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The Role of the ncRNA RgsA in the Oxidative Stress Response and Biofilm Formation in *Azotobacter vinelandii*

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Abstract *Azotobacter vinelandii* is a soil bacterium that forms desiccation-resistant cysts, and the exopolysaccharide alginate is essential for this process. *A. vinelandii* also produces alginate under vegetative growth conditions, and this production has biotechnological significance. Poly-β-hydroxybutyrate (PHB) is another polymer synthetized by *A. vinelandii* that is of biotechnological interest. The GacS/A two-component signal transduction system plays an important role in regulating alginate production, PHB synthesis, and encystment. GacS/A in turn controls other important regulators such as RpoS and the ncRNAs that belong to the Rsm family. In *A. vinelandii*, RpoS is necessary for resisting oxidative stress as a result of its control over the expression of the catalase Kat1. In this work, we characterized a new ncRNA in *A. vinelandii* that is homologous to the P16/RsgA reported in *Pseudomonas*. We found that the expression of *rgsA* is regulated by GacA and RpoS and that it was essential for oxidative stress resistance. However, the activity of the catalase Kat1 is unaffected in *rgsA* mutants. Unlike those reported in *Pseudomonas*, RgsA in *A. vinelandii* regulates biofilm formation but not polymer synthesis or the encystment process.

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Introduction

In the last decade, small non-coding RNAs (ncRNAs) have gained great relevance in bacteriology. The ncRNAs are involved in many and diverse processes such as the stress response, motility, biofilm formation, control of metabolic fluxes, virulence, and many others [37]. In recent years, massive-scale RNA sequencing (RNAseq) and in silico genomic analysis have revealed hundreds of bacterial ncRNAs, but at present, the function of only a few of them is fully understood [27].

Genomic studies in *Pseudomonas* species revealed that one of the most ubiquitous ncRNAs is P16, also named RgsA [13]. In *Pseudomonas fluorescens* and *Pseudomonas syringae*, RgsA is related to the oxidative stress response, and in both cases, the ncRNA acts as a positive regulator [14, 28]. In *P. syringae, rgsA* mutants showed enhanced resistance to heat stress [28]. In *P. aeruginosa*, *P. fluorescens*, and *P. syringae,* the P16/*rgsA* ncRNA gene is transcriptionally controlled by GacA and the RpoS sigma factor [14, 28]. The two-component signal transduction system GacS/GacA is conserved in γ proteobacteria, and in many species, Gacs/A directly controls the expression of ncRNAs belonging to the Rsm family. The Rsm ncRNAs counteract the repressive activity of RsmA, an RNA-binding protein that functions as a translational repressor of its target genes [20]. Rsm ncRNAs show several stem-loop structures with a GGA-binding motif at the loops, which are the binding sites of RsmA [20]. The RgsA/P16 predicted secondary structure shows a single stem-loop structure with a GGA motif and resembles the Rsm ncRNA, but there are reports that ruled out a connection between RsgA and RsmA [14]. Contrary to observations for the *rsm* ncRNA genes, the regulatory region of *rsgA* does not contain a *gacA*-dependent activating sequence.

Azotobacter vinelandii is a soil bacterium that, under adverse environmental conditions, forms desiccation-resistant cysts [34]. Alginate is an exopolysaccharide that is essential for cyst formation [34]. Also vegetative cells produce alginate with other purposes. In this condition, alginate is proposed to act as a protective barrier against heavy metal toxicity or as a hydrophilic barrier that provides protection against adverse environmental conditions. Alginate is used for a variety of industrial purposes as a stabilizing or gelling agent [9]. Poly-β-hydroxybutyrate (PHB) is also synthetized by *A. vinelandii* and functions as an intracellular carbon and energy storage material [33]; it is a polyester of biotechnological interest because it can be used as a substrate to manufacture biodegradable plastics [12]. In *A. vinelandii,* GacS/GacA is involved in the control of alginate and PHB production [6]. A mutation in *gacS* abrogates both alginate and PHB synthesis and also significantly reduces the encystment capacity [6]. GacS/A in *A. vinelandii* regulates the transcription of the ncRNA genes *rsmZ1* and *rsmZ2*. RsmZ1 and RsmZ2 partially relieve the repression carried out by RsmA over alginate synthesis [24]. On the other hand, GacA positively controls the transcription of the gene *rpoS* [7], encoding the stationary phase sigma factor essential for oxidative stress resistance in *A. vinelandii* [10, 32].

A. vinelandii possesses two catalases called Kat1 (also named CCC and encoded by the *cccA* gene) and Kat2 (encoded by *katG*) [31]. It was previously shown that Kat1 activity is necessary for survival under oxidative stress; its enzymatic activity is RpoS dependent and is predominantly observed in the stationary growth phase [32]. To date, it is not clear how RpoS controls Kat1 activity, and it is unknown whether σ^S directly regulates *cccA* transcription or if other types of regulation exist. Although Kat2 is expressed in the exponential growth phase, Kat2 activity is also detected in the stationary phase of cultures with high aeration [32]. A third type of catalase activity (called Kat3) can be detected; Kat3 is proposed as an isoform of Kat2, and its activity is detected under similar conditions to Kat2 [31, 32].

Recently, physiological studies in *A. vinelandii* revealed that the presence of endogenous or exogenous reactive oxygen species (ROS) is an important condition to promote biofilm formation [36]. On the other hand, general catalase activity is enhanced in biofilm-embedded cells compared with planktonic bacteria [36]. However, currently, there are no reports of genetic regulatory elements related to biofilm formation.

In this work, we report the identification and characterization of the RsgA/P16 homologue in *A. vinelandii*. As in *Pseudomonas* spp, *rgsA* is regulated by GacA and RpoS, and it is involved in oxidative stress resistance. However, in contrast to that reported in *Pseudomonas,* we show here that RgsA regulates biofilm formation but that this effect is not related to the synthesis of alginate.

Materials and Methods

Microbiological Procedures

The bacterial strains and plasmids used in this study are listed in Table 1. The medium and growth conditions were as follows: *A. vinelandii* was grown at 30 °C in Burk's nitrogen-free salts medium [18] supplemented with 2 % sucrose (BS) or peptone yeast extract with 2 % sucrose (PY). *E. coli* strain DH5α was grown on Luria–Bertani medium (LB) at 37 °C. Antibiotic concentrations used (in micrograms per milliliter) for *A. vinelandii* and *E. coli,* respectively, were as follows: tetracycline, 20 and 20; ampicillin (Ap), not used and 100; nalidixic acid (Nal) and gentamycin (Gm), 1.5 and 10. *A. vinelandii* transformation was carried out as previously described [2]. The encystment assay was carried out as described by Moreno et al. [25].

Nucleic Acid Procedures

DNA isolation, cloning, and Northern blot procedures were carried out as described previously [30]. The *A. vinelandii* DJ genome sequence was used for designing the oligonucleotides used for PCR amplifications. The DreamTaq polymerase (Thermo Fisher Scientific) was used for PCR amplifications.

Cloning the *A. vinelandii rgsA* Locus

A 1.4-kb DNA fragment containing *rgsA* was amplified by PCR from *A. vinelandii* AEIV and DJ chromosomal DNA with the primers DcomrgsA (5′-GTTGCAGCGCCAGCG ACTTATG-3′) and RcomrgsA (5′-CGAGACCGGGCTG GACTATCACTA-3′). This fragment was cloned into pGEM-T Easy (Promega). The plasmids generated were named pGE*rgsA*1.4 and pGDJ*rsgA*1.4 and were used to determine the nucleotide sequences of the *rgsA* locus in the AEIV and DJ strains, respectively.

Generation of *A. vinelandii rgsA* Mutants

To generate *A. vinelandii rsgA* mutants, an *rsgA* deletion was generated by inverse PCR using the primers FinvrsgA (5′-GGAAGGTACCAGCGGGCGAC-3′) and RinvrgsA (5′-AGAGGAGGTACCGTCGTGGAT-3′), which contain *Kpn*I sites. Inverse PCR was carried out with pGE*rgsA*1.4 as the template. The resulting fragment was digested with

Table 1 Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristics	Source or reference
A. vinelandii strains		
DJ	*algU*, non-mucoid, sequenced strain	[35]
AEIV	mucoid, wild-type	[21]
AEIV*rgsA*	AEIV with an *rgsA*::Gm mutation	This work
AEIV*gacA*	AEIV with a *gacA*::Gm mutation	[24]
AEIV*rpoS*	AEIV with an *rpoS*::Sm mutation	[24]
DJ*rsgA*	DJ with an *rgsA*::Gm mutation	This work
DJ*gacA*	DJ with *gacA*::Gm mutation	[17]
DJ*rpoS*	DJ with *rpoS*::Sm mutation	[17]
AEIV$rgsA^{-/+}$	AEIV*rgsA* complemented in cis which carried wild-type *rgsA* gen recombined in melA locus.	This work
DJ$rgsA^{-/+}$	DJ*rgsA* complemented in cis which carried wild-type *rgsA* gen recombined in *melA* locus.	This work
E. coli strains		
DH5α	F^- _80*lac*ZΔM15 Δ (*lac*ZYA-*argF*) U169 *recA1endA1 hsd*R17(r_k^-, m_k^+) *phoAsup* E44 *thi*-1 *gyr*A96 *rel*A1 _-	Invitrogen
Plasmids		
pGEM-T Easy	PCR cloning vector	Promega
pGErgsA1.4	pGEM-T Easy carrying 1.4 kb *rsgA* locus from the strain AEIV cloned by PCR	This work
pGDJrgsA1.4	pGEM-T Easy carrying 1.4 kb *rsgA* locus from the strain AEIV cloned by PCR	This work
pGrsgA1.2	pGDJrgsA1.4with a 160 bp deletion of *rsgA*	This work
pGrsgA1.2Gm	pGrsgA1.2with an Gm cassette inserted instead of *rgsA*	This work
pG0.9rgsA	pGEM-T Easy carrying 0.9 kb *rsgA* locus from the strain AEIV cloned by PCR	This work
pUMATc	Integrative cloning vector	[10]

*Kpn*I and ligated to generate the plasmid pGrsgA1.2, which has a 160 bp deletion of the *rsgA* locus. This plasmid was digested with *Kpn*I, and a Gm cassette from pBSL41 [1] was ligated to generate pGrsgA1.2Gm. The *rsgA* deletion and the cassette insertions in the recombinant plasmid were verified by PCR and DNA sequencing. Plasmid pGrsgA1.2Gm, which is unable to replicate in *A. vinelandii*, was introduced into strains AEIV and DJ. Transformants resistant to Gm were isolated and confirmed by PCR analysis to carry the *rgsA*::Gm mutation (data not shown). The resultant mutants were named AEIV*rgsA* and DJ*rsgA*.

Complementation of *rsgA* Mutants

To complement the *rgsA* mutants, a 0.9-kb DNA fragment that contains the *rsgA* gene plus its 0.5 kb upstream sequence containing the promoter and URS region was amplified by PCR using AEIV chromosomal DNA and the primers DcomrgsA and RsonrgsA (5′-TCGCCCGCTGATACTT TC-3′). This fragment was cloned into pGEM-T Easy (Promega) to generate pG0.9rgsA. To delimit the *rgsA* gene with its regulatory region, a fragment was excised with *Stu*I (insertion site) and *Pst*I (vector site) cuts. The 0.35 kb fragment (*Stu*I-*Pst*I) was cloned into the pUMATc vector [10]. The resultant plasmid was called pUMATcrsgA. pUMATc is an integrative suicidal vector that promotes the integration of the cloned DNA in the *melA* locus of the *A. vinelandii* chromosome. Plasmid pUMATcrsgA was introduced by transformation into strains AEIV*rgsA* and DJ*rgsA*. Transformants resistant to Tc were isolated and confirmed by PCR analysis to carry the *rgsA* wild-type gene recombined in the *melA* locus using the primers DsonrgsA (5′-ACTGACGTGGCATTTCCC-3′) and RsonrgsA (data not shown). The resultant recombinants were named AEIV$rgsA^{-/+}$ and DJ$rgsA^{-/+}$.

Oxidative Stress Assay and Heat Survival Assay

The assay was carried out as reported by Cocotl-Yañez et al. [11]. To carry out the heat survival assay, once cells were resuspended to standardize the cellular concentration, the cultures were incubated at 48 °C, taking samples prior to incubation and after 30, 60, and 120 min. The colony-forming units (CFU) in each sample were determined by serial dilutions.

Biofilm Assay

Biofilm formation in microtiter plates was quantified by crystal violet staining as reported by O'Toole and Kolter [26], with some modifications; we normalized biofilm formation to the concentration of protein instead, using optical density (OD) as growth parameter. Biofilm formation was reported as OD/mg of protein.

Analytical Methods

The protein concentration was determined by the Lowry method [23]. Alginate production was determined as previously described [3]. The PHB content of the bacteria was assayed by the method of Law and Slepecky [22]. The zymographic catalase analysis was conducted as described by Woodbury et al. [38].

Results

Identification of the *A. vinelandii rgsA* Gene

To identify ncRNAs in the *A. vinelandii* DJ genome, we analyzed the intergenic regions with the INFERNAL algorithm using the Rfam database as a comparison pattern (http://rfam.xfam.org/). One region located within nucleotides 1479172–1479292 of the DJ chromosome sequence presented 56 % identity with the *rsgA* homologue of *P. fluorescens*. The predicted secondary structure of the *A. vinelandii* RsgA ncRNA was similar to the *Pseudomonas* RgsA, showing only one stem-loop structure with a predicted GGA motif in the loop. *rgsA* was located between a gene encoding a putative TatD family hydrolase (Avin_15000) and a gene encoding a putative IS4 transposase (Avin_15010). The genome of the DJ strain has been sequenced and published [35] and is considered the type strain of *A. vinelandii*. This strain does not produce alginate due to an IS in the *algU* gene, which is essential for alginate production [25]. Hence, in this study, we worked with the AEIV strain, a wild-type mucoid strain of *A. vinelandii* [21].

The DNA fragments from the *A. vinelandii* AEIV and DJ strains containing the *rgsA* gene, including its promoter and terminator sequences, were amplified by PCR and cloned as described in Materials and Methods. Sequence analysis revealed 100 % identity between the two *A. vinelandii* strains. To detect *rsgA* transcripts, Northern blot analysis was conducted and confirmed the presence of the ncRNA in the AEIV and DJ strains (Fig. 1b).

GacA and RpoS are Required for Efficient *rgsA* Expression

In *P. fluorescens*, *P. aeruginosa*, and *P. syringae,* RpoS controls *rgsA* transcription [14, 28]. To investigate whether RpoS controls the expression of the *rgsA* gene in *A. vinelandii*, Northern blot analysis was conducted using RNA extracted from the *rpoS* mutants derived from the AEIV and DJ strains. As shown in Fig. 1b, the expression of *rgsA* was not detected in the *rpoS* mutants.

GacA regulation of *rgsA* is a common phenomenon in *Pseudomonas* spp.; however, the *rsgA* regulatory regions do not show the typical *gacA*-dependent activating sequences. Interestingly, these regulatory regions show a conserved sequence called the URS (Upstream Regulatory Sequence). This box is proposed to serve as the binding site of an unknown regulator [14]. Likewise, in *A. vinelandii,* the *rsgA* regulatory region also contains an URS box (Fig. 1a). As shown in Fig. 1b, *rgsA* transcripts were detected in the AEIV*gacA* and DJ *gacA* mutants. In both cases, *rgsA* expression was diminished but not completely abrogated.

The ncRNA RsgA is an Important Factor in Oxidative Stress Resistance

It was reported in *P. fluorescens* and *P. syringae* that RgsA is important for resisting oxidative stress. To investigate the role of RsgA in oxidative stress, an *rsgA* mutant derived from strain AEIV and carrying an *rgsA* deletion was constructed as described in Materials and Methods. The absence of the *rgsA* gene was confirmed by Northern blot analysis (Fig. 1b). We tested the survival of the *rsgA* mutant of *A. vinelandii* upon hydrogen peroxide exposure. As shown in Fig. 2a, the AEIV*rgsA* mutant exhibited enhanced sensitivity to hydrogen peroxide. The wild-type strain showed a survival of approximately 20 % after 300 mM H_2O_2 exposure, while the *rsgA* mutant showed a survival rate of approximately 4 %. Similar data were obtained for the DJ*rgsA* mutant (data not shown). Genetic complementation of the *rgsA* mutant with a wild-type copy of the *rsgA* gene integrated into the chromosome restored the survival of the mutant, confirming the positive effect of *rsgA* on oxidative stress resistance.

In *A. vinelandii,* the role of RpoS and Kat1 in the survival of oxidative stress has been well documented. We hypothesized that Kat1 catalase activity could be influenced by RgsA. To test this hypothesis, we carried out zymographic analysis to detect catalase activity in the *A. vinelandii* AEIV*rgsA* mutant. As shown in Fig. 2b, no differences in Kat1 activity were observed, indicating that RsgA is necessary for oxidative stress resistance in a Kat1-independent manner.

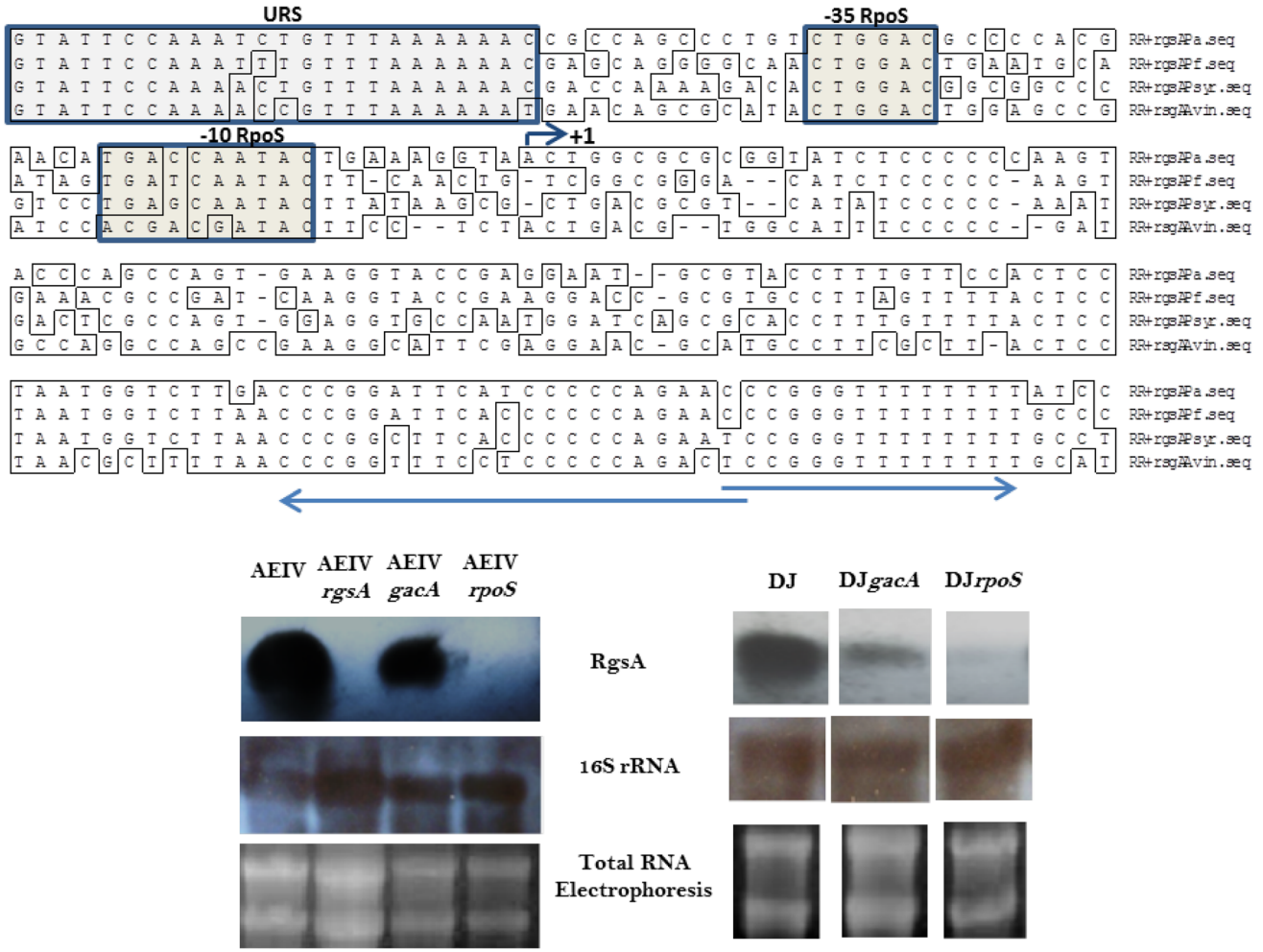

Fig. 1 **a** Alignments between *P. aeruginosa rgsA*, *P. fluorescens rgsA*, and *P. syringae rgsA* with *A. vinelandii rsgA* homologue. $^{+}1$ putative transcription start and URS *boxes* are indicated. The −10 and −35 σ^{S} putative promoter sequences are *boxed*. A predicted terminator sequence is indicated by divergent *arrows*. **b** Northern blot analysis of *rgsA* in AEIV, AEIV*rgsA*, and AEIV*rgsA*$^{-/+}$ (*left panel*) and DJ, DJ*rgsA*, and DJ *rgsA*$^{-/+}$ (*right panel*). The RNAs were isolated from stationary phase cultures (48 h of incubation in Burk's sucrose medium). All blots were re-probed with a 16S rRNA probe to confirm that similar amounts of total RNA were analyzed

Additionally, we determined the high-temperature resistance response for the AEIV*rgsA* mutant. Contrary to published findings in *P. syringae,* differences between the *rgsA* mutant and its parental strain were not observed (Fig. 3).

RsgA Controls Biofilm Formation

As mentioned above, in *A. vinelandii,* an increase in ROS promotes biofilm formation [36]. Therefore, we investigated whether *rgsA* was involved in biofilm generation; the experiment was conducted as described in Materials and Methods. As expected, the AEIV*rgsA* mutant showed enhanced capacity for developing biofilms in both rich and minimal media (Fig. 4). The complementant strain (AEIV*rgsA*$^{-/+}$) showed a similar behavior to the wild type.

RgsA is not Involved in Alginate Production, PHB Biosynthesis, or Encystment in *A. vinelandii*

In *A. vinelandii,* the two metabolites controlled by the GacS/A system are alginate and PHB [6, 7]. To investigate whether RgsA was involved in these GacA-regulated-pathways, we measured both polymers in *rgsA*. As shown in Table 2, alginate production by the AEIV*rgsA* mutant showed wild-type levels, indicating that RsgA is not involved in the regulation of this polymer. Likewise, PHB in the DJ genetic background was not affected by the *rsgA* mutation, DJ*rgsA,* indicating that RgsA is not involved in the control that GacA exerts upon these polymers.

As both GacA and RpoS are essential for cyst formation and since these regulators control *rsgA* expression, the possible involvement of RgsA in this process was also

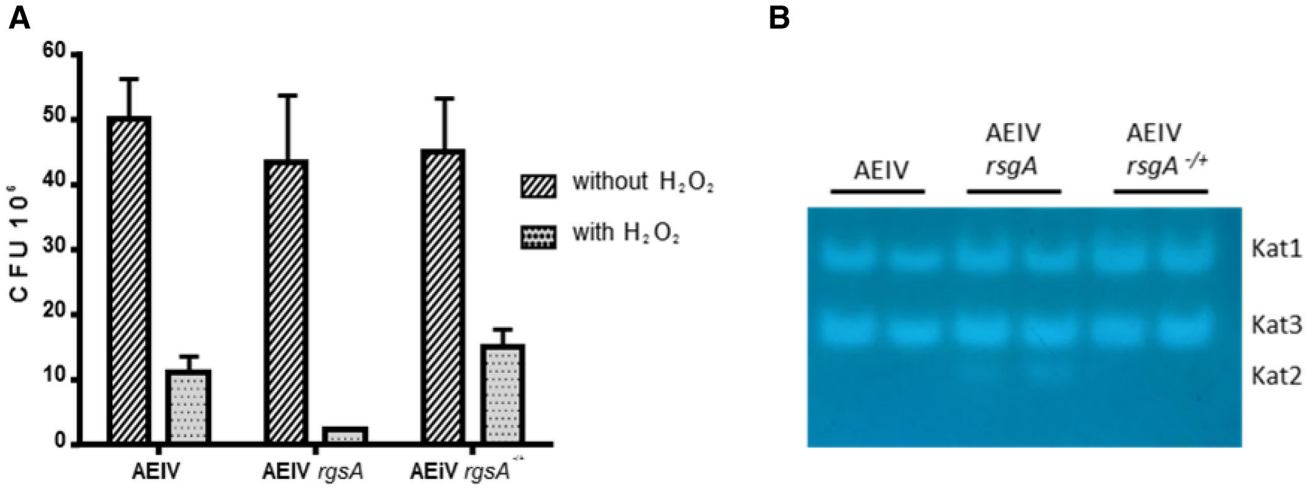

Fig. 2 Oxidative stress response and catalases activities in *A. vinelandii* strains. **a** Resistance to 300 mM of hydrogen peroxide of the strains AEIV, AEIV*rgsA*, and AEIV*rgsA*$^{-/+}$, and the number of colony former units (CFU) after exposure to peroxide are shown. The bars represent the statistical media of six measures and its standard deviation. **b** Zymographic analyses of catalases activity. In situ catalase zymographs carried out with 50 µg of cell lysate from cultures of the strains AEIV, AEIV*rgsA*, and AEIV*rgsA*$^{-/+}$, separated by non-denaturing PAGE. The three bands corresponding to different catalases activities are indicated

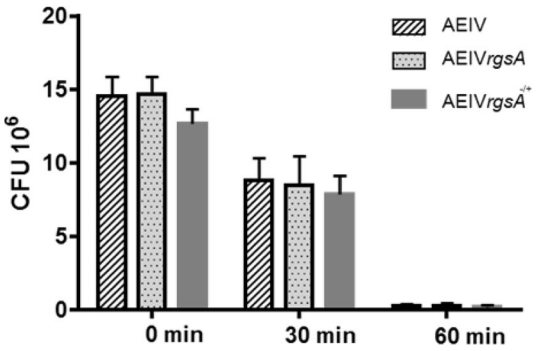

Fig. 3 Heat survival assay in AEIV, AEIV*rgsA*, and AEIV*rgsA*$^{-/+}$ strains. The media of the CFU number after exposure to 48 °C, the intervals of exposition in minutes (min) are shown in the *Y* axis. The *bars* represent the statistical media of six measures and its standard deviation

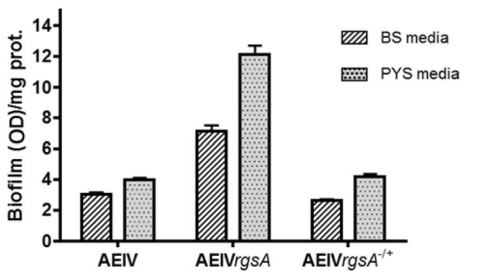

Fig. 4 Biofilm formation of AEIV, AEIV*rgsA*, and AEIV*rgsA*$^{-/+}$ strains on polystyrene microtitrer plates. The determinations were carried out in minima medium (BS) and rich medium (PYS) as reported by O'Toole and Kolter [26]. The data represent the media of six individual experiments with its statistical standard deviation

evaluated. As shown in Table 2, the encystment capacity of the mutant AEIV*rgsA* was not affected, indicating that RsgA is not involved in this phenomenon.

Discussion

In this work, we reported the first study of *rgsA* outside of *Pseudomonas* spp. The *Azotobacter* and *Pseudomonas* groups belong to the Pseudomonadaceae family. *In silico* analysis revealed *rsgA* homologues in three *A. vinelandii* strains (DJ, CA and CA6). In this work, we characterized an *rgsA* homologue in a fourth strain (AEIV). We also localized an *rsgA* homologue in the recently published genome of *Azotobacter chrococcum* [29]. In all cases, the gene downstream of *rgsA* is a *tatD* homologue. The gene upstream of *rgsA* varies between the two *Azotobacter* species; in *A. vinelandii,* the 5′ neighbor is a transposase belonging to the IS4 family and in *A. chrococcum,* it is a class V aminotransferase. Our bioinformatics search did not find *rgsA* homologues outside of Pseudomonadaceae family, suggesting that RsgA is an ncRNA that is only related to this phylogenetic family.

The RgsA stem-loop structure with a GGA motif resembles the secondary structures of the Rsm ncRNAs. However, the current interaction model between RsmZ and RsmA involves two contiguous GGA motifs that bind an RsmA dimer, so it is unlikely that RgsA interacts with RsmA. The role of the RgsA GGA motif remains to be elucidated. Otherwise, in *P. syringae,* RgsA interacts with

Table 2 Polymers production and encystment in *A. vinelandii* strains

Strain	Alginate production (μg/mg of protein)[a], BS medium	PHB production (μg/mg of protein)[b] PY medium	Encystment (%)
AEIV	2.424 ± 0.161	ND	9.2 ± 1.1
AEIV*rgsA*	2.528 ± 0.039	ND	8.8 ± 1.4
AEIV*rgsA*$^{-/+}$	2.393 ± 0.266	ND	9.5 ± 1.8
DJ	ND	0.612 ± 0.024	ND
DJ*rsgA*	ND	0.671 ± 0.065	ND
DJrsgA$^{-/+}$	ND	0.625 ± 0.031	ND

ND not determined

[a] Alginate production was determined in cells grown for 48 h (stationary phase) in Burk's liquid medium (BS) with 2 % sucrose

[b] PHB content was determined in cells grown for 48 h in PY liquid medium supplemented with 2 % sucrose. In both media, the cultures were incubated at 30 °C with 210 rpm of shaking. All the measurements were done in sextuplicate; the median and standard deviation are shown

the RNA chaperone Hfq, which suggests that this chaperone promotes the interaction between the ncRNAs and their mRNA targets [28]. The predicted gene Avin 7540 of the *A. vinelandii* DJ genome encodes a putative homologue of Hfq, which could mediate RgsA activity; whether an RgsA–Hfq interaction occurs and its functional mechanism with its mRNA targets remain to be established.

The zymographic analysis of the catalases of *A. vinelandii* suggests that there is no regulatory relation between Kat1 activity and RgsA. The zymogram reveals predominant activity of Kat3, which is an isoform of Kat2; a similar phenomenon has been described by Sandercock et al. [32] in the late stationary phase of cultures with high aeration. The authors suggest that the Kat2/Kat3 activities occur in response to high levels of ROS caused by the high aeration. Therefore, the presence of Kat3 activity in the zymogram gel (Fig. 2b) could be due to increased ROS in the culture conditions. Interestingly, only a faint Kat2 band was detected in the *rgsA* mutant and was not present in the complemented strain, probably as a compensatory response to the defective oxidative stress response of the mutant.

Mucoid strains of *A. vinelandii* produce an alginate-dense biofilm [15]. Recently, Villa et al. [36] have shown that *A. vinelandii* responds to the increase in ROS by enhancing biofilm formation. As expected, we showed that RgsA is necessary to contend with oxidative stress and that its absence promotes a major biofilm formation. Further, the enhanced catalase activity in biofilms produced by cells expressing the alkyl hydroperoxide reductase gene (*ahpC*) is increased in the presence of endogenous ROS [36]; it would be interesting to determine the *ahpC* expression expression in *rgsA* mutants.

In *P. fluorescens* [16], *P. syringae* [8], and *A. vinelandii* [7], GacA has been shown to control *rpoS* expression and to regulate the transcription of ncRNAs from the Rsm family [20]. Therefore, the *gacA* mutant phenotypes could be related to RpoS, the Rsm system or both. In *A. vinelandii,* GacS/A controls alginate and PHB production, and this regulation is explained partially by the control exerted over RsmZ1 and RsmZ2 [24]; so it could be that RgsA will control the synthesis of polymers, fact that was ruled out in this study. Thus, it is possible that GacA will control some targets exclusively through RpoS.

In *A. vinelandii,* the *rpoS* mutant cysts completely lacked the exine and intine layers of the cyst capsule and are unable to resist desiccation [11]. Interestingly, inactivation of *rpoS* does not prevent alginate synthesis. Therefore, the inability of the *rpoS* mutant to form the capsule layers is not caused by the absence of alginate [11]. Based on the findings of Cocotl-Yanez et al. [11] it is likely that other genes controlled by RpoS are essential for cyst formation. *rgsA* could be one of these genes, although the encystment assay we developed ruled out this hypothesis. Similarly, in *P. syringae,* RpoS controls virulence genes independent of RgsA [28]. In both bacteria, the data suggest the existence of alternative regulation pathways controlled by RpoS, and one of these (RpoS–RgsA) controls the oxidative stress response. It remains to be determined whether other functions regulated by the RpoS–RsgA pathway exist, as well as its target genes.

In order to establish possible interaction targets of RsgA, we carried out a search using the IntaRNA algorithm (http://rna.informatik.uni-freiburg.de/IntaRNA/Input.jsp) [5]. The software found two interesting targets in the genome sequence of the DJ strain: HexR1 (Avin_27270) and AlgW (Avin_12950). HexR1 is a transcriptional regulator of Entner–Doudoroff pathway (ED). In *Pseudomonas putida*, HexR1 has been related with the increasing of NADPH production under oxidative stress. This fact suggests that the ED pathway regulates the cell redox status resulting in improved tolerance to oxidative stress [19]. AlgW is a homologue of the protease HtrA,

interestingly, in *P. aeruginosa* AlgW is also involved in the resistance to oxidative stress [4].

In summary, in *A. vinelandii*, the expression of *rgsA* is controlled by RpoS and GacA. Besides, we found that RgsA is an important factor to resist the oxidative stress and plays a significant role in the biofilm formation.

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