Two-component system CbrA/CbrB controls alginate production in *Azotobacter vinelandii*

Elva Quiroz-Rocha, † Fernando Bonilla-Badía, † Valentina García-Aguilar, ‡ Liliana López-Pliego, †
Jade Serrano-Román, † Miguel Cocotl-Yañez, ‡ Josefina Guzmán, † Carlos L. Ahumada-Manuel, †
Luis Felipe Muriel-Millán, † Miguel Castañeda, ‡ Guadalupe Espín † and Cinthia Nuñez †,*

Abstract

*Azotobacter vinelandii*, belonging to the *Pseudomonadaceae* family, is a free-living bacterium that has been considered to be a good source for the production of bacterial polymers such as alginate. In *A. vinelandii* the synthesis of this polymer is regulated by the Gac/Rsm post-transcriptional regulatory system, in which the RsmA protein binds to the mRNA of the biosynthetic *alg* gene, inhibiting translation. In several *Pseudomonas* spp. the two-component system CbrA/CbrB has been described to control a variety of metabolic and behavioural traits needed for adaptation to changing environmental conditions. In this work, we show that the *A. vinelandii* CbrA/CbrB two-component system negatively affects alginate synthesis, a function that has not been described in *Pseudomonas aeruginosa* or any other *Pseudomonas* species. CbrA/CbrB was found to control the expression of some alginate biosynthetic genes, mainly *algD* translation. In agreement with this result, the CbrA/CbrB system was necessary for optimal *rsmA* expression levels. CbrA/CbrB was also required for maximum accumulation of the sigma factor RpoS. This last effect could explain the positive effect of CbrA/CbrB on *rsmA* expression, as we also showed that one of the promoters driving *rsmA* transcription was RpoS-dependent. However, although inactivation of *rpoS* increased alginate production by almost 100 %, a *cbrA* mutation increased the synthesis of this polymer by up to 500 %, implying the existence of additional CbrA/CbrB regulatory pathways for the control of alginate production. The control exerted by CbrA/CbrB on the expression of the RsmA protein indicates the central role of this system in regulating carbon metabolism in *A. vinelandii*.

INTRODUCTION

*Azotobacter vinelandii* is a free-living bacterium member of the *Pseudomonadaceae* family [1]. It has been considered to be a good source for the production of polymers of industrial importance, such as the polysaccharide alginate and the polyester poly-β-hydroxybutyrate [2–4].

Alginate, which is synthesized from fructose-6-phosphate, is a linear polymer composed of variable amounts of β-D-mannurionate and its C-5 epimer α-L-guluronate linked by 1–4 glycosidic bonds [2]. In *Pseudomonas aeruginosa*, the production of alginate has been extensively studied due to its role in the pathogenesis of lung infection in cystic fibrosis patients [5]. The biochemistry and genetics of alginate biosynthesis are highly conserved between *P. aeruginosa* and *A. vinelandii* [5, 6]. The main biosynthetic *alg* gene cluster (*algD-8–44 K-J-G-X-L-I-V-F-A*) is headed by *algD*. In addition to the *algD* promoters, internal promoters upstream of *algD* and *algG* have been identified in *A. vinelandii* [3]. Transcription and translation of the *A. vinelandii* *algD* gene, encoding a key enzyme in this pathway, are highly regulated. Transcription is dependent on the stressresponse sigma factors AlgU and RpoS [7–9], whereas *algD* translation is dependent on the Gac/Rsm post-translational regulatory system [10]. In gammaproteobacteria, the Gac/Rsm signal transduction pathway regulates primary and secondary metabolism [11]. The GacS/GacA two-component system (TCS) activates...
the transcription of several small RNAs (sRNAs) of the RsmZ-Y (CsrB-C) family that antagonize the activity of the RsmA (CsrA) protein, a global repressor of genes that are expressed in the stationary growth phase [12, 13]. In *A. vinelandii*, the signal transduction pathway Gac/Rsm controls alginate production. RsmA was found to bind to the *algD* mRNA at a site overlapping the ribosome-binding site [10], and the GacA activator is required for transcriptional activation of the RsmZ-Y sRNAs, relieving the repressing activity of RsmA on *algD* translation [10, 14].

The histidine kinase (HK) CbrA and the response regulator (RR) CbrB constitute a TCS that, together with the Crc system, controls carbon metabolic flow in *Pseudomonas* species, allowing the preferential utilization of good carbon sources and establishing a healthy carbon/nitrogen balance [15–17]. CbrB, belonging to the NtrC family of RR, activates the transcription of sRNAs of the CrcX, CrcY or CrcZ family [18–20]. While the Crc protein, together with the chaperone Hfq, forms a complex with target mRNAs and inhibits translation [17, 21], the Crc sRNAs antagonize such activity. There is evidence indicating that not all activities of the CbrA/CbrB TCS are mediated by the Crc system, as *cbrB* and *crcZ* mutants of *P. aeruginosa* do not exhibit identical phenotypes [15, 16]. The CbrA/CbrB system has several functions besides the control of catabolic pathways. In *Pseudomonas* species such as *P. aeruginosa* and *P. putida*, this system is involved in the regulation of swarming, biofilm formation, cytotoxicity and antibiotic and stress resistance [22, 23].

In the present work, we identified an *A. vinelandii cbrA::miniTn5* mutant by its alginate-overproducing phenotype. Its characterization showed that the TCS CbrA/CbrB of *A. vinelandii* has a negative effect on alginate production by positively affecting *rsmA* mRNA levels. In agreement with this result, *algD* translation was found to be derepressed in a *cbrA* mutant. Moreover, we identified the sigma factor RpoS as one of the intermediaries in the regulatory cascade controlling *rsmA* expression by the CbrA/CbrB TCS.

**METHODS**

**Strains and cultivation conditions**

The bacterial strains and plasmids used in the present work are listed in Table 1. The *A. vinelandii* wild-type strain AEIV [24] was used in this study. Mutant GG15 was identified in a random miniTn5 mutant bank derived from strain AEIV due to its highly mucoid phenotype on plates of solid medium. This mutant carries the miniTn5 transposon inserted within the gene *cbrA* (Avin42670) [25].

*A. vinelandii* was grown in Burk’s nitrogen-free salts supplemented with 20 g L⁻¹ of sucrose (Burk’s sucrose medium) at 30 °C [26]. The composition of the growth medium and the culture conditions have been reported elsewhere [27]. *Escherichia coli* DH5α [28] was grown on Luria–Bertani (LB) medium at 37 °C [29]. The antibiotic concentrations (in µg ml⁻¹) used for *A. vinelandii* and *E. coli*, respectively, were: spectomycin, 50 and 100; tetracycline, 15 and 10; ampicillin, 100 (not used for *A. vinelandii*); gentamicin, 1 and 10.

*A. vinelandii* transformation was carried out as described [30]. To ensure double reciprocal recombination and allelic exchange, mutants constructed by reverse genetics were generated by transforming *A. vinelandii* cells with linear DNA carrying the desired mutation. At least two independent transformation events were conducted, and transformants were selected using the corresponding antibiotic. Three representative transformants were confirmed by PCR analysis to carry the desired mutation, and only one was chosen for further studies. Due to the polyplody of *A. vinelandii*, the absence of wild-type alleles in the resulting strains was verified by PCR amplification of the corresponding locus. To this end, chromosomal DNA, purified from candidates grown in the absence of the antibiotic, was used as the DNA template to favour enrichment of possible wild-type chromosomes.

**Standard techniques**

DNA isolation and cloning were conducted as described [31]. The *A. vinelandii* genome sequence is available [32] and this sequence was used for designing the oligonucleotides used for PCR amplifications. The sequence of the oligonucleotides used in this study is listed in Table 2. High-fidelity Phusion DNA polymerase (Thermo Fisher Scientific) was used for all PCR amplifications, and they were confirmed by DNA sequencing. DNA sequencing was performed with fluorescent dyeoxy terminators using a cycle sequencing method and the 3130xl Genetic Analyzer from Applied Biosystems.

**Construction of mutants CFB03 (**cbrB::Sp**)**

To construct an AEIV derivative carrying a mutation within the *cbrB* gene (Avin42680), an internal fragment of *cbrB* of 754 bp was PCR-amplified using oligonucleotides cbrB-F and cbrB-R (Table 1), and sub-cloned into the pMOSBlue vector (GE Healthcare). An Sp<sup>r</sup> resistance cassette derived from plasmid pHP45Ω [33] was inserted into the *SmaI* site located within the *cbrB* fragment, producing plasmid pFB02. This plasmid was linearized with the *PstI* restriction enzyme and was used to transform competent cells of the wild-type strain AEIV, selecting double recombinants Sp<sup>r</sup>. A representative mutant was confirmed by PCR amplification of the *cbrB* locus, followed by sequencing or endonuclease restriction patterns, to carry the *cbrB::Sp* mutation and to lack wild-type copies of *cbrB* (data not shown); this mutant was named CFB03 (Fig. S1, available in the online Supplementary Material).

**Genetic complementation of mutant GG15**

To complement the GG15 *cbrA::miniTn5* mutant with a wild-type copy of the gene *cbrA*, a Gm resistance cassette derived from plasmid pBSL141 [34] was ligated into the multiple cloning site of plasmid pCN48 carrying the *cbrA* gene [25]. The resultant plasmid was named pLS04. This plasmid, unable to replicate in *A. vinelandii*, was used to...
Table 1. Relevant strains and plasmids used in the present work

<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype/Relevant characteristics</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. vinelandii</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEIV (also name E strain)</td>
<td>Wild-type strain</td>
<td>[24]</td>
</tr>
<tr>
<td>GG15</td>
<td>miniTn5 AEIV derivative, identified by its highly mucoid phenotype. cbrA::miniTn5, Sp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>[25]</td>
</tr>
<tr>
<td>JS05</td>
<td>GG15 derivative, complemented with a wild-type copy of cbrA integrated into the chromosome. Gm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>CFB03</td>
<td>cbrB::Sp mutant derived from AEIV</td>
<td>This work</td>
</tr>
<tr>
<td>EQR02</td>
<td>AEIV derivative carrying an insertion of a Sp&lt;sup&gt;+&lt;/sup&gt; cassette within the cbrA gene</td>
<td>[25]</td>
</tr>
<tr>
<td>AED–gusA</td>
<td>AEIV derivative carries a chromosomal algD–gusA transcriptional fusion. Gm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>CbrAD–gusA</td>
<td>AEIV derivative carries a chromosomal algD–gusA translational fusion. Gm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>AED–algD</td>
<td>AEIV derivative carries a chromosomal algD–gusA transcriptional fusion. Sp&lt;sup&gt;+&lt;/sup&gt;; Gm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>AEd–gusA</td>
<td>AEIV derivative carries a chromosomal algD–gusA translational fusion. Sp&lt;sup&gt;+&lt;/sup&gt;; Gm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>AEdrpoS</td>
<td>AEIV derivative carries an rpoS:Sp mutation. Sp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>[36]</td>
</tr>
<tr>
<td>AEdgacA</td>
<td>AEIV derivative carries a gacA::Gm mutation</td>
<td>[10]</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<td></td>
</tr>
<tr>
<td>pET1.2/blunt</td>
<td>PCR cloning vector; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Thermo Fisher Scientific</td>
</tr>
<tr>
<td>pMOSHue</td>
<td>Cloning vector; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>GE Healthcare</td>
</tr>
<tr>
<td>pGEMT Easy</td>
<td>Cloning vector; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Promega</td>
</tr>
<tr>
<td>pBSL141</td>
<td>Source of the Gm&lt;sup&gt;+&lt;/sup&gt; cassette</td>
<td>[34]</td>
</tr>
<tr>
<td>pH4501–Sp</td>
<td>Source of the Sp cassette</td>
<td>[33]</td>
</tr>
<tr>
<td>pCN48</td>
<td>pMOSHue vector derivative carries a 2.8 kb fragment containing the cbrA gene</td>
<td>[25]</td>
</tr>
<tr>
<td>pLS04</td>
<td>pCN48 derivative carries a Gm&lt;sup&gt;+&lt;/sup&gt; cassette as a selection marker at a BamHI site of the polylinker</td>
<td>This work</td>
</tr>
<tr>
<td>pEB02</td>
<td>pMOSHue derivative carries a cbrB:Sp mutation</td>
<td>This work</td>
</tr>
<tr>
<td>pUMAZ2</td>
<td>pUMATgusAT vector derivative carries a prsmZ2–gusA transcriptional fusion</td>
<td>This work</td>
</tr>
<tr>
<td>pUMATgusAT</td>
<td>Vector for the construction of gusA transcriptional fusions into the A. vinelandii chromosome</td>
<td>[36]</td>
</tr>
<tr>
<td>pUMATgusAPT</td>
<td>Vector for the construction of gusA translational fusions into the A. vinelandii chromosome</td>
<td>[35]</td>
</tr>
<tr>
<td>pUMATgusATGm</td>
<td>pUMATgusAT derivative. Source of the gusA–Gm cassette. Carries a Gm cassette cloned into the HindIII site downstream of the gusA gene</td>
<td>This work</td>
</tr>
<tr>
<td>pUMATgusAPTGm</td>
<td>pUMATgusAPT derivative. Source of the gusA–Gm cassette. Carries a Gm cassette cloned into the HindIII site downstream of the gusA gene</td>
<td>This work</td>
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<tr>
<td>pGD2A</td>
<td>pGEMT Easy derivative carries a 2.4 kb fragment containing algD</td>
<td>This work</td>
</tr>
<tr>
<td>pGD–gusA</td>
<td>pGD2.4 derivative carries an algD–gusA transcriptional fusion. Gm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pGD–algD</td>
<td>pGD2.4 derivative carries an algD–gusA translational fusion. Gm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pfrmA</td>
<td>pET1.2/blunt derivative, carries the regulatory region of rsmA</td>
<td>This work</td>
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</table>

Transform competent cells of mutant GG15, selecting transformants Gm<sup>+</sup>. The presence of the pLS04 plasmid integrated into the chromosome before the cbrA::miniTn5 insertion in mutant GG15 was confirmed by PCR, followed by endonuclease restriction patterns (data not shown). This mutant was named JS05 (Fig. S2).

**Construction of strains carrying a prsmZ2–gusA transcriptional fusion**

A DNA fragment carrying the promoter region of rsmZ2 (prsmZ2) was excised from plasmid pSAHFUTS-Z2 [14] and cloned into the EcoRI site of pUMATgusAT, producing plasmid pUMAZ2. The vector pUMATgusAT is useful for the construction of gusA transcriptional fusions that can be directed to the chromosome after a double recombination event within the melA locus. A Tc<sup>+</sup> cassette adjacent to the gusA gene served as a selection marker [35]. The wild-type strain AEIV and the GG15 mutant were transformed with pUMAZ2 previously linearized with the NdeI endonuclease, and Tc<sup>+</sup> transformants were selected. The AEIV derivative carrying the prsmZ2–gusA transcriptional fusion was named EQR101Z2, while that derived from mutant GG15 was named EQR103Z2. The presence of the prsmZ2–gusA transcriptional fusions in the chromosome was confirmed by PCR amplification followed by endonuclease restriction pattern.
Table 2. Sequence of the oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Nucleotide sequence (5’–3’)</th>
</tr>
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<tbody>
<tr>
<td>RT-2 upRsmA</td>
<td>GTCGGAGAGAACCCTCATGG</td>
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<tr>
<td>RT-2 downRsmA</td>
<td>GACCTCTTGTGGTGCATTGA</td>
</tr>
<tr>
<td>upR-ppoS</td>
<td>AGGATGGTCGAGACGATGAG</td>
</tr>
<tr>
<td>dwR-ppoS</td>
<td>TCCAGGCGCCCTAGTGATGTC</td>
</tr>
<tr>
<td>F-1(cbrA)</td>
<td>GCCCTACCAACTGTGCTCC</td>
</tr>
<tr>
<td>R-1(cbrA)</td>
<td>GCTGATAGCAGTCGAACGC</td>
</tr>
<tr>
<td>cbrB-F</td>
<td>CTGGCAGCTGCTATCCG</td>
</tr>
<tr>
<td>cbrB-R</td>
<td>GCAGTTACCGAGATCGACTG</td>
</tr>
<tr>
<td>RsmAPrim</td>
<td>GACTGTCACATCCATCAC</td>
</tr>
<tr>
<td>UprsmAHind</td>
<td>CAAAGGCTTGGTGGGTATGAT</td>
</tr>
<tr>
<td>DwrsmA Xho</td>
<td>CACTCGAGTGCGTTGCGCT</td>
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<tr>
<td>algD-RT3-F</td>
<td>TCTGCACTGCGCTACAGG</td>
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<tr>
<td>algD-RT3-R</td>
<td>GCCGCTTATGCTTACGCG</td>
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<tr>
<td>alg8-RT-F</td>
<td>CCACTGGAATCTACATCC</td>
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<tr>
<td>alg8-RT-R</td>
<td>GAAACACTGGGAAGATCG</td>
</tr>
<tr>
<td>algC-RT-F</td>
<td>GACGTGCTGCGAGCTTGGAT</td>
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<tr>
<td>algC-RT-R</td>
<td>ATGGGCGCGCCGATGAG</td>
</tr>
<tr>
<td>UpalgD</td>
<td>GACGTGCTGCGAGCTTGGAT</td>
</tr>
<tr>
<td>DSalgD</td>
<td>GTCGGGAGAGAACCCTCATGG</td>
</tr>
</tbody>
</table>

Construction of transcriptional and translational algD–gusA fusions

To generate algD–gusA transcriptional and translational fusions within the native algD gene, we constructed gusA-Gm cassettes for transcriptional (gusA–Gm) or translational (gusA–Gm) fusions. A HindIII fragment carrying the Gm interposon from vector pBSL141 [34] was ligated to the HindIII-digested plasmids pUMATcngusAT [36] and pUMATcngusAPT [35] to generate the plasmids pUMATcngusATGm and pUMATcngusAPTGm, respectively (Figs S3 and S4). The generated transcriptional (gusA-Gm) and translational (gusA-Gm) cassettes can be released with either the SacI or the SacI restriction enzyme.

A 2.4 kb fragment containing algD was amplified by PCR using the primers UpalgD and DSalgD and it was subsequently cloned into the pGEMT Easy Vector (Promega), producing plasmid pGD2.4 (Table 1). SacI fragments carrying the gusA-Gm and gusA-Gm cassettes were ligated to plasmid pGD2.4 previously digested with the same enzyme, disrupting the algD gene within codon 96. The resulting plasmids, called pGD-gusA and pGD-gusA, respectively, were verified by PCR analysis and DNA sequencing to carry the cassettes inserted in the same orientation as that of algD transcription. In the case of plasmid pGD-gusA, we also verified the correct construction of the algD–gusA translational fusion. Plasmids pGD-gusA and pGD-gusA were made linear with SphI endonuclease and used to transform competent cells of the strain AEIV. Double recombinants Gm were selected and verified by PCR analysis and DNA sequencing to carry the desired constructions within the algD locus (data not shown). Furthermore, segregation of the algD–gusA fusions to all the chromosomal copies was verified by PCR analysis using the primers UpalgD and DSalgD, confirming the absence of wild-type algD alleles. The strains generated, carrying transcriptional or translational algD–gusA fusions, were named AED-gusA and AED-gusA, respectively.

To construct cbrA mutants carrying transcriptional and translational fusions of algD with the reporter gene gusA, the strains AED-gusA and AED-gusA were employed. The mutant EQR02 (cbrA::Sp) was transformed with chromosomal DNA from the strains AED-gusA and AED-gusA and transformants Gm were selected. The resulting cbrA::Sp derivative strains were named CbrAD-gusA and CbrAD-gusA, respectively. PCR amplification of the algD locus, followed by DNA sequencing, confirmed the corresponding constructions and their segregation to all the chromosomal copies of A. vinelandii (data not shown).

Quantitative real-time reverse transcription (qRT-PCR)

A. vinelandii strains were cultured in Burk’s sucrose liquid medium. The cells were collected by centrifugation, and the total RNA was extracted as described [37]. Genomic DNA contamination was removed with DNase I (Thermo Fisher Scientific). Details of cDNA synthesis and qRT-PCR amplification conditions are reported elsewhere [30]. qRT-PCR assays were performed with a Light Cycler 480 II instrument (Roche), using the Maxima SYBR Green/ROX qPCR Master Mix (2X) kit (Thermo Fisher Scientific). The sequences of the primer pairs used for the genes algD (algD-RT-F and algD-RT-R), alg8 (alg8-RT-F and alg8-RT-R), algC (algC-RT-F and algC-RT-R), rsmA (RT-upRsmA and RT-downRsmA), rpoS (upR-ppoS and dwR-ppoS) and gyrA (gyrAforward and gyrA reverse) are listed in Table 2. These primers were designed using the Primer3 program (http://bioinfo.ut.ee/primer3/), with an optimal length of 20 bases and a melting temperature of 60°C. Verification of specific single-product amplification by melting-curve analyses validated each primer set. Thereafter the efficiency of PCR was estimated by developing standard curves for each amplicon using dilution series of the cDNA corresponding to the reference sample. cDNAs derived from the reference and experimental samples were amplified using quantities within the linear range of the standard curve. Three biological replicates (independent cell cultures) were performed with three technical replicates for each one. Similar results were obtained for the transcription of all measured genes in the repetitions. As in previous reports, relative mRNA transcript levels were determined in relation to gyrA (Avin15810) mRNA [27, 30, 38]. A non-template control for each reaction was included for each gene. The quantification technique used to analyse the generated data was the 2ΔΔCT method reported previously [39].

Primer extension assays

The transcriptional start site of rsmA was mapped by primer extension analysis. Total RNA was prepared as previously reported [37] from the A. vinelandii wild-type strain and its
**RESULTS**

**Mutants lacking the HK CbrA or the RR CbrB overproduce alginate**

In a different work, we reported the characterization of the *A. vinelandii* TCS CbrA/CbrB on carbon catabolite repression. It was achieved by characterizing a *cbrA::miniTn5* mutant, named GG15, generated by random mutagenesis of the wild-type strain AEIV. Our results demonstrated that the CbrA/CbrB system is conserved between *A. vinelandii* and *Pseudomonas* species and heads a regulatory cascade that, along with the translational regulatory system Hfq/Crc, controls carbon catabolite repression [25].

The *A. vinelandii* mutant GG15 (*cbrA::miniTn5*) was originally identified by its alginate-overproducing phenotype under diazotrophic growth on plates of Burk’s sucrose medium. In the present work, the effect of the TCS CbrA/CbrB on alginate production was investigated. As shown in Fig. 1(a), there was a statistically significant increase of approximately fivefold in specific alginate production by the mutant GG15 in liquid Burk’s sucrose medium relative to its parental AEIV strain. Genetic complementation of the mutant GG15 with a wild-type copy of *cbrA* integrated into the chromosome was conducted; the resulting strain,
named JS05, showed wild-type levels of alginate. In addition, the mutant EQR02 carrying a cbr::Sp mutation and generated by reverse genetics [25] showed alginate levels (5170 µg alginate per mg of protein) similar to those of the mutant GG15, thus indicating that the HK CbrA exerts a negative effect on the production of this polymer.

The A. vinelandii cbrA locus is highly conserved with respect to Pseudomonas species [25]. The cbrB gene, encoding the CbrA cognate RR, is located immediately downstream of cbrA. To investigate the role of the RR CbrB in alginate production, the strain CF03, carrying a cbrB::Sp mutation, was also constructed. As shown in Fig. 1(a), this mutant exhibited 10-fold higher levels of alginate production than the wild-type strain and twofold higher levels than the cbrA mutant GG15. Collectively, these data indicated that the TCS CbrA/CbrB negatively affects alginate production in A. vinelandii.

Fig. 1(b) shows the growth kinetics of the strains AEIV, GG15, CF03 and JS05 cultured in liquid Burk’s sucrose medium. The cbrB::Sp mutant CF03, but not the cbrA::miniTn5 mutant GG15, showed an 8 h lag phase and reached a lower protein concentration than the parental strain AEIV. This result indicated that the lack of the RR CbrB is deleterious for cell growth under our assay conditions. For this reason, we continued to study the role of the CbrA/CbrB system principally by characterizing the effect of the HK CbrA.

Effect of CbrA on the expression of alg genes

We next investigated whether the increase in alginate production observed in mutant cbrA was due to higher expression of alginate biosynthetic genes. qRT-PCR assays were conducted to estimate algD, alg8 and algC expression using total RNA extracted from cells grown for 24 h. As shown in Fig. 2(a), alg8 mRNA levels increased threefold in the cbrA::miniTn5 mutant, but the levels of algD and algC transcripts were approximately twofold higher with respect to the wild-type strain; as expected, in the complemented JS05 strain, expression levels of the mRNA of these alg genes were reduced to wild-type levels.

The small increase in algD transcription in the cbrA mutant was somewhat unexpected, as alginate overproduction is commonly associated with elevated algD expression [27, 30, 45]. Therefore, expression of the algD gene was investigated using transcriptional (algD–gusA) and translational (algD¢–gusA) fusions constructed by inserting the corresponding gusA–Gm cassettes into the algD gene. As shown in Fig. 2(b) with respect to the wild-type strain, algD showed a slight increase in transcription, of less than twofold in the cbrA genetic background, whereas the activity of the algD¢–gusA translational fusions was higher along the entire growth curve in the cbrA genetic background, and such an increase was more pronounced (about fivefold) at the exponential growth phase relative to the wild-type strain (Fig. 2c). Taken together, these data indicated that in addition to affecting the transcription of alg8 and algC negatively, the HK CbrA affects the expression of algD negatively, mainly at the translational level.

The TCS CbrA/CbrB is required for maximum transcription of the gene rsmA

It was previously shown that in A. vinelandii, RsmA binds to the algD mRNA, inhibiting translation, and that the repressor activity of RsmA is antagonized by sRNAs of the RsmZ and RsmY families [10, 14]. Because algD translation was increased in the cbrA genetic background, we explored whether the control of algD translation by the TCS CbrA/CbrB was directly involved in the RsmA protein. Therefore, the effect of CbrA on rsmA gene expression was determined. rsmA mRNA levels were assessed by qRT-PCR using total RNA extracted from cells grown in Burk’s sucrose medium for 24 h. In both the cbrA and cbrB mutants, there were statistically significant reductions in the levels of rsmA mRNA (Fig. 3a). However, this reduction was more pronounced in the cbrB genetic background, in which the levels of the rsmA mRNA decreased by approximately 70% with respect to the parental AEIV strain. This result is consistent with the higher alginate levels observed for the mutant cbrB when compared to the mutant cbrA.

In addition, the activity of the GacA/GacS system was evaluated by determining the expression of rsmZ2, a gene under the control of GacA [10, 14]. To this end, a transcriptional fusion of the rsmZ2 promoter with the gusA reporter gene (prsmZ2–gusA) was employed. Derivatives of the wild-type strain AEIV and mutant EQR02 (cbrA::Sp) carrying this transcriptional fusion were cultured in Burk’s sucrose medium, and the activity of β-glucuronidase was measured along the growth curve. The activity of the rsmZ2 promoter in the cbrA genetic background was not affected and showed an expression pattern similar to that in the wild-type strain (Fig. 3b). As expected, such activity was abolished in a gacA genetic background. This result indicated that the cbrA mutation does not affect the activity of RsmA by means of increasing rsmZ transcription.

The sigma factor RpoS directs the transcription of the gene rsmA

As the TCS CbrA/CbrB was necessary for maximum rsmA expression, and since CbrB activates RpoN-dependent promoters, we investigated the nature of the promoter(s) driving rsmA transcription. To this end, rsmA primer extension analysis was conducted using total RNA extracted from the A. vinelandii wild-type strain. As shown in Fig. 4(a), a transcription start site was identified 83 nt upstream of the ATG translation initiation codon. An analysis of the region allowed the identification of a consensus sequence for putative RpoS-dependent promoters (CAATACT) at the −10 region [46, 47] (Fig. 4b). Thus, we carried out a primer extension assay using RNA extracted from the rpoS mutant. As shown in Fig. 4(a), a transcription initiation at this site was not detected. Additionally, qRT-PCR assays using total RNA extracted from the strain AEpoS showed that expression of rsmA diminished by 40% relative to the wild-type

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strain (Fig. 3a). Taken together, these data indicate the existence of an RpoS-dependent promoter driving rsmA transcription.

Because we were unable to detect RpoN-dependent promoters at the regulatory region of rsmA, we hypothesized that the effect of the TCS CbrA/CbrB on rsmA transcription could be due, in part, to it indirectly affecting the expression of RpoS. We next analysed this possibility.

**TCS CbrA/CbrB is required for optimal accumulation of RpoS**

To investigate the possible effect of the TCS CbrA/CbrB on rpoS expression, the levels of the rpoS mRNA were assessed by qRT-PCR in the wild-type strain AEIV and in its derivative mutants cbrA and cbrB. As shown in Fig. 5(a), there were statistically significant reductions of approximately 40 and 80% in the rpoS mRNA levels in the cbrA and the cbrB mutants, respectively, when compared to those of the wild-type strain.

Western blot analysis using antibodies against the A. vinelandii RpoS protein were also conducted. As shown in Fig. 5(b), RpoS protein levels were reduced by approximately 30% in the cbrA mutant with respect to the wild-type strain, whereas in the JS05-complemented strain, the RpoS protein was restored to levels similar to those observed in the wild-type strain. The change in RpoS expression was more pronounced in the cbrB mutant, with a reduction of approximately 70%, which was in agreement with the results obtained by qPCR. Taken together, these results imply that the TCS CbrA/CbrB exerts a positive effect on RpoS expression.

The data presented above suggest the functioning of a CbrA/B-RpoS-RsmA regulatory cascade for the control of alginate production. Therefore, the question of whether RpoS is the only intermediary in this regulatory pathway was raised. Thus, the alginate phenotype was analysed in the rpoS mutant AErpoS. As has been reported for other A. vinelandii strains [48], the levels of alginate in the rpoS mutant AErpoS were significantly higher when compared to the wild-type strain (1685 versus 1090 μg of protein⁻¹, respectively) (Fig. 1a). However, this increase was not as high as that observed in the cbrA or cbrB mutants, indicating that in addition to the CbrA/B-RpoS-RsmA cascade, the TCS CbrA/CbrB controls alginate production by one or more additional pathways.

**DISCUSSION**

In the present work, we show that the A. vinelandii TCS CbrA/CbrB exerts a negative effect on alginate synthesis. Interestingly, this role has not been described in *Pseudomonas* spp. Although the biochemistry of alginate production is well conserved between *P. aeruginosa* and *A. vinelandii*, remarkable differences exist in terms of its regulation. As opposed to *P. aeruginosa*, the algD gene in *A. vinelandii* is a target of the translational repressor protein RsmA [10]. GacA is necessary to activate the transcription of the sRNAs of the Rsm family, which counteract RsmA activity [14]. Therefore, in *A. vinelandii* gacA mutants alginate production is blocked due to translational repression of algD by RsmA [7, 10]. Differences in the regulation of alginate synthesis could be attributed to the different roles that alginate plays in the physiology of these members of the Pseudomonadaceae family. In *A. vinelandii*, alginate is a structural part of the layers surrounding differentiated cells called cysts and is essential for their resistance to desiccation. However, this differentiation process does not occur in *Pseudomonas* spp.

In *A. vinelandii*, the cbrA mutant showed a significant five-fold increase in alginate production compared with the parental AEIV strain, while the cbrB mutant exhibited twofold higher alginate levels relative to those of mutant cbrA. This behaviour is expected, as in the absence of an HK the
be attributed to increased transcription of some alg genes, such as alg8 and algD, but mainly to increased algD translation.

As mentioned above, the translation of algD in *A. vinelandii* is under the control of the RsmA translational repressor protein, which binds to the leader of the algD mRNA, forming a stable ribonucleoprotein complex [10]. Accordingly, in the cbrA mutant, showing from two- to fivefold higher algD translational levels along the growth curve, *rsmA* transcripts were 40% reduced. The difficulty of isolating an *rsmA* null mutant in *A. vinelandii* was reported previously [10]. The observed growth defect of the mutant cbrB in Burk’s sucrose medium might be related to the strong reduction in *rsmA* mRNA levels (of approximately 70%) causing 10-fold higher alginate production levels relative to its parental AEIV strain.

Primer extension analysis indicated that *rsmA* transcription initiates from an RpoS promoter. However, our data suggest the existence of additional promoter(s) driving *rsmA* expression, as in an *rpoS* mutant *rsmA* mRNA levels were only reduced by 40%. In fact, the *rsmA* gene in *E. coli* is transcribed from five promoters, and one of them is RpoS-dependent [51].

The positive effect of the CbrA/CbrB system upon *rsmA* expression might be indirect, since we did not identify putative CbrB binding sites in the operator/promoter region of *rsmA*. Therefore, the effect of CbrA/CbrB on RpoS expression was investigated. Both Western blot analysis and qPCR assays indicated that the TCS CbrA/CbrB
was necessary for maximum expression of RpoS, as the rpoS mRNA and RpoS protein levels were reduced in the cbrA and cbrB mutants. Although the positive effect of CbrA/CbrB on RpoS was clear, this effect was partial and would not completely explain the effect of CbrA/CbrB on rsmA transcription. In support of this result, the specific alginate production by the rpoS mutant AErpoS was not comparable to that of the mutant cbrA. Taken together, these data revealed the existence of the regulatory cascade CbrA/CbrB-RpoS-RsmA for the control of alginate production, but also provided evidence suggesting that CbrA/CbrB controls RsmA expression through an alternative mechanism (Fig. 6).

In a different study, we reported that the CbrA/CbrB-Crc/Hfq system is functionally conserved in A. vinelandii with respect to its role in the process of carbon catabolite repression in Pseudomonas spp. [25]. Although glucose uptake in A. vinelandii occurs through a novel GluP transporter that is uncommon in Pseudomonas species, we found that its expression was under the control of the CbrA/CbrB-Crc/Hfq system [25]. In E. coli, the existence of regulatory connections between the carbon catabolite repression and Csr (Rsm) systems has been established. The EIIA^nc regulatory protein of the PTS system and the cAMP-CRP transcriptional factor influence the expression and accumulation of the sRNAs CsrB and CsrC, which antagonize the activity of CsrA (RsmA) protein. This allows the Csr system to respond to the carbon nutritional status of the bacterium and reorganize the metabolism of the cell [52, 53]. Whether in A. vinelandii the Crc/Hfq system is an intermediary in the regulation of rsmA expression and alginate production by the TCS CbrA/CbrB remains to be investigated.

The concerted action of the signalling systems CbrA/CbrB and GacS/GacA to control the activity and the amount of the RsmA protein, which in turn regulates alginate production, indicates that these systems have a central role in regulating carbon metabolism in A. vinelandii. Exploring the function of the A. vinelandii CbrA/CbrB-Crc/Hfq system in alginate production would contribute to our understanding of the regulation of the carbon metabolic flow in this bacterium.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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