Chapter 4
Azotobacter vinelandii Small RNAs: Their Roles in the Formation of Cysts and Other Processes

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Abstract *Azotobacter vinelandii* is a soil bacterium that undergoes differentiation to form cysts resistant to desiccation. Alginate, polyhydroxybutyrate (PHB) and alkylresorcinols (AR) are structural components of the cysts. The synthesis of these compounds has been shown to be under control of the Gac/Rsm signal transduction pathway. This pathway includes eight small non-coding RNAs (RsmZ1-7 and RsmY), and the translational repressor protein RsmA. Binding of RsmA to Rsm-RNAs inhibits its repressor activity. Here, we review and discuss the roles of sRNAs in the formation of cysts and other processes in *A. vinelandii*. We also report a search for genes encoding small RNAs in the *A. vinelandii* genome. Two new sRNAs potentially related to the control of cyst components synthesis were identified in this search.

4.1 Introduction

Azotobacter vinelandii is a soil nitrogen fixing soil bacterium related to *Pseudomonas* species to the extent, that it has been proposed to belong to this genus (Ozen and Ussery 2012; Rediers et al. 2004). Additionally 50 % of *A. vinelandii* genes encode proteins that show a similarity of 67 % or higher with a *Pseudomonas* homologues (Gonzalez-Casanova et al. 2014). In spite of close relationship, *A. vinelandii* shows important phenotypic features that are not observed in most *Pseudomonas* species, including the ability to fix nitrogen under aerobic conditions,

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a high respiration rate, and to undergo a differentiation process to form desiccation resistant cysts. *A. vinelandii* produce the exo-polysaccharide alginate, the intracellular polyester polyhydroxybutyrate (PHB), and the phenolic lipids alkylresorcinols (AR) and alkylpyrones (AP). Alginate, PHB, AR, and AP are structural components of cysts. Mature cysts consist of a cell known as central body, covered by a capsule composed of two layers named the intine and exine. Numerous PHB granules are always observed within the central body, alginate is a major component of the capsule, and AR and AP that are synthesized upon encystment induction replace the phospholipids of the cyst membranes and are components of the exine (Segura et al. 2014).

The expression of genes involved in the synthesis of cyst components mentioned above has been shown to be under the control of small non-coding RNAs (sRNAs) (Hernandez-Eligio et al. 2012; Manzo et al. 2011; Muriel-Millan et al. 2014; Romero et al. 2016). sRNAs are present in diverse organisms (i.e. bacteria, archaea and eukaryotes). In prokaryotes sRNAs have been linked to specialized housekeeping functions, to several stress situations, to pathogenicity and in catabolic carbon repression. The roles played by most sRNAs in the regulation of bacterial gene expression are exerted at a post-transcriptional level, where they activate or repress translation, or by stabilizing or destabilizing mRNAs (Waters and Storz 2009).

Four classical sRNAs, named Ffs, SsrA, RnpB SsrS play essential housekeeping roles and the genes encoding these RNAs have been identified in most bacterial genomes (Sonnleitner and Haas 2011). Their presence in *A. vinelandii* has not been reported.

Most sRNAs regulate gene expression at the level of translation mainly by two general mechanisms: The most common mechanism involves short, imperfect base-pairing interactions with the 5' end of mRNAs (Brantl 2002; Storz et al. 2011). The sRNA-mRNA base pairing mechanism requires a chaperon protein called Hfq. One example is the *Escherichia coli* RyhB RNA and *Pseudomonas* PrrF1-PrrF2 sRNAs (Masse and Gottesman 2002; Wilderman et al. 2004), These RNAs are involved in iron homeostasis, repressing the expression of some genes encoding nonessential proteins that use iron (Salvail and Masse 2012). RyhB regulates its targets by the formation of sRNA-mRNA complexes stabilized by the chaperone Hfq. A RhyB/PrrF homolog, known as ArrF (**A**zotobacter **r**egulatory RNA involving Fe) is present in the *A. vinelandii* genome, and is involved in iron homeostasis and also in the control of PHB synthesis (Jung and Kwon 2008; Muriel-Millan et al. 2014). Other sRNA that interacts with Hfq is RgsA (Park et al. 2013). Recently a homolog of RgsA has been characterized in *A. vinelandii*, in this bacterium RgsA is an important factor to resist oxidative stress, and plays a role in biofilm formation (Huerta et al. 2016).

The other regulatory mechanism involves binding of the sRNAs to a small protein that acts as translational repressor. One example involves the regulatory sRNAs named CsrB and CsrC in *E. coli* or RsmZ, RsmY and RsmX in *Pseudomonas* species. These sRNAs bind to the CsrA and RsmA proteins respectively. These proteins repress translation by binding their target mRNAs. The

proteins encoded in mRNAs that are targets the CsrA/RsmA, are involved in numerous and diverse functions (Lapouge et al. 2008). Seven *rsmZ* genes and one *rsmY* as well as the gene encoding the RsmA protein are present in the *A. vinelandii* genome. Although two RsmY RNAs: named RsmY1 and RsmY2 were first reported (Manzo et al. 2011; Setubal et al. 2009), they were later shown to constitute a single gene (Hernandez-Eligio et al. 2012). In *A. vinelandii* the Rsm-RNAs are involved in regulating the synthesis of the cyst components alginate, PHB and AR (Hernandez-Eligio et al. 2012; Manzo et al. 2011; Romero et al. 2016).

Other example of a regulatory mechanism involving sRNAs and a translation repressor protein has been reported in *Pseudomonas* species, where the sRNA named CrcZ is involved in carbon catabolic repression. Here the small protein Crc binds the CrcZ RNAs, and also to the 5' leader of mRNAs having the sequence GGG to repress their translation. Hfq is required for the binding of the small Crc protein to its target mRNAs or to CrcZ sRNA (for a Review see (Sonnleitner and Haas 2011)). Two copies of CrcZ are present in the *A. vinelandii* genome (Filiatrault et al. 2013).

Here, we present a review and a discussion of the mechanisms by which sRNAs control the synthesis of cyst components and report the results of a search for sRNAs encoded in the *A. vinelandii* genome.

4.2 Search of Genes Encoding sRNAs in the *A. vinelandii* Genome

In order to identify genes for sRNAs in the *A. vinelandii* DJ genome, we generated a database file of its intergenic regions. The intergenic sequences were analyzed with the INFERNAL algorithm using the Rfam database as comparison pattern (http://rfam.xfam.org/), using the tool "batch sequence search" of this server. The query intergenic sequences were obtained from the *A. vinelandii* DJ genome sequence (http://img.jgi.doe.gov/). The analysis identified 30 potential sRNAs. This search also revealed the presence of 10 riboswitches or *in cis* RNA element, 15 self-catalytic ribozymes, a CRISPR element, and 3 RNA thermometers (ROSE) element 3 (data not shown). The 30 sRNAs, their Rfam identifiers, and their position in the chromosome are listed in Table 4.1, and include the four sRNAs ubiquitous in bacteria: SsrS, SsrA, Ffs and RnpB (Sonnleitner and Haas 2011). SsrS or 6S is an RNA that specifically associates with RNA polymerase holoenzyme containing the sigma70 factor. This interaction represses expression from sigma70-dependent promoters during stationary phase (Wassarman 2007); SsrA, also known as 10Sa and tmRNA (transfer-messenger RNA), participates in a mechanism known as trans-translation. The tmRNA forms a ribonucleoprotein complex (tmRNP) together with Small Protein B (SmpB), Elongation Factor Tu (EF-Tu), and ribosomal protein S1. The tmRNA and its associated proteins bind to bacterial ribosomes, which have stalled in the middle of protein biosynthesis; it

recycles the stalled ribosome, adds a proteolysis-inducing tag to the unfinished polypeptide, and facilitates the degradation of the aberrant messenger RNA (Keiler 2015); Ffs is a 4.5S RNA which is a component of a signal recognition particle, that promotes the insertion of membrane proteins into the cytoplasmic membranes (Ulbrandt et al. 1997); RnpB is the 10Sb RNA subunit of the catalytic ribonuclease RNase P that is present in almost all living cells. Transfer RNA (tRNAs) genes are transcribed as precursors with extra residues at their 5' and 3' ends that have to be removed to generate functional tRNAs. In bacteria the RNAse P is responsible for removing the extra 5' residues (for a review see Kirsebom 2007). Due to its essential nature, it is expected that the functions of these RNAs in *A. vinelandii* is similar to those described above.

As expected, the previously identified and characterized RsmZ1-7, RsmY (Hernandez-Eligio et al. 2012; Manzo et al. 2011), the ArrF (Jung and Kwon 2008), the RgsA (Huerta et al. 2016), and the two CrcZ sRNAs were found in this search and are listed in Table 4.1. Interestingly a new copy of *rsmZ*, here named *rsmZ8* was also identified, raising to nine the number of Rsm-RNAs present in *A. vinelandii*.

Homologs to the *P. aeruginosa* P11, P14, P15, P20 (PhrS), P26, P27, P31 sRNAs, that were predicted by the bioinformatic program SRNApredict2, and confirmed by Northern blot analysis in this bacterium (Livny et al. 2006), were identified in the *A. vinelandii* genome Table 4.1. P20 (PhrS) promotes the expression of *pqsR*, an important regulator to establish the quorum sensing mediated by quinones in *P. aeruginosa* (Sonnleitner and Haas 2011). As *A. vinelandii* does not possess quorum-sensing systems related with quinones, PhrS must have a different function in this bacterium. The functions of P11, P14, P15, P26, P27, P31 are unknown.

An sRNA homologue to Spot 42 sRNA of *E. coli* (Beisel and Storz 2011) is present in *P. aeruginosa* where it was named Pseudomon 1. Recently it was renamed as ErsA and was shown to regulate the expression of the alginate, biosynthetic gene *algC* at a posttranscriptional level (Ferrara et al. 2015).

IsrK is present in *Salmonella typhimurium*, the gene encoding this sRNA is located within a virulence island and is a host-induced gene (Padalon-Brauch et al. 2008).

Finally, the other sRNAs found in *A. vinelandii* are; a homologue of t44 of unknown function, found in enteric bacteria and in *Pseudomonas* (Tjaden et al. 2002), and two C4 RNAs. C4 antisense molecules are related with the presence of P1 and P7 temperate phages in bacteria (Citron and Schuster 1990).

Several of the sRNAs listed in the Table 1 such as IsrK, RgsA, Pseudomon-1 (EsrA), CsrZ and PhrS have been shown to interact and mediate its function with the chaperone Hfq (Ferrara et al. 2015; Park et al. 2013; Sonnleitner and Blasi 2014; Sonnleitner and Haas 2011; Zhang et al. 2003). Therefore, in *A. vinelandii* Hfq the predicted product of gene Avin7540 could mediate the activity of the same RNAs.

4.3 RsmZ and RsmY SRNAs Involved in Synthesis of Cyst Components and Cyst Resistance to Desiccation

In many γ-proteobacteria the conserved two-component GacS-GacA system (named BarA-UvrY in *E. coli*) controls the expression of one or more genes encoding small RNAs. These RNAs bind small proteins named RsmA or CsrA that act as translational repressors. Thus the GacS-GacA system controls the post-transcriptional regulatory system that involves the translation repressor protein RsmA (CsrA) and the sRNAs RsmZ, RsmY (CsrB) (Lapouge et al. 2008). As in other γ-proteobacteria in *A. vinelandii* GacA the protein acting as a transcriptional activator is required for the transcription of the genes encoding the eight Rsm-sRNAs (RsmZ1, RsmZ2, RsmZ3, RsmZ4, RsmZ5, RsmZ6, RsmZ7 and RsmY (Hernandez-Eligio et al. 2012; Manzo et al. 2011). In agreement with this requirement, the Rsm-sRNAs genes possess a GacA binding box within their regulatory region (Manzo et al. 2011). In the search carried out in this study, we found an additional *rsmZ* gene (*rsmZ8*). Although the nucleotide sequence of RsmZ8 is the least conserved among the RsmZs, its secondary structure is well conserved (data not shown). An inspection of the regulatory region of *rsmZ8* revealed the presence of GacA binding sites (Table 4.2).

4.3.1 Rsm-RNAs and Alginate Production

In *A. vinelandii* as in *Pseudomonas* species the genes encoding the enzymes that participate in alginate biosynthesis (with the exception of *algC* which encodes for the second enzyme of the pathway) constitute a cluster headed by *algD*, which encodes GDP-mannose dehydrogenase, the key enzyme of the pathway (Campos et al. 1996). Alginate is essential for cyst formation as mutants unable to produce this polysaccharide are impaired in cyst development (Campos et al. 1996; Mejia-Ruiz et al. 1997). Early, we reported that inactivation of the *gacS* or *gacA* gene impaired alginate synthesis (Castañeda et al. 2000, 2001).

The RsmZ1 and RsmZ2 RNAs were shown to interact with the RsmA protein by RNA gel mobility shift experiments, and RsmA was shown to bind to a 200 nt transcript, corresponding to the 5' leader of the *algD* mRNA that includes a putative RsmA binding site (Manzo et al. 2011). Thus, It was concluded that in the *gacA* mutant the absence of Rsm RNAs allows, the free RsmA protein to bind the *algD* mRNA inhibiting its translation. Indeed, transcription of a single Rsm RNA (RsmZ1) from a GacA-independent promoter, restored alginate synthesis to the *gacA* mutant (Manzo et al. 2011). These studies clearly indicated the important role of the Rsm-RNAs acting as RsmA anti-repressors to allow translation of *algD* and the synthesis of alginate an essential component of the cyst capsule.

4.3.2   Rsm-RNAs and Alkylresorcinols Production

In *A. vinelandii* alkylresorcinols replace the phospholipids in the cysts membranes and are components of the exine, the outer layer of the cyst envelope. Mutants impaired in AR synthesis produce cysts with a defective exine (Segura et al. 2009). The biosynthesis of alkylresorcinols is also under the control of the Gac-Rsm system. This control is exerted at the level of *arpR* expression. ArpR is the transcriptional activator of the alkylresorcinol biosynthetic operon *arsABCD* (Romero et al. 2013). Inactivation of *gacA* impaired the synthesis of AR, and mutations in *rsmZ1* diminished the synthesis of these phenolic lipids. Additionally the RsmA protein was shown to bind to a transcript corresponding to the 5' leader of the *arpR* mRNA (Romero et al. 2016). Taken together these data indicated the essentiality of Rsm RNAs to release the repression of *arpR* mRNA translation exerted by RsmA.

4.3.3   Rsm-RNAs and PHB Production

Numerous PHB granules are present in mature cysts, and mutants unable to synthesize PHB produce cysts that lack PHB granules but resist desiccation (Segura et al. 2003) indicating that PHB is not essential for cyst formation or germination. However, this result does not rule out the possibility that under natural conditions PHB plays an important role in the formation and/or survival of cysts. Encystment in laboratory conditions is induced in Burk's medium containing 0.2 % of γ-hydroxybutyrate as sole carbon source, thus it is likely that in soil, this compound is provided by the degradation of PHB. Similar to alginate and AR genes, the expression of the PHB biosynthetic and regulatory genes is controlled by the Gac-Rsm pathway, as inactivation of *gacA* impaired PHB synthesis. The PHB biosynthetic operon *phbBAC* is activated by the transcriptional activator PhbR. RsmA was shown to interact with RNAs corresponding to the *phbB* and *phbR* mRNA leaders. Additionally the stability of *phbB* and *phbR* transcripts is increased in a *rsmA* mutant strain (Hernandez-Eligio et al. 2012). Therefore as in the case of alginate, and AR the Rsm RNAs are also required to antagonize the repressor activity of RsmA on translation of *phbR* and *phbB* mRNAs.

4.3.4   Expression of Hsp20 an Essential Protein for Cyst Resistance to Desiccation Is Regulated at a Posttranscriptional Level

The small heat shock protein Hsp20 is one of the most abundant proteins in *A. vinelandii* cysts and is present in low levels in vegetative cells (Cocotl-Yañez et al. 2014). However, transcription of *hsp20* is higher in vegetative cells than in cysts,

Table 4.1 Genes encoding sRNA found in silico in *A. vinelandii*

sRNA	Rfam identifiers	Localization	Reference
Classical sRNAs			
6S (ssrS)	RF00013	Intergenic 4809934..4810217	Wassarman (2007)
tmRNA (10Sa, *ssrA*)	RF00023	Intergenic 4355922..4356427	Keiler (2015)
Bacteria_small_SRP (4.5Sm *ffs*)	RF00169	Intergenic 1969172..1970650	Siu et al. (2007)
RNase P RNA (P28, 10Sb, *rnpB*)	RF00010	Intergenic 1278334..1279056	Kirsebom (2007)
sRNAs that sequester RNA-binding proteins			
PrrB_RsmZ (RsmZ1)	RF00166	Intergenic 385392..385847	Manzo et al. (2011)
PrrB_RsmZ (RsmZ2)		Intergenic 1671372..1672144	Manzo et al. (2011)
PrrB_RsmZ (RsmZ3)		Intergenic 396386..397732	Manzo et al. (2011)
PrrB_RsmZ (RsmZ4)		Intergenic 399749..401572	Manzo et al. (2011)
PrrB_RsmZ (RsmZ5)		Intergenic 422104..422515	Manzo et al. (2011)
PrrB_RsmZ (RsmZ6)		Intergenic 1692051..1692868	Manzo et al. (2011)
PrrB_RsmZ (RsmZ7)		Intergenic 843721..844218	Manzo et al. (2011)
PrrB_RsmZ (RsmZ8)		Intergenic 116768..117043	This work
RsmY	RF00195	Intergenic 444526..445166	Manzo et al. (2011)
CrcZ (CrcZ1)	RF01675	Intergenic 4303112..4303775	Filiatrault (2013)
CrcZ (CrcZ2)		Intergenic 452629..452762	Filiatrault (2013)
Base pairing sRNAs			
Pseudomon-1 (Spot 42, ErsA)	RF01719	Intergenic 64850..65144	Ferrara et al. (2015)
P20 (PhrS)	RF01673	Intergenic 3126790..3126965	Sonnleitner et al. (2011)
Prrf1 (ArrF)	RF00444	Avin 42580	Muriel-Millan et al. (2014)
Isrk	RF01394	Intergenic 3302323..3303960	Padalon-Brauch et al. (2008)
C4	RF01695	Intergenic 3302323..3303960	Citron and Schuster (1990)

Table 4.1 (continued)

sRNA	Rfam identifiers	Localization	Reference
C4	–	Intergenic 4107181..4107534	–
RgsA (P16)	RF00628	Intergenic 1479161..1479523	Huerta et al. 2016
Others sRNAs			
T44	RF00127	Intergenic 3946329..3946592	Tjaden et al. (2002)
P11	RF00625	Intergenic 2336465..2336727	Livny et al. (2006)
P14	RF01669	Intergenic 2306571..2306897	Livny et al. (2006)
P15	RF00627	Avin 33220	Livny et al. (2006)
P26	RF00630	Intergenic 593666..593879	Livny et al. (2006)
P27	RF01674	Intergenic 592518..592714	Livny et al. (2006)
P31	RF01676	Intergenic 4335150..4335335	Livny et al. (2006)

but translation is much higher under encystment conditions than in vegetative cells, indicating a post-transcriptional regulation mechanism. Indeed the *hsp20* Shine-Dalgarno possesses a potential RsmA binding site that matched the RsmA binding sites present in the *algD*, *phbR,* and *arpR* transcripts (Cocotl-Yañez et al. 2014), suggesting that expression of Hsp20 is under Rsm-RNAs control. Mutations of *hsp20* did not affect the synthesis of cyst components nor the morphology of the cyst, however wild type-like cysts formed by the *hsp20* mutant were unable to resist desiccation (Cocotl-Yañez et al. 2014).

By the mean of allowing the expression of genes involved in the synthesis of cyst component alginate, AR and PHB as well as the *hsp20* gene essential for cyst desiccation resistance, the Rsm-RNAs play an important role in the differentiation process to produce cysts. A model for the control of cyst formation by the Rsm-RNAs is shown in Fig. 4.1.

4.3.5 Role of Rsm-RNAs Reiterated Genes

The presence of multiple copies of Rsm-RNAs in *A. vinelandii* raises the question about their biological role. One possibility is to provide with a range of levels of these regulatory RNAs (doses). This range could be achieved by differential expression under different conditions. This in turn will result in a wide range of levels of the free RsmA repressor. Indeed although *rsmZ1-7* and *rsmY* genes and
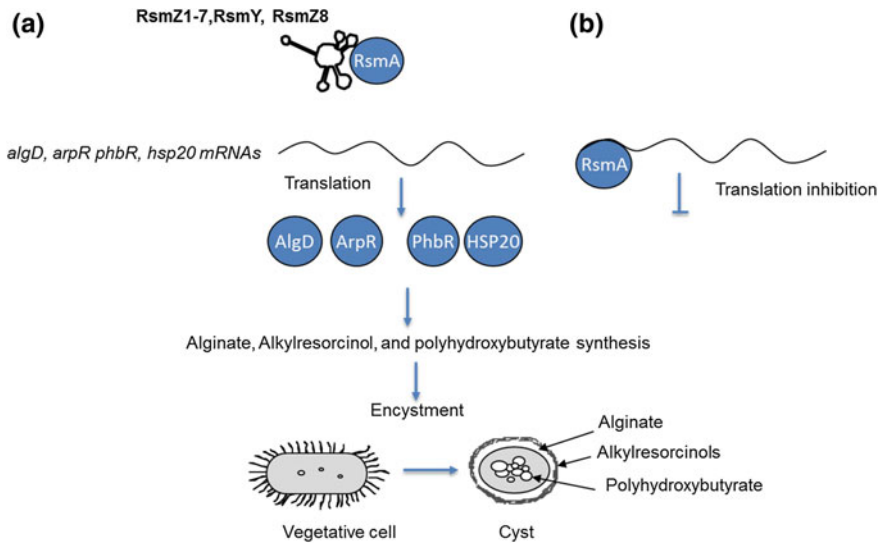
Fig. 4.1 Model for the control of cyst formation by the Rsm-RNAs. **a** The binding of RsmA to the Rsm-RNAs inhibits its translation repression function, allowing expression of the AlgD, ArpR PhbR and Hsp20 proteins, the synthesis of alginate, alkylresorcinol and PHB proteins, and the formation of cysts. **b** Binding of RsmA to the 5' UTRs of the *algD*, *arpR*, *phbR* and *hsp20* mRNAs inhibits their translation

probably *rsmZ8*, are all activated by GacA, their expression levels varies among them (Hernandez-Eligio et al. 2012). This could be related to the position of the GacA binding sequences, respect to the transcription start site, as well as the % of AT (Table 4.2). In fact, regulators with affinity to AT rich sequences like IHF and H-NS have been related with differential expression of Rsm-RNAs genes in *Pseudomonas* species (Brencic et al. 2009; Humair et al. 2010).

The effect of the RNA structure-affinity on the binding to the protein RsmA can also provide the cells with an even higher range of free RsmA levels. In a model of RsmA (CsrA)-RNAs interaction, the greater number of stem-loop structures, the more RsmA molecules sequestered is expected (Babitzke and Romeo 2007; Heroven et al. 2012). Indeed in the *A. vinelandii* Rsm-RNAs the numbers of stem-loops structures vary from four to nine (Fig. 4.2, Table 4.2). Differences in their ΔG, and therefore their stability are also predicted (Table 4.2). Other factor affecting the level of Rsm RNAs is the turn over, as their enzymatic degradation is related to their structure (Seyll and Van Melderen 2013).

In summary, we propose that the redundancy of Rsm-RNAs allows the cell to have a wide range of RsmA levels in response to different environmental or metabolic signals including those that induce differentiation. In fact, redundancy in *A. vinelandii* is higher than in any *Pseudomonas* species, where the number of Rsm-RNAs varies from two to five (Moll et al. 2010). Thus, the presence of nine
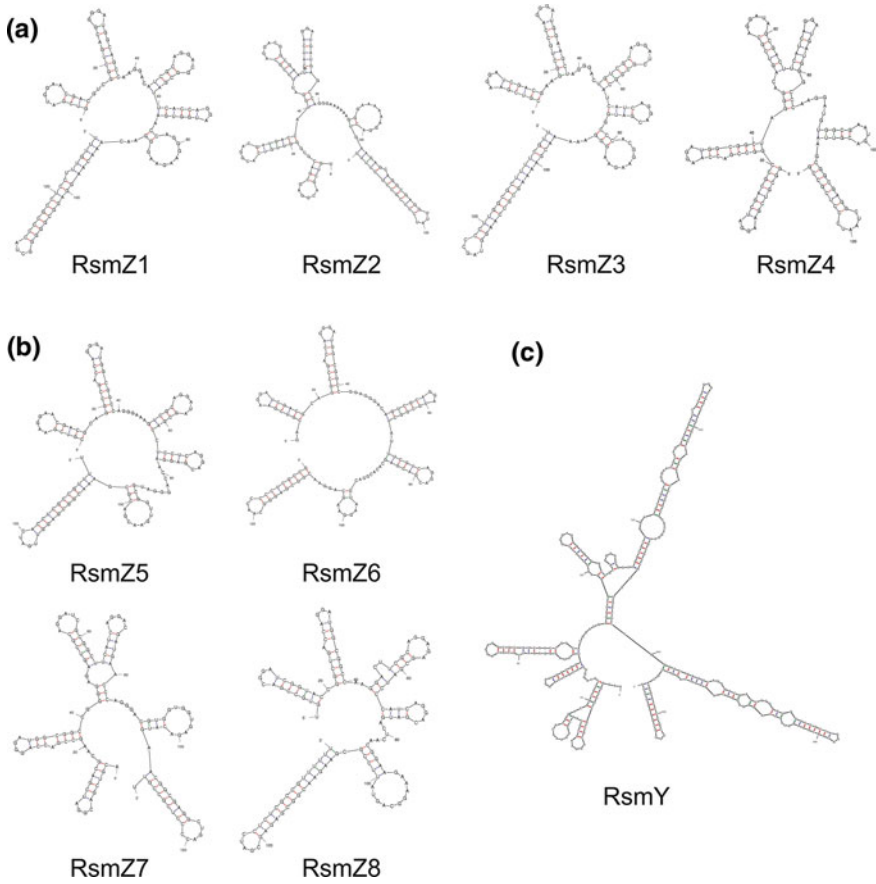
Fig. 4.2 Stem-loop structures of *A. vinelandii* Rsm small RNAs. **a** RsmZ1, RsmZ2, RsmZ3 and RsmZ4. **b** RsmZ5, RsmZ6, RsmZ7 and RsmZ8. **c** RsmY

Table 4.2 *A. vinelandii* Rsm-RNAs properties and characteristics

Rsm sRNA	Number of stem-loops with GGA motif	ΔG (kcal/mol)	GacA box position (pb)	AT content (%) of rsm genes regulatory regions
RsmZ1	5	−61.85	−175, −155	45
RsmZ2	5	−50.36	−181, −163	50
RsmZ3	5	−68.04	−82, −64	40
RsmZ4	4	−65.40	−80, −62	42
RsmZ5	4	−66.05	−80, −62	36
RsmZ6	4	−60.34	−81, −63	39
RsmZ7	5	−63.26	−80, −62	33
RsmZ8	4	−65.27	−75, −57	44
RsmY	9	−148.90	−75, −57	37

Rsm-RNAs in *A. vinelandii* could be related to their involvement in cyst formation, a feature not observed in its close relatives, the *Pseudomonas*.

4.4 ArrF Controls Iron Homeostasis and PHB Synthesis in *A. vinelandii*

Iron acquisition is controlled by the Fur (**F**erric **u**ptake **r**epressor) protein, which represses the transcription of many genes, including those associated with iron uptake, like siderophores and receptors (Escolar et al. 1999). Under high concentrations of iron, Fur forms a complex with Fe^{2+}, and binds to "iron boxes" present in its target genes, repressing their transcription. When iron is limited, Fur is unable to bind iron boxes. Fur also represses the transcription of RyhB in *E. coli*, and of ArrF in *A. vinelandii* (Jung and Kwon 2008; Masse and Gottesman 2002). RhyB represses the expression of genes encoding non-essential proteins that use iron (Salvail and Masse 2012). RhyB sRNA forms sRNA-mRNA complexes stabilized by Hfq. RhyB also exerts a positive regulation on the expression of *shiA*. In this case the rhyB-mRNA pairing disrupt an inhibitory structure allowing translation (Prevost et al. 2007).

Iron is essential for different biological processes in *A. vinelandii* including nitrogen fixation, respiration, and nitrogenase protection (Page and Huyer 1984; Page and von Tigerstrom 1982). ArrF was shown to negatively regulate the expression of *sodB* gene encoding Fe-containing superoxide dismutase and FeSII protein (Jung and Kwon 2008), which is known to mediate the conformational protection of nitrogenase enzyme against oxygen inactivation. Iron also affects PHB accumulation in this bacterium (Pyla et al. 2009). Under Iron limitation transcription of *arrF* increased significantly. The expression of PhbR, the transcriptional activator of PHB synthesis also increased post- transcriptionally in iron limitation, suggesting that iron limitation increases translation of *phbR* through ArrF (Muriel-Millan et al. 2014). The study of the role of ArrF in the control of PHB was carried out in vegetative cells. Thus its role in encystment remains to be investigated.

4.5 Roles of EsrA sRNA in *Pseudomonas*

In *P. aeruginosa* and *A. vinelandii* the alginate biosynthetic, gene *algC* is transcribed from a promoter dependent on AlgU, a homologue of the RpoE alternative sigma factor (Gaona et al. 2004; Zielinski et al. 1991). Therefore, inactivation of *algU* in *A. vinelandii* abrogates alginate synthesis and encystment (Moreno et al. 1998). In *P. aeruginosa* the EsrA RNA regulates expression of AlgC at a post-transcriptional level and, *esrA* as *algC* is transcribed from a AlgU promoter (Ferrara et al. 2015). As shown in Table 1 an EsrA homolog sharing 78 % of identity with *Pseudomonas esrA* is present in *A. vinelandii*. Interestingly conserved −10 and −35

boxes that correspond to an AlgU promoter are present in the regulatory region of the *A. vinelandii esrA*. These observations suggest that in *A. vinelandii* ErsA sRNA may be involved in the control of alginate synthesis.

4.6   CrcZ and Its Role in Carbon Catabolic Repression in *Pseudomonas* Species

Carbon catabolic repression is a mechanism that allows bacteria to catabolize preferred carbon sources for most efficient growth. In enteric bacteria this feature is controlled by the phosphoenolpyruvate (PEP): carbohydrate phosphotransferase system (carbohydrate PTS) by the mean of controlling cAMP-dependent transcriptional activation (for a review see Lengeler and Jahreis 2009).

In *Pseudomonas* and in *A. vinelandii* PTS plays no role in catabolic repression (Filiatrault et al. 2013; Moreno et al. 2015). Instead, in *Pseudomonas* species *aeruginosa*, *putida*, and *syringae* the control of carbon catabolic repression involves the two-component system CbrBA, the sRNAs CrcX, CrcY or CrcZ, and the Crc protein. The expression of the genes encoding Crc sRNAs is dependent on the alternative sigma factor RpoN and the CbrB transcriptional activator. Crc is a small protein that recognizes and bind mRNAs encoding proteins involved in catabolic pathways of less preferred carbon sources. This binding results in repression of translation. In the presence of Hfq, CrcZ sRNA binds the translation repressor Crc. When *Pseudomonas* grows on a less preferred carbon source, CbrB activates transcription of CrcZ that sequesters the Crc protein allowing translation of mRNAs that are Crc targets. On the other side, in the presence of a preferred source, CbrAB activity is inhibited, resulting in low levels of CrcZ sRNA and repression of mRNA by Crc.

The *crbAB*, *crc* genes, and two copies encoding sRNA CrcZ are present in the *A. vinelandii* genome (Filiatrault et al. 2013; Setubal et al. 2009). The high conservation of the catabolic repression CbrAB/Crc system genes between *Pseudomonas* and *A. vinelandii* suggest the same function in this bacterium. If this was the case, The CbrAB/Crc system is likely to have an effect on the synthesis of alginate and PHB as the precursors for their synthesis (fructose 6P and acetyl-CoA respectively) are originated from the catabolism of the carbon source provided.

4.7   Outlook

Several important aspects of the encystment process that undergoes *A. vinelandii*, such as the control of gene expression that leads to the synthesis of important cyst components have been shown to be under Rsm-RNAs control. However, more details about their individual expression and their interaction with RsmA remain to be investigated. We do not rule out that in addition to the Rsm-RNAs other RNAs

like EsrA a regulator of the alginate biosynthetic, gene *algC* in *P. aeruginosa* also participate in the control of alginate synthesis, an essential component of mature cysts.

Another important subject that remains to be investigated is the behavior of *A. vinelandii* cysts or vegetative cells in natural soil conditions. It is well known that bacteria are killed by viruses (bacteriophages), and are predated by other bacteria and eukaryotic organisms as protozoan, amoebas and fungi. Bacteria has developed anti-depredator strategies, one of them is the synthesis of exo-polysaccharides, as mutant strains impaired in exo-polysaccharide production are more susceptible to bacteriovores (Matz et al. 2004). Whether alginate producing, cells of *A. vinelandii* are more resistant to predators than alginate minus mutants remains to be investigated. However, It has been reported that *A. vinelandii* cysts (that possess an alginate capsule), are more resistant to predation by *Agromyces ramosus* than vegetative cells (Casida 1983). Whether cysts are also more resistant to eukaryotic predators like protozoan remains to be tested. Thus, in *A. vinelandii* sRNAs are essential for the production of exo-polysaccharide and for cyst formation, two features that may play essential roles in the survival of this bacterium in soil.

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