Roles of RpoS and PsrA in cyst formation and alkylresorcinol synthesis in *Azotobacter vinelandii*

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_Azotobacter vinelandii_ is a soil bacterium that undergoes differentiation to form cysts that are resistant to desiccation. Upon induction of cyst formation, the bacterium synthesizes alkylresorcinols that are present in cysts but not in vegetative cells. Alternative sigma factors play important roles in differentiation. In *A. vinelandii*, AlgU (sigma E) is involved in controlling the loss of flagella upon induction of encystment. We investigated the involvement of the sigma factor RpoS in cyst formation in *A. vinelandii*. We analysed the transcriptional regulation of the _rpoS_ gene by PsrA, the main regulator of _rpoS_ in *Pseudomonas* species, which are closely related to *A. vinelandii*. Inactivation of _rpoS_ resulted in the inability to form cysts resistant to desiccation and to produce cyst-specific alkylresorcinols, whereas inactivation of _psrA_ reduced by 50 % both production of alkylresorcinol and formation of cysts resistant to desiccation. Electrophoretic mobility shift assays revealed specific binding of PsrA to the _rpoS_ promoter region and that inactivation of _psrA_ reduced _rpoS_ transcription by 60 %. These results indicate that RpoS and PsrA are involved in regulation of encystment and alkylresorcinol synthesis in *A. vinelandii*.

INTRODUCTION

*Azotobacter vinelandii* is a soil bacterium that undergoes a differentiation process in its life cycle, resulting in cyst formation. 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. When growing on glucose or sucrose, cysts are formed at late stationary phase by 0.01 % of the cells (Lin & Sadoff, 1968). However, encystment can be induced in a higher percentage of cells (10 % or more) by removing glucose or sucrose from exponentially growing cultures and replacing the glucose or sucrose with either n-butanol or β-hydroxybutyrate as the sole carbon source (Lin & Sadoff, 1968).

The extracellular polysaccharide alginate is a major component of the exine and intine layers of the cyst (Sadoff, 1975) and is essential for the differentiation process; mutations in alginate biosynthetic genes impair the formation of cysts (Campos _et al._, 1996; Mejía-Ruiz _et al._, 1997). 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 & Sadoff, 1983). Alkylresorcinols play a structural role in the exine, as strains carrying mutations in alkylresorcinol biosynthetic genes produce cysts with a defective exine. However, despite the defective exine layer, the cysts remain resistant to desiccation (Segura _et al._, 2009).

Alternative sigma factors play important roles in bacterial differentiation and adaptation to a variety of environmental conditions. In *A. vinelandii*, the sigma factor AlgU participates in the encystment process by controlling both alginate synthesis (Moreno _et al._, 1998) and the loss of flagella that occurs upon cyst formation (León & Espín, 2008). Mechanistically, AlgU promotes the transcription of the _algC_ gene, which is involved in alginate biosynthesis, and _cydR_, which is involved in repressing transcription of the flagella regulator FlhDC (Gaona _et al._, 2004; León & Espín, 2008).

The sigma factor RpoS is a central regulator during stationary phase in bacteria (for a recent review see Navarro Llorens _et al._, 2010). In *Pseudomonas* species, which are phylogenetically closely related to *A. vinelandii* (Setubal _et al._, 2009), RpoS regulates quorum sensing, virulence and many stationary phase genes (Schuster _et al._, 2008).

**Abbreviations:** EMSA, electrophoretic mobility shift assay; qRT-PCR, quantitative RT-PCR.

A supplementary table of primer sequences is available with the online version of this paper.
Regulation of rpoS in Pseudomonas differs from its regulation in Escherichia coli (Venturi, 2003). In Pseudomonas aeruginosa and Pseudomonas putida, rpoS is transcribed from a typical sigma 70-dependent promoter located within the upstream nlpD gene. Transcription of rpoS increases upon entry into stationary phase and is activated by PsrA, a regulator of the TetR family (Kojic & Venturi, 2001; Kojic et al., 2002). PsrA specifically binds to DNA regions in the rpoS and psrA promoters containing C/G AAAAC N2-4 GTTTG/C sequences (Kojic et al., 2002). PsrA activates the exsCEBA operon of the type III secretion system (Shen et al., 2006) involved in the secretion of exotoxins. PsrA also acts as an auto-repressor, and as a repressor of the fadBA5 β-oxidation operon (Kojic & Venturi, 2001; Kang et al., 2008). Repression of fadBA5 is relieved by PsrA binding to long-chain fatty acids (Kang et al., 2008), which also decreases rpoS and exsC expression (Kang et al., 2009).

In the cyst-forming bacterium A. vinelandii, RpoS is required for the activation of one of the three promoters driving transcription of the alginate biosynthetic gene algD (Castañeda et al., 2001), and for activation of one of the two promoters of phbB, a gene involved in polyhydroxybutyrate biosynthesis (Peralta-Gil et al., 2002). Under non-desiccatizing solid-medium growth conditions, the non-mucoid A. vinelandii strain UW136 remained viable for 16.5 years, while its rpoS mutant strain remained viable for only 10 months (Sandercock & Page, 2008). RpoS was also required for survival under oxidative stress and in conditions of either carbon or nitrogen starvation (Sandercock & Page, 2008).

As mentioned above, a low percentage of encystment occurs in late stationary phase A. vinelandii cultures. Therefore, it is possible that RpoS is involved in the encystment process. The aim of this study was to investigate the role of RpoS in the formation of cysts and the role of PsrA in the regulation of rpoS expression in A. vinelandii.

### METHODS

**Microbiological procedures.** Bacterial strains and plasmids used are listed in Table 1. Oligonucleotides used are listed in Supplementary Table S1, available with the online version of this paper. A. vinelandii was grown at 30 °C in Burk’s nitrogen-free salts medium (Kennedy et al., 1986) supplemented with 2% sucrose (BS) or 0.2% n-butanol (BBOH). E. coli strains DH5α and Top10 were grown on Luria–Bertani medium (LB) at 37 °C. Antibiotic concentrations used for A. vinelandii and E. coli, respectively, were as follows: ampicillin, 100 µg ml⁻¹; nalidixic acid, 30 and 0 µg ml⁻¹; spectinomycin, 50 and 50 µg ml⁻¹; and kanamycin, 1 and 30 µg ml⁻¹. Transformation and conjugation of A. vinelandii were carried out as previously described by Page & von Tigerstrom (1978) and Bali et al. (1992). Alkylresorcinol production was measured as described previously (Segura et al., 2003).

**Construction of psrA mutant strain.** Primers upLexA and lwPsrA were used to amplify a PCR fragment of 1.5 kb containing the

### Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference or source</th>
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<tr>
<td><strong>A. vinelandii strains</strong></td>
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<tr>
<td>ATCC 9046</td>
<td>Wild-type</td>
<td>Laboratory collection</td>
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<tr>
<td>CNS59</td>
<td>ATCC 9046 derivative carrying an rpoS::Sp mutation</td>
<td>Castañeda et al. (2001)</td>
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<td>ATCC 9046 derivative carrying a psrA::Sp mutation</td>
<td>This study</td>
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<td>This study</td>
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<tr>
<td>CNS59/pBBR1MCS-2</td>
<td>CNS59 harbouring plasmid pBBR1MCS-2</td>
<td>This study</td>
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<td>OV11</td>
<td>SW136 derivative carrying an ara::Tn5::gusA40 mutation</td>
<td>Segura et al. (2009)</td>
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<td><strong>E. coli strains</strong></td>
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</tr>
<tr>
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<td>Hanahan (1983)</td>
</tr>
<tr>
<td>Top10</td>
<td>F’ mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (StrR) endA1 supG</td>
<td>Invitrogen</td>
</tr>
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<td>LMG194</td>
<td>F’ ΔlacX74 galE thi rpsL ΔphoA (Pvu II) Δara714 leu::Tn10</td>
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<td><strong>Plasmid</strong></td>
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</tr>
<tr>
<td>pMOSBlue</td>
<td>Cloning vector</td>
<td>Amersham</td>
</tr>
<tr>
<td>pBAD-TOPO</td>
<td>Cloning and expression vector</td>
<td>Invitrogen</td>
</tr>
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<td>pBBR1MCS-2</td>
<td>Cloning vector with a kanamycin resistance cassette</td>
<td>Kovach et al. (1995)</td>
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<tr>
<td>pMPO1</td>
<td>pBSL97 derivative with an Sp-gusA cassette</td>
<td>Peralta-Gil et al. (2002)</td>
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<td>pASpsRA</td>
<td>pMOSBlue containing a 1.5 bp fragment with the psrA gene</td>
<td>This study</td>
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<td>pASpsRA::Sp</td>
<td>pASpsRA containing a psrA::Sp mutation</td>
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<td>pMrpoS</td>
<td>pMOSBlue containing 413 bp corresponding to the rpoS promoter region</td>
<td>This study</td>
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<td>pMrpoS-2</td>
<td>pMOSBlue containing 622 bp corresponding to the rpoS promoter region</td>
<td>This study</td>
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<td>pMpsrA</td>
<td>pMOSBlue containing 317 bp corresponding to the psrA promoter region</td>
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<td>pMpsrAhis</td>
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<td>This study</td>
</tr>
<tr>
<td>pSMrpoS</td>
<td>pBBR1MCS-2 containing the rpoS gene under the Km promoter</td>
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complete psrA gene by using chromosomal DNA of A. vinelandii ATCC 9046 as a template. This fragment was ligated into the pMO5Blue vector (Amersham) to obtain plasmid pASpsrA. An XhoI digest of pASpsrA was ligated to a XhoI fragment containing the spectinomycin cassette excised from vector pMP01. This generated plasmid pASpsrA::Sp, which was transformed into strain ATCC 9046. A spectinomycin-resistant transformant (ATpsrA) generated by a double recombination event was isolated and confirmed as carrying the psrA::Sp mutation by Southern blot analysis and PCR by using primers upLecA and lpPsrA (data not shown).

**Construction of plasmids pMrpoS, pMrpoS-2, pMpsrA and pSMrpoS.** A 413 bp fragment corresponding to the promoter region of rpoS (nucleotides −281 to +132), a 622 bp fragment of the promoter region of rpoS (nucleotides −404 to +208) and a 317 bp fragment of the promoter region of psrA (nucleotides −225 to +92) were amplified by PCR using primers rpoSmiguF/rpoSmiguR, emsaSup/dwRT-rpoS and psrAmiguF/psrAmiguR, respectively. The products were cloned into pMO5Blue in accordance with the manufacturer’s instructions (Amersham), resulting in plasmids pMrpoS, pMrpoS-2 and pMpsrA. A promoter-less rpoS gene flanked by HindIII and BamHI restriction sites was amplified by PCR by using primers rpoScod-up and rpoScod-lw. The product was cloned into plasmid pBR1MCS-2 digested with the HindIII and BamHI enzymes. This produced plasmid pSMrpoS with rpoS under the control of the kanamycin promoter, pSMpsrA, which was able to replicate in A. vinelandii, was transferred by conjugation into strain CNS59 to produce strain CNS59/pSMrpoS.

**Nucleic acid procedures.** DNA isolation, cloning and random primer procedures were carried out as described by Sambrook (1989). DNA sequencing was done with a Perkin Elmer/Applied Biosystems DNA sequencer.

**Quantitative RT-PCR (qRT-PCR).** Expression of rpoS and psrA was measured by qRT-PCR, as previously reported (Noguez et al., 2008). For RNA extraction, cultures were grown in BS or BBOH media. Cells were collected at the stationary phase of growth. The primers used for the qRT-PCR assays (Supplementary Table S1) were as follows: upRT-rpoS/dwRT-rpoS for rpoS expression, upRT-psrA/dwRT-psrA for psrA expression and fw-gyrA/rev-gyrA for gyrA expression. Expression of gyrA was used as an internal control to normalize the results. All assays were performed in triplicate. The data are presented as fold changes (mean ± SD) of mRNA levels of the mutant strain relative to those of the wild-type.

**Cloning and purification of His6-PsrA.** To express and purify PsrA, a 714 bp fragment corresponding to the psrA gene lacking the first and last codons was amplified by PCR using high fidelity Taq polymerase (Invitrogen), and primers uppsrAprot and dwpsrAprot. The resulting PCR product was ligated into plasmid pBAD-TOPO (Invitrogen). The ligation mix was transformed into E. coli Top10 (Invitrogen) and a transformant carrying plasmid pMpsrAhis was isolated. The plasmid was isolated, sequenced to confirm the correct transcriptional start sites were rpoSprim1 and psrAprim, and the cDNAs were end-labelled with [32P]-dATP by using polynucleotide kinase (Roche). The sequencing ladders were generated with the same primers by using a Thermo Sequenase Cycle Sequencing kit (USB) and plasmid pMrpoS, pMrpoS-2 or pMpsrA as template. High-resolution S1 nuclease mapping was carried out as previously reported (Peralta-Gil et al., 2002). This fragment was obtained by PCR using primers emsaRini and emsaRbo2. DNA binding reactions were carried out at room temperature for 25 min, and the samples were then subjected to native PAGE in 6% polyacrylamide gels in a buffer containing 90 mM Tris, 90 mM H3BO3 and 2 mM EDTA. The gel was dried and radioactive signals were detected by autoradiography.

**Regulation of rpoS and psrA transcription**

**DNA gel mobility shift assays.** A 225 bp and a 263 bp DNA fragment, corresponding to the intergenic rpoS-psrD and psrA–lexA intergenic regions, respectively, were amplified from A. vinelandii ATCC 9046 chromosomal DNA by PCR using primers emsaSup/ emsaSdnw for the rpoS region and emsaSup/psrAprim for the psrA region. DNA binding reactions were carried out in a total volume of 20 µl. The reactions contained the DNA binding buffer [10 mM Tris/ HCl, 50 mM KCl, 1 mM DTT, 0.5 mM EDTA, 5% (v/v) glycerol and 10 µg BSA µl−1], and 100 nM of each DNA fragment was labelled with [32P]-dCTP and variable amounts of PsrA (0–3 µM). As a negative control a 245 bp fragment of DNA corresponding to the promoter region of phbR was used (Peralta-Gil et al., 2002). This fragment was obtained by PCR using primers emsaRini and emsaRbo2. DNA binding reactions were carried out at 42 °C by using AMV reverse transcriptase (Roche) with primers rpoSprim1, emsaSdnw and psrAprim, and the cDNAs were end-labelled with [32P]-dCTP by using polynucleotide kinase (Roche). The sequencing ladders were generated with the same primers by using a Thermo Sequenase Cycle Sequencing kit (USB) and plasmid pMrpoS, pMrpoS-2 or pMpsrA as template. High-resolution S1 nuclease mapping was carried out as previously reported (Peralta-Gil et al., 2002). The labelled primers used to map the rpoS transcriptional start sites were rpoSprim1 and emsaSdnw and the unlabelled primers were rpoSprim1-S1up and emsaSup for Pr1 and Pr2, respectively; these primers generated a 302 and 225 bp rpoS probe. For psrA, the labelled primer was psrAprim and the unlabelled primer was emsaSup, generating a 263 bp psrA probe.

**Encystment and electron microscopy assays.** Encystment was induced by transferring washed cells from 36 h BS liquid cultures to BBOH medium plates. Desiccation-resistance assays were carried out as described previously (Campos et al., 1996; Segura et al., 2009). Cells were collected after 5 days in the induction medium and were desiccated at 30 °C on 0.2 µm membranes for 5 days. Surviving cells, quantified by viable cell count, were considered mature cysts. Electron microscopy was carried out as previously reported (Mejia-Ruiz et al., 1997).

**Hydrogen peroxide sensitivity.** This assay was conducted as described by Sanders and Page (2008), with some modifications. A. vinelandii cultures were grown to stationary phase (48 h) in liquid BS medium. At this time the cultures were centrifuged, and the cell pellet was washed twice with sterile Burk’s buffer salts to remove the alginate. Cells were resuspended in an appropriate volume to obtain (approximately) 2.0 × 106 c.f.u. ml−1. Hydrogen peroxide was added to a final concentration of 300 mM, and after 20 min of incubation at 30 °C, viable cells were determined by serial dilutions to detect survival above 0.001%.

**RESULTS**

**Encystment in the rpoS and psrA mutants**

To study the effect of rpoS and psrA mutations on encystment, strain ATpsrA carrying a psrA::Sp mutation was constructed as described in Methods. When grown in...
BS medium, the psrA mutant strain ATpsrA and rpoS mutant strain CNS59 showed growth rates and alginate production similar to the parental wild-type strain ATCC 9046 (data not shown). To induce synchronous encystment, A. vinelandii psrA and rpoS mutant strains were cultured in liquid BS medium and then transferred to BBOH plates. The rpoS mutant strain CNS59 was unable to produce mature cysts resistant to desiccation; however, the psrA mutant formed mature desiccation-resistant cysts, although to a reduced percentage compared with the wild-type (Table 2). Electron microscopic examination of the cysts formed by the rpoS and psrA mutants was performed (Fig. 1a). Cysts of the psrA mutant strain were similar to mature cysts of the wild-type strain ATCC 9046; cysts were composed of the compacted cell (central body containing mature cysts of the wild-type strain ATCC 9046; cysts were (Fig. 1a). Cysts of the mutant strain CNS59 formed mature desiccation-resistant cysts, whereas colonies of the rpoS mutant developed a red colour, indicative of the presence of alkylresorcinols, whereas colonies of the rpoS or arsA mutant (carrying a mutation in the alkylresorcinol biosynthetic gene arsA, used as a negative control) remained white (Fig. 1b). Alkylresorcinols were quantified in these strains after 5 days of incubation in BBOH liquid medium. Whereas the psrA mutant strain ATpsrA produced 50% of the alkylresorcinols produced by the wild-type strain ATCC 9046, no alkylresorcinols were detected in the rpoS mutant strain CNS59 (Table 2), confirming the inability of this strain to produce these phenolic lipids. Plasmid pSMrpoS carrying a wild-type copy of rpoS but not the vector pBBR1MCS-2 restored the ability of strain CNS59 to encyst and to produce alkylresorcinols (Fig. 1b, Table 2). These results indicate that RpoS and PsrA play a role in the formation of mature cysts that are resistant to desiccation.

Sensitivity of rpoS and psrA mutants to hydrogen peroxide

In strain UW, a non-mucoid derivative of the A. vinelandii strain OP, inactivation of rpoS reduced survival to hydrogen peroxide stress, and stationary phase cells were rapidly killed by exposure to 15 mM H₂O₂ (Sandercock & Page, 2008). Hydrogen peroxide sensitivity was tested in the wild-type strain ATCC 9046 and its rpoS and psrA mutant derivatives. Following 20 min of exposure to 300 mM H₂O₂ there was 86 ± 8% survival of wild-type cells and 95 ± 5% survival of psrA mutant cells. However, no viable cells of the rpoS mutant strain CNS59 were recovered. Plasmid pSMrpoS, but not vector pBB1MCS-2, restored survival of strain CNS59 to 75 ± 5%. Taken together, these results suggest that RpoS, but not PsrA, plays a role in survival of A. vinelandii following hydrogen peroxide stress.

### Sensitivity of rpoS and psrA mutants to hydrogen peroxide

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Encystment (%)</th>
<th>Alkylresorcinols [μg (mg protein)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 9046</td>
<td>Wild-type</td>
<td>13.9 ± 2.6</td>
<td>12.3 ± 0.3</td>
</tr>
<tr>
<td>CNS59</td>
<td>rpoS::Sp</td>
<td>&lt;0.0001</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ATpsrA</td>
<td>rpsA::Sp</td>
<td>7.6 ± 1.9</td>
<td>5.8 ± 0.06</td>
</tr>
<tr>
<td>CNS59/pSMrpoS</td>
<td>rpoS::Sp/rpoS⁺</td>
<td>8.2 ± 3.3</td>
<td>5.1 ± 0.07</td>
</tr>
<tr>
<td>CNS59/pBBR1MCS-2</td>
<td>rpoS::Sp/pBBR1MCS-2</td>
<td>&lt;0.0001</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

**PsrA is required for maximal rpoS transcription and binds to the rpoS promoter region**

The negative effect on encystment and alkylresorcinol production exerted by the rpoS and psrA mutants suggested that similar to *Pseudomonas* species, PsrA could be an activator of rpoS transcription (Kojic & Ventura, 2001). Indeed, the intergenic nlpD–rpoS region, the 3' end of the nlpD gene are highly conserved among *P. aeruginosa*, *P. putida* and *A. vinelandii*; furthermore, the palindromic motif C/GAAAC N2-4 GTTTG/C, to which PsrA binds in the rpoS regulatory region of *P. aeruginosa* (Kojic et al., 2002), is present in *A. vinelandii* (see Fig. 3b). Expression of rpoS in *A. vinelandii*, as determined by Northern blot analysis, is under growth phase regulation. Highest expression was found in stationary phase whereas a low-level of expression was detected in exponentially growing cells (Castañeda et al., 2001; Sandercock & Page, 2008).

To investigate the role of PsrA in the regulation of rpoS, qRT-PCR was used to determine the effect of the psrA::Sp mutation on the transcription of rpoS. The level of rpoS mRNA was determined in cells harvested at stationary phase. The relative expression level (0.37 ± 0.1 in BS; 0.34 ± 0.11 in BBOH) of rpoS mRNA was reduced in the psrA mutant compared with the wild-type (1.0), regardless of whether the cells were vegetative or cysts.

To determine whether PsrA binds to the rpoS promoter region, an electrophoretic mobility shift assay (EMSA) was performed with PsrA. A DNA fragment of 225 bp containing the putative PsrA binding sites in the rpoS upstream region was amplified by PCR and subjected to an EMSA with purified PsrA. A DNA fragment of 225 bp, containing the putative PsrA binding sites in the rpoS promoter region, was amplified by PCR and subjected to an EMSA with purified PsrA as described in Methods.

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**Table 2. Encystment and alkylresorcinol production in A. vinelandii**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Encystment (%)</th>
<th>Alkylresorcinols [μg (mg protein)⁻¹]</th>
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<td>ATpsrA</td>
<td>rpsA::Sp</td>
<td>7.6 ± 1.9</td>
<td>5.8 ± 0.06</td>
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<td>CNS59/pSMrpoS</td>
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Fig. 2(a) shows the binding of PsrA to this fragment, but not to a non-specific 245 bp DNA fragment corresponding to the phbR promoter region (Peralta-Gil et al., 2002) used as a negative control. Competition assays using increasing concentrations of the unlabelled 225 bp fragment were carried out as controls to demonstrate specificity (Fig. 2b). These results indicate that PsrA directly activates the expression of rpoS.

Identification of the rpoS and psrA transcription start sites

We identified the transcription start sites upstream of the rpoS and psrA genes by primer extension analysis with total RNA isolated from stationary-phase cultures of strain ATCC 9046 grown on BS medium as described in Methods. The primers for mapping the promoters are shown in Supplementary Table S1 (available with the online version of this paper). Two transcriptional start sites were identified for rpoS (Fig. 3a). They were located 68 and 358 nt upstream of the ATG start codon, defining the Pr1 and Pr2 promoters (Fig. 3b). These two start sites have also been detected by primer extension analysis with the wild-type strain ATCC 9046, the psrA mutant ATpsrA, the rpoS mutant CNS59 and the complemented strain CNS59/pSMrpoS. Strain rpoS with plasmid vector CNS59/pBBR1MCS-2 and the arsA mutant OV11, which are unable to produce alkylresorcinols, were used as controls. Cells were induced to encyst on BBOH medium for 5 days before staining.

For psrA, two transcriptional start sites, located 21 and 35 nt upstream of psrA, were also identified by primer extension (Fig. 4), and by S1-mapping analysis in strain UW136 (data not shown), confirming the presence of the Pr1 and Pr2 promoters. Notably, the transcription start site of Pr2 is located 48 nt downstream of the putative PsrA binding sites (Fig. 3b), similar to the Pseudomonas rpoS transcription start site reported by Kojic et al. (2002).

PsrA auto-regulation

In P. aeruginosa, PsrA negatively regulates its own expression by binding to the consensus PsrA binding sequences located between the −18 and +20 region of the psrA promoter (Kojic et al., 2002). We determined the
effect of the psrA mutation on the transcription of psrA in vivo by qRT-PCR. In the psrA mutant strain ATpsrA, the Sp-gusA cassette was inserted between nucleotides 240 and 241 of the 720 nt psrA coding sequence. Thus, we were able to monitor psrA transcription in this strain by qRT-PCR by using oligonucleotides corresponding to the first 100 nt of the psrA 5’ coding region. Inactivation of psrA increased the relative level of the psrA mRNA by fivefold (4.93 ± 2) compared with the wild-type strain (1.0). We next determined whether A. vinelandii PsrA binds to its own promoter region to mediate the observed increase in psrA transcription. A 263 bp DNA fragment (RRpsrA), corresponding to the psrA–lexA intergenic region, was amplified by PCR and subjected to EMSA with purified PsrA. Fig. 5(a) shows the binding of PsrA to RRpsrA DNA. Competition assays using increasing concentrations of unlabelled RRpsrA were carried out as a specificity control (Fig. 5b). These results indicate that PsrA specifically interacts with its own promoter region to act as a repressor. No consensus sequences for PsrA binding (C/GAAAC N2-4 GTTTG/C) were identified in the A. vinelandii psrA regulatory region. However, the sequence CAAA, corresponding to the −10 sequence of the Psr1 promoter (Fig. 4), could be the PsrA binding site for repression of psrA.

### DISCUSSION

Here, we describe some phenotypic characteristics of rpoS and psrA mutants and show evidence of the involvement of these regulators in the process of encystment in A. vinelandii. When cells of the rpoS mutant strain CNS59 were induced to differentiate, the cysts completely lacked the exine and intine layers and were unable to form capsulated cyst cells. Consistent with this observation, rpoS mutant cysts were unable to resist desiccation. An early electron microscopy study of the development of A. vinelandii cysts (Wyss et al., 1961) revealed that the exine appears 36–48 h after encystment induction, after which the exine thickens and the intine is formed between the exine and the central body. This time point seems to correlate with the stage at which cyst development is blocked in the rpoS mutant. Interestingly, it seems to be the same stage at which encystment stops in mutants that are unable to synthesize alginate, an essential component of the intine and exine layers of the cysts. However, inactivation of rpoS did not prevent alginate synthesis (Castaneda et al., 2001; this study, data not shown). Therefore, the inability of the rpoS mutant to form the intine and exine layers is not caused by the absence of alginate. The involvement of RpoS in the control of encystment is also shown by the
inability of the rpoS mutant strain CNS59 to synthesize cyst-specific alkylresorcinols (Segura et al., 2009). However, the mechanism of alkylresorcinol regulation remains unknown. The lack of alkylresorcinols is not responsible for the inability of the rpoS mutant CNS59 to resist desiccation or to form mature cysts; we previously reported that 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 indicate that other genes under the control of RpoS are essential for cyst formation.

In P. aeruginosa, PsrA has roles as an activator of rpoS (Kojic & Venturi, 2001) and as an auto-repressor (Kojic et al., 2002). This study showed that, similarly, in A. vinelandii PsrA also has a dual role as an activator of rpoS expression and as an auto-repressor. However, inactivation of psrA in A. vinelandii reduced transcription of rpoS by

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**Fig. 3.** (a) Identification of transcription start sites of rpoS by primer extension analysis by using total RNA isolated from strain ATCC 9046. (b) Alignment of the upstream sequences of the rpoS gene from P. aeruginosa and A. vinelandii. The sequences recognized by PsrA are shown in grey boxes. The rpoS promoters from P. aeruginosa (Ppa) and A. vinelandii (Pr1 and Pr2) are indicated. The transcription start site (+1), the nlpD stop codon (TGA) and the rpoS ATG translation start codon are shown in bold. Arrows indicate the start sites of transcription.

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**Fig. 4.** Primer extension analysis of the psrA gene and comparison of the sequence of the promoter region of psrA from P. putida and A. vinelandii. The +1, −10 and −35 positions are shown. The sequences recognized by PsrA in P. putida are indicated as grey boxes and in A. vinelandii as a white box. Arrows indicate the start sites of transcription.
60%, whereas in Pseudomonas species psrA-null mutants show an 80% reduction in rpoS promoter activity (Kojic & Venturi, 2001). Thus, the level of activation of rpoS by PsrA seems to be lower in A. vinelandii than in Pseudomonas. In A. vinelandii the rpoS mutation caused an inability to encyst, to produce alkylresorcinols and to resist hydrogen peroxide-induced stress, whereas for the psrA mutant only a reduction in the synthesis of alkylresorcinols and in the formation of mature cysts were observed. We therefore conclude that although PsrA positively activates rpoS expression, the psrA mutant produced a level of RpoS protein that allowed encystment and alkylresorcinol synthesis, although to a reduced level.

A single promoter driving transcription of rpoS has been identified in Pseudomonas (Kojic et al., 2002). In contrast, two transcription start sites were identified for the rpoS gene in A. vinelandii. One of these promoters (Pr2) is similar to the promoter identified in Pseudomonas; it is located far upstream of the rpoS start codon within the nlpD coding region and is close to the PsrA binding sites. The differences observed in the level of regulation of rpoS by PsrA between Pseudomonas and Azotobacter could be related to the presence of a second promoter directing transcription of rpoS in A. vinelandii. Additionally, the difference in rpoS regulation might be related to the requirement of RpoS for encystment in A. vinelandii, a process not carried out by Pseudomonas species.

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**REFERENCES**


Regulation of rpoS and psrA transcription


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