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Research Letter

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RpoS controls the expression and the transport of the AlgE1-7 epimerases in *Azotobacter vinelandii*

S. Moreno[1], H. Ertesvåg[2], S. Valla[2,†], C. Núñez[1], G. Espin[1] and M. Cocotl-Yañez[1,3,*]

[1]Departamento de Microbiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México. Av. Universidad 2001, Col. Chamilpa, Cuernavaca, Morelos. C.P. 62210, México, [2]Department of Biotechnology and Food Science, NTNU-Norwegian University of Science and Technology, Sem Sælands vei 6/8, 7491 Trondheim, Norway and [3]Departamento de Microbiología y Parasitología, Facultad de Medicina, Universidad Nacional Autónoma de México. Ciudad de México. C.P. 04510, México

*Corresponding author: Departamento de Microbiología y Parasitología, Facultad de Medicina, Universidad Autónoma de México. Av. Universidad 3000, Cd. Universitaria, 04510 Coyoacán, Ciudad de México. Tel: +52-555622 9201; E-mail: miguel.cocotlyanez@iibiomedicas.unam.mx

One sentence summary: Regulation of algE1-7 and eexDEF genes by RpoS.

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[†]Deceased

ABSTRACT

Azotobacter vinelandii produces differentiated cells, called cysts, surrounded by two alginate layers, which are necessary for their desiccation resistance. This alginate contains variable proportions of guluronate residues, resulting from the activity of seven extracytoplasmic epimerases, AlgE1–7. These enzymes are exported by a system secretion encoded by the *eexDEF* operon; mutants lacking the AlgE1-7 epimerases, the EexDEF or the RpoS sigma factor produce alginate, but are unable to form desiccation resistant cysts. Herein, we found that RpoS was required for full transcription of the *algE1-7* and *eexDEF* genes. We found that the AlgE1-7 protein levels were diminished in the *rpoS* mutant strain. In addition, the alginate produced in the absence of RpoS was more viscous in the presence of proteases, a phenotype similar to that of the *eexD* mutant. Primer extension analysis located two promoters for the *eexDEF* operon, one of them was RpoS-dependent. Thus, during encysting conditions, RpoS coordinates the expression of both the AlgE1-7 epimerases and the EexDEF protein complex responsible for their transport.

Keywords: alginate; epimerases; azotobacter encystment; RpoS

INTRODUCTION

Azotobacter vinelandii is a nitrogen-fixing soil bacterium that produces the industrially widely used exopolysaccharide alginate (Bulen, Burns and LeComte 1964; Pacheco-Leyva, Pezoa and Diaz-Barrera 2016), a linear co-polymer composed of 1→4 linked β-D-mannuronic acid (M) and its C-5-epimer α-L-guluronic acid (G). The G residues in alginates are the result of a polymer-level epimerization process catalyzed by mannuronan C-5-epimerases. In alginate-producing bacteria, epimerization is carried out by a periplasmic enzyme which is encoded by *algG* (Chitnis and Ohman 1990; Franklin *et al.* 1994; Rehm, Ertesvåg and Valla 1996). In addition to *algG*, the *A. vinelandii* genome encodes a family of seven epimerases, AlgE1–7, that are exported to the cell surface and are released into the extracellular environment (Ertesvåg *et al.* 1994, 1995; Svanem *et al.* 1999). These epimerases are structurally unrelated to AlgG. AlgE1–6

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can be used to epimerize M-rich alginates *in vitro*. Each of these enzymes introduces different patterns of G residues into their substrates; *in vitro* studies have shown that AlgE4 introduces alternating MG blocks, while the remaining enzymes can generate G blocks of varying lengths (Ertesvåg *et al.* 1999). AlgE7 is a bifunctional epimerase and lyase (Svanem *et al.* 1999).

Azotobacter vinelandii undergoes a differentiation process resulting in the formation of cysts resistant to desiccation. A mature cyst consists of a contracted cell, known as the central body, which is surrounded by a capsule made up of a laminated outer layer called the exine and an inner layer called the intine. Alginate is the major component of both layers of the cyst (Sadoff 1975) and it is essential for the differentiation process; mutations in alginate biosynthetic genes impair the encystment process (Campos *et al.* 1996; Mejía-Ruíz *et al.* 1997). Vegetatively growing *A. vinelandii* cells also produce alginates that are released into the extracellular environment.

The exine is characterized by being structured and rigid with alginates rich in GG block sequences. The intine, in contrast, is less structured and its alginates consist mostly of MG and MM blocks (Page and Sadoff 1975). The AlgE1-7 epimerases are essential for the differentiation process; *A. vinelandii* strain MS163171, in which all *algE* genes were inactivated, was found to be unable to form functional cysts. The MS163171 cells induced for encystment lacked the rigid cyst coat characteristic of the wild type and were unable to resist desiccation (Steigedal *et al.* 2008). Inactivation of the *eexDEF* gene cluster encoding a type I transport system for the AlgE epimerases resulted in the absence of epimerase activity in culture supernatants and in the formation of cysts unable to resist desiccation with morphology similar to the MS163171 strain (Gimmestad *et al.* 2006).

Other components of the cyst are the alkylresorcinols, which replace the phospholipids of the cyst membranes during differentiation and are components of the exine layer (Reusch and Sadoff 1983). The sigma factor RpoS is essential for alkylresorcinol synthesis and for cyst formation; similar to the MS163171 strain and *eexDEF* mutants, inactivation of *rpoS* resulted in cysts that completely lacked of the exine and intine layers and were unable to resist desiccation (Cocotl-Yañez *et al.* 2011; Romero *et al.* 2013). Since inactivation of *rpoS* did not prevent alginate synthesis (Castañeda *et al.* 2001), the inability of this mutant to form the intine and exine layers is not caused by the absence of this polymer. Although alkylresorcinols play a structural role in the exine layer, they are not essential for either cyst formation or desiccation resistance (Segura *et al.* 2009). These results suggest that some genes essential for proper cyst formation are under the control of RpoS. In a previous work, the proteome of an *rpoS* mutant undergoing differentiation was established and was compared to that of the wild type strain (Cocotl-Yañez *et al.* 2014). We reported that RpoS controls a small heat shock protein, Hsp20, that is essential for cyst desiccation resistance. Moreover, we found that expression of AlgE1 and AlgE6 proteins was downregulated in the *rpoS* mutant strain (Cocotl-Yañez *et al.* 2014). In this study, we investigated the role of the RpoS on the expression of *algE1-7* genes and the *eexDEF* operon encoding the transport system for the export of the AlgE1-7 epimerases.

MATERIALS AND METHODS

Microbiological procedures

Bacterial strains, plasmids and oligonucleotides used are listed in Table S1, Supporting Information. Medium and growth conditions were as follows: *A. vinelandii* was grown at 30°C in Burk's nitrogen-free salts medium (Kennedy *et al.* 1986) supplemented with 2% sucrose (BS) for vegetative conditions, 0.2% n-butanol (BB) for encystment conditions. *Escherichia coli* strains DH5α was grown on Luria-Bertani medium at 37°C. Antibiotic concentrations used (μg mL^{-1}) for *A. vinelandii* and *E. coli*, respectively, were as follows: ampicillin (Ap), 0 and 100; nalidixic acid (Nal), 30 and 0; spectinomycin (Sp), 50 and 50; kanamycin (Km), 1 and 30; gentamicine (Gm), 1 and 10. Transformation and conjugation of *A. vinelandii* were carried out as previously described (Page and von Tigerstrom 1978; Bali, Blanco and Hill 1992). Cultivation for alginate production was performed using liquid RA1 medium (pH7.0) (Gimmestad *et al.*2006) containing 0.1 mL/L each of alkalase 2.4 L and neutrase 0.5 L (proteases) from Novo Nordisk.

Nucleic acid procedures

DNA and RNA isolation and cloning procedures were carried out as previously described (Sambrook, Fritsch and Maniatis 1989). Chromosomal DNA used as template for PCRs was obtained from *A. vinelandii* AEIV wild-type strain. DNA sequencing was done with a Perkin Elmer/Applied Biosystems DNA Sequencer. The sequence of all the primers used in this work is shown in Table S2, Supporting Information.

Construction and complementation of rpoS mutant strain

Plasmid pSMS7, which is unable to replicate in *A. vinelandii*, was introduced into strain AEIV. The spectinomycin-resistant transformant (AErpoS) (Cocotl-Yañez *et al.* 2014) was generated by a double recombination event and confirmed as carrying the *rpoS*::Sp mutation by PCR using the primers RTupRpoS and RT-2downRpoS (data not shown).

pSMrpoS, which is able to replicate in *A. vinelandii*, was transferred by conjugation into strain AErpoS to produce AErpoS/pSMrpoS.

Analyses of epimerase production

Western blot assays were performed as described previously by Høidal *et al.* (2000) with some modifications. *Azotobacter vinelandii* was grown at 30°C in Burk's medium supplemented with sucrose for 30 h. The cultures were centrifuged, and the cells were washed with $MgSO_4$ and transferred to BB medium plates for 5 days. Cells were collected and washed with 1 mL of 50 mM Tris pH 7.8 centrifuged for 10 min, resuspended in 0.5 mL of 50 mM Tris, 3 mM Na_2EDTA and shaken at 250 r.p.m. for 15 min to allow for cyst rupture and enhanced release of central bodies. The solution was centrifuged and the supernatant represents the epimerases associated with the central body surface. The proteins were blotted as described for Høidal *et al.* (2000) using the anti-AlgE4 antibody.

Quantitative RT-PCR

Expression of *eexD*, *algE1* and *algE1-6* were measured by quantitative RT-PCR (qRT-PCR), as previously reported (Noguez *et al.* 2008). RNA was extracted from cultures grown in BS medium for 30 h. The primers used for the qRT-PCR assays were as follows: FwRT-eexD/RvRT-eexD for *eexD* expression, FwRT-algE1-6/RvRT-algE1-6 for measuring the combined expression of *algE1-6* FwR-algE1/RvRT-algE1 for *algE1* expression and fw-gyrA/rev-gyrA for *gyrA* expression. Relative mRNA transcript levels were determined in relation to *gyrA* mRNA as described earlier (Noguez *et al.* 2008). All real-time PCR reactions were performed with three biological replicates (independent cultures) with three technical replicates for each one. The quantification technique used to analyze the data was the $2^{-\Delta\Delta CT}$ method and the data are presented as fold changes (mean ± SD) of mRNA levels of mutant strain relative to those of the wild type (Livak and Schmittgen 2001).

Primer extension analysis assays

A 1001-pb fragment corresponding to the promoter region of *eexD* was amplified by PCR using primers UpeexDrr and Dweex-Drr. The product was cloned into pJET1.2/blunt resulting in pM-CeexD. Total RNA was isolated from AEIV and AErpoS cultures grown for 30 h in BS. Primer extension experiments were carried out at 42°C using avian myeloblastosis virus reverse transcriptase (Roche) with the primer eexDP1 and the cDNAs were end-labeled with (γ-^{32}P)-dATP using polynucleotide kinase (Roche). The sequencing ladders were generated with the same primers using a Thermo Sequenase Cycle Sequencing kit (USB) and plasmid pMCeexD as template.

RESULTS

Analysis of AlgE epimerase levels in the *rpoS* mutant

Considering that inactivation of *rpoS* gene in *A. vinelandii* resulted in the formation of cysts lacking the intine and exine layers and thus, unable to resist desiccation, we investigated the possible role of the RpoS sigma factor on regulating the expression of the seven secreted *A. vinelandii* epimerases (AlgE1-7).

The presence of the seven AlgE epimerases in proteins associated to the central body of the *rpoS* mutant cells (AErpoS) induced for encystment was investigated by Western blot assays using antibodies against the mannuronan C-5 epimerase AlgE4. Since all AlgE-epimerases are modular enzymes and the ranges of homology between them are from 50% to 100% (Ertesvåg *et al.* 1995), all seven proteins can be detected using this antibody (Høidal *et al.* 2000). As shown in Fig. 1, in the *rpoS* mutant strain the AlgE epimerases are present in very low concentrations as compared to the wild-type strain. Genetic complementation of mutant AErpoS with a wild-type copy of the *rpoS* gene, expressed *in trans*, restored the accumulation of AlgE1-7. As expected, this effect was not observed for the same strain carrying the empty vector pBBR1MCS-2 used as a negative control. These results indicated that RpoS controls the expression of the AlgE1-7 epimerases.

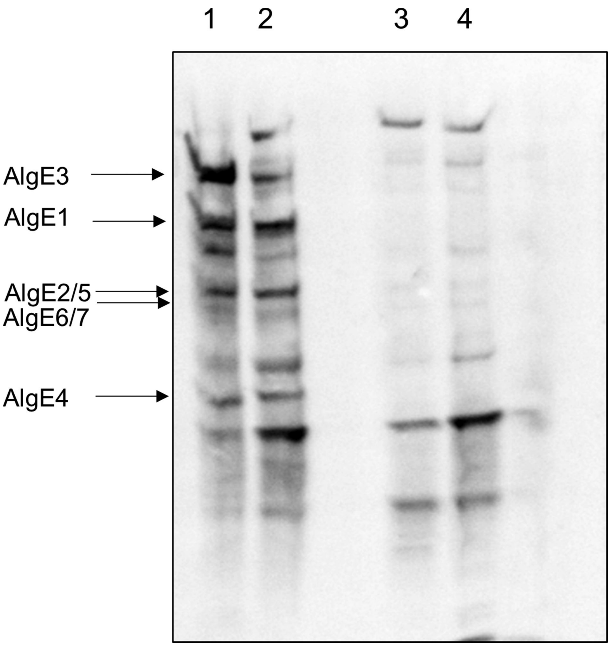


Figure 1. Western blot analysis to detect the AlgE1-7 epimerases. Cell surface protein extracts of the wild-type AEIV (1), AErpoS/pSMrpoS (2), AErpoS (3) and AErpoS/pBBR1MCS-2 (4) strains were used. The proteins were separated on a 4%–12% gradient SDS-PAGE gel. The anti-AlgE4 antibody (Høidal 2000) was used as primary antibody.

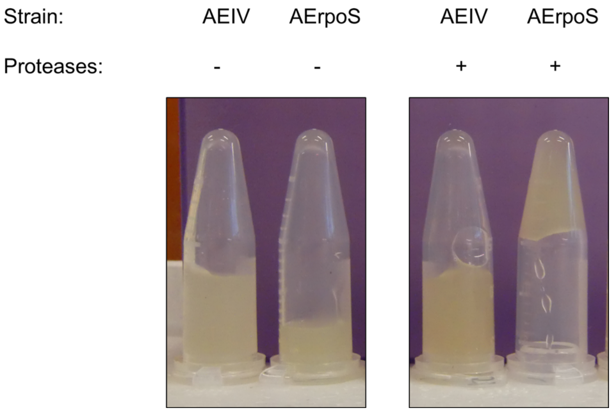


Figure 2. Visualization of the viscosity in the RA1 culture medium after growing the wild-type strain AEIV and its *rpoS* mutant derivative AErpoS, without (left) or with added proteases (right).

The absence of RpoS results in an alginate of higher viscosity

In a previous work, Gimmestad *et al.* (2006) reported that relative to the wild-type strain, an *eexDEF* mutant produced an alginate of higher molecular weight. When the cells were cultivated in the presence of proteases, this difference became very pronounced, leading to a visible change in viscosity. The molecular basis of this phenotype is not totally understood, but the authors proposed that since the polymeric surface coat was missing in the *eex* mutants, alginate lyases normally embedded in the surface coat might become more susceptible to proteolysis by proteases. This could hypothetically prevent alginate polymer degradation. Thus, if as shown above, RpoS positively affects expression of the AlgE1-7 epimerases, the *rpoS* mutant would be expected to have a similar phenotype in which the alginate lyases could be degraded by proteases added to the culture medium resulting in alginates of higher viscosity. To investigate this, strains AErpoS and the wild-type AEIV were grown in RA1 medium supplemented with proteases as previously reported (Gimmestad *et al.* 2006). As shown in Fig. 2, the viscosity of the alginates produced by mutant *rpoS* was higher than that of the wild-type strain. This result suggested the involvement of RpoS on the control of *eexDEF* transcription, and we next explored this possibility.

Effect of RpoS on the transcription of the *eexDEF* operon

In order to assess the mRNA levels of the *eexDEF* operon, qPCR assays were conducted using total RNA extracted from vegetative cultures of the wild-type strain AEIV and from mutant AErpoS. As shown in Fig. 3, the levels of the *eexD* transcript were

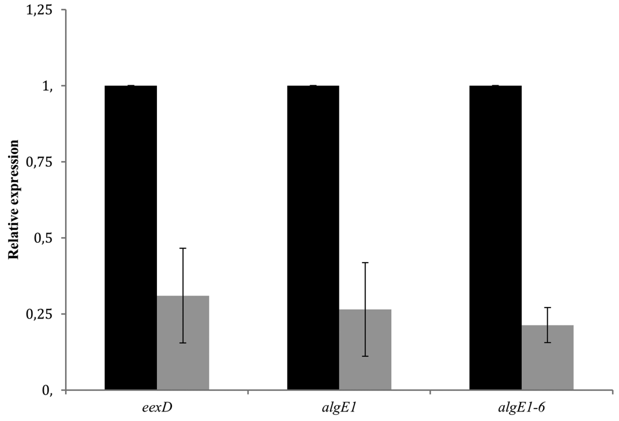


Figure 3. mRNA relative levels of *eexD*, *algE1* and *algE1-6* genes in the wild-type strain (black bars) and in its isogenic mutant AErpoS (grey bars) determined by qRT-PCR. The data are presented as fold changes of mRNA levels of each gene in the *rpoS* mutant strain relative to that of the parental strain, AEIV. Error bars represent standard deviations of three independent experiments.

diminished about 60% in the *rpoS* mutant relative to the wild-type strain indicating that RpoS is required for full expression of the *eexDEF* operon.

The transcription start site for the *eexD* gene was determined by primer extension using total RNA isolated from strains AErpoS and AEIV cells grown in vegetative medium. Two transcriptional start sites were identified for *eexDEF* operon (Fig. 4). The promoter P1$_{eexD}$ was located 40 nucleotides upstream of the ATG start codon; however, no consensus sequence for the -10 and -35 region was observed. The promoter P2$_{eexD}$ was located 100 nucleotides upstream of the ATG start codon of *eexD*. The -10 region of this start site has the CTACAAT sequence that is similar to other RpoS-dependent promoters previously identified in *A. vinelandii* (Castañeda *et al.* 2001; Peralta-Gil *et al.* 2002; Cocotl-Yañez *et al.* 2014). Strikingly, in the *rpoS* mutant transcription of *eexD* was only initiated from the P1$_{eexD}$ promoter but not from the P2$_{eexD}$, as no primer extension product initiating from this site was detected. Together, these results indicate that RpoS recognizes the P2$_{eexD}$ promoter for transcription initiation of the *eexDEF* operon.

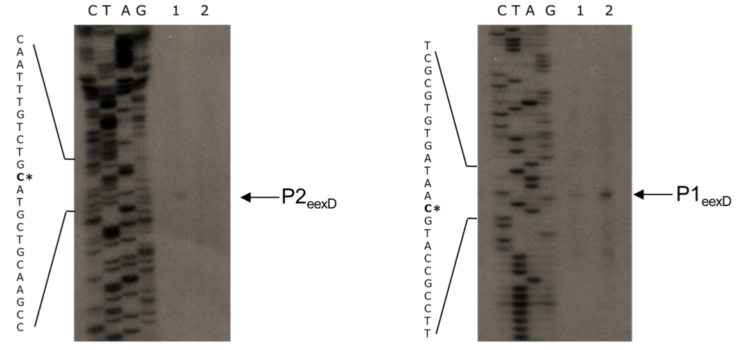
Transcription of the epimerases genes *algE1-7* are under the control of RpoS

Next, we investigated the role of RpoS in the expression of the epimerases genes. This assay was conducted using a primer pair that is able to anneal to the six epimerases. This primer pair does not detect the epimerase-like lyase gene *algE7* or *algY* encoding an epimerase-like protein of unknown function (Svanem *et al.* 1999). Additionally, the expression of *algE1* was measured, using a primer pair specific for this gene. The relative levels of the *algE* mRNAs was determined by qPCR in the *rpoS* mutant AErpoS, and were compared to those of the wild-type strain. As shown in Fig. 3, the relative transcript levels of *algE1* and of *algE1-6* in the *rpoS* mutant strain were approximately 25%–40% of those produced by the wild-type strain. Thus, taken together, these results imply that during encysting conditions RpoS coordinates the expression of both, the AlgE1-7 epimerases and the EexDEF protein complex responsible for their transport.

DISCUSSION

In bacteria, alternative sigma factors play a role in different processes, including cell differentiation. In *A. vinelandii*, the stationary phase sigma factor RpoS is essential to form mature cysts.

(A)



(B)

```
                                                        P2eexD
                                                -------------------------
TTTCGACTCTGTTTTTTTTGCCTTGCCTTATCTCTTCTTGCCTACAATTTGTCTGCATGCTGCAAGCCGGATTTTTCATGCGGGATGATGG
                                           -10         +1

CAATTTGATGGCTTGATGCCATTCGTTAAACGGACTGAGCTATGCGTAGCCGAAGGAATATGGCTGTCAAGGCGCGAAGAAACATTTGGTC
                               P1eexD
   -------------------------------------------------------
GGAACGGGAGACATCCGGTCTTTGGGGCTCGGATCGCGTGTGATAACGTACCGCCTTTATTTTTTTACCTTATCCGAGCGACACAGTATG
        -35                        -10       +1
```

Figure 4. (**A**) Identification of the transcriptional start sites of *eexD* by primer extension analysis using total RNA isolated from strain AEIV (lane 1) and from strain AErpoS (lane 2) in vegetative growing conditions. Arrows indicate the start sites of transcription. (**B**) Sequence of the regulatory region of *eexD*. The two transcription initiation sites (+1) are indicated along with the -10 and -35 regions of the identified promoters (P1eexD and P2eexD). The ATG translational start codon is show in italics.

In an early report, the influence of RpoS on alginate production was established (Castañeda *et al.* 2001). RpoS recognizes one of the promoters driving *algD* expression, encoding the key enzyme of the alginate biosynthetic pathway. As RpoS exerts a partial effect on *algD*, the *rpoS* mutant produces alginate, yet it is unable to form cysts resistant to desiccation. In a previous work, we found that under encysting conditions AlgE1 and AlgE6 proteins were downregulated in the *rpoS* mutant strain AErpoS (Cocotl-Yañez *et al.* 2014). In this work, we have identified both the *algE1-7* epimerase genes and the epimerase transporter genes *eexDEF* as targets of RpoS.

RpoS seems to equally affect the expression of the seven *algE* genes, as the accumulation of the AlgE1-7 epimerases was reduced on the surface of cells lacking this sigma factor. Accordingly, qRT-PCR assays showed that the amounts of the *algE1-6* mRNAs were diminished by 60% in the *rpoS* genetic background. The amount of *algE1* mRNA showed the same reduction suggesting a general effect of RpoS on the transcription of the *algE1-6* genes. Little is known about the transcriptional regulation of the *algE* genes; *in silico* analysis suggests that they probably are arranged in single transcriptional units; however no consensus sequences for either RpoD or RpoS type promoters were identified (Ertesvåg *et al.* 1995; Svanem *et al.* 1999). Thus, it remains to be investigated whether or not RpoS directly regulates the transcription of these genes.

RpoS was also found to partially regulate expression of the *eexDEF* operon. In agreement with this result, the *rpoS* mutant phenocopied the *eexDEF* mutants, as both strains produce an alginate of higher molecular mass when proteases were added to the medium. Transcription of the *eexD* gene was initiated from two promoters but only the distal one ($\mathrm{P2}_{eexD}$) was RpoS dependent. This result is in agreement with the qRT-PCR analysis, showing a 60% reduction in *eexD* mRNA in the absence of RpoS. The biological significance of the $\mathrm{P1}_{eexD}$ promoter is unknown but might be related to the different roles of alginates with high G content, apart from structuring the mature cyst envelope. It was previously reported that in the presence of high oxygen concentrations, *A. vinelandii* forms an alginate capsule on the surface of the cell with greater G proportion, which serves as a barrier for O_2 transfer into the cell (Sabra *et al.* 2000). In this context, full activation of the *eexDEF* operon may be influenced by additional regulatory inputs.

As opposed to *A. vinelandii*, in *Pseudomonas spp.*, AlgG is the major enzyme that carries out the epimerization process and has a structural function in transporting the polymer out of the cell (Gimmestad *et al.* 2003; Jain *et al.* 2003). The *algG* gene from *Pseudomonas* is transcribed in operon with *algD* and their expression is mainly controlled by the transcriptional factor AlgR and the AlgU sigma factor (Kato and Chakrabarty 1991; Mohr *et al.* 1992; Hershberger *et al.* 1995). *algG* is also found in *A. vinelandii*; however, the encoded enzyme shows low epimerization activity *in vivo* and *in vitro* (Rehm *et al.* 1996; Steigedal *et al.* 2008).

The present work expands our knowledge about the role of RpoS during the differentiation process of *A. vinelandii*. Besides controlling the expression of the protein Hsp20 or the production of alkylresorcinols, RpoS influences the properties of the alginate chains by affecting the amount and the transport of the AlgE1-7 epimerases essential for structuring the cysts coat. Taken together, our results indicate that RpoS is a master regulator of the encystment process in *A. vinelandii*.

SUPPLEMENTARY DATA

Supplementary data is available at *FEMSLE* online.

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