The GacS/GacA signal transduction system of *Pseudomonas aeruginosa* acts exclusively through its control over the transcription of the RsmY and RsmZ regulatory small RNAs

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Abstract

We report here the results of an analysis of the regulatory range of the GacS/GacA two-component system in *Pseudomonas aeruginosa*. Using microarrays, we identified a large number of genes that are regulated by the system, and detected a near complete overlap of these genes with those regulated by two small RNAs (sRNAs), RsmY and RsmZ, suggesting that the expression of all GacA-regulated genes is RsmY/Z-dependent. Using genome-wide DNA-protein interaction analyses, we identified only two genomic regions that associated specifically with GacA, located upstream of the *rsmY* and *rsmZ* genes. These results demonstrate that in *P. aeruginosa*, the GacS/GacA system transduces the regulatory signals to downstream genes exclusively by directly controlling the expression of only two genes *rsmY* and *rsmZ*. These two sRNAs serve as intermediates between the input signals and the output at the level of mRNA stability, although additional regulatory inputs can influence the levels of these two riboregulators. We show that the A+T-rich DNA segment upstream of *rsmZ* is bound and silenced by MvaT and MvaU, the global gene regulators of the H-NS family. This work highlights the importance of post-transcriptional mechanisms involving sRNAs in controlling gene expression during bacterial adaptation to different environments.

INTRODUCTION

Two-component regulatory systems, consisting of sensor kinase and response-regulator pairs have emerged as key mediators of successful adaptation of microorganisms to changing environments. These systems are capable of assimilating a wide range of environmental stimuli into different outputs, ranging from gene expression and motility to enzymatic activity (Gao *et al.*, 2007). The majority of responses are associated with the control of gene expression, utilizing the ability of phosphorylated (or de-phosphorylated) response-regulators to recognize and bind to specific DNA sequences located upstream of regulated genes and thus influence their transcription. As such, the two-component systems often participate in complex networks where they control the expression of other regulatory factors, including transcriptional regulators and small regulatory RNAs (sRNAs) (Valverde and Haas, 2008).

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In a number of human and plant pathogens, a conserved two-component system regulates a broad range of virulence and stress-responsive genes (Lapouge *et al.*, 2008). This family of regulatory systems is referred to as GacS (LemA)/GacA in *Pseudomonas*, *Erwinia* and *Vibrio fisherii* species, VarS/VarA in *Vibrio cholerae*, BarA/UvrY in *E. coli* and BarA/SirA in *Salmonella*, and LetS/LetA in *Legionella*. These systems regulate the expression of a variety of phenotypes including production of extracellular enzymes, secreted toxins, quorum sensing molecules, various metabolic functions and motility. Where examined, transcription of small trans-acting regulatory RNAs of the RsmY/Z CsrB/C family has been shown to depend on these regulators. Since these sRNAs interact with and modulate the activity of the translation repressors of the RsmA/CsrA family, the phenotypes regulated by the GacS/GacA system and its homologs could be controlled not only at the level of transcription initiation by the response-regulator but also through mRNA turnover influenced by the interaction of the sRNAs with RsmA. In many cases, deletion of the regulatory sRNAs results in phenotypes that are similar to those of mutants in the GacS/GacA system. Such observations have been reported for *rsmY rsmZ* and *gacA* mutants in *P. aeruginosa* (Kay *et al.*, 2006), *rsmB* and *gacA* mutants in *E. carotovora* (Liu *et al.*, 1998), *csrB csrC* and *uvrY* mutants in *E. coli* (Weilbacher *et al.*, 2003), *csrB csrC* and *sirA* mutants in *S. enterica* (Fortune *et al.*, 2006), *rsmX rsmY rsmZ* and *gacA* mutants in *P. fluorescens* (Kay *et al.*, 2005), and most recently for *rsmY rsmZ* and *letA* mutants in *Legionella pneumophila* (Sahr *et al.*, 2009). This suggests that the GacS/GacA system and its homologs control expression of their target genes mainly through the regulation of the expression of the sRNA genes. However, the possibility that the systems directly regulate other types of genes cannot be excluded. In fact, in *L. pneumophila*, LetA regulates expression of flagellar genes by a mechanism that appears to be independent of *rsmY* and *rsmZ* (Sahr *et al.*, 2009).

In *Pseudomonas aeruginosa* the GacS/GacA two-component system controls the reciprocal expression of acute and chronic virulence determinants (Goodman *et al.*, 2004). In this organism, the GacS/GacA signal-transduction pathway itself is regulated by opposing activities of two additional sensor kinases, RetS and LadS (Goodman *et al.*, 2004; Ventre *et al.*, 2006). We have recently shown that RetS forms inactive heterodimers with GacS, preventing phosphorylation of the response regulator GacA (Goodman *et al.*, 2009). In a microarray analysis of transcript levels in *P. aeruginosa rsmA* mutant, we have identified hundreds of genes whose expression levels were affected by the absence of RsmA, and many of these included genes previously shown to be regulated by RetS and LadS (Brencic and Lory, 2009; Goodman *et al.*, 2004; Ventre *et al.*, 2006). This suggested that RetS/GacS/GacA and RsmY/RsmZ/RsmA may be part of a single signal-transduction pathway, where RsmY and RsmZ serve as the regulatory intermediates between the input signals and the regulatory output at the levels of mRNA stability, and therefore that the only direct targets of GacA regulation are the two sRNAs. To test this idea, we have undertaken a study to identify all genes that are controlled by GacA and, among those, to identify the ones whose transcription is controlled by GacA directly. We found that in *P. aeruginosa*, GacS/GacA directly regulates only two genes, *rsmY* and *rsmZ*, and therefore that the transcripts of the hundreds of genes that are regulated by the RetS/GacS/GacA system are regulated by RsmY- and RsmZ-dependent modulation of RsmA activity. We also present evidence that GacA regulates *rsmY* and *rsmZ* by directly binding to sequences upstream of these genes. Furthermore, we show that the H-NS family of DNA-binding proteins MvaT and MvaU associate with the chromosomal region corresponding to *rsmZ* and also influence *rsmZ* expression. In summary, this study shows that in *P. aeruginosa* and possibly in other bacteria that possess orthologs of the GacS/GacA system, the output of the signal transduction pathway mediated by this two-component system is entirely directed towards controlling translation initiation or mRNA stability.

RESULTS

Gene regulation by RetS requires GacS

We have examined two known phenotypes regulated by RetS in a full range of mutants predicted to influence the downstream components of the RetS regulatory pathway (Figure 1). As predicted, a reciprocal regulation of transcription of the *exoS* gene, encoding a protein exported by the type III secretion (T3S) system, and formation of static biofilm was observed in strains with a deletion of the *retS* and *rsmA* genes compared to *P. aeruginosa gacA*, *rsmY*, *rsmZ* mutants, and to the *rsmYZ* double mutant. Examination of the phenotypes of the *rsmY* and *rsmZ* mutants showed that both mutations had an effect on *exoS* expression and on biofilm production (Figure 1). A somewhat stronger effect was observed in *P. aeruginosa* with a mutation in the *rsmY* gene, compared to the strain lacking *rsmZ*, however, a double *rsmYZ* deletion was required in order to observe a phenotype that equaled that of the *gacA* mutant. Similar results were previously reported for the RetS/GacS/A/RsmY/Z/A regulation of another T3S gene, *exsD* (Brencic and Lory, 2009). On the other hand, overexpression of either one of the small RNAs from a constitutively active exogenous promoter was sufficient to completely shut off the expression of *exoS* or to induce development of a robust biofilm that did not depend on the presence of *gacA* (Figure 2). When *rsmY* or *rsmZ* were expressed from a plasmid under the control of their respective native promoters, inhibition of *exoS* transcription was observed only with the *rsmY* construct, while in the same context, expression of *rsmZ* had no effect. Similarly, in the biofilm assay, expression of *rsmY* and *rsmZ* from their native promoters did not appear to significantly affect biofilm formation, although in the *gacA* mutant, the *rsmY* construct allowed for biofilm formation above the background. In general, *rsmY* and *rsmZ* seem to be redundant in function and deletion of both *rsmY* and *rsmZ* results in phenotypes that are similar to those of a *gacA* mutant. The stronger effect of *rsmY* on the two phenotypes is consistent with the higher levels of *rsmY* expression relative to those of *rsmZ* (Brencic and Lory, 2009;Kay et al., 2006).

These results are also consistent with the recently-proposed model in which RetS is an antagonist of the GacS-catalyzed phosphorylation of the response-regulator GacA, and where transcription of the sRNAs RsmY and RsmZ is activated by the GacS/GacA two-component system (Goodman *et al.*, 2009). In addition to the T3S genes and the genes involved with biofilm production, RetS is known to regulate several hundred *P. aeruginosa* genes, many of which are also involved in virulence. We wondered whether or not all of these genes are regulated by RetS through the GacS/GacA pathway. We analyzed and compared transcription profiles of the *P. aeruginosa retS gacS* double mutant to the previously published profiles of the wild type and the *retS* mutant strains (Goodman *et al.*, 2004). Comparison of the transcript levels in the *retS* and *retS gacS* mutant strains to the levels in the wild type strain showed that the 218 genes that were up-regulated in the *retS* mutant, were down-regulated in the *retS gacS* double mutant (Figure S1, Table S1). On the other hand, the 275 that were down-regulated in the *retS* strain were up-regulated in the *retS gacS* strain. This result confirms that GacS acts downstream of RetS and suggests that RetS acts exclusively through its control of the GacS/A system. The fact that the effect of the *retS gacS* double mutation was less pronounced compared to the effect of the *retS* mutation (Figure S1, Table S1), suggests that the effect of GacS on gene expression under the conditions that were used in the experiment is not significant. This is not surprising; the experiment was conducted with cultures in the log phase (at OD $_{600}$ of 0.5) when *rsmY* and *rsmZ* are expressed at very low levels (Brencic and Lory, 2009). At that time point, a mutation in *retS* results in a dramatic increase in the levels of *rsmY* and *rsmZ*, while in the *gacS* mutant, the resulting lack of expression of the sRNAs is not very different from the low levels of expression of the two sRNAs in the wild type strain. This rationale was also considered in the design of the experiments with the *gacA* and *rsmYZ* mutants described

below, which were consequently conducted with stationary phase cultures. In summary, the results of the microarray analysis provide strong evidence that the regulation of all RetS-regulated genes is *gacS*-dependent. Since GacS phosphorylates GacA (Goodman *et al.*, 2009), and GacA controls transcription of the sRNAs RsmY and RsmZ, the entire RetS regulon is very likely regulated through RsmY and RsmZ.

Regulation of all GacA-controlled genes is dependent on RsmY and RsmZ

We next wondered whether or not the regulation of all of the GacA-controlled genes in *P. aeruginosa* is dependent on RsmY and RsmZ. Therefore, we examined the role of *rsmY* and *rsmZ* in the regulation of GacA-controlled genes by comparing mRNA levels of a wild type *P. aeruginosa* to those of a *gacA* mutant and of an *rsmYZ* double mutant (Figure 3, Table S2). A total of 241 genes showed changes in their transcript levels by more than two-fold in at least one of the two mutants (Table S2). The affected genes included many virulence factors, including the T3S and the type IV pili genes, which were upregulated in the two mutants, and genes located on the type VI secretion islands HSI-I and HSI-III, which were downregulated (Figure 3B–C). Overall, the transcriptomes of the two strains were almost indistinguishable (Figure 3A), suggesting that all of the GacA-regulated genes are controlled through the RsmY/Z system.

There were 13 genes whose transcript levels were not affected in the *gacA* mutant, yet they were significantly different from the wild type levels in the *rsmYZ* mutant (Figure 3A, Table S3). For some of these genes, the standard deviation for the two replicates in the *rsmYZ* strain was rather large and this could explain this finding (Table S3 and data not shown). On the other hand, this result could suggest that the effect of RsmY and/or RsmZ on some of these genes can be influenced by factors other than GacA, such as the RNA chaperone Hfq, which is known to affect RsmY stability (Sonnleitner *et al.*, 2006). The transcriptome analysis identified only one gene whose transcript concentration changed in the *gacA* mutant but was unaffected in the *rsmYZ* mutant (Figure 3A and 4D). Levels of *trpC* were increased in the *gacA* mutant, but did not change in the *rsmYZ* double mutant, suggesting that *trpC* is regulated by GacA independently of *rsmY* and *rsmZ*. The *trpC* gene is the last gene in a three-gene operon involved in tryptophan biosynthesis. The transcript levels for other two genes in the operon, *trpG* and *trpD*, were also increased in the *gacA* mutant and not affected in the *rsmYZ* double mutant, although because of their high P-value, these two genes did not pass our stringency cut-off. We constructed *trpG-lacZ* and *trpC-lacZ* reporter fusions and tested their expression in wild type and *gacA* and *rsmYZ* mutant backgrounds (Data not shown.). These fusions were expressed at the same levels in all three strains and therefore we conclude that the result is a false positive. In summary, our analysis found that the same genes are controlled by the GacS/GacA two-component system and by the RsmY/Z post-transcriptional mechanism. Therefore, it appears that regulation of all GacA-regulated genes is dependent on RsmY and RsmZ.

GacA directly controls transcription of only two genes, *rsmY* and *rsmZ*

To confirm the above results and identify all *P. aeruginosa* genes that are directly regulated by GacA, we performed genome-wide ChIP-on-chip analysis using a *P. aeruginosa retS* mutant strain that expresses an epitope-tagged version of GacA from the native *gacA* locus. This strain has a fully active GacS/GacA phosphorelay pathway, and expresses GacA with a vesicular stomatitis virus glycoprotein (VSV-G) epitope tag fused to its carboxy terminus (Figure 4D). For the experiment, cells were grown for approximately 4 hours (at OD_{600} of 1.4). At this time point, RsmY and RsmZ begin to accumulate and it is therefore expected that *rsmY* and *rsmZ* are actively transcribed (Brencic and Lory, 2009). We used anti-VSV-G antibody-coated agarose beads to capture the VSV-G-tagged GacA (GacA-V) with bound DNA fragments, and these fragments were then hybridized to a high density DNA

oligonucleotide microarray representing the *P. aeruginosa* (PAO1) genome. To obtain a genome-wide view of GacA-V-bound probes, data for three biological replicates were averaged and used to generate an enrichment map of fragments represented by probes corresponding to the *P. aeruginosa* chromosome. Using a stringent cut-off threshold of 99.9% (see Experimental Procedures), we identified a total of four putative binding regions present in all three replicates, and denoted by peaks in Figure 4A. Enrichment of DNA fragments at the *rsmY* and *rsmZ* loci was observed. In addition, regions corresponding to the intergenic region between PA2004 and PA2005, and the region corresponding to PA4554 were also enriched by the GacA-V immunoprecipitation.

To validate the enrichment of the specific regions identified by ChIP-on-chip, we carried out chromatin immunoprecipitation (ChIP) with GacA-V in both the *retS* mutant, and in wild type *P. aeruginosa*; in the wild type strain phosphorylation and thus activation of the GacA response regulator is blocked by RetS. Although three of the regions that were enriched in the ChIP-on-chip analysis were detected in the ChIP experiment, only fragments containing the *rsmY* and *rsmZ* genes were bound in a RetS-responsive manner (Figure 4C). PA4554 was not enriched in the ChIP experiments, and represents a false positive result in the ChIP-on-chip data. Such a result is most likely due to an amplification and/or hybridization bias at this chromosomal location (Figure 4C). In order to further assess the specificity of the binding, we also carried out a mock ChIP experiment, where beads coated with antibody to the VSV-G tag were used to capture crosslinked DNA from *P. aeruginosa* expressing untagged GacA. In the mock experiment, significant enrichment of a fragment containing the PA2004-PA2005 intergenic region was detected, while no *rsmY*-or *rsmZ*-containing DNA fragments were detected (Figure 4C). Therefore the enrichment of the PA2004-PA2005 intergenic region in the ChIP-on-chip experiment is the result of a non-specific interaction between this region and reagents used in the IP reactions. Furthermore, when *lacZ* reporter fusions were constructed using promoter-containing fragments from each of the regions showing enhanced GacA binding, no difference in promoter activity was detected in the wild type, *gacA* or *retS* mutants using the DNA fragment from the PA2004-PA2005 intergenic region in either orientation (Figure 4D). On the other hand, transcription of *rsmY* and *rsmZ* was strongly dependent on *gacA* and enhanced by the *retS* mutation (Figure 4D). In summary, our analysis of GacA binding to the *P. aeruginosa* chromosome DNA suggests that the RetS/GacS/GacA pathway regulates global gene expression by directly controlling transcription of only two genes specifying the sRNAs RsmY and RsmZ.

Features of the *rsmY* and *rsmZ* regulatory regions and the role of MvaT and MvaU

In *P. aeruginosa* a conserved region upstream of the *rsmY* and *rsmZ* promoters has been identified, consisting of the sequences TGTAAGCATTAACTTACA and TGTAAGCCAAGGCTTACA, respectively (Heeb *et al.*, 2002; Kay *et al.*, 2006; Kulkarni *et al.*, 2006). Variations of these sequences can be also found upstream of *csrB/csrC*, and *rsmY/rsmZ* genes in other microorganisms and may represent upstream activation sites (UASs), recognized by the phosphorylated forms of the GacA/UvrY/LetA family of regulatory proteins (Kulkarni *et al.*, 2006). Indeed, in *L. pneumophila*, LetA bound to fragments containing these sequences (Sahr *et al.*, 2009), and in *P. fluorescens* CHA0, a deletion of this sequence caused a marked reduction in the expression of *rsmZ* and completely abolished the control by GacA (Heeb *et al.*, 2002).

We constructed transcriptional *lacZ* fusions using truncated fragments of the upstream regulatory regions of *rsmY* and *rsmZ*. For both *rsmY* and *rsmZ*, activities of those reporter fusions that lacked the conserved UAS site were reduced to the levels detected in the *gacA* mutant (Figure 5). For *rsmY*, fusion to a fragment extending 21 bps upstream of the UAS was as active and GacA-dependent, as the fusion ending 303 bps upstream of the UAS, suggesting that it contained all the elements necessary for the full expression of *rsmY*. In the

case of *rsmZ*, fusions to fragments extending as far as 61 bps upstream of the UAS showed only partial activity when compared to the fragment that ended 285 bps upstream of the UAS, suggesting that the sequences considerably further upstream of the conserved UAS element play a role in the activation of *rsmZ* expression (Figure 5). Overall, these results confirm that the predicted UAS is required for the GacA-dependent expression of the *rsmY* and *rsmZ* genes and these regulatory sequences most likely represent the binding sites for phosphorylated GacA.

The differences in length and structure of the sequences upstream of the *rsmY* an *rsmZ* genes that are required for the expression of the two sRNAs, and the overall lower levels of expression of *rsmZ* compared to *rsmY* (Brencic and Lory, 2009), suggest that factors other than GacA may play a role in the regulation of the expression of each of the two sRNAs. We have previously used ChIP-on-chip analyses to identify interactions of MvaT and MvaU, two members of the H-NS family of global regulatory proteins, with regions on the *P. aeruginosa* chromosome (Castang *et al.*, 2008). Among hundreds of chromosomal fragments, bound to MvaT, MvaU, or both, immunoprecipitation with either one of these proteins enriched for fragments containing the *rsmZ* gene. In contrast, we detected no enrichment of probes near the *rsmY* gene (Figure 6A). In order to test the influence of MvaT and MvaU on *rsmZ* expression, we introduced *rsmY* and *rsmZ* transcriptional *lacZ* fusions into *mvaT* and *mvaU* mutants of *P. aeruginosa*. In the *mvaT* mutant, expression of the *rsmZ-lacZ* fusion was increased about two fold throughout the course of growth, while the expression of the *rsmY-lacZ* construct was unaffected by the absence of MvaT (Figure 6B). The lack of any effect of the *mvaU* deletion on *rsmZ* expression is consistent with the observations made for the roles of MvaT and MvaU in expression of other target genes. The two proteins are functionally redundant, and a deletion of *mvaU* results in an increase in the amount of MvaT in the cell. It has been proposed that this compensatory effect could be sufficient to mask any role MvaU might play in the repression of target genes (Vallet-Gely *et al.*, 2005).

MvaT and MvaU are known to preferentially bind AT-rich regions of the chromosome (Castang *et al.*, 2008). We examined the A+T composition of the *rsmY* and *rsmZ* coding regions and of the regions between the UAS element and the transcription start site of each sRNA (Figure 6C). The 174-bp region between the GacA binding site and the *rsmZ* transcriptional start site is highly enriched for A and T (55%) compared to the overall composition of various *P. aeruginosa* genomes, with an average % (A+T) content of 34%, as well as compared to the A+T-composition of the *rsmZ* coding sequence (39%) (Figure 6C). Therefore, it is likely that this region represents one or more MvaT or MvaU binding sites. Both the *rsmY* coding sequence and the *rsmY* upstream region are also enriched for A and T (45%), although the enrichment is not as striking and the A+T compositions of two regions are indistinguishable (Figure 6C).

DISCUSSION

RsmY and RsmZ-like small regulatory RNAs (sRNAs) act by antagonizing the activities of RNA-binding translational regulators of the CsrA/RsmA family (Babitzke and Romeo, 2007; Lapouge *et al.*, 2008; Lucchetti-Miganeh *et al.*, 2008). RsmA/CsrA proteins bind to structured segments at the 5' ends of target mRNAs, and prevent or facilitate translational initiation, which is reversed following sequestration of RsmA by the sRNAs. The transcription of genes encoding the Csr/Rsm sRNAs in all γ-proteobacteria is controlled by a two-component regulatory system, consisting of a sensor-kinase and response regulator, BarA and UvrY, BarA and SirS, LetS and LetA, GacS and GacA in *E. coli*, *Salmonella*, *Legionella* and *Pseudomonas* species, respectively. In *P. aeruginosa* the RsmY/Z/A post-transcriptional regulatory system controls the expression of over 500 genes (Brencic and

Lory, 2009; Burrowes *et al.*, 2006) and the transcription of RsmY and RsmZ depends on the GacS/GacA pathway, which is antagonized by the RetS sensor kinase (Goodman *et al.*, 2009). We have shown that the expression of two selected reciprocally controlled phenotypes (type III secretion and biofilm formation) is coordinated such that a mutant in *retS* phenocopies a mutation in *rsmA*, and the phenotypes resulting from a *gacA* mutation are identical to those observed in strains where the genes for *rsmY* and *rsmZ* have been deleted. We also compared transcript levels in the *retS* mutant and *retS gacS* double mutant to those in wild type cells. For each gene, we found that changes in transcript levels caused by the *retS* mutation depended on the presence of GacS. On the other hand, comparison of mRNA levels in a *gacA* mutant and *rsmYZ* double mutant showed that transcript concentrations for all genes were almost indistinguishable between the two strains. Combined, these results provided evidence that the components of the RsmA, RsmY and RsmZ post-translational regulatory system may be the primary, if not the sole mediators of the signals transduced by the RetS/GacS/GacA pathway. To test this hypothesis further, we carried out ChIP-on-chip analysis to determine the genome-wide distribution of GacA binding sites. We detected specific GacA association only to regions adjacent to the *rsmY* and *rsmZ* genes, and we showed that this association is positively influenced by a *retS* mutation. We therefore conclude that phosphorylation of GacA by GacS results in transcription of only two genes, and the broad effect on mRNA levels influenced by GacS/GacA (and therefore RetS) is due to the activities of two sRNAs RsmY and RsmZ on the translational regulator RsmA. It is important to note, however, that not all of the genes that are affected by the *retS*, *gacA* or *rsmYZ* mutations are expected to be directly regulated by RsmA via mRNA binding. In fact, the effect of the RetS/GacS/A/RsmY/Z/A on many of these genes, including the T3S genes, is likely to be a consequence of RsmA-mediated regulation of transcriptional regulators of those genes (Brencic and Lory, 2009).

The two-component systems that regulate transcription of the Csr/Rsm family of sRNAs control a variety of cellular functions in different microorganisms. It is conceivable that in most instances the regulatory effect will be achieved by control of transcription of the sRNA genes with the subsequent post-transcriptional effect of these regulatory RNAs on CsrA/RsmA translational regulators bound to their mRNA targets. However, unlike what we have observed for the GacS/A system in *P. aeruginosa*, in other bacterial species, direct binding of the response regulator to promoters other than those for the sRNAs has been demonstrated. In *Salmonella enterica* ser. Typhimurium, SirA directly controls the expression of *csrB* and *csrC*, but also the expression of the HilA and HilC transcription factors (Teplitski *et al.*, 2003). Furthermore, the *Salmonella* BarA/SirA system can affect the expression of the operon encoding the type I fimbriae by both direct transcriptional and indirect post-transcriptional mechanisms. The transcription of the *fim* genes is directly regulated by SirA, and the same regulatory protein also controls the translation and/or stability of the *fim* gene transcripts through its regulation of *csrB* and *csrC* transcription (Teplitski *et al.*, 2006). In *Legionella pneumophila*, the expression of genes involved in flagellar functions is regulated by the LetS/LetA system independently of RsmY and RsmZ (Sahr *et al.*, 2009). Therefore, utilizing genome-wide technologies such as ChIP-on-chip analyses, a more careful examination of direct interactions of the response regulators of the GacA/UvrY/SirA/LetA family with their regulatory targets could identify more complex regulatory networks in diverse organisms, capable of amplifying or directing the flow of input signals towards specific groups of genes. However, in *P. aeruginosa* the GacA regulatory activity is limited to only two promoters.

Given the membrane localization and a prominent periplasmic domain of the two-component sensor kinases, it is expected that the GacS/GacA/RsmY/Z-type regulatory pathways are controlled by external signals that regulate the autokinase activity of the sensor-kinase. However, in certain instances, additional regulatory elements were shown to

influence the expression of the Csr/Rsm small RNAs. In *Erwinia caratovora*, the FlhDC complex, the master regulator of flagella production, positively regulates the expression of the response regulator GacA and represses the transcription of HexA, a LysR-like protein shown to control the expression of the sRNA gene *rsmB* (Cui *et al.*, 2008; Mukherjee *et al.*, 2000). In *E. coli*, the CsrD protein that contains GGDEF and EAL domains regulates the levels of the small RNAs CsrB and CsrC by controlling their degradation rates (Suzuki *et al.*, 2006). In *P. aeruginosa*, the RNA chaperone Hfq binds and stabilizes RsmY but not RsmZ and therefore influences RsmY levels post-transcriptionally (Sonnleitner *et al.*, 2006).

The transcription rates of the *rsmY* and *rsmZ* genes in *P. aeruginosa* are clearly distinct; *rsmY* is expressed at approximately two-fold higher levels than *rsmZ*. We have shown that a considerably longer region upstream of the conserved UAS element is required for the expression of *rsmZ* compared to the region that is required for the expression of *rsmY*. Furthermore, there is a distinct difference in the location of the conserved UAS element relative to the transcription start sites of the two small RNAs. The UAS site is located 57 upstream of *rsmY* and 174 bps upstream of *rsmZ* (Table S4). This pattern appears to be conserved among other organisms phylogenetically related to *P. aeruginosa*, including *Pseudomonas putida*, *Pseudomonas fluorescens*, and *Pseudomonas syringae* (Table S4). The location of the UAS relative to the *rsmY* and *rsmZ* genes in *L. pneumophila* is also similar to those in the Pseudomonaceae, while this pattern is not conserved in *E. coli*, *Salmonella typhimurium*, *Vibrio cholerae* or *Yersinia pestis*, where the distances between the UAS sequences and the genes encoding sRNAs CsrB, CsrC ranges between 150 to 211 bp. The differences in length and structure of the sequences upstream of the *rsmY* an *rsmZ* genes that are required for the expression of the two sRNAs, and the overall difference in the levels of expression of *rsmZ* and *rsmY* (Brencic and Lory, 2009) suggest that additional factors may be contributing to the control of the expression of each of the two sRNAs.

We have shown that a region of DNA adjacent to *rsmZ*, but not *rsmY*, associates with MvaT and MvaU, two histone-like nucleoid structuring proteins related to H-NS (Castang *et al.*, 2008), and that MvaT repressed transcription of *rsmZ* while it had no effect on the expression of *rsmY*. Similar to H-NS binding to sequences with a strong bias towards adenine and thymine deoxyribonucleotides (A and T), MvaT and MvaU preferentially associate with regions of the chromosome where the % (A+T) is relatively high (Castang *et al.*, 2008). We found that the 174-bp region between the GacA binding site and the *rsmZ* transcriptional start site is highly enriched for A and T (55%) compared to the overall composition of various *P. aeruginosa* genomes, with an average % A+T of 34% (Mathee *et al.*, 2008; Stover *et al.*, 2000). Therefore, it is likely that this region contains one or more MvaT or MvaU binding sites, whose occupancy results in transcriptional repression. Since repression of gene expression by H-NS-like proteins is often counteracted by the activities of specific transcriptional regulators, it is conceivable that GacA performs this anti-silencing function, or that displacement of bound MvaT or MvaU is carried out by an as yet unidentified transcription factor. The differences in regulation of cellular concentrations of RsmY and RsmZ may provide a clue to understanding the benefits of having two seemingly redundant RNAs. In principle, this arrangement may allow a more efficient and precise regulatory response via a gene dosage effect by providing more possibilities for integrating various signals resulting in the regulation of the overall levels of the sRNAs.

Recent recognition of sRNAs as key intermediates in stress and nutritional responses in bacteria highlighted the importance of post-transcriptional control mechanisms for successful environmental adaptation. In several microorganisms, up to a hundred different sRNAs have been identified (Altuvia, 2007) and given the likelihood of each regulating more than a single mRNA, these elements can account for the regulation of expression of a substantial fraction of the transcriptome. Indeed, the global impact of the RetS/Gac/Rsm

signal transduction pathway acting through *rsmY* and *rsmZ* may greatly exceed the range of many prokaryotic signal transduction pathways that act through transcriptional regulators.

EXPERIMENTAL PROCEDURES

Plasmids, Strains and Culture Conditions

All strains used in this study were derived from *P. aeruginosa* strain PAK (D. Bradley) or PAO1 (A. Rietsch). Primer sequences and a detailed description of cloning procedures used for construction of *lacZ* fusions, deletion mutants and other strains used in the study are provided in Supporting experimental procedures. *P. aeruginosa* strains were maintained in Luria-Bertani (LB) broth with antibiotics as required (150 µg/ml carbenicillin, 75 µg/ml gentamicin, 25 µg/ml Irgasan, 50 µg/ml tetracycline). Growth incubations were performed at 37°C at 300 rpm shaking in baffled flasks for transcriptional profiling or in culture tubes for β-galactosidase assays and chromatin immunoprecipitation analyses.

Transcriptional Profiling

For the *retS/gacS* experiment, the cultures were grown and processed exactly as described by Goodman and colleagues (Goodman *et al.*, 2004), such that direct comparisons between the *gacS retS* transcriptome and the previously published *retS* and wt transcriptomes were possible. For the *gacA/rsmYZ* experiment, duplicate cultures were grown overnight in LB. These cultures were inoculated at an optical density at 600 nm (OD_{600}) of 0.01 into LB and grown for 7 hours (OD_{600} ~ 6.0). RNA was extracted, reverse transcribed, fragmented, and labeled as described (Wolfgang *et al.*, 2003) with one exception; at the time of harvesting, 20% of the culture volume of stop solution (95% ethanol, 5% phenol, pH 4.7) was added. The processed samples were hybridized to Affymetrix GeneChip *P. aeruginosa* Microarrays (Affymetrix), and the chips were washed and scanned according to the provided protocol. The 5,678 probe sets specific to strain PAK were filtered for statistically significant differences (Student's t test p value ≤ 0.05), signal above noise level (Present call in at least two samples), and a minimum 2-fold change using commercially available software (GeneSpring).

β-galactosidase Assays

All measurements of β-galactosidase activity were carried out in duplicates or triplicates starting with overnight cultures. Each experiment was performed at least twice. Overnight cultures were grown in LB with antibiotics as appropriate. Cultures were diluted 1:100 into LB, and grown for 4 or 7 h before being assayed. For the time course of *rsmY* and *rsmZ* expression, cultures were sampled at each time point and assayed immediately. The amounts of β-galactosidase activity were quantified as described (Sambrook *et al.*, 1989). In experiments involving the type III secretion genes, assay cultures were grown in calcium-depleted conditions which were achieved by addition of nitrilotriacetic acid and $CaCl_2$, each to a final concentration of 10 mM.

Biofilm Assay

In a modification of the biofilm ring assay (O'Toole and Kolter, 1998), overnight cultures were diluted to OD_{600} =0.0025 in LB and triplicate 1-ml aliquots were dispensed into glass tubes. Following static incubation in a humidified 37°C chamber, the media was removed and tubes were washed gently with distilled water.

Chromatin Immunoprecipitation (ChIP)

5-ml volumes of LB broth were inoculated at a starting OD_{600} of ~0.03, and grown at 37 °C with aeration, to an OD_{600} of ~1.4. Crosslinking and ChIP were then performed as described previously (Castang *et al.*, 2008).

ChIP-on-Chip

Triplicate cultures of PAO1 Δ*retS* GacA-V, each grown in eight×5-ml volumes of LB as described above, were used for chromatin immunoprecipitation. Approximately 250 ng of ChIP and input DNA from each replicate was subjected to one round of amplification using the BioPrime Array CGH Genomic Labeling System kit (Invitrogen). Resulting sample DNA was labeled and hybridized to custom *P. aeruginosa* PAO1 high-density oligonucleotide arrays, by Roche NimbleGen, Inc., as previously described (Castang *et al.*, 2008).

ChIP-on-Chip Data Analysis

To control for any nonspecific enrichment, the average log2 values from three mock VSV-G ChIP-on-chip experiments were subtracted from each array prior to data analysis (Castang *et al.*, 2008). Data from each array were smoothed using a sliding window average, with a window size of 6 probes and a step size of 3 probes. Following smoothing, a fixed percentile cut-off method was used to determine "peaks" of GacA-V enrichment (Castang *et al.*, 2008). The threshold level used for analysis was set at 99.9% and only peaks that were isolated in all three arrays were considered to be significant. Such a stringent cut-off was used as we were unable to validate enrichment in regions that were isolated using lower threshold levels (data not shown).

ChIP Quantitative PCR

qPCR was performed using i*Taq* SYBR green with ROX (BioRad) and an Applied Biosystems StepOnePlus detection system. ChIP fold enrichment values were calculated as described (Castang *et al.*, 2008), and represent the relative abundance of a sequence of interest compared to a negative control region. All ChIP fold enrichment values represent the average of two biological replicates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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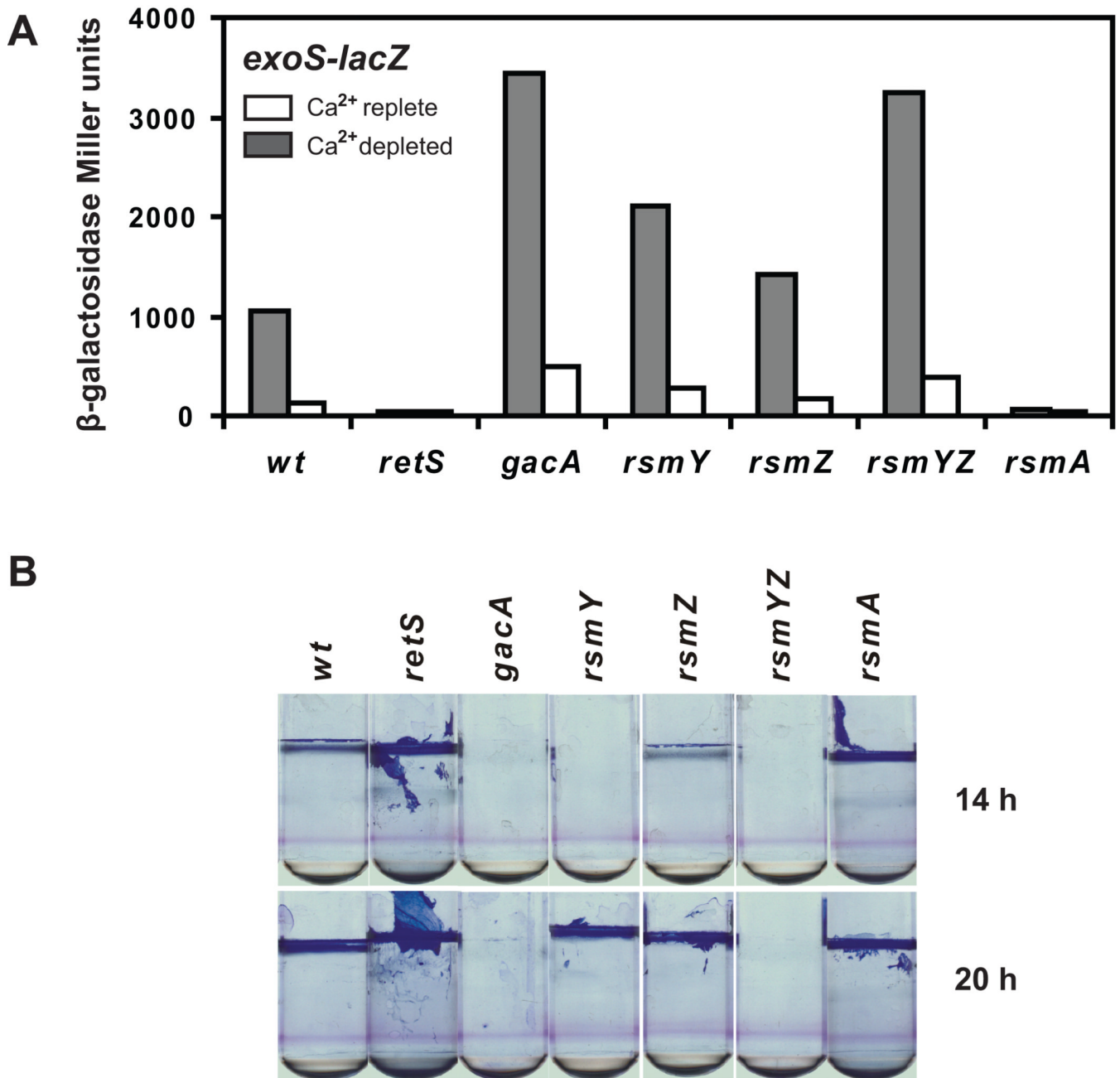
Figure 1. Effect of mutations in the RetS/GacS/A/RsmY/Z/A pathway on expression of a T3S gene *exoS* and on development of biofilm

A. Miller assays were used to measure expression of the *exoS-lacZ* fusion in wild type and mutant strains of *P. aeruginosa*. As induction of the T3S genes occurs under conditions of low calcium, cultures were grown in Ca^{2+}-depleted (grey bars) and Ca^{2+}-replete (white bars) LB media, at 37°C for 4 hours.

B. Biofilm phenotypes were measured in wild type and mutant strains of *P. aeruginosa* as the attachment of bacteria to tubes and formation of biofilm rings and pellicles after 14 and 20 h static incubation at 30°C.
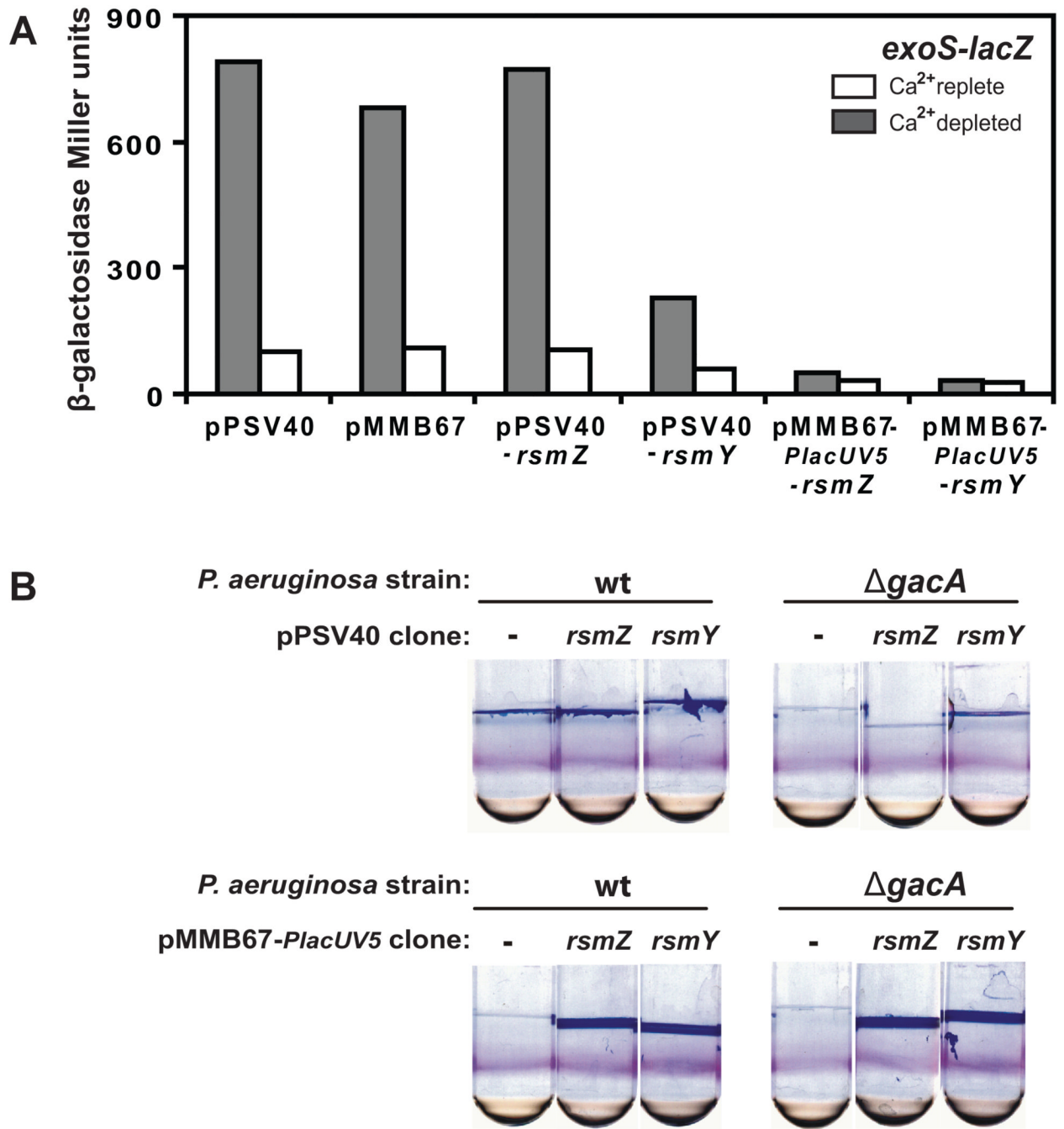
Figure 2. Effect of RsmY and RsmZ overexpression on expression of the *exoS* gene and on biofilm development
A. Expression of the *exoS* gene was measured using Miller assays in *P. aeruginosa* strains carrying an *exoS-lacZ* reporter fusion and *rsmY* or *rsmZ* cloned with their native promoter into a promoterless plasmid pPSV40, or under the control of a constitutively active PlacUV5 promoter in a derivative of the pMMB67 plasmid (See Experimental procedures). As induction of the T3S genes occurs under conditions of low calcium, cultures were grown in Ca^{2+}-depleted (grey bars) and Ca^{2+}-replete (white bars) LB media, at 37°C for 4 hours.
B. Biofilm phenotypes were measured in *P. aeruginosa* wild type and *gacA* mutant strains with *rsmY* or *rsmZ* cloned with their native promoter into a promoterless plasmid pPSV40,

or under the control of a constitutively active PlacUV5 promoter in a derivative of the pMMB67 plasmid. Biofilms were scored as the attachment of *P. aeruginosa* to tubes and formation of the biofilm rings and pellicles after 20 h static incubation at 30°C.
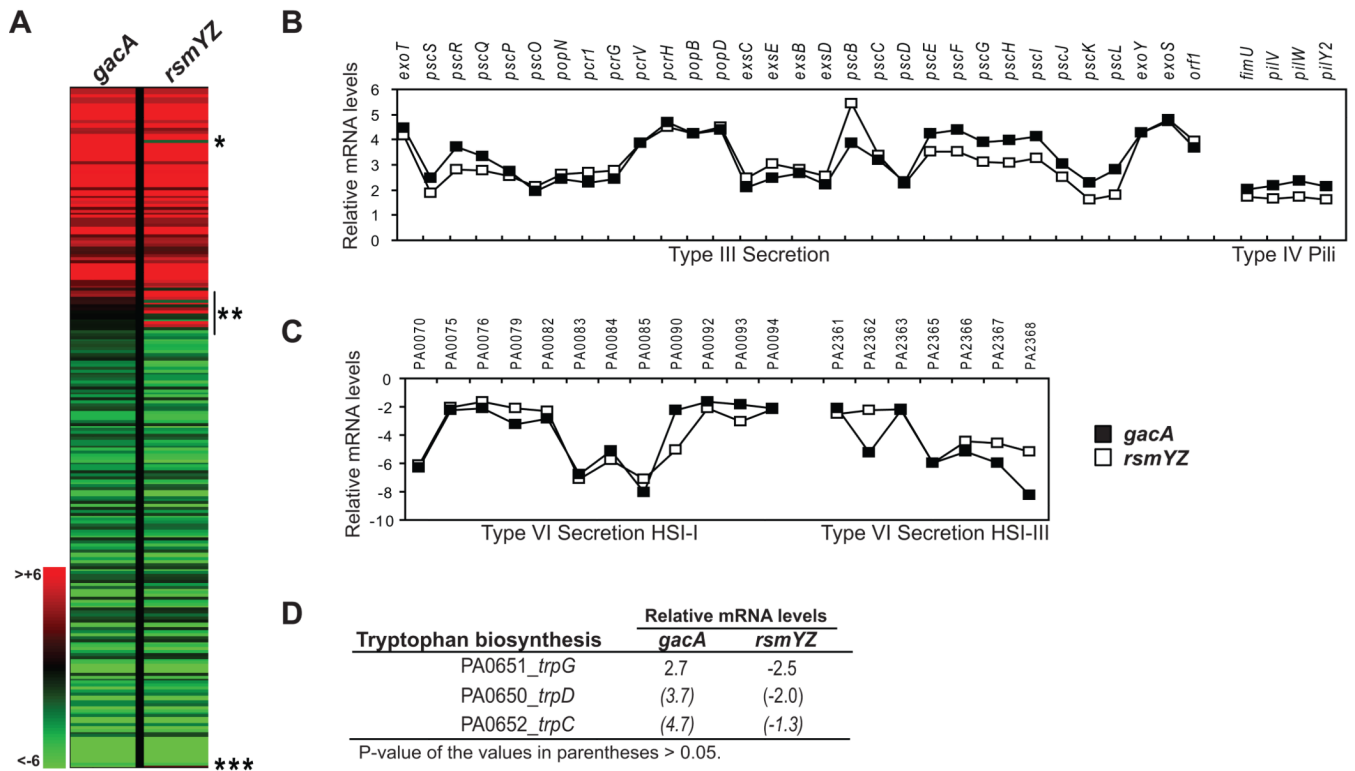
Figure 3. Comparison of mRNA levels relative to wild type between *P. aeruginosa gacA* and *rsmYZ* mutants

A. Heatmap of the 241 genes whose mRNA levels changed more than two fold relative to wild type in at least one of the two mutants (See text and Table S1 for detail). Red color represents genes that were upregulated relative to wild type, green represents genes that were downregulated relative to wild type (see color scale on the left). * *trpC* gene, which was upregulated in the *gacA* strain but was not affected in the *rsmYZ* strain (See text for detail); ** genes whose expression was affected in the *rsmYZ* strain only (See text and Table S3 for detail); *** *gacA* gene; downregulation in the *gacA* strain is artificial as this gene was deleted in this strain.

B–D. mRNA levels of selected genes in the *gacA* and *rsmYZ* mutants relative to wild type. B. Type III secretion genes and Type IV pili genes were upregulated in both *gacA* and *rsmYZ* mutants. C. Genes encoded in the Type VI secretion islands HSI-I and HSI-III were downregulated in both *gacA* and *rsmYZ* mutants. Open symbols – *gacA* mutant; Closed symbols – *rsmYZ* mutant. D. Genes encoded in the *trp* operon were upregulated in the *gacA* mutant and downregulated in the *rsmYZ* mutant (See text for detail).
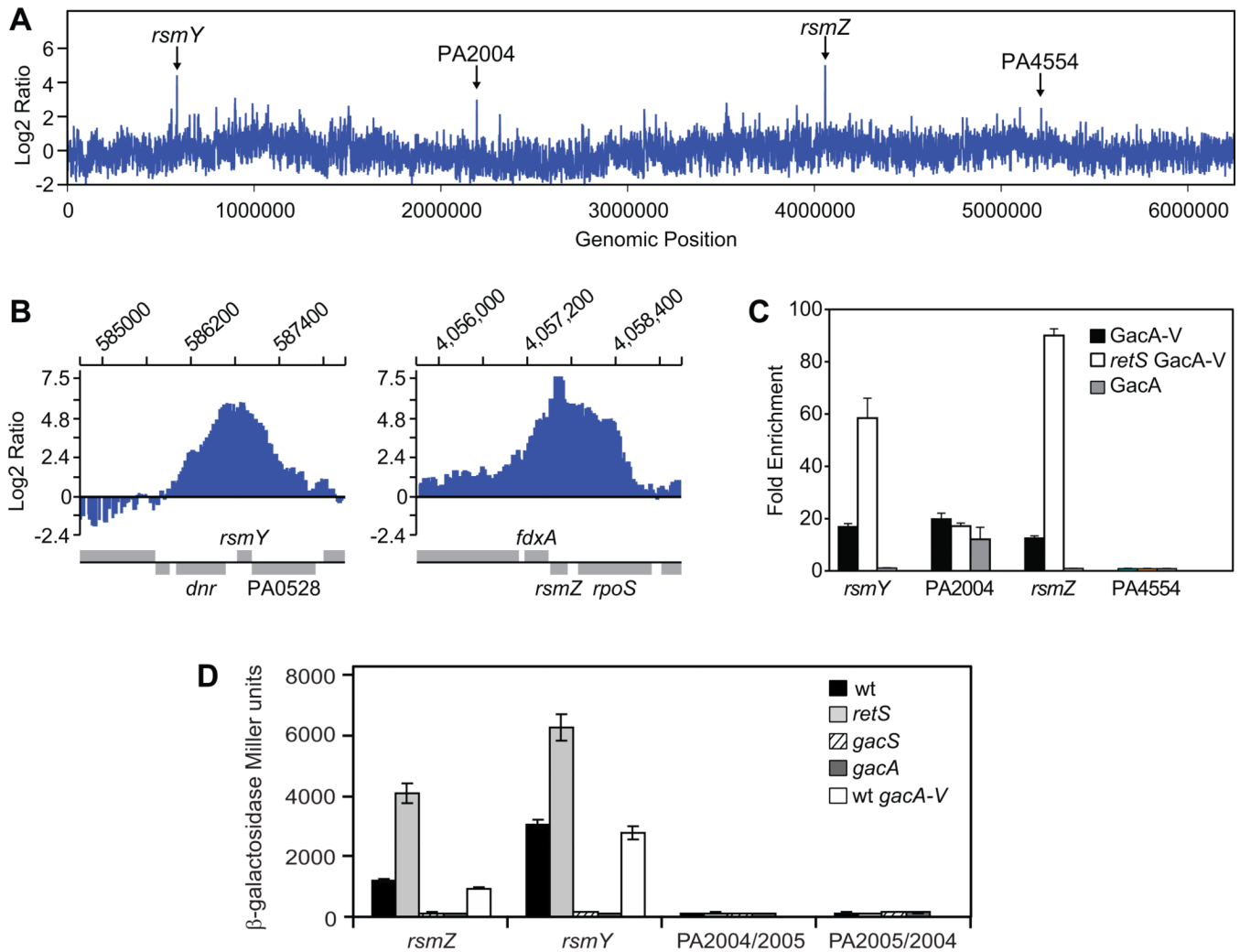
Figure 4. Identification of GacA binding sites in the *P. aeruginosa* genome
A. Results of genome-wide ChIP-on-chip analysis using a *P. aeruginosa retS* strain with
VSV-G-tagged GacA (GacA-V). Anti-VSV-G-tag antibody was used to capture GacA-V
with bound DNA fragments, which were then used to analyze a high density DNA
oligonucleotide microarray representing the *P. aeruginosa* genome. To obtain a genome-
wide view of GacA-V-bound probes, data for the three biological replicates were averaged
and were used to generate an enrichment map of fragments represented by probes
corresponding to genes on the *P. aeruginosa* chromosome. Indicated are the four loci where
enrichment of co-immunoprecipitated DNA fragments was observed.
B. Higher resolution of the *rsmZ* and *rsmY* genomic loci where the enrichment of DNA
fragments was detected. Genes corresponding to the enriched regions are represented below
the charts above the horizontal line if their orientation is left to right, and below the
horizontal line if their orientation is right to left.
C. Chromatin immunoprecipitation (ChIP) using a wild type *P. aeruginosa* and a *retS*
mutant expressing VSV-G tagged GacA (GacA-V), and a wild type strain expressing
untagged GacA (mock ChIP). Quantitative PCR was used to quantify the co-
immunoprecipitated DNA fragments using *rsmZ*-, *rsmY*-, PA2004/PA2005-, and PA4554-
specific primers.

D. Activities of a *lacZ* reporter fused to DNA fragments that were enriched in the ChIP-on-chip experiment. The expression of the *lacZ* fusions was measured as β-galactosidase activity (in Miller units) in the *P. aeruginosa* wild type and *retS*, *gacS*, and *gacA* mutant strains. The PA2004/PA2005 intergenic region was tested in both orientations. For *rsmY* and *rsmZ* fusions, the expression was also measured in the strain expressing *gacA* with a carboxy-terminal VSV-G tag (*gacA-V*). The assays were conducted with cultures at 7 hours of growth.

A

B
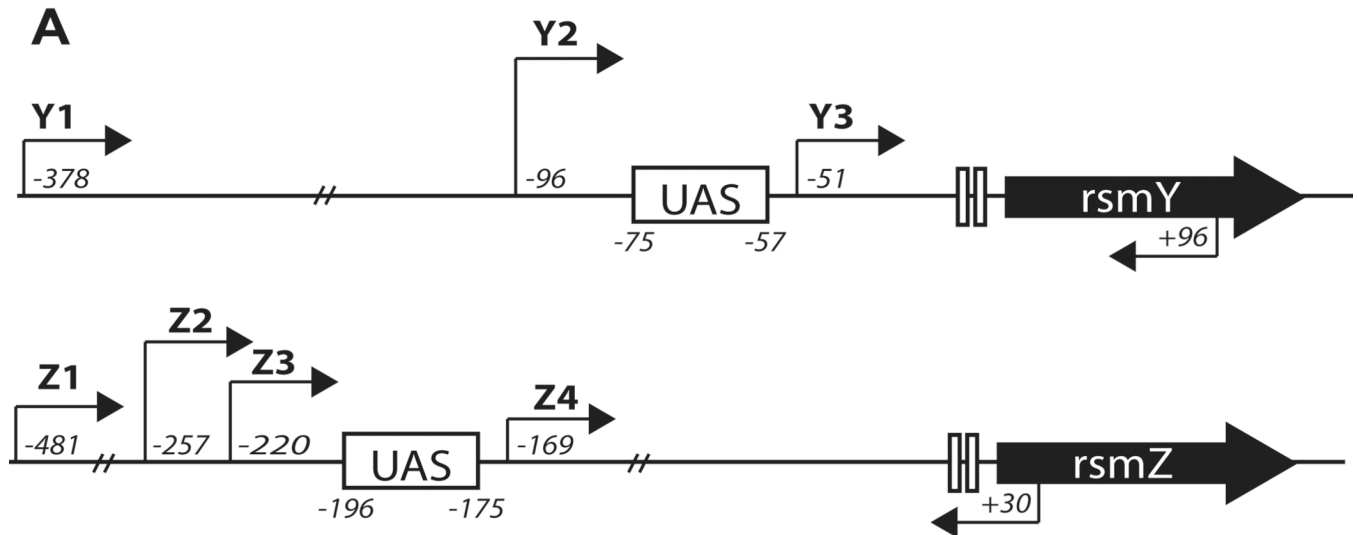
rsmY	Miller Units of β-galactosidase activity	
fragment	wt	*gacA*
Y1	1834 (+/-120)	61 (+/-0.7)
Y2	1920 (+/-82)	61 (+/-0.7)
Y3	97 (+/-7)	58 (+/-2.8)

rsmZ	Miller Units of β-galactosidase activity	
fragment	wt	*gacA*
Z1	1290 (+/-120)	71 (+/-0.4)
Z2	314 (+/-16)	60 (+/-0.8)
Z3	255 (+/-27)	61 (+/-3.8)
Z4	67 (+/-3)	58 (+/-0.6)

Figure 5. Location of the putative GacA regulatory sites upstream of the *rsmY* and *rsmZ* genes
A. Arrows indicate the location of primers used to amplify the sequences that were fused to a *lacZ* reporter. Italicized numbers indicate the position of the beginning (top arrows) and the end (bottom arrows) nucleotide of each fragment relative to the transcription start site of *rsmY* or *rsmZ*.
B. Activities of a *lacZ* reporter fused to various fragments upstream of the *rsmY* and *rsmZ* genes as indicated in A. The activities were measured in *P. aeruginosa* wild type and *gacA* mutant strains and are shown as Miller Units of β-galactosidase activity (with standard deviation shown in parentheses). Cultures were grown for 7 hours at 37°C before the assay.
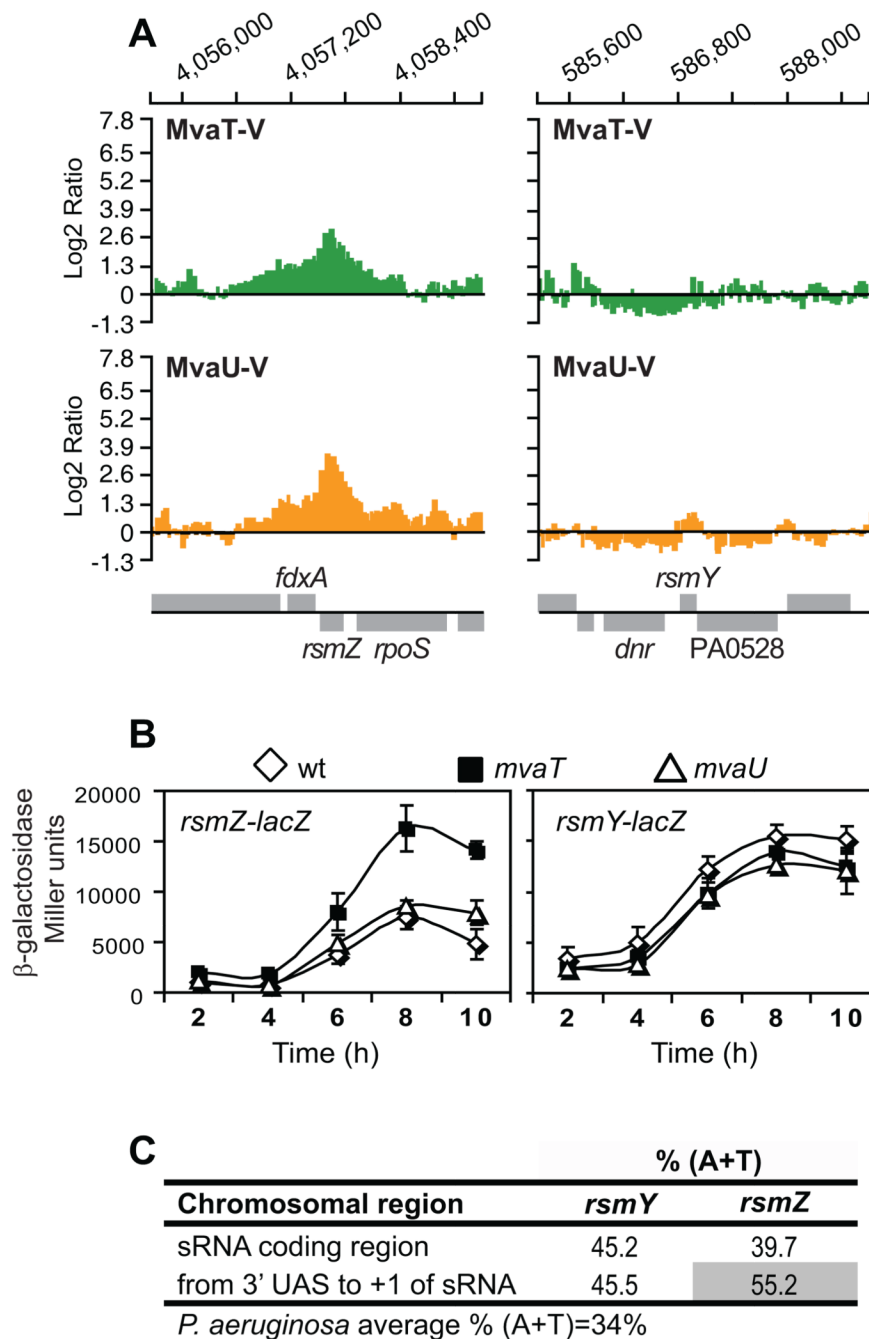
Figure 6. Role of MvaT and MvaU in the regulation of *rsmY* and *rsmZ*
A. VSV-G-tagged MvaT (MvaT-V) and MvaU (MvaU-V) associate with intergenic and coding sequences of *rsmZ* (left panel) but not of *rsmY* (right panel). Genes corresponding to the enriched regions are represented below the charts above the horizontal line if their orientation is left to right, and below the horizontal line if their orientation is right to left.
B. Effect of *mvaT* and *mvaU* deletions on the expression of *rsmZ* (left panel) and *rsmY* (right panel), as measured by Miller assays using *rsmZ-lacZ* and *rsmY-lacZ* reporter fusions.
C. The region between 3' of the UAS site and transcription start site (+1) of *rsmZ* is significantly enriched for A and T compared to the overall composition of various *P. aeruginosa* genomes and compared to the *rsmZ* coding sequence.