Effect of Overexpressing *rsmA* from *Pseudomonas aeruginosa* on Virulence of Select Phytotoxin-Producing Strains of *P. syringae*

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ABSTRACT

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The GacS/GacA two-component system functions mechanistically in conjunction with global post-transcriptional regulators of the RsmA family to allow pseudomonads and other bacteria to adapt to changing environmental stimuli. Analysis of this Gac/Rsm signal transduction pathway in phytotoxin-producing pathovars of *Pseudomonas syringae* is incomplete, particularly with regard to *rsmA*. Our approach in studying it was to overexpress *rsmA* in *P. syringae* strains through introduction of pSK61, a plasmid constitutively expressing this gene. Disease and

colonization of plant leaf tissue were consistently diminished in all *P. syringae* strains tested (pv. *phaseolicola* NPS3121, pv. *syringae* B728a, and BR2R) when harboring pSK61 relative to these isolates harboring the empty vector pME6031. Phaseolotoxin, syringomycin, and tabtoxin were not produced in any of these strains when transformed with pSK61. Production of protease and pyoverdine as well as swarming were also diminished in all of these strains when harboring pSK61. In contrast, alginate production, biofilm formation, and the hypersensitive response were diminished in some but not all of these isolates under the same growth conditions. These results indicate that *rsmA* is consistently important in the overarching phenotypes disease and endophytic colonization but that its role varies with pathovar in certain underpinning phenotypes in the phytotoxin-producing strains of *P. syringae*.

The ability to sense and rapidly respond to changing environmental conditions is a critical feature of opportunistic organisms such as phytopathogenic bacteria. Phytopathogenic bacteria have multiple lifestyles, surviving in diverse environments as pathogens in plants but also as epiphytes on plant surfaces or saprophytes in soil and water. Expression of virulence factors and behaviors associated with virulence must be coordinated with appropriate environmental cues for effective disease development

and energy conservation when conditions favor the pathogenic lifestyle (56). Common systems in gram-negative bacteria involved in the detection and response to environmental cues are two-component signal transduction systems that consist of a histidine kinase (HK) sensor and a cognate response regulator (RR) protein. As with other gram-negative bacteria, plant-pathogenic bacteria can possess multiple two-component systems. For example, the fire blight pathogen *Erwinia amylovora* Ea1189 possesses 20 putative HK and 24 putative RR genes; *Pseudomonas syringae* pathogens of bean (*P. syringae* pv. *syringae* B728a and *P. syringae* pv. *phaseolicola* 1448A) and tomato (*P. syringae* pv. *tomato* DC3000) possess 68 to 70 putative HK and 92 to 95 putative RR genes, respectively; and *Xanthomonas* pathogens of citrus (*Xanthomonas axonopodis* pv. *citri*), cruciferous plants (*X. campestris* pv. *campestris*), and solanaceous plants (*X. campestris* pv. *vesicatoria*) possess 40 to 60 putative HK and 50 to 61 putative RR genes (48,67,87). One of the most studied two-component systems in plant pathogens is the GacS/GacA system (27), one of eight ancient core two-component systems in the family Enterobacteriaceae (66).

The HK GacS (initially called LemA) was first reported in the bean pathogen *P. syringae* pv. *syringae* B728a (33,86). The RR GacA was described shortly thereafter in the biological control bacterium *P. fluorescens* strain CHA0 (47). Subsequently, components of GacS/GacA two-component systems were reported in many of the gram-negative bacteria examined and, in most cases, shown to positively regulate the production of exoproducts important for biological activity (27). The GacS/GacA two-component system was initially thought to act directly on the transcription of target genes involved in the production of the various exoproducts; however, it is now known that the GacS/GacA two-

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component system functions mechanistically in conjunction with the Rsm post-translational regulatory network of secondary metabolism which incorporates RNA-binding proteins (RsmA homologues) and small, untranslated regulatory RNAs (27,80,82). The RNA-binding protein RsmA, first discovered in *Escherichia coli* as a carbon storage regulator (74), was then described in the plant pathogen *Erwinia carotovora* subsp. *carotovora*, in which an *rsmA* mutant was found to produce higher amounts of extracellular enzymes (pectate lyase isozymes, cellulase, polygalacturonase, and protease), was hypervirulent, but produced similar levels of the *N*-acyl homoserine lactone (HSL) quorum-sensing signaling molecules compared with the parental strain (9). Overexpression of *rsmA* in *Erwinia* spp. either fully or partially suppressed the production of extracellular enzymes, HSL, and exopolysaccharides (EPS), and suppressed virulence in host plants, hypersensitive response (HR) in non-host plants, and swarming (9,13,58). In *Erwinia* spp., the production of extracellular enzymes, the ability to incite the HR in non-host plants, and pathogenicity are regulated by the GacS/GacA two-component system as well (12,17,22).

In *P. syringae*, the GacS/GacA two-component system is the master regulator of secondary metabolism (10,45). The GacS/GacA two-component system accomplishes this by regulating quorum sensing and the σ factors RpoS, RpoN, and HrpL (10). A large body of evidence demonstrates that, with few exceptions, important bacterial processes such as the production of quorum-sensing compounds, phytotoxins and antibiotics, extracellular proteases, fluorescent pigments, and alginate, as well as swarming, biofilm formation, lesion formation, sensitivity to reactive oxygen intermediates, disease development, and host-plant colonization are under the regulation of the GacS/GacA two-component system in *P. syringae* pathovars (2,10,15,16,32–34,40,41,52,68,69,73,83,85,86). HR in non-host plants caused by *P. syringae* pv. *tomato* DC3000 is also under the regulation of the GacS/GacA two-component system (79).

Based on genome analysis, the Gac/Rsm signal transduction pathway in *P. syringae* phytotoxin-producing pathovars should be similar to that of *P. fluorescens*, because multiple homologues of *rsmA* and of the three sRNA genes *rsmZ*, *rsmY*, and *rsmX* are present (28,43). In addition, and not reported in other γ -proteobacteria, *P. syringae* pathovars possess up to five *rsmX* homologues (57). In *P. syringae* pv. *tomato* DC3000, GacA was shown to control the transcription of *rsmZ*, *salA* (*rsmY*), and another sRNA gene identified as *rsmB* (10). Nevertheless, the detailed role for the Rsm system in the regulation of exoproducts and processes associated with pathogenicity and virulence of phytotoxin-producing *P. syringae* strains has yet to be described (45). The objective of this study was to extend the analysis of the Gac/Rsm signal transduction pathway in phytotoxin-producing strains of *P. syringae* by determining the role of *rsmA* in regulation of phytotoxin production and other traits associated with pathogenicity in pvs. *phaseolicola* and *syringae* and in a tabtoxin-producing strain of *P. syringae*. Our approach was to overexpress *rsmA* through introduction of *rsmA* from *P. aeruginosa* on a low-copy-number plasmid and determine the impact of *rsmA* overexpression on traits important to disease. It is convenient to study RsmA regulation using overexpression approaches as opposed to the use of knock-out mutants because some bacteria, including pseudomonads, contain two or more *rsmA* alleles with redundant functions (45).

MATERIALS AND METHODS

Microbial strains, plasmids, and culture conditions. A description of fungal and bacterial strains and plasmids used in this study is given in Table 1. Spontaneous, rifampicin (Rif)-resistant mutants were isolated from populations of pv. *syringae* B728a and pv. *phaseolicola* NPS3121 after plating overnight cultures onto King's Medium B (KB) (39) agar plates containing Rif at

50 μ g/ml essentially as described (55). Strain NPS3121 was chosen over *P. syringae* pv. *phaseolicola* 1448A, whose genome has been sequenced (35), for this study because strain NPS3121 has had the most in-depth work performed with regard to the GacS/GacA two-component system (15,27,72). A Rif-resistant colony for each pathovar was selected (B728aR and NPS3121R) that caused disease symptoms on bean similar to the wild type. Where appropriate, media were supplemented with tetracycline (Tc) and Rif at 10 and 50 μ g/ml, respectively.

General molecular techniques. Genomic DNA isolation was done using the Qiagen Genomic Midi Kit according to conditions of the manufacturer (Qiagen Inc., Valencia, CA). Plasmid DNA isolation, restriction enzyme digestion, ligation, electrophoresis, hybridization, and transformation procedures were performed as previously described (77). Electroporation was essentially as described (77), where the electroporation apparatus was set to deliver an electrical pulse of 25 μ F, 2.5 kV, and 200 Ω with a field strength of 12.5 kV/cm for 4.5 to 5 ms.

Polymerase chain reaction (PCR) primers for amplification of *gacS* (GACS-F/GACS-R), *gacA* (GACA-F/GACA-R), *rsmA* (RSMA-F/RSMA-R; NPS3121-RSMA-9F/NPS3121-RSMA-R), and *rsmY* (RSMY-F/RSMY-R) are listed in Table 1. These primers were designed based on sequences in conserved regions from the respective genes in *P. syringae* pv. *syringae* B728a (accession number CP000075), *P. syringae* pv. *tomato* DC3000 (accession number AE016853), and *P. syringae* pv. *phaseolicola* 1448A (accession number CP0000580) using Primer3 (75). PCR amplifications of *gacS*, *gacA*, *rsmA*, and *rsmY* were performed in a total of 25 μ l. Reaction mixtures consisted of GoTaq Colorless Master Mix (Promega Corp., Madison, WI), 0.8 μ M each primer, and 40 ng of bacterial DNA or 1 μ l of a bacterial cell suspension. The reactions were performed in an Applied Biosystems 2720 Thermal Cycler. For PCR with all primers in amplifications of *P. syringae* genes, denaturation was at 94°C for 5 min for the first cycle and 94°C for 30 s for each subsequent cycle. Annealing and elongation were at 55°C for 30 s and 72°C for 30 s, respectively, for 25 cycles. Reactions were terminated after a final 7-min elongation at 72°C. Amplicons from PCR amplifications for *gacS* and *gacA* were purified using a Qiagen QIAquick PCR Purification Kit and sequenced directly using the respective PCR primers. Amplicons from PCR amplifications for *rsmA* and *rsmY* were purified, cloned into pCR4-TOPO (Invitrogen Corp., Carlsbad, CA), and sequenced using M13F and M13R primers following the manufacturer's recommendations.

Plasmid pSK61 was constructed in multiple stages using plasmid intermediates pBluescript (Agilent Technologies, Inc., Santa Clara, CA), pSK9, pSK13, pSK82, pSK59, pME6031, and pME6032 (Table 1; Fig. 1). Plasmid pSK9 was constructed by amplifying the upstream region of *rsmA* from *P. aeruginosa* PAO1 using primers RSMPO1 and RSMAPA2, digesting the resulting PCR product with *EcoRI* and *XhoI*, and subsequently ligating this fragment into *EcoRI*-*XhoI*-digested pBluescript. PCR was carried out in a total volume of 20 μ l containing 100 ng of genomic DNA from *P. aeruginosa* PAO1, 1 μ M each primer, 250 μ M each dNTP, 5 U of GoTaq DNA Polymerase, and the recommended buffer provided by the manufacturer (Promega Corp.). After an initial DNA denaturation step of 5 min at 95°C, 30 amplification cycles of 95°C for 40 s, 50°C for 30 s, and 72°C for 30 s were performed. Plasmid pSK13 was constructed by amplifying *rsmA* and downstream regions from *P. aeruginosa* PAO1 using primers RSMAPA3 and RSMAPA4, digesting the resulting PCR product with *BamHI* and *XbaI*, and subsequently ligating this fragment into *BamHI*-*XbaI*-digested pBluescript. The PCR conditions for this amplification were the same as used in the construction of pSK9, except for the extension time, which was increased to 45 s. Absence of nucleotide misincorporations in the PCR amplicons cloned in pBluescript were verified by sequencing. Plasmid pSK82 resulted from a triple ligation between *EcoRI*-*XbaI*-

digested pSK9, the *EcoRI-BamHI-lacI^Q* P_{tac} fragment from pME6032 (26), and the 503-bp *BamHI-XbaI-rsmA* fragment from pSK13. Plasmid pSK59 resulted from the ligation of the 634-bp *XhoI-SspI*-P_{tac}-*rsmA* fragment from pSK82 into *XhoI-SmaI*-digested pSK13. Plasmid pSK61 was constructed by ligating the 792-bp *XhoI-SacI*-P_{tac}-*rsmA* fragment from pSK59 to *XhoI-SacI*-digested pME6031 and constitutively expressing *rsmA* from the *tac* promoter.

Real-time PCR. For total RNA extraction, *P. syringae* strains B728a, BR2R, and NPS3121 harboring plasmid pSK61 or pME6031 were grown in 5 ml of Kapadnis-Baseri medium (29 g of Bacto proteose peptone, 1.5 g of K₂HPO₄, 8 ml of glycerol, and 0.74 g of MgSO₄ per liter) containing Tc at 28°C and 250 rpm for 48 h. Cultures (1 ml) were centrifuged at 5,000 × g for 5 min at 22°C and the pellets frozen at -80°C until use. Total RNA was extracted from these cell pellets using the Qiagen RNeasy Protect Bacteria Reagent Kit according to the manufacturer's recommendations (Qiagen Inc.) and subsequently treated with Ambion TURBO DNase using the manufacturer's recommendations (Ambion Inc., Austin, TX). The quantity and quality of the DNase-treated RNA were determined using an Experian chip (Bio-Rad, Hercules, CA). cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad) and 25 ng of this cDNA was used per well for PCR amplification.

The probes and primers for real-time PCR (Table 1) were designed using Primer Express software (Applied Biosystems Inc., Foster City, CA). The DNA gyrase subunit B gene of *P. syringae* pv. *phaseolicola* strain ISPaVe596 (accession number EF203074) was chosen as the virulence-independent, single-copy housekeeping gene probe for determining basal expression (44) and *rsmA* from *P. aeruginosa* PAO1, resident gene in pSK61 (bases 991,013 to 991,198, accession number AE004091), was used as probe for analysis of expression of *rsmA* in *P. syringae* strains transformed with pSK61 or pME6031. Both probes were TaqMan MGB with 5'-TET and 3'-quencher NFQ-MGB (Applied Biosystems Inc.). PCR was performed with an ABI PRISM 7700 sequence detector system (Applied Biosystems Inc.). The final concentrations of both forward and reverse primers were 0.9 μM and the probe concentration was 0.24 μM for reactions for each of the respective genes. The amplification conditions were as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s followed by 60°C for 1 min. Fluorescence signals measured during amplification were processed post amplification and considered positive if the fluorescence intensity was >20-fold greater than the standard deviation of the baseline fluorescence (14). The observed cycle threshold (C_t) values were analyzed using Statview 5.0 for Macintosh (Abacus Concepts, Berkeley, CA) and Excel (Windows, version 7). The effect of pSK61 transformation

TABLE 1. Microbial strains, plasmids, and primers

Strain, plasmid, primer, or probe	Relevant characteristics ^y	Reference or source
Strains		
<i>Escherichia coli</i> K12 25922	Indicator strain for phaseolotoxin and tabtoxin production	ATCC ^z
<i>Geotrichum candidum</i>	Indicator strain for syringomycin production	24
<i>Pseudomonas syringae</i> pv. <i>phaseolicola</i> NPS3121	Phaseolotoxin-producing strain	63
<i>P. syringae</i> pv. <i>phaseolicola</i> NPS3121R	Spontaneous Rif ^R mutant of NPS3121	This study
<i>P. syringae</i> pv. <i>syringae</i> B728a	Syringomycin-producing strain	41
<i>P. syringae</i> pv. <i>syringae</i> B728aR	Spontaneous Rif ^R mutant of B728a	This study
<i>P. syringae</i> BR2R	Tabtoxin producing strain; Rif ^R	71
Plasmids:		
pBluescript	Cloning vector; Ap ^R	Agilent Technologies, Inc.
pCR4-Topo	Cloning vector; Ap ^R ; Kan ^R	Invitrogen Corp.
pME6031	pVS1-p15A shuttle cloning vector for gram-negative bacteria; Tc ^R	29
pME6032	<i>lacI^Q</i> -P _{tac} expression vector derived from pME6031; Tc ^R	26
pSK9	Contains upstream region of <i>rsmA</i> from <i>P. aeruginosa</i> PAO1 cloned in pBluescript; Ap ^R	This study
pSK13	Contains <i>rsmA</i> and downstream region from <i>P. aeruginosa</i> PAO1 cloned in pBluescript; Ap ^R	This study
pSK82	pSK9 containing the <i>lacI^Q</i> P _{tac} fragment from pME6032 and the 503-bp <i>BamHI-XbaI-rsmA</i> fragment from pSK13; Ap ^R	This study
pSK59	pSK13 containing the 634-bp <i>XhoI-SspI</i> P _{tac} fragment from pSK82; Ap ^R	This study
pSK61	pME6031 containing the 792-bp P _{tac} - <i>rsmA</i> fragment from pSK59; constitutive expression of <i>rsmA</i> driven from the <i>tac</i> promoter; Tc ^R	This study
Primers		
RSMPOA1	TATGAATTCATGCTGATTCTGACTCGTCG; <i>rsmA</i> upstream forward primer	30
RSMAPA2	TATCTCGAGCTTCGAAATATGGCGCACTC; <i>rsmA</i> upstream reverse primer	This study
RSMAPA3	TATTCTAGACAATGTGGAAGTCGACATGAT; <i>rsmA</i> downstream forward primer	This study
RSMAPA4	CACGCGAATATTTTCAGGACA; <i>rsmA</i> downstream reverse primer	This study
GACS-F/GACS-R	CAACGAGATTCTCGACTTTTCG TTGAGGGATTCTTCTTGTCTTC	This study
GACA-F/GACA-R	TTTTTGTTATGGTGATTGGCATTTCGT GGGTCATGTTTCAGGCGCTGG	This study
RSMA-F/RSMA-R	CTCgaattcATGCTGATTCTGACTCG ATAActcgagTTAATGGCTTGGTT	This study
NPS3121-RSMA-9F/9R	GGTCATATGCTGATTCTGACTCGT CGCGGATCCTTAATGGCTTGGTTC	This study
RSMY-F/RSMY-R	ATAggtaccATGGACGTAGCGCAGGAAG CGCaagcttAAAACGCGACAGCAAGAAAACC GTAAACGACGGCCAG CAGGAAACAGCTATGAC	This study Invitrogen Corp.
Real-time polymerase chain reaction:		
gyrB-28F/gyrB-144R (primers)	TGGAACGACAGCTTCAACGA GTAGTTGTTTCAGGTTACGCGTCA	This study
gyrB-58 MGB T (probe)	TGCTTCACCAACAACAT	This study
rsmA-35F/rsmA-159R (primers)	TGATGGTAGGTGACGACGTCA TTTCTGGATGCGCTGGTAAAT	This study
rsmA-109 MGB T (probe)	CCGAAGGAAGTCGCC	This study

^y Ap^R, Kan^R, Rif^R, and Tc^R = resistant to ampicillin, kanamycin, rifampicin, and tetracycline, respectively; IPTG = isopropyl-β-D-1-thiogalactopyranoside.

^z American Type Culture Collection, Manassas, VA.

on *rsmA* gene expression (C_t values) was evaluated by analysis of variance (ANOVA) (SAS, Inc., Cary, NC) that compared C_t values obtained for the respective induced (pSK61 transformed) and background (pME6031 transformed) conditions. Means were separated using least squares difference, with $P \leq 0.05$ considered to be statistically significant. Real-time PCR treatment reactions were replicated three times. The expression of *rsmA* was adjusted with respect to the constitutive *gyrB* gene using the formula $\Delta\Delta C_t = \Delta C_t (rsmA) - \Delta C_t (gyrB)$. Finally, the fold-increment expression of *rsmA* in the pSK61-transformed strains was calculated in terms of $2^{\Delta\Delta C_t}$ values (65).

Disease assay. Bean (*Phaseolus vulgaris* L. 'Bush Blue Lake 274') seed was planted in 4-in. pots containing Promix BX (Premier Tech Horticulture, Quakertown, PA 18951) and grown in growth chambers at 28 and 23°C with a 16-h photoperiod (photon flux density of 450 $\mu\text{mol}/\text{m}^2/\text{s}$). Plants were watered as needed and fertilized with a nitrogen-phosphorous-potassium (20:20:20) solution at 3.2 mg/ml the day prior to treatment application. Overnight cultures of *Pseudomonas syringae* strains (50 ml) harboring pSK61 or pME6031, grown at 28°C and 250 rpm in KB broth supplemented with Tc, were pelleted by centrifugation, washed with 10 mM MgCl_2 , repelleted, and resuspended in 10 mM MgCl_2 to an optical density at 600 nm (OD_{600}) of 0.01 for B728a strains and BR2R strains ($\approx 2 \times 10^7$ CFU/ml), and 0.03 for NPS3121 strains ($\approx 6 \times 10^7$ CFU/ml). For inoculation, 11-day-old bean plants with fully expanded primary leaves and emerging trifoliate leaves were dipped into a 1-liter *P. syringae*-cell suspension containing 0.02% Silwet L-77 (added immediately before treating plants) for 30 s with gentle swirling (84). Bean plants dipped into 10 mM MgCl_2 with 0.02% Silwet L-77 served as controls. Immediately after treating, plants were transferred to humidity boxes within the growth chamber. The plants were removed from the humidity boxes after 24 h, arranged in a completely randomized design in the growth chamber, and incubated under the conditions described above. Bean plants were examined for disease symptoms at 4 days after inoculation (DAI) for B728a and BR2R treatments and 5 DAI for NPS3121 treatments. Experiments were performed three times for each strain, with six replicate plants per treatment.

Bean leaf colonization assay. Bean plants were grown and bacterial treatments applied as described for the disease assays, with the exception that the Rif-resistant strains B728aR, BR2R, and NPS3121R harboring plasmid pME6031 or pSK61 were used. Experiments with treatments from each pathovar were run independently from those involving treatments from other pathovars. Treatments were replicated six times and arranged in a completely randomized design. Bean leaves were analyzed for colonization at 0 DAI for treatments with all three strains, again at 4 DAI for treatments with strains B728aR and BR2R, and at 5 DAI for treatments with strain NPS3121R. Populations of *P. syringae* strains were determined by a modification of the method of Sabartnam and Beattie (76). One of the first primary leaves, with petiole attached, was removed from the plant and soaked in 1% sodium hypochlorite containing 0.01% Silwet L-77 for 5 min with shaking at 125 rpm. The leaf was subsequently washed twice

with sterile water and patted dry with a sterile paper towel, and four 1.0-cm-diameter discs were sampled from across the leaf approximately half-way down the midrib. Leaf discs were transferred to a Duall 22 glass tissue homogenizer (Kontes Glass Co., Vineland, NJ) and homogenized in 5 ml of 10 mM MgCl_2 . One leaf was sampled from each plant. Homogenates were serially diluted and plated onto KB agar supplemented with Tc and Rif using a spiral platter. The plates were incubated at 28°C and colonies counted after 2 or 3 days of growth. Mean $\log_{10}$ CFU/cm² of leaf for each treatment at each time was determined, ANOVA was carried out, and differences among means were estimated using least significant difference in Statgraphics Plus 5 (Manugistics, Inc., Rockville, MD). All experiments were analyzed independently ($n = 6$) because there were significant experiment effects with experiments containing BR2R treatments ($P < 0.0001$) and NPS3121R treatments ($P < 0.0001$). Experiments containing B728aR could be analyzed together because there was no significant experiment effect ($P = 0.37$) but they were analyzed independently for consistency.

HR assay. Tobacco (*Nicotiana glauca* 'Xanthi') plants were started in 4-by-8-in. flats containing Jiffy Mix (Alwaysgrows Greenhouse Supplies, Sandusky, OH) and grown in growth chambers at 28 and 23°C with a 16-h photoperiod (photon flux density of 450 $\mu\text{mol}/\text{m}^2/\text{s}$). After 21 days, plants were transplanted into 10-in. pots containing Promix BX, watered as needed, and fertilized with a nitrogen-phosphorous-potassium (20:20:20) solution at 3.2 mg/ml every fourth watering. Various dilutions of bacterial suspensions in 10 mM MgCl_2 were infiltrated into individual leaf panels of mature leaves using a blunt syringe. Control treatments received 10 mM MgCl_2 without cells. Leaves were observed for the HR 3 days after treatment. There were three replicates per experiment and experiments were performed three times.

Phytotoxin assays. Phaseolotoxin production was assayed essentially as described (81). *P. syringae* pv. *phaseolicola* NPS3121 strains were grown and cell suspensions prepared as above and adjusted to an OD_{600} of 0.04. These cell suspensions (10 μl) were spotted onto minimal medium (MM) agar plus 0.5% glycerol and incubated at 18°C for 48 h prior to being overlaid with MM containing 7.5% agar and the indicator strain *Escherichia coli* 25922. Plates were incubated an additional 24 h at 37°C and examined for zones of inhibition of growth of *E. coli* 25922. To determine that the inhibition of growth of *E. coli* 25922 was due to phaseolotoxin, parallel assays were conducted in which the overlay medium was supplemented with 0.05 mM L-arginine. Tabtoxin production by *P. syringae* BR2R strains was assayed as for phaseolotoxin production, with the exception that BR2R strains were incubated at 28°C prior to being overlaid with *E. coli* 25922. To determine that the inhibition of growth of *E. coli* 25922 was due to tabtoxin, parallel assays were conducted in which the overlay medium was supplemented with 0.075 mM L-glutamine (23,51). Syringomycin production by *P. syringae* pv. *syringae* B728a strains was assayed essentially as described (24). Cell suspensions (10 μl) were spotted on potato dextrose agar and the plates were incubated at 28°C. After incubation for 48 h, these

Fig. 1. Physical map of pSK61. Plasmid pSK61 constitutively expresses *rsmA* and is derived from the p15A-pVS1 shuttle vector pME6031. *oriV*, regions containing origins of replication and stability determinants; *P_{tac}*, *tac* promoter; *T₄*, T4 transcription terminator; *tetR*, tetracycline-regulated resistance genes. All figure details are drawn to scale. Details for pSK61 are also in Materials and Methods.

plates were sprayed with a mycelial suspension of *Geotrichum candidum*, incubated an additional 4 days at 28°C, and examined for inhibition of growth by *G. candidum*; zones of inhibition of *G. candidum* were indicative of syringomycin production (24). Phytotoxin assays were performed three times, with three replicates per treatment.

Protease plate assay. Strains B728a, BR2R, and NPS3121 harboring either pME6031 or pSK61 were grown on KB agar with Tc for 2 days, rinsed, and resuspended in 10 mM MgCl₂ to a density of $\approx 10^9$ cells/μl. Cell suspensions (2 μl) were applied to a filter disc (3/4-in. diameter) (Schleicher and Schuell, Keene, NH) at the center of NYG skim milk agar plates (42) and incubated at 26°C for 18 h. Plates were examined visually for zones of clearing. Experiments were performed two times, with three replicates per treatment.

Pyoverdine assay. Bacterial cells (5 μl), from cultures prepared as in the phytotoxin assays, were spotted onto mannitol-glutamate (MG) agar medium (37) containing Tc. Plates were incubated at 28°C for 3 days prior to examination for fluorescent pigment production (pyoverdine) under illumination with ultraviolet light. Experiments were performed two times, with three replicates per treatment.

Swarming assay. Swarming motility was assessed on semisolid KB plates that contained 0.4% agar as described previously (40). For this assay, bacterial cells were prepared as described for the protease plate assay. Sterile filter discs (3/4-in. diameter) (Schleicher and Schuell) were placed in the center of semisolid KB agar plates and inoculated with 2 μl of the bacterial suspensions. These plates were subsequently incubated at 26°C for 18 h and examined visually for swarming. Experiments were performed two times, with three replicates per treatment.

Alginate assay. Bacterial suspensions were prepared as described for the phytotoxin assays. These suspensions (100 μl) were spread on the inner three-quarters of KB agar plates amended with Tc and the plates were subsequently incubated in plastic containers for 48 h at 28°C. Cells from these agar plates were suspended in 0.85% NaCl and the EPS extracted as described previously (8). Alginate in the EPS was quantified by a modified carbazole assay (53) and expressed as uronic acid equivalents after interpolation of a uronic acid standard curve. Total protein from the cell pellet was determined by the method of Bradford (5), and alginate was reported as micrograms of uronic acid equivalents per milligram of total cellular protein. Experiments were performed three times with three replicates per treatment

with BR2R and B728a strains, and five times with three replicates per treatment with NPS3121 strains. Mean micrograms of uronic acid equivalents per milligram total cellular protein was determined, ANOVA was carried out, and differences among means were estimated with the Student's *t* test (SAS Inc.). Experiments were analyzed independently (*n* = 3) because there were significant experiment effects with experiments containing BR2R treatments (*P* = 0.0084), B728a treatments (*P* = 0.0002), and NPS3121 treatments (*P* = 0.0022).

Biofilm assay. Bacterial strains were grown overnight in KB broth supplemented with 0.5% casamino acids and Tc, harvested by centrifugation, washed with 10 mM MgCl₂, centrifuged again, and resuspended to an OD₆₀₀ of 0.04 in M63 basal salts (62) supplemented with glucose (0.2%) and casamino acids (0.5%) (61). These bacterial suspensions (100 μl) were added to sterile microtiter dishes and allowed to grow statically at 28°C for 24 h. Samples were processed and biofilms quantitated as previously described (54). The experiments were performed four times with BR2R, three times with B728a, and eight times with NPS3121, with five replicates per treatment. Mean OD₆₀₀ was determined for each treatment, ANOVA was carried out, and differences among means were estimated with the Student's *t* test in SAS. All experiments were analyzed independently (*n* = 5) because there were significant experiment effects with experiments containing BR2R treatments (*P* < 0.0001), B728a treatments (*P* = 0.03), and NPS3121 treatments (*P* = 0.0005).

RESULTS

Identification of major GacS/A-RsmA pathway components in phytotoxin-producing *P. syringae* strains. PCR confirmed the presence of *gacS*, *gacA*, *rsmY*, and *rsmA* in strains BR2R and NPS3121 and *rsmY* in B728a (Table 2). *gacS*, *gacA*, and *rsmA* were detected in *P. syringae* pv. *syringae* B728a in prior work (18). These genes code for the HK GacS, the RR GacA, the small regulatory RNA RsmY, and the global posttranscriptional regulator RsmA. The additional small regulatory RNAs RsmX and RsmZ, which are thought to be functionally redundant with RsmY (36), were not targeted in these PCR experiments. Together *gacA*, *gacS*, *rsmY*, and *rsmA* comprise major components of the GacS/A-RsmA signal transduction pathway (45).

Transformation with pSK61 results in overexpression of *rsmA* in *P. syringae* strains. Plasmid pSK61 (Fig. 1) is stably maintained in vitro and constitutively expresses *rsmA* of *P.*

TABLE 2. Detection of GacS/A-RsmA signal transduction pathway genes in *Pseudomonas syringae* strains B728a, BR2R, and NPS3121^a

Strain, gene	Primer	PCR size (expected/detected) ^y	Sequence accession ^w	Comment
B728a				
<i>gacA</i>	ND	ND/ND	CP000075	Published
<i>gacS</i>	ND	ND/ND	CP000075	Published
<i>rsmA</i>	ND	ND/ND	CP000075	Published
<i>rsmY</i>	RSMY-F/R	140 bp/Yes	CP000075	Published
BR2R				
<i>gacA</i>	GACA-F/R	710 bp/Yes	JN655683	97% identity with <i>gacA</i> from Psp 1448A ^x
<i>gacS</i>	GACS-F/R	555 bp/Yes	JN655684	96% identity with <i>gacS</i> from Psp1448A
<i>rsmA</i>	RSMA-F/R	207 bp/Yes	JN655685	98% identity with <i>RsmA</i> from Psp 1448A
<i>rsmY</i>	RSMY-F/R	140 bp/Yes	JN655686	99% identity with <i>rsmY</i> from Psp 1448A
NPS3121				
<i>gacA</i>	GACA-F/R	710 bp/Yes	JN655687	98% identity with <i>gacA</i> from Psp 1448A
<i>gacS</i>	GAC-F/R	555 bp/Yes	JN655688	99% identity with <i>gacS</i> from Psp1448A
<i>rsmA</i>	NPS3121-RSMA-9F/R	204 bp/No	Not submitted ^y	116 bases with 93% identity with B728a was obtained ^z
<i>rsmY</i>	RSMY-F/R	140 bp/Yes	JN655689	99% identity with <i>rsmY</i> from Psp1448A

^a ND = not determined in this study. Gene was detected in this strain in a prior study. Published = sequence previously published (18).

^y Polymerase chain reaction (PCR) product size.

^w GenBank accession number.

^x Psp 1448A, *P. syringae* pv. *syringae* strain 1448A (GenBank accession number CP000058.1).

^y PCR and DNA sequence analysis was hampered by the preponderance of insertion elements in NPS3121.

^z A portion of the sequence of the amplicon was highly similar to a section of *rsmA*, while the remaining sequence was similar to a transposable element that exists in the genome of NPS3121 in multiple copies.

aeruginosa PAO1 from the P_{tac} promoter. In pilot experiments, 70 ± 50 , 85 ± 13 , and $70 \pm 19\%$ of the populations of BR2R, B728a, and NPS3121, respectively, contained pSK61 after 48 h of incubation in KB broth without Tc selection when cultures were initially inoculated with BR2R(pSK61), B728a(pSK61), and NPS3121(pSK61), respectively. Estimations of percent population containing pSK61 were made from data derived from dilution-plating onto KB agar with and without Tc. Amino acid sequence analysis of *rsmA* from PAO1 and the three phytotoxin-producing strains of *P. syringae* included in this study revealed that the residues important for maintaining RsmA structure and that form the potential RNA-binding site are conserved among these strains (data not shown) (30). Introduction of pSK61 into *P. syringae* strains B728a, BR2R, and NPS3121 resulted in elevated levels of *rsmA* mRNA in these strains (Table 3). Levels of *rsmA* mRNA were significantly greater in B728a, BR2R, and NPS3121 harboring pSK61 than when these strains harbored the empty cloning vector pME6031. In contrast, levels of the single-copy, housekeeping-gene control *gyrB* were similar between individual strain pairs harboring pSK61 or pME6031. The mean C_t values for *gyrB* and *rsmA* expression, the adjusted incremental C_t values ($\Delta\Delta C_t$) of *rsmA* expression with respect to *gyrB* expression, and the fold-increase ($2^{\Delta\Delta C_t}$) of *rsmA* expression due to pSK61 for each of the three *P. syringae* strains are presented in Table 3. Using *gyrB* primers and probe, no product could be traced after 36 cycles of PCR from the cDNA of DNase-treated total RNA of the three *P. syringae* strains using the PCR conditions detailed in the Materials and Methods section, indicating the absence of detectable genomic DNA contamination in these total RNA preparations (data not shown).

Overexpression of *rsmA* results in diminished disease and internal leaf colonization in all *P. syringae* strains tested. Pot experiments were conducted in the growth chamber to determine the impact of overexpression of *rsmA* on disease on bean by the *P. syringae* strains used in this study. Disease on bean plants was considerably reduced in *P. syringae* pv. *syringae* B728a, *P. syringae* pv. *phaseolicola* NPS3121, and *P. syringae* BR2R due to overexpression of *rsmA* by pSK61 (Fig. 2). The treated leaves of bean plants inoculated with B728a harboring the empty cloning vector pME6031 were severely diseased within 4 DAI whereas bean plants inoculated with B728a overproducing RsmA due to the presence of pSK61 were unaffected (Fig. 2). *P. syringae* BR2R and *P. syringae* pv. *phaseolicola* NPS3121 harboring pME6031 and producing wild-type levels of RsmA were not as aggressive on bean as strain B728a(pME6031) (Fig. 2). However, overexpression of *rsmA* from pSK61 resulted in diminished disease with strains BR2R and NPS3121 and resembled bean leaves treated with negative controls.

Colonization of internal portions of bean leaves by *P. syringae* strains was significantly reduced due to overexpression of *rsmA* from pSK61 (Fig. 2; Supplementary Table 1). In all cases, populations of strains harboring pSK61 were similar to or greater than those harboring pME6031 at the time of inoculation. Populations of strains harboring pSK61 were significantly lower ($P < 0.05$) than these strains harboring the empty vector pME6031 at 4 DAI for B728aR and BR2R strains or 5 DAI for NPS3121R strains. Populations of Rif-resistant bacteria were below detectable limits in all experiments in the noninoculated controls.

An in planta pilot plasmid stability experiment was conducted with strains BR2R(pSK61), B728a(pSK61), and NPS3121(pSK61) with bean plants prepared as with these disease and internal leaf colonization assays. At 3 DAI, 17 ± 41 , 75 ± 26 , and $24 \pm 9\%$ of the populations of BR2R, B728a, and NPS3121, respectively, contained pSK61. Percentage of the populations of these strains containing pSK61 was determined by dilution-plating onto KB agar with and without Tc.

The impact of overexpressing *rsmA* on the HR varied with *P. syringae* pathovar. HR in tobacco leaves was noticeably reduced with strains BR2R and NPS3121 when harboring pSK61 relative to these strains harboring pME6031 (Fig. 3). However, there was no difference in HR induced by B728a when transformed with either pSK61 or pME6031.

Overexpression of *rsmA* results in substantially reduced phytotoxin production by all *P. syringae* strains tested. Little to no phytotoxin was detected from *P. syringae* pv. *phaseolicola* NPS3121, *P. syringae* pv. *syringae* B728a, and *P. syringae* BR2R harboring pSK61 (Fig. 4). Zones of inhibition of *E. coli* 25992, indicative of phaseolotoxin production (81), were substantially greater with strain NPS3121 harboring the empty vector pME6031 than when this strain harbored pSK61. There was no apparent zone of inhibition of this *E. coli* indicator strain with NPS3121(pSK61). In parallel assays, when the overlay medium containing the indicator *E. coli* strain was supplemented with 0.05 mM L-arginine, there were no zones of inhibition in treatments containing NPS3121(pME6031) (data not shown), demonstrating that the inhibition was due to phaseolotoxin. Phaseolotoxin is an inhibitor of L-ornithine carbamyltransferase, creating a phenotypic requirement for L-arginine (81). Overexpression of *rsmA* from pSK61 also had a very strong effect on tabtoxin production by strain BR2R. There was a large zone of inhibition in the lawn of *E. coli* 25992 around the BR2R(pME6031) colony whereas there was a much smaller and more opaque zone of inhibition around BR2R(pSK61). In parallel assays, when the overlay medium containing the *E. coli* indicator strain contained 0.075 mM L-glutamine, there were no zones of inhibition in treatments containing BR2R(pME6031) (data not

TABLE 3. Real-time polymerase chain reaction (PCR) estimates of *rsmA* and DNA gyrase subunit B (*gyrB*) expression in *Pseudomonas syringae* pv. *syringae* B728a, *P. syringae* BR2R, and *P. syringae* pv. *phaseolicola* NPS3121 containing plasmids pSK61 or pME6031

Strain pair	Average C_t^w		ΔC_t^x	$\Delta\Delta C_t^y$	Fold-incremental expression <i>rsmA</i> ^z
	<i>rsmA</i>	<i>gyrB</i>			
B728a(pME6031)	35.2 ± 1.8 A	21.2 ± 0.3 A	14.1	...	...
B728a(pSK61)	15.6 ± 0.0 B	21.0 ± 0.2 A	-5.3	19.4	6.7×10^5
BR2R(pME6031)	33.4 ± 1.1 C	20.5 ± 0.1 A	12.8	...	...
BR2R(pSK61)	15.7 ± 0.0 B	20.4 ± 0.1 A	-4.8	17.6	2.0×10^5
NPS3121(pME6031)	30.9 ± 0.1 D	21.2 ± 0.1 A	9.7	...	...
NPS3121(pSK61)	16.6 ± 0.1 B	21.7 ± 0.3 A	-5.1	14.9	2.9×10^4

^w C_t = cycle number at which the DNA concentration in the PCR reaction crossed the detection threshold. This provided a relative measure of the concentration of the target mRNA in the PCR reaction (the lower the cycle number, the higher the initial mRNA concentration). The DNA gyrase subunit B gene of *P. syringae* pv. *phaseolicola* strain ISPaVe596 (accession number EF203074) was chosen as the virulence-independent, single-copy housekeeping gene probe for determining basal expression (44) and *rsmA* from *P. aeruginosa* PAO1, resident gene in pSK61 (bases 991,013 to 991,198, accession number AE004091), was used as probe for analysis of expression of *rsmA* in pSK61- and pME6031-transformed *P. syringae* strains. Values reported are the average of three replicate C_t values. Values within a column followed by the same letter are not significantly different at $P \leq 0.05$.

^x $\Delta C_t = C_t(\text{rsmA}) - C_t(\text{gyrB})$.

^y $\Delta\Delta C_t = (\Delta C_t \text{ in pME6031-transformed cells}) - (\Delta C_t \text{ in pSK61-transformed cells})$.

^z Fold-incremental expression of *rsmA* in *P. syringae* strains transformed with pSK61 over background *rsmA* expression (strains transformed with pME6031).

shown), indicating that inhibition was due to tabtoxin. Tab-toxinine- β -lactam, the degradation product of tabtoxin, is a potent inhibitor of glutamine synthetase, resulting in a phenotypic requirement for L-glutamine (3,23). Zones of inhibition of *G. candidum*, indicative of syringomycin production (24), were considerably greater with strain B728a harboring the empty cloning vector pME6031 than when this strain harbored pSK61. There was no apparent zone of inhibition of this *G. candidum* indicator strain with B728a(pSK61). There are no known metabolites that can reverse the fungal growth inhibition effects of syringomycin.

Overexpression of *rsmA* results in diminished protease and pyoverdine production in all *P. syringae* strains tested that exhibited these phenotypes. Colonies of *P. syringae* strain BR2R and *P. syringae* pv. *syringae* B728a on NYG skim milk agar produced noticeably less protease due to overproduction of RsmA by pSK61 (Fig. 5). Zones of clearing on these plates due to proteolytic degradation of milk protein were substantially reduced in both strains when harboring pSK61 compared with these strains harboring pME6031. Protease production by *P. syringae* pv. *phaseolicola* NPS3121 was not detected with these assays (data not shown). Pyoverdine production on MG agar medium was also reduced in *P. syringae* strains due to overexpression of *rsmA* mediated by pSK61 (Fig. 6). Zones of fluorescence, indicative of pyoverdine production, were substantially smaller for strains B728a, BR2R, and NPS3121 when these strains harbored pSK61 instead of pME6031.

Overexpression of *rsmA* results in diminished swarming in all *P. syringae* strains tested that exhibited this phenotype. Swarming motility was also severely reduced in *P. syringae* strains B728a and BR2R due to overexpression of *rsmA* by pSK61 (Fig. 7). Regions of swarming on KB plates containing 0.4% agar were substantially greater with strains B728a and BR2R containing the empty vector pME6031 than when these strains harbored pSK61. *P. syringae* pv. *phaseolicola* NPS3121 containing either plasmid pSK61 or pME6031 failed to swarm under the conditions of this assay.

The impact of *rsmA* overexpression on alginate production and biofilm production varied with *P. syringae* pathovar. Alginate production, expressed as micrograms of uronic acid equivalents per milligram of protein, was severely reduced in *P. syringae* strain BR2R and *P. syringae* pv. *syringae* strain B728a due to overexpression of *rsmA* from pSK61 (Table 4). Alginate production trended lower with NPS3121(pSK61) relative to NPS3121(pME6031), with micrograms of uronic acid equivalents per milligram of protein being lower with NPS3121(pSK61) in four of five experiments. However, alginate production was significantly lower with NPS3121(pSK61) in only three of five experiments (Table 4).

The impact of RsmA overproduction on in vitro biofilm production, as determined using the crystal violet method, varied with the *P. syringae* pathovar tested (Table 4). Biofilm production was not significantly reduced for *P. syringae* BR2R when harboring plasmid pSK61 in four of four experiments relative to

Fig. 2. A to C, Disease and D, internal leaf colonization assays with *Pseudomonas syringae* strains containing pSK61 or pME6031. Disease assay with strains of A, *P. syringae* pv. *syringae* B728a; B, *P. syringae* BR2R; or C, *P. syringae* pv. *phaseolicola* NPS3121. In each subfigure (A to C), bean plants were dipped into the test strain harboring the empty cloning vector pME6031 in 10 mM MgCl₂ with 0.02% Silwet L-77 (left plant) or the test strain harboring pSK61 in 10 mM MgCl₂ with 0.02% Silwet L-77 (right plant). Bacterial cells in these suspensions were at optical densities at 600 nm of 0.01 for the B728a and BR2R treatments and 0.03 for the NPS3121 treatment. Negative controls (not shown) were solutions of 10 mM MgCl₂ containing 0.02% Silwet L-77 without bacteria. Following inoculation, plants were held for 24 h in humidity chambers and then maintained under normal growth-chamber conditions. Photos were taken 4 days after inoculation (DAI) for the B728a and BR2R treatments and 5 DAI for the NPS3121 treatment. SK, pSK61; ME, pME6031. Pictures are representative of all six replicates in three experiments with these strains. D, Internal leaf colonization assay with strains B728a, BR2R, and NPS3121 containing pME6031 (ME) or pSK61 (SK). Log₁₀ CFU/cm² of bean leaf tissue was determined at the time of inoculation (0 DAI) and 4 DAI for BR2R and B728a strains and 5 DAI for NPS3121 strains by spiral plating onto selective media for each strain. Values are the mean of a single experiment with six replicates per treatment ($n = 6$). Means were separated using least significant difference where values followed by the same letter are not significantly different ($P \leq 0.05$). Comparisons were made within and between columns for a particular strain.

BR2R (pME6031). However, biofilm production was considerably reduced in the presence of pSK61 with strain B728a in three of three experiments. The impact of RsmA overexpression on in vitro biofilm production by strain NPS3121 was inconsistent. Biofilm production trended lower with strain NPS3121 when harboring pSK61 in six of eight experiments but was significantly lower when harboring pSK61 in only four of eight experiments relative to when this strain harbored pME6031.

DISCUSSION

RsmA is a member of the RsmA/CsrA family of RNA-binding proteins that mediate post-transcriptional control of gene expression in bacteria. When acting as negative effectors of gene expression, these small (≈ 7 -kDa) proteins recognize and bind to specific sites located in the 5' leader sequence of target mRNAs, thereby blocking the interaction of the ribosomal binding site on the mRNA molecule with the 30S ribosomal subunit, ultimately reducing translation and mRNA stability (6,45,46,78). The mechanistic link between RsmA and the GacS/GacA two-component system within the Gac/Rsm signal transduction pathway was demonstrated at the molecular level in *Pseudomonas* spp. in studies conducted with *P. fluorescens* (1,4). Upon activation, the GacS/GacA two-component system was shown to switch on expression of *rsmX*, *rsmY*, and *rsmZ*, genes that encode the small regulatory RNAs RsmX, RsmY, and RsmZ (45). RsmX, RsmY, and RsmZ, in turn, antagonize RsmA by competing with the target mRNAs for the binding site on RsmA (6). In experiments presented here (Table 2) and elsewhere (2,15,18,27,72), major components of the Gac/Rsm signal transduction pathway were shown to be present in strains B728a, BR2R, and NPS3121 producing the phytotoxins syringomycin, tabtoxin, and phaseolotoxin, respectively. However, the linkage between the GacS/GacA two-component system and RsmA has never been demonstrated at the phenotype level in these strains or any other

pathovar of *P. syringae*. According to the accepted model, mutations in *gacA* or *gacS* should provide phenotypes that are consistent with those obtained with the overexpression of *rsmA* (45). Through mutagenesis, it was shown that *gacS* or *gacA* in *P. syringae* pv. *syringae* B728a were important for lesion formation on bean; for production of protease, syringomycin, and alginate; and for swarming on swarm media (40,72,73,85,86). Likewise, it was shown that *gacS* was important in *P. syringae* BR2R for lesion formation on bean and for production of exoprotease and tabtoxin (2,27). Here, we show that overexpression of *rsmA* from pSK61 results in these same phenotype changes in *P. syringae* pv. *syringae* B728a and *P. syringae* BR2R as those previously reported for *gacS* or *gacA* mutations of these strains. These results are expected when there is a molecular link between the GacS/GacA two-component system and RsmA in these strains.

The situation with *P. syringae* pv. *phaseolicola* NPS3121 has been less clear. Prior work with NPS3121 indicated that mutations in *gacS* (*lemA*) did not affect water-soaked lesion formation on bean or phaseolotoxin production (72), seemingly in contradiction to numerous studies with other *P. syringae* strains that demonstrated a critical role for the GacS/GacA two-component system in achieving full virulence and pathogenicity (27,45). Recent work with *gacS* and *gacA* mutants of NPS3121 has demonstrated that *gacS* or *gacA* mutations in this strain no longer produce phaseolotoxin (15). This recent study in conjunction with our work not only demonstrate the linkage between the GacS/GacA two-component system and RsmA at the phenotype level in NPS3121 with regard to phaseolotoxin production but also that NPS3121 is like other strains of *P. syringae* where the Gac/Rsm signal transduction pathway is required for full virulence and pathogenicity (45). It has been hypothesized that the Tn5 insertion in *gacS* in the work of Rich et al. (72) left the GacS phosphotransferase activity intact (15).

rsmA was consistently important in regulating the overarching phenotypes disease and epiphytic colonization in the phytotoxin-

Fig. 3. Effects of *rsmA* overexpression on the hypersensitive response in tobacco by *Pseudomonas syringae* toxin-producing strains. Strains were transformed either with the empty vector pME6031 (ME) or with pSK61 (SK) which provides for the constitutive expression of *rsmA*. Areas next to the letters A, B, and C were inoculated with cell suspensions that were not diluted, diluted 1:2, or diluted 1:10 with 10 mM MgCl_2 , respectively. The area next to the letter D was inoculated with 10 mM MgCl_2 (no cells). Optical densities at 600 nm of undiluted cell suspensions were 0.1 for strains B728a and BR2R, and 0.3 for NPS3121. Photos were taken 3 days after inoculating the leaves. Results are representative of all three replicates from each of three experiments performed with each strain.

Fig. 4. Phytotoxin production by *Pseudomonas syringae* BR2R, B728a, and NPS3121 harboring plasmids pME6031 or pSK61. Toxin production was assayed using indicator strains where zones of inhibition are indicative of phytotoxin production. Plasmid pSK61 (SK) contains *rsmA* under constitutive expression while pME6031 (ME) is the empty cloning vector. See Materials and Methods for assay conditions. Pictures are representative of all three replicates in three experiments with these strains.

Fig. 5. Protease production by *Pseudomonas syringae* pv. *syringae* B728a and *P. syringae* BR2R harboring plasmids pME6031 or pSK61. Protease production was assayed using skim milk agar plates where zones of clearing are indicative of protease activity. Plasmid pSK61 (SK) provides constitutive expression of *rsmA* while pME6031 (ME) is the empty cloning vector. Pictures were taken after 18 h of incubation at 26°C and are representative of all three replicates in two experiments with these strains.

producing strains of *P. syringae* but the role of *rsmA* varied with pathovar in certain underpinning phenotypes. RsmA played an important role in regulation of disease on bean plants because overexpression of *rsmA* from pSK61 resulted in a considerable reduction in disease caused by strain B728a and resulted in a reduction in disease caused by strains NPS3121 and BR2R (Fig. 2). Likewise, RsmA played a regulatory role in colonization of bean leaves. Populations of strains B728a, BR2R, and NPS3121 at 4 to 5 DAI were all significantly lower in bean leaves inoculated with strains harboring pSK61 than with strains transformed with the empty vector pME6031 (Fig. 2). However, the role of RsmA varied among these strains with regard to some of the underpinning phenotypes that contribute to colonization and disease. For example, the role of RsmA in alginate production and biofilm formation varied with the strain tested. Additionally, the impact of overexpression of *rsmA* on HR in the nonhost tobacco in these phytotoxin-producing strains varied with the pathovar tested (Fig. 3). This is consistent with other studies, where the phenotypes regulated by the Gac/Rsm signal transduction pathway have been shown to vary widely among different bacterial species (45). HR in non-host plants caused by *P. syringae* pv.

Fig. 6. Pyoverdinin production by *Pseudomonas syringae* pv. *syringae* B728a, *P. syringae* BR2R, and *P. syringae* pv. *phaseolicola* NPS3121 harboring plasmids pME6031 (ME) or pSK61 (SK). Pyoverdinin production was assayed on mannitol-glutamate medium, where zones of fluorescence are indicative of pyoverdinin production. Plasmid pSK61 contains *rsmA* under constitutive expression while pME6031 is the empty cloning vector. Pictures were taken after 3 days of incubation at 28°C and are representative of all three replicates in two experiments with these strains.

tabaci (strain 6605) and *P. syringae* pv. *syringae* is independent of regulation by the GacS/GacA two-component system (52,59) whereas HR in non-host plants caused by *P. syringae* pv. *tomato* DC3000 is under the regulation of GacS/GacA two-component system (79). Nevertheless, the effect of *rsmA* overexpression on

HR and alginate production by B728a(pSK61) was similar to that of a mutation in *gacS* in B728a (79,85).

P. syringae produces two major EPS: levan, a β -(2,6) polyfructan with extensive branching through β -(1,4) linkages; and alginate, a copolymer of O-acetylated β -(1,4)-linked D-man-

Fig. 7. Swarming by *Pseudomonas syringae* pv. *phaseolicola* NPS3121, *P. syringae* pv. *syringae* B728a, and *P. syringae* BR2R harboring plasmids pME6031 (ME) or pSK61 (SK). Plasmid pSK61 provides constitutive expression of *rsmA* while pME6031 is the corresponding empty cloning vector. Pictures were taken after 18 h of incubation at 26°C and are representative of all three replicates in two experiments with these strains.

TABLE 4. Production of alginate and biofilm by strains of *Pseudomonas syringae*^x

Experiment	Strains			Strains			Strains		
	BR2R (pME6031)	BR2R (pSK61)	<i>P</i> > <i>t</i>	B728a (pME6031)	B728a (pSK61)	<i>P</i> > <i>t</i>	NPS3121 (pME6031)	NPS3121 (pSK61)	<i>P</i> > <i>t</i>
Alginate^y									
1	49.4 ± 2.7	3.3 ± 0.5	<0.0001	13.5 ± 1.4	4.7 ± 1.1	0.001	10.2 ± 0.3	2.7 ± 0.2	0.0045
2	127.0 ± 19.8	1.6 ± 0.6	0.0004	9.2 ± 0.7	2.5 ± 0.3	0.0001	2.9 ± 0.3	2.7 ± 1.0	0.81
3	339.4 ± 14.8	2.6 ± 0.3	<0.0001	9.9 ± 0.9	2.8 ± 1.1	0.0009	3.1 ± 0.4	1.6 ± 0.3	0.005
4	...	...	...	...	...	...	5.2 ± 0.7	6.4 ± 2.6	0.5
5	...	...	...	...	...	...	4.7 ± 0.1	1.9 ± 1.1	0.01
Biofilm^z									
1	0.6 ± 0.1	0.5 ± 0.1	0.43	0.9 ± 0.1	0.5 ± 0.1	0.0003	1.9 ± 0.1	2.5 ± 0.1	0.013
2	0.3 ± 0.0	0.4 ± 0.0	0.03	0.9 ± 0.0	0.5 ± 0.0	0.0001	1.4 ± 0.3	1.4 ± 0.3	0.96
3	0.4 ± 0.1	0.4 ± 0.1	0.3	1.4 ± 0.1	0.4 ± 0.1	0.0002	0.7 ± 0.2	0.4 ± 0.1	0.009
4	0.4 ± 0.0	0.4 ± 0.0	0.46	...	...	...	1.9 ± 0.1	0.3 ± 0.1	0.0001
5	...	...	...	...	...	...	2.8 ± 0.4	2.2 ± 0.5	0.054
6	...	...	...	...	...	...	0.6 ± 0.2	0.2 ± 0.1	0.009
7	...	...	...	...	...	...	2.0 ± 0.8	1.2 ± 0.3	0.079
8	...	...	...	...	...	...	2.0 ± 0.6	1.3 ± 0.7	0.12

^x BR2R is *P. syringae* BR2R, B728a is *P. syringae* pv. *syringae* B728a, and NPS3121 is *P. syringae* pv. *phaseolicola* NPS3121. Plasmid pSK61 contains *rsmA* under constitutive expression while pME6031 is the empty cloning vector.

^y Alginate production was determined by a modified carbazole assay (53) and expressed as mean uronic acid equivalents per milligram of total cellular protein with standard deviation. Differences between means were estimated using the Student's *t* test. Experiments contained three replicates per treatment and were analyzed independently.

^z Biofilm production was determined using the Crystal Violet method (54) and expressed as mean optical density at 600 nm per treatment with standard deviation. Differences between means were estimated using the Student's *t* test. Experiments contained five replicates per treatment and were analyzed independently.

nuronic acid and its C-5 epimer, L-guluronic acid (20,21, 25,31,49,60). The structural genes for alginate production and their organization in the *P. syringae* pathovars examined are nearly identical to those of the structural gene cluster in *P. aeruginosa* (9,11,18,35,64). Previous studies on EPS produced by *P. syringae* indicated that alginate was the major EPS produced in planta (19). The uronic acid polymer component of EPS from *P. syringae* pv. *syringae* FF5 was shown to consist solely of alginate (38), and there are no reports that EPS produced by *P. syringae* contains any other uronic acid polymer. Consequently, an assay that utilizes carbazole, which reacts with uronic acid (53), has been commonly used to estimate alginate production in *P. syringae* pathovars.

Many of the important molecular features of the Gac/Rsm signal transduction pathway are known and it has been established that the Gac/Rsm signal transduction pathway is the master regulator in *P. syringae* (10,45). However, small variations in the Gac/Rsm signal transduction pathway between species have been reported. In *Erwinia* spp., a nontranslated *rsmB* RNA binds to RsmA and neutralizes its regulatory effects (50); the levels of *rsmB* RNA are positively regulated by the GacS/GacA two-component system (12). Comparable postranslational systems regulate exoproduct production and biological activity in the pseudomonad opportunistic pathogen *P. aeruginosa* and the biological control strains *P. fluorescens* CHA0 and Pf-5 (6,7,80, 82). However, the Gac/Rsm signal transduction pathway varies slightly in these pseudomonads from that of *Erwinia* spp., in that they include multiple sRNAs (RsmZ and RsmY in *P. aeruginosa* and RsmZ, RsmY, and RsmX in *P. fluorescens*) and *P. fluorescens* produces at least one additional RsmA homologue, RsmE, with at least partially redundant function with RsmA. In *P. fluorescens* CHA0, a single mutation in *rsmA* was insufficient for the complete derepression of factors regulated by the Gac/Rsm signal transduction pathway. A double mutant with mutations in both *rsmA* and *rsmE* was required to achieve the same effect (70). Clearly more studies, such as the one reported here, are required to further define the mechanism by which RNA-binding proteins function within the Gac/Rsm signal transduction pathway to regulate disease and other lifestyles in *P. syringae*.

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