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 extracytoplasmic function (ECF) sigma factors play an important role in stress adaptation, we analysed the role of ECF sigma factor (also known as RpoE or $\sigma^E$) 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.

**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; Zahrani, 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, $\sigma^{70}$ and $\sigma^{54}$. The majority of sigma factors belong to the $\sigma^{70}$ 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.

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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 extracytoplasmic compartment of the cell and hence were called extracytoplasmic 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 (1O2) by quenching and reacting with 1O2 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 1O2 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 1O2 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 Zn2+ 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 NH4Cl 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 OD600. All the chemicals used for growing bacteria were from Hi-media while incubator shaker and growth was monitored by measuring OD600

**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′-F/rpoE′-R and rpoE′-F/rpoEs′-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 PstI and BglII at the 5’ and 3’ ends of amplicon A, and restriction sites for BglII and EcoRI at the 5’ and 3’ ends of amplicon B. After amplification, these fragments were digested with PstI–BglII and BglII–EcoRI, respectively, and inserted between the PstI and EcoRI sites of pSUP202. The resulting plasmid (pMN2) was cleaved with BglII and ligated to a BamHI-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 (rpoE1F/rpoER). The rpoE disruption mutant of Sp7 was designated rpoEKm.

**Construction of an in-frame rpoE deletion mutant (ΔrpoE) 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 (ΔrpoE) 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 rpoEA1F/rpoEA1R and rpoEB1F/rpoEB1R 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 PstI and BglII at the 5’ and 3’ ends of amplicon A1, and restriction sites for BglII and EcoRI at the 5’ and 3’ ends of amplicon B1. After amplification, these fragments were digested with PstI–BglII and BglII–EcoRI, respectively, and inserted between the PstI and EcoRI 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 (rpoE1F/ rpoER).

**Growth and stress sensitivity test.** The differences in the sensitivity of A. brasilense Sp7 and its ΔrpoE mutant (ΔrpoE) 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, parquat, 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 OD600 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 OD600.
Growth inhibition assays were performed on LB agar plates to examine the sensitivity of *A. brasilense* WT, ΔrpoE 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 μl 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 μl 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) (μg RNA)−1 for 30 min at 37 °C followed by phenol extraction and ethanol precipitation. The cycling conditions were 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 *rpoEF*I/*rpoE*R1 and *chrR*F1/*chrRR1* 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 *EcoR*I–*BamHI* and cloned in the pBT bait plasmid, which encodes the full-length bacterial phage λ cl repressor protein under the control of the IPTG inducible *lacUV5* promoter, resulting in pBT-*rpoE*. Likewise, the 700 bp *chrR* gene was digested with *EcoR*I–*XhoI* and cloned in pTRG target plasmid to fuse with the N-terminal domain of the x-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 μM IPTG, 80 μg X-Gal mL−1 and 200 μM phenethyl 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 rpoEP2 and chrRP2 primers with genomic DNA of A. brasilense Sp7. The amplified product was digested with XbaI–BamHI, purified and ligated with the similarly digested expression vector pET15b. *E. coli* DH5α was transformed with the ligation mix and the transformants were selected on Luria agar containing ampicillin (100 µg ml⁻¹). The pet15b clones harbouring *rpoE–chrR* genes were confirmed by restriction digestion and nucleotide sequencing, and designated pNGI. *E. coli* BL21λ (DE3) pLysS competent cells were then transformed with pNGI, and the transformants were selected on LB agar supplemented with ampicillin and chloramphenicol (25 µg 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 pNGI1 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* BL21λ (DE3) pLysS harbouring pNGI (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 (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 µl 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 (rpoEIR). 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 rpoEII2 primer (complementary to the region upstream of the rpoEI 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 rpoEIII3 primer, which is complementary to a region further upstream of the rpoEII 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 (rpoEP1 and rpoEP2) were amplified by PCR and cloned separately as well as together between Stul and KpnI 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 rpoEP1 and rpoEP2) was amplified by using the rpoEP2 : F/rpoEP1 : R primer set. The rpoEP1 (−76 to +63 of TSS1) and rpoEP2 (−64 to +65 of TSS2) were separately amplified using rpoEP1 : F/rpoEP1 : R and rpoEP2 : F/rpoEP2 : R primer sets (Supplementary Table S1), respectively. All three amplified products were digested with Stul–KpnI, and ligated with a similarly digested pRKK200 vector. *E. coli* DH5α was then transformed with the ligation mix and the transformants were selected on LB agar supplemented with spectinomycin (100 µg ml⁻¹). After confirmation of recombinant plasmids by sequencing, the constructs were designated pSK4 [rpoEP1 : P1 + P2 : lacZ fusion], pSK5 (rpoEP1 : lacZ fusion) and pSK6 (rpoEP2 : lacZ fusion). These constructs were finally conjugatively mobilized into the *A. brasilense* strains via *E. coli* S17.1 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 µM 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 (ΔrpoE)**

A mutant of *A. brasilense* Sp7 was constructed by inserting a Km’ 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' 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' 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 (ΔrpoE) was generated by deleting >95% of rpoE from the rpoE: Km strain by exchanging the chromosomally located rpoE: Km copy with a ΔrpoE 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' 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 ΔrpoE 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' gene cassette) of the inactivated rpoE. Further, in order to examine whether the deletion of rpoE in the ΔrpoE 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, ΔrpoE and Car-1 by amplifying rpoE and chrR with RNA samples isolated from Sp7, rpoE: Km, ΔrpoE and Car-1. RT-PCR analysis revealed that the RNA sample from the ΔrpoE 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 ΔrpoE 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 ΔrpoE mutant. However, insertion of the Km' 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 ΔrpoE mutant, we characterized its phenotype.

The obvious phenotypic difference observed in the ΔrpoE 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 ΔrpoE mutant were creamy. This difference in the colour of the colonies could only be observed when Sp7, ΔrpoE 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 ΔrpoE mutant, it produced carotenoids and acquired the light pink colour that was seen in the wild-type Sp7 (data not shown).

Since Sp7, ΔrpoE 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 ΔrpoE but not in Car-1 (Fig. 2b). The zone of inhibition around ΔrpoE was significantly larger than that formed by Sp7, indicating a clear difference in the sensitivity of ΔrpoE 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
decreased and methylene blue sensitivity increased in the order Car-1–Sp7–ΔrpoE.

To examine the role of RpoE in the growth and tolerance to other stresses, we compared the growth of ΔrpoE with that of Sp7 and ΔrpoE/pAT5 (expressing basal levels of RpoE from the uninduced lac promoter), with and without specific stresses. In the absence of any stress, the ΔrpoE mutant grew considerably slower than the parent and ΔrpoE (pAT5), indicating the involvement of RpoE in controlling some housekeeping functions (Fig. 3a). Of the general stresses such as desiccation, heat, salinity and ethanol, ΔrpoE 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 ΔrpoE 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 ΔrpoE mutant to PEG-200 and heat was observed (data not shown). Since the difference between carotenoid content of Sp7 and ΔrpoE is very small, the lack of significant difference in the susceptibility of Sp7 and ΔrpoE 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 ¹O₂ 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/chrR 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
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 TATCC at −35 and CGCCTA 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 rpoE1) having TGAACA at −35 and AACAC 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 rpoE1 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 (rpoE1 and rpoE2) were amplified separately and together, and
Role of RpoE in *A. brasilense*

Insertion of promoterless *lacZ* reporter in pRRK200 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, ΔrpoE and Car-1) harbouring promoter: *lacZ* fusions (pSK4, pSK5 and pSK6). These three *A. brasilense* strains (Sp7, ΔrpoE 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, ΔrpoE(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 ΔrpoE 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 ΔrpoE) harbouring pRRK200, pSK5 or pSK6. *rpoEP2* activity was significantly induced in Sp7 in response to stationary phase, NaCl (250 mM), ethanol (1.5%), methylene blue (10 μM) and heat (40 °C), whereas the ΔrpoE 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 °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 synthesise 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 ΔrpoE mutant produced carotenoids by complementing the defect, which reconfirmed the role of *rpoE* in carotenoid biosynthesis. The ability of *A. brasilense* strains to synthesise carotenoids correlated well with their tolerance against methylene blue. Carotenoids have been shown to confer resistance to methylene-blue-mediated stress in *R. sphaeroides* by preventing the harmful effects of ¹O₂ (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 ΔrpoE 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 ΔrpoE mutant.
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 σE 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* (σE) 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 σF 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 σE 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 *A. brasilense*.

**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.
The promoter activities were determined by measuring the β-galactosidase activity 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 ΔrpoE 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 (GGCGTA) 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 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 is an open question. In view of the physicochemical properties of ChrR, 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), prtl/prlR 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.

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>β-Galactosidase activity (Miller units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vector (pRKK200)</td>
</tr>
<tr>
<td>Control</td>
<td>Sp7</td>
</tr>
<tr>
<td>Stationary phase</td>
<td>7.0 (0.3)</td>
</tr>
<tr>
<td>NaCl (250 mM)</td>
<td>6.8 (0.4)</td>
</tr>
<tr>
<td>Ethanol (1.5 %)</td>
<td>7.2 (0.3)</td>
</tr>
<tr>
<td>Methylene blue (10 μM)</td>
<td>6.9 (0.5)</td>
</tr>
<tr>
<td>Heat (42 °C)</td>
<td>7.1 (0.2)</td>
</tr>
</tbody>
</table>

The promoter activities were determined by measuring the β-galactosidase activity of Sp7 and ΔrpoE harbouring pSK5 or pSK6 after growth in the presence of different stresses. Data shown are the mean (±SD) from three replicates.
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 ¹O₂ so as to cope with the stress generated by elevated levels of ¹O₂. 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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