An extra-cytoplasmic function sigma factor and anti-sigma factor control carotenoid biosynthesis in *Azospirillum brasilense*

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Strains Sp7 and Cd of *Azospirillum brasilense*, a plant growth-promoting rhizobacterium, differ in synthesis of carotenoids. While colonies of strain Sp7 have a white–cream colour on plates, colonies of strain Cd are orange–pink coloured because of the synthesis of carotenoids. Screening of a mini-Tn5 mutant library of *A. brasilense* Sp7 revealed two orange–pink-coloured mutants that produced carotenoids. Cloning and sequencing of the Tn5 flanking region in both the carotenoid-producing mutants of Sp7 revealed insertion of Tn5 in an ORF encoding anti- σ factor, a ChrR-like protein. The upstream region of the Tn5-mutated ORF contained another ORF that encoded an extra-cytoplasmic function (ECF)-class σ factor (σ^E , RpoE). When the nucleotide sequences of the corresponding ORFs from the carotenoid-producing strain Cd were analysed, the sequence of the Cd σ^E was identical to that of the carotenoid non-producing strain Sp7, but the Cd anti- σ^E ORF had a deletion that caused frame shifting and creation of a stop codon. This resulted in the premature termination of the protein, which was about 7 kDa smaller than the Sp7 anti- σ^E . Cloning of Sp7 anti- σ^E in a broad-host-range expression vector and expression in *A. brasilense* Cd and in the anti- σ^E knockout mutant of *A. brasilense* Sp7 resulted in the inhibition of carotenoid synthesis. Similarly, cloning and overexpression of *A. brasilense* Sp7 σ^E in *A. brasilense* Sp7 resulted in the production of carotenoids. These observations clearly indicate that carotenoid synthesis in *A. brasilense* is controlled by σ^E with its cognate anti- σ^E .

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INTRODUCTION

Members of the genus *Azospirillum* are nitrogen-fixing bacteria that live in close association with the roots of several important crop plants and grasses, and stimulate the growth of their host plant by producing phytohormones and siderophores (Steenhoudt & Vanderleyden, 2000). Taxonomically, they are closely related to *Rhodospirillum rubrum* and *Magnetospirillum magnetotacticum* of

the family Rhodospirillaceae (Woese *et al.*, 1982). Currently, the genus consists of 10 species, including *Azospirillum brasilense*, *Azospirillum lipoferum*, *Azospirillum halopraeferens*, *Azospirillum amazonense*, *Azospirillum irakense*, *Azospirillum largimobile*, *Azospirillum doebereineriae*, *Azospirillum oryzae*, *Azospirillum melinis* and *Azospirillum canadense* (Mehnaz *et al.*, 2007). Some of the strains of *A. brasilense* and *A. lipoferum* show orange–pink pigmentation due to the presence of carotenoids (Tarrand *et al.*, 1978). Within *A. brasilense*, two strains (Cd and Sp7) differ from each other with regard to their carotenoid content. While Cd produces carotenoids, giving an orange–pink colour to the colonies, Sp7 lacks detectable levels of carotenoids (Tarrand *et al.*, 1978; Nur *et al.*, 1981). Strains Sp7 and Cd are genetically very closely related. It has even been suggested that strain Cd is a reisolat following plant inoculation with Sp7 (Nur *et al.*, 1981).

Abbreviation: ECF, extra-cytoplasmic function.

The GenBank/EMBL/DDBJ accession numbers for the σ^E and anti- σ^E sequences of *A. brasilense* are DQ324853 and DQ324854, respectively.

Three supplementary figures showing phylogenetic trees for the amino acid sequences of ChrR and ECF- σ^E , and the effects of the expression of *rpoE* and *chrR* in *Azospirillum brasilense*, are available with the online version of this paper.

Although *A. brasilense* Sp7 is not reported to produce carotenoids, carotenoid-producing mutants (spontaneous or induced by chemical mutagens or Tn5) of this strain have been isolated (Tarrand *et al.*, 1978; Abdel-Salam & Klingmueller, 1987). The synthesis of carotenoids in these mutants is triggered by oxygen stress (Hartmann & Hurek, 1988), and their presence has been shown to improve oxygen tolerance for nitrogen fixation at high dissolved oxygen concentrations (12 μ M) compared with their pigmentless parent.

Besides the enzymes that detoxify superoxide and hydrogen peroxide, carotenoids in *A. brasilense* strain Cd are implicated in protection against oxidative damage owing to their ability to quench singlet oxygen and possibly oxygen radicals (Hartmann & Hurek, 1988; Nur *et al.*, 1981, 1982). The fact that nitrogen fixation is inhibited in *A. brasilense* Cd at high dissolved oxygen concentrations upon inhibition of carotenoid synthesis by diphenylamine suggests that carotenoids protect nitrogenase from oxidative damage (Nur *et al.*, 1981).

On the basis of similarity to the absorption spectra of carotenoids from *Halobacterium cutirubrum*, the carotenoids of *A. brasilense* Sp7 mutants and those present in the *A. brasilense* Cd strain were thought to be of the bacterioruberin type (Nur *et al.*, 1981). The long-chain C-50 bacterioruberin-type carotenoids have been isolated from several extremophilic bacteria and Archaea, including species of *Halobacterium*, *Haloferax* (D'Souza *et al.*, 1997) and psychrotrophic bacteria [*Micrococcus roseus* (Jagannadham *et al.*, 1991) and *Arthrobacter agilis* (Fong *et al.*, 2001)]. These organisms require adaptive mechanisms to cope with extreme environmental conditions, and bacterioruberin has been shown to contribute to their adaptation to and survival in extreme environments. The presence of these carotenoids may also reinforce the membrane bilayer to reduce O₂ diffusion in the cytoplasm (Wisniewska & Subczynski *et al.*, 1998) and raise the hydrophobic barrier for polar molecules and ions.

While screening a mini-Tn5 mutant library of *A. brasilense* Sp7, two mutants, Car-1 and Car-2, that were orange–pink coloured due to the production of carotenoids, were identified. Activation of the synthesis of carotenoids in the two Tn5 mutants suggested to us that a repressor of carotenoid biosynthesis had been inactivated in these strains. Although carotenoid-producing mutants of *A. brasilense* Sp7 have been isolated and their phenotype analysed, the genetic basis of carotenoid production has not been investigated (Hartmann & Hurek, 1988). In this study we have shown that inactivation of the genetic locus encoding anti- σ factor in *A. brasilense* Sp7 leads to carotenoid production in the mutants. The occurrence of a deletion in the ORF of the gene encoding anti- σ factor in a naturally carotenoid-producing strain, *A. brasilense* Cd, further corroborated the role of an extra-cytoplasmic function σ factor and its cognate anti- σ factor in controlling carotenoid production in *A. brasilense*.

METHODS

Bacterial strains, plasmids, chemicals and growth conditions.

A. brasilense strains were grown in minimal medium for *A. brasilense* (MMAB; Vanstockem *et al.*, 1987) containing malate and NH₄Cl (10 mM) as sole source of carbon and nitrogen, respectively. Plasmid pCMPG8003 (Nagarajan *et al.*, 2007) was used to generate random insertions of a transposon (Tn5) in *A. brasilense* Sp7. Another plasmid, pJDN30 (Newman *et al.*, 1999), containing a fusion of *rpoE* P1 promoter with *lacZ* on a broad-host-range vector, was conjugatively mobilized via *Escherichia coli* S.17-1 into *A. brasilense* Sp7 and the Car-1 mutant (Vanstockem *et al.*, 1987). Assays of β -galactosidase activity were performed in triplicate as in Newman *et al.* (2001), except that the cells were grown aerobically with shaking in MMAB medium. All chemicals used for growing bacteria were from Hi-media, while methanol (HPLC grade) was from Merck.

Screening of the mutant library, cloning and characterization of Tn5 mutations.

A mutant library of *A. brasilense* Sp7 containing 6800 mutants generated by random insertion of mTn5*gusA-oriV* Tn5 was screened visually for orange–pink coloured colonies on minimal agar plates containing 40 μ g kanamycin ml⁻¹. Total genomic DNA isolated with the aid of a GFX DNA purification kit (Amersham Pharmacia Biotech) was digested with restriction endonucleases (*Nde*I or *Xho*I) that do not cut mTn5*gusA-oriV*. The presence of *oriV* in the mTn5 cassette facilitated direct cloning by ligating the restriction-endonuclease-digested genomic DNA using T4 DNA ligase (Invitrogen) without a cloning vector. The *Nde*I fragment containing an mTn5 insertion in the genomic region of the Car-1 mutant was self-ligated and transformed into *E. coli* DH5 α , and the resulting plasmid was designated pTN1. A partial sequence of the genomic DNA region flanking both sides of the mTn5 insertion was determined by using the primers 5'-CTCCTTAGCTAGTCAGGTACCG-3', which binds close to the *Nof*I site of *gusA*, and 5'-GTCTGACGCTCAGTGGAAAC-3', which binds in the *oriV* region of mTn5. DNA sequence similarities were examined using the BLASTX program at NCBI (Altschul *et al.*, 1997). The CLUSTAL W program (Thompson *et al.*, 1994) was used to align the target sequences with similar sequences retrieved from the databases, and a dendrogram was constructed using the neighbour-joining method (Saitou & Nei, 1987) with the program MEGA version 3.0 (Kumar *et al.*, 2004). The robustness of the inferred trees was evaluated by 500 bootstrap resamplings.

Cloning of *rpoE* and *chrR* genes in the expression vector pET15b.

PCR amplified *rpoE* and *chrR* genes were cloned in the expression vector, pET15b digested with *Nde*I and *Bam*HI. The *rpoE* gene was PCR-amplified by using the primers *rpoE*-FNde (5'-GGAATTCATATGCAGGATCTTGGTTCCCC-3'), with a *Nde*I site located upstream of the start codon, and *rpoE*-RBam (5'-CGGGATCTCATCGGGAGTCCCTCATG-3'), with a *Bam*HI site located downstream of the stop codon (restriction sites are underlined). Similarly, the *chrR* gene was amplified by using the primers *arpoE*-FNde (5'-ACTCCCGCATATGACCGTGCCACCCACC-3') and *chrR*-RBam (5'-CGGGATCTCTCAGAACTTCACAAACGGGTGAGG-3'). The amplicons were digested with *Nde*I and *Bam*HI, PCR-purified and ligated with the similarly digested expression vector pET15b. *E. coli* DH5 α was transformed with the ligation mix and transformants were selected on Luria agar containing 100 μ g ampicillin ml⁻¹. The nucleotide sequences of the clones of *rpoE* and *chrR* were determined, and showed the expected size of inserts.

pET15b clones harbouring *rpoE* and *arpoE* genes from *A. brasilense* Sp7 and *A. brasilense* Cd were designated pAT1 and pAT2 (*rpoE*), and pAT3 and pAT4 (*chrR*), respectively (see Table 1). These clones were then transferred into *E. coli* BL21 λ DE3 (pLysS). The clones were grown overnight in LB medium with 25 μ g chloramphenicol ml⁻¹.

Table 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant properties	Source or reference
Strains		
<i>E. coli</i> DH5 α	$\Delta lacU169$ <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thiL</i> <i>relA1</i>	Gibco-BRL
<i>E. coli</i> S17-1	Sm ^r , <i>recA</i> , <i>thi</i> , <i>pro</i> , <i>hsdR</i> ⁻ M+RP4:2-Tc:Mu:Km Tn7, <i>λpir</i>	Nagarajan <i>et al.</i> (2007)
<i>E. coli</i> BL21 λ DE3(pLysS)	<i>ompT</i> <i>hsdS</i> (<i>r_B⁻ m_B⁻</i>) <i>dcm</i> + Tet ^r <i>endA</i> <i>gal</i> λ (DE3)	Novagen
<i>A. brasilense</i> Sp7	Carotenoid-non-producing strain	Nur <i>et al.</i> (1981)
<i>A. brasilense</i> Cd	Carotenoid-producing strain	Nur <i>et al.</i> (1981)
Car-1	Carotenoid-producing <i>A. brasilense</i> Sp7 mutant with mTn5 insertion	This work
Car-2	Carotenoid-producing <i>A. brasilense</i> Sp7 mutant with mTn5 insertion	This work
Plasmids		
pCMPG8003	Ap ^r Km ^r , mTn5 <i>gusA-oriV</i> in pUT vector	Nagarajan <i>et al.</i> (2007)
pET15b T7	Expression vector	Novagen
pTN1	Self-ligated <i>NdeI</i> fragment containing mTn5OriV and flanking genomic region from Car-1	This work
pMMB206	Cm ^r , broad-host-range low-copy-number expression vector	Morales <i>et al.</i> (1991)
pAT1	<i>rpoE</i> gene from <i>A. brasilense</i> Sp7 cloned into <i>NdeI</i> and <i>Bam</i> HI sites of pET15b	This work
pAT2	<i>rpoE</i> gene from <i>A. brasilense</i> Cd cloned into <i>NdeI</i> and <i>Bam</i> HI sites of pET15b	This work
pAT3	<i>chrR</i> gene from <i>A. brasilense</i> Sp7 cloned into <i>NdeI</i> and <i>Bam</i> HI sites of pET15b	This work
pAT4	<i>chrR</i> gene from <i>A. brasilense</i> Cd cloned into <i>NdeI</i> and <i>Bam</i> HI sites of pET15b	This work
pAT5	<i>rpoE</i> gene from <i>A. brasilense</i> Sp7 cloned into <i>Eco</i> RI and <i>Bam</i> HI sites of pMMB206	This work
pAT6	<i>chrR</i> gene from <i>A. brasilense</i> Sp7 cloned into <i>Eco</i> RI and <i>Bam</i> HI sites of pMMB206	This work
pJDN30	<i>rpoE</i> P1 promoter of <i>R. sphaeroides</i> cloned into <i>lacZ</i> fusion reporter vector pRKK200	Newman <i>et al.</i> (1999)

and 100 μ g ampicillin ml⁻¹, and inoculated into fresh medium so as to give an OD₆₀₀ of 0.03. Cultures were then incubated with shaking at 25 °C until they attained OD₆₀₀ 0.3. At this stage, IPTG was added to give a final concentration of 1 mM to induce the expression of the cloned gene. Aliquots of the cultures were taken every 30 min, and were mixed with TCA to hydrolyse the cells and kept at 4 °C to precipitate the proteins. Equal amount of proteins from each sample were loaded and resolved on 15% SDS-PAGE gels. After having optimized the conditions for induction, a 30 ml culture volume was used to induce the expression of proteins. Cultures induced for 3 h were pelleted and used for extraction and purification of induced proteins using Ni-NTA resin, as described in the QIAexpress manual.

Cloning of *rpoE* and *chrR* genes in the broad-host-range expression vector pMMB206, and conjugative mobilization into *A. brasilense*. PCR-amplified *rpoE* and *chrR* genes were cloned into the broad-host-range expression vector pMMB206, digested with *Eco*RI and *Bam*HI. The *rpoE* gene was PCR-amplified by using the primers *rpoE*-FEco (5'-GGAATTCAATGCAGGATCTTGGTTCCCC-3'), with an *Eco*RI site (underlined) located upstream of the start codon, and *rpoE*-RBam (5'-CGGGATCCTCATCGGGAGTCCCTCATG-3'), with a *Bam*HI site (underlined) located downstream of the stop codon. Similarly, the *chrR* gene was amplified by using the primers *chrR* *rpoE*-FEco (5'-GGAATTCAATGACCGTGCCCA-CCCACCATC-3') and *rpoE*-RBam (5'-CGGGATCCTCAGAACTTCAACAACGGGTTGAGG-3'). The amplicons were digested with *Eco*RI and *Bam*HI, PCR-purified and ligated with the similarly digested pMMB206. *E. coli* DH5 α was transformed with the ligation mix and transformants were selected by blue/white selection on Luria agar plates containing 1 mM IPTG, 80 μ g X-Gal ml⁻¹ and 25 μ g chloramphenicol ml⁻¹. Nucleotide sequences of the clones from white colonies harbouring inserts of the expected size were determined. The clones harbouring *rpoE* (pAT5) and *chrR* (pAT6) genes from *A. brasilense* Sp7 were transferred into *E. coli* S17-1, and

then conjugatively mobilized into *A. brasilense* Sp7, the Car-1 mutant and *A. brasilense* Cd, as described above.

Spectrophotometric analysis for carotenoids. The bacterial cultures were allowed to grow to stationary phase with shaking at 150 r.p.m. in an incubator-shaker at 30 °C in MMAB medium. Cells were then pelleted and washed with saline solution (0.85% NaCl in water). Fresh cells were suspended in methanol repeatedly to extract the pigments whenever required to characterize them by absorption spectra (Jagannadham *et al.*, 1991). Pigment extraction was carried out at room temperature, and all glassware was covered with aluminium foil to protect the pigments from light. UV-visible absorption spectra between 300 and 600 nm of the pigments dissolved in methanol were recorded with a Hitachi U-2000 spectrophotometer. To study the effects of cloned *rpoE* and *chrR*, *A. brasilense* cultures were grown in 50 ml LB medium for 24 h with appropriate antibiotics and IPTG, if required. The growth of the cultures was estimated by recording OD₆₀₀, after which the cell density was equalized by suitable dilution. Cultures (40 ml) with equal OD₆₀₀ values were used for the extraction of carotenoids that were quantified by measuring A₄₈₅.

Effect of growth phase and oxidative stress on heterologous expression of *rpoE-lacZ* fusion in *A. brasilense*. In order to examine the effect of growth phase on induction of the *Rsp rpoE* promoter in *A. brasilense*, we performed β -galactosidase assays on exponential-phase as well as stationary-phase cells of *A. brasilense* strains harbouring pJDN30. To examine the effect of different reactive oxygen-species-producing chemicals, *A. brasilense* strains harbouring pJDN30 were exposed to oxidative stress by supplementing the mid-exponential-phase cultures with 2 mM H₂O₂, 1 mM paraquat or 10 μ M methylene blue and growing them for another 2 h. Untreated cells were used as a control. At the time of assay, the number of cells in treated and control cultures was equalized by diluting them with fresh medium. β -Galactosidase assay (Miller, 1972) was performed

Fig. 1. Absorption spectra of methanol extracts prepared from *A. brasiliense* Sp7 and its Car-1 mutant.

with 1 ml equalized culture in triplicate for each sample on two different occasions.

RESULTS

Isolation and identification of Tn5 mutants leading to carotenoid production in *A. brasiliense* Sp7

While screening a library of 6800 mutants of *A. brasiliense* Sp7 generated by a mini-Tn5 derivative containing a promoterless *gusA* reporter along with the *oriV* and *nptII* genes, on agar plates, two pink-coloured colonies representing mutants (designated Car-1 and Car-2) were observed. Expression of GusA was not observed in either mutant, possibly due to the insertion of Tn5 in the orientation wherein the promoterless *gusA* did not fuse with any promoter of *A. brasiliense*. When absorption spectra of methanol extracts from the two Tn5 mutants were compared with their parent, they showed carotenoid-specific peaks (Fig. 1). It was also noted that aerobically grown cultures of the two Tn5 mutants (Car-1 and Car-2)

produced carotenoids only during late-exponential phase to stationary phase, but not in early exponential phase (data not shown).

The genetic locus inactivated by Tn5 insertion in the two mutants (Car-1 and Car-2) was cloned, and the identity of the inactivated locus in each mutant was determined by nucleotide sequencing of genomic DNA flanking the site of Tn5 insertion. When plasmid pTN1 (harbouring the Tn5-mutagenized genomic region from the Car-1 mutant) was electroporated into *A. brasiliense* Sp7, the resulting transformants produced red-pigmented colonies. PCR amplification using primers specific for the *chrR* gene failed to produce any amplicon with the genomic DNA extracted from red-pigmented colonies, whereas an amplicon of ~700 bp was observed in the case of Sp7 genomic DNA. This confirmed that the *chrR* gene in the red-pigmented colonies was replaced by the Tn5-mutated *chrR* allele (data not shown). The deduced amino acid sequence of the ORF carrying the Tn5 insertion in *A. brasiliense* Sp7 (231 aa) showed closest relatedness to the anti- σ factor-encoding genes of *R. rubrum* (ZP_00267593, 224 aa) and *M. magnetotacticum* (ZP_00056407, 215 aa) (Supplementary Fig. S1). Among the other closely related anti- σ factors, the deduced amino acid sequence of the putative anti- σ factor from *A. brasiliense* showed very high similarity (40% identity) with the ChrR protein of *R. sphaeroides* (ZP_00004392, 213 aa), which has been thoroughly investigated genetically, structurally and functionally (Campbell *et al.*, 2007; Newman *et al.*, 2001). In view of this, the anti- σ factor protein of *A. brasiliense* Sp7 was designated Abr ChrR. The sites of Tn5 insertion in the Car-1 and Car-2 were 216 and 456 nt downstream of the start codon of the anti- σ ORF, respectively (Fig. 2). Sequence analysis revealed that the Tn5 insertion was oriented such that the promoterless *gusA* could not be read from the promoter driving *chrR*, explaining the *gus*⁻ phenotype.

Primer walking on both sides of Tn5 revealed that the ORF of the anti- σ was preceded by another ORF of 191 aa, which showed very high similarity to an ECF- σ factor (COG1595), σ^E or RpoE (Fig. 2). The nucleotide sequences downstream of the anti- σ -encoding region revealed

Fig. 2. Genetic map of the mutated locus involved in carotenoid overproduction in *A. brasiliense* Sp7. Grey arrows represent identified ORFs and white arrows represent high-homology regions. ORFs: 1, Fe-S cluster redox enzyme; 2, hypothetical protein (ZP00268580); 3, ECF- σ factor; 4, anti- σ factor; 5, rRNA methylase; 6, L-asparaginase II.

significant similarities with the genes encoding rRNA methylase and L-asparaginase II. Upstream of the ORF of ECF- σ , regions having significant similarities to a hypothetical protein of *Nitrosomonas europaea* (ZP_00268580), followed by a gene encoding a redox enzyme containing a Fe-S cluster, were identified.

Analysis of the nucleotide sequences of putative anti- σ (named the *chrR* gene) and ECF- σ^E (named the *rpoE* gene) region of *A. brasilense* revealed that both genes were very closely linked, to the extent that the start codon of the anti- σ ORF overlapped with the stop codon of the σ^E ORF, and thus they were probably transcribed together from a promoter located upstream of the ORF of σ^E . The 191 amino acids of the putative ECF- σ factor of *A. brasilense* showed maximum similarity (46% identity) to that of RpoE from *R. sphaeroides* (ZP_00004391, 181 aa; Fig. 3a), *R. rubrum* (ZP_00267592, 236 aa) and *M. magnetotacticum* (ZP_00056406, 225 aa) (Supplementary Fig. S2) which are located upstream of the ORF of their anti- σ partners (COG3806). Interestingly, the single-nucleotide overlap of the start codon of the ORF of anti- σ with the stop codon of the ORF of σ in *A. brasilense* was also observed in the *R.*

rubrum and *M. magnetotacticum* genomes. In *R. sphaeroides*, however, the ORFs of these two genes were distinct from each other without overlap of the start and stop codons.

Comparison of the translated ORF (191 aa) of the putative ECF- σ factor of *A. brasilense* Sp7 revealed maximum similarity with *R. sphaeroides* in the whole of region 2, including 2.1, 2.2, 2.3 and 2.4, and in region 4.2. An examination of the ORF of anti- σ of *A. brasilense* (Fig. 3b) revealed the presence of eight cysteine residues. In *R. sphaeroides*, ChrR possesses four cysteine residues, of which the two residues located at positions 35 and 38 at the N-terminal end constitute a conserved Hx₃Cx₂C zinc-binding domain, whereas the other two cysteine residues are located at positions 187 and 189 at the C-terminal end (Campbell *et al.*, 2007; Newman *et al.*, 2001). Both these cysteine-containing domains of ChrR in *R. sphaeroides* have been shown to be involved in interaction with σ^E . Besides the two cysteines located at the 36th and 39th positions at the N terminal, which constitute the Hx₃Cx₂C zinc-binding domain in the ChrR of *A. brasilense*, the two other C-terminal cysteines also aligned well with positions

(a) (ECF-sigma factor)

<i>R. sphaeroides</i>	MTDK-----SDRTDVALMRAIRDHRDEAAFAELFQHFAPKVGFLMKSGSVASQAEEC	54
<i>A. brasilense</i>	MQDLGSPAAGADRT-FEELLTAVGRDRDRAAFAALFGHFAPKRLKAYLRQGCAGGAEEL	59
	* * * * * : * * * * * : * * * * * : * * * * * : * * * * * : * * * * *	
	<.....1.2.....>.....2.1.....>.....2.2.....>	
<i>R. sphaeroides</i>	AQDVMAVWQKAHLFDPSRASVATWIFTIARNRRIDGLRKDRQP--EPEDLFWGPDSEPD	112
<i>A. brasilense</i>	VQDVMLLVWRAETDPTQASAGTWVETIARNRRIDALRREQRPEIDPADPTLVDPDMES	119
	. * * * * * * * : * * : * * : * * : * * : * * : * * : * * : * * : * * : *	
	<.....2.3.....>.....2.4.....>.....3.0.....>.....3.1.....>	
<i>R. sphaeroides</i>	QADVEMQOENARLGRAIARLPEAQRALIERAFFGDLTHRELAETGLPLGTIKSRIRLA	172
<i>A. brasilense</i>	ADDGVAARETAGRLRAALKTLPEQADLLRLAYFEDKPHSLISAEQGIPLGTVKSRRLRA	179
	* : : * * * * * : * * * * * : * * * * * : * * * * * : * * * * * : * * * * *	
	/3.2.....>.....4.1.....>.....4.2.....>	
<i>R. sphaeroides</i>	LDRLRQHMS---	181
<i>A. brasilense</i>	MERLRKAMRDSR	191
	: * * * * * :	
	>	

(b) (Anti-sigma factor)

<i>R. sphaeroides</i>	MTIR-HHVS DALLTAYAGTLSEAFSLVVATHLSLCDECRARAGALDAVGGSLMEETAPV	59
<i>A. brasilense</i>	MTVPTHHPGDTLLIDYAGGALGEAASLIVATHLALCPCCRLNVAEMEAVGGALLESIEPE	60
	* * : * * : * * : * * : * * : * * : * * : * * : * * : * * : * * : * * : *	
<i>R. sphaeroides</i>	ALSEGSLASVMAQLDRQIQRPAPARRADPR-----APAPLADYVGRRLIEDVWRWT	109
<i>A. brasilense</i>	EVDPACLETVLAVLARLDLPLPRAPRPKLVCQPAEVPLLPPLRRYVGSLSRLPWKR	120
	: . . * : * : * * : * . . * : * * * * * . : * :	
<i>R. sphaeroides</i>	LGGGVQRQAILPTGG-----EAIARLLWIPGGQAVPDHGRGLELTLVLQGAFRDETD	161
<i>A. brasilense</i>	LMRGMDCYDIPLGSGSGYDSERRGKARIMRLASGVGPPHHTHRGIELTLVLDDGGFTDDL	180
	* * : * * : * * : * * : * * : * * : * * : * * : * * : * * : * * : * * : *	
<i>R. sphaeroides</i>	RFGAGDIEIADQELEHTPVAERGLDCICLAATDAPLRFNSFLPKLVQPFPR	213
<i>A. brasilense</i>	QFARGDLSVADDSVRHRPVADE-EGCLCLAVTDAPLHFTGALGLILNPFVKF	231
	: * . * * : * * : * * : * * : * * : * * : * * : * * : * * : * * : * * : *	

(c) Promoter

	-35	-10	+1
<i>R. sphaeroides</i>	CTTTTCCCGCCTCCGCCTGATCCAGACTGGCCCGGCCCGCTAAGAGGACGTTAAGATCACGGC		
<i>A. brasilense</i>	ATTTCGTATCGAAAGCCGGTGATCCGATGGCCCGCGGGGTGCGTAGAAGACCCATCGAACCTCAAGT		

Fig. 3. (a) CLUSTAL W alignment of the deduced amino acid sequence of σ^E of *A. brasilense* with that of *R. sphaeroides*. (b) CLUSTAL W alignment of the deduced amino acid sequence of anti- σ^E of *A. brasilense* with that of *R. sphaeroides*. (c) Comparison of σ^E promoter motifs (underlined) of *R. sphaeroides* with nucleotide sequences located in the 5' untranslated region of the σ^E ORF in *A. brasilense* (underlined letters show similarity with the promoter motifs of *R. sphaeroides*).

187 and 189 of the *R. sphaeroides* protein. In agreement with the high similarity of the ORFs of σ^E and anti- σ^E between *A. brasilense* and *R. sphaeroides*, when we compared the nucleotide sequences in the untranslated region upstream of the σ^E , a typical σ^E -dependent promoter structure of *R. sphaeroides* having -35 (TGATCC) and -10 (TAWww) regions separated by 18 nt (Newman *et al.*, 1999) was also present upstream of the ORF of σ^E in *A. brasilense* (Fig. 3c).

Comparison of the nucleotide sequence of the *rpoE* and *chrR* genes and the size of anti-sigma factor protein from *A. brasilense* Sp7 and *A. brasilense* Cd

CLUSTAL W alignment of the 576 bp ORF of *rpoE* from Sp7 and Cd showed that both the sequences were identical (data not shown). Analysis of the sequences of the *chrR* gene from Sp7 and Cd revealed that the size of the gene in Sp7 was 696 nt, while in Cd it was 695 nt. CLUSTAL W alignment of the *chrR* gene sequence from both the strains showed that nucleotide 466 of the *chrR* gene was missing in Cd (Fig. 4a). The occurrence of a run of four Cs around the point of

deletion, as observed in this case, is normally a hotspot for frameshift mutations. A further analysis of the sequences downstream revealed that the deleted nucleotide changed the reading frame and resulted in the creation of a stop codon after seven amino acids. This is expected to reduce the size of anti- σ factor from 231 aa in Sp7 to 162 aa in Cd.

The primers used for PCR amplification and cloning of the ORF of the *chrR* gene from Sp7 in pET15b were also used to amplify and clone the same gene from Cd. When the proteins expressed from IPTG-induced cultures of *E. coli* BL21 λ DE3 (pLysS) carrying plasmids pAT3 (Sp7 sequence) and pAT4 (Cd sequence) were resolved by SDS-PAGE, it was observed that the overexpressed protein from pAT4 was about 7 kDa smaller than that expressed from pAT3 (Fig. 4b). The His-tagged anti- σ factor protein from Sp7 was expected to be of 249 aa (~ 27 kDa), whereas the truncated protein of Cd was expected to be of 183 aa (~ 20 kDa).

Effect of expressing *rpoE* and *chrR* genes

The *rpoE* and *chrR* genes, PCR-amplified from *A. brasilense* Sp7 genomic DNA, were cloned into the *EcoRI* and *BamHI*

Fig. 4. (a) CLUSTAL W alignment of the nucleotide sequence of the PCR-amplified *chrR* region from *A. brasilense* Sp7 and *A. brasilense* Cd showing a single deletion (highlighted in grey) at nucleotide 466 in Cd. (b) SDS-PAGE of total proteins extracted from *E. coli* BL21 λ DE3 (pLysS) harbouring plasmids pAT3 (lane 1) and pAT4 (lane 3). Lane 2 contains a molecular mass marker (BenchMark pre-stained protein ladder, Invitrogen). Indications of the molecular masses of relevant marker proteins are on the right of the figure.

sites of the broad-host-range expression vector pMMB206, resulting in plasmids pAT5 and pAT6, respectively. Both these plasmids were transferred into *E. coli* S17-1 and conjugatively mobilized into two *A. brasilense* strains. *A. brasilense* Sp7 harbouring pAT5 showed slight orange–pink colouration indicating carotenoid synthesis (data not shown), but in the presence of 1 mM IPTG, the cells produced an intense orange–pink colour showing overproduction of carotenoids. Wild-type *A. brasilense* Sp7 with or without IPTG did not produce carotenoids. On the other hand, *A. brasilense* Car-1 harbouring pAT6 displayed a slight orange–pink colour showing reduced synthesis of carotenoids compared with *A. brasilense* Car-1 (Supplementary Fig. S3). Addition of 1 mM IPTG completely inhibited the production of carotenoids by *A. brasilense* Car-1 harbouring pAT6. Quantitative estimation of carotenoids also showed that when *rpoE* was expressed in *A. brasilense* Sp7 via pAT5, the carotenoid content increased considerably. There was no increase in carotenoid content in *A. brasilense* Sp7 harbouring pMMB206 or pAT6 (expressing *chrR*). On the other hand, expression of *rpoE* via pAT5 showed very little increase in carotenoid content in the mutant Car-1. However, expression of *chrR* via pAT6 showed a considerable reduction of carotenoids in the Car-1 mutant (Fig. 5).

Effect of growth phase and reactive oxygen species on heterologous expression of the *rpoE* P1 promoter of *R. sphaeroides* in *A. brasilense*

The strong sequence similarity of the ORFs of σ^E and anti- σ^E of *A. brasilense* with those of *R. sphaeroides*, and also of

Fig. 5. Effect of *rpoE* (via pAT5) and *chrR* (via pAT6) expression on carotenoid content in *A. brasilense* Sp7 (empty bars) and Car-1 mutant (filled bars). Carotenoid content was determined by measuring A_{485} of methanol extracts prepared from stationary-phase cultures grown with 1 mM IPTG, as described in Methods. Error bars indicate SD from three replicates.

the *rpoE* P1 promoter sequence with that present upstream of the ORF of σ^E in *A. brasilense* Sp7, prompted us to investigate whether the σ^E -activated promoter of *R. sphaeroides*, *rpoE* P1, can be activated in *A. brasilense*. A transcriptional *lacZ* fusion of *R. sphaeroides* *rpoE* P1 promoter in a broad-host-range vector was conjugatively mobilized into *A. brasilense* Sp7 and the Car-1 mutant. Assay of the β -galactosidase activity of the transconjugants showed that *R. sphaeroides* *rpoE* P1 was not activated in *A. brasilense* Sp7 but was activated in the Car-1 mutant, in which activation occurred during both exponential and stationary phases of growth (Fig. 6a). Interestingly, the expression of *R. sphaeroides* *rpoE* P1 promoter in the Car-1 mutant was more pronounced during stationary phase than in the exponential phase. When the effect of three different reactive oxygen species, superoxide, peroxide and singlet oxygen, on the heterologous expression of the *Rsp rpoE* P1 promoter was examined, paraquat did not show any effect, hydrogen peroxide showed very little increase, but methylene blue showed a pronounced increase in β -galactosidase activity (Fig. 6b).

DISCUSSION

Out of a pool of 6800 mTn5 mutants of *A. brasilense* Sp7 we found two carotenoid-producing mutants. Both had insertions of mTn5 in *chrR*, the ORF encoding anti- σ^E . This can be explained by hypothesizing that expression of the carotenoid biosynthetic genes in *A. brasilense* Sp7 is σ^E -dependent (Anthony *et al.*, 2005). Normally *A. brasilense* Sp7 has very low σ^E activity and low expression of its target genes (e.g. carotenoid-biosynthesis genes) because it has a functional anti- σ^E . Inactivation of anti- σ^E in the two mTn5 mutants, Car-1 and Car-2, should activate σ^E and drive the expression of its target genes, resulting in the synthesis of significant levels of carotenoids. Consistent with this explanation, supplying intact *chrR* resulted in complete inhibition of carotenoid synthesis, and overexpression of σ^E resulted in carotenoid production. Analysis of the promoter regions of the carotenoid biosynthetic genes of the *A. brasilense* genome did not show the presence of the consensus σ^E -dependent -35 sequence (TGATCC), indicating an indirect control of carotenoid biosynthetic genes by σ^E .

These results led us to hypothesize that the naturally carotenoid-producing *A. brasilense* Cd strain might harbour mutation(s) in either *rpoE* or *chrR*. Indeed, the *chrR* gene in strain Cd had a single nucleotide deletion which resulted in a truncated protein (about 7 kDa smaller than that of Sp7), whereas the *rpoE* gene sequence was identical in both the strains. In view of the earlier observations that the N-terminal 70–80 amino acid residues of the anti- σ factor RseA and the first 85 amino acid residues of ChrR are sufficient to inhibit σ^E (Campbell *et al.*, 2007), it might be expected that truncation at the C-terminal end would not inhibit the σ^E -sequestering ability of Abr ChrR. However, in *R. sphaeroides* ChrR it has been

Fig. 6. (a). Heterologous expression of *R. sphaeroides* *rpoE* P1::lacZ transcriptional fusion (pJDN30) in *A. brasilense* Sp7 and the Car-1 mutant during mid-exponential phase (empty bars) and stationary phase (filled bars). (b) Effect of hydrogen peroxide (5 mM), paraquat (1 mM) and methylene blue (10 μ M) on β -galactosidase activity of Rsp *rpoE* P1::lacZ fusion. β -Galactosidase assays were performed three times in triplicate. Error bars indicate SD from three replicates. Sp7, empty wild-type strain; Sp7 *rpoE*, Sp7 carrying pJDN30; Car-1, empty mutant strain; Car-1 *rpoE*, Car-1 carrying pJDN30.

shown clearly that replacement of the two cysteines located at positions 187 and 189 of ChrR with serine produces a mutant protein that fails to inhibit σ^E activity (Newman *et al.*, 2001). Possibly, these two cysteines at the C-terminal end participate in maintaining the native structure of ChrR, and hence the truncated ChrR in the Cd strain may not be functional. These observations have clearly established that inactivation of the *chrR* gene by mutations (either by Tn5 insertion in the Car-1 and Car 2 mutants or by a nucleotide deletion in strain Cd) resulted in the carotenoid synthesis. The carotenoid-synthesizing phenotype was reversed by expressing the *chrR* gene in the Car-1 mutant as well as in *A. brasilense* Cd.

The operon that encodes σ^E in *A. brasilense* is similar to that in *R. sphaeroides*. The σ^E -dependent regulatory components of *A. brasilense* also showed strong similarity with those of *R. sphaeroides*. This included high similarity of the ORFs of σ^E and anti- σ^E , similarity in the promoter structure and the occurrence of conserved cysteine residues in the ORF of anti- σ^E that are involved in interacting with σ^E . The amino acid sequence of the σ_4 conserved domain of *A. brasilense* σ^E and the corresponding -35 promoter region showed 100% similarity to that of *R. sphaeroides*. On the other hand, the relatively lower degree of similarity in the -10 promoter sequence implied relatively lower similarity with the σ_2 domain of region 2 (Missiakas & Raina, 1998). Conservation of the -35 sequence and its distance from the -10 sequence in *A. brasilense* and *R.*

sphaeroides is important for promoter specificity and recognition by σ^E (Newman *et al.*, 1999). The absence of similarity in the -10 sequences has been suggested to account for the co-existence of multiple members of the σ^E subfamily (Missiakas & Raina, 1998). The anti- σ^E of *A. brasilense* Sp7 also showed similarity with the ChrR anti- σ^E of *R. sphaeroides* by lacking membrane-spanning domains and possessing a zinc binding domain (Hx₃Cx₂C) in the N-terminal region. Recent structural studies of ChrR have revealed a two-domain architecture consisting of an N-terminal anti- σ domain (ASD) that sequesters σ^E by binding to Zn²⁺ in order to inhibit σ^E -dependent transcription, and a C-terminal domain that adopts a cupin fold and coordinates an additional Zn²⁺ to carry out a transcriptional response (Campbell *et al.*, 2007). The strong homology between the ChrR and anti- σ of *A. brasilense* and presence of Zn²⁺ binding domains in the ASD clearly suggest that the anti- σ belongs to the ZAS (Zn²⁺ anti-sigma) subfamily of group IV anti-sigmas.

Although ORFs of σ^E and anti- σ^E in *A. brasilense* showed maximum similarity with those of *R. sphaeroides*, σ^E of *R. sphaeroides* does not appear to directly control the biosynthetic genes of carotenoids as they lack σ^E -dependent promoters (Anthony *et al.*, 2005). It has been clearly established that the *rpoE*P1 promoter in *R. sphaeroides* is σ^E -dependent. In view of the strong similarity of the σ^E and of the putative promoter sequences located in the untranslated region of σ^E of *A. brasilense* with those of *R. sphaeroides*, it

was expected that σ^E -dependent promoters of *R. sphaeroides* should be activated in *A. brasilense* as well. Activation of the σ^E -activated promoter, *rpoE* P1 of *R. sphaeroides* in the Car-1 mutant confirmed that the *rpoE* P1 promoter can indeed be identified and activated *in vivo* by the free σ^E . Carotenoid biosynthesis has earlier been shown to be regulated by ECF σ factor in the Gram-negative bacterium *Myxococcus xanthus* (Browning *et al.*, 2003) and some Gram-positive bacteria belonging to the genus *Streptomyces* (Takano *et al.*, 2005). In *M. xanthus*, carotenoid synthesis is regulated in response to copper and blue light; the latter is sensed by a membrane-bound anti- σ factor, CarR, followed by the transduction of the signal to the ECF σ factor CarQ, which in turn drives the expression of the *carQRS* operon and of the *crtI* gene, depending upon exposure to light and dark conditions (Browning *et al.*, 2003). The synthesis of carotenoids in the Gram-positive genus *Streptomyces*, however, may be constitutive, light-dependent or cryptic. Earlier genetic studies of carotenoid production in two species of *Streptomyces*, *Streptomyces setonii* (Kato *et al.*, 1995) and *Streptomyces griseus* (Schumann *et al.*, 1996), have shown that carotenogenesis is cryptic. Carotenoid production in these two species is induced by an increased copy number of a gene that encodes a stress-response σ factor, σ^{CrtS} , suggesting the involvement of this σ factor in the transcription of carotenoid biosynthetic genes. On the other hand, *Streptomyces coelicolor* A3 does not have a *crtS* homologue in its genome, and its light-induced carotenoid synthesis is under the control of an ECF σ factor, σ^{LitS} (Takano *et al.*, 2005).

We observed that *A. brasilense* Sp7 did not show any notable σ^E activity during exponential or stationary phase, but when anti- σ^E was mutated the mutant showed notable σ^E activity during exponential phase, which increased further during stationary phase. This result is similar to that in *E. coli*, where σ^E is activated upon entry into stationary phase, due to starvation, by the alarmone ppGpp, even if its cognate anti- σ factor is inactivated (Costanzo & Ades, 2006; Costanzo *et al.*, 2008). Therefore, stationary phase could be one of the conditions during which *rpoE* is activated only in anti- σ mutants of *A. brasilense*. σ^E and anti- σ^E of *A. brasilense* Sp7 showed maximum similarity to σ^E and ChrR of *R. sphaeroides*, respectively, and their regulatory behaviour was also similar. As observed in *R. sphaeroides* (Anthony *et al.*, 2005), methylene blue induced σ^E activity, whereas hydrogen peroxide and paraquat did not. Interestingly, in *A. brasilense* methylene blue increased the σ^E activity substantially, whether or not an anti- σ was present. Thus, out of the two well-studied zinc anti- σ factors, ChrR of *R. sphaeroides* and RsrA of *S. coelicolor*, the *A. brasilense* anti- σ factor is similar to RsrA in having seven cysteines, but its ability to respond to methylene blue and not to paraquat or hydrogen peroxide is more like that of ChrR of *R. sphaeroides* (Campbell *et al.*, 2007).

In this report we have clearly established that the inactivation of anti- σ^E in *A. brasilense* leads to carotenoid synthesis in a

σ^E -dependent manner. The operon that includes the *rpoE* gene in *A. brasilense* is bicistronic and similar to that of *R. sphaeroides*. However, carotenoid synthesis in *M. xanthus* and *Streptomyces* species is controlled by *rpoE*, which is part of a polycistronic operon and controlled by light and copper. Since none of the carotenoid biosynthetic genes contained a consensus σ^E -dependent promoter, it is expected that σ^E controls carotenoid synthesis indirectly through intermediate regulators. We are currently investigating the environmental conditions/signals that can induce carotenoid synthesis and the alternative σ factors that might be involved in σ^E -dependent control of carotenoid biosynthesis in *A. brasilense*.

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