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ORIGINAL PAPER

Transcriptional Study of the RsmZ-sRNAs and Their Relationship to the Biosynthesis of Alginate and Alkylresorcinols in *Azotobacter vinelandii*

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Abstract

The GacS/A system in Azotobacter vinelandii regulates alginate and alkylresorcinols production through RsmZ1, a small regulatory RNA (sRNA) that releases the translational repression of the algD and arpR mRNAs caused by the RsmA protein. In the Pseudomonadaceae family, the Rsm-sRNAs are grouped into three families: RsmX, RmsY and RsmZ. Besides RsmZ1, *A. vinelandii* has six other isoforms belonging to the RsmZ family and another one to the RsmY. Environmental signals controlling rsmsRNAs genes in *A. vinelandii* are unknown. In this work, we present a transcriptional study of the *A. vinelandii* rsmZ1–7-sRNAs genes, whose transcriptional profiles showed a differential expression pattern, but all of them exhibited their maximal expression at the stationary growth phase. Furthermore, we found that succinate promoted higher expression levels of all the rsmZ1–7 genes compared to glycolytic carbon sources. Single mutants of the rsmZ-sRNAs family were constructed and their impact on alginate production was assessed. We did not observe correlation between the alginate phenotype of each rsmZ-sRNA mutant and the expression level of the corresponding sRNA, which suggests the existence of additional factors affecting their impact on alginate production. Similar results were found in the regulation exerted by the RsmZ-sRNAs on alkylresorcinol synthesis.

Keywords *Azotobacter* · Alginate · Alkylresorcinols · RsmZ · GacS/A

Introduction

The free-living bacteria face constant environmental changes; for survival, they require the adaptation of their metabolism through a flexible genic regulation. One of the signal transduction systems involved in such adaptation is the Gac-Rsm system, which is composed of the two-component system (TCS) GacS/A and the Rsm post-transcriptional regulatory system [1]. The TCS GacS/A has been extensively studied in *Pseudomonas* spp. and in several enterobacteria, in which TCS controls transcription of small regulatory RNAs (sRNAs) from the Rsm regulatory system (also called Csr, outside the *Pseudomonadaceae*). Generally, the Rsm system has two or more sRNAs, and a protein that acts as a post-transcriptional repressor (RsmA/CsrA). The Rsm-sRNAs have stem-loop structures containing GGA motifs counteracting the repressor activity of the RsmA protein. When Rsm-sRNAs are absent, the RsmA protein binds over or near the ribosome binding site of its mRNA target blocking translation and promoting its degradation [1].

In *Erwinia carotovora* and *Pseudomonas fluorescens,* the Rsm (Csr) system modulates the production of extracellular enzymes, secondary metabolites and motility [2, 3]. In *P. aeruginosa* it controls the quorum sensing system, exoproduct synthesis, motility and biofilm formation [4]. Moreover, in *Escherichia coli* the system controls the expression of genes involved in carbon metabolism, motility, biofilm

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formation and the production of the exopolysaccharide PGA (poly-β-1,6-*N*-acetyl-D-glucosamine) [5–8], while in *Salmonella* it controls the expression of invasion genes [9].

Rsm-sRNAs have been classified into three families, RsmX, RsmY and RsmZ [10]. The presence of two or more sRNAs of the Rsm family has been reported in some bacterial species. In bacteria of the *Pseudomonadaceae* family, the number of Rsm-sRNAs varies from two in *P. aeruginosa* (RsmZ and RsmY) to five in *Pseudomonas syringae* (five isoforms of RsmX) [10–13]. *A. vinelandii* is the bacterium with the highest number of Rsm-sRNAs reported to date, with seven sRNAs belonging to the RsmZ family (RsmZ1–RsmZ7) and one of the RsmY family [14, 15]. Several authors have proposed that such sRNAs act synergically to counteract the negative effect of RsmA [1, 16, 17].

The TCS GacS/A controls the expression of thousands of genes; however the only identified genes directly regulated by this TCS are those encoding the Rsm/Csr-sRNAs [1, 18, 19]. Generally, genes encoding the Rsm/Csr-sRNAs have GacA regulatory boxes located upstream from the transcription start site [13, 18]. Thus, conditions activating the TCS GacS/GacA are commonly identified by their effect on the *rsm*/*csr* sRNA gene transcription. In *E. coli, csrB* expression responds to short-chain organic acids such as acetate or formate [20] and the *csrB* and *csrC* expressions are upregulated during growth in a nutrient-poor medium [21]. In *P. fluorescens*, Krebs cycle intermediaries are related to the stimulus triggering Rsm-sRNAs transcription [22]. In this bacterium, transcriptional activation of *rsmZ* during transition from exponential to stationary growth phase also responds to an extracellular compound, whose nature is still unknown [23].

Global regulators such as IHF, H–NS and CRP are also involved in the control of the *rsm* (*csr*) gene transcription [18, 19]. IHF in *P. fluorescens* and MvaT/MvaU (H–NS homologues) in *P. aeruginosa* are involved in the *rsmZ* regulation.

Azotobacter vinelandii is a nitrogen-fixing soil bacterium that belongs to the *Pseudomonadaceae* family and has the capacity to form desiccation-resistant cysts [24]. In this bacterium, the GacS/A-Rsm pathway controls the alginate biosynthesis, a linear co-polymer of ß-D-mannuronic and its C-5 epimer α-L-guluronic acid [25]. This polymer has a broad range of applications as gelling, stabilizing and thickening agents in many industries. Alginate is produced under vegetative growing conditions and during the differentiation process leading to the formation of cysts. The GacS/A-Rsm systems control the *algD* gene expression encoding a GDP-mannose dehydrogenase, the key enzyme of the alginate biosynthetic pathway [14]. RsmA recognizes the *algD* mRNA leader blocking translation, while GacA is necessary for the Rsm-sRNAs expression, which in turn counteracts the RsmA translational repression effect. In *A. vinelandii*, the roles of sRNAs RsmZ1 and RsmZ2 in alginate production have been studied; individual mutations in both genes significantly diminished alginate production and *rsmZ1* over-expression restored to alginate synthesis in a *gacA* mutant [14].

Other metabolites controlled by GacA and RsmZ1 are the alkylresorcinols (ARs), a family of phenolic lipids, which are important components of the membrane of *A. vinelandii* cysts. Mutations in *gacA* and *rsmZ1* impaired the synthesis of ARs [26]. The genes necessary to produce ARs are encoded by the *arsABCD* operon. This operon is activated by ArpR, a LysR-type transcriptional regulator. RsmA represses *arpR* expression by binding to its mRNA; thus, the positive role of GacA and RsmZ in the biosynthesis of ARs is explained by their negative effect on RsmA activity.

As in other bacteria, transcription of all the *rsmZ-Y* sRNAs genes in *A. vinelandii* is GacA-dependent [14, 15]. Interestingly, the location of putative GacA-binding boxes varies among the different *rsmZ*-sRNAs genes, suggesting different modes of regulation. While the GacA-binding box for *rsmZ1* and *rsmZ2* was located at positions ranging from − 175 to − 155 and − 181 to − 163, relative to the transcription start site, the GacA boxes in the *rsmZ3, rsmZ4, rsmZ5, rsmZ6* and *rsmZ7* genes were found at positions − 50 to − 80 bp [27]. However, the conditions promoting transcription of *A. vinelandii rsm*-sRNAs genes are still unknown. In this work, we studied the effect of different types of carbon sources on the expression of the *rsmZ1-7* genes and the possible relationship between the expression of these genes with the production of alginate and ARs.

Materials and Methods

Microbiological Procedures

Bacterial strains and plasmids used in this study are listed in Table 1. The *A. vinelandii* wild-type E strain (also named AEIV) was used in this study [28]. *A. vinelandii* was grown at 30 °C in Burk's nitrogen-free salts medium [29] supplemented with 20 g/L of sucrose (BS), fructose (BFru), glucose (BGlu) or succinate (BSucc). *E. coli* strain DH5-α was grown in Luria–Bertani medium (LB) [30] at 37 °C. Antibiotic concentrations used (in μg/ml) for *A. vinelandii* and *E. coli*, respectively, were as follows: tetracycline (Tc), 40 and 20; kanamycin (Km), 4 and 20; gentamicin (Gm), 1.5 and 10; streptomycin (Sm) 2 and 20; ampicillin (Ap), not used and 100; nalidixic acid (Nal), 10 and 10. *A. vinelandii* transformation was carried out as previously described [31] with some modifications as reported previously [32].

Table 1 Strains and plasmids used in this study

Strains or plasmid	Description	Reference or source
A. vinelandii strains		
E	Mucoid, wild-type strain, also named AEIV	[28]
DJ also named UW 136	Rifampicin-resistant strain derived from the UW	[24]
EZ*1*	E strain derivative carrying a Δ*rsmZ1*::Gm mutation	[14]
EZ2	E strain derivative carrying a Δ*rsmZ2*::Gm mutation	[14]
EZ3	E strain derivative carrying a Δ*rsmZ3*::Gm mutation	This study
EZ4	E strain derivative carrying a Δ*rsmZ4*::Km mutation	This study
EZ5	E strain derivative carrying a Δ*rsmZ5*::Sm mutation	This study
EZ6	E strain derivative carrying a Δ*rsmZ6*::Gm mutation	This study
EZ7	E strain derivative carrying a Δ*rsmZ7*::Gm mutation	This study
EZ1T	E strain derivative, carrying a P*rsmZ1-gusA* transcriptional fusion	This study
EZ2T	E strain derivative, carrying a P*rsmZ2-gusA* transcriptional fusion	This study
EZ3T	E strain derivative, carrying a P*rsmZ3-gusA* transcriptional fusion	This study
EZ4T	E strain derivative, carrying a P*rsmZ4-gusA* transcriptional fusion	This study
EZ5T	E strain derivative, carrying a P*rsmZ5-gusA* transcriptional fusion	This study
EZ6T	E strain derivative, carrying a P*rsmZ6-gusA* transcriptional fusion	This study
EZ7T	E strain derivative, carrying a P*rsmZ7-gusA* transcriptional fusion	This study
E*gyrA*T	E strain derivative, carrying a P*gyrA-gusA* transcriptional fusion	This study
Escherichia coli strains		
DH5α	*supE44 DlacU169 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Invitrogen
Plasmids		
pBSL99	Source of the Gm^r cassette	[34]
pBSL130	Source of the Sm^r cassette	[34]
pBSL99	Source of the Km^r cassette	[34]
pSAHFUSTs-Z1	Source of the *rsmZ1* promoter	[15]
pSAHFUSTs-Z2	Source of the *rsmZ2* promoter	[15]
pSAHFUSTs-Z3	Source of the *rsmZ3* promoter	[15]
pSAHFUSTs-Z4	Source of the *rsmZ4* promoter	[15]
pSAHFUSTs-Z5	Source of the *rsmZ5* promoter	[15]
pSAHFUSTs-Z6	Source of the *rsmZ6* promoter	[15]
pSAHFUSTs-Z7	Source of the *rsmZ7* promoter	[15]
pGEM-T Easy	PCR cloning vector	Promega
pUMATc*gusA*T	Plasmid for construction of *gusA* transcriptional fusions	[35]
pGEM*rsmZ3*	pGEM-T Easy carrying the *rsmZ3* gene cloned by PCR	This study
pGEM*rsmZ4*	pGEM-T Easy carrying the *rsmZ4* gene cloned by PCR	This study
pGEM*rsmZ5*	pGEM-T Easy carrying the *rsmZ5* gene cloned by PCR	This study
pGEM*rsmZ6*	pGEM-T Easy carrying the *rsmZ6* gene cloned by PCR	This study
pGEM*rsmZ7*	pGEM-T Easy carrying the *rsmZ7* gene cloned by PCR	This study
pGEMΔ*rsmZ3*	pGEM*rsmZ3* carrying a deletion of *rsmZ3* gene by inverse PCR	This study
pGEMΔ*rsmZ4*	pGEM*rsmZ4* carrying a deletion of *rsmZ4* gene by inverse PCR	This study
pGEMΔ*rsmZ5*	pGEM*rsmZ5* carrying a deletion of *rsmZ5* gene by inverse PCR	This study
pGEMΔ*rsmZ6*	pGEM*rsmZ6* carrying a deletion of *rsmZ6* gene by inverse PCR	This study
pGEMΔ*rsmZ7*	pGEM*rsmZ7* carrying a deletion of *rsmZ7* gene by inverse PCR	This study
pGEMΔ*rsmZ3*-Gm	pGEM*rsm*ΔZ3 with a Gm cassette instead of *rsmZ3* gene	This study
pGEMΔ*rsmZ4*-Km	pGEM*rsm*ΔZ4 with a Km cassette instead of *rsmZ4* gene	This study
pGEMΔ*rsmZ5*-Sm	pGEM*rsm*ΔZ5 with a Sm cassette instead of *rsmZ5* gene	This study
pGEMΔ*rsmZ6*-Gm	pGEM*rsm*ΔZ6 with a Gm cassette instead of *rsmZ6* gene	This study
pGEMΔ*rsmZ7*-Gm	pGEM*rsm*ΔZ7 with a Gm cassette instead of *rsmZ7* gene	This study
pUMATc*gusA*T-*rsmZ1*	pUMATc*gusA*T with a P*rsmZ1-gusA* transcriptional fusion	This study
pUMATc*gusA*T-*rsmZ2*	pUMATc*gusA*T with a P*rsmZ2-gusA* transcriptional fusion	This study

Table 1 (continued)

Strains or plasmid	Description	Reference or source
pUMATc*gusA*T-*rsmZ3*	pUMATc*gusA*T with a P*rsmZ3-gusA* transcriptional fusion	This study
pUMATc*gusA*T-*rsmZ4*	pUMATc*gusA*T with a P*rsmZ4-gusA* transcriptional fusion	This study
pUMATc*gusA*T-*rsmZ5*	pUMATc*gusA*T with a P*rsmZ5-gusA* transcriptional fusion	This study
pUMATc*gusA*T-*rsmZ6*	pUMATc*gusA*T with a P*rsmZ6-gusA* transcriptional fusion	This study
pUMATc*gusA*T-*rsmZ7*	pUMATc*gusA*T with a P*rsmZ7-gusA* transcriptional fusion	This study
pUMATc*gusA*T-*gyrA*	pUMATc*gusA*T with a P*gyrA-gusA* transcriptional fusion	This study

Nucleic Acid Procedures

DNA isolation and cloning procedures were carried out as described previously [33]. The *A. vinelandii* DJ genome sequence was used to design the oligonucleotides used for PCR amplifications. The DreamTaq polymerase (Thermo Fisher Scientific) and Vent DNA polymerase (NEB) were used for PCR amplifications.

Cloning the *A. vinelandii rsmZ3, rsmZ4, rsmZ5, rsmZ6* and *rsmZ7* Genes

Fragments of approximately 2 kb containing the *rsmZ3, rsmZ4, rsmZ5, rsmZ6* and *rsmZ7 loci* were amplified by PCR using the primers specified in Table S1; chromosomal DNA from the wild-type mucoid strain E was used as DNA template. The amplified fragments were individually cloned into pGEM-T Easy (Promega), which generated pGEM*rsmZ3*, pGEM*rsmZ4*, pGEM*rsmZ5*, pGEM*rsmZ6* and pGEM*rsmZ7* plasmids. These plasmids were used to determine the nucleotide sequence of the *rsmZ3, rsmZ4, rsmZ5, rsmZ6* and *rsmZ7 loci* of the *A. vinelandii* E strain.

Generation of *rsmZ3, rsmZ4, rsmZ5, rsmZ6* and *rsmZ7* Mutants Derived from the E Strain

A schematic representation of the procedure to generate deletion mutants in each of the *rsmZ3–7* genes is depicted in Fig. S1. Individual *rsmZ*-sRNA deletions were generated by inverse PCR using the primers specified in Table S1. These primers contain either *Xho*I or *Kpn*I sites used in subsequent steps to insert a selection marker (an antibiotic-resistance cassette). Inverse PCRs were carried out using pGEM*rsmZ3*, pGEM*rsmZ4*, pGEM*rsmZ5*, pGEM*rsmZ6* or pGEM*rsmZ7* plasmids as DNA template. The resulting PCR fragments were digested with *Xho*I or *Kpn*I endonucleases and were re-ligated generating pGEMΔ*rsmZ3*, pGEMΔ*rsmZ4*, pGEMΔ*rsmZ5*, pGEMΔ*rsmZ6* and pGEMΔ*rsmZ7* plasmids, respectively. These plasmids were linearized with *Kpn*I (pGEMΔ*rsmZ4*, pGEMΔ*rsmZ5* and pGEMΔ*rsmZ6*) or *Xho*I (pGEMΔ*rsmZ3* and pGEMΔ*rsmZ7*) and were ligated to Gm-, Km- or Sm-resistance cassettes [34] excised with the corresponding endonuclease. The generated plasmids were named pGEMΔ*rsmZ3*-Gm, pGEMΔ*rsmZ4*-Km, pGEMΔ*rsmZ5*-Sm, pGEMΔ*rsmZ6*-Gm and pGEMΔ*rsmZ7*-Gm (Table 1). These plasmids, unable to replicate in *A. vinelandii*, were linearized by excision with *Sca*I restriction enzyme to ensure double homologous recombination event and allelic exchange. Afterwards, they were introduced into E strain by transformation. Double recombinants were selected on plates of BS medium amended with the corresponding antibiotic. The presence of the desired Δ*rsmZ* mutation and the absence of the corresponding wild-type *rsmZ* allele were verified by PCR (data not shown). The E derivative mutants carrying individual deletions of *rsmZ3–7* genes were named EZ3 (Δ*rsmZ3*::Gm), EZ4 (Δ*rsmZ4*::Km), EZ5 (Δ*rsmZ5*::Sm), EZ6 (Δ*rsmZ6*::Gm) and EZ7 (Δ*rsmZ7*::Gm) (Table 1).

Construction of P*rsmZ-gusA* Transcriptional Fusions

Fragments containing promoter regions from *rsmZ1, rsmZ2, rsmZ4, rsmZ5, rsmZ6, rsmZ7* (P*rsmZ*) were obtained from pSAHFUSTs-Z1–7 plasmids [15], as *EcoR*1 fragments, except for in *rsmZ3*, which was obtained by excision with *Xba*I. P*rsmZ1–7* were individually cloned into the integrative vector pUMATc*gus*AT [35], generating P*rsmZ-gusA* transcriptional fusions (Tc^r) for each gene. This vector directs the integration of the *gusA* fusion into the neutral *melA locus* [35]. The resulting plasmids (named pUMATc*gusA*T-*rsmZ1* to -*rsmZ7*) were linearized with *Sca*I endonuclease and introduced by transformation into E strains. Double recombinants Tc^r carrying the individual P*rsmZ-gusA* fusion integrated into the chromosome were isolated and confirmed by PCR analysis. The resulting strains were named EZ1T (P*rsmZ1-gusA*), EZ2T (P*rsmZ2-gusA*), EZ3T (Pr*smZ3-gusA*), EZ4T (P*rsmZ4-gusA*), EZ5T (P*rsmZ5-gusA*), EZ6T (Pr*smZ6-gusA*), EZ7T (P*rsmZ7-gusA*) (Table 1).

Construction of *gyrA-gusA* Transcriptional Fusion

A fragment of 130 pb containing the regulatory region of the *A. vinelandii gyrA* gene (P*gyrA*) (Avin_RS07245) was PCR amplified using primers DgyrXbaI and RgyrEcoRI, which

carry *Xba*I and *Eco*RI artificial sites, respectively. The P*gyrA* was cloned into the pUMATc*gusA*T vector as an *Eco*RI-*Xba*I fragment, generating a P*gyrA-gusA* transcriptional fusion. This plasmid was made linear with *Sca*I endonuclease and was transformed into the E strain. Double recombinants Tc^r were isolated and confirmed by PCR to carry the P*gyrA-gusA* transcriptional fusion into the chromosome. One representative transformant was chosen for further studies and was named E*gyrA*T (Table 1).

Analytical Methods

Alginate production was determined using spectrophotometric determination of the uronic acids with carbazole [36]. The synthesis of ARs was measure as reported previously [37]. β-glucuronidase activity was measured as reported by Wilson et al. [38]; 1U corresponds to 1 nmol of p-nitrophenyl-β-D-glucuronide hydrolyzed per min per mg of protein. Protein was determined by the Lowry method [39].

Results and Discussion

Transcriptional Profile of the *rsmZ1-7 sRNAs* Genes

In *Pseudomonadaceae*, it is common to find two or more Rsm-sRNAs of the RsmZ, RsmY or RsmX family. However, the presence in a bacterium of reiterated alleles of the same family is uncommon. The presence of multiple alleles of *rsmX* has been reported in *P. syringae* [10]. *A. vinelandii* is the only bacterium having multiple alleles of the *rsmZ* family; however, the physiological significance of such reiteration has not been explored. *rsmZ1–7* genes are located in different regions of the chromosome; *in silico* analysis suggests that they are contained in monocistronic operons. The genetic arrangement of the *rsmZ1–7 loci* is shown in Figure S2.

In contrast to the DJ strain, the E strain (also named AEIV) is a wild-type isolate of *A. vinelandii* [24, 28]. In order to study the transcriptional regulation of the *A. vinelandii rsmZ1–7* genes, we constructed a series of P*rsmZ1–7-gusA* transcriptional fusions, as described in Materials and Methods, and they were tested in the genetic background of the wild-type E strain. For this purpose, DNA fragments of approximately 2 kb containing the regulatory and structural region of each one of the *rsmZ1–7* genes from the wild-type mucoid strain E were amplified and sequenced. The DNA sequence of PCR products showed from 98 to 100% identity with the corresponding chromosomal region of the DJ strain already sequenced [24]. For this reason, we used the *rsmZ1–7*-sRNAs regulatory regions derived from the DJ strain [15] to generate the P*rsmZ-gusA* transcriptional fusions to be tested in the E strain.

Strains carrying the P*rsmZ1–7-gusA* transcriptional fusions were cultivated in Burk's Sucrose medium (BS) and showed similar growth kinetics (Fig. 1A), which allowed the comparative analysis among the different P*rsmZ-gusA* fusions. The transcription of every *rsmZ*-sRNA gene, indirectly estimated by the activities of the P*rsmZ1–7-gusA* fusion, showed a similar temporal pattern (Fig. 1b,c), reaching its maximum expression at the stationary growth phase (36 h). A similar fact has been reported in *P. fluorescens* and *P. aeruginosa*, in which *rsmZ* expression was delayed showing its greatest point at the stationary phase [3, 4]. Interestingly, in *Pseudomonas* spp. the expression of *rsmY* and *rsmX* correlates with the cell growth [4, 12].

Although all the *rsmZ* alleles showed a similar transcriptional pattern throughout the growth curve, the activity of the different *rsmZ* promoters was very variable. Those of the *rsmZ1, rsmZ3* and *rsmZ5* showed lower expression levels (Fig. 1B) when compared to the activity derived from *rsmZ2*, *rsmZ4, rsmZ6* and *rsmZ7* promoters (Fig. 1C). *rsmZ1* showed the lowest expression pattern while *rsmZ2* presented the highest expression throughout the growth curve.

Differences in the expression levels of the *rsmZ* alleles may reflect differences in their regulatory regions, including the location of the GacA-binding box. The regulatory regions of *rsmZ* alleles could be grouped in two sets: those with large regulatory regions (*rsmZ1* and *rsmZ2*) and those with short regulatory regions (*rsmZ3–7*). Although the regulatory regions from *rsmZ1* and *rsmZ2* have similar lengths, their sequences are not conserved which may explain the observed differences in their expression (Fig. S3). In both cases, however, the sequences in between the GacA-binding box and the transcription start site have an unusual high AT content (*A. vinelandii* has a rich GC genome) suggesting the binding of regulators with affinity for AT-rich sequences, such as H–NS and IHF. Indeed, using the bioinformatics tools, Virtual Footprint-Prodoric (http://prodoric.tu-bs.de/vfp/vfp_promoter.php) and Softberry-BPROM (http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb), we found putative IHF and H–NS binding sites in the regulatory region of *rsmZ2* and *rsmZ1*, respectively (Fig. S3). Similar regulatory regions have been reported for the homologous *rsmZ* genes from *Pseudomonas* spp [13, 18]. In *P. fluorescens*, IHF binds to the regulatory region of *rsmZ* with high affinity [18], while in *P. aeruginosa*, H–NS binds to an AT-rich sequence in the *rsmZ* regulatory region repressing its expression [13]. Another factor that may influence the differential expression is the conservation degree of the palindromic GacA-binding box (**TGTAAG**NN**AT**NN**CTTACA**) [27]. As shown in Figure S3, except for *rsmZ1*, the regulatory regions of all *rsmZ* genes showed conserved GacA boxes. Since *rsmZ1* showed the lowest activity, it suggests that the conservation of the GacA box determines the transcriptional level of the individual

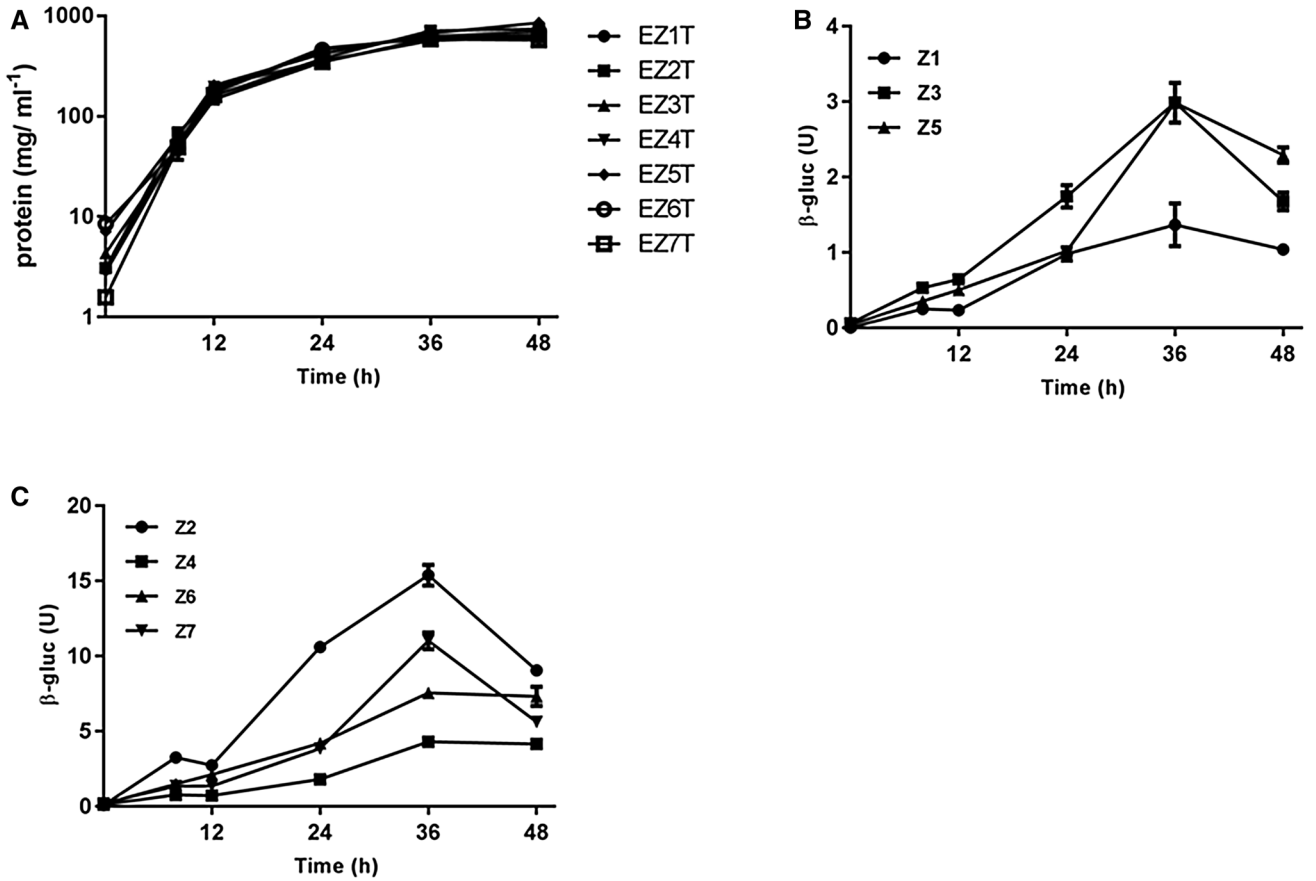

Fig. 1 Expression profiles of *rsmZ1–7* sRNAs genes in *A. vinelandii*. (**a**). Growth kinetics of the strains carrying the individual *rsmZ1–7-gusA* transcriptional fusions (EZ1T–EZ7T). Promoter activity of the *rsmZ1* (Z1), *rsmZ3* (Z3) and *rsmZ5* (Z5) (**b**) or *rsmZ2* (Z2), *rsmZ4* (Z4), *rsmZ6* (Z6) and *rsmZ7* (Z7) (**c**) genes, measured along the growth curve, using the *rsmZ1–7-gusA* transcriptional fusions. Cells were grown in Burk's sucrose medium. Bars of standard deviation from three independent experiments (biological replicates) are shown

rsmZ genes. However, as the expression of the *rsmZ2–7* transcriptional fusions was highly variable, it also implies that GacA is necessary but not sufficient to establish a suitable expression of the *rsmZ1–7*-sRNAs genes.

The Type of the Carbon Source Influences the Transcription of the *rsmZ*-sRNAs

In *E. coli*, the Csr system controls carbon metabolism, and different types of carbon sources (glycolytic or gluconeogenic) modify the expression of *csrB* and *csrC* [40]. In *P. fluorescens*, the presence in the culture medium of Krebs cycle intermediaries, such as succinate or malate, promotes the *rsm*-sRNAs expression [22]. Therefore, we explored the effect of different types of carbon sources on the *A. vinelandii rsmZ*-sRNAs expression. For this purpose, the strains carrying *rsmZ1–7-gusA* transcriptional fusions were grown in Burk's minimal medium amended with glucose (BGluc) or fructose (BFru), as glycolytic carbon sources, or with succinate (BSucc), as a gluconeogenic carbon source (Fig. 2).

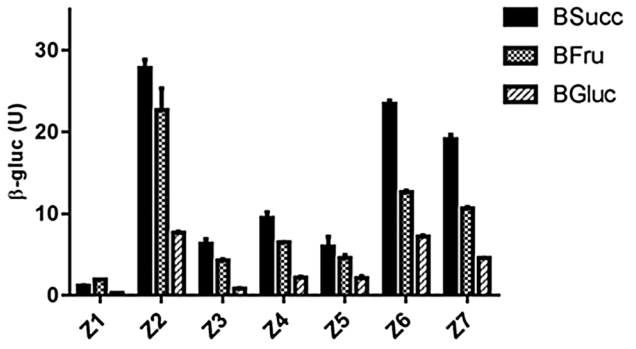

Fig. 2 Effect of glycolytic or gluconeogenic carbon sources on the *rsmZ1–7* sRNAs expression. Promoter activity of the *rsmZ1–7* genes was assessed using the transcriptional fusions of Fig. 1. Cells were grown in, Burk's minimal medium amended with fructose (BFru), glucose (BGlu) or succinate (BSucc). Cells were grown to the stationary phase (48 h). Bars of standard deviation from three independent experiments (biological replicates) are shown

Interestingly, the expression of all the *rsmZ* genes was higher in the presence of succinate than in the glycolytic condition. Regardless of the tested carbon source, *rsmZ2, rsmZ6* and *rsmZ7* showed the highest expression levels while *rsmZ1* presented the lowest expression (Fig. 2), a pattern previously observed in the presence of sucrose (Fig. 1b, c). In order to rule out a possible effect of the different carbon sources on the expression of the *gusA* reporter gene, a transcriptional P*gyrA-gusA* fusion was constructed as described in Materials and Methods. In previous reports, the expression of *gyrA* in *A. vinelandii* has been shown to be constitutive under different growing conditions [15, 41]. As expected, the expression of the P*gyrA-gusA* transcriptional fusion was constitutive and was not affected by the type of carbon source (Fig. S4). This result clearly indicated that the activity of the P*rsmZ1–7-gusA* constructions reflects the activity of the fused promoters.

The *rsmZ*-sRNA expression levels were different when the cells were cultivated in fructose or glucose, even though these glycolytic substrates could produce similar amounts of Krebs cycle metabolites. This result suggests the existence of an additional signal responsible for the increased *rsmZ1–7* expression levels in the presence of fructose (Fig. 2). In *A. vinelandii*, there are clear differences in the uptake and metabolism of these hexoses; glucose uptake is carried out by the GluP transporter and is oxidized through the Entner–Doudoroff pathway [42, 43]. In the case of fructose, the presence of *fruA* (Avin_RS05545), *fruB* (Avin_RS05535) and *fruK* (Avin_RS05530) genes in the genome of several strains of *A. vinelandii* suggests that, as in *Pseudomonas putida*, fructose is assimilated through the PTS^{fru} system that, upon conversion to fructose 1–6 bisphosphate, following the Embden–Meyerhof glycolytic pathway [44]. Hence, the differences in the RsmZ-sRNAs expression between fructose and glucose could be attributed to differences in the assimilation and metabolism of these sugars. Besides controlling sugar assimilation, the PTS^{Fru} system is involved in other regulatory processes [45, 46]. In *P. putida*, the PTS^{Fru} system is related to the PTS^{Ntr} (nitrogen-related PTS), a non-canonical PTS system reported in several *Pseudomonadaceae* that lack the subunit needed for carbohydrate transport, so this PTS system is not involved in carbohydrate assimilation. Early studies suggested that the PTS^{Ntr} system had a role in nitrogen metabolism; however, recent data indicated that it has several regulatory functions [35, 47–49]. In *P. putida* the two PTS branches are connected establishing a cross-talk process. *A. vinelandii* has a PTS^{Ntr} homologue system that controls the production of secondary metabolites such as PHB and ARs [50]. Therefore, fructose is likely to trigger a regulatory pathway that affects the *rsmZ*-sRNAs expression. On the other hand, fructose assimilation by the PTS^{Fru} generates fructose 1-P, which is the metabolic negative effector of FruR/Cra regulator. In *E. coli*, FruR/Cra controls a large number of genes related to carbon metabolism [51], a similar fact might occur in *P. putida* [52]. The *A. vinelandii* genome has a putative FruR/Cra homologue, which could be involved in the regulation of *rsmZ*-sRNAs genes in response to the metabolic state of the cell. To support this idea, a regulatory link between FruR and CsrA has been recently reported [53].

Mutations in *rsmZ*-sRNAs Genes Differentially Affect Alginate Production

The importance of the sRNAs RsmZ1 and RsmZ2 in the regulation of alginate production in *A. vinelandii* has been previously established [14]. In the absence of RsmZ1 or RsmZ2, alginate production diminished 70%. Therefore, we investigated the role of the RsmZ-sRNAs in alginate production in cells grown in the gluconeogenic and glycolytic carbon sources previously used. To do this, we used the individual *rsmZ1* and *rsmZ2* mutants previously constructed [14], and generated independent deletions of the sRNAs *rsmZ3, rsmZ4, rsmZ5, rsmZ6* and *rsmZ7* genes, as described in Materials and Methods. These mutants were constructed by deleting the *rsmZ3–7* genes and inserting an antibiotic-resistance cassette. Given the monocistronic nature of the predicted operons containing the *rsmZ1–7* genes (Fig. S2), it is unlikely that this strategy generates polar effects on downstream genes.

Mutants carrying individual deletions of the *rsmZ1–7* genes were grown in Burk's minimum medium in the presence of the different types of carbon sources and the production of alginate was determined (Fig. 3). Absence of any of these sRNAs did not abrogate alginate production. In glucose-grown cells, the sRNAs RsmZ1–5 were necessary for maximum alginate production. Interestingly, in cells grown in fructose the individual positive effect of these sRNAs was more accentuated than in glucose-grown cells (Fig. 3a,b), which might be associated with their observed enhanced expression in fructose (Fig. 2). The lack of RsmZ1 and RsmZ4 almost completely inhibited alginate production, whereas the absence of the remaining sRNAs reduced the levels of this polymer from 3 to 5 times.

The effect of the *rsmZ*-sRNAs mutations in alginate production was also tested in succinate as carbon source (Fig. 3c). Under this condition, the lack of RsmZ2 totally impaired alginate synthesis, which correlated with its high expression level observed in this carbon source (Fig. 2). Absence of RsmZ1 and RsmZ4 diminished the production of this polymer by 70 and 80%, respectively, which also implies an important role of these two sRNAs under this condition. Finally, the absence of RsmZ3, Z5, Z6 or Z7 only reduced the synthesis of alginate about 25% suggesting a minor role of these sRNAs in succinate.

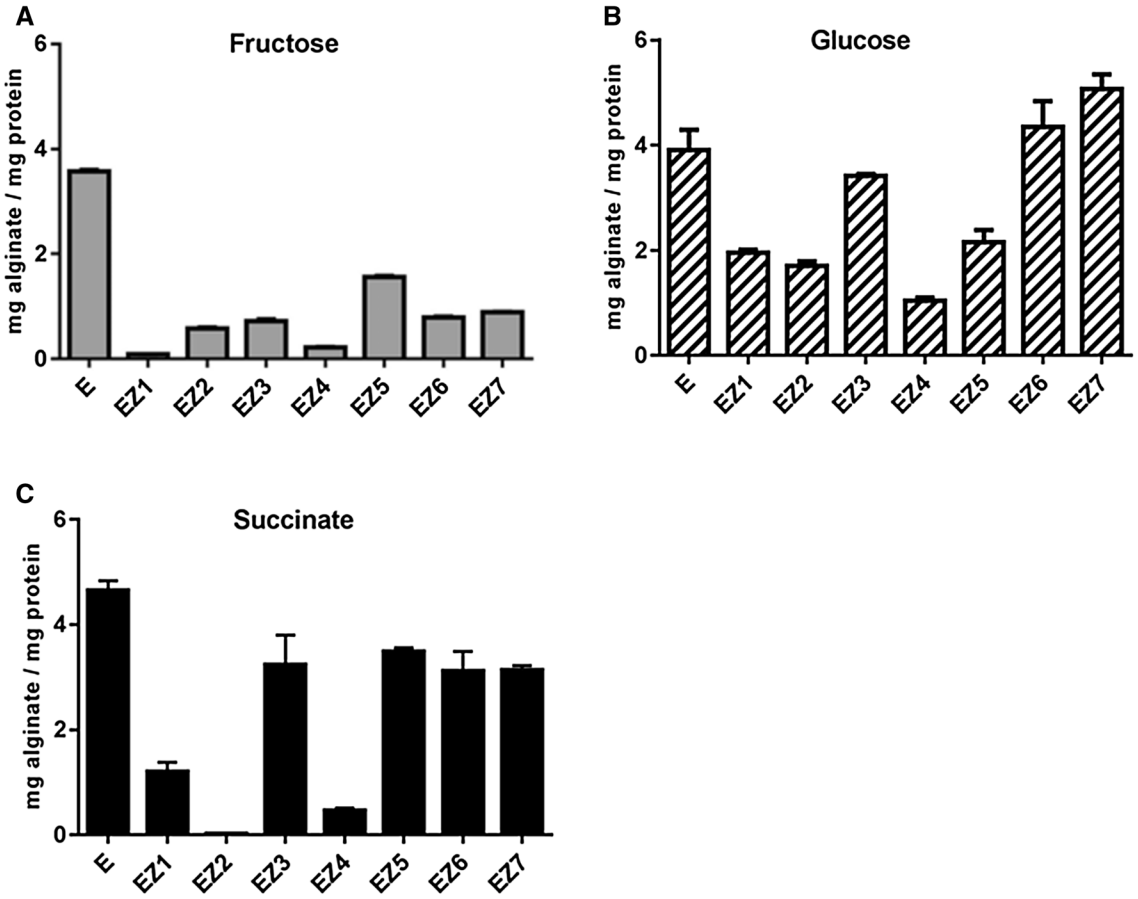

Fig. 3 Alginate production in *rsmZ1-7* sRNAs mutants. The production of alginate was determined in cells of the wild-type strain E and in its derivatives carrying individual deletions of the *rsmZ1-7* sRNAs (EZ1–EZ7). Cells were grown for 48 h (stationary phase) in Burk's minimal media with fructose (**a**), glucose (**b**) or succinate (**c**) as the sole carbon source. Bars of standard deviation from three independent experiments (biological replicates) are shown

Our results indicated that only RsmZ1, RsmZ2 and RsmZ4 had a consistent positive effect on alginate production in the presence of the three carbon sources tested (Fig. 3). However, it was not related to the extent of expression of these sRNAs. While *rsmZ2* showed the highest expression levels, the expression of *rsmZ1* was low in either glycolytic or gluconeogenic carbon sources (Fig. 2). Furthermore, expression of *rsmZ6* and *rsmZ7* was always higher than that of *rsmZ1* but their effects on alginate synthesis were marked only in the presence of fructose (Fig. 3a), but moderated in succinate (Fig. 3c). Collectively, our data indicated the absence of a direct correlation between alginate production and the expression of the *rsmZ*-sRNAs. It suggests the existence of additional factors involved in the regulatory functions of the RsmZ-sRNAs. It is possible that the sRNAs have different affinities for the RsmA protein. In *E. coli*, the RsmZ functional homologues, CsrB and CrsC have important differences in their affinities for CsrA [16]. Stability of each *rsmZ*-sRNA is another factor to consider for understanding their functional differences. Additionally, the predicted ΔG values of the different *A. vinelandii rsmZ*-sRNAs rank from − 50.36 to 68.04 kcal / mol [27]. Enzymatic degradation is another factor that affects the sRNAs turnover. The RNase E cleavage sites present in the RsmZ-sRNAs from *Pseudomonas* spp. are conserved in the RsmZ1–7-sRNAs of *A. vinelandii*, suggesting that their accumulation is influenced by this process (Fig. S3). In *P. fluorescens*, the interaction of the Rsm-sRNA with the RsmA protein prevents its enzymatic degradation [54]. Similarly, in *P. aeruginosa*, the interaction of Hfq with RsmY, but not with RsmZ or RsmX, protects against RNase E [55]. The existence of these mechanisms controlling the turnover of the RsmZ-sRNA in *A. vinelandii* deserves further investigation.

Effect of the RsmZ1–7 sRNAs on the Production Of Alkylresorcinols

In *A. vinelandii*, ARs are other metabolites controlled by the Gac–Rsm system; these aromatic lipids are produced

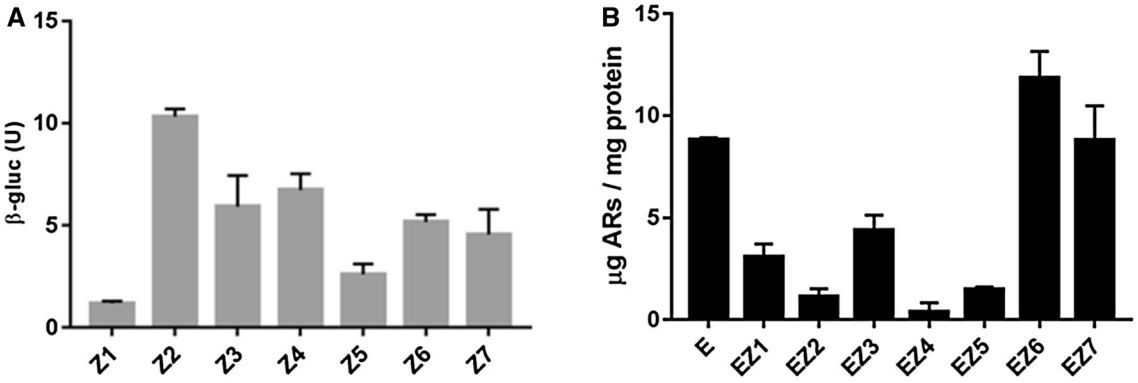

Fig. 4 Expression of the *rsmZ1-7* sRNAs genes in the presence of *n*-butanol and their effect on alkylresorcinols production. **a** The promoter activity of *rsmZ1-7*genes was assessed using the transcriptional fusions of Fig. 1. Cells were grown for 5 days in Burk's minimal media amended with *n*-butanol as the sole carbon source. **b** The effect of each of the RsmZ-sRNAs on alkylresorcinols (ARs) accumulation was determined after 5 days of growth in liquid Burk's-butanol media. Bars of standard deviation from three independent experiments (biological replicates) are shown

during the differentiation process leading to the formation of cysts resistant to desiccation. Cyst biogenesis could be induced in laboratory conditions with *n*-butanol as the sole carbon source. In strain SW, an *rsmZ1* mutant showed a strong reduction in the transcription of *arsA*, a biosynthetic ARs gene, suppressing the production of these lipids. The strain SW is a derivative of the non-mucoid strain DJ, which carries a natural IS insertion within *algU*, encoding a stress sigma factor essential for alginate production [24, 56]. The strain SW was generated by genetic complementation of the DJ strain with a wild-type copy of *algU* integrated into its chromosome [57].

Since *n*-butanol triggered the accumulation of ARs in an RsmZ1-dependent manner, the possible link between this carbon source and the expression of the RsmZ1–7 sRNAs was evaluated in the wild-type strain E. For this reason, expression levels of each sRNA were determined in cells grown in liquid Burk's medium with *n*-butanol as the sole carbon source, for 5 days (Fig. 4a). As in other carbon sources, the expression of the RsmZ-sRNAs varied greatly, *rsmZ2* showed the maximum expression and *rsmZ1* and *rsmZ5* showed the lowest expression levels. The transcriptional levels of *rsmZ3, rsmZ4, rsmZ6* and *rsmZ7* were somewhat similar.

Later, the effect of each sRNA in ARs production was determined. In contrast to the pronounced effect shown by RsmZ1 on ARs production in the SW strain, absence of RsmZ1 in the E strain caused a reduction of 70%. Interestingly, absence of RsmZ2, RsmZ4 or RsmZ5 resulted in a marked reduction of about 83 to 95% in ARs accumulation. It is worth noting that although *rsmZ6* and *rsmZ7* expressions were higher than that of *rsmZ1*, absence of RsmZ6 or RsmZ7 did not reduce the synthesis of ARs and a significant increase was observed in mutant *rsmZ6* (Fig. 4b).

Altogether, our results indicated that the expression levels of the *rsmZ1-7*-sRNAs in ARs accumulating conditions did not correlate with their individual effect on the synthesis of these lipids.

Concluding Remarks

A. vinelandii has the uncommon characteristic of harbouring large numbers of highly similar carbohydrate metabolism homologues, which are proposed to confer adaptive benefits with respect to certain environmental factors and life styles [58]. The presence of multiple sRNAs of the RsmZ family in *A. vinelandii*, might be related to this trait, which may provide a versatile adaptability through a highly flexible Rsm system.

We found no correlation between the extent of the transcription of the individual RsmZ-sRNA and its corresponding effect on the production of either alginate or ARs. Our data revealed that additional factors, besides to the transcriptional control, influence the different regulatory roles of the RsmZ-sRNAs. These factors could respond to diverse stimuli, signals or conditions generating a better and versatile response of the Rsm system.

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