Carotenoid biosynthetic pathways are regulated by a network of multiple cascades of alternative sigma factors in *Azospirillum brasilense* Sp7

Ashutosh Kumar Rai[1], Ashutosh Prakash Dubey[1], Santosh Kumar[1], Debashis Dutta[2], Mukti Nath Mishra[1], Bhupendra Narain Singh[2] and Anil Kumar Tripathi[1*]

[1]School of Biotechnology, Faculty of Science, Banaras Hindu University, Varanasi-221005, India

[2]Division of Microbiology, Central Drug Research Institute, Lucknow-226015, India

*Address correspondence to:

Anil Kumar Tripathi,
School of Biotechnology, Faculty of Science,
Banaras Hindu University, Varanasi-221005, India.
Email: tripathianil@rediffmail.com;
Fax: +542-368693/2368174

Running Title: Regulation of carotenoid synthesis by cascades of sigma factors

Abstract

Carotenoids constitute an important component of the defense system against photooxidative stress in bacteria. In *Azopsirillum brasilense* Sp7, a non-photosynthetic rhizobacterium, carotenoid synthesis is controlled by a pair of extracytoplasmic function sigma factors (RpoEs) and their cognate zinc-binding anti-sigma factors (ChrRs). Its genome harbours two copies of the gene encoding geranylgeranyl pyrophosphate synthase (CrtE), the first critical step in the carotenoid biosynthetic pathway in bacteria. Inactivation of each of two the *crtE* paralogs found in *A. brasilense* caused reduction in carotenoid content, suggesting their involvement in carotenoid synthesis. However, effect of *crtE1* deletion was more pronounced than that of *crtE2*. Out of the five paralogs of *rpoH* in *A. brasilense*, overexpression of *rpoH1* and *rpoH2* enhanced carotenoid synthesis. Promoters of *crtE2* and *rpoH2* were found to be dependent on RpoH2 and RpoE1, respectively. Using a two-plasmid system in *E. coli*, we have shown that *crtE2* gene of *A. brasilense* Sp7 is regulated by two cascades of sigma factors, one consisting of RpoE1and RpoH2, and the other consisting of RpoE2 and RpoH1. In addition, expression of *crtE1* was upregulated indirectly by RpoE1 and RpoE2. This study shows, for the first time in any carotenoid-producing bacterium, that the regulation of carotenoid biosynthetic pathway involves a network of multiple cascades of alternative sigma factors.

Importance Carotenoids play very important role in coping with photooxidative stress in prokaryotes and eukaryotes. Although extracytoplasmic function (ECF) sigma factors are known to directly regulate the expression of carotenoid biosynthetic genes in bacteria, regulation of carotenoid biosynthesis by cascade/s of sigma factors had not been reported. This study provides the first evidence of the involvement of multiple cascades of sigma factors in the regulation of carotenoid synthesis in any bacterium by showing the regulation of a gene encoding

geranylgeranyl pyrophosphate synthase (*crtE2*) by RpoE1→RpoH2→CrtE2 and RpoE2→RpoH1→CrtE2 cascades in *A. brasilense*. It also provides an insight in to existence of additional cascade/s regulating expression of another paralog of *crtE*.

Introduction

Photosynthetic as well as non-photosynthetic organisms encounter photooxidative stress caused by the generation of highly reactive singlet oxygen in the presence of light and oxygen. Singlet oxygen reacts with a wide range of cellular macromolecules including proteins, lipids, DNA and RNA, leading to the formation of reactive substances such as organic peroxides and sulfoxides (1). Mechanisms to cope with the photooxidative stress have been investigated in a range of photosynthetic and non-photosynthetic microorganisms. These mechanisms include the use of quenchers, such as carotenoids, which interact either with excited photosensitizer molecules or singlet oxygen itself to prevent damage of cellular molecules (2). Carotenoids are a widely distributed class of structurally diverse yellow, orange or red pigmented compounds (tetraterpenoids) consisting of a polyene hydrocarbon chain derived from eight isoprene units. Modifications like cyclization and desaturations of C_{40} backbone result in a variety of divergent chemical structures produced in eukaryotes as well as prokaryotes (3).

Conversion of farnesyl pyrophosphate to geranylgeranyl pyrophosphate (GGPP) by GGPP synthase (CrtE) is a critical step before carotenoid biosynthesis begins in bacteria. The first two committed steps in the biosynthesis of carotenoids include conversion of GGPP into phytoene by a phytoene synthase (CrtB) followed by phytoene desaturation via phytoene dehydrogenase (CrtI). These three steps in the carotenoid biosynthetic pathway and associated enzymes are common in the carotenoid producing organisms (4), suggesting evolutionary conservation of the early steps of this pathway (5). The regulation of their expression, however, may differ in different bacteria (6). While *Rhodobacter capsulatus* and *Erwinia herbicola* (7) produce carotenoids constitutively, *Myxococcus xanthus* (8, 9), *Flavobacterium dehydrogenans* (10), *Sulfolobus* spp. (11) and *Streptomyces coelicolor* (12) produce carotenoids in a

93 photoinducible manner. In non-photosynthetic bacteria, such as *M. xanthus* (a Gram-negative
94 bacterium) (13) and *S. coelicolor* (a Gram-positive bacterium) (12), carotenoid synthesis is
95 regulated by light via an extra-cytoplasmic function (ECF) sigma factor and its cognate anti-
96 sigma factor. In *S. griseus,* carotenoid synthesis is also regulated by an ECF sigma factor, but the
97 stimulus responsible for its induction is not known yet (14).

98 *Azospirillum brasilense* is a non-photosynthetic, plant growth-promoting rhizobacterium,
99 which belongs to the family *Rhodospirillaceae* of α-proteobacteria. Since it inhabits the
100 rhizosphere as well as soil, it has to cope with the fluctuations in its environment. It synthesizes
101 bacterioruberin-type of carotenoids (15). Recently, we reported that carotenoid synthesis is
102 induced by light, and regulated by a pair of ECF-sigma factors (RpoE1 and RpoE2) in this
103 bacterium (16, 17, 18). In addition, we have also shown that a heat-shock sigma factor (RpoH2
104 or σ^H) is involved in the photooxidative stress response in this bacterium (19). These
105 observations suggested that, besides RpoE1-ChrR1, RpoH2 might also be involved in the
106 regulation of carotenoid biosynthesis in *A. brasilense* Sp7. Whether RpoE1 and RpoE2 regulate
107 the carotenoid biosynthetic genes directly, and whether RpoH2 is also involved in this
108 regulation, is not known yet. Using *crtE*, the first gene of the carotenoid biosynthetic pathway,
109 we have shown here that the carotenoid biosynthetic pathway in *A. brasilense* is regulated by a
110 network of multiple cascades of RpoE and RpoH sigma factors.

111 ## Materials and Methods

112 **Bacterial strains, plasmids, chemicals and growth conditions.** Plasmids and strains used in
113 this study are described in Table 1. *Escherichia coli* DH5α and *E. coli* S17-1 were grown in
114 Luria Bertani (LB) medium at 37 ^{o}C. *A. brasilense* was grown in minimal malate medium (20) as
115 well as in LB medium at 30 ^{o}C. Media used for bacterial growth were from Hi-Media (Mumbai,

India), and enzymes used for DNA manipulation and cloning were from New England Biolabs. Primer sequences are given in Suppl. Table 1.

Prediction of promoter regions of *rpoH2* and *crtE* in *A. brasilense*. Upstream nucleotide sequences of *rpoH2* and *crtE* paralogs were analyzed by online promoter prediction tool, BPROM (21), to identify putative promoters. Upstream nucleotide sequences were also searched manually to find RpoE and RpoH2 promoter elements using the consensus sequences reported earlier in *A. brasilense* and related bacteria (22, 23).

Construction of mutants and RpoH expressing plasmids. The *rpoH2*, *crtE1* and *crtE2* genes of *A.brasilense* Sp7 were insertionally inactivated via allele replacement by using a suicide plasmid vector (pSUP202) as described earlier (24, 25). Entire coding regions of the *rpoH* paralogs were cloned in a broad host range expression vector (pMMB206) as described earlier (16, 26).

Determination of transcription start site (TSS). The 5'ends of the mRNAs of *crtE1*, *crtE2* and *rpoH2* were identified by 5'RACE as described earlier (25). Briefly, 2µg RNA was reverse transcribed to cDNA using gene-specific reverse primer 1 (GSP1), followed by cDNA purification and poly(dA)-tailing. Poly(dA)-tailed cDNA was then PCR amplified using GSP2 and Oligo-dT anchor primers. The amplicon so obtained was submitted to the next round of nested PCR using Anchor and GSP3 primers. Final PCR product was then cloned in pGEM-T Easy vector (Promega), and nucleotide sequences determined by the chain termination method.

Construction of promoter::*lacZ* fusions. Upstream regions (approximately 500 bp) of the start codon of the selected genes were PCR amplified, and the amplicons inserted in pCZ750 vector (27) using XbaI and HindIII to construct promoter::*lacZ* transcriptional fusions. Constructs were confirmed by sequencing, and mobilized in *A. brasilense* by the biparental conjugation method

(16). Nucleotide sequences of the -10 and -35 elements of the *rpoH2* promoter (CG to GC and TGA to ACT, respectively) were mutated by the protocol for one-step site-directed mutagenesis (28). The -35 region of *crtE2* promoter was deleted by overlap extension PCR technique (29).

Estimation of promoter activity. Promoter activity was monitored by measuring the β-galactosidase activity (30) of *A.brasilense* Sp7 and its mutants harbouring different promoter::*lacZ* fusions. An *E. coli* DH5α based two-plasmid system (31) was used to identify *A. brasilense* promoters that were directly activated by specific alternative sigma factors. We used this method by transforming *E. coli* DH5α with two recombinant plasmids, a pMMB206 derivative with a gene encoding an alternative sigma factor fused to the tacUV5 promoter, and a pCZ750 derivative harbouring promoter region of a target gene transcriptionally fused to the *lacZ* reporter, and measuring the β-galactosidase activity of the transformed cells.

Real-Time PCR. Total RNA from *A. brasilense* strains was isolated, quantified and quality tested, as reported earlier (25). The cDNA was synthesized from 2 µg RNA using cDNA synthesis kit (Fermentas, Germany). Real-time PCR was performed using SYBR Green I (Roche, USA) in a Light Cycler 480 II (Roche, USA) according to manufacturer's instructions, using *rpoD* as an endogenous control. Relative expression level was compared by $2^{-\Delta\Delta C}{}_{T}$ method (32).

Extraction and estimation of carotenoids. *A. brasilense* strains were grown in the LB medium upto the stationary phase. Pellets from the equal volume of equal OD_{600nm} cultures were washed twice with saline, resuspended in 5 ml 100% methanol, and carotenoids extracted by shaking the suspension overnight at 180 rpm at 25 ^{0}C in the Oakridge tubes covered with aluminum foil (16). Concentrated methanolic extracts of carotenoids were spotted on TLC plate, resolved in and visualized (15). Absorption spectra of the carotenoids extracted in methanol were recorded using

a 300-800 nm range in a UV-VIS spectrophotometer (V630, Jasco, Japan). Carotenoids were extracted and estimated three times. Student's *t* tests were performed for statistical comparisons, and *P* values of <0.05 were considered to show statistically significant differences in carotenoid contents.

Results

Two CrtE paralogs are involved in carotenoid synthesis in *A. brasilense*. In carotenoid producing bacteria, the first step of the carotenoid biosynthetic pathway is catalyzed by GGPS (encoded by *crtE* gene). Since *A. brasilense* genome harbours two paralogs of this gene, *crtE1* (locus tag: AzoBR_180071) and *crtE2* (locus tag: AzoBR_p1140021), we examined their involvement in carotenoid synthesis by mutating each of them separately in a naturally carotenoid-overproducing strain, *A. brasilense* Cd. Qualitative analysis of carotenoids by TLC revealed 4 spots which were common in the extracts of *A. brasilense* Cd and its two mutants, *crtE1::km* and *crtE2::km* (data not shown). The intensity of the spots in case of *crtE1::km* mutant, however, was very weak in comparison to that detected in *crtE2:::km* or *A. brasilense* Cd. Absorption spectra of the methanolic extracts of the two mutants revealed that *crtE1* inactivation led to approximately 75% reduction in the carotenoid content, whereas *crtE2* inactivation reduced carotenoids only by <25% (*P* <0.03) (Fig. 1A and Suppl. Fig. 1) indicating an involvement of both the CrtEs in carotenoid biosynthesis.

***crtE* promoters are regulated indirectly by RpoEs.** We have shown earlier that overexpression of *rpoE1* or *rpoE2* leads to an enhancement in carotenoids level in *A. brasilense* Sp7 (16, 17). To examine if *crtEs* are regulated directly by RpoE1, we analyzed the effect of RpoE1 expression on the activation of *crtE* promoters by mobilizing *crtE1*::*lacZ* (pAPD3) and *crtE2*::*lacZ* (pAPD4) fusions in *A. brasilense* Sp7, and its anti-sigma mutant, Car-1 (a *chrR1*::Tn*5* mutant

overexpressing RpoE1 due to the inactivation of anti-sigma factor ChrR1), followed by estimating the β-galactosidase activity. Promoter assays revealed that activity from both the promoters in *A. brasilense* Sp7 was almost equal. Both the promoters showed considerably higher activity in the Car-1 mutant than that observed in *A. brasilense* Sp7. Further, the activity of *crtE1* promoter in the Car-1 mutant was relatively high (Fig. 1B). Although these results indicate an RpoE1-dependent regulation of *crtE*s, they do not confirm if RpoE1 regulates the expression of *crtE1* directly.

To examine if the *crtE*s are regulated directly by RpoE, we used a two-plasmid system (31). The principle of the two-plasmid system is based on the ability of the *A. brasilense* alternative sigma factors, expressed via an expression vector, to bind and recruit *E. coli* DH5α RNA polymerase to initiate transcription from the *A. brasilense* promoters fused to a *lacZ* reporter gene in a compatible vector (Fig. 2A). For this, we transformed *crtE1*::*lacZ* and *crtE2*::*lacZ* fusion plasmids into *E.coli* DH5α individually, and these transformants were then further transformed with *rpoE1* and *rpoE2* expressing plasmids, pAT5 and pNG6, respectively. β-galactosidase assays of these combinations showed that neither RpoE1 nor RpoE2 activated *crtE1* or *crtE2* promoters in *E.coli* DH5α (Fig. 2B), suggesting an indirect mode of RpoE-dependent regulation of *crtEs* in *A. brasilense*.

***crtE2* has an RpoH-dependent promoter.** Absence of any direct regulation of *crtE*s by RpoE prompted us to look for RpoE-dependent intermediate regulator(s) that might regulate *crtE*s directly. In order to identify alternative sigma factors directly regulating *crtE*s, we examined the upstream DNA regions of both the *crtE* genes for recognizable promoter elements. This analysis failed to find any RpoE1-dependent promoter (25) in the upstream region of both the *crtEs,* which supports the lack of direct regulation of both these genes by RpoE1. The upstream regions

of *crtE1* and *crtE2*, however, revealed the presence of **CCTTGC**-N_{17}-C**TA**TGC and **CCTTGC**-N_{17}-CA**TA**AT sequences, respectively, which showed strong resemblance to the RpoH-dependent promoter consensus (conserved residues are in boldface type). To validate this prediction, we determined the TSS to identify promoter elements of both the *crtE*s which revealed that -35 (CCTTGC) and -10 (C**TA**TGC) hexamers of only *crtE2* match with the RpoH-dependent consensus, while possible -35 (TTGACC) and -10 (TTATGC) hexamers of *crtE1* did not match with the RpoH-dependent promoter consensus (Fig. 3A and B). This indicated that *crtE2* might be regulated in an RpoH-dependent manner.

RpoH1 and RpoH2 directly activate *crtE*2 promoter. Identification of an RpoH-dependent promoter in the *crtE2* upstream region prompted us to use the two-plasmid system to examine which of the five RpoH paralogs, encoded in the genome of *A. brasilense* (19), directly regulate *crtE* genes. Since *rpoH* gene of *E.coli* is expressed under heat-shock condition, expression of β-galactosidase in *E.coli* DH5α can be observed only if an *A. brasilense* RpoH is able to drive the expression of *lacZ* from the *crtE1* or *crtE2* promoters. Each RpoH paralog was expressed in *E.coli* DH5α harbouring *crtE1:lacZ* or *crtE2:lacZ* to examine their involvement in the transcription of *crtE* genes. Promoter activity showed that only *crtE2:lacZ* fusion was activated by both, RpoH1 and RpoH2 (Fig. 2B); *crtE1* promoter does not seem to be activated by any of the RpoH paralog. When we examined the ability of *A. brasilense* RpoH2 to activate a derivative of the promoter of *crtE2:lacZ* fusion, in which -35 hexamer was deleted (Fig. 3B), only negligible β-galactosidase activity was seen (Fig. 3C). Since we have an *rpoH2*::*km* mutant of *A. brasilense* we compared the activity of *crtE2* promoter in the *rpoH2*::*km* mutant and its parent. The promoter activity of *crtE2* was very low in the *rpoH2*::*km* mutant than that observed in the parent, showing an RpoH-dependent regulation of *crtE2 in vivo* (Fig. 3D). These observations

confirmed that *crtE2* is regulated directly by RpoH1 and RpoH2, and that the promoter elements identified for *crtE2* are actually used for this regulation.

Expression of RpoH1 and RpoH2 enhances the carotenoid synthesis. RpoH1 and RpoH2-dependent regulation of *crtE2* suggested a positive role of these RpoHs in the regulation of carotenoid synthesis of *A. brasilense*. To confirm this, we expressed each of the five RpoH sigma factors in *A. brasilense* Sp7, and estimated their effects on the carotenoid content. Fig. 4A shows that the overexpression of *rpoH1* and *rpoH2* caused very significant increases in carotenoid content in *A. brasilense* Sp7; RpoH2 causing maximum synthesis of carotenoids. Carotenoid content in the strains of *A. brasilense* Sp7 overexpressing *rpoH3*, *rpoH4* and *rpoH5* were not significantly different from that in *A. brasilense* Sp7. These results confirm the role of RpoH1 and RpoH2 in the regulation of carotenoid synthesis in *A. brasilense*.

RpoE1 and RpoE2 directly regulate *rpoH* promoters. After establishing a direct regulation of *crtE2* by RpoH1 as well as RpoH2, we hypothesized that carotenoid synthesis in *A. brasilense* might be indirectly regulated by RpoEs, using RpoH1 or RpoH 2 as intermediate regulators. To confirm this hypothesis, we used the two-plasmid system once again to examine whether RpoE1 and RpoE2 directly regulate the *rpoH* paralogs. Fig. 4B shows that the activities of the *rpoH1:lacZ* and the *rpoH4:lacZ* fusions, respectively, were upregulated about 3-fold by RpoE2. Similarly, the activity of *rpoH2:lacZ* and *rpoH5:lacZ* fusions was upregulated by more than 3-fold by RpoE1. These observations indicate that *rpoH1* and *rpoH4* promoters are directly regulated by RpoE2, whereas *rpoH2* and *rpoH5* promoters are directly regulated by RpoE1. The activity of *rpoH3:lacZ* fusion, however, does not seem to be affected by the expression of RpoE1 or RpoE2.

253 ***rpoH2* has RpoE1-dependent promoter.** Since RpoH2 directly regulates *crtE2*, and the
254 expression of *rpoH2* seems to be directly regulated by RpoE1, we attempted to examine whether
255 *rpoH2* harbours an RpoE1-depenedent promoter. Determination of the *rpoH2* TSS revealed a
256 "G" located 163 nucleotides upstream of the start codon, and TGATCC and CGTATG as
257 possible -35 and -10 elements, respectively, with a space of 16 nucleotides (Fig. 5A). The
258 promoter elements of *rpoH2* predicted on the basis of TSS matched well with the earlier reported
259 promoter sequence recognized by the RpoE1 sigma factor (25). In order to prove further that the
260 predicted -35 and -10 elements are indeed utilized by RpoE1 sigma factor for driving the
261 transcription of *rpoH2*, we generated three site-directed mutants of the *rpoH2* promoter; in one,
262 TGA of the -35 element was replaced with ACT; in the second, CG of the -10 element was
263 replaced by GC; and in the third, -35 element was deleted completely (Fig. 5B). The *lacZ* fusion
264 with the *rpoH2* native promoter gave the maximum and minimum activity in the Car-1 and
265 *rpoE1*::*km* mutant, respectively, indicating a dependence of *rpoH2* promoter on RpoE1. The
266 derivatives of *rpoH2*:*lacZ* fusion carrying three different mutant versions of the promoters
267 showed very low activity even in Car-1 mutant, clearly indicating that the identified -35 and -10
268 elements were the essential promoter elements of the RpoE1-dependent regulation of *rpoH2*.
269 To prove the RpoE1-dependent direct regulation of *rpoH*2 promoter and that the -35 and
270 -10 elements are real promoter elements, we used the two-plasmid system to examine the ability
271 of RpoE1 to directly activate the *lacZ* fused native *rpoH2* promoter and any of the *rpoH2*
272 promoter mutant versions . Fig. 5D shows that RpoE1 was able to directly activate *lacZ* fused
273 *rpoH2* native promoter whereas it failed to activate any of the mutant versions of *rpoH2*
274 promoter. These observations further established that TGATCC and CGTATG are the -35 and -
275 10 elements of the *rpoH2* promoter, respectively, and these elements are utilized as promoters by

RpoE1 for the transcription of *rpoH2*. We also examined transcript levels of *rpoH2* in *A. brasilense* Sp7, *rpoE1::km* and Car-1 mutants by real-time PCR, and reproducibly observed that *rpoH2* expression was upregulated >5- fold in Car-1, but downregulated by about 0.5 fold in the *rpoE1::km* mutant, in comparison to that in *A. brasilense* Sp7 (Suppl. Fig. 2). This result further corroborated our inference about an RpoE1-dependent regulation of *rpoH2*, as *rpoH2* transcript level was maximum in Car-1, as it overexpresses RpoE1 due to autoregulation in the absence of ChrR.

Discussion

In our studies on the regulation of carotenoid biosynthesis in *A. brasilense,* we have shown earlier that two ECF-sigma factors, RpoE1 and RpoE2, positively regulate carotenoid synthesis in *A. brasilense* Sp7 (16, 17, 18, 25). Whether carotenoid biosynthetic genes are controlled directly or indirectly by RpoEs was not known. In this study, we have shown that carotenoid synthesis in *A. brasilense* is controlled by RpoE indirectly via RpoH. Although RpoH2 acts as a component of the RpoE→RpoH2 cascade regulating photooxidative stress response in *Rhodobacter sphaeroides,* carotenoid biosynthetic genes were not regulated by this cascade. Carotenoid biosynthetic genes were shown to be directly regulated by ECF sigma factors in *S. coelicolor and M. xanthus.* While ECF sigma factor σ^{LitS} was shown to regulate the expression of carotenoid biosynthetic genes directly in *S. coelicolor* (12), the ECF sigma factor CarQ, essential for light-induced carotenogenesis in *M. xanthus,* was shown to directly activate the promoters of two carotenoid biosynthetic genes (33). This study, however, provides the first evidence of the regulation of carotenoid biosynthesis by a cascade of RpoE-RpoH sigma factors in any bacterium.

298 We used a two-plasmid system to identify promoters of *A. brasilense* genes that are
299 recognized by the alternative sigma factors of *A. brasilense* in *E.coli*. Such a system was used
300 earlier to identify *Mycobacterium tuberculosis* genes directly regulated by σ^F, and of
301 *Staphylococcus aureus* genes activated by σ^B (34, 35). The advantage of using a two-plasmid
302 system lies in *E.coli* providing a noise-free genetic background to analyze the expression of
303 genes, as native RpoH and RpoE sigma factors of *E.coli* are expressed only during heat-shock
304 conditions.

305 Observations that both the *crtE*s are involved in carotenoid biosynthesis, and that
306 RpoE1/RpoE2 sigma factors activate the promoters of the two *crtE*s in *A. brasilense* suggest that
307 RpoEs may control carotenoid biosynthesis in *A. brasilense*, most likely, by the regulation of
308 *crtE* genes. The inability of RpoE1/RpoE2, however, to activate either *crtE* promoters in *E.coli*
309 background suggests that *crtE* genes are indirectly regulated by these sigma factors. A clue about
310 the regulation of *crtE* genes was provided by the *in silico* analysis of their upstream regions,
311 which predicted RpoH-dependent promoters, and hence indicated towards an RpoH-mediated
312 regulation of these genes. Confirmation of the promoters of *crtE* on the basis of TSS revealed
313 that the prediction was, at least, valid for *crtE2*. By showing that *crtE2* expression was directly
314 regulated by RpoH1 and RpoH2, our data from two-plasmid system-based promoter activation
315 indicated an involvement of RpoH1 and RpoH2 in the regulation of carotenoid biosynthesis in *A.*
316 *brasilense*. This was further corroborated by an increase in the carotenoid content due to the
317 overexpression of RpoH in *A. brasilense*.

318 On the basis of these as well as our previous observations (16, 18) it can be hypothesized
319 that the RpoEs may indirectly regulate *crtE2* via RpoH1/RpoH2 to control carotenoid synthesis
320 in *A. brasilense*. And, if so, these RpoHs should be regulated directly or indirectly by RpoEs.

Our data from the two-plasmid system reveal direct regulation of *rpoH2* and *rpoH5* by RpoE1, and of *rpoH1* and *rpoH4* by RpoE2, and confirm occurrence of at least two cascades regulating *crtE2* which include RpoE1→RpoH2→CrtE2 and RpoE2→RpoH1→CrtE2. Furthermore, mutagenesis of *crtE2* and *rpoH2* promoters followed by their promoter activity assays provided another level of evidence for RpoE1→RpoH2→CrtE2 cascade by revealing that: i) RpoE1 activates the native promoter of *rpoH2* but not those having mutations in -10 and -35 elements of *rpoH2* promoter; and similarly ii) deletion of -35 element of *crtE2* promoter abolishes regulation by RpoH2.

By demonstrating a major role of *crtE1* in carotenoid biosynthesis in *A. brasilense*, and strong upregulation of *crtE1* promoter in the Car-1 mutant, in which RpoE1 is constitutively overexpressed, our observations clearly indicate towards existence of an even more important RpoE-dependent carotenoid regulatory cascade in *A. brasilense*. Although *crtE1* is also regulated indirectly by RpoE1, the regulatory cascade involved in this case seems to be RpoH-independent, as none of the RpoH paralogs could directly activate *crtE1* promoter in *E.coli* background. These observations suggest RpoE-mediated regulation of the two *crtE* paralogs through different regulatory cascades involving different types of intermediate alternative sigma factors to control carotenoid biosynthesis in *A. brasilense*. This provides an insight into the flexibility of regulation of carotenoid biosynthesis in *A. brasilense* to integrate and respond to different kinds of stimuli which may require carotenoids to cope with the fluctuations in its environment. For example, in *Salmonella typhimurium,* a cascade of sigma factors linking σ^E, σ^H and σ^S allows the integration of diverse environmental signals to result in the expression of a common stress response (36).

343 Although alternative sigma factors can act independently, a considerable level of overlap
344 between the genes controlled by alternative sigma factors was demonstrated in *B. burgdorferi*
345 (37, 38). Our previous study showed that *A. brasilense* RpoE1 and RpoE2 are highly
346 homologous, and hence, they not only regulate carotenoid synthesis, but also the expression of
347 several common genes in *A. brasilense* (18). Data from this work shows that although RpoE1
348 and RpoE2 both regulate carotenoid biosynthesis in *A. brasilense*, they bring about this
349 regulation through different RpoH sigma factors (RpoE1 regulating RpoH2/5 and RpoE2
350 regulating RpoH1/4) indicating recognition of different promoters by the two RpoEs. An
351 overlapping function of these RpoEs is manifested by RpoH1 and RpoH2-mediated regulation of
352 a common gene (*crtE2*). A similar overlap of functions between RpoH1 and RpoH2 has also
353 been demonstrated in *R. sphaeroides* (39) and *S. meliloti* (40). Furthermore, RpoE1/RpoE2-
354 mediated regulation of RpoH4/5 that are not involved in carotenoid regulation provide an insight
355 into the utilization of RpoE1→RpoH4 and RpoE2→RpoH5 regulatory cascades for responding
356 to other stresses, such as salinity and organic stresses in which an involvement of RpoE has been
357 shown (25).

358 Since *A. brasilense* is a non-photosynthetic bacterium, it may not be as vulnerable to
359 photooxidative stress/damage as photosynthetic bacteria like *R. sphaeroides*. However, being a
360 soil bacterium, it may be exposed to the damaging effects of light while swimming under
361 submerged soil conditions. Inducibility of carotenoid synthesis in response to light might thus be
362 a desirable and beneficial trait in *A. brasilense*. On the basis of this study, and our earlier
363 observations, we propose a model of regulation of carotenoid biosynthesis in *A. brasilense* (Fig.
364 6). In the absence of light, RpoE sigma factor remains bound to its cognate anti-sigma factor
365 (ChrR). The singlet oxygen produced in the presence of light may react with the two paralogs of

ChrR, and alter their conformation to release their cognate RpoE sigma factors, which can express their target genes including *rpoH2* and *rpoH1*. RpoH2 and RpoH1, in turn, express their own target genes including *crtE2,* which encodes geranyl geranyl pyrophosphate synthetase to catalyze the first step of the carotenoids biosynthesis. However, identification of the RpoE-regulated alternative sigma factor which directly regulates the expression of *crtE1* is needed to develop a better understanding of carotenoid biosynthesis in *A. brasilense*

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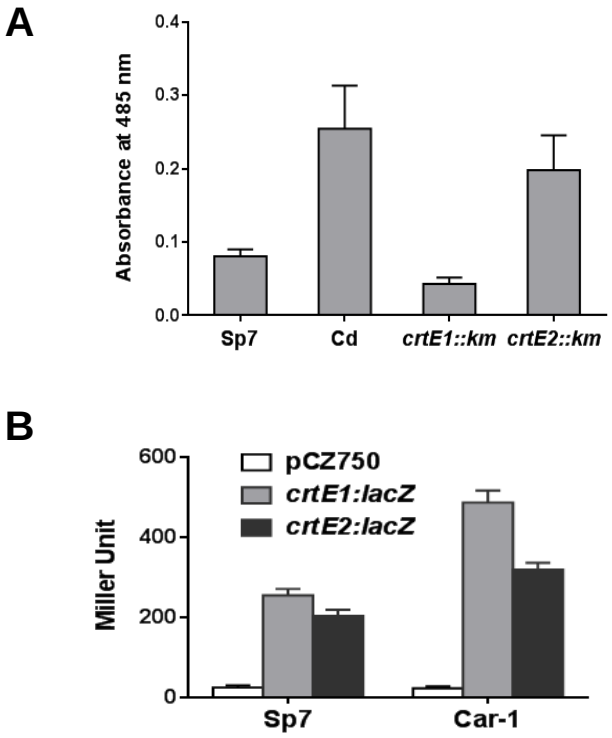

Figure 1. (A) Effect of *crtE1* and *crtE2* gene inactivation on carotenoid content in a carotenoid overproducing strain *A. brasilense* Cd. The carotenoid content was compared by measuring absorption maxima at 485nm of the methanolic extracts of the strains/mutants. The carotenoid content of Cd is significantly different from *crtE1::km* and *crtE2::km* ($P < 0.03$). **(B)** β-galactosidase activity due to *lacZ* fusions with the promoters of *crtE1* (pAPD3) and *crtE2* (pAPD4) in *A.brasilense* Sp7 and Car1. The error bar shows the standard deviation from the three replicates.

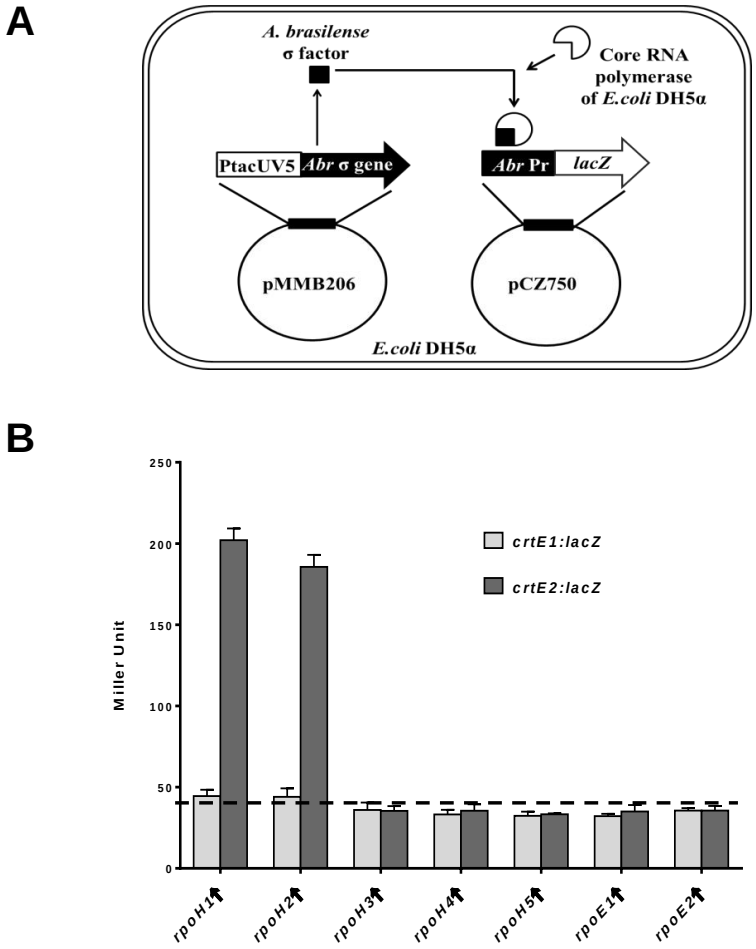

Figure 2. (A) Schematic presentation of the principle of two-plasmid system. A gene encoding *A. brasilense* sigma factor (*Abr* σ) is cloned downstream of the inducible tacUV5 promoter in pMMB206 vector. Upon induction by IPTG, *A. brasilense* sigma factor is expressed from the tacUV5 promoter. The *A. brasilense* sigma factor expressed in *E.coli* then brings *E.coli* core RNA polymerase binding to the *A. brasilense* promoter (*Abr* Pr), cloned upstream of the *lacZ* reporter in pCZ750 vector. If *A. brasilense* target promoter is recognized

by the *A. brasilense* sigma factor expressed in *E.coli* DH5α, β-galactosidase will be expressed, which can be assayed **(B)** Effect of the expression of *rpoH* and *rpoE* paralogs of *A. brasilense* on the β-galactosidase activity of *E.coli* DH5α harbouring *crtE1:lacZ* (pAPD3) or *crtE2:lacZ* (pAPD4) fusions. Vertical arrows indicate expression of the genes. The dashed base line shows the β-galactosidase activity of *E.coli* DH5α harbouring empty vector. Error bars show the standard deviation from the three replicates.

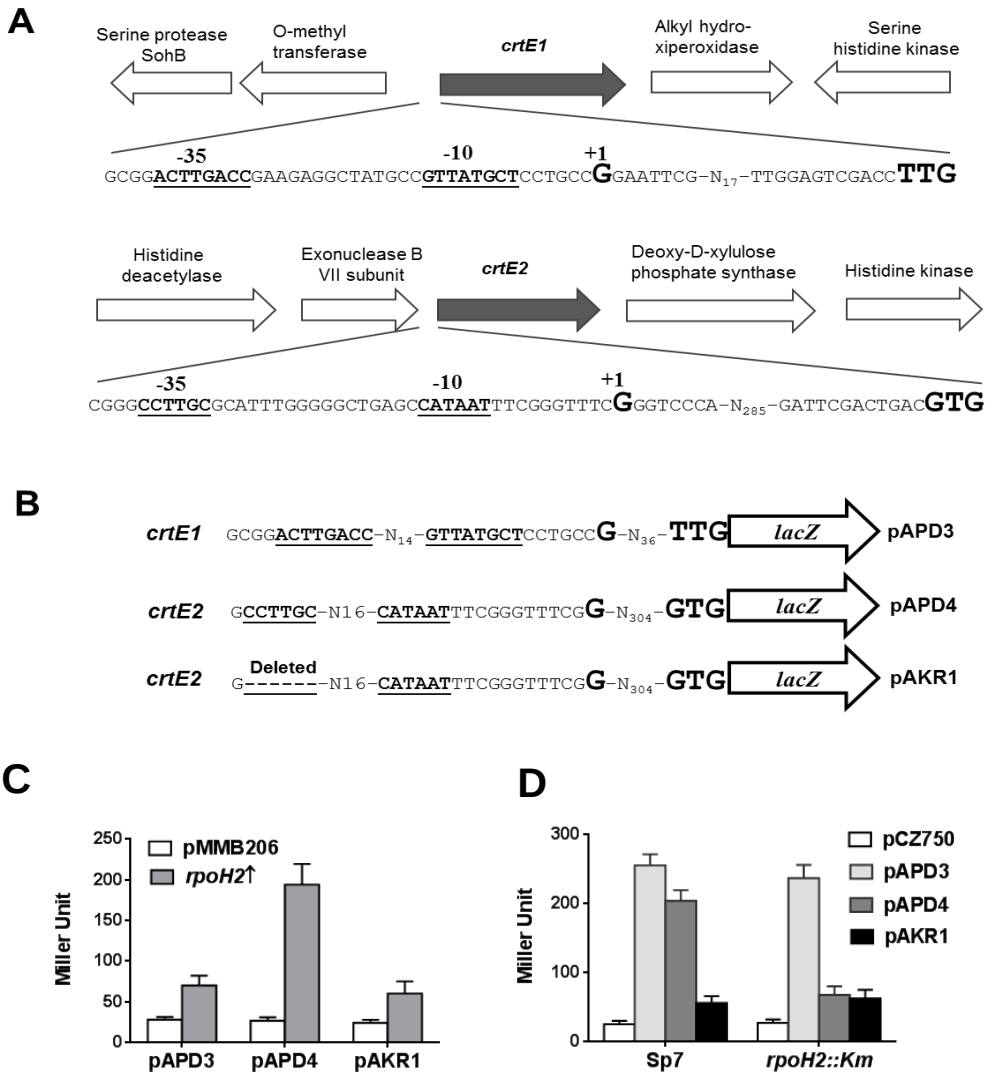

Figure 3. (A) Organization of *A. brasilense crtE1* and *crtE2* loci and possible -35 and -10 elements (bold and underlined) of *crtE1* and *crtE2* genes predicted on the basis of identified of TSS (shown as +1 and in larger and bold fonts); start codons are shown in larger and bold font. **(B)** Representation of the promoter::*lacZ* fusions showing promoter elements of *crtE1* (pAPD3) and *crtE2* (pAPD4) as well as a derivative of *crtE2* promoter having deletion of -35 element (pAKR1). **(C)** Effect of *rpoH2* overexpression (indicated by an upward arrow) on the

β-galactosidase activity of *A. brasilense crtE* promoter::*lacZ* fusions using two-plasmid system in *E.coli* DH5α. Error bar shows the standard deviation from the three replicates. **(D)** β-galactosidase activity of the *lacZ* fusions fused with the promoters of *crtE1* (pAPD3), *crtE2* (pAPD4) and -35 element deletion derivative of *crtE2* promoter (pAKR1) in *A.brasilense* Sp7 and *rpoH2::km* mutants.

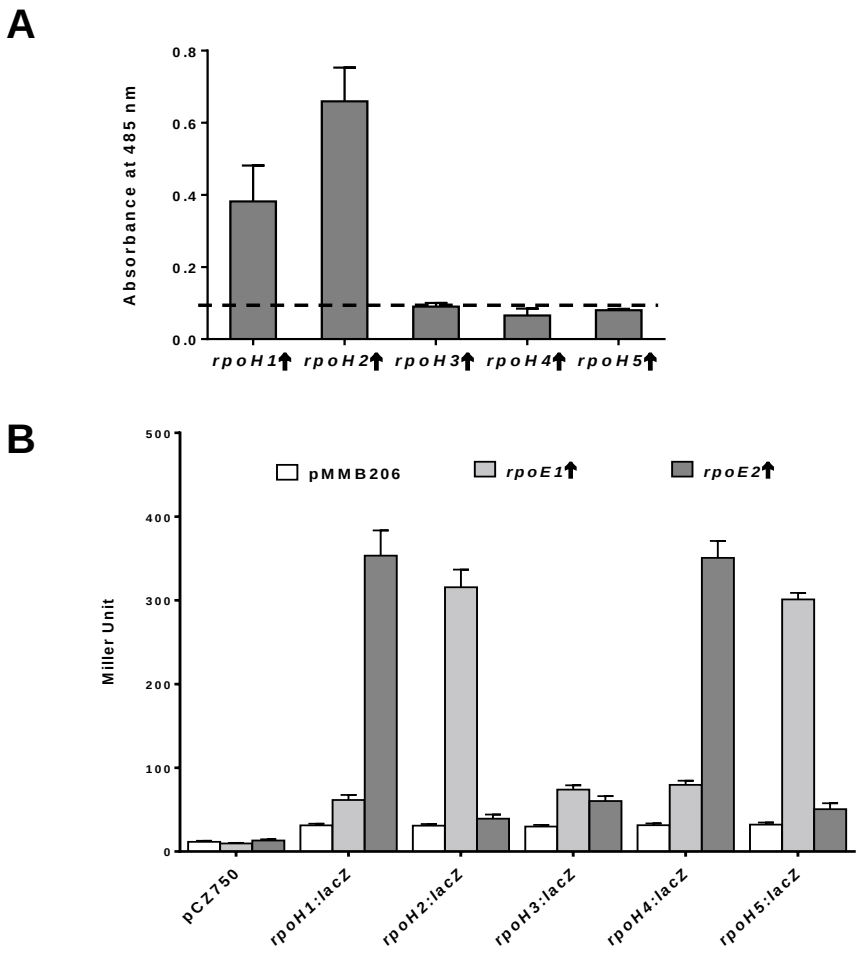

Figure 4. (A) Effect of overexpression of the *rpoH* paralogs (indicated by upward arrows) on the carotenoid content of *A. brasilense* Sp7. Carotenoid content was compared by measuring absorption maxima at 485nm of the methanolic extracts of the strains. line shows the carotenoid content in *A. brasilense* Sp7 harbouring the empty vector. **(B)** Effect of overexpression of *rpoE1* (pAT5) or *rpoE2* (pNG6) on the β-galactosidase activity from *lacZ* fusions with the promoters of five *rpoH* paralogs of *A. brasilense* using two-plasmid system in *E.coli* DH5α. pCZ750 and pMMB206 are the vectors used for constructing *lacZ* fusions and expressing sigma factor genes, respectively. Error bar shows the standard deviation from the three replicates.

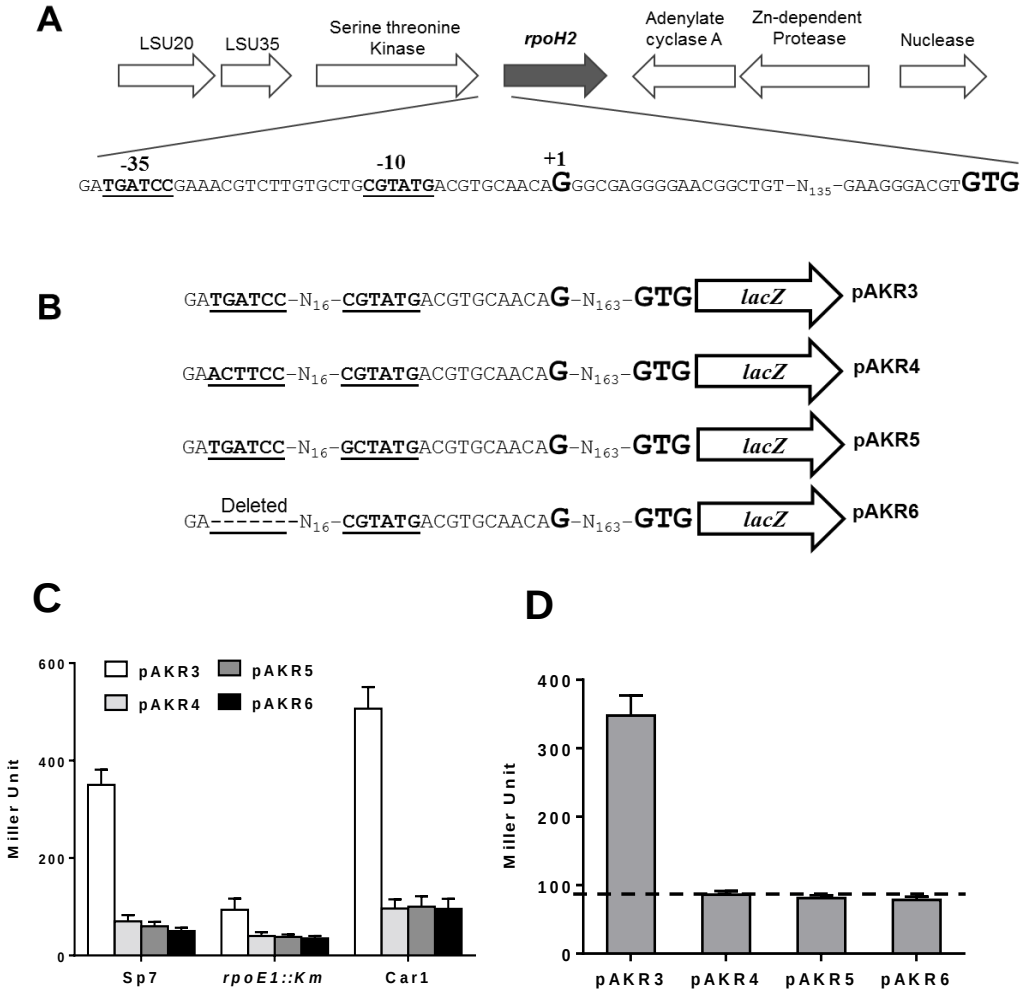

Figure 5. (A) Organization of *A. brasilense rpoH2* locus. The -35 and -10 elements (shown in bold fonts and underlined) of the *rpoH2* gene predicted after the determination of transcription start site (shown as +1 in bold and larger font). *rpoH2* start codon (GTG) is shown in bold and larger font. **(B)** Schematic diagram showing *rpoH2:lacZ* fusion (pAKR3) and its derivatives with mutated -35 element (pAKR4), mutated -10 element (pAKR5) and with deletion of -35 element (pAKR5). **(C)** β-galactosidase activity due to *rpoH2:lacZ* fusion

and its derivatives in *A. brasilense* Sp7, and its mutants *rpoE1::km* and Car1 (*chrR1::Tn5*). **(D)** Effect of overexpression of *A. brasilense rpoE1*on the β-galactosidase activity from *A. brasilense rpoH2* promoter::*lacZ* fusion and its mutant derivatives using two-plasmid system in *E.coli* DH5α. The dashed base line shows the β-galactosidase activity in *E. col*i DH5α expressing *A. brasilense rpoE1*and promoterless *lacZ* gene on pCZ750.

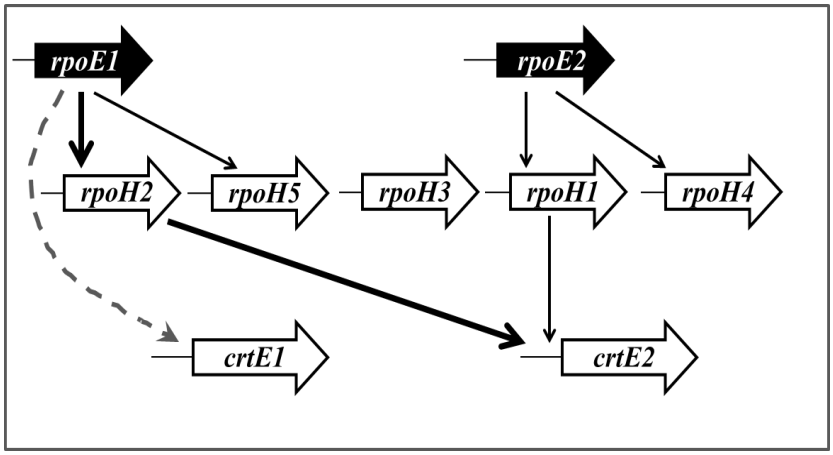

Figure 6. Proposed network of alternative sigma factors regulating the expression of *crtE2* gene of the carotenoid biosynthetic pathway in *A. brasilense Sp7*. In response to appropriate stimuli, RpoE1 regulates RpoH2 and RpoH5, while RpoE2 regulates RpoH1 and RpoH4. RpoH1 and RpoH2 initiate the transcription of a common gene, *crtE2*, to regulate the carotenoid biosynthesis in *A. brasilense*. *crtE1* promoter is also activated in an RpoE1-dependent manner but via unknown intermediate regulator(s). Thick arrows indicate the cascade established in this study by mutation studies as well as by using a two-plasmid system. Thin arrows show the cascades inferred from promoter activity assays using the two-plasmid system. The dotted arrow indicates an indirect regulation of *crtE1* by RpoE1 via unknown intermediate sigma factor(s).

Table 1. Bacterial strains and plasmids used in this study

Strains or plasmids	Relevant properties	Reference or Source
Bacterial Strains		
E. coli DH5α	Δ*lacU*169 *hsdR*17 *recA1 endA*1 *gyrA*96 *thiL relA1*	Gibco-BRL
E. coli S17-1	Sm^r *recA thi pro hsdR* RP4-2(Tc::Mu; Km:: Tn*7*)	24
A. brasilense Sp7	Wild-type strain	15
A. brasilense Cd	Wild-type carotenoid producing strain	15
Car-1	Carotenoid producing *chrR1:Tn5* mutant of *A. brasilense* Sp7	16
rpoE1::km	*A. brasilense* Sp7 *rpoE1* gene disrupted by the insertion of kanamycin resistance gene cassette	25
rpoH2::km	*A. brasilense* Sp7 *rpoH2* gene disrupted by the insertion of kanamycin resistance gene cassette	19
crtE1::km	*A. brasilense* Cd *crtE1* gene disrupted by the insertion of kanamycin resistance gene cassette	This Work
crtE2::km	*A. brasilense* Cd *crtE2* gene disrupted by the insertion of kanamycin resistance gene cassette	This Work
Plasmids		
pSUP202	ColE1 replicon, mobilizable, suicide vector for *A. brasilense*, Ap^r, Cm^r, Tc^r	24
pUC4K	Vector containing kanamycin resistance gene cassette	GE Healthcare
pAPD1	*crtE1*::*km* in pSUP202	This work
pAPD2	*crtE2*::*km* in pSUP202	This work
pCZ750	Tet^r, Amp^r; *lacZ* fusion reporter vector	27
pAPD3	*crtE*1 promoter sequence of *A. brasilense* Sp7 fused with *lacZ* in pCZ750	This work
pAPD4	*crtE*2 promoter sequence of *A. brasilense* Sp7 fused with *lacZ* in pCZ750	This work
pAKR1	*crtE2* promoter (deleted -35) region of *A. brasilense* Sp7 fused with *lacZ* in pCZ750	This work
pAKR2	*RpoH1* promoter sequence of *A. brasilense* Sp7 fused with *lacZ* in pCZ750	This work
pAKR3	*RpoH2* promoter sequence of *A. brasilense* Sp7 fused with *lacZ* in pCZ750	This work
pAKR4	*rpoH2* promoter -35 region (TGA to ACT) of *A. brasilense* Sp7 fused with *lacZ* in pCZ750	This work
pAKR5	*rpoH2* promoter -10 region (CG to GC) of *A. brasilense* Sp7 fused with *lacZ* in pCZ750	This work
pAKR6	*rpoH2* promoter (deleted -35) region of *A. brasilense* Sp7 fused with *lacZ* in pCZ750	This work
pAKR7	*rpoH3* promoter sequence of *A. brasilense* Sp7 fused with *lacZ* in pCZ750	This work
pAKR8	*rpoH4* promoter sequence of *A. brasilense* Sp7 fused with *lacZ* in pCZ750	This work
pAKR9	*rpoH5* promoter sequence of *A. brasilense* Sp7 fused with *lacZ* in pCZ750	This work
pMMB206	Cm^r, broad host range, low copy number, expression vector	26
pAT5	*rpoE1* gene of *A. brasilense* Sp7 cloned in pMMB206 vector	16
pNG6	*rpoE2* gene of *A. brasilense* Sp7 cloned in pMMB206 vector	17
pAKR9	*rpoH1* gene of *A. brasilense* Sp7 cloned at EcoRI and PstI in pMMB206 vector	This work
pSNK10	*rpoH2* gene of *A. brasilense* Sp7 cloned in pMMB206 vector	19
pAKR11	*rpoH3* gene of *A. brasilense* Sp7 cloned in pMMB206 vector	This work
pAKR12	*rpoH4* gene of *A. brasilense* Sp7 cloned in pMMB206 vector	This work
pAKR13	*rpoH5* gene of *A. brasilense* Sp7 cloned in pMMB206 vector	This work