 and RsmA4 in PstDC3000 resulted in reduced pyoverdine production (Fig. S2A) and protease activities (Fig. S2B). Overexpression of RsmA2 and RsmA3 led to decreased ability in utilizing γ-amino butyric acid (GABA), whereas overexpression of RsmA1 and RsmA4 resulted in enhanced GABA utilization (Fig. S3A). On the other hand, motility was slightly decreased only in PstDC3000 overexpressing RsmA3 (Fig. S3B). Overexpressing of CsrA from E. amylovora in PstDC3000 led to similar phenotypic changes with those of overexpressing RsmA2 of PstDC3000 (Figs S2 and S3). The deduced amino acid sequence of CsrA of E. amylovora shared relatively higher identities and similarities to that of the RsmA2 than to RsmA3 of PstDC3000 (Fig. S1; Table S1). We also observed that mutation of the rsmA1 gene alone did not affect GABA utilization, protease activity and pyoverdine production (except a minor increase in motility) (Figs S4 and S5), suggesting that RsmA1 plays a very minimal role, as previously reported (Ferreiro et al., 2018). Based on these results, we mainly focused on RsmA2, RsmA3 and RsmA4 for the major part of our studies.

**RsmA2 and RsmA3 are required for virulence and bacterial growth in planta**

We inoculated plants using an infiltration method with a very low concentration of initial inoculum and found that PstDC3000, all three overexpression strains, three single mutants (rsmA2, rsmA3, rsmA4) and two double mutants (rsmA2/rsmA4 and rsmA3/rsmA4) exhibited similar disease symptoms (Fig. 1A,B). Interestingly, the rsmA2/rsmA3 double mutant and the rsmA2/rsmA3/rsmA4 triple mutant exhibited dramatically reduced symptoms, including both necrotic spots and the amount of chlorosis (Fig. 1B). Virulence of the rsmA2/
RsmA binding affinities and roles in virulence

The rsmA3/rsmA4 triple mutant could be restored by complementation with either the rsmA2 or rsmA3 gene, but not with the rsmA4 gene (Fig. 1C). Virulence of the rsmA2/rsmA3 double mutant could partially be recovered by either rsmA2 or rsmA3 (Fig. 1C). These findings suggest that both RsmA2 and RsmA3 are required for virulence.

Fig. 1 Virulence of Pseudomonas syringae pv. tomato DC3000, rsmA overexpression, rsmA mutants and complementation strains. (A) Disease symptoms caused by PstDC3000(pUCP18), PstDC3000(pRsmA2), PstDC3000(pRsmA3) and PstDC3000(pRsmA4) overexpression strains in tomato leaves. (B) Symptoms caused by PstDC3000 and the rsmA2, rsmA3, rsmA4, rsmA2/rsmA3, rsmA2/rsmA4, rsmA3/rsmA4 and rsmA2/rsmA3/rsmA4 mutants in tomato leaves. (C) Symptoms caused by complementation strains of the rsmA2/rsmA3 and the rsmA2/rsmA3/rsmA4 mutants in tomato leaves. Pictures were taken at 7 days post-inoculation. The experiment was repeated three times and similar results were obtained.
We also monitored bacterial growth in planta at 0, 1, 3 and 5 days post-inoculation (dpi). No significant difference was found among PstDC3000, three overexpressed strains and three single mutants as well as the rsmA2/rsmA4 and the rsmA3/rsmA4 double mutants (Fig. 2A,B). Bacterial growth for the rsmA2/rsmA3 double mutant and the rsmA2/rsmA3/rsmA4 triple mutant was about 5–10-fold lower than those of all other strains (Fig. 2B), and bacterial growth could be completely rescued in the triple mutant by expression of either the rsmA2 or rsmA3 gene, but not the rsmA4 gene (Fig. 2C). Expression of the rsmA2 or rsmA3 gene in the rsmA2/rsmA3 double mutant could partially restore bacterial growth in tomato leaves (Fig. 2D). In order to rule out the possibility that the defect of bacterial growth in planta was due to defects in their abilities to grow in vitro, we determined bacterial growth in KB medium. Though some mutants showed delay in growth, all the mutants reached a similar level to the wild-type after 24 h of growth (Fig. S6). All the overexpression strains and mutants could still elicit HR on non-host tobacco leaves (Fig. S7). Overall, these results support the finding that RsmA2 and RsmA3 play an important role in the interaction of PstDC3000 with tomato plants, and suggest that these proteins might have functional redundancy.

Expression of T3SS, coronatine and alginate genes in PstDC3000 is regulated by RsmA2, RsmA3 and RsmA4 to varying degrees

In order to determine how mutation of the rsmA genes affects virulence, expression of selected virulence-related genes in five mutants was quantified. First, expression of avrE and hrpL in all five mutants was reduced (Fig. 3), especially in the rsmA2/rsmA3 double mutant and the rsmA2/rsmA3/rsmA4 triple mutant, where expression of avrE and hrpL was more than 30–50- and 250–1000-fold lower than that of the wild type (WT), respectively (Fig. 3). In the single mutant, the effect of the rsmA3 gene on expression of hrpL and avrE genes was much stronger than that of the rsmA2 and rsmA4 genes. These results suggest that RsmA2, RsmA3 and RsmA4 synergistically influence the expression of T3SS genes in PstDC3000, and further indicate that RsmA3 plays a major role.

Expression of coronatine-related genes, i.e. the corR and cfl genes, was also reduced in four of the five mutants. Expression of the corR and cfl genes in the rsmA2/rsmA3 double mutant was decreased about 3- and 2.2-fold than that of the wild type, respectively, while in the rsmA2/rsmA3/rsmA4 triple mutant, they were also down-regulated about 6.7- and 4.8-fold, respectively (Fig. 3). Both corR and cfl gene expression was slightly down-regulated in the rsmA2 and rsmA3 mutants, but not in the rsmA4 mutant (Fig. 3). These results suggest that RsmA2 and RsmA3 synergistically regulate coronatine gene expression in PstDC3000, whereas RsmA4 might also have a minor role when both RsmA2 and RsmA3 are absent.

Expression pattern for the algQ gene was reduced in the rsmA3, rsmA2/rsmA3 and rsmA2/rsmA3/rsmA4 mutants (Fig. 3). However, expression of algK gene was increased in all three mutants lacking the rsmA3 gene. Expression of algK was up-regulated about 11-fold in the rsmA3 mutant and more than 30-fold in both the rsmA2/rsmA3 double mutant and the rsmA2/rsmA3/rsmA4 triple mutant than that in the WT (Fig. 3). These results suggest that different regulation mechanisms exist for the algQ and algK genes in PstDC3000 by the RsmA proteins.

Protease activities are influenced by RsmA2, RsmA3 and RsmA4

Overexpression of RsmA in PstDC3000 exhibited reduced protease activities compared to that of PstDC3000 (Figs 4A and S8A). In contrast, the rsmA2, rsmA3 and rsmA4 deletion mutants all exhibited increased protease activities compared to PstDC3000 (Figs 4B and S8B). The protease activities of the rsmA2/rsmA4 and rsmA3/rsmA4 mutants were also slightly increased (Figs 4B and S8B), whereas the protease activities for the rsmA2/rsmA3, rsmA2/rsmA3/rsmA4 and rsmA1/rsmA2/rsmA3/rsmA4 mutants were similar to each other and increased to the level of the rsmA3 single mutant (Figs 4B, S4B and S8B). Complementation of the rsmA2/rsmA3/rsmA4 and rsmA2/rsmA3 mutant with the rsmA2 gene partially restored protease activities, whereas complementation with the rsmA3 gene led to reduced protease activities (Figs 4CD and S8C). In conclusion, these results indicate that RsmA3 plays a major role in regulating protease activity, whereas RsmA2 and RsmA4 also negatively influence protease activity in PstDC3000.

The effect of RsmA proteins on pyoverdine production

One characteristic of fluorescent pseudomonads is the production of pyoverdine as a siderophore and virulence-related signal molecule (Imperi et al., 2009). Similar to the protease activity results, overexpression of rsmA2, rsmA3 or rsmA4 led to varying reduced pyoverdine production, where overexpression of the rsmA3 gene exhibited the strongest negative effect (Figs 5A and S9A). In contrast, deletion of the rsmA2 and rsmA3 genes, but not the rsmA4 or rsmA1 genes, led to increased pyoverdine production (Figs S5B, S4A and S9A). Furthermore, pyoverdine production in the rsmA2/rsmA4 and rsmA3/rsmA4 double mutants was similar to that in the rsmA2 and rsmA3 single mutants, respectively (Figs S5B and S9B). When both rsmA2 and rsmA3 were deleted, pyoverdine production in the rsmA2/rsmA3 double mutant and the rsmA2/rsmA3/rsmA4 triple mutants was significantly lower compared to DC3000, but similar to that of the rsmA3 overexpression strains (P < 0.05, Figs S5A, S8B and S9A, B). However,
Fig. 2  Bacterial growth of *Pseudomonas syringae* pv. *tomato* DC3000, *rsmA* overexpression, *rsmA* mutants and complementation strains in tomato. (A) PstDC3000, PstDC3000(pUCP18), PstDC3000(pRsmA2), PstDC3000(pRsmA3) and PstDC3000(pRsmA4) overexpression strains. (B) PstDC3000 and the *rsmA2*, *rsmA3*, *rsmA4*, *rsmA2/rsmA3*, *rsmA2/rsmA4*, *rsmA3/rsmA4* and *rsmA2/rsmA3/rsmA4* mutants. (C) PstDC3000, the *rsmA2/rsmA3/rsmA4* mutant and its complementation strains. (D) PstDC3000, the *rsmA2/rsmA3* and its complementation strains. Bacterial growth was monitored at 0, 1, 3 and 5 days post-inoculation. Vertical bars represent standard deviations. The experiment was repeated three times and similar results were obtained.
The rsmA1/rsmA2/rsmA3/rsmA4 quadruple mutant exhibited increased pyoverdine production compared to the rsmA2/rsmA3 double and rsmA2/rsmA3/rsmA4 triple mutants (Fig. S4A). Complementation of the rsmA2/rsmA3/rsmA4 mutant with either rsmA2 or rsmA4, but not the rsmA3 gene, partially restored pyoverdine production to the wild-type level (Figs 5C and S9C). Similarly, complementation of the rsmA2/rsmA3 mutant by expressing the rsmA2, but not the rsmA3 gene, partially recovered its pyoverdine production (Figs 5D and S9C). These results indicate that all four RsmA proteins influenced pyoverdine production in PstDC3000 to some degree. These results also suggest that expression levels of RsmA2 and RsmA3, especially RsmA3, might be important in pyoverdine production, and RsmA1 might also play a role in pyoverdine production when RsmA2, RsmA3 and RsmA4 are all deleted.

RsmA3 negatively regulates motility in PstDC3000

In contrast to protease activity and pyoverdine production, motility was only slightly decreased in PstDC3000 overexpressing the rsmA3 gene and slightly increased in the single rsmA1 and rsmA3 mutants and the rsmA3/rsmA4 double mutant (Figs S3B, S5B, S10A,B and S11A,B). Similar to pyoverdine production, when both rsmA2 and rsmA3 were deleted, motility of the rsmA2/rsmA3 double mutant, the rsmA2/rsmA3/rsmA4 triple mutant and the rsmA1/rsmA2/rsmA3/rsmA4 quadruple mutant was significantly reduced as compared to other strains (P < 0.05, Figs S5B, S10B and S11B). Motility can be rescued for the rsmA2/rsmA3 double mutant and the rsmA2/rsmA3/rsmA4 triple mutant by the rsmA2 or rsmA4 gene, but not the rsmA3 gene as described above for pyoverdine production (Figs S10C and S11CD). These results indicate that although RsmA1 may also suppress motility, RsmA3 plays a dominant role in regulating motility in PstDC3000, and also suggest that RsmA2 could affect motility when interacting with RsmA3.

The effect of RsmA on GABA utilization

Non-protein amino acid GABA is highly abundant in tomato apoplast and PstDC3000 can utilize GABA as a sole carbon and nitrogen source, thus utilization of GABA might affect its survival in planta (Rico and Preston, 2008). Overexpression of the rsmA2 and rsmA3 genes in PstDC3000 led to decreased ability in utilizing GABA, whereas overexpression of the rsmA4 gene resulted in enhanced GABA utilization (Fig. 6A). In contrast, deletion of the rsmA2 and rsmA3 genes increased or decreased GABA utilization, respectively, whereas no effect was found in the rsmA1 and rsmA4 single mutants or in the rsmA2/rsmA4 and rsmA3/rsmA4 double mutants and the rsmA1/rsmA2/rsmA3/rsmA4 quadruple mutant (Figs 6B and S5A). Similar
**Fig. 4** Diameter of halo zones of protease activities. (A) PstDC3000, PstDC3000(pUCP18), PstDC3000(pRsmA2), PstDC3000(pRsmA3) and PstDC3000(pRsmA4) overexpression strains. (B) PstDC3000 and the rsmA2, rsmA3, rsmA4, rsmA2/rsmA3, rsmA2/rsmA4, rsmA3/rsmA4 and rsmA2/rsmA3/rsmA4 mutants. (C) PstDC3000, the rsmA2/rsmA3/rsmA4 mutant and its complementation strains. (D) PstDC3000, the rsmA2/rsmA3 mutant and its complementation strains. All strains were grown on NYG agar plates containing 0.75% skimmed milk at room temperature. Diameters were measured after 24 h of incubation. Vertical bars represent standard deviations. Bars marked with the same letter are not significantly different (P < 0.05). The experiment was repeated three times with three replicate and similar results were obtained.
Fig. 5 Quantification of pyoverdine production. (A) PstDC3000, PstDC3000(pUCP18), PstDC3000(pRsmA2), PstDC3000(pRsmA3) and PstDC3000(pRsmA4) overexpression strains. (B) PstDC3000 and the rsmA2, rsmA3, rsmA4, rsmA2/rsmA3, rsmA2/rsmA4, rsmA3/rsmA4 and rsmA2/rsmA3/rsmA4 mutants. (C) PstDC3000, the rsmA2/rsmA3/rsmA4 mutant and its complementation strains. (D) PstDC3000, the rsmA2/rsmA3 mutant and its complementation strains. Pyoverdine production was quantified by measuring the absorbance at OD405 of culture supernatants diluted 2:1 in 100 mM Tris-HCl (pH 8.0) and normalized at OD600 of bacterial suspensions. Data were presented as relative fluorescence levels (A405/A600). All strains were grown in MG medium at 28 °C for 24 h. Vertical bars represent standard deviations. Bars marked with the same letter are not significantly different (P < 0.05). The experiment was repeated three times with three replicates and similar results were obtained.
Fig. 6 Growth of Pseudomonas syringae pv. tomato DC3000, rsmA overexpression, rsmA mutants and complementation strains in GABA. (A) PstDC3000, PstDC3000(pUCP18), PstDC3000(pRsmA2), PstDC3000(pRsmA3) and PstDC3000(pRsmA4) overexpression strains. (B) PstDC3000 and the rsmA2, rsmA3, rsmA4, rsmA2/rsmA3, rsmA2/rsmA4, rsmA3/rsmA4 and rsmA2/rsmA3/rsmA4 mutants. (C) PstDC3000, the rsmA2/rsmA3/rsmA4 mutant and its complementation strains. (D) PstDC3000, the rsmA2/rsmA3 mutant and its complementation strains. All the strains were grown in modified MG medium (replacing mannitol and l-glutamic acid in MG medium with 10 mM γ-amino butyric acid) at 28 °C and bacterial growth was monitored by measuring OD_{600} at 24 h. Vertical bars represent standard deviations. Bars marked with the same letter were not significantly different (P < 0.05). The experiment was repeated three times with three replicates and similar results were obtained.
to motility and pyoverdine production, when both the rsmA2 and rsmA3 genes were deleted, GABA utilization in the rsmA2/rsmA3 double mutant and the rsmA2/rsmA3/rsmA4 triple mutant was significantly decreased compared to other strains, but was similar to that of the rsmA3 overexpression strain ($P < 0.05$, Fig. 6A,B). Complementation of the rsmA2/rsmA3/rsmA4 mutant with either the rsmA2 or rsmA4 gene increased GABA utilization to the same level as the rsmA4 overexpression strain (Fig. 6A,C), whereas complementation with the rsmA3 gene led to GABA utilization as low as that of the rsmA3 overexpression strain (Fig. 6A,C). Surprisingly, when the rsmA2/rsmA3 double mutant complemented with either the rsmA2 or rsmA3 gene, it resulted in significantly decreased ability in utilizing GABA ($P < 0.05$, Fig. 6D). These results indicate that all four RsmA proteins influence GABA utilization to varying degrees in PstDC3000. Furthermore, when RsmA2, RsmA3 and RsmA4 were all absent, RsmA1 could also influence GABA utilization, suggesting that the interaction between these four RsmA proteins in influencing GABA utilization is very complicated.

Expression of the rsmA genes in PstDC3000

In order to give a glimpse of the complex interaction among RsmA proteins in PstDC3000, we first determined the expression of the rsmA genes using reverse transcription quantitative real-time PCR (qRT-PCR) in HMM medium. Expression of rsmA2 was decreased about 2-fold in the rsmA3 single mutant compared to that of the wild-type and rsmA4 mutant (Fig. 7A). No significant change was observed for the rsmA3 gene in the mutant strains tested. However, in the rsmA2/rsmA3 double mutant, expression of the rsmA4 and rsmA1 genes was slightly down-regulated, whereas expression of the rsmA1 gene was significantly down-regulated in the rsmA2/rsmA3/rsmA4 triple mutant ($P < 0.05$, Fig. 7A). These results suggest that RsmA3 positively regulates rsmA2 expression. Furthermore, the results also suggest that RsmA2 and RsmA3 might synergistically activate the expression of the rsmA4 gene, whereas RsmA2, RsmA3 and RsmA4 synergistically promote the expression of the rsmA1 gene.

Western blot analyses showed that the abundance of the RsmA2 and RsmA4 proteins was decreased about 30% and 50% in the rsmA3 single mutant strain, respectively, whereas the abundance of RsmA3 protein remained mostly unchanged in the rsmA2 and rsmA4 single mutants (Figs 7B and S12A). Furthermore, the abundance of the RsmA2 protein was significantly decreased (70% less) in the rsmA2/rsmA3 double mutant and the rsmA2/rsmA3/rsmA4 triple mutant as compared to the WT, whereas the abundance of the RsmA4 protein was not detectable in the rsmA2/rsmA3 double mutant and the rsmA2/rsmA3/rsmA4 triple mutant (Figs 7C and S12B). Interestingly, the abundance of the RsmA3 protein was also decreased about 30% in the rsmA2/rsmA3 double mutant, but only slightly decreased in the rsmA2/rsmA3/rsmA4 triple mutant (Figs 7C and S12B). These results suggest that RsmA3 influences the expression of RsmA2 and RsmA4 proteins. These results also suggest that RsmA2 might self-regulate itself and reciprocally influence RsmA3 expression, whereas RsmA2 and RsmA3 together might also affect the expression of RsmA4 proteins.

RsmA proteins have distinct binding affinities to ncsRNAs

To compare RNA-binding affinities to different ncsRNAs, four RsmA proteins (RsmA1, RsmA2, RsmA3 and RsmA4) were purified and subject to RNA gel shift assays (Fig. 8). Since previous sequence analysis of five rsmX ncsRNAs revealed the same secondary structure with five GGA motifs in the hairpin loop (Moll et al., 2010), only rsmX1 and rsmX5 as well as rsmY and rsmZ were selected for the analysis. In all four ncsRNAs tested, a band shift was observed at 40 nM for RsmA2 and RsmA3, at 320 nM for RsmA1, and at 640 nM for RsmA4. These results indicate that all ncsRNAs of PstDC3000 exhibit similar binding affinity to different RsmA homologues, while RsmA homologues have distinct binding affinities to ncsRNAs in the following order from strongest to weakest: RsmA2 = RsmA3 > RsmA1 > RsmA4.

DISCUSSION

Since 1993, the CsrA/RsmA homologues have been extensively studied in human and plant pathogens as well as plant-associated microorganisms. In a previous report, three paralogues, i.e. csrA1, csrA2 and csrA3 in PstDC3000, were evaluated for their roles in motility, alginate biosynthesis, syringafactin production and virulence, and the authors concluded that CsrA1 to CsrA3 were not required for virulence (Ferreiro et al., 2018). However, in our study we demonstrated that RsmA2 and RsmA3 were required for virulence in PstDC3000 and bacterial growth in planta, and RsmA4 might also play a minor role in virulence. We also provided evidence that RsmA2 and RsmA3 regulate genes at both transcriptional and post-transcriptional levels and interactions between themselves are very complicated. Moreover, we showed that RsmA proteins in PstDC3000 exhibited distinct binding affinities to ncsRNAs, which might explain the distinguishing role each RsmA protein plays in affecting various phenotypes.

RsmA (CsrA) plays a critical role in virulence among many pathogenic bacteria (Ancona et al., 2016; Andrade et al., 2014; Barnard et al., 2004). It has been reported that deletion of a single csrA gene in PstDC3000 did not affect virulence, but bacterial growth was reduced in the csr2 and csr3 mutants (Ferreiro et al., 2018). In our study, we confirmed that deletion of a single rsmA gene did not change its ability to cause disease. However, we found that deletion of both rsmA2 and
rsmA significantly affects disease symptom development and bacterial growth, suggesting that both RsmA2 and RsmA3 are required for PstDC3000 virulence and bacterial growth in planta. These results also suggest that RsmA proteins in PstDC3000 exhibit function redundancies in controlling virulence factors.

The T3SS and phytotoxin coronatine are major pathogenicity and virulence factors in PstDC3000, respectively (Zhao et al., 2003). The ability of PstDC3000 to multiply in plant tissue and promote symptom development is dependent on the translocation of many effector proteins to target specific host proteins and interfere with plant innate immune signalling systems (Feng and Zhou, 2012; Mudgett, 2005). The non-host specific toxin coronatine increases disease severity by suppressing stomata closure and promoting the jasmonic acid (JA) signalling pathway to suppress salicylic acid (SA)-mediated defence responses (Brooks et al.,...
Our results show that transcription levels of avrE, hrpL, corR and cif are positively regulated by RsmA proteins at various degrees, indicating that RsmA proteins synergistically regulate virulence factors and contribute to virulence, and further suggesting that RsmA proteins in PstDC3000 have function redundancy. We also demonstrated that RsmA2 and RsmA3 play a major role, whereas RsmA1 and RsmA4 play a minor role in virulence.

In addition, our results show that RsmA3 negatively regulates algK gene, which is responsible for alginate synthesis, whereas RsmA2 positively affect algK expression. However, expression of algK increased drastically in mutants deleting both RsmA2 and RsmA3. Our results are consistent with previous reports that algD is up-regulated in both the rsmA mutant of P. aeruginosa and the csrA mutant of PstDC3000 (Burrowes et al., 2006; Ferreiro et al., 2018). In contrast, expression of algQ, encoding a global regulatory protein of alginate biosynthesis (Ambrosi et al., 2005; Kim et al., 1998; Schlichtman et al., 1995), was significantly decreased when both rsmA2 and rsmA3 were deleted, indicating that RsmA3 and RsmA2 synergistically regulate AlgQ at transcriptional level. It is possible that AlgK is negatively regulated by AlgQ, which needs to be further verified.

The potential interplay between different Rsm proteins has been investigated in some Pseudomonas strains (Kay et al., 2005; Morris et al., 2013; Zha et al., 2014). In P. fluorescens, RsmE expression was negatively regulated by RsmA and RsmE, which regulate itself (Reimmann et al., 2005). Both RsmA and RsmE, which are closely related to RsmA2 and RsmA3, respectively, negatively affect their own expression in P. putida (Huertas-Rosales et al., 2016). Furthermore, RsmA and RsmF translation is repressed by specific binding of RsmA to rsmA and rsmFM mRNA in vitro at post-transcriptional level in P. aeruginosa (Marden et al., 2013). In our study, one of the novel findings is that RsmA3 positively regulates RsmA2 at transcriptional level. This result might explain why many phenotypes were most significant when both RsmA2 and RsmA3 were absent. In other words, RsmA3 probably is on the top of the RsmA regulatory cascade in PstDC3000. Interestingly, our results suggest that expression of the rsmA1 gene, which is present in most Pseudomonas strains, might be suppressed by the synergistic action of RsmA2, RsmA3 and RsmA4 at transcriptional level. In addition, we demonstrated that in the rsmA3 mutant, the abundance of the RsmA2 and RsmA4 protein was significantly decreased, indicating that RsmA3 positively affects RsmA2 and RsmA4 at post-transcriptional and translational levels. Furthermore, the abundances of the RsmA2, RsmA3 and RsmA4 proteins were all decreased in rsmA2 and rsmA3 double mutants as compared to the wild-type and the rsmA3 single mutant, further suggesting that RsmA2 and RsmA3 might synergistically and reciprocally influence the expression of RsmA2, RsmA3 and RsmA4 proteins at the post-transcriptional level. Future studies should focus on

Fig. 8 RsmA proteins exhibit distinct binding affinities to ncsRNAs. To compare RNA-binding affinity to different ncsRNAs (RsmX1, RsmX5, RsmY and RsmZ), four RsmA proteins (RsmA1, RsmA2, RsmA3 and RsmA4) were purified and subjected to RNA gel shift assays. Different concentrations of four proteins (nM) are indicated above each lane. The red frame shows the minimal concentration of proteins where band shift could be observed.
are much stronger than those of RsmA1 and RsmA4. These re-
in P. fluorescens have similar binding affinities to ncsRNAs (Kay
results are consistent with previous findings that RsmA and RsmE
we demonstrated that RsmA proteins in PstDC3000 modulate
merous GGA motifs, sequester their functions (Vakulskas et al.,
structure within the 5’ UTR, whereas ncsRNAs, containing nu-
rein P. fluorescens have similar binding affinities to ncsRNAs (Kay
et al., 2005; Reimmann et al., 2005). These results also provide
evidence as why RsmA2 and RsmA3 are more important than
RsmA1/RsmA4 in regulating various phenotypes. The question
that remains unanswered is, since the major residues in binding
to GGA motif are well conserved among these RsmA proteins,
why do they display distinct binding affinities to ncsRNAs?

In summary, the RsmA/CsrA family protein has long been
deemed an important and pleiotropic post-transcriptional reg-
ulator in many bacteria and is extensively involved in the gene
regulatory network (Vakulskas et al., 2015). In our current study,
we demonstrated that RsmA proteins in PstDC3000 modulate
virulence and bacterial growth in planta, and regulate protease
activity, pyoverdine production, utilization of GABA and mo-
activity, pyoverdine production, utilization of GABA and mo-
tional levels; however, the exact regulatory mechanism remains
unknown. In the future, it is worth exploring potential direct
or indirect regulation pathways as well as interaction among
the RsmA proteins. Furthermore, identifying direct targets of
RsmA proteins in the regulation of virulence factors should be
a priority.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study are listed in
Table 1. Pseudomonas syringae pv. tomato strains were cultured on
King’s medium B (KB). For T3SS gene expression, an hrp-inducing
minimal medium (HMM), supplemented with 10 mM fructose as
carbon source, was used (Chatnaparat et al., 2015). Luria-Bertani
(LB) broth was utilized for routine growth of E. coli strain at 37 °C.
Antibiotics were used at the following concentrations when ap-
propriate: 100 μg/mL rifampicin, 50 μg/mL kanamycin, 100 μg/mL
ampicillin, 15 μg/mL tetracycline and 100 μg/mL spectinomycin.
All the primers used are listed in Supplementary Table S2.

Construction of deletion mutants

Deletion mutations of rsmA1, rsmA2, rsmA3, rsmA4, rsmA2/
rsmA3, rsmA2/rsmA4, rsmA3/rsmA4, rsmA2/rsmA3/rsmA4

and rsmA1/rsmA2/rsmA3/rsmA4 were generated using splice
overlap extension mutagenesis as described previously
(Chatnaparat et al., 2015). Briefly, in a first round of PCRs,
about 1 kb upstream and downstream fragments of rsmA1,
rsmA2, rsmA3 and rsmA4 in PstDC3000 were amplified using
primers unique to these regions (Table S2). The two PCR pro-
ducts contained ends overlapping with sequences at both ends
of the kanamycin resistance cassette (Datsenko and Wanner,
2000). The FRT-Km-FRT forward and reverse primers were
used amplified FRT-flanked kanamycin cassette. In a subse-
quent overlap extension PCR, these three fragments were am-
plied into a single fragment. The final fragment was cloned
into the pTok2 suicide vector digested with SmaI, resulting in
pTok2-rsmA1del, pTok2-rsmA2del, pTok2-rsmA3del and
pTok2-rsmA4del, respectively. To generate marker-less mu-
tants, plasmid pFLP2-omega expressing FLP recombinase was
introduced into the mutant and transformants were plated on
KB plates containing spectinomycin, resulting in the loss of the
kanamycin resistance cassette. To generate double mutants,
plasmid pTok2-rsmA4del or pTok2-rsmA2del was transferred
into the rsmA2 and rsmA3 marker-less mutants. For generating
the rsmA2/rsmA3/rsmA4 triple and rsmA1/rsmA2/rsmA3/
rsmA4 quadruple mutants, the plasmids pTok2-rsmA2del and
pTok2-rsmA1del were transferred into the rsmA3/rsmA4 and
rsmA2/rsmA3/rsmA4 marker-less mutant strains, respectively.

Complementation of mutants and generation of
overexpression strains

For complementation of the rsmA mutants, a 1 kb fragment
containing the native promoter and the rsmA genes was ampli-
ﬁed by PCR and cloned into the pUCP18 vector to yield plasmids
pRsmA1, pRsmA2, pRsmA3 and pRsmA4. The resulting plasmids
were sequenced at the University of Illinois at Urbana-Champa-
genome sequencing facility. The ﬁnal plasmids were introduced into
the corresponding marker-less deletion mutants and PstDC3000
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Table 1  Bacterial strains and plasmids used in this study.

| Strains, plasmids | Description | Reference or source |
|-------------------|-------------|---------------------|
| **Pseudomonas syringae pv. tomato** | | |
| DC3000 | Wild type, Rif spontaneous resistance | |
| ΔrsmA1(Pspto1629) | Km<sup>+</sup>, rsmA1::Kan, DC3000 derivative | This study |
| ΔrsmA2(Pspto1844) | Km<sup>+</sup>, rsmA2::Kan, DC3000 derivative | This study |
| ΔrsmA3(Pspto3566) | Km<sup>+</sup>, rsmA3::Kan, DC3000 derivative | This study |
| ΔrsmA4(Pspto3943) | Km<sup>+</sup>, rsmA4::Kan, DC3000 derivative | This study |
| ΔrsmA2/rsmA4 | Km<sup>+</sup>, rsmA2 and rsmA4::Kan, DC3000 derivative | This study |
| ΔrsmA3/rsmA4 | Km<sup>+</sup>, rsmA3 and rsmA4::Kan, DC3000 derivative | This study |
| ΔrsmA2/rsmA3 | Km<sup>+</sup>, rsmA3 and rsmA2::Kan, DC3000 derivative | This study |
| ΔrsmA2/rsmA3/rsmA4 | Km<sup>+</sup>, rsmA3, rsmA4 and rsmA2::Kan, DC3000 derivative | This study |
| ΔrsmA1/rsmA2/rsmA3/rsmA4 | Km<sup>+</sup>, rsmA3, rsmA4, rsmA2 and rsmA1::Kan, DC3000 derivative | This study |
| **E. amylovora** | | |
| Ea1189 | Wild type, isolated from apple | Wang et al., 2010 |
| **E. coli** | | |
| DH10B | F<sup>−</sup> mcrAΔ(mrr-hsdRMS-mcrBC)Φ80lacZΔM15ΔlacX74 recA1 endA1 araΔ139 (ara, leu)7697 galU galK λ—rpsL (StrR) nupG | Invitrogen (Carlsbad, CA, USA) |
| BL21(DE3) | ompT hsdS<sub>B</sub>(rB mB<sup>+</sup>) gal dcm (DE3) | Invitrogen (Carlsbad, CA, USA) |
| **Plasmids** | | |
| pUCP18 | E. coli-Pseudomonas shuttle vector, Ap<sup>+</sup> | Norrander et al., 1983 |
| pTok2 | CoIE1 replicon, suicide plasmid, Tc<sup>+</sup> | Kitten and Willis, 1996 |
| pKD13 | FRT-Kan-FRT, oriR6K, Ap<sup>+</sup>, Km<sup>+</sup> | Datsenko and Wanner, 2000 |
| pFLP2-omega | Suicide vector encoding flp recombinase, sacB, Sp<sup>+</sup> | Chatnaparat et al., 2015 |
| pGEM-T Easy | PCR cloning vector, Ap<sup>+</sup> | Promega |
| pET-42b | E. coli His-tag expression vector, Amp<sup>+</sup> | Novagen |
| pTok2-rsmA1del | rsmA1::Kan from overlapping PCR cloned into pTok2, Tc<sup>+</sup>, Km<sup>+</sup> | This study |
| pTok2-rsmA2del | rsmA2::Kan from overlapping PCR cloned into pTok2, Tc<sup>+</sup>, Km<sup>+</sup> | This study |
| pTok2-rsmA3del | rsmA3::Kan from overlapping PCR cloned into pTok2, Tc<sup>+</sup>, Km<sup>+</sup> | This study |
| pTok2-rsmA4del | rsmA4::Kan from overlapping PCR cloned into pTok2, Tc<sup>+</sup>, Km<sup>+</sup> | This study |
| pRsmA1 | 995-bp fragment containing rsmA1 gene with native promoter cloned into pUCP18, Ap<sup>+</sup> | This study |
| pRsmA2 | 989-bp fragment containing rsmA2 gene with native promoter cloned into pUCP18, Ap<sup>+</sup> | This study |
| pRsmA3 | 989-bp fragment containing rsmA3 gene with native promoter cloned into pUCP18, Ap<sup>+</sup> | This study |
| pRsmA4 | 995-bp fragment containing rsmA4 gene with native promoter cloned into pUCP18, Ap<sup>+</sup> | This study |
| pCsrA | 894-bp fragment containing csrA gene with native promoter from E. amylovora Ea1189 cloned into pUCP18, Ap<sup>+</sup> | This study |
| pRsmA2-His6 | 827-bp fragment containing rsmA2 gene with native promoter and C-terminal His-tag in pUCP18, Ap<sup>+</sup> | This study |
| pRsmA3-His6 | 827-bp fragment containing rsmA3 gene with native promoter and C-terminal His-tag in pUCP18, Ap<sup>+</sup> | This study |
| pRsmA4-His6 | 833-bp fragment containing rsmA4 gene with native promoter and C-terminal His-tag in pUCP18, Ap<sup>+</sup> | This study |

(Continues)
room temperature and the motility of bacterial cells was visually examined at 24 and 48 h post-inoculation. The experiments were performed three times, with three biological replicates per treatment.

Pyoverdine product was detected in mannitol–glutamate (MG) medium as previously described (Ambrosi et al., 2005; Chatnaparat et al., 2015; Imperi et al., 2009; Park et al., 2010). Bacterial cells of overnight cultures in KB were washed in PBS, resuspended to an OD600 of 0.05 in MG medium and incubated with shaking at 28 °C for 24 h. Pyoverdine was quantified by measuring the absorbance at 405 nm of culture supernatants diluted 2:1 in 100 mM Tris-HCl (pH 8.0) and normalized with OD600. To visualize pyoverdine product, bacterial cells were re-suspended in PBS to a final concentration of OD 600 = 0.3, and 2 μL bacterial suspensions were spotted onto MG agar plates. Plates were incubated at 28 °C and observed under UV light after 48 h. The experiments were performed three times, with three biological replicates per treatment.

For protease activity, all strains were grown in KB overnight, rinsed and resuspended in PBS to a density of OD600 = 1. Bacterial suspensions (2 μL) were applied onto NYG (peptone yeast glycerol medium; 5 g/L peptone, 3 g/L yeast extract, 20 g/L glycerol) agar plates containing 0.75% skimmed milk. Plates were incubated at room temperature for 3 days prior to examination and measurement of the diameter of halo zones. The experiments were repeated three times, with three biological replicates each.

For GABA utilization assays, bacterial cells of overnight cultures in KB were washed and resuspended to OD 600 = 0.02 in modified MG medium by replacing mannitol and l-glutamic acid in MG medium with 10 mM GABA (Chatnaparat et al., 2015). Cells were grown overnight at 28 °C, and bacterial growth was monitored by measuring OD600. The experiments were repeated three times, with three biological replicates each.

Virulence assay and bacterial growth in tomato

Tomato, Solanum lycopersicum 'Big Daddy Hybrid' plants were grown in a greenhouse. About 3–4 weeks after transplanting, leaves were infiltrated with bacterial suspension at about 5 × 10⁴ CFU/mL (diluted from original suspension at OD600 = 0.1) using a needleless syringe. Bacteria were recovered from plants by taking three samples from three leaves at the site of infiltration using a disk punch (three disks per strain) at 0, 1, 3, 5 days post-inoculation (dpi). Leaf disks were homogenized by mechanical disruption using pestles in PBS. Serial 10-time dilutions of the tissue homogenates were plated on LB plates, and the number of CFUs per disk (cm²) was calculated. For virulence assay, disease symptoms were recorded at 7 dpi. The experiment was repeated three times.

RNA isolation and reverse transcription quantitative real-time PCR

After 6 h incubation in HMM at 18 °C, 4 mL of RNA protect reagent (Qiagen, Hilden, Germany) was added to 2 mL of bacterial culture mixed by vortex and incubated at room temperature for 5 min. Cells were harvested by centrifugation and RNA was extracted using RNeasy® mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. DNase I treatment was performed with TURBO DNA-free kit (Ambion, TX, USA) and RNA was quantified using Nano-drop ND100 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA). One microgram of total RNA was reverse transcribed using Superscript III reverse transcriptase (Invitrogen,
Carlsbad, CA, USA) following the manufacturer’s instructions. One microgram of cDNA was used as the template for reverse transcription quantitative real-time PCR (qRT-PCR). PowerUp SYBR® Green PCR master mix (Applied Biosystems, CA, USA) was used to detect gene expression of selected genes. qRT-PCR amplifications were performed using the StepOnePlus Real-Time PCR system (Applied Biosystems, CA, USA) under the following conditions: 50 °C for 2 min and 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The dissociation curve was measured after the programme was completed and gene expression was analysed with the relative quantification (ΔΔCt) method using the rpoD gene as an endogenous control. The experiment was repeated three times, and three technical replicates were included for each of the two biological samples per experiment.

Western blot

The DNA fragments containing the native promoters and coding sequences of the rsmA2, rsmA3 and rsmA4 genes with a 6-His tag at the C-terminus were cloned into pUCP18. The resulting plasmids were transformed by electroporation into the PstDC3000 and mutants. For western blot, equal amounts of bacterial cells grown in HMM containing 10 mM fructose at 18 °C for 24 h were collected. Cell lysates were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). After blocking with 5% milk in PBS, membranes were probed with 1.0 μg/mL rabbit anti-His antibodies (GenScript, Piscataway, NJ, USA), followed by horseradish peroxidase-linked anti-rabbit IgG antibodies (Amersham Bioscience, Uppsala, Sweden) diluted 1:10 000. Immunoblots were developed using enhanced chemiluminescence reagents (Pierce, Rockford, IL, USA) and visualized using an ImageQuant LAS 4010 CCD camera (GE Healthcare, Piscataway, NJ, USA).

Statistical analysis

Statistical comparison among different strains or conditions was performed by one-way ANOVA and the Student–Newman–Keuls test (P = 0.05) to analyse the data.

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COMPETING INTERESTS

The authors have declared that no competing interests exist.

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**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of this article at the publisher’s web site:

**Fig. S1** (A) Phylogenetic tree of RsmA/CsrA proteins from *E. coli*, *S. enterica*, *E. amylovora* and *Pseudomonas* strains. (B) Alignment of deduced amino acids of different RsmA/CsrA proteins in *E. amylovora* and three *Pseudomonas* strains. The deduced amino acid sequences of RsmA/CsrA proteins were aligned and analysed by GeneDoc software (Nicholas et al., 1997). Phylogenetic tree of RsmA/CsrA proteins was made by MEGA5 (Tamura et al., 2001). The GenBank accession numbers are *E. coli* CsrA: BAA16558; *S. enterica* subsp. enterica CsrA: NP_461747; *E. amylovora* CFBP1430 CsrA: CBA19758; Host factor-I: CBA23141; *P. aeruginosa* PA01 RsmA: AAO4294; *P. aeruginosa* UCSPP-PA14 RsmN: BAK92751; *P. fluorescens* FI13 RsmA: ABW16952; RsmE: ABW16953; *P. fluorescens* A506 RsmE: AFJ58988; *P. syringae* pv. syringae B728a RsmA1:YP_236820; RsmA2:YP_236624; RsmA3:YP_236409; *P. syringae* pv. tomato DC3000 RsmA1:AAO55149; RsmA2:AAO5536; RsmA3:AAO57040; RsmA4:AAO57041; RsmA5:YP_003355050.

**Fig. S2** Effect of RsmA of *P. syringae* pv. tomato DC3000 and CsrA of *E. amylovora* on pyoverdine production and protease activity. (A) Pyoverdine production by *PstDC3000* (*PrsmA1*) and *PstDC3000* (*PcsrA1*) overexpression strains and compared with *PstDC3000* (*PUCP18*), *PstDC3000* (*PrsmA2*), *PstDC3000* (*PrsmA3*) and *PstDC3000* (*PcsrA4*) overexpression strains. (B) Protease activity by *PstDC3000* (*PrsmA1*) and *DC3000* (*PcsrA*) overexpression strains and compared with *PstDC3000* (*PUCP18*), *PstDC3000* (*PrsmA2*), *PstDC3000* (*PrsmA3*) and *PstDC3000* (*PcsrA4*) overexpression strains. Vertical bars represent standard deviations. One-way ANOVA and the Student–Newman–Keuls test (*P = 0.05*) were used to analyse the data. Bars marked with the same letter are not significantly different (*P < 0.05*). The experiment was repeated three times and similar results were obtained.
Fig. 53 Effect of RsmA1 of P. syringae pv. tomato DC3000 and CsrA of E. amylovora on GABA utilization and swimming motility. (A) Growth of PstDC3000(pRsmA1) and PstDC3000(pCsrA) overexpression strains as compared with PstDC3000(pUCP18), PstDC3000(pRsmA2), PstDC3000(pRsmA3) and PstDC3000(pRsmA4) overexpression strains in GABA. All the strains were grown in modified MG medium (replacing mannotol and L-glutamic acid in MG medium with 10 mM GABA) at 28 °C and bacterial growth was monitored by measuring OD600 at 24 h. Vertical bars represent standard deviations. Bars marked with the same letter are not significantly different (P < 0.05). The experiment was repeated three times and similar results were obtained. (B) Motility of PstDC3000(pRsmA1) and PstDC3000(pCsrA) overexpression strains compared with PstDC3000(pUCP18), PstDC3000(pRsmA2), PstDC3000(pRsmA3) and PstDC3000(pRsmA4) overexpression strains. Vertical bars represent standard deviations. One-way ANOVA and the Student–Newman–Keuls test (P = 0.05) were used to analyse the data. Bars marked with the same letter are not significantly different (P < 0.05). The experiment was repeated three times and similar results were obtained.

Fig. 54 Effect of the rsmA1 mutant and the rsmA1/rsmA2/rsmA3/rsmA4 quadruple mutant of P. syringae pv. tomato DC3000 on pyoverdine production and protease activity. (A) Pyoverdine production by the rsmA1 mutant and the rsmA1/rsmA2/rsmA3/rsmA4 quadruple mutant and compared with PstDC3000, PstDC3000(pRsmA1), the rsmA2/rsmA3 and the rsmA2/rsmA3/rsmA4 mutant strains. (B) Protease activity by the rsmA1 mutant and the rsmA1/rsmA2/rsmA3/rsmA4 quadruple mutant and compared with PstDC3000, PstDC3000(pRsmA1) and the rsmA2/rsmA3 and rsmA2/rsmA3/rsmA4 mutant strains. Vertical bars represent standard deviations. One-way ANOVA and the Student–Newman–Keuls test (P = 0.05) were used to analyse the data. Bars marked with the same letter are not significantly different (P < 0.05). The experiment was repeated three times and similar results were obtained.

Fig. 55 Effect of the rsmA1 mutant and the rsmA1/rsmA2/rsmA3/rsmA4 quadruple mutant of P. syringae pv. tomato DC3000 on GABA utilization and motility. (A) Growth of the rsmA1 mutant and the rsmA1/rsmA2/rsmA3/rsmA4 quadruple mutant as compared with PstDC3000, PstDC3000(pRsmA1) and the rsmA2/rsmA3 and rsmA2/rsmA3/rsmA4 mutant strains. All the strains were grown in modified MG medium (replacing mannotol and L-glutamic acid in MG medium with 10 mM GABA) at 28 °C and bacterial growth was monitored by measuring OD600 at 24 h. Vertical bars represent standard deviations. Bars marked with the same letter are not significantly different (P < 0.05). The experiment was repeated three times and similar results were obtained. (B) Motility of the rsmA1 mutant and the rsmA1/rsmA2/rsmA3/rsmA4 quadruple mutant as compared with PstDC3000, PstDC3000(pRsmA1) and the rsmA2/rsmA3 and rsmA2/rsmA3/rsmA4 mutant strains. Vertical bars represent standard deviations. One-way ANOVA and the Student–Newman–Keuls test (P = 0.05) were used to analyse the data. Bars marked with the same letter are not significantly different (P < 0.05). The experiment was repeated three times and similar results were obtained.

Fig. 56 Growth of P. syringae pv. tomato DC3000, rsmA overexpression and mutant strains. (A) PstDC3000, PstDC3000(pUCP18), PstDC3000(pRsmA1), PstDC3000(pRsmA2), PstDC3000(pRsmA3), PstDC3000(pRsmA4) and PstDC3000(pCsrA) overexpression strains. (B) PstDC3000 and the rsmA2, rsmA3 and rsmA4 mutants. (C) PstDC3000 and the rsmA2/rsmA3, rsmA2/rsmA4, rsmA3/rsmA4 and rsmA2/rsmA3/rsmA4 mutants. All the strains were grown in KB at 28 °C. Overnight cultures of PstDC3000, mutants and overexpression strains as well as complementation strains were harvested and resuspended to OD600 = 0.01 in fresh KB medium. Bacterial strains were grown at 28 °C, and aliquots of the culture were taken every 2 h for 24 h. Bacterial growth for each strain was determined by measuring OD600. The experiments were performed in triplicate and repeated three times and similar results were obtained. Vertical bars represent standard deviations.

Fig. 57 HR assay on tobacco leaves. PstDC3000, the rsmA overexpression and rsmA mutant strains were infiltrated into 8-week-old tobacco leaves. PBS was used as negative control. Photographs were taken 24 h post-infiltration. The experiment was repeated three times and similar results were obtained. Overnight cultures of bacterial strains were harvested by centrifugation, resuspended in 1/2× PBS and adjusted to OD600 = 0.1. Bacterial suspension was infiltrated into tobacco leaves (Nicotiana tabacum) by needleless syringe. Infiltrated plants were kept in a humid growth chamber and HR symptoms were recorded at 24 h post-infiltration. The experiment was repeated three times.

Fig. 58 Protease activity by P. syringae pv. tomato DC3000, rsmA overexpression, rsmA mutants and complementation strains. (A) PstDC3000(pUCP18), PstDC3000(pRsmA2), PstDC3000(pRsmA3) and PstDC3000(pRsmA4) overexpression strains. (B) PstDC3000 and the rsmA2, rsmA3, rsmA4, rsmA2/rsmA3, rsmA2/rsmA4, rsmA3/rsmA4 and rsmA2/rsmA3/rsmA4 mutants. (C) PstDC3000, the rsmA2/rsmA3 and rsmA2/rsmA3/rsmA4 mutants and their complementation strains. Protease activity was measured at room temperature using NYG agar plates containing 0.75% skimmed milk where halo zones are indicative of protease activities. Pictures were taken at 72 h post-incubation. The experiment was repeated three times and similar results were obtained.

Fig. 59 Pyoverdine production by P. syringae pv. tomato DC3000, rsmA overexpression, rsmA mutants and complementation strains. (A) PstDC3000(pUCP18), PstDC3000(pRsmA2), PstDC3000(pRsmA3) and PstDC3000(pRsmA4) overexpression strains. (B) PstDC3000(pRsmA1) and PstDC3000(pCsrA) overexpression strains compared with PstDC3000(pUCP18), PstDC3000(pRsmA2), PstDC3000(pRsmA3) and PstDC3000(pCsrA) overexpression strains. Vertical bars represent standard deviations. One-way ANOVA and the Student–Newman–Keuls test (P = 0.05) were used to analyse the data. Bars marked with the same letter are not significantly different (P < 0.05). The experiment was repeated three times and similar results were obtained.

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strains. (B) PstDC3000 and the rsmA2, rsmA3, rsmA4, rsmA2/rsmA3, rsmA2/rsmA4, rsmA3/rsmA4 and rsmA2/rsmA3/rsmA4 mutants. (C) PstDC3000, the rsmA2/rsmA3 and rsmA2/rsmA3/rsmA4 mutants and their complementation strains. Pyoverdine production was visualized on MG plates under the UV light, where intensities of fluorescence were indicative of pyoverdine production. All strains were grown on MG plates at 28 °C. Pictures were taken at 48 h post-incubation. The experiment was repeated three times and similar results were obtained.

Fig. S10 Motility of P. syringae pv. tomato DC3000, rsmA overexpression, rsmA mutants and complementation strains. (A) PstDC3000, PstDC3000(pUCP18), PstDC3000(pRSmA2), PstDC3000(pRSmA3), PstDC3000(pRSmA4) overexpression strains and the rsmA2, rsmA3 and rsmA4 single mutant strains. (B) The rsmA2/rsmA3, rsmA2/rsmA4, rsmA3/rsmA4 and rsmA2/rsmA3/rsmA4 mutants. (C) PstDC3000, the rsmA2/rsmA3 and rsmA2/rsmA3/rsmA4 mutants and complementation strains. All strains were grown on 0.3% KB agar plates at room temperature. Pictures were taken at 24 h and 48 h post-inoculation. The experiment was repeated three times and similar results were obtained.

Fig. S11 Diameter of movement circle of P. syringae pv. tomato DC3000, rsmA overexpression, rsmA mutants and complementation strains. (A) PstDC3000, PstDC3000(pUCP18), PstDC3000(pRSmA2), PstDC3000(pRSmA3) and PstDC3000 (pRSmA4) overexpression strains and the rsmA2, rsmA3 and rsmA4 single mutant strains. (B) PstDC3000 and the rsmA2/ rsmA3, rsmA2/rsmA4, rsmA3/rsmA4 and rsmA2/rsmA3/rsmA4 mutants. (C) PstDC3000, the rsmA2/rsmA3/rsmA4 mutant and its complementation strains. (D) PstDC3000, the rsmA2/rsmA3 mutant and its complementation strains. All strains were grown on 0.3% KB agar plates at room temperature. Diameters of the movement circles were measured at 24 h and 48 h post-incubation. Vertical bars represent standard deviations. One-way ANOVA and the Student–Newman–Keuls test (P = 0.05) were used to analyse motility diameter data. Bars marked with the same letter are not significantly different (P < 0.05). The experiment was repeated three times and similar results were obtained.

Fig. S12 Duplicated SDS-PAGE gel stained using Coomassie Blue as loading control. (A) 1, marker; 2, DC3000(pRSmA2-His); 3, ∆rsmA2(pRSmA2-His); 4, ∆rsmA3(pRSmA2-His); 5, ∆rsmA4(pRSmA2-His); 6, DC3000(pRSmA3-His); 7, ∆rsmA2(pRSmA3-His); 8, ∆rsmA3(pRSmA3-His); 9, ∆rsmA4(pRSmA3-His); 10, DC3000(pRSmA4-His); 11, ∆rsmA2(pRSmA4-His); 12, ∆rsmA3(pRSmA4-His); 13, ∆rsmA4(pRSmA4-His). (B) 1, marker; 2, DC3000(pRSmA2-His); 3, DC3000(pRSmA3-His); 4, DC3000(pRSmA4-His); 5, ∆rsmA2/A3(pRSmA2-His); 6, ∆rsmA2/A3(pRSmA3-His); 7, ∆rsmA2/A3/A4(pRSmA3-His); 8, ∆rsmA2/A3/A4(pRSmA2-His); 9, ∆rsmA2/A3/A4(pRSmA3-His); 10, ∆rsmA2/A3/A4(pRSmA4-His).

Table S1 Comparison of deduced amino acids of different RsmA/CsrA proteins in E. amylovora and three Pseudomonas strains.

Table S2 Primers used in this study.