AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Bacteriology®

RESEARCH ARTICLE

RNase E Promotes Expression of Type III Secretion System Genes in *Pseudomonas aeruginosa*

Josh S. Sharp,[a,b] Arne Rietsch,[c] Simon L. Dove[a]

[a]Division of Infectious Diseases, Boston Children's Hospital, Harvard Medical School, Boston, Massachusetts, USA
[b]Biology Department, Northern Michigan University, Marquette, Michigan, USA
[c]Department of Molecular Biology and Microbiology, Case Western Reserve University, Cleveland, Ohio, USA

ABSTRACT *Pseudomonas aeruginosa* is an important opportunistic pathogen that employs a type III secretion system (T3SS) to inject effector proteins into host cells. Using a protein depletion system, we show that the endoribonuclease RNase E positively regulates expression of the T3SS genes. We also present evidence that RNase E antagonizes the expression of genes of the type VI secretion system and limits biofilm production in *P. aeruginosa*. Thus, RNase E, which is thought to be the principal endoribonuclease involved in the initiation of RNA degradation in *P. aeruginosa*, plays a key role in controlling the production of factors involved in both acute and chronic stages of infection. Although the posttranscriptional regulator RsmA is also known to positively regulate expression of the T3SS genes, we find that RNase E does not appreciably influence the abundance of RsmA in *P. aeruginosa*. Moreover, we show that RNase E still exerts its effects on T3SS gene expression in cells lacking all four of the key small regulatory RNAs that function by sequestering RsmA.

IMPORTANCE The type III secretion system (T3SS) is a protein complex produced by many Gram-negative pathogens. It is capable of injecting effector proteins into host cells that can manipulate cell metabolism and have toxic effects. Understanding how the T3SS is regulated is important in understanding the pathogenesis of bacteria with such systems. Here, we show that RNase E, which is typically thought of as a global regulator of RNA stability, plays a role in regulating the T3SS in *Pseudomonas aeruginosa*. Depleting RNase E results in the loss of T3SS gene expression as well as a concomitant increase in biofilm formation. These observations are reminiscent of the phenotypes associated with the loss of activity of the posttranscriptional regulator RsmA. However, RNase E-mediated regulation of these systems does not involve changes in the abundance of RsmA and is independent of the known small regulatory RNAs that modulate RsmA activity.

KEYWORDS GacA, global regulatory networks

Pseudomonas aeruginosa is a pathogenic bacterium capable of causing a number of opportunistic infections. *P. aeruginosa* infections are a major cause of morbidity and mortality in patients with cystic fibrosis (1). Patients with wounds or burns are also susceptible to *P. aeruginosa* infection (2). Urinary tract and lung infections with this organism are common in hospitalized patients who require catheters or ventilator-assisted breathing (2). *P. aeruginosa* infections are especially difficult to treat because these bacteria have a naturally high level of resistance to antibiotic treatment (1–3).

Prominent among those virulence factors that *P. aeruginosa* uses to infect the host is the type III secretion system (T3SS). *P. aeruginosa* uses its T3SS to inject effector proteins into host cells (4); the toxic effectors delivered by the T3SSs are critical for the organism to establish an acute infection and are important for the survival of the

Citation Sharp JS, Rietsch A, Dove SL. 2019. RNase E promotes expression of type III secretion system genes in *Pseudomonas aeruginosa*. J Bacteriol 201:e00336-19. https://doi.org/10.1128/JB.00336-19.

Editor Victor J. DiRita, Michigan State University

Address correspondence to Josh S. Sharp, jsharp@nmu.edu.

Received 14 May 2019
Accepted 26 August 2019
Accepted manuscript posted online 3 September 2019
Published 21 October 2019

bacterium inside the host, as well as for the systemic spread of the bacterium within the host (5).

Effector secretion via the T3SS is triggered by host-cell contact or, *in vitro*, by removing calcium from the growth medium (6, 7). Triggering of effector secretion results in a concomitant upregulation of T3SS gene expression, which results from the activation of the master activator of T3SS gene transcription, ExsA (8). If effector secretion is off, ExsA is sequestered by the antiactivator protein ExsD, allowing only low-level expression of T3SS genes (9). Upon triggering of effector secretion, the sensor protein ExsE is exported via the T3SS, freeing it from the chaperone protein ExsC and allowing ExsC to bind ExsD, thereby releasing ExsA such that it can activate the transcription of the T3SS genes (Fig. 1A) (8, 53).

ExsA expression is positivity regulated by cyclic AMP (cAMP) levels in the cell. The cAMP-dependent DNA binding protein Vfr has been shown to bind to the ExsA promoter and activate its transcription (10). ExsA is also positively regulated at the posttranscriptional level. The RNA helicase DeaD promotes the translation of ExsA by likely reducing secondary structures within the ExsA mRNA (11).

Virulence gene expression in *P. aeruginosa* is tied into the global regulatory networks of the cell (8, 51). One of the most important of these networks is controlled by the Gac/RsmA system, which reciprocally controls the expression of genes involved in acute and chronic infection (12, 13) (Fig. 1B). In particular, the sensor kinase GacS controls the activity of the response regulator GacA, which in turn positively regulates the expression of the *rsmY* and *rsmZ* genes encoding the small RNAs RsmY and RsmZ, respectively (14). These two small RNAs exert their influence on gene expression by binding to and modulating the activity of the RNA-binding protein RsmA, which is a posttranscriptional regulator that typically acts to repress the translation of target transcripts and influences the expression of hundreds of genes (15). For example, RsmA acts to repress type VI secretion system (T6SS) gene expression but activates T3SS gene expression (15, 16). This signaling cascade is subject to further fine control by two hybrid sensor kinases, RetS and LadS, with RetS serving to inhibit the activity of GacS (12, 13, 17) and LadS serving to promote the activity of GacS (18).

In many Gram-negative bacteria, the endonuclease RNase E is highly conserved and is essential for cell survival (19, 20). In *Escherichia coli*, RNase E is the principal enzyme involved in mRNA degradation and can interact with polynucleotide phosphorylase, as well as the RNA helicase RhlB and the glycolytic enzyme enolase, to form a high-molecular-weight complex referred to as the degradosome (21).

In several pathogenic bacteria, RNase E has been implicated in the control of virulence gene expression. In *Escherichia coli* O157:H7, RNase E deficiency has been linked to reduced stx_2 phage replication (22). In *Yersinia* spp., RNase E is thought to positively regulate the expression of genes encoding the type III secretion system and influence intracellular survival (23). However, assessing the contribution of RNase E to the control of gene expression in pathogenic bacteria is complicated by the fact that the protein is often essential. Here, we show that depletion of RNase E in *P. aeruginosa* results in decreased expression of T3SS genes and increased biofilm formation and expression of genes encoding the T6SS. Our findings suggest that RNase E plays a critical role in the control of both acute and chronic virulence factors in *P. aeruginosa*.

RESULTS

Use of a ClpXP-based protein depletion system to study RNase E in *P. aeruginosa*. In *E. coli*, RNase E is essential for viability (19, 20), and transposon insertion sequencing experiments suggest that RNase E is essential in *P. aeruginosa* strain PAO1 as well as other strains of *P. aeruginosa* (24, 25). We therefore sought to determine whether we could study the effects of RNase E on the expression of virulence genes in *P. aeruginosa* using a previously described ClpXP protease-based protein depletion system (26, 27) (Fig. 2A). This system requires the fusion of a small peptide tag, referred to here as DAS4, to the C terminus of a protein of interest, in this case, RNase E. The

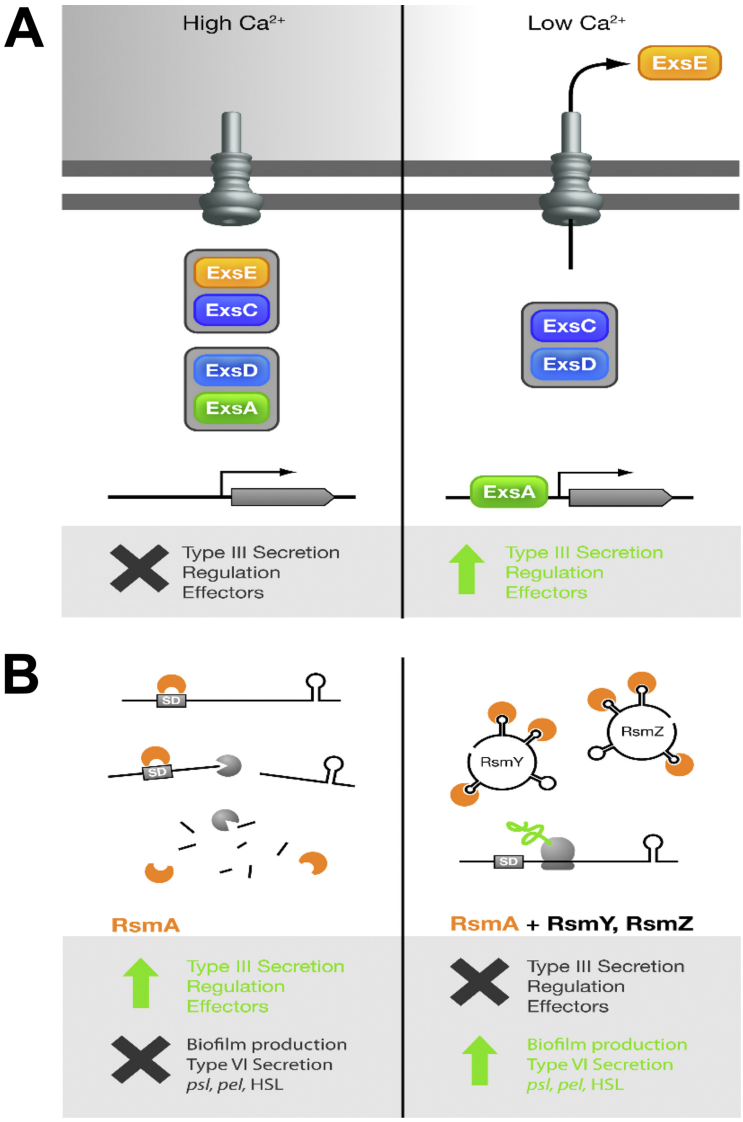

FIG 1 Models of the control of type III secretion in *P. aeruginosa* and the control of RsmA activity. (A) Model of type III secretion system regulation in *P. aeruginosa*. Under conditions of high calcium (Ca^{2+}) concentrations (left), ExsE forms a complex with ExsC, ensuring that ExsA forms a complex with ExsD, thus preventing expression of the type III secretion genes. In response to low calcium (right), ExsE is secreted, allowing ExsC to form a complex with ExsD, thus allowing ExsA to activate transcription from target promoters. In both panels, the gray lines correspond to the outer membrane (uppermost line) and inner membrane. (B) Reciprocal regulation of acute and chronic virulence determinates by the posttranscriptional regulator RsmA and the sRNAs RsmY and RsmZ. When RsmA (depicted in orange) recognizes GGA target sites within, or in close proximity to, Shine-Dalgarno sequences (SD) on target mRNAs, translation is inhibited, which can result in the degradation of target transcripts. This promotes expression of the T3SS genes (indicated by the arrow) and inhibits biofilm formation as well as expression of T6SS genes (indicated by the cross). Under conditions in which the production of the sRNAs RsmY and RsmZ is upregulated, these sRNAs interact with RsmA, thus sequestering it from target mRNA species, resulting in their translation. This serves to repress T3SS gene expression but enhance biofilm formation and T6SS gene expression. HSL, homoserine lactone.

DAS4 tag contains a low-affinity binding site for ClpX and a high-affinity binding site for the adapter protein SspB. The DAS4-tagged protein is bound by SspB and shuttled to the ClpXP protease for degradation (27). The rate of degradation of the tagged protein by ClpXP is therefore determined by the concentration of SspB in the cell (27). The RNase E depletion strain we constructed (PAO1 Δ*sspB rne-VDAS4*) carries a deletion of the native *sspB* gene and contains an RNase E gene that is modified such that it produces RNase E with both a vesicular stomatitis virus glycoprotein (VSV-G) epitope

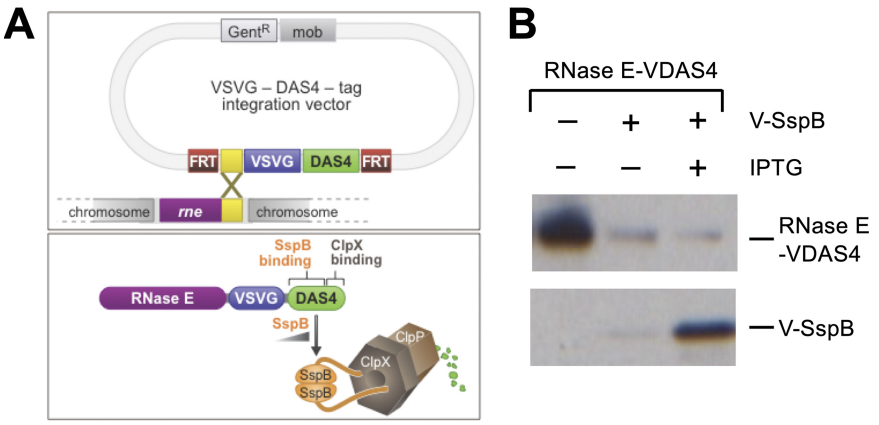

FIG 2 ClpXP-based controllable protein degradation system. (A) Top, diagram of the VSV-G-DAS4 tag integration vector and its use in tagging RNase E with a VSV-G epitope and DAS4 depletion tag. Gent^R, gentamicin resistant. Bottom, schematic representation of the ClpXP-based protein depletion system. Degradation of the RNase E-VDAS4 protein is depicted. (B) Western blot showing that RNase E depletion is dependent on *sspB* expression. V corresponds to the VSV-G epitope tag. The DAS4 tag allows for depletion of RNase E-VDAS4 in a manner dependent upon the intracellular concentration of the SspB adapter protein that directs RNase E-VDAS4 to the ClpXP protease complex. SspB synthesis is under the control of an IPTG-inducible promoter. FRT, FLP recombinase.

tag and a DAS4 tag at its C terminus (RNase E-VDAS4) (Fig. 2A). The PAO1 Δ*sspB rne-VDAS4* depletion strain was first transformed with plasmid pV-SspB, which directs the production of SspB with an N-terminal VSV-G tag (V-SspB) under the control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter. Figure 2B shows that the IPTG-inducible synthesis of V-SspB results in the depletion of RNase E in cells of the PAO1 Δ*sspB rne-VDAS4* strain. We note that even in the absence of IPTG, the low level of SspB expressed from the *lac* promoter on the pPSV38 vector results in appreciable depletion of RNase E; however, the addition of IPTG results in further depletion of RNase E.

Depletion of RNase E antagonizes the expression of T3SS genes. Because RNase E has been implicated in controlling the expression of T3SS genes in *Yersinia* spp., we sought to determine whether RNase E controlled the expression of T3SS genes in *P. aeruginosa* (23). Expression of the *P. aeruginosa* T3SS genes can be induced by growing cells in low-calcium medium (6, 28). To quantify the effect of RNase E depletion on T3SS gene expression, a PAO1 Δ*sspB* Δ*exoS*::*lacZ* reporter strain was generated. This strain has the T3SS effector gene *exoS* replaced by *lacZ*, which encodes β-galactosidase. Activation of the T3SS in cells of the PAO1 Δ*sspB* Δ*exoS*::*lacZ* reporter strain results in increased expression of *lacZ*, suggesting that the lack of SspB did not prevent the induction of *exoS* expression under conditions of calcium limitation (Fig. 3A). The PAO1 Δ*sspB* Δ*exoS*::*lacZ* reporter strain therefore serves as the control strain for comparison to when RNase E is depleted. PAO1 Δ*sspB* Δ*exoS*::*lacZ* was transformed with either pPSV38 (empty vector) or pPSV38-*sspB*. The pSV38-*sspB* plasmid expresses SspB under the control of an IPTG-inducible promoter. The T3SS in the PAO1 Δ*sspB* Δ*exoS*::*lacZ* reporter strain was activated when grown in low-calcium medium in either the absence or presence of SspB (Fig. 3A). However, when SspB was expressed in a PAO1 Δ*sspB* Δ*exoS*::*lacZ rne-VDAS4* reporter strain, *lacZ* expression decreased 18-fold. This indicated that under conditions of RNase E depletion, T3SS production was significantly reduced (Fig. 3A).

RNase E-mediated regulation of the T3SS is not dependent on the small regulatory RNA RsmY, RsmZ, RsmV, or RsmW. In *P. aeruginosa*, the small regulatory RNAs RsmY and RsmZ have been shown to inhibit T3SS gene expression (15, 29, 30). We reasoned that depletion of RNase E might inhibit the expression of T3SS genes by increasing the stability of either RsmY, RsmZ, or both of these small RNAs (sRNAs). The T3SS is activated under low-calcium growth conditions in PAO1 Δ*sspB* Δ*exoS*::*lacZ*

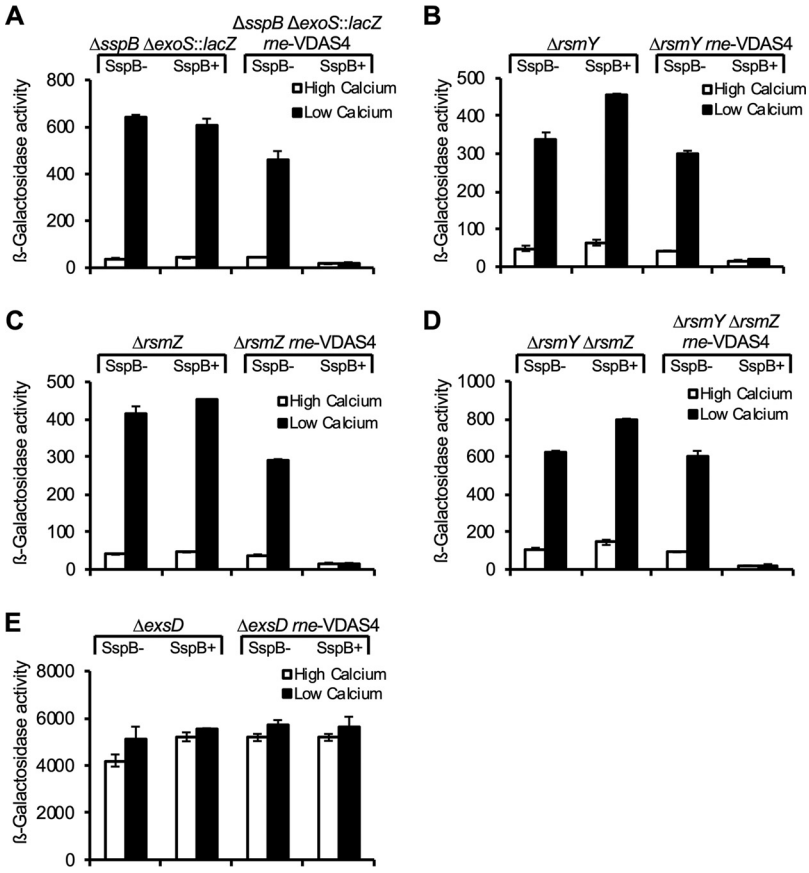

FIG 3 RNase E is required for type III secretion gene expression in *P. aeruginosa*. Shown is the quantification of Δ*exoS*::*lacZ* expression in cells of the Δ*sspB* mutant reporter strain and the *rne-VDAS4* mutant derivative containing either empty vector (−SspB) or a SspB expression vector (+SspB). IPTG was added to both the −SspB and +SspB conditions. Various strain genetic backgrounds are as listed above each panel.

reporter strains lacking *rsmY*, *rsmZ*, or both *rsmY* and *rsmZ* in either the absence or presence of SspB (Fig. 3B to D). When SspB was expressed in a PAO1 Δ*sspB* Δ*exoS*::*lacZ rne-VDAS4* reporter strain also lacking *rsmY*, *rsmZ*, or *rsmYZ*, *lacZ* expression decreased 15- to 18-fold (Fig. 3B to D). RNase E-dependent regulation of the T3SS therefore is not dependent on *rsmY* or *rsmZ*.

Recent findings indicate that the activity of RsmA is also subject to control by the sRNAs RsmV and RsmW that, like RsmY and RsmZ, exert their regulatory effects through interaction with RsmA (29, 31). The results depicted in Fig. S1 in the supplemental material indicate that under low-calcium growth conditions, depletion of RNase E results in reduced expression of the *exoS-lacZ* reporter gene in quadruple-mutant cells lacking *rsmY*, *rsmZ*, *rsmV*, and *rsmW*. Thus, the regulation of T3SS gene expression is independent of any of the sRNAs that are currently known to limit the activity of RsmA.

RNase E depletion does not influence the expression of T3SS genes in cells lacking *exsD*. ExsD represses T3SS gene expression by sequestering the transcription activator ExsA (9, 32, 33) (Fig. 1A). *P. aeruginosa* cells that lack *exsD* therefore constitutively express the T3SS genes (Fig. 3E). We found that *exoS* expression was not affected by RNase E depletion in cells of an Δ*exsD* mutant (Fig. 3E), indicating that the mechanism by which RNase E regulates the expression of the T3SS genes cannot suppress activation by the liberated ExsA in cells of the Δ*exsD* mutant.

Depletion of RNase E decreases the abundance of the *exsA* transcript. ExsA is the major transcription factor responsible for the activation of T3SS gene expression (8,

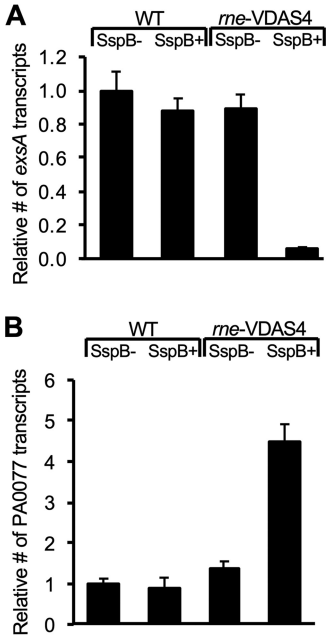

FIG 4 Reciprocal regulation of type III and type VI secretion by RNase E. Effects of RNase E depletion on target gene expression are shown. qRT-PCR was used to measure the indicated transcript abundance relative to the wild-type (WT) strain containing empty vector (SspB−). Error bars represent relative expression values calculated from ±1 standard deviation (SD) from the mean $\Delta\Delta C_T$.

32–34). We hypothesized that decreased T3SS gene expression in RNase E-depleted cells is due to decreased expression of *exsA*. PAO1 Δ*sspB* Δ*exoS*::*lacZ* and PAO1 Δ*sspB* Δ*exoS*::*lacZ rne-VDAS4* cells were grown under low-calcium conditions which should induce the transcription of *exsA* and activate the expression of T3SS-related genes. The abundance of the *exsA* transcript was measured by quantitative real-time PCR (qRT-PCR) (Fig. 4A). Under low-calcium conditions, *exsA* transcripts were detected in both reporter strains. When SspB synthesis is induced by the addition of IPTG to the growth medium in PAO1 Δ*sspB* Δ*exoS*::*lacZ rne-VDAS4* cells, RNase E is depleted, and *exsA* expression decreases 12-fold (Fig. 4A). This decrease in *exsA* expression would explain the decrease in *exoS* expression in RNase E-depleted cells.

RNase E depletion decreases T3SS protein secretion and cytosolic T3SS-associated regulatory proteins. In order to confirm the gene expression phenotype, we monitored secretion of effector and translocator proteins by assaying export of the effector protein ExoT and the translocator protein PopD. Wild-type PAO1 produces PopD and ExoT and secretes both proteins into the culture supernatant in a T3SS-dependent manner when grown under low-calcium conditions (Fig. 5). Depletion of RNase E in the PAO1 strains containing DAS4-tagged RNase E expressing SspB resulted in decreased production of PopD and ExoT in the cells and decreased secretion of both proteins (Fig. 5). We also monitored levels of the regulatory proteins ExsA and ExsD. In wild-type PAO1 cells, PopD, ExsA, and ExsD levels all increase when the cells are grown in low-calcium medium (Fig. 5). When RNase E is depleted in RNase E-VDAS4-tagged strains expressing SspB, ExsA, and ExsD, protein levels decreased (Fig. 5), consistent with the *exoS* reporter expression data (Fig. 3).

Depletion of RNase E results in increased expression of a type 6 secretion system gene. Studies of RetS and LadS in *P. aeruginosa* indicate that many virulence factors are reciprocally regulated. For example, the T3SS is RetS activated and LadS repressed, while biofilm formation and the T6SS are LadS activated and RetS repressed (12, 13, 18). We hypothesized that if RNase E depletion results in repression of the T3SS, it might also result in activation of the genes associated with the T6SS. PAO1 Δ*sspB* Δ*exoS*::*lacZ* and PAO1 Δ*sspB* Δ*exoS*::*lacZ rne-VDAS4* cells were grown under low-calcium

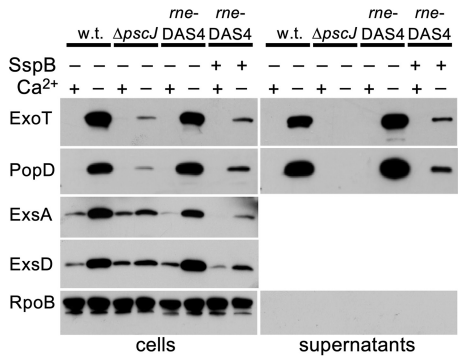

FIG 5 RNase E depletion decreases type III effector protein secretion. The effects of RNase E depletion on the production of T3SS regulators ExsA and ExsD, as well as production and export of a T3SS translocator protein (PopD), and the effector ExoT were monitored by Western blotting. Amounts of ExsA and ExsD, T3SS regulatory proteins, are also decreased under RNase E depletion conditions. RpoB was detected as a fractionation control. The Δ*pscJ* mutant strain does not assemble a functional T3SS. w.t., wild type.

conditions, which should activate expression of the T3SS. The level of T6SS activation was measured by monitoring the expression of *icmF1* (PA0077) by qRT-PCR (Fig. 4B). *icmF1* was chosen because it is the last gene of an operon that encodes part of the H1 T6SS. *icmF1* also is required for the secretion of Hcp1, which makes up part of the T6SS (35). Under low-calcium conditions, *icmF1* transcripts were detected at low levels in both reporter strains. When SspB was expressed in PAO1 Δ*sspB* Δ*exoS::lacZ rne-VDAS4* and RNase E is depleted, *icmF1* expression increased 4-fold (Fig. 4B). Taken together, our findings suggest that RNase E plays an important role in T3SS activation while repressing a gene associated with the T6SS. RNase E may therefore reciprocally regulate T3SS and T6SS.

Depletion of RNase E results in increased biofilm formation. Since RNase E reciprocally regulates the T3SS and the T6SS, we hypothesized that biofilm formation would be upregulated under conditions of RNase E depletion. Twenty-four-well culture plates containing LB with 20 mM IPTG were inoculated with the *P. aeruginosa* strains indicated in Fig. 6. Following incubation, the culture supernatant was removed, and adherent cells were stained with crystal violet. Biofilm formation was quantitated based on crystal violet retention. Wild-type PAO1 containing pPSV38 or pSspB produced equal amounts of biofilm (Fig. 6). Depletion of RNase E in the PAO1 strains containing

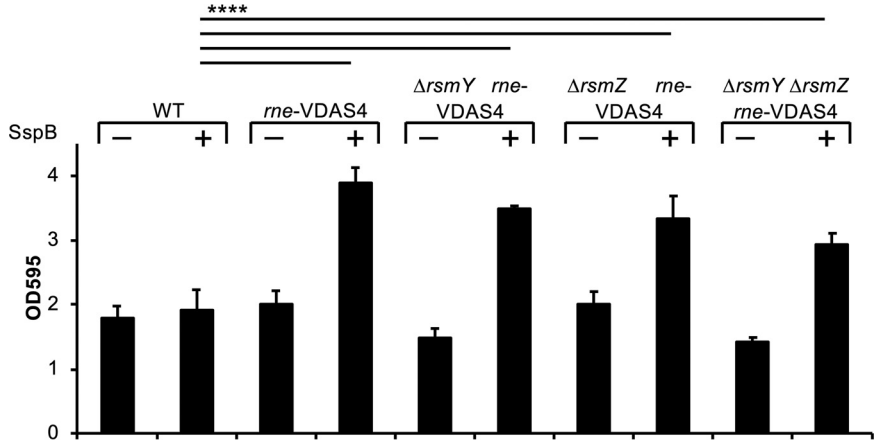

FIG 6 Depletion of RNase E results in increased biofilm formation. Cells were grown in 24-well plastic tissue culture plates for 16 h at 37°C. After incubation, cells were removed and attached biofilms strained with crystal violet. Biofilm staining was quantified by dissolving the crystal violet in methanol and measuring the OD_{595}. Samples were compared by one-way analysis of variance (ANOVA), using Dunnett's *post hoc* test. Significant differences are indicated by a line connecting the data points. ****, $P < 0.0001$.

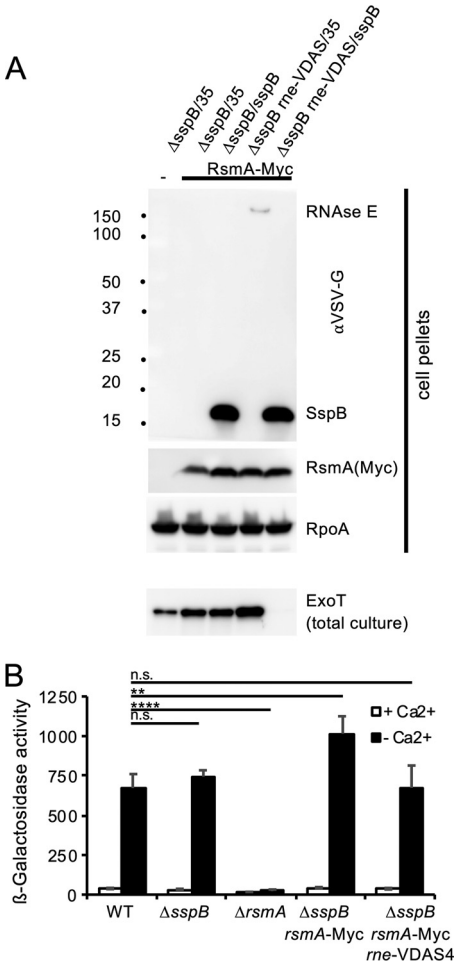

FIG 7 RNase E depletion does not alter RsmA abundance. (A) RsmA abundance was monitored in strains producing a Myc-tagged version of the protein. The relevant genotype of each strain is indicated above the blot. SspB was supplied from a plasmid, where indicated, or the strain was transformed with the relevant vector control (pPSV35 [denoted "35"]). All strains were grown in the absence of calcium to induce expression of the type III secretion genes. Since this also induces effector secretion, ExoT production was monitored by running total culture samples, rather than cell pellets. RpoA was detected as a loading control. The size standards, indicated at the left, are in kilodaltons. (B) Tagging of RsmA does not interfere with RsmA function. The expression of *exoS* was monitored in the presence or absence of calcium using a *lacZ* reporter gene, as described above. The relevant genotype is listed below the columns. *β*-Galactosidase activity is recorded in Miller units and represents an average of the results from three independent experiments with standard deviation. Samples were compared by one-way ANOVA, using Dunnett's *post hoc* test. Specific comparisons are indicated by a line connecting the data points. ****, $P < 0.0001$; **, $P < 0.01$; n.s., not significant.

DAS4-tagged RNase E expressing SspB resulted in approximately 2-fold increases in biofilm formation (Fig. 6). This increase in biofilm formation was not dependent on *rsmY* or *rsmZ* (Fig. 6). The data in Fig. 6 support the idea that RNase E also reciprocally regulates T3S and biofilm formation.

RNase E depletion does not influence RsmA abundance. Since the regulatory phenotype of RNase E depletion is consistent with modulation of the Gac/RsmA signal transduction system, we examined whether RNase E depletion interferes with RsmA production. The chromosomal copy of *rsmA* was modified to encode a version of RsmA that is tagged at the C terminus with 2 repeats of the Myc epitope tag. Tagging of RsmA did not interfere with RsmA function (Fig. 7A). We found that depletion of RNase E did not appreciably alter the abundance of RsmA (Fig. 7B), suggesting that the effect of RNase E depletion on T3SS gene expression cannot be explained through an effect of RNase E on RsmA abundance.

DISCUSSION

Using a ClpXP-based protein depletion system, we have shown that the endoribonuclease RNase E positively regulates expression of the T3SS genes in *P. aeruginosa*. Depletion of RNase E does not appear to influence the abundance of RsmA, a posttranscriptional regulator that is known to positively regulate T3SS gene expression. Furthermore, the effects of RNase E on T3SS gene expression are independent of the RsmY, RsmZ, RsmV, and RsmW sRNAs that serve to antagonize RsmA activity. We also find that RNase E negatively regulates the expression of a T6SS gene and limits biofilm formation in this organism.

To study the effects of RNase E on the T3SS, we took a targeted protein depletion approach (Fig. 2). By expressing RNase E with a C-terminal VDAS4 tag, we could control ClpXP-dependent degradation of RNase E by IPTG-inducible expression of the SspB adapter protein. When SspB is expressed, it shuttles the VDAS4-tagged RNase E to the ClpXP for degradation (Fig. 2). When RNase E-VDAS4 was expressed in cells in the absence of SspB and grown in calcium-containing medium, the T3SS remained suppressed. Switching these cells to low-calcium medium resulted in the expression of the T3SS. However, depletion of RNase E-VDAS4, by expression of SspB, resulted in cells that maintained suppression of the T3SS, even in low-calcium medium, which should normally trigger expression of the T3SS (Fig. 3).

Since RNase E is predicted to control the degradation of many RNAs in the cell, we considered the possibility that depletion of RNase E leads to increased stability of the sRNAs RsmY and RsmZ. These sRNAs are known to bind and inhibit the activity of RsmA, a key posttranscriptional regulator required for expression of the T3SS genes (36, 37); by depleting RNase E and stabilizing RsmY and RsmZ, free RsmA levels in the cell could in principle be reduced, and the T3SS would be repressed. Indeed, the exoribonuclease polynucleotide phosphorylase (PNPase) has been shown to influence the expression of T3SS gene expression in *P. aeruginosa* by influencing the stability of RsmY and RsmZ (38). To address this possibility, we depleted RNase E in strains lacking either *rsmY*, *rsmZ*, or both. However, depletion of RNase E in these mutant strains still resulted in reduced expression of the T3SS genes in low-calcium medium (Fig. 3A to D). These results establish that RNase E does not regulate the T3SS through a mechanism that is dependent upon *rsmY* or *rsmZ*.

RsmV and RsmW are two recently discovered sRNAs that, like RsmY and RsmZ, sequester RsmA and inhibit its activity (31, 39). Depletion of RNase E could potentially stabilize either RsmV, RsmW, or both, thus inhibiting RsmA activity. When RsmV or RsmW was deleted in a strain lacking RsmY and RsmZ, expression of an *exoS-lacZ* reporter was still reduced upon RNase E depletion (see Fig. S1 in the supplemental material). These results indicate that the RNase E-mediated regulation of the T3SS does not require RsmY, RsmZ, RsmV, or RsmW. However, our findings do not exclude the possibility that RNase E exerts its effects by altering the abundances of other RNA species that act as molecular sponges for RsmA.

We considered the possibility that RNase E regulates T3SS gene expression by influencing the abundance of RsmA which is required for T3SS gene expression. If RNase E depletion leads to decreased RsmA in the cell, that would decrease T3SS gene expression. RNase E depletion did not result in a decrease in the abundance of RsmA in the cell (Fig. 7), suggesting that RNase E does not exert its effects on T3SS gene expression through positive effects on the abundance of RsmA.

RNase E is required for efficient production of the master regulator of T3SS gene expression, ExsA. Both *exsA* transcript and ExsA protein abundance are reduced when RNase E is depleted (Fig. 4A and 5). This suggests that RNase E influences the expression of the T3SS by controlling the abundance of the ExsA transcription factor. ExsA activity is held in check through sequestration by the antiactivator protein ExsD. The decreased T3SS gene expression we observed in low-calcium medium was reversed in a strain background lacking ExsD (Fig. 3E). These data suggest that the feedforward loop that exists in the absence of ExsD (since ExsA upregulates its own expression as well) may

be sufficient to overcome the defect in T3SS gene expression we observed upon depletion of RNase E.

Since the T3SS is often reciprocally regulated with the T6SS and biofilm formation, we measured the effects of RNase E depletion on the expression of *icmF1* (PA0077), which is part of the H1 T6SS. While RNase E depletion resulted in decreased expression of *exsA*, it resulted in an increase in the expression of *icmF1* (Fig. 4B). These findings suggest that RNase E participates in a pathway that reciprocally regulates the T3SS and T6SS. Similarly, depletion of RNase E resulted in an approximately 2-fold increase in biofilm formation. Consistent with our observations regarding T3SS gene regulation, the increase in biofilm formation upon RNase E depletion was not dependent on two known regulators of biofilm formation, RsmY and RsmZ (Fig. 5). These data suggest that RNase E has multiple influences on regulating virulence factor gene expression.

We have established that RNase E plays a role in positively regulating the expression of T3SS genes, but exactly how RNase E exerts its regulatory effects is unclear. It is striking that RNase E appears to have activities reminiscent of RsmA. Although RNase E influences T3SS gene expression independently of sRNAs that are known to limit the activity of RsmA, and although RNase E does not positively influence the abundance of RsmA, our findings raise the possibility that RsmA and RNase E act in concert with one another. Indeed, RsmA is a RNA-binding protein that typically acts to repress the translation of target mRNA species (40). By influencing the translation of target mRNA species, RsmA may render these species accessible to degradation by RNase E. It was shown previously that the metabolic state of *P. aeruginosa* can control T3SS assembly, presumably through an effect on the envelope (41, 42). We cannot rule out the possibility that the depletion of RNase E influences the metabolic state of the cell, thereby affecting T3SS assembly, and, by extension, the ability to trigger T3SS gene expression by removing calcium from the growth medium.

RNase E is typically thought of as a global regulator of RNA stability in bacterial cells. Here, we have shown that RNase E can influence the expression of genes involved in the virulence of *P. aeruginosa*. Depletion of RNase E from the cell reduces T3SS gene expression and protein abundance. While further investigation is needed to fully elucidate the mechanism by which RNase E controls T3SS gene expression, our data support recent findings that RNases are important players in the control of virulence gene expression in a variety of pathogenic bacteria (23, 42–46).

MATRIALS AND METHODS

Bacterial strains. The recipient strain for all plasmid constructions was *E. coli* DH5αF′IQ (Invitrogen). For strain construction, plasmids were transferred into *P. aeruginosa* via conjugation utilizing *E. coli* SM10 λpir. Bacterial cultures were grown in lysogeny broth (LB) or on LB plates containing 15 g/liter agar. For *P. aeruginosa* strains, gentamicin (Gent; 30 μg/ml), carbenicillin (200 μg/ml), or tetracycline (Tet; 35 μg/ml) was used for selection when required. For *P. aeruginosa*, cells were grown in liquid LB cultures containing 10 mM $MgCl_2$ and 0.5 mM $CaCl_2$ (LB-MC). Where indicated, type III secretion was triggered by removing calcium from the medium by the addition of EGTA (5 mM final concentration; LB-MC-EGTA). A list of strains and plasmids can be found in Table 1.

Construction of depletion strains. Table 2 contains a list of primers used in this study. The construct for deleting the *sspB* gene (PA4427) was previously described (26). Briefly, the flanking regions of *sspB* were amplified by the PCR and spliced together with overlap extension PCR. The resulting PCR product was cloned into plasmid pEXG2 (41), as described previously (26), yielding plasmid pEXG2-Δ*sspB*. This plasmid, along with recipient strains PAO1 and PAO1 Δ*exoS*::*lacZ*, was then used to create the following strains containing in-frame deletions of *sspB* by allelic exchange: PAO1 Δ*sspB* and PAO1 Δ*exoS*::*lacZ* Δ*sspB*. Th deletions were confirmed by PCR.

The constructs for deleting the genes for *rsmW* (PA4570.1), *rsmY* (PA0527.1), and *rsmZ* (PA3621.1) were made by amplifying 1-kb regions flanking *rsmW*, *rsmY*, or *rsmZ* by PCR and then splicing the flanking regions together by overlap extension PCR. The deletion was in-frame and contained the linker sequence 5′-ATGGCGGCCGCTTAA-3′. The resulting PCR product was cloned on an XbaI/EcoRI fragment into plasmid pEXG2, yielding plasmids pEXG2-Δ*rsmW*, pEXG2-Δ*rsmY*, and pEXG2-Δ*rsmZ*, respectively. These plasmids along with recipient strain PAO1 Δ*exoS*::*lacZ* Δ*sspB* were then used to create the following strains containing in-frame deletions of *rsmW*, *rsmY*, and *rsmZ* by allelic exchange: PAO1 Δ*exoS*::*lacZ* Δ*sspB* Δ*rsmY*, PAO1 Δ*exoS*::*lacZ* Δ*sspB* Δ*rsmZ*, PAO1 Δ*exoS*::*lacZ* Δ*sspB* Δ*rsmY* Δ*rsmZ*, and PAO1 Δ*exoS*::*lacZ* Δ*sspB* Δ*rsmW* Δ*rsmY* Δ*rsmZ*. The deletions were confirmed by PCR.

The VSV-G-DAS4 tag (VDAS4 tag) integration vector pVDIV was described previously (26). Plasmid pVDIV-*rne* was constructed by amplifying approximately 300 bp of the 3′ end of the *rne* (PA2976)

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
RP1831	PAO1F (wild-type PAO1)	3
RP1868	PAO1F Δ*exoS*::GL3	This study
JS1869	PAO1F Δ*exoS*::GL3 Δ*sspB*	This study
JS1870	PAO1F Δ*exoS*::GL3 Δ*sspB rne-VDAS4*	This study
JS1871	PAO1F Δ*exoS*::GL3 Δ*sspB rne-VDAS4* Δ*rsmY*	This study
JS1872	PAO1F Δ*exoS*::GL3 Δ*sspB rne-VDAS4* Δ*rsmZ*	This study
JS1873	PAO1F Δ*exoS*::GL3 Δ*sspB rne-VDAS4* Δ*rsmY* Δ*rsmZ*	This study
RP2093	PAO1F Δ*exoS*::GL3 Δ*rsmA*	This study
RP10346	PAO1F Δ*exoS*::GL3 Δ*sspB rsmA*-Myc	This study
RP10351	PAO1F Δ*exoS*::GL3 Δ*sspB rsmA*-Myc *rne-VDAS4*	This study
PAO1F	Δ*exoS*::GL3 Δ*sspB rne*-VDAS4 Δ*rsmY* Δ*rsmZ* Δ*rsmV* Δ*rsmW*	This study
Primers		
pPSV35	Expression plasmid with *lacIQ*, *lacUV5* promoter, Gent resistance, *colE1* and *Pseudomonas* origins of replication, *oriT*	43
pEXG2	Allelic exchange vector, *colE1* origin; *oriT* Gent^r *sacB*	43
pEXG2-Δ*rsmA*	pEXG2 with Δ*rsmA* allele	This study
pEXG2-Δ*rsmY*	pEXG2 with Δ*rsmY* allele	This study
pEXG2-Δ*rsmZ*	pEXG2 with Δ*rsmZ* allele	This study
pEXG2-Δ*rsmV*	pEXG2 with Δ*rsmV* allele	This study
pEXG2-Δ*rsmW*	pEXG2 with Δ*rsmW* allele	This study
pEXG2-*rsmA*-Myc	pEXG2 with *rsmA* fused at the 3′ end to 2 copies of the Myc tag	This study
pV-*sspB*	pPSV35 vector directing IPTG-inducible expression of SspB with a N-terminal VSV-G tag	10
pVDIV	Integration vector to incorporate C-terminal VDAS4 tag	10
pVDIV-*rne*	Integration vector to incorporate C-terminal VDAS4 tag to *rne*	This study

containing a 5′ HindIII site and a 3′ NotI site. This allowed cloning of the 3′ end of *rne* into pVDIV cut with HindIII and NotI; the portion of the *rne* gene was cloned such that they were in-frame with the DNA specifying the VDAS4 tag. This plasmid together with recipient strains PAO1 Δ*sspB*, PAO1 Δ*exoS*::*lacZ* Δ*sspB*, PAO1 Δ*exoS*::*lacZ* Δ*sspB* Δ*rsmY*, PAO1 Δ*exoS*::*lacZ* Δ*sspB* Δ*rsmZ*, PAO1 Δ*exoS*::*lacZ* Δ*sspB* Δ*rsmY* Δ*rsmZ*, and PAO1 Δ*exoS*::*lacZ* Δ*sspB* Δ*rsmW* Δ*rsmY* Δ*rsmZ* were used to create strains PAO1 Δ*sspB rne-VDAS4*, PAO1 Δ*exoS*::*lacZ* Δ*sspB rne-VDAS4*, PAO1 Δ*exoS*::*lacZ* Δ*sspB* Δ*rsmY rne-VDAS4*, PAO1 Δ*exoS*::*lacZ* Δ*sspB* Δ*rsmZ rne-VDAS4*, PAO1 Δ*exoS*::*lacZ* Δ*sspB* Δ*rsmY* Δ*rsmZ rne-VDAS4*, and PAO1 Δ*exoS*::*lacZ* Δ*sspB* Δ*rsmW* Δ*rsmY* Δ*rsmZ rne-VDAS4* by integration of plasmid into the chromosome. The plasmid backbone was excised by the expression FLP recombinase from pFLP2.

SspB expression vectors. Plasmid pV-SspB directs IPTG-inducible expression of SspB with an N-terminal VSV-G tag and was previously described (26).

Depletion of RNase E. Strains containing VSV-G-DAS4-tagged RNase E were transformed with pV-SspB. IPTG (1 mM final concentration) was added to liquid cultures to induce the expression of SspB and subsequent degradation of RNase E by the ClpXP protease.

β-Galactosidase assays. *P. aeruginosa* cultures were grown at 37°C to log phase in LB-MC medium. One milliliter of that initial culture was then added to 1 ml LB-MC medium (+Ca) and 1 ml of LB-MC medium containing 5 mM EGTA (−Ca). These cultures were incubated, and β-galactosidase assays were performed essentially as described in references 47 and 52.

RNA isolation and cDNA synthesis. *P. aeruginosa* strains were grown with aeration at 37°C in LB-MC (+Ca) or LB-MC-EGTA (−Ca) medium containing 30 μg/ml gentamicin and 1 mM IPTG. Triplicate cultures of each strain were inoculated to a starting optical density at 600 nm (OD_{600}) of 0.01 and grown to a final OD_{600} of ~0.5. RNA isolation and cDNA synthesis were performed essentially as described previously (48).

Quantitative real-time PCR. The abundance of target transcripts relative to that of the *clpX* transcript was determined by quantitative real-time reverse transcription-PCR (RT-PCR) using the iTaq SYBR green kit (Bio-Rad). cDNAs were amplified by real-time PCR utilizing an ABI Prism 7000 system (Applied Biosystems). PCR primer specificities were confirmed by melting curve analyses (Table 2). Relative transcript abundance was determined using the comparative threshold cycle (C_T) method ($\Delta\Delta C_T$), as described previously (49). The values presented in Fig. 4 are the averages of 3 real-time RT-PCR amplifications from three independent RNA isolations. Error bars represent the relative expression values calculated from ±1 standard deviation from the mean $\Delta\Delta C_T$.

Biofilm assays. Twenty-four-well culture plates (VWR) containing 10 ml LB broth with 20 mM IPTG and appropriate antibiotic selection were inoculated with the *P. aeruginosa* strains to a starting OD_{600} of 0.03. Plates were incubated at 37°C for 16 h without shaking. Following incubation, the culture supernatant was removed, and adherent cells were stained with 0.1% crystal violet for 15 min. Biofilm assays and quantitation were performed essentially as described in reference 50.

Western blots. Overnight cultures were grown in high-salt LB medium with Gent and, if needed (*rne-VDAS*), 100 μg/ml tetracycline and then diluted 1:200 (*rne-VDAS* and *sspB* 1:100) into high-salt LB medium with 15 μg/ml Gent, 1 mM IPTG, and 200 μg/ml Tet for *rne-VDAS* strains. Cultures were grown for 1.5 h, at which point EGTA was added to 5 mM and incubated for another 2.5 h. For total culture

TABLE 2 Primers used in this study

Name	Sequence	Description
rsmA5-1	AAAAAgaattcCGACACCAACATCGCCAAGGTT	*rsmA* deletion, removing codons 3–59, outside primer, EcoRI site (lowercase)
rsmA5-2	AACTCGAGCCGCAAGCATGCTGAACAGCATTCCTTTCTCCTCACGCGAA	Pair with rsmA5-1
rsmA3-1	TTCAGCATGCTTGCGGCTCGAGTTAACCATTAATTTTTATCTAATTTT	Pair with rsmA3-2
rsmA3-2	AAAAAaagcttTAACGCTTGTTTTACCGTGAAAGA	Outside primer, HindIII site (lowercase)
rsmA-Myc-5-2	CTCCGAGATCAGTTTCTGCTCGCCCAAATCTTCTTCAGAAATCAACTTTTGTTCATGGTTTGGCTCTTGATCTTTCTC	Fuse 2× Myc tag to 3′ end of *rsmA*, pair with rsmA5-1
rsmA-Myc-3-1	GAAGAAGATTTGGGCGAGCAGAAACTGATCTCGGAGGAGGACCTGCAATGATTTTTATCTAATTTTCCCTTTGCA	Pair with rsmA3-2
rsmV-5-1	AAAAAtctagaATGGGTTTCCGGCCAGTTAGC	*rsmV* deletion 5′ flank outside primer, XbaI site (lowercase)
rsmV-5-2	GGATAGCGGGAGGGGATGAGGTTGGAAATCTACCAAGC	Pair with rsmV-5-1
rsmV-3-1	GCTTGGTAGATTTCCAACCTCATCCCCTCCCGCTATCC	Pair with rsmV-3-2
rsmV-3-2	AAAAAaagcttCCGCTGGCGATCAGGTACAGCAC	*rsmV* deletion, 3′ flank outside primer, HindIII site (lowercase)
rsmW-5-1	AAAAAtctagaGCCCTGGACTATACCGCCAAC	*rsmW* deletion 5′ flank outside primer, XbaI site (lowercase)
rsmW-5-2	GGAAAGACCGGGACATGAGGGCTCAGCGCAGGGCGCGGGCGTAG	Pair with rsmW-5-1
rsmW-3-1	CTACGCCCGCGCCCTGCGCTGAGCCCTCATGTCCCGGTCTTTCC	Pair with rsmW-3-2
rsmW-3-2	AAAAAaagcttGGTCGAACTCTTCGAAGCTGTA	*rsmW* deletion, 3′ flank outside primer, HindIII site (lowercase)
delrsmY F1	ATATggatccGGTGGCCACGTAGTTCGGGG	*rsmY* deletion 5′ flank outside primer, BamHI site (lowercase)
delrsmY R2	GCGGTTTTCCTCGGGCAATAAGGTTTGAAGATTACGCATCTCTG	*rsmY* reverse inner primer
delrsmY F3	TGCGTAATCTTCAAACCTTATTGCCCGAGGAAAACCGCGTCGCT	*rsmY* forward inner primer
delrsmY R4	ATATctcgagCTGCTCACCGGCAACCTGGA	*rsmY* deletion 3′ flank outside primer, XhoI site (lowercase)
delrsmZ F1	ATATggatccCGAGCTGCTGCAGGATGACG	*rsmZ* deletion 5′ flank outside primer, BamHI site (lowercase)
delrsmZ R2	ACGAGTAAAACGGCAGGCAAACAGGAGTGATATTAGCGATTCC	*rsmZ* reverse inner primer
delrsmZ F3	CGCTAATATCACTCCTGTTTGCCTGCCGTTTTACTCGTCGCCAA	*rsmZ* forward inner primer
delrsmZ R4	ATATctcgagGCCCGCGGCAAGCTCTCGAT	*rsmZ* deletion 3′ flank outside primer, XhoI site (lowercase)
rneVDAS4-F1	ATATggatccAGGACGAGCAGGACGATACCGATG	Forward primer to fuse VDAS4 tag to *rne* 3′ end, BamHI site (lowercase)
rneVDAS4-R2	CCTAGGTCAGCTGGCGTCGGCGTAGTTCTCGCTGTA	*rne* reverse inner primer to fuse VDAS4 tag to *rne* 3′ end
rneVDAS4-F3	GCCAGCTGACCTAGGGTGACAGGCTGAAAAAAGGG	*rne* forward inner primer to fuse VDAS4 tag to *rne* 3′ end
rneVDAS4-R4	ATATgaattcCTGACCCGTGACGTCGAGGCGCTG	Reverse primer to fuse VDAS4 tag to *rne* 3′ end, EcoRI site (lowercase)
PA0077-RT F1	ATCGACAGCCTGCTGGAAGACAT	PA0077 (*icmF1*) forward primer for real-time PCR
PA0077-RT R1	TGGTGGAGTTGACCACGTTCTTCA	PA0077 (*icmF1*) reverse primer for real-time PCR
exsA-RT F1	ATGCAAGGAGCCAAATCTCTTGG	*exsA* forward primer for real-time PCR
exsA-RT R1	TCAGTTATTTTTAGCCCGGCATTC	*exsA* reverse primer for real-time PCR

samples, 75 μl of culture was removed and mixed with 25 μl of 4$\times$ SDS sample buffer. Cell pellets from 1 ml of culture were also collected, resuspended in 1$\times$ sample buffer to an OD of 5, and lysed. Supernatant samples were collected by removing 500 μl of culture supernatant after pelleting cells and precipitating secreted proteins by added trichloroacetic acid to 10%. Precipitates were collected by centrifugation and washed with acetone to remove residual trichloroacetic acid before being resuspended in 1$\times$ SDS sample buffer, also normalized to a culture with an OD of 5.

Four percent to 12% Bis-Tris NuPAGE gels (Invitrogen) were utilized to separate purified proteins and cell lysates. Western blotting was performed as previously described (28). Polyclonal rabbit anti-VSV-G (Sigma-Aldrich) and peroxidase-conjugated goat anti-rabbit IgG (Sigma-Aldrich) antibodies were used to detect the VSV-G tag. In some experiments, samples were separated on 12% TGX protein gels (Bio-Rad), transferred to polyvinylidene difluoride (PVDF) membranes using a Pierce Power Station semidry transfer apparatus (Thermo), and blocked using 5% nonfat milk resuspended in Tris-buffered saline (20 mM Tris-HCl [pH 7.5], 150 mM NaCl) with 0.05% Tween 20 (TBS-T). All antibody incubations were performed in the same buffer. Primary antibodies to the Myc tag and VSV-G tag were purchased commercially (Sigma-Aldrich), as well as the antibody directed against the RNA polymerase alpha subunit (BioLegend). Affinity-purified antibodies to PopD, ExoT, ExsA, and ExsD were raised against purified His-tagged proteins in rabbits and affinity purified (Covance). Primary antibodies were detected with species-specific secondary antibodies conjugated to horseradish peroxidase (Sigma-Aldrich), using the Advansta Quantum chemiluminescent substrate.

Statistical analysis. Statistical analysis was performed using the Prism software package, version 6 (GraphPad).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00336-19.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

ACKNOWLEDGMENTS

This work was funded in part by National Institutes of Health grants AI069007 and AI118955 to S.L.D., NIH grant EY022052 to A.R., and NIH grant T32-AI07061-32 to J.S.S.

We thank Bryan McGuffie for designing the artwork for Fig. 1 and Renate Hellmiss for the artwork in Fig. 2.

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