RESEARCH LETTER – Physiology & Biochemistry

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

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

Editor: Jana Jass

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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, Paezoa and Díaz-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 alglnates are the result of a polymer-level epimerization process catalyzed by mannuronic 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

Received: 30 May 2018; Accepted: 27 August 2018
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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 (Értesvág 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-Ruiz 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 (Réusch and Sadoff 1983). The sigma factor RpoS is essential for alkylresorcinol synthesis and for cyst formation; similar to the MS163171 strain (Gimmestad et al. 2006). Growth of the MS163171 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. 1997; 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 down-regulated 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 Tiggesen 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 alcalase 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 AEV 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 (AEIVrpoS) (Cocotl-Yañez et al. 2014) was generated by a double recombination event and confirmed as carrying the rps::Sp mutation by PCR using the primers RTPupRpoS and RT2downRpoS (data not shown).

pSMrpoS, which is able to replicate in A. vinelandii, was transferred by conjugation into strain AEIVrpoS to produce AEIVrpoS/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 succrose for 30 h. The cultures were centrifuged, and the cells were washed with MgSO₄ 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 Na₂EDTA 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 FwRT-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 ($\gamma$-32P)-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 manuronan C-5 epimerase AlgE4. Since all AlgE-epimerases are modular enzymes and the ranges of homology between them are from 50% to 100% (Ertesv˚ag 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.

**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 strain 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 P1eexD was located 40 nucleotides upstream of the ATG start codon; however, no consensus sequence for the -10 and -35 region was observed. The promoter P2eexD 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; Cocot-Yañez et al. 2014). Strikingly, in the rpoS mutant transcription of eexD was only initiated from the P1eexD promoter but not from the P2eexD, as no primer extension product initiating from this site was detected. Together, these results indicate that RpoS recognizes the P2eexD 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.
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 algE-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 (P2exd) 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 P1exd promoter is unknown but might be related to the different roles of alginites 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 O2 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.

ACKNOWLEDGEMENTS

We thank Paul Gaytán, Jorge Yañez, Eugenio López and Santiago Becerra for DNA synthesis and sequencing. We also thank A. Trejo for technical support. This article is dedicated to the memory of Prof. Svein Valla.

FUNDING

This work was supported by Consejo Nacional de Ciencia y Tecnología-CONACYT [grant number 255212], Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica—PAPIIT [grant number 207017] and the Research Council of Norway [grant 221576].

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