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4   **RsmV a small non-coding regulatory RNA in *Pseudomonas aeruginosa***

5   **that sequesters RsmA and RsmF from target mRNAs**

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ABSTRACT

The Gram-negative opportunistic pathogen *Pseudomonas aeruginosa* has distinct genetic programs that favor either acute or chronic virulence gene expression. Acute virulence is associated with twitching and swimming motility, expression of a type III secretion system (T3SS), and the absence of alginate, Psl, or Pel polysaccharide production. Traits associated with chronic infection include growth as a biofilm, reduced motility, and expression of a type VI secretion system (T6SS). The Rsm post-transcriptional regulatory system plays an important role in the inverse control of phenotypes associated with acute and chronic virulence. RsmA and RsmF are RNA-binding proteins that interact with target mRNAs to control gene expression at the post-transcriptional level. Previous work found that RsmA activity is controlled by at least three small, non-coding regulatory RNAs (RsmW, RsmY, and RsmZ). In this study, we took an in-silico approach to identify additional sRNAs that might function in the sequestration of RsmA and/or RsmF and identified RsmV, a 192 nt transcript with four predicted RsmA/RsmF consensus binding sites. RsmV is capable of sequestering RsmA and RsmF in vivo to activate translation of *tssA1*, a component of the T6SS, and to inhibit T3SS gene expression. Each of the predicted RsmA/RsmF consensus binding sites contribute to RsmV activity. Electrophoretic mobility shifts assays show that RsmF binds RsmV with >10-fold higher affinity than RsmY and RsmZ. Gene expression studies revealed that the temporal expression pattern of RsmV differs from RsmW, RsmY, and RsmZ. These findings suggest that each sRNA may play distinct roles in controlling RsmA and RsmF activity.

IMPORTANCE

The role of RsmF in post-transcriptional control of gene expression remains enigmatic. While numerous *rsmA*-dependent phenotypes are more pronounced in an *rsmAF* double mutant, deletion of *rsmF* alone has only modest effects. Understanding mechanisms that control RsmF activity will provide insight into additional roles for RsmF. In the current study we identify

63   RsmV as an sRNA that controls RsmA and RsmF activity, and show that RsmV, RsmW, RsmY,

64   and RsmZ are differentially expressed during growth.

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INTRODUCTION

67   *Pseudomonas aeruginosa* is a Gram-negative opportunistic pathogen that can cause

68   acute infections in the immunocompromised and chronic infections in individuals with cystic

69   fibrosis (CF) (1, 2). Acute *P. aeruginosa* maladies include skin and soft tissue infections,

70   ventilator associated pneumonia (VAP), and urinary tract infections. *P. aeruginosa* isolated from

71   acute infections are typically motile, non-mucoid, and toxigenic. Acute infections by multi-drug

72   resistant *P. aeruginosa* are difficult to resolve and can progress to sepsis resulting in a high rate

73   of morbidity and mortality (3). Chronic *P. aeruginosa* infections are most common in CF patients

74   and result from a variety of mutations in the CFTR ion channel that result in dehydrated and

75   thickened mucus, and physiochemical changes in the airway surface fluid that result in a

76   clearance defect (4). The persistence of *P. aeruginosa* in the CF airways is associated with

77   adaptive changes including loss of motility, growth as a biofilm, mucoidy, and loss of some

78   acute virulence functions (5, 6). The coordinate transition from an acute to a chronic infection

79   phenotype is regulated by a variety of global regulatory networks including the Rsm system (7).

80   The Rsm system controls ~10% of the *P. aeruginosa* genome including the type III and

81   type VI secretions, exopolysaccharides important for biofilm formation, and motility (8, 9). The

82   Rsm system includes two small RNA-binding proteins (RsmA and RsmF/RsmN) and at least

83   three small non-coding RNAs (RsmW, RsmY, and RsmZ) that function by sequestering RsmA

84   and RsmF from mRNA targets. RsmA and RsmF are part of the CsrA family and regulate gene

85   expression at the post-transcriptional level. RsmA and RsmF are 31% identical at the amino

86   acid level and both rely on a conserved arginine residue for RNA-binding activity (13, 16). RsmA

87   and RsmF directly interact with mRNA targets to positively or negatively alter translation

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88   efficiency and/or mRNA stability (8, 10, 11). The RsmA and RsmF bindings site on target

89   mRNAs commonly overlap the ribosome binding site and consist of a conserved 5'-

90   CANGGAYG sequence motif (where N is any nucleotide, the underlined GGA is 100%

91   conserved, and Y is either a cytosine or uracil) that presents the GGA sequence in the loop

92   portion of a stem-loop structure (12-15). While RsmA is able to bind mRNA targets with a single

93   CANGGAYG sequence (12), RsmF differs in that high affinity binding is only observed with

94   mRNAs targets possessing at least two CANGGAYG consensus binding sites (12). Although

95   RsmA and RsmF share some targets in common, the full extent of overlap between the

96   regulons is unknown (13, 16). The RsmF regulon, however, was recently determined using a

97   pull-down method to identify 503 target RNAs (17).

98   The RNA-binding activity of RsmA is controlled by the small non-coding RNAs RsmW,

99   RsmY and RsmZ (13, 18). RsmW, RsmY, and RsmZ each have multiple CANGGAYG binding

100   sites that allow for sequestration of RsmA from target mRNAs (19). It is unclear whether RsmW,

101   RsmY, and RsmZ are the only sRNAs that function in the sequestration of RsmA or whether

102   RsmW, RsmY, or RsmZ are the primary sRNAs that function in sequestration of RsmF. The

103   affinity of RsmF for RsmY and RsmZ is 10-fold lower than RsmA. In this study, we sought to

104   identify additional sRNAs that regulate RsmA and RsmF activity, and identified RsmV, a 192 nt

105   transcript that has four CANGGAYG sequences presented in stem-loop structures. We

106   demonstrate that RsmV is able to sequester RsmA and RsmF in vivo, that full RsmV activity is

107   dependent upon each of the four CANGGAYG sequences, and that RsmV demonstrates a

108   temporal expression pattern that is distinct from RsmW, RsmY, and RsmZ. We propose a model

109   wherein each sRNA plays differential and distinct roles in control of the Rsm system.

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RESULTS

Identification of RsmV as a sequestering RNA for RsmA and RsmF. We took an in-silico approach to identify candidate sRNAs that might control RsmA and/or RsmF activity *in vivo*. A prior SELEX study concluded that optimal RNA-binding activity by RsmF requires RNA targets with at least two GGA sequences presented in the loop portion of stem-loop structures. Transcriptome studies have identified ~500 potential sRNAs in *P. aeruginosa* (28, 29). The secondary structure of each sRNA was predicted using mFold and then examined for the presence of ≥2 GGA sequences presented in stem-loop structures. One sRNA candidate had six GGA sequences (Fig. 1A). Each GGA sequence demonstrated a ≥60% match to the full RsmA/RsmF consensus binding site (CAnGGAyG) (Fig. 1B). Four of the GGA sequences (designated sites 2, 3, 5 and 6) are predicted by mFold to be presented in stem-loop structures (Fig. 1A). The gene encoding the sRNA is located in the intergenic region between *mucE* and *apqZ,* and has been designated *rsmV* (Fig. 1C). A search of the Pseudomonas Genome Database indicates that the *rsmV* sequence is highly conserved in >100 sequenced *P. aeruginosa* genomes and is absent from the genomes of other Pseudomonads.

The RNAseq study that identified *rsmV* concluded that the RNA is 192 nt long (29). To verify the *rsmV* transcription start site, cDNA was generated using a primer within the gene (Fig. 2A). The cDNA was then used in a PCR reaction with primers positioned just upstream of and at the predicted start site. Whereas the primer positioned at the start site generated the expected product, the primer located just upstream did not generate a product. This finding is consistent with the *rsmV* transcription start site identified in the Wurtzel RNAseq study (29). There is no identifiable transcriptional terminator downstream of *rsmV*. To verify the 3' boundary, cDNA was generated from total cellular RNA with primers positioned at the predicted 3' end of *rsmV* and at several downstream positions as shown in Fig. 1C. The resulting cDNAs were then used as

135   templates in PCR reactions with primers (Fprimer and Rprimer) positioned within the gene (Fig.

136   1C). The cDNA primer positioned within the *aqpZ* coding region (primer 4) did not yield a

137   product. The cDNA primers positioned upstream of *aqpZ* (primers 1, 2, and 3) all yielded

138   products, and the strongest product was observed with cDNA generated at the predicted 3'

139   terminus of *rsmV* using primer 2 (Fig. 2B). The weaker PCR products produced from cDNA

140   generated with primers 2 and 3 may represent transcriptional read through.

141   **RsmV interacts with and controls RsmA and RsmF activity.** The presence of four

142   predicted GGA sequences in stem-loop structures is consistent with RsmV serving as a

143   sequestering sRNA for RsmA and/or RsmF. To test this prediction we measured binding using

144   EMSA experiments. Full length RsmV was synthesized *in vitro*, radiolabeled at the 5' end, and

145   incubated with purified RsmA$_{His}$ or RsmF$_{His}$ prior to electrophoresis on non-denaturing gels.

146   RsmA$_{His}$ formed high affinity binding products with RsmV (K_{eq} 14 nM) and two distinct binding

147   complexes were evident (Fig. 3A). Those products could reflect binding of multiple RsmA$_{His}$

148   dimers or differential interactions with multiple sites on the RsmV probe. RsmF also bound the

149   RsmV probe with high affinity (K_{eq} 2 nM), but only a single binding complex was detected (Fig.

150   3A).

151   With evidence that both RsmA and RsmF interact with RsmV we next examined whether

152   RsmV can sequester RsmA/RsmF in vivo. RsmA and RsmF have inverse effects on expression

153   of the type VI (T6SS) and type III (T3SS) secretion systems (13). We used the previously

154   described P$_{tssA1'-'lacZ}$ translational reporter as surrogate for regulatory control of T6SS (13) and

155   P$_{exsD-lacZ}$ transcriptional reporter as marker for the T3SS (30). RsmA/RsmF directly bind the

156   *tssA1* leader region to inhibit translation (12, 13) and positively regulate T3SS gene expression

157   through a mechanism that remains to be defined (13). In a mutant lacking *rsmV*, *rsmY*, and

158   *rsmZ*, RsmA/RsmF availability is high resulting in repression of P$_{tssA1'-'lacZ}$ reporter activity and

159   high levels of P$_{exsD-lacZ}$ reporter activity (Fig. 3B-C). Plasmid expressed RsmV resulted in

160   significant activation of P$_{tssA1'-'lacZ}$ reporter activity and inhibition of the P$_{exsD-lacZ}$ reporter. Both of

161   these findings are consistent with RsmV serving a role in RsmA/RsmF sequestration. When

162   compared to the previously identified sequestering RNAs RsmW, RsmY, and RsmZ (13, 31),

163   RsmV demonstrated activity comparable to RsmY when using the $P_{tssA1'-'lacZ}$ reporter (Fig. 3B)

164   and had the strongest inhibitory activity for the $P_{exsD-lacZ}$ reporter (Fig. 3C).

165   To determine whether RsmV preferentially sequesters either RsmA or RsmF, the $P_{tssA1'-}$

166    $_{'lacZ}$ translational reporter was introduced into $\Delta rsmAVYZ$ and $\Delta rsmFVYZ$ mutant backgrounds.

167   RsmA is more active than RsmF resulting in stronger repression of $P_{tssA1'-'lacZ}$ reporter activity in

168   the $\Delta rsmFVYZ$ mutant when compared to the $\Delta rsmAVYZ$ background for strains carrying the

169   vector control (pJN105) (Fig. 4A vs 4B). In the $\Delta rsmFVYZ$ mutant, where repression of $P_{tssA1'-'lacZ}$

170   activity is attributable to RsmA, RsmV demonstrated relatively weak suppressive activity when

171   compared to RsmW, RsmY, and RsmZ (Fig. 4A). A similar picture emerged when using the

172    $\Delta rsmAVYZ$ background to examine RsmF sequestration in that RsmV demonstrated the

173   weakest suppressive activity (Fig. 4B). Thus, RsmV is capable of sequestering both RsmA and

174   RsmF and appears to lack strong preference for one vs the other under the conditions tested.

175   **Contribution of GGA sites 2, 3, 5 and 6 to RsmV activity.** The RsmV primary

176   sequence contains six GGA sequences, four of which (GGA2, 3, 5, and 6) may be presented in

177   the loop portions of stem-loop structures (Fig. 1A). To determine which GGA sites are important

178   for RsmV regulatory activity each of the GGA sequences in stem-loop structures was changed

179   to CCU. The activity of each mutant RNA was tested using the $P_{tssA1'-'lacZ}$ translational and P_{exsD-}

180    $_{lacZ}$ transcriptional reporters. The GGA4 and GGA6 mutant RNAs demonstrated a significant loss

181   of regulatory activity for the $P_{tssA1'-'lacZ}$ translational reporter when compared to wt RsmV (Fig.

182   5A). In contrast, each of the GGA sites was required for full regulatory control of the $P_{exsD-lacZ}$

183   transcriptional reporter (Fig. 5B). The most likely explanation for the differential requirement for

184   the GGA2 and GGA5 sites is that the $P_{exsD-lacZ}$ reporter is more sensitive to changes in RsmA

185   availability relative to the $P_{tssA1'-'lacZ}$ reporter.

186   The simplest interpretation of the reporter findings is that the mutant RNAs with altered

187   activity have reduced capacity to sequester RsmA/RsmF. To test this prediction, binding assays

188   were performed with radiolabeled RNA probes. RsmA bound each of the single GGA

189   substitution mutants with affinities similar to or greater than wt RsmV (Fig. 5C, Table 1, Fig. S1).

190   Whereas two distinct products are formed upon RsmA binding to the wt, GGA2, and GGA5

191   probes, only a single product was observed for the GGA3 and GGA6 probes. This is noteworthy

192   as the mutant GGA3 and GGA6 RNAs also demonstrated a defect in activation of the $P_{tssA1'-'lacZ}$

193   translational reporter (Fig. 5A). RsmF also bound each of the mutant probes with high affinity,

194   with the exception of GGA6, which was significantly reduced (Fig. 5D, Table 1, Fig. S1). Given

195   that RsmA and RsmF are homodimers with two RNA binding sites (one from each monomer),

196   and that each mutant RNA still has three potential GGA interaction sites, high affinity binding to

197   the mutant probes was not unexpected. We thus generated a probe bearing CCU substitutions

198   at all four sites (Quad) and found that RsmA (K_{eq} >27 nM) and RsmF (K_{eq} >243 nM) were

199   unable to bind (Fig. 5C-D, Table 1, Fig. S1) or exert regulatory control over the $P_{tssA1'-'lacZ}$

200   translational and $P_{exsD-lacZ}$ transcriptional reporters. We conclude that the primary, if not

201   exclusive, sites for RsmA/RsmF binding are GGA2, 3, 5, and 6.

202   **Role of RsmV in vivo.** Data presented thus far have relied upon plasmid-expressed

203   RsmV, which may result in RNA levels that exceed the native level expressed by cells under

204   physiologically relevant conditions. To address the effect of RsmV expressed at native levels on

205   the output of the Rsm system we generated an in-frame *rsmV* deletion mutant (Δ*rsmV*) and

206   measured $P_{exsD-lacZ}$ reporter activity. When compared to wt cells, the Δ*rsmV* demonstrated a

207   modest but significant increase in reporter activity (Fig. 6A). This increase in reporter activity is

208   consistent with reduced sequestration of RsmA and/RsmF, both of which have a positive effect

209   on T3SS gene expression. By comparison, $P_{exsD-lacZ}$ reporter activity is also elevated in an

210   Δ*rsmYZ* double mutant. The higher level of reporter activity in the Δ*rsmYZ* mutant is consistent

211   with the data presented in Fig. 3C showing that RsmY and RsmZ each have stronger effects on

212   activation of $P_{exsD-lacZ}$ reporter activity relative to RsmV.

213   A second approach to test the relevance of RsmV in vivo involved precipitation

214   experiments with histidine-tagged RsmA or RsmF. A $\Delta rsmAF$ double mutant transformed with

215   either $RsmA_{His}$ or $RsmF_{His}$ expression plasmids was cultured to mid-log phase and then rapidly

216   subjected to precipitation with Ni^{2+}-agarose beads and isolation of bound RNA. The presence of

217   specific RNAs was detected from the entire pool of bound RNAs by qRT-PCR. Positive controls

218   were the known RsmA/RsmF targets RsmY, RsmZ, and the *tssA1* leader region (13). Negative

219   controls included two mRNAs (*lolB* and *rnpB*) that are not known targets of RsmA or RsmF, and

220   Ni^{2+}-agarose beads alone. Whereas no enrichment of the *lolB* or *rnpB* mRNAs was detected,

221   there was significant enrichment of the *tssA1* mRNA and the RsmV, RsmY, and RsmZ sRNAs

222   by both RsmA and RsmF (Fig. 6B).

223   **Differential expression of RsmV, RsmW, RsmY, and RsmZ.** The in vivo data

224   demonstrate that RsmV, RsmW, RsmY, and RsmZ are each capable of sequestering RsmA and

225   RsmF. We hypothesized that differential expression of the RNAs might allow cells to fine-tune

226   the output of the Rsm system. To test for differential expression, RNA samples were collected

227   from cells cultured to OD_{600} readings of 0.5 (early log phase), 1.0 (mid-log phase), 2.0, 5.0, and

228   7.0 (late stationary phase). The amount of each RNA detected by qRT-PCR at early log phase

229   was normalized to 1.0 and the reported values for each subsequent time point are relative to

230   those values (Fig. 7). Both RsmY and RsmZ showed a transient increase in expression at mid-

231   log phase, followed by a decrease at OD_{600} readings of 2.0 and 5.0, and then a significant

232   increase in late stationary phase (OD_{600} 7.0) The expression pattern for RsmW was delayed

233   until the OD_{600} reached 2.0 but demonstrated the highest fold changes in expression at OD_{600}

234   2.0 and 5.0, and then approached the fold changes observed for RsmY and RsmZ at OD_{600} 7.0.

235   By contrast, RsmV demonstrated a slow but steady increase throughout the growth curve but

236   was the least dynamic of the four RNAs. The observed differences in expression patterns are

237   consistent with the hypothesis that the sRNAs may serve distinct roles in RsmA/RsmF

238   sequestration based upon their timing of expression.

239   One mechanism to account for the differential expression of RsmV, RsmW, RsmY, and

240   RsmZ is by distinct transcription factors. Transcription of *rsmY* and *rsmZ* is controlled by the

241   GacAS two component system (20). A previous study found that GacAS does not control *rsmW*

242   transcription (31). To determine whether *rsmV* transcription is regulated by GacA, a P$_{rsmV-lacZ}$

243   transcriptional reporter was integrated at the ΦCTX phage attachment site of wt cells and a

244     $\Delta gacA$ mutant. Whereas P$_{rsmY-lacZ}$ and P$_{rsmZ-lacZ}$ reporter activity demonstrate strong *gacA*-

245   dependence, P$_{rsmV-lacZ}$ activity showed no difference between wildtype and the *gacA* mutant (Fig.

246   S2).

247

248   **DISCUSSION**

249   The primary RsmA/RsmF sequestering RNAs in *Pseudomonas aeruginosa* are RsmY

250   and RsmZ. In addition to RsmY/RsmZ, RsmW plays a smaller role in the sequestration of RsmA

251   (31) and can also sequester RsmF (Fig. 2). RsmV represents a fourth RsmA/RsmF

252   sequestering RNA in *P. aeruginosa.* RsmV shares sequence and structural characteristics with

253   RsmY and RsmZ including multiple GGA motifs (6), four of which are likely presented in stem-

254   loop structures. RsmY and RsmZ are also the primary sequestering RNAs in *P. fluorescens*

255   (now *P. protegens)* (18). At least one additional sRNA, RsmX, also contributes to Rsm control in

256   *P. protegens* (32)*. The involvement of multiple sequestering sRNAs in the control of CsrA/RsmA

257   activity is common. CsrB is the primary CsrA-sequestering RNA in *E. coli* and contains 18 GGA

258   motifs (33). Other *E. coli* sRNAs can also sequester CsrA including CsrC and McaS (34, 35).

259   CsrC has a structure similar to CsrB but with fewer GGA motifs (34). McaS is an sRNA that

260   basepairs with some mRNAs involved in curli and flagella synthesis and can also sequester

261   CsrA via two GGA motifs (35). In addition to sRNAs, the 5' untranslated region of mRNAs can

262   also function in the sequestration of CrsA (36).

263   The relative activities of RsmV, RsmW, RsmY, and RsmZ were compared by expressing

264   each sRNA from an arabinose-inducible expression vector. Plasmid expressed RsmV activated

265    $P_{tssA1'-'lacZ}$ reporter activity and inhibited $P_{exsD-lacZ}$ reporter activity (Fig. 3B-C). RsmV had activity

266   comparable to RsmY for activation of the $P_{tssA1'-'lacZ}$ reporter activity and the strongest effect on

267   activation for $P_{exsD-lacZ}$ reporter activity. RsmA and RsmF both bind to RsmV with high affinity in

268   vitro (Fig. 3A), and the affinity of RsmF for RsmV is at least 10-fold higher than for RsmY and

269   RsmZ (12, 13). Although the affinity of RsmF for RsmV is higher in vitro, RsmV does not seem

270   to show preferential activity towards RsmF over RsmA in vivo (Fig. 4). The reason for this is

271   unclear but may reflect differences between the in vitro and in vivo binding conditions. A

272   difference between in vitro and in vivo conditions was also evident when the RsmV GGA

273   mutants were examined. Whereas each of single GGA substitution mutants demonstrated

274   altered regulatory control of $P_{exsD-lacZ}$ and/or $P_{tssA1'-'lacZ}$ reporter activity (Fig. 5A-B), the binding

275   affinity of RsmA and RsmF was relatively unaffected by the single GGA substitutions (Table 1).

276   A similar trend was observed in a previous mutagenesis study of RsmY and RsmZ wherein the

277   in vivo activity did not strictly correlate with in vitro binding (37). It was speculated that other

278   RNA binding proteins, such as Hfq, may prevent binding to suboptimal sites in vivo.

279   RsmV activity is clearly evident when expressed from a plasmid (Fig. 3). A role for RsmV

280   when expressed at native levels from the chromosome was also detected. Deletion of *rsmV*

281   resulted in a modest but significant increase in T3SS reporter activity (Fig. 6A) and co-

282   purification experiments found that RsmV interacts with RsmA and RsmF (Fig. 6B). Both of

283   these findings suggest that RsmV can compete with RsmY and RsmZ for RsmA/RsmF binding

284   in wt cells (Fig. 6B). Unclear is whether conditions exist where *rsmV* transcription is elevated

285   and might result in more pronounced phenotypes. Transcription of *rsmY* and *rsmZ* is directly

286   controlled by the GacA/S two-component system, a highly conserved system in Gamma-

287   proteobacteria (20, 38, 39). GacS is a sensor kinase whose activity is controlled by two orphan

288   kinases, RetS and LadS (40-43). Additional regulators interact with and alter the effect of RetS

289   on GacS (44, 45). SuhB regulates *rsmY* and *rsmZ* transcription indirectly by altering *gacA* levels

290   (24). The phosphotransfer protein, HptB, regulates *rsmY* and *rsmZ* transcription when *P.*

291   *aeruginosa* is grown on a surface (22, 23). Other regulators contribute to *rsmY* and *rsmZ*

292   transcription through mechanisms that do not alter GacS/GacA activity. MvaT, a H-NS like

293   protein, binds A+T rich regions of DNA and silences *rsmZ* transcription, while BswR, a

294   transcriptional regulator, counteracts negative regulation of *rsmZ* by MvaT (20, 21). Recently,

295   MgtE, a magnesium transporter, was shown to alter *rsmY* and *rsmZ* transcription, yet a

296   mechanism of action is yet to be defined (25).

297   Neither *rsmV* nor *rsmW* are under positive transcriptional control of the GacAS system

298   (31) (Fig. S2). GacA may repress *rsmW* transcription through an indirect mechanism (30).

299   RsmW expression appears to be highest during stationary phase in minimal media, which may

300   be more biologically representative of a biofilm (31). RsmW is encoded directly downstream of

301   PA4570, a protein of unknown function. RsmW and PA4570 are likely co-transcribed and

302   separated by an RNase cleavage event. Determining the transcriptional regulation of PA4570

303   may provide insight into *rsmW* transcriptional control. A search for potential promoters upstream

304   of *rsmV* predicted binding sites for the transcriptional activators RhlR, AlgU, and FleQ. mRNA

305   levels for *rsmV,* however, were unaffected in PA14 transposon mutants within each of those

306   genes relative to wild type as measured by qRT-PCR (data not shown). Additional studies will

307   be required to determine how *rsmV* and *rsmW* transcription is controlled, and if RsmV plays a

308   larger role in regulating RsmA and/or RsmF activity under a different set of growth conditions.

309   RsmX, RsmY, and RsmZ in *P. protogens* are differentially expressed, thus contributing

310   to a mechanism of fine-tuning RsmA and RsmE activity (32). Expression of *P. protogens* RsmX

311   and RsmY occurs in parallel during exponential growth while RsmZ expression is delayed (32).

312   This may allow cells to fine-tune expression of these sRNAs based on the environmental

313   conditions. We propose a similar scenario for expression of RsmV, RsmW, RsmY, and RsmZ in

314   *P. aeruginosa*. The differences in binding affinities for RsmA/RsmF, timing of gene expression,

315   and expression levels of the sRNAs may provide a mechanism of fine-tuning the expression of

316   genes under control of the Rsm system.

317

318   **METHODS AND MATERIALS**

319   **Strain and plasmid construction**. Routine cloning was performed with *E. coli* DH5α

320   cultured in LB-Lennox medium with gentamycin (15 μg/ml) as required. *P. aeruginosa* strain

321   PA103 and the Δ*gacA*, Δ*rsmYZ* mutants were reported previously (Table 1) (47). The in-frame

322   Δ*rsmV* deletion mutant was constructed by allelic exchange. The upstream and downstream

323   flanking regions (~800 bp) of *rsmV* were generated by PCR using primer pairs 118845409-

324   118845410 and 118845411-118845412. The PCR products were cloned into pEXG2 (48) and

325   the resulting construct was mobilized into wild type PA103 and the Δ*rsmYZ,* Δ*rsmAYZ,* and

326   Δ*rsmFYZ* mutant by conjugation. Merodiploids were resolved by sucrose counter-selection as

327   previously described (49). The RsmV expression plasmid was constructed by positioning the

328   *rsmV* transcription start site immediately downstream of the P$_{BAD}$ promoter start site using the

329   Gibson assembly method (New England Biolabs). Briefly, the P$_{BAD}$ promoter region from

330   pJN105 (primer pair 117830775-117830776) and *rsmV* (primer pair 118845423-118845424)

331   were amplified by PCR and then assembled into the MluI and SacI digested pJN105 (50).

332   pRsmV vectors bearing single GGA to CCT substitutions, or various combinations therefore,

333   were assembled using the Gibson method from gene blocks listed in Table 2 and cloned into

334   the NruI and PvuI sites of pJN105 as outlined in Table 3. The *rsmV* transcriptional reporter

335   (primer pair 150592489-150592490) includes 500 nucleotides upstream of the *rsmV*

336   transcription start site. The *rsmV* reporter was integrated into the CTX phage attachment site in

337   WT and *gacA* strains.

338   **β-Galactosidase Assays**. PA103 strains were grown overnight at 37ºC in LB containing

339   80 μg/ml gentamicin as required. The next day strains were diluted to an absorbance (A_{600}) of

340   0.1 in tryptic soy broth (TSB) for measurement of *tssA1'-'lacZ* reporter activity or TSB

341   supplemented with 100 mM monosodium glutamate, and 1% glycerol for measurement of P$_{exsD}$-

342    $_{lacZ}$ reporter activity. Arabinose (0.4%) was also added to induce *rsmV* expression from the P$_{BAD}$

343   promoter. The cultures were incubated at 37ºC and harvested when the A_{600} reached 1.0. β-

344   galactosidase activity was assayed with the substrates ortho-nitrophenyl-galactopyranoside

345   (ONPG) as previously described (51) or chlorophenol red-β-D-galactopyranoside (CPRG).

346   CPRG activity was determined by measuring product formation at 578 nM and using an

347   adaptation of the Miller equation: CPRG units = (A_{578}/culture A_{600}/time /culture vol [ml]) x 1000.

348   CPRG and Miller units are reported as the average of at least three independent experiments

349   with error bars representing the standard deviation (SD).

350   **Electrophoretic mobility shift assays**. DNA templates encoding wildtype *rsmV* or

351   *rsmV* bearing point mutations within the GGA sequences were PCR amplified and used as

352   templates for in vitro generation of RNA probes. RNA probes were end-labeled with [γ-^{32}] ATP

353   as previously described (13). Purified RsmA or RsmF, as described previously (13), were

354   incubated with the RNA probes at the indicated concentrations in 1X binding buffer (10 mM Tris-

355   HCl pH [7.5], 10 mM MgCl$_2$, 100 mM KCl), 3.25 ng/μl total yeast tRNA (Life Technologies), 10

356   mM DTT, 5% (vol/vol) glycerol, 0.1 units RNAse Out (Life Technologies). Reactions were

357   incubated at 37º C for 30 min, and then mixed with 2 μl of gel loading buffer II (Life

358   Technologies) and immediately subjected to electrophoresis on 7.5 % (wt/vol) native

359   polyacrylamide glycine gels (10 mM Tris-HCl pH[7.5], 380 mM glycine, 1 mM EDTA) at 4º C.

360   Imaging was performed using an FLA-7000 phosphorimager (Fujifilm), and analyzed using

361   MultiGuage v3.0 software.

362   **RNA enrichment experiments**. Strain PA14 Δ*rsmAF* carrying either an empty vector

363   control, pRsmA$_{His6}$, or pRsmF$_{His6}$ was grown in TSB supplemented with 20 mM MgCl$_2$, 5 mM

364   EGTA, 15 µg/ml gentamicin, and 0.1% arabinose to mid log phase, chilled, and pelleted for

365   immediate lysis. Cells were lysed under native conditions to retain protein structure (Qiagen

366   QIAexpressionist manual native purification buffer recipe) supplemented with 2.5 mM

367   ribonucleoside vanadyl complex (NEB) to inhibit RNAse activity, 1 mg/mL lysozyme, and 0.1%

368   Triton X-100. Lysis was completed by freeze-thaw cycles. Lysates were treated with 10 uL RQ-1

369   RNase-free DNAse and cleared by centrifugation. An aliquot was removed from the cleared

370   lysate for total RNA isolation and preserved in Trizol, and the remaining lysate was incubated

371   with Ni-NTA agarose at 4°C for 1 hour under non-denaturing binding conditions. Ni-NTA

372   agarose was then loaded into a column and washed 3 times with non-denaturing binding buffer

373   containing 10 mM imidazole. Protein and associated RNAs were eluted in 4 fractions with 250

374   mM imidazole and 4 fractions with 500 mM imidazole. Protein-containing fractions from the

375   RsmA$_{His6}$, or RsmF$_{His6}$ expressing strains, and an equivalent volume from the vector control

376   strain, were treated with TRIzol (Thermofisher) and RNA was extracted according to the

377   manufacturer's protocol. RNA was treated with RQ-1 RNase-free DNase and concentrated

378   using a RNA Clean and Concentrator kit (Zymo). First strand cDNA was synthesized using

379   Superscript II (ThermoFisher) according to manufacturer's protocol with Random Primer 9

380   (NEB). The copy number of the indicated genes was determined by qPCR using SYBR Green

381   Master Mix (Bio-rad).

382   **Statistical analyses.** One-way ANOVA was performed using Prism 6.0 (GraphPad

383   Software, Inc., La Jolla, CA).

384

385   **ACKNOWLEDGEMENTS**

388

389

390

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549   mechanism regulates expression of the Pseudomonas aeruginosa type III secretion
550   system. Mol Microbiol **53:**297-308.

551

552

553   Table 1. RsmA and RsmF affinities for wt and mutant RsmV

554

RNA	RsmA	RsmF
wt RsmV	14 ± 4[a]	2 ± 0.2
GGA2	3 ± 3	1 ± 0.6
GGA3	1 ± 0.4	5 ± 3
GGA5	3 ± 3	8 ± 13
GGA6	9 ± 11	>243
Quad	>27	>243

562   [a] apparent equilibrium binding constant (nM)

579   **FIGURE LEGENDS**

580   **Figure 1.** Predicted structure of RsmV and genomic context. (*A*) Predicted mFold structure of

581   RsmV. *P. aeruginosa* RsmV secondary structure determined by mFold modeling. Each of the

582   six GGA sequences are highlighted in red and numbered by order of appearance from the 5'

583   end of the sequence. (*B*) Alignment of each GGA site to the full RsmA/RsmF consensus binding

584   site. The GGA sites are 100% conserved (red) and other conserved portions of the consensus

585   are highlighted in blue. (*C*) The genome context of *rsmV,* located between *mucE* and *aqpZ*.

586   The positions of the primer used to generate cDNA for the experiment in Fig. 2B are labeled 1-

587   4. Primers used to generate PCR products in Fig. 2B are labeled Fprimer and Rprimer.

588

589   **Figure 2.** Verification of the *rsmV* 5' and 3' boundaries. (*A*) RNA purified from wt cells was used

590   to generate cDNA using the indicated 3' primer. The cDNA was then used in PCR reactions with

591   the same 3' primer and 5' primers positioned just upstream of or at the predicted start of *rsmV*

592   transcription. Genomic DNA (gDNA) served as a positive control. (*B*) Verification of the *rsmV*

593   termination site. cDNA was generated using primers 1-4 as shown in Fig. 1C. The cDNA was

594   then used in PCR reactions with the indicated primer sets in Fig. 1C. Genomic DNA (gDNA)

595   served as a positive control.

596

597   **Figure 3.** RsmV binding and regulatory activity. (*A*) RsmV was radiolabeled and used in

598   electrophoretic mobility shift assays with purified RsmA (lanes 2-5) and RsmF (lanes 7-10) at

599   the indicated concentrations. The position of the unbound RsmV probe is indicated. (*B-C*) Effect

600   of RsmV on *tssA1'-'lacZ* translational reporter (*B*) and P$_{exsD-lacZ}$ transcriptional reporter (C)

601   activities. Strains consisting of a Δ*rsmVYZ* mutant transformed with either a vector control

602   (pJN105) or the indicated sRNA expression plasmids were cultured in the presence of 0.4%

603   arabinose to induce expression of the respective RNAs and assayed for ß-galactosidase

604   activity. The reported values represent the average of at least three experiments with the

605   standard error indicated. * *P*-value <0.05 relative to the vector control.

606

607   **Figure 4.** Sequestration of RsmA or RsmF by the RsmV, RsmW, RsmY and RsmZ regulatory

608   RNAs. (*A-B*) Either Δ*rsmAVYZ* (*A*) or Δ*rsmFVYZ* (*B*) quadruple mutants carrying the P$_{lac-tssA1'-'lacZ}$

609   translational reporter was transformed with either a vector control (pJN105) or RsmV, RsmW,

610   RsmY and RsmZ expression plasmids. The resulting strains were cultured in the presence of

611   0.4% arabinose to induce expression of the respective RNAs and assayed for ß-galactosidase

612   activity. Reported values represent the average of at least three experiments with the standard

613   error indicated. * *P*-value <0.05 relative to the vector control.

614

615   **Figure 5. Functional analyses of the RsmV mutants**. The PA103 Δ*rsmVYZ* mutant carrying

616   the (*A*) P$_{lac}$ *tssA1'*-'*lacZ* translational reporter or (*B*) *P$_{exsD}$-lacZ* transcriptional reporter were

617   transformed with either a vector control (pJN105) or the indicated RsmV expression plasmids.

618   The resulting strains were cultured in the presence of 0.4% arabinose to induce expression of

619   the respective RNAs and assayed for ß-galactosidase activity. Reported values represent the

620   average of at least three experiments with the standard error indicated. * *P*-value <0.05 relative

621   to the vector control. (*C-D*) EMSA experiments with wt RsmV and the indicated mutant

622   radiolabeled probes. 40 nM RsmA (*C*) or RsmF (*D*) were incubated with the indicated probes,

623   subjected to non-denaturing gel electrophoresis, and phosphorimaging. The positions of the

624   unbound probes are indicated.

625

626   **Figure. 6.** In vivo activity of RsmV. (*A*) Strain PA103 (wt), and the Δ*rsmV* and Δ*rsmYZ* mutants

627   carrying the *P$_{exsD}$-lacZ* transcriptional reporter were cultured under inducing conditions for T3SS

628   gene expression and assayed for ß-galactosidase activity. * *P*-value <0.05 relative to wt. (*B*) A

629   Δ*rsmAF* mutant transformed with either a vector control, or pRsmA$_{His}$ or pRsmF$_{His}$ expression

630   vectors was cultured and subjected to rapid purification of pRsmA$_{His}$ or pRsmF$_{His}$ and bound

631   RNAs. Select RNAs (as indicated) were quantified from the purified RNA pool by qRT-PCR and

632   reported as fold change relative to the vector control. The coding sequences for *lolB* and *rnpB*

633   were included as negative controls and *tssA1* served as a positive control. Reported values

634   represent the average of at least three replicates with the standard error reported. **P*-value

635   <0.05 when compared to expression of the wild type vector is indicated.

636

637   **Figure 7.** Expression profiles of RsmV, RsmW, RsmY, and RsmZ during a growth curve. RNA

638   was isolated from wt cells harvested at the indicated A$_{600}$ readings and used as template in RT-

639   qPCR experiments with primers specific to *rsmV, rsmW, rsmY*, and *rsmZ*. The reported values

640   for each RNA are relative to the measurement of the sample collected at A$_{600}$ 0.5. The data

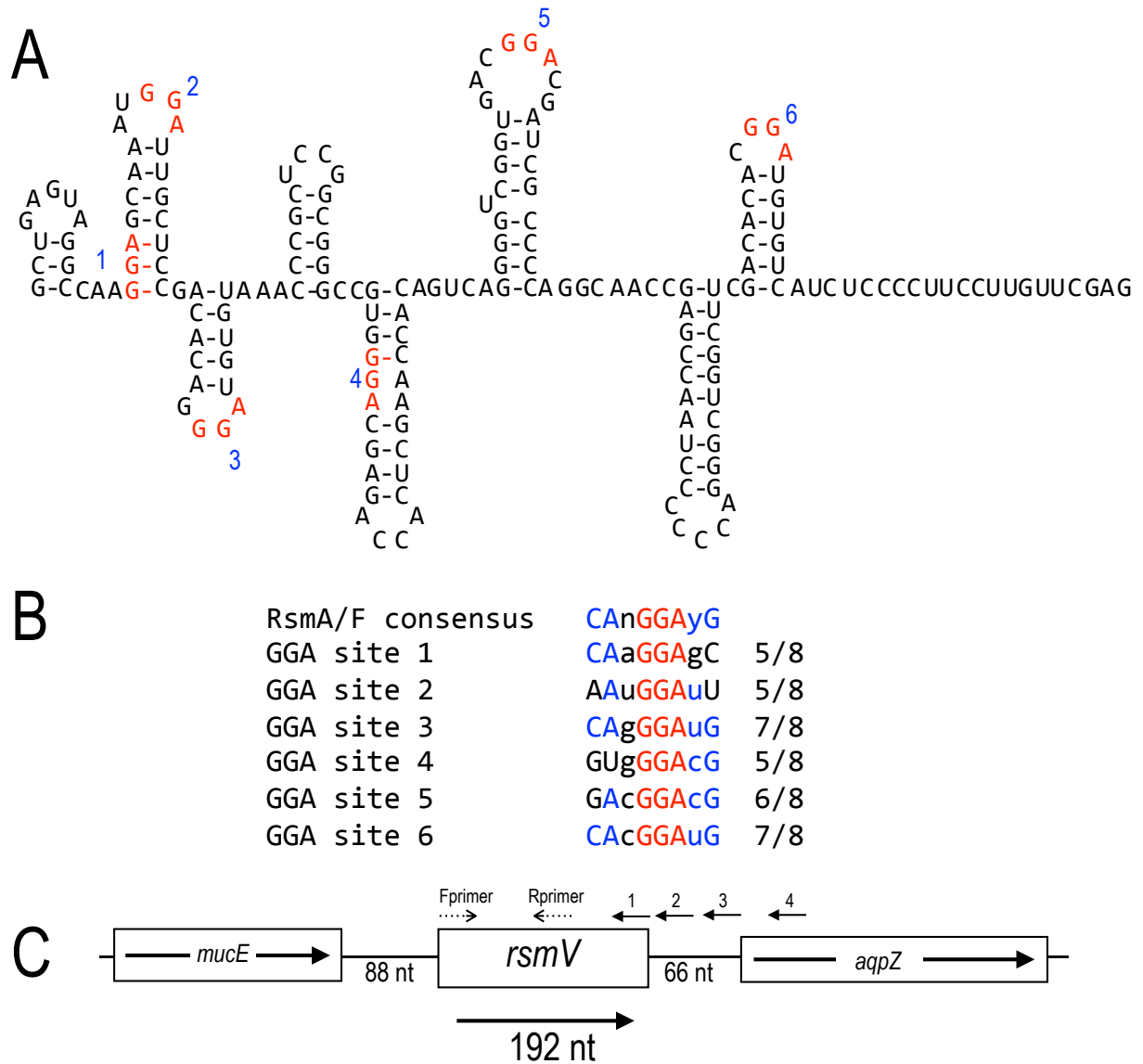641   represent the average of at least three replicates.

642

643

Figure 1

A

| PCR template | N/A | cDNA | gDNA | cDNA | gDNA |

Primer locations

5' → 3' ← | 5' → 3' ←
rsmV | *rsmV*

B

200 bps →

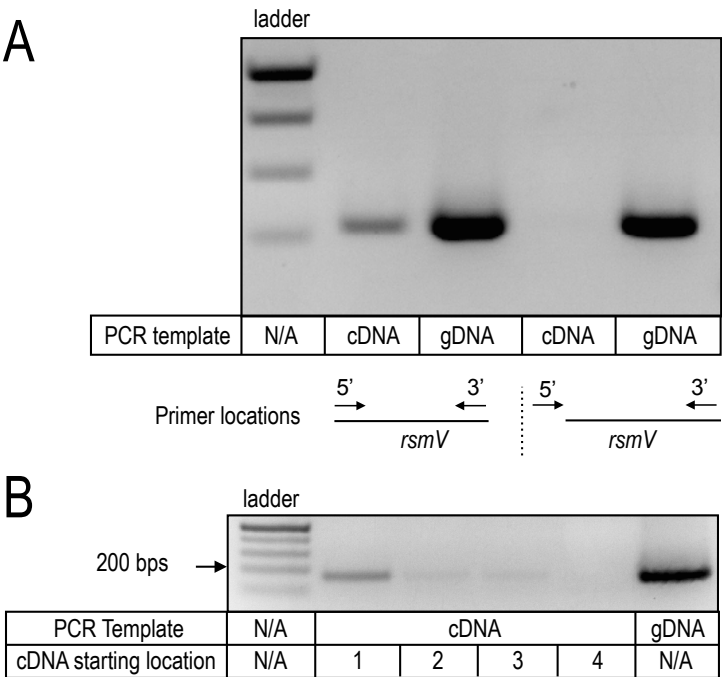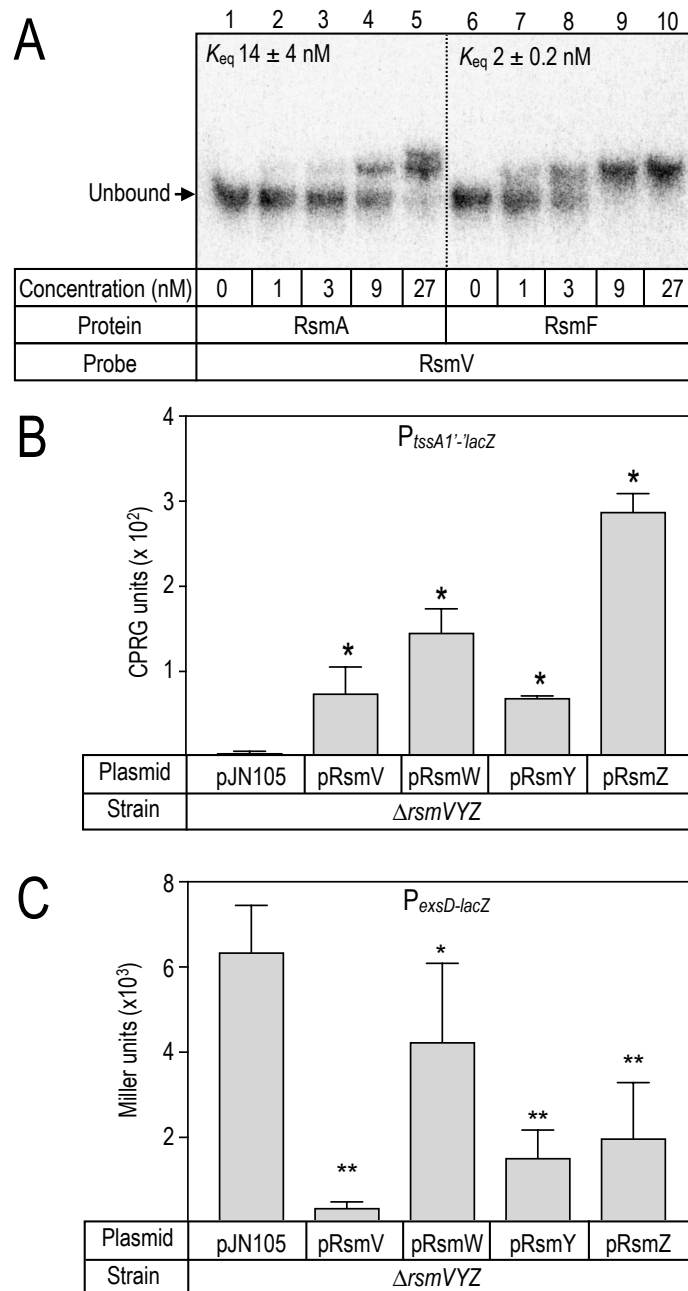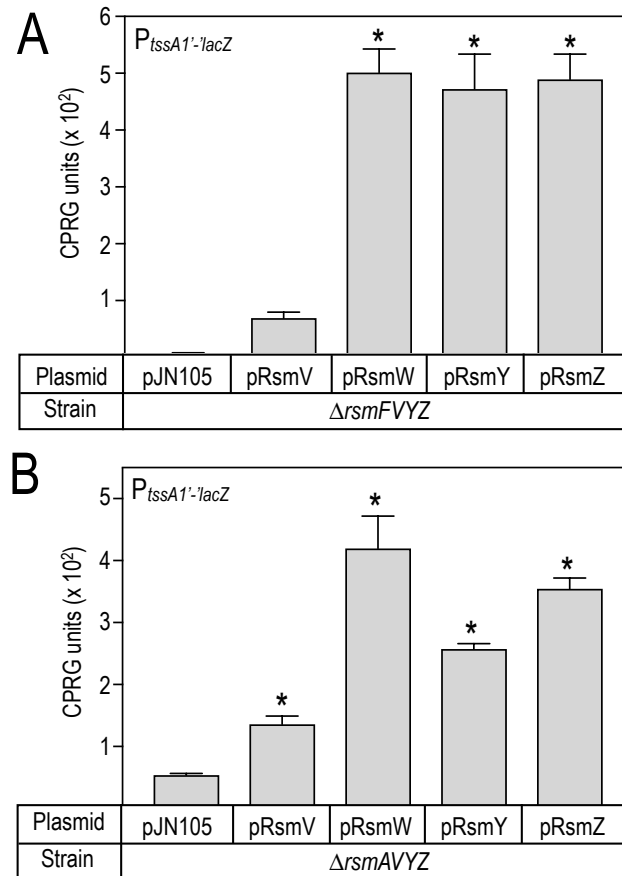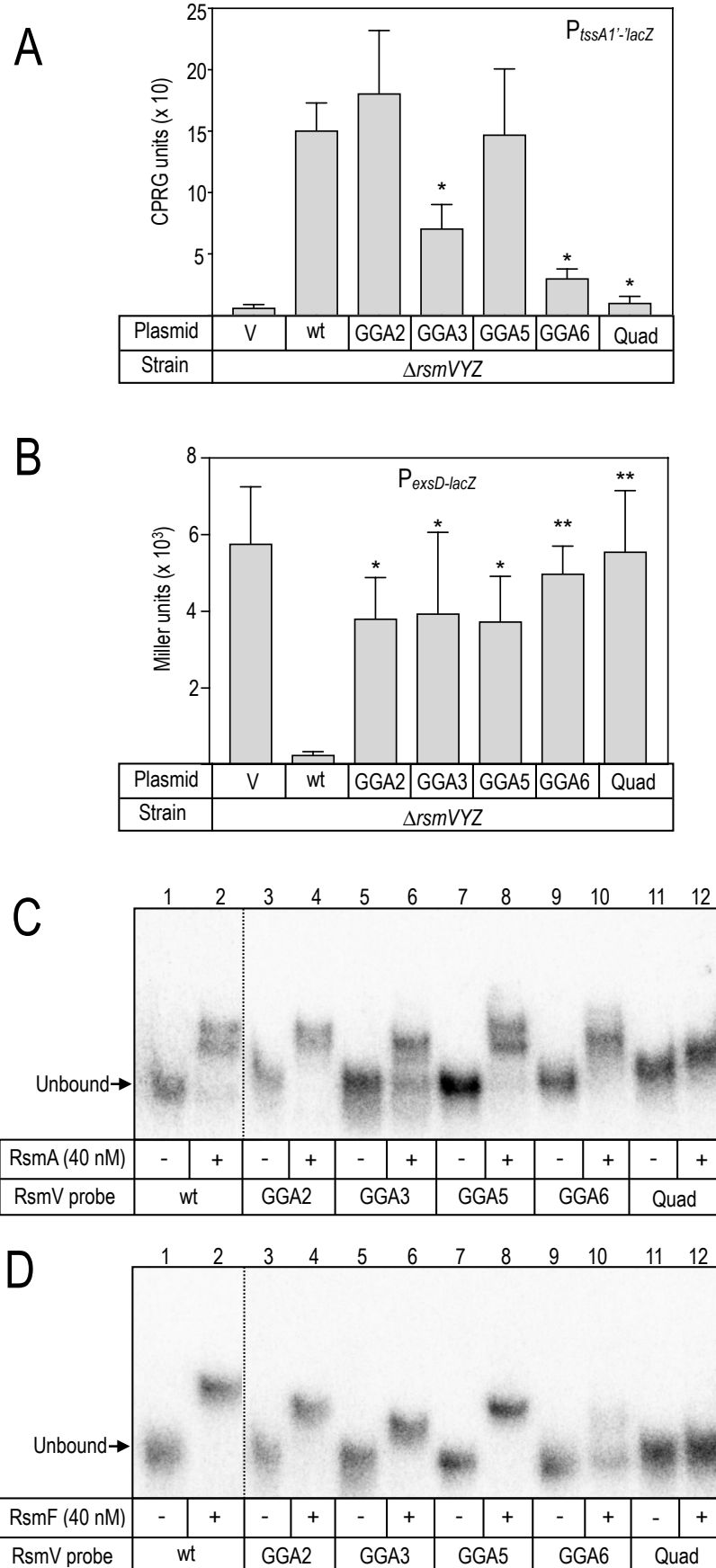| PCR Template | N/A | cDNA | | | | gDNA |
| cDNA starting location | N/A | 1 | 2 | 3 | 4 | N/A |

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7