An extracytoplasmic function sigma factor cotranscribed with its cognate anti-sigma factor confers tolerance to NaCl, ethanol and methylene blue in *Azospirillum brasilense* Sp7

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Azospirillum brasilense, a plant-growth-promoting rhizobacterium, is exposed to changes in its abiotic environment, including fluctuations in temperature, salinity, osmolarity, oxygen concentration and nutrient concentration, in the rhizosphere and in the soil. Since extra-cytoplasmic function (ECF) sigma factors play an important role in stress adaptation, we analysed the role of ECF sigma factor (also known as RpoE or σ^E) in abiotic stress tolerance in *A. brasilense*. An in-frame *rpoE* deletion mutant of *A. brasilense* Sp7 was carotenoidless and slow-growing, and was sensitive to salt, ethanol and methylene blue stress. Expression of *rpoE* in the *rpoE* deletion mutant complemented the defects in growth, carotenoid biosynthesis and sensitivity to different stresses. Based on data from reverse transcriptase-PCR, a two-hybrid assay and a pull-down assay, we present evidence that *rpoE* is cotranscribed with *chrR* and the proteins synthesized from these two overlapping genes interact with each other. Identification of the transcription start site by 5' rapid amplification of cDNA ends showed that the *rpoE*–*chrR* operon was transcribed by two promoters. The proximal promoter was less active than the distal promoter, whose consensus sequence was characteristic of RpoE-dependent promoters found in alphaproteobacteria. Whereas the proximal promoter was RpoE-independent and constitutively expressed, the distal promoter was RpoE-dependent and strongly induced in response to stationary phase and elevated levels of ethanol, salt, heat and methylene blue. This study shows the involvement of RpoE in controlling carotenoid synthesis as well as in tolerance to some abiotic stresses in *A. brasilense*, which might be critical in the adaptation, survival and proliferation of this rhizobacterium in the soil and rhizosphere under stressful conditions.

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INTRODUCTION

Azospirillum brasilense is a Gram-negative, nitrogen fixing, alphaproteobacterium that lives in close association with the roots (rhizosphere) of several important crop plants and grasses, and stimulates the growth of the host plant by producing phytohormones (Baldani *et al.*, 1979; Steenhoudt & Vanderleyden, 2000; Tarrand *et al.*, 1978). Different strains of *A. brasilense* differ in their ability to colonize different hosts under variable soil conditions and also in the extent of their root colonization. Although the ability to accumulate polyhydroxyalkanoates has been shown to be

important in the survival and multiplication of *A. brasilense* in the soil and rhizosphere (Kadouri *et al.*, 2005), the mechanisms used in achieving significant numbers in the rhizosphere or inside roots to elicit a plant-growth-promoting effect under stressful conditions are not fully known.

Bacteria that live in the rhizosphere experience variations in temperature, salinity, osmolarity, pH and availability of nutrients and oxygen (van Veen *et al.*, 1997; Zahran, 1999). They regulate expression of different sets of genes to adapt to different environmental conditions. In response to a specific stimulus, the bacterial sigma factors alter the pattern of gene expression by changing the affinity and specificity of RNA polymerase to different promoters at the time of initiation of transcription (Heimann, 2002). Based on similarities in the amino acid sequence, structure and mechanism of action, the sigma factors are divided into two families, σ^{70} and σ^{54} . The majority of sigma factors belong to the σ^{70} family, which is further divided into four

Abbreviations: 5' RACE, 5' random amplification of cDNA ends; ECF, extracytoplasmic function; Km, kanamycin; PEG, polyethylene glycol; RT-PCR, reverse transcriptase-PCR; Tc, tetracycline; TSS, transcription start site.

A supplementary table, showing primer sequences, and a supplementary figure, showing RT-PCR results, are available with the online version of this paper.

groups (Heimann, 2002; Lonetto *et al.*, 1992). Among them, Group 4 sigma factors were initially thought to be involved in responses to changes in the extra-cytoplasmic compartment of the cell and hence were called extra-cytoplasmic function (ECF) sigma factors, a subfamily composed of divergent members. The members of the ECF sigma family are involved in a wide range of environmental responses and generally regulate the genes whose products deal with the extracytoplasmic functions (Heimann, 2002).

Carotenoids are known to prevent the harmful effects of singlet oxygen ($^1\text{O}_2$) by quenching and reacting with $^1\text{O}_2$ or by quenching the triplet state of bacteriochlorophyll *a* (Ziegelhoffer & Donohue, 2009). The role of ECF sigma factors in the synthesis of carotenoids has been investigated in a photosynthetic bacterium *Rhodobacter sphaeroides* as well as in a non-photosynthetic bacterium *Myxococcus xanthus*. The level of $^1\text{O}_2$ in *R. sphaeroides* is sensed by a cytosolic anti-sigma factor, ChrR, which regulates the activity of RpoE. Although carotenoid biosynthesis is not under the control of RpoE in *R. sphaeroides*, the damage from $^1\text{O}_2$ is prevented by carotenoids as well as by genes that are under the control of RpoE. In *M. xanthus*, however, the carotenoid synthesis increases in response to blue light via CarQ, an ECF sigma factor (Browning *et al.*, 2003).

We have previously reported that a mutation in the gene encoding an anti-sigma factor (ChrR) belonging to the Zn^{2+} anti-sigma family (Thirunavukkarasu *et al.*, 2008) caused overproduction of carotenoids in *A. brasilense*. The present study shows that besides its role in carotenoid biosynthesis in *A. brasilense*, the *rpoE* gene encoding an ECF sigma factor is required for tolerating some abiotic stresses. Here, we show that the *rpoE* gene located upstream of *chrR* is cotranscribed with *chrR*, and that the ChrR anti-sigma factor can physically sequester RpoE. By determining the transcription start site we have also identified two promoters and examined their induction in response to abiotic stresses.

METHODS

Bacterial strains, plasmids, chemicals and growth conditions.

The bacterial strains and plasmids used in this study are shown in Table 1. MMAB medium containing 37 mM malate and 10 mM NH_4Cl as the sole source of carbon and nitrogen, respectively, was used to grow *A. brasilense* strains (Vanstockem *et al.*, 1987). Cultures were grown at 30 °C with shaking at 150 r.p.m. in an orbital incubator shaker and growth was monitored by measuring OD_{600} . All the chemicals used for growing bacteria were from Hi-media while ethanol (HPLC grade) was from Merck. Plasmid isolation kits and gel elution or purification kits were from Qiagen and Promega, respectively; restriction enzymes were from New England Biolabs.

Construction of an *rpoE* disruption (*rpoE*:Km) mutant in *A. brasilense* Sp7. To generate an *rpoE* disruption mutant of Sp7 by homologous recombination, an *rpoE* disruption plasmid (pMN3) was made and conjugatively mobilized in *A. brasilense* Sp7 through

Escherichia coli S.17-1. The *rpoE* region was amplified in two parts, amplicons A (1300 bp) and B (1100 bp), using *rpoE*5':F/*rpoE*5':R and *rpoE*3':F/*rpoE*3':R primer sets, respectively (all primers are listed in Supplementary Table S1, available with the online version of this paper). The primers were designed in order to generate restriction sites for *Pst*I and *Bgl*II at the 5' and 3' ends of amplicon A, and restriction sites for *Bgl*II and *Eco*RI at the 5' and 3' ends of amplicon B. After amplification, these fragments were digested with *Pst*I-*Bgl*II and *Bgl*II-*Eco*RI, respectively, and inserted between the *Pst*I and *Eco*RI sites of pSUP202. The resulting plasmid (pMN2) was cleaved with *Bgl*II and ligated to a *Bam*HI-digested 1.4 kb kanamycin (Km) resistance cassette excised from pUC4K (GE Healthcare) to generate the *rpoE* disruption plasmid, pMN3.

E. coli S.17-1 transformed with pMN3 was used as the donor in a biparental mating experiment; *A. brasilense* Sp7 was the recipient. Exconjugants were selected on MMAB plates supplemented with 40 μg Km ml^{-1} on which neither strain could grow; the donor, *E. coli* S.17-1, could not grow due to proline auxotrophy and the recipient, *A. brasilense*, could not grow due to its Km sensitivity. The exconjugants that grew on selection plates were analysed for the insertion of the Km resistance cassette in the *rpoE* gene by PCR using *rpoE*-gene-specific primers (*rpoE*:F/*rpoE*:R). The *rpoE* disruption mutant of Sp7 was designated *rpoE*:Km.

Construction of an in-frame *rpoE* deletion mutant (Δ *rpoE*) of *A. brasilense* Sp7.

An in-frame *rpoE* deletion plasmid (pMN5) was constructed and conjugatively mobilized in *rpoE*:Km through *E. coli* S.17-1 to obtain the *rpoE* deletion mutant (Δ *rpoE*) of *A. brasilense* Sp7 by homologous recombination. Upstream and downstream flanking regions of the *rpoE* ORF were amplified in two parts, amplicons A1 (1500 bp) and B1 (1350 bp), using *rpoE*A1:F/*rpoE*A1:R and *rpoE*B1:F/*rpoE*B1:R primer sets, respectively. Both amplicons A1 and B1 had only 15 bp from the 5' and 3' regions of *rpoE*. The primers were designed so as to generate restriction sites for *Pst*I and *Bgl*II at the 5' and 3' ends of amplicon A1, and restriction sites for *Bgl*II and *Eco*RI at the 5' and 3' ends of amplicon B1. After amplification, these fragments were digested with *Pst*I-*Bgl*II and *Bgl*II-*Eco*RI, respectively, and inserted between the *Pst*I and *Eco*RI sites of pSUP202 to generate pMN5. pMN5 was conjugatively mobilized in the *A. brasilense* Sp7 disruption mutant (*rpoE*:Km) via *E. coli* S.17-1, and the single cross-over exconjugants were selected on MMAB plates supplemented with 40 μg tetracycline (Tc) ml^{-1} and 40 μg Km ml^{-1} . The exconjugants were grown in MMAB liquid medium without any antibiotic to provide favourable condition for the double cross-over event to occur. The double cross-over mutants were selected on the basis of Tc and Km sensitivity. The deletion of the *rpoE* gene in Tc and Km sensitive exconjugants was further confirmed by PCR and reverse transcriptase (RT)-PCR using *rpoE*-gene-specific primers (*rpoE*:F/*rpoE*:R).

Growth and stress sensitivity test. The differences in the sensitivity of *A. brasilense* Sp7 and its σ^E mutant (Δ *rpoE*) to different stresses such as salinity, exposure to reactive oxygen species, desiccation, heat and organic stress were examined at sublethal levels of NaCl, hydrogen peroxide, methylene blue, paraquat, polyethylene glycol (PEG-200 and -6000), ethanol and heat. The stress sensitivity of strains was examined as described previously (da Silva Neto *et al.*, 2007). Briefly, pre-cultures of *A. brasilense* strains grown overnight in LB broth were diluted to OD_{600} 0.1 in MMAB and further grown for 4 h at 30 °C (early exponential phase). Cultures of each strain were then equally divided, and one flask of each strain was supplemented with either 250 mM NaCl, 1.5 % ethanol, 3.0 % PEG-200 or 5.7 % PEG-6000 or incubated at 40 °C, while the other was incubated at 30 °C in the absence of any treatment as the control. The cultures were allowed to grow to stationary phase with shaking at 150 r.p.m. at 30 °C. Growth was monitored at fixed intervals by measuring OD_{600} .

Table 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics	Reference or source
<i>E. coli</i> strains		
DH5 α	$\Delta lacU169$ <i>hsdR17 recA1 endA1 gyrA96 thiL relA1</i>	Gibco-BRL
S.17-1	Sm ^r , <i>recA thi pro hsdR</i> ⁻ M ⁺ RP4:2-Tc:Mu:Km Tn7 λ pir	Simon <i>et al.</i> (1983)
BL21 λ (DE3) pLysS	<i>ompT hsdS</i> (r _B ⁻ m _B ⁻) <i>dcm</i> ⁺ <i>endA gal</i> λ BL21(DE3)	Novagen
<i>A. brasilense</i> strains		
Sp7	Wild-type strain	Nur <i>et al.</i> (1981)
Car-1	Carotenoid-producing <i>chrR</i> :Tn5 mutant of <i>A. brasilense</i> Sp7	Thirunavukkarasu <i>et al.</i> (2008)
<i>rpoE</i> :Km	<i>A. brasilense</i> Sp7 <i>rpoE</i> gene disrupted by insertion of Km ^r cassette	This work
Δ <i>rpoE</i>	<i>A. brasilense</i> Sp7 <i>rpoE</i> gene deleted	This work
Plasmids		
pSUP202	ColE1 replicon, mobilizable plasmid, suicide vector suitable for <i>A. brasilense</i> , Ap ^r , Tc ^r , Cm ^r	Simon <i>et al.</i> (1983)
pRKK200	Km ^r , Sp ^r , <i>lacZ</i> fusion reporter vector	Karls <i>et al.</i> (1999)
pUC4K	Vector containing Km cassette	GE Healthcare
pTRG	Target plasmid, BacterioMatch II two-hybrid system	Agilent Technologies
pBT	Bait plasmid, BacterioMatch II two-hybrid system	Agilent Technologies
pAT5	<i>rpoE</i> gene from <i>A. brasilense</i> Sp7 cloned into <i>EcoRI</i> and <i>BamHI</i> sites of pMMB206	Thirunavukkarasu <i>et al.</i> (2008)
pET15b	Expression vector, Ap ^r	Novagen
pMN2	Fragment A and B of <i>rpoE</i> gene from <i>A. brasilense</i> cloned into the <i>PstI/EcoRI</i> site of pSUP202	This work
pMN3	<i>rpoE</i> disruption plasmid; kanamycin resistance gene (<i>apt</i>) from pUC4K cloned into the <i>BglII</i> site of pMN2	This work
pMN5	<i>rpoE</i> deletion plasmid; <i>rpoE</i> upstream and downstream region cloned into the <i>PstI/EcoRI</i> site of pSUP202	This work
pNG1	ORF of <i>rpoE</i> – <i>chrR</i> genes from <i>A. brasilense</i> Sp7 cloned into the <i>NdeI/BamHI</i> site of pET15b	This work
pBT- <i>rpoE</i>	<i>rpoE</i> gene from <i>A. brasilense</i> Sp7 cloned into the <i>EcoRI/BamHI</i> site of pBT	This work
pRTG- <i>chrR</i>	<i>chrR</i> gene from <i>A. brasilense</i> Sp7 cloned into the <i>EcoRI/XhoI</i> site of pRTG	This work
pSK4	<i>A. brasilense</i> <i>rpoE</i> promoter region (<i>rpoEP1</i> + <i>rpoEP2</i>) cloned into the <i>KpnI/StuI</i> site of pRKK200	This work
pSK5	<i>rpoEP1</i> cloned in the <i>KpnI/StuI</i> site of pRKK200	This work
pSK6	<i>rpoEP2</i> cloned in the <i>KpnI/StuI</i> site of pRKK200	This work

Growth inhibition assays were performed on LB agar plates to examine the sensitivity of *A. brasilense* WT, Δ *rpoE* and Car-1 (*chrR*:Tn5; Thirunavukkarasu *et al.*, 2008) to different oxidative stresses. Precultures of these strains were allowed to grow to late exponential phase in LB broth with respective antibiotics. A 100 μ l pre-culture of each strain was mixed with 5 ml LB agar (with only 0.5% agar), poured on top of the LB agar (1.5% agar) and allowed to solidify. After 15 min, a 10 μ l drop of either 5 mM methylene blue or 2 mM H₂O₂ or 1 mM paraquat was placed in the centre of each agar plate and the plates were incubated for 3 days in white light at 30 °C.

RNA extraction and RT-PCR. Total RNA was extracted from the *A. brasilense* cultures grown to late-exponential phase (OD₆₀₀ 2.5–2.8) using TRIzol reagent (Invitrogen). Cells were further treated with 0.05 U RNase-free DNase I (NEB) (μ g RNA)⁻¹ for 30 min at 37 °C followed by phenol extraction and ethanol precipitation. RT-PCR was carried out with 1–1.5 μ g RNA using a one-step RT-PCR kit (Qiagen) according to the manufacturer's instructions. The cycling conditions used were 50 °C for 30 min; 95 °C for 15 min; 30 cycles of 95 °C for 30 s, 52–58 °C (according to the primer used in the reaction) for 30 s and 72 °C for 1 min; followed by incubation at 72 °C for 10 min. Negative controls were included to check for DNA contamination.

Analysis of the protein–protein interactions between RpoE and ChrR

Bacterial two-hybrid assay. The BacterioMatch two-hybrid system (Stratagene) was used to study the *in vivo* protein–protein interactions between RpoE and ChrR. The complete coding regions of *rpoE* and *chrR* were amplified by using *rpoE*:F1/*ropE*:R1 and *chrR*:F1/*chrR*:R1 primer sets, respectively. The primers were provided with extra 5' sequences to generate the required restriction sites at the ends of the amplified products. The 576 bp *rpoE* gene fragment amplified by PCR was digested with *EcoRI*–*BamHI* and cloned in the pBT bait plasmid, which encodes the full-length bacterial phage λ cI repressor protein under the control of the IPTG inducible *lacUV5* promoter, resulting in pBT-*rpoE*. Likewise, the 700 bp *chrR* gene was digested with *EcoRI*–*XhoI* and cloned in pTRG target plasmid to fuse with the N-terminal domain of the α -subunit of RNA polymerase, resulting in pTRG-*chrR*. The clones were verified by PCR amplification, restriction digestion and sequencing.

The level of transcriptional activation of the *lacZ* reporter in various cotransformants was qualitatively screened on LB agar supplemented with 25 μ M IPTG, 80 μ g X-Gal ml⁻¹ and 200 μ M phenylethyl β -D-thiogalactoside (a β -galactosidase inhibitor). The interaction of fusion proteins encoded by pBT-LGF2 and pTRG-GAL11P, provided in the

BacterioMatch II two-hybrid system, was used as a positive control whereas one empty plasmid (pBT or pTRG) and one recombinant plasmid served as a negative control. The quantitative measurement of β -galactosidase activity for each plasmid combination was carried out in sets of three independent cotransformants to confirm *in vivo* protein–protein interaction. The cotransformants were grown at 30 °C to the mid-exponential phase in the presence of IPTG and the β -galactosidase inhibitor in the LB medium. Aliquots were collected from the exponential phase cultures and the number of cells in each culture was equalized on the basis of their OD₆₀₀ at the time of assay. The β -galactosidase assay, as described by Miller (1972), was performed twice with 1.5 ml equalized culture for each sample.

Pull-down assay. The ORFs of the genes encoding RpoE and ChrR were amplified as a single amplicon by PCR using *rpoE*:F2 and *chrR*:R2 primers with genomic DNA of *A. brasilense* Sp7. The amplified product was digested with *Nde*I–*Bam*HI, purified and ligated with the similarly digested expression vector pET15b. *E. coli* DH5 α was transformed with the ligation mix and the transformants were selected on Luria agar containing ampicillin (100 μ g ml^{−1}). The pET15b clones harbouring *rpoE*–*chrR* genes were confirmed by restriction digestion and nucleotide sequencing, and designated pNG1. *E. coli* BL21 λ (DE3) pLysS competent cells were then transformed with pNG1, and the transformants were selected on LB agar supplemented with ampicillin and chloramphenicol (25 μ g ml^{−1}).

The physical interaction between RpoE and ChrR was monitored by passing the soluble extract, from *E. coli* cells that expressed both His-RpoE and -ChrR, over Ni-NTA agarose under non-denaturing conditions, and analysing the eluted fraction for the presence of ChrR protein along with His-RpoE. For the overexpression of His-RpoE and -ChrR, the *E. coli* cells harbouring pNG1 were cultured overnight in LB medium containing ampicillin and chloramphenicol at 37 °C, diluted 1:100 with the same medium and incubated at 28 °C with shaking at 150 r.p.m. When OD₆₀₀ reached ~0.4, expression of the above proteins was induced by adding 1 mM IPTG and cells were grown for an additional 6 h at 28 °C. The cells were harvested by centrifugation, resuspended in the lysis buffer (50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 10 mM imidazole, 1 mM PMSF, 0.1 % Triton X-100), lysed with lysozyme (1 mg ml^{−1}) followed by sonication at 4 °C by six 10 s bursts alternated with a 10 s cooling period between each burst. The lysate was centrifuged (10 000 *g* for 30 min at 4 °C) and the resulting supernatant was mixed with Ni-NTA resin (Qiagen) with gentle shaking for 1 h. The non-specifically bound proteins were removed by washing the resin twice with the wash buffer (50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 20 mM imidazole, 0.1 % Triton X-100). The bound proteins were eluted with elution buffer (50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl and 250 mM imidazole). All purification steps were carried out at 4 °C. The eluted fractions were resolved by SDS-PAGE and visualized by Coomassie staining.

Detection of His-tagged RpoE by Western immunoblotting. The crude lysates from uninduced and induced cultures of *E. coli* BL21 λ (DE3) pLysS harbouring pNG1 (expressing RpoE–ChrR) were resolved by 12.5 % SDS-PAGE and electroblotted onto PVDF membrane (Millipore). The blot was blocked with 5 % non-fat dry milk (in 1 × PBST) for 1 h, exposed to a mouse monoclonal anti-6 × His antibody (Sigma) used at a 1:12 000 dilution in 1 × PBST and incubated at room temperature for 1 h with gentle shaking. The blot was washed three times with 1 × PBST for 10 min each at room temperature with gentle shaking. Polyclonal rabbit anti-mouse IgG–horseradish peroxidase, the secondary antibody, (Sigma) was used at a 1:9000 dilution in 1 × PBST to probe the blot for 1 h. The blot was once again washed three times with 1 × PBST for 10 min each and developed by using DAB solution (6 mg DAB in 10 ml PBST) containing 100 μ l H₂O₂ (3 %). The blots were incubated in this

solution until the bands on the blot were visible. The colour development was terminated by washing the blots in PBST.

5' random amplification of cDNA ends (RACE). The transcription start site (TSS) of the *rpoE* gene was determined by using 5' RACE using the 3'/5' RACE kit, 2nd Generation (Roche). Briefly, total RNA was isolated from the cells taken from the stationary phase cultures of Car-1, and treated with DNase I as described above. The *rpoE* transcript was reverse transcribed into cDNA using an *rpoE*-gene-specific primer (*rpoE*:R). The cDNA was purified and a poly(dA) tail was added at the 3' end. The resulting poly(dA)-tailed cDNA was amplified by PCR using the oligo (dT)-anchor primer provided with the kit [to anneal at the poly(dA) tail] and *rpoE*:R2 primer (complementary to the region upstream of the *rpoE*:R binding site). The amplicons from the first PCR were used as the template in the second PCR using the anchor primer provided by the kit (to anneal at a region generated by oligo (dT)-anchor primer at the 3' end of cDNA) and the *rpoE*:R3 primer, which is complementary to a region further upstream of the *rpoE*:R2 binding site. The amplified products obtained from the second PCR were ligated into the pGEM-T Easy vector (Promega) and the nucleotide sequence of several distinct clones was determined by using an ABI-PRISM, 310 Genetic Analyzer (Applied Biosystems).

Construction of promoter:*lacZ* fusions. The promoters of the *rpoE* gene (*rpoEP*1 and *rpoEP*2) were amplified by PCR and cloned separately as well as together between *Stu*I and *Kpn*I site of pRKK200 to construct promoter:*lacZ* fusions (transcriptional fusions). The complete *rpoE* promoter region from −264 to −35 of the predicted translational start site (encompassing *rpoEP*1 and *rpoEP*2) was amplified by using the *rpoEP*2:F/*rpoEP*1:R primer set. The *rpoEP*1 (−76 to +63 of TSS1) and *rpoEP*2 (−64 to +65 of TSS2) were separately amplified using *rpoEP*1:F/*rpoEP*1:R and *rpoEP*2:F/*rpoEP*2:R primer sets (Supplementary Table S1), respectively. All three amplified products were digested with *Stu*I–*Kpn*I, and ligated with a similarly digested pRKK200 vector. *E. coli* DH5 α was then transformed with the ligation mix and the transformants were selected on LB agar supplemented with spectinomycin (100 μ g ml^{−1}). After confirmation of recombinant plasmids by sequencing, the constructs were designated pSK4 [*rpoEP*(P1 + P2):*lacZ* fusion], pSK5 (*rpoEP*1:*lacZ* fusion) and pSK6 (*rpoEP*2:*lacZ* fusion). These constructs were finally conjugatively mobilized into the *A. brasilense* strains via *E. coli* S.17.1 and exconjugants were selected on MMAB plates supplemented with spectinomycin.

β -Galactosidase assay. The β -galactosidase assay (Miller, 1972) was performed using *A. brasilense* strains harbouring pRKK200, pSK4, pSK5 or pSK6. The *A. brasilense* strains harbouring the above promoter:*lacZ* fusions were grown in MMAB medium to mid-exponential phase and then grown for a further 2 h in the presence of different stress agents either by supplementing with 250 mM NaCl, 1.5 % ethanol, 2 mM H₂O₂, 1 mM paraquat, 10 μ M methylene blue, 3.0 % PEG-200 or 5.7 % PEG-6000 or by incubating at 40 °C. Untreated cultures were taken as controls. At the time of assay, the number of cells in each culture was equalized by diluting with the fresh medium or the medium supplemented with respective stress agents. The assay was performed with 1 ml equalized culture in triplicate for each sample on two different occasions.

RESULTS

Confirmation and phenotypic characterization of the *rpoE* deletion mutant (Δ *rpoE*)

A mutant of *A. brasilense* Sp7 was constructed by inserting a Km^r gene cassette in the ORF encoding *rpoE* (designated

rpoE:Km) by exchanging the wild-type allele with the disrupted copy via standard homologous recombination. The ORF of *rpoE* was amplified using *rpoE*-specific primers using the genomic DNA of the parental strain (Sp7) and of the *rpoE*:Km mutant as templates, producing amplicons of the expected sizes (576 bp and 2.0 kb, respectively), and confirming the insertion of the Km^r gene cassette in the *rpoE* ORF (data not shown). Since *rpoE* is located upstream of *chrR* and the organization of the two genes suggested their cotranscription, it was logical to assume that the insertion of the Km^r gene cassette in the *rpoE* ORF might exert a polar effect on the expression of *chrR* in the *rpoE*:Km mutant.

In order to examine the phenotype resulting only due to the inactivation of *rpoE*, an in-frame *rpoE* deletion mutant was constructed without affecting the expression of *chrR*. The *rpoE* deletion mutant ($\Delta rpoE$) was generated by deleting >95% of *rpoE* from the *rpoE*:Km strain by exchanging the chromosomally located *rpoE*:Km copy with a $\Delta rpoE$ copy. After mobilization of the deletion plasmid (pMN5), single cross-overs were initially obtained in the presence of Tc and Km, and the second cross-over mutant (which had lost the integrated plasmid as well as the major part of *rpoE* along with the Km resistance gene) was subsequently selected by screening for Tc and Km sensitivity. Only five of 48 Tc^r single cross-over mutants showed Tc and Km sensitivity, indicating that the double cross-over event might have taken place.

The deletion of *rpoE* in these double cross-over mutants was confirmed by amplification of *rpoE* using *rpoE*-specific primers using the genomic DNA of the parental strain (Sp7) and the $\Delta rpoE$ mutants as templates. The parental strain produced an amplicon of the expected size (576 bp); however, the Tc and Km sensitive mutants did not produce either a 576 bp *rpoE* amplicon (confirming the deletion of *rpoE*, data not shown) or the 2.0 kb amplicon (confirming the loss of the Km^r gene cassette) of the inactivated *rpoE*. Further, in order to examine whether the deletion of *rpoE* in the $\Delta rpoE$ mutant had any polar effect on the expression of *chrR* located downstream of *rpoE*, the presence and absence of the transcripts of *rpoE* and *chrR* was examined in Sp7, *rpoE*:Km, $\Delta rpoE$ and Car-1 by amplifying *rpoE* and *chrR* with RNA samples isolated from Sp7, *rpoE*:Km, $\Delta rpoE$ and

Car-1. RT-PCR analysis revealed that the RNA sample from the $\Delta rpoE$ mutant did not produce any amplicon with *rpoE*-specific primers while *chrR*-specific primers produced an amplicon of similar size (~700 bp) when the RNA samples extracted from $\Delta rpoE$ and Sp7 were used as template (Fig. 1). However, the RNA sample from *rpoE*:Km did not produce *rpoE* and *chrR* amplicons. These results indicated that the in-frame deletion of *rpoE* did not have a polar effect, as the *chrR* transcript was present in the $\Delta rpoE$ mutant. However, insertion of the Km^r gene cassette in *rpoE* had a polar effect on the transcription of the downstream *chrR* gene, as the *chrR* transcripts were absent in the *rpoE*:Km mutant. After confirming the expression of *chrR* transcripts in the $\Delta rpoE$ mutant, we characterized its phenotype.

The obvious phenotypic difference observed in the $\Delta rpoE$ mutant compared with the parent Sp7 was the lack of carotenoids in the former. While the colonies of Sp7 were light orange and those of Car-1 (an anti-sigma mutant) were pink, the colonies of the $\Delta rpoE$ mutant were creamy. This difference in the colour of the colonies could only be observed when Sp7, $\Delta rpoE$ and Car-1 were compared on the same plate (Fig. 2a). When the plasmid pAT5, expressing the *rpoE* gene via a *lac* promoter from a broad-host-range vector pMMB206 (Thirunavukkarasu *et al.*, 2008), was mobilized in the $\Delta rpoE$ mutant, it produced carotenoids and acquired the light pink colour that was seen in the wild-type Sp7 (data not shown).

Since Sp7, $\Delta rpoE$ and Car-1 had very obvious differences in their carotenoid content, we examined whether the presence or absence of carotenoids resulting from the inactivation of *chrR* and *rpoE* could change the sensitivity of *A. brasilense* strains to the reactive oxygen species. *A. brasilense* strains showed a notable difference in their sensitivity only to methylene blue (and not to hydrogen peroxide or paraquat), as a clear zone of growth inhibition was observed in Sp7 and $\Delta rpoE$ but not in Car-1 (Fig. 2b). The zone of inhibition around $\Delta rpoE$ was significantly larger than that formed by Sp7, indicating a clear difference in the sensitivity of $\Delta rpoE$ compared with Sp7 to ¹O₂ generated by 5 mM methylene blue. The sensitivity of the three strains to methylene blue correlated well with their carotenoid-producing ability. The carotenoid content

Fig. 1. RT-PCR amplification of *rpoE* and *chrR* with the RNA samples isolated from Sp7, *rpoE*:Km, $\Delta rpoE$ and Car-1. 16S rRNA was amplified to confirm equal amounts of RNA samples.

Fig. 2. (a) LB plate showing colonies of *A. brasilense* Sp7, $\Delta rpoE$ and Car-1 which display differently coloured colonies. (b) Growth inhibition assay of *A. brasilense* Sp7, $\Delta rpoE$ and Car-1 using methylene blue.

decreased and methylene blue sensitivity increased in the order Car-1–Sp7– $\Delta rpoE$.

To examine the role of RpoE in the growth and tolerance to other stresses, we compared the growth of $\Delta rpoE$ with that of Sp7 and $\Delta rpoE/pAT5$ (expressing basal levels of RpoE from the uninduced *lac* promoter), with and without specific stresses. In the absence of any stress, the $\Delta rpoE$ mutant grew considerably slower than the parent and $\Delta rpoE$ (pAT5), indicating the involvement of RpoE in controlling some housekeeping functions (Fig. 3a). Of the general stresses such as desiccation, heat, salinity and ethanol, $\Delta rpoE$ showed a significantly higher sensitivity to 250 mM NaCl and 1.5% ethanol compared with Sp7 (Fig. 3b and c). Expression of *rpoE* in the $\Delta rpoE$ strain via pAT5 complemented the defects when RpoE was expressed at basal levels from the uninduced *lac* promoter (Fig. 3d).

No significant difference in the sensitivity of the parent and $\Delta rpoE$ mutant to PEG-200 and heat was observed (data not shown). Since the difference between carotenoid content of Sp7 and $\Delta rpoE$ is very small, the lack of significant difference in the susceptibility of Sp7 and $\Delta rpoE$ strains to PEG-200 and heat is consistent with our earlier observation that carotenoids are mainly responsible for this difference in susceptibility (Mishra *et al.*, 2008). Altogether, these results indicate that RpoE is partly involved in growth under optimal conditions and plays a major role in tolerating stresses caused by salt, ethanol and $^1O_2$ in *A. brasilense*.

Cotranscription of *rpoE* and *chrR* genes, and interaction of RpoE and ChrR proteins

An analysis of the sequences of *rpoE* and *chrR* in *A. brasilense* Sp7 revealed that the two genes are not only juxtaposed but also overlap by four nucleotides. The start codon (AUG) of *chrR* overlaps the stop codon (UGA) of

rpoE at the junction sequence, AUGA, which strongly suggested the possibility of cotranscription of the two genes. RT-PCR analysis revealed that *rpoE*- and *chrR*-specific primers produced amplicons of ~600 and ~700 bp, respectively, corresponding to the expected sizes of *rpoE* and *chrR* (Supplementary Fig. S1, available with the online version of this paper). However, RT-PCR with *rpoE*:F/*chrR*:R primers gave an amplicon of ~1.3 kb indicating that both the genes were transcribed together as a single transcript.

The molecular interaction between RpoE and ChrR was studied by using the BacterioMatch II two-hybrid system using *E. coli* as a host (Kumar *et al.*, 2009). The transformants carrying the two recombinant plasmids, one expressing RpoE and the other expressing ChrR, displayed a LacZ⁺ phenotype and produced blue colonies on X-Gal plates, indicating an interaction between the two proteins. Quantitative β -galactosidase assay of the cotransformants also showed that the strength of the *in vivo* interactions between RpoE and ChrR was almost the same as that observed in the positive control (Fig. 4a). The activity was considerably lower in the negative control harbouring pBT and pTRG-*chrR*.

In view of the overlap between the start codon of *chrR* and the stop codon of *rpoE*, we had anticipated that the two genes might be not only cotranscribed but also translationally coupled (Thirunavukkarasu *et al.*, 2008). In order to avoid the problems resulting from the unequal solubility and stability of the two interacting partner proteins when overexpressed separately (Thakur *et al.*, 2010), we examined the physical interaction between RpoE and ChrR by cloning both genes (*rpoE*–*chrR*) together in pET15b to co-express (i) His–RpoE from the Shine–Dalgarno (SD) sequence of the vector and (ii) wild-type ChrR from its native SD sequence located at the 3' end of the *rpoE*. We observed that the amount of the two overexpressed and eluted proteins was unequal (Fig. 4b, ii and iii). Another

Fig. 3. Comparison of the growth of Sp7, $\Delta rpoE$ and $\Delta rpoE/pAT5$ strain ($\Delta rpoE$ mutant complemented with $rpoE$ expressing plasmid pAT5) without stress (a) and with stress (b–d). In (b–d), strains [Sp7 (b), $\Delta rpoE$ (c), $\Delta rpoE/pAT5$ (d)] were grown without stress or with either 250 mM NaCl or 1.5 % ethanol (EtOH). Error bars indicate SD from the three replicates.

co-expression study of sigma factors and anti-sigma factors has shown that the expression of the promoter distal gene is usually lower than the promoter proximal gene, resulting in a substoichiometric complex of the two co-expressed proteins (Thakur *et al.*, 2010). Since only His–RpoE can directly bind to the Ni-NTA agarose, presence of ChrR along with His–RpoE in the eluted fraction indicates that ChrR formed a complex with RpoE (Fig. 4b, iii). In the control experiments, however, when the soluble extract from *E. coli* cells expressing only wild-type ChrR (using pET9a vector) was passed over Ni-NTA agarose, no protein was observed in the eluted fraction, suggesting that wild-type ChrR is unable to bind to the Ni-NTA agarose (data not shown). The Western immunoblot (Fig. 4b, i) showed that RpoE, which cross-reacted with the anti-6 × His antibody, was expressed at considerably higher levels than ChrR.

Determination of the *rpoE* TSS

The TSS of *rpoE* was determined by 5' RACE using RNA samples isolated from the anti-sigma knockout mutant, Car-1, which expresses the *rpoE* gene constitutively. In the 5' RACE experiment, amplicons of two different sizes, indicating two transcription start sites for *rpoE*, were obtained. Both the amplicons were cloned in pGEM-T and sequenced (Fig. 5a). The majority of the clones (more than

80 %) showed a TSS located at position –199 (TSS2) relative to the predicted translational start site (Fig. 5a, i, and 5b) while few clones (less than 20 %) showed a TSS located at position –100 (TSS1) relative to the predicted translational start site (Fig. 5a, i, and 5b). An analysis of the region upstream of the identified TSSs (Fig. 5b) for corresponding promoter elements revealed the presence of TGATCC at –35 and CGCGTA at –10 of TSS2 with a spacing of 14 nt, upstream of TSS2. The upstream region of TSS1 showed a different type of promoter (designated *rpoEP1*) having TGAACA at –35 and AAACAC at –10 of TSS1 with a spacing of 17 nt. The number of clones showing the start of transcription from TSS1 was much smaller compared to those showing transcription from TSS2 in Car-1, in which RpoE is not inactivated by ChrR. This indicated that either *rpoEP1* may be regulated by a sigma factor other than RpoE or that it is a weak RpoE-regulated promoter.

Regulation of the *rpoE* promoters

The occurrence of more than one TSS and respective promoter elements prompted us to study the regulation of the two promoters and their RpoE dependence, as several *rpoE* orthologues are known to be positively autoregulated. For this purpose, both the promoter regions (*rpoEP1* and *rpoEP2*) were amplified separately and together, and

Fig. 4. (a) β -Galactosidase activity of the BacterioMatch two-hybrid system cotransformed with pBT/pTRG-*chrR* (negative control), pBT-LGF2/pTRG-GAL11P (positive control) and pBT-*rpoE*/pTRG-*chrR* showing the interaction of RpoE and ChrR. Error bars show SD. (b) (i) Immunoblot showing a protein band cross-reacting with anti-6 $\times$ His antibody. (ii) SDS-PAGE showing proteins in the soluble extracts of *E. coli* BL21 λ (DE3) pLysS harbouring pNG1. U, Uninduced; I, induced; M, protein molecular mass marker. (iii) SDS-PAGE showing wild-type ChrR co-purified with His-RpoE after passing the soluble extract from *E. coli* cells expressing both His-RpoE and wild-type ChrR over Ni-NTA agarose in non-denaturing conditions. The eluted fraction (lane 2) was resolved by using SDS-PAGE to examine the presence of ChrR with His-RpoE. Lane 1 shows protein molecular mass marker.

inserted upstream of the promoterless *lacZ* reporter in pRKK200 to make transcriptional fusions with individual promoters as well as with the two promoters together. To examine the RpoE dependence of *rpoE* promoters, a β -galactosidase assay was performed with *A. brasilense* strains (Sp7, Δ *rpoE* and Car-1) harbouring promoter: *lacZ* fusions (pSK4, pSK5 and pSK6). These three *A. brasilense* strains (Sp7, Δ *rpoE* and Car-1) harbouring pSK5 (*rpoEP1*) showed almost equal β -galactosidase activities, indicating that the product of *rpoE* (RpoE) was not involved in controlling *rpoEP1* (Fig. 6). On the other hand, Δ *rpoE*(pSK6) did not show significant β -galactosidase activity, while Sp7(pSK6) and Car-1(pSK6) showed significant and high β -galactosidase activity, respectively (Fig. 6), indicating that the *rpoEP2* promoter was regulated in an RpoE-dependent manner. The β -galactosidase activities of Car-1 cells harbouring either pSK4 (*rpoEP1* + *rpoEP2*) or pSK6 (*rpoEP2*) were almost equal and more than threefold higher than that

shown by *rpoEP1*. This indicated that, in the presence of free RpoE, the transcription of *rpoE* occurred mainly from the *rpoEP2* promoter. On the other hand, the Δ *rpoE* mutants harbouring either pSK4 or pSK5 (*rpoEP1*) had equal β -galactosidase activities, suggesting that in the absence of RpoE, expression of *rpoE* occurred from the *rpoEP1* promoter. The promoter activity of these three constructs in Sp7 showed that both the promoters were active and were used to regulate *rpoE* expression under optimal conditions.

To examine the inducibility of *rpoE* promoters in response to different stresses, we measured the effect of different stress agents on the β -galactosidase activity of *A. brasilense* strains (Sp7 and Δ *rpoE*) harbouring pRKK200, pSK5 or pSK6. *rpoEP2* activity was significantly induced in Sp7 in response to stationary phase, NaCl (250 mM), ethanol (1.5 %), methylene blue (10 μ M) and heat (40 $^{\circ}$ C), whereas the Δ *rpoE* strain showed no induction (Table 2). On the other hand, induction of *rpoEP1* activity was not observed in response to any of the above stresses in any strain. These results again indicated that only *rpoEP2* and not *rpoEP1* is RpoE-dependent, and transcription of *rpoE* is positively regulated from *rpoEP2* in response to stationary phase, NaCl, ethanol, methylene blue and heat (40 $^{\circ}$ C) in an autoregulated manner. There was no significant induction of β -galactosidase activity in Car-1 harbouring either pSK5 or pSK6, suggesting that the promoters in Car-1 are constitutively expressed.

DISCUSSION

A. brasilense Sp7 was earlier reported to be a carotenoidless strain (Nur *et al.*, 1981; Tarrand *et al.*, 1978). However, we had shown that either inactivation of *chrR* (gene encoding anti-sigma factor ChrR) or overexpression of *rpoE* (gene encoding ECF sigma factor RpoE) caused overproduction of carotenoids in *A. brasilense* Sp7 (Thirunavukkarasu *et al.*, 2008). The present study clearly shows that *A. brasilense* Sp7 does synthesize basal levels of carotenoids which are abolished upon inactivation of the ECF sigma factor gene *rpoE*. Expressing *rpoE* via a low-copy broad-host-range expression vector in a Δ *rpoE* mutant produced carotenoids by complementing the defect, which reconfirmed the role of *rpoE* in carotenoid biosynthesis. The ability of *A. brasilense* strains to synthesize carotenoids correlated well with their tolerance against methylene blue. Carotenoids have been shown to confer tolerance to methylene-blue-mediated stress in *R. sphaeroides* by preventing the harmful effects of $^1\text{O}_2$ (Anthony *et al.*, 2004). Since the *A. brasilense* *chrR*:Tn5 mutant overproduced carotenoids, no growth inhibition was seen with 5 mM methylene blue. However, the maximum growth inhibition observed in the case of the Δ *rpoE* mutant might have been the consequence of the accumulation of higher levels of singlet oxygen and resulting cellular damage due to the carotenoid deficiency. However, *A. brasilense* Sp7 produced basal levels of carotenoids and hence displayed less growth inhibition than that seen in the Δ *rpoE* mutant.

Fig. 5. Determination of the TSS of *rpoE* by 5' RACE. (a) Electropherograms showing TSS1 (i) and TSS2 (ii) are representatives of results from sequencing of several distinct clones obtained after 5' RACE experiments. (b) Schematic representation of the genetic organization of the *rpoE-chrR* chromosomal region of *A. brasilense* and sequences upstream of the *rpoE* translational start site (bold and italicized). TSSs (blue text; indicated by +1) and their respective promoter regions are boxed.

Studies in different Gram-negative bacteria like *Caulobacter crescentus* (Alvarez-Martinez *et al.*, 2007), *R. sphaeroides* (Anthony *et al.*, 2005) and *Xylella fastidiosa* (da Silva Neto *et al.*, 2007) have shown that ECF sigma factors are required for tolerating different extra-cytoplasmic stresses but are not essential for survival under optimal growth conditions. On the other hand, the σ^E of *E. coli*, a typical example of ECF sigma factors, is necessary for survival of cells even during optimal growth conditions (De Las Peñas *et al.*, 1997). Our results suggest that the product of *rpoE* (σ^E) in *A. brasilense*, although not essential for survival, is required for optimal growth. It is also apparent that RpoE is involved in the salt and ethanol tolerance in *A. brasilense*. The role of ECF sigma factors σ^T and AlgU in tolerating salt stress has been demonstrated in *C. crescentus* and *Pseudomonas fluorescens*, respectively (Alvarez-Martinez *et al.*, 2007; Schnider-Keel *et al.*, 2001). Few ECF sigma factors like SigV of *Enterococcus*

faecalis and σ^E of *X. fastidiosa* have also been shown to be involved in ethanol tolerance (Benachour *et al.*, 2005; da Silva Neto *et al.*, 2007).

Generally, the activities of ECF sigma factors are regulated in a coordinated way by controlling their expression at the transcriptional level and activity at the post-translational level. To maintain this coordinated regulation, in most cases the genes encoding sigma factors are positively autoregulated, providing a mechanism for rapid amplification of signal and shutdown of the response due to the co-expression of its negative regulator anti-sigma factor (Heimann, 2002; Lonetto *et al.*, 1992; Alba & Gross, 2004). An anti-sigma factor negatively regulates the activity of an ECF sigma factor by sequestering it and making it unavailable for binding to the core enzyme. Our RT-PCR data show that the *rpoE* gene is cotranscribed with *chrR* in

Fig. 6. β -Galactosidase activity of mid-exponential phase cells of *A. brasilense* strains Sp7, $\Delta rpoE$ and Car-1 harbouring pSK4, pSK5 or pSK6. Assays were performed in triplicate twice; error bars indicate SD.

A. brasilense. The results of two-hybrid and pull-down assays show that like other anti-sigma factors, ChrR also physically binds to RpoE to sequester it in order to regulate its activity at the post-translational level. But, how the activity of ChrR is regulated in response to different stresses in *A. brasilense* is an open question. In view of the redox sensitivity of the zinc anti-sigma factors ChrR of *R. sphaeroides* and RsrA of *Streptomyces coelicolor* (Campbell *et al.*, 2007), it is likely that ChrR of *A. brasilense* might also sense and respond to the redox status in the cell.

The gene organization such as that in *rpoE*–*chrR* of *A. brasilense* often results in the translation of two proteins by a translational coupling which executes tighter control on the stoichiometry of the interacting proteins (Norman *et al.*, 2000). Similar organization of regulatory gene pairs also occurs in the case of *amiC/amiR* in *P. aeruginosa* (Norman *et al.*, 2000), *prtI/prtR* in *P. fluorescens* (Burger *et al.*, 2000), *rsbW/sigB* and *rsbV/rsbW* in *Listeria monocytogenes* (Becker *et al.*, 1998). Such a mechanism locally increases the number of ribosomes close to the initiation codon of the

downstream gene (*chrR* in this case), which could reinitiate translation of the downstream gene even in the absence of a strong Shine–Dalgarno sequence.

ECF sigma factors, in general, are positively autoregulated in response to different stresses. In contrast with this generalization, it was recently shown that *rpoE* in *X. fastidiosa* is not autoregulated and its transcription is under the control of σ^{70} (da Silva Neto *et al.*, 2007). Our data indicate that the transcription of *A. brasilense rpoE* is initiated from two different transcription start sites (TSS1 and TSS2). The proximal promoter (*rpoEP1*) corresponding to TSS1 is RpoE-independent as its activity was not affected by inactivation of *rpoE* in the $\Delta rpoE$ strain. However, the activity of the distal promoter (*rpoEP2*) was notably RpoE-dependent. The –35 and –10 elements of the distal promoter also represent an RpoE-dependent promoter structure similar to that found in *R. sphaeroides*, with –35 (TGATCC) and –10 (GCCGTA) regions separated by 14 nt (Dufour *et al.*, 2008). This result is in agreement with our previous observation that the σ^E -dependent promoter of *R. sphaeroides* is expressed at high efficiency in the *A. brasilense* anti-sigma mutant, Car-1 (Thirunavukkarasu *et al.*, 2008). A recent analysis of the amino acids in region 4.2 of RpoE of *R. sphaeroides* as well as in other bacteria indicated that a KSR-RLA amino acid stretch is required for interaction with the –35 consensus, TGATCC (Dufour *et al.*, 2008). The amino acid sequence of region 4.2 of RpoE of *A. brasilense* also revealed the presence of a KSR-RLA stretch (Thirunavukkarasu *et al.*, 2008), indicating the identical nature of the interaction between RpoE and its cognate –35 consensus (TGATCC) in *R. sphaeroides* and *A. brasilense*.

Expression of sigma factors from more than one promoter has been shown to occur in *P. aeruginosa* and *S. coelicolor* wherein five and four promoters, respectively, are present, but only two (P_1 and P_3) of five *algU* promoters in *P. aeruginosa* (Schurr *et al.*, 1995) and only one (*sigHP2*) of four in *S. coelicolor* (Sevciková *et al.*, 2001) were autoregulated. The autoregulated promoters were induced in

Table 2. Effect of stationary phase, salt, ethanol, methylene blue and heat on the induction of *rpoE* promoters (*rpoEP1* and *rpoEP2*)

The promoter activities were determined by measuring the β -galactosidase activity of Sp7 and $\Delta rpoE$ harbouring pSK5 or pSK6 after growth in the presence of different stresses. Data shown are the mean ($\pm$ SD) from three replicates.

Treatment	β -Galactosidase activity (Miller units)					
	Vector (pRKK200)		pSK5 (<i>rpoEP1</i>)		pSK6 (<i>rpoEP2</i>)	
	Sp7	$\Delta rpoE$	Sp7	$\Delta rpoE$	Sp7	$\Delta rpoE$
Control	7.0 (0.3)	8.9 (0.5)	24.6 (2.5)	21.7 (1.2)	29.4 (2.8)	10.2 (1.3)
Stationary phase	6.8 (0.4)	6.6 (0.3)	23.6 (1.7)	23.5 (2.4)	68.3 (4.7)	13.4 (0.7)
NaCl (250 mM)	7.2 (0.3)	7.3 (0.6)	24.7 (3.2)	22.6 (0.9)	73.5 (3.2)	9.8 (0.8)
Ethanol (1.5 %)	6.9 (0.5)	6.8 (0.4)	25.8 (2.1)	23.7 (1.8)	82.7 (4.1)	10.6 (0.3)
Methylene blue (10 μ M)	7.1 (0.2)	9.1 (0.7)	24.5 (1.9)	20.8 (2.6)	65.4 (5.3)	11.2 (0.5)
Heat (42 °C)	6.6 (0.3)	6.8 (0.2)	26.7 (3.3)	24.9 (1.4)	76.7 (3.8)	12.7 (2.1)

response to environmental stresses such as heat shock. In *A. brasilense* too, only the RpoE-dependent promoter (*rpoEP2*) was induced in response to stress caused by stationary phase, salt, ethanol, heat and methylene blue. Thus, the basal level of carotenoids present in *A. brasilense* Sp7 may be largely due to the constitutive expression from the *rpoEP1*, whereas *rpoEP2* is induced under stressful conditions.

Our present and previous observations (Thirunavukkarasu *et al.*, 2008) show that the RpoE–ChrR system of *A. brasilense*, a non-photosynthetic bacterium, resembles that present in the well-characterized photosynthetic bacterium *R. sphaeroides* (Newman *et al.*, 1999; Anthony *et al.*, 2005) because (i) the *rpoE* gene is located upstream of *chrR* and makes a single transcriptional unit for *rpoE–chrR*; (ii) ChrR is a cytosolic anti-sigma factor and has a zinc-binding motif; (iii) ChrR physically interacts with RpoE to negatively regulate the activity of RpoE; (iv) the promoter consensus sequences recognized by RpoE are similar; (v) the transcription of the *rpoE–chrR* operon is autoregulated; and (vi) the transcription of *rpoE* is induced in response to exposure to $^1\text{O}_2$ so as to cope with the stress generated by elevated levels of $^1\text{O}_2$. Despite these similarities, the RpoE–ChrR system of *A. brasilense* differs from that of *R. sphaeroides* because (i) carotenoid synthesis is under the control of RpoE; (ii) the *rpoE* and *chrR* ORFs overlap by 4 nt and seem to be translationally coupled; (iii) transcription of the *rpoE–chrR* operon is regulated from two different promoters, one of which is RpoE-dependent; and (iv) RpoE plays a positive role in tolerating salt and ethanol stress. Further, while *R. sphaeroides* and *A. brasilense* possess a cytosolic ChrR-like anti-sigma factor, carotenoid synthesis in the latter is RpoE-dependent. These unique features of *A. brasilense* suggest that the regulation of RpoE–ChrR in *A. brasilense* may be more complex than that in *R. sphaeroides*. The study of ECF sigma factors in *A. brasilense* will help to better understand the mechanism of abiotic stress tolerance in *A. brasilense*, which might be critical in the adaptation, survival and proliferation of this bacterium in the rhizosphere and the soil under stressful conditions.

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