AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Bacteriology

Carotenoid Biosynthetic Pathways Are Regulated by a Network of Multiple Cascades of Alternative Sigma Factors in *Azospirillum brasilense* Sp7

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ABSTRACT

Carotenoids constitute an important component of the defense system against photooxidative stress in bacteria. In *Azospirillum brasilense* Sp7, a nonphotosynthetic 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 harbors 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 *crtE* paralogs found in *A. brasilense* caused reduction in carotenoid content, suggesting their involvement in carotenoid synthesis. However, the effect of *crtE1* deletion was more pronounced than that of *crtE2* deletion. 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 *Escherichia coli*, we have shown that the *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 a 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 one or multiple cascades 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 into existence of an additional cascade or cascades regulating expression of another paralog of *crtE*.

Photosynthetic as well as nonphotosynthetic 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 nonphotosynthetic 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 photoinducible manner. In nonphotosynthetic

Received 12 June 2016 **Accepted** 30 July 2016

Accepted manuscript posted online 22 August 2016

Citation Rai AK, Dubey AP, Kumar S, Dutta D, Mishra MN, Singh BN, Tripathi AK. 2016. Carotenoid biosynthetic pathways are regulated by a network of multiple cascades of alternative sigma factors in *Azospirillum brasilense* Sp7. J Bacteriol 198:2955–2964. doi:10.1128/JB.00460-16.

Editor: C. W. Mullineaux, Queen Mary, University of London

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/JB.00460-16.

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
Strains		
E. coli		
DH5α	Δ*lacU169 hsdR17 recA1 endA1 gyrA96 thiL relA1*	Gibco-BRL
S17-1	Sm^r *recA thi pro hsdR* RP4-2(Tc::Mu Km::Tn*7*)	24
A. brasilense		
Sp7	Wild-type strain	15
Cd	Wild-type carotenoid-producing strain	15
Car-1	Carotenoid-producing *chrR1*::Tn*5* mutant of *A. brasilense* Sp7	16
rpoE1::Km mutant	*A. brasilense* Sp7 *rpoE1* gene disrupted by insertion of Km^r gene cassette	25
rpoH2::Km mutant	*A. brasilense* Sp7 *rpoH2* gene disrupted by insertion of Km^r gene cassette	19
crtE1::Km mutant	*A. brasilense* Cd *crtE1* gene disrupted by insertion of Km^r gene cassette	This work
crtE2::Km mutant	*A. brasilense* Cd *crtE2* gene disrupted by insertion of Km^r 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	*crtE1* promoter sequence of *A. brasilense* Sp7 fused with *lacZ* in pCZ750	This work
pAPD4	*crtE2* 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

bacteria, such as *M. xanthus* (a Gram-negative bacterium) (13) and *S. coelicolor* (a Gram-positive bacterium) (12), carotenoid synthesis is regulated by light via an extracytoplasmic function (ECF) sigma factor and its cognate anti-sigma factor. In *Streptomyces griseus*, carotenoid synthesis is also regulated by an ECF sigma factor, but the stimulus responsible for its induction is not known yet (14).

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

MATERIALS AND METHODS

Bacterial strains, plasmids, chemicals, and growth conditions. The plasmids and strains used in this study are described in Table 1. *Escherichia coli* DH5α and *E. coli* S17-1 were grown in Luria Bertani (LB) medium at 37°C. *A. brasilense* was grown in minimal malate medium (20) as well as in LB medium at 30°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 Table S1 in the supplemental material.

Prediction of promoter regions of *rpoH2* and *crtE* in *A. brasilense*. Upstream nucleotide sequences of *rpoH2* and *crtE* paralogs were analyzed by the 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 the TSS. To determine the transcription start site (TSS), the 5′ ends of the mRNAs of *crtE1*, *crtE2*, and *rpoH2* were identified by 5′ rapid amplification of cDNA ends (RACE) as described earlier (25). Briefly, 2 μg 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. The final PCR product was then cloned in the pGEM-T Easy vector (Promega), and nucleotide sequences were 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 were inserted into the 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 the *crtE2* promoter was deleted by the 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 harboring 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 PtacUV5 promoter and a pCZ750 derivative harboring the 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 a cDNA synthesis kit (Fermentas, Germany). Real-time PCR was performed using SYBR green I (Roche) in a Light Cycler 480 II (Roche) according to the manufacturer's instructions, using *rpoD* as an endogenous control. The relative expression level was compared by the threshold cycle ($2^{-\Delta\Delta CT}$) method (32).

Extraction and estimation of carotenoids. *A. brasilense* strains were grown in the LB medium up to the stationary phase. Pellets from equal volumes of cultures with equal optical densities at 600 nm (OD_{600}) were washed twice with saline and resuspended in 5 ml 100% methanol, and carotenoids were extracted by shaking the suspension overnight at 180 rpm at 25°C in Oakridge tubes covered with aluminum foil (16). Concentrated methanolic extracts of carotenoids were spotted onto a thin-layer chromatography (TLC) plate, resolved, and visualized (15). Absorption spectra of the carotenoids extracted in methanol were recorded using a 300- to 800-nm range in a UV-visible (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 the *crtE* gene). Since the *A. brasilense* genome harbors 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 that were common in the extracts of *A. brasilense* Cd and its two *crtE1*::Km and Cd *crtE2*::Km mutants (data not shown). The intensity of the spots in case of the *crtE1*::Km mutant, however, was very weak in comparison to that detected in the *crtE2*::Km mutant or *A. brasilense* Cd. Absorption spectra of the methanolic extracts of the two mutants revealed that *crtE1* inactivation led to an approximately 75% reduction in the carotenoid content, whereas *crtE2* inactivation reduced carotenoids only by <25% ($P < 0.03$) (Fig. 1A; see Fig. S1 in the supplemental material), indicating involvement of both of the CrtE proteins in carotenoid biosynthesis.

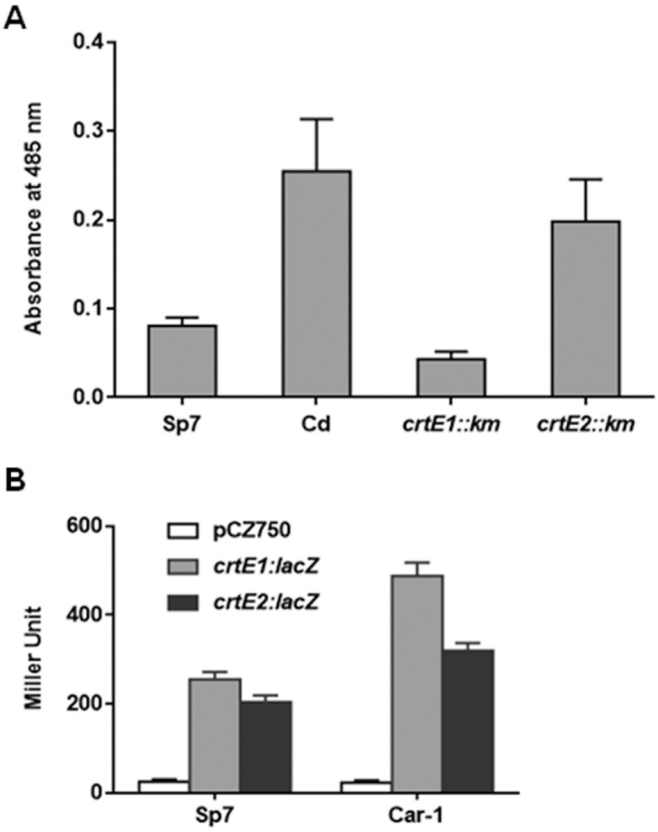

FIG 1 (A) Effect of *crtE1* and *crtE2* gene inactivation on carotenoid content in a carotenoid-overproducing strain, *A. brasilense* Cd. The carotenoid content was compared by measuring absorption maxima at 485 nm of the methanolic extracts of the wild-type or mutant strains. The carotenoid content of Cd is significantly different from those of the *crtE1*::Km and *crtE2*::Km mutants ($P < 0.03$). (B) β-Galactosidase activity due to *lacZ* fusions with the promoters of *crtE1*(pAPD3) and *crtE2*(pAPD4) in *A. brasilense* Sp7 and Car-1. Error bars show standard deviations from three replicates.

***crtE* promoters are regulated indirectly by RpoEs.** We have shown earlier that overexpression of *rpoE1* or *rpoE2* leads to enhancement in the carotenoid level in *A. brasilense* Sp7 (16, 17). To examine if *crtE* genes 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) fu-

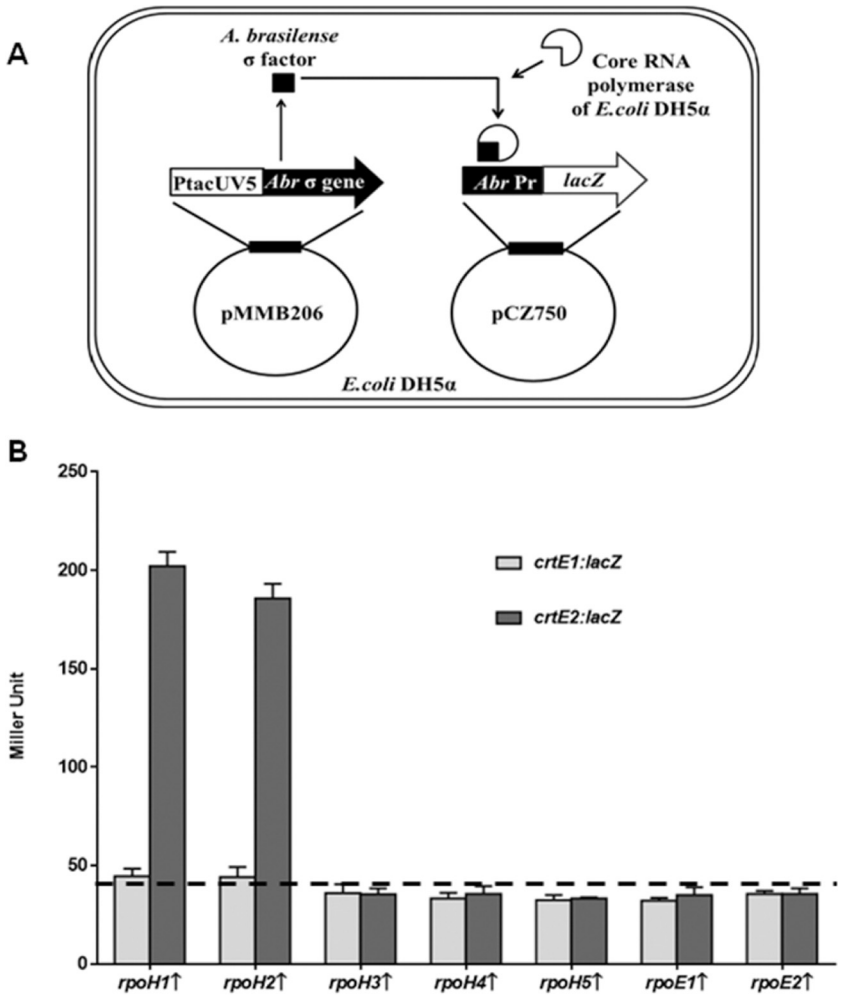

FIG 2 (A) Schematic presentation of the principle of the two-plasmid system. A gene encoding *A. brasilense* sigma factor (*Abr* σ) is cloned downstream of the inducible PtacUV5 promoter in the pMMB206 vector. Upon induction by IPTG, the *A. brasilense* sigma factor is expressed from the PtacUV5 promoter. 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 the *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 harboring *crtE1*::*lacZ*(pAPD3) or *crtE2*::*lacZ*(pAPD4) fusions. Vertical arrows indicate expression of the genes. The dashed baseline shows the β-galactosidase activity of *E. coli* DH5α harboring empty vector. Error bars show standard deviations from three replicates.

sions 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 the activities from both promoters in *A. brasilense* Sp7 were almost equal. Both promoters showed considerably higher activity in the Car-1 mutant than that observed in *A. brasilense* Sp7. Furthermore, the activity of the *crtE1* promoter in the Car-1 mutant was relatively high (Fig. 1B). Although these results indicate an RpoE1-dependent regulation of *crtE* genes, they do not confirm if RpoE1 regulates the expression of *crtE1* directly.

To examine if the *crtE* genes 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 *crtE* genes in *A. brasilense*.

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

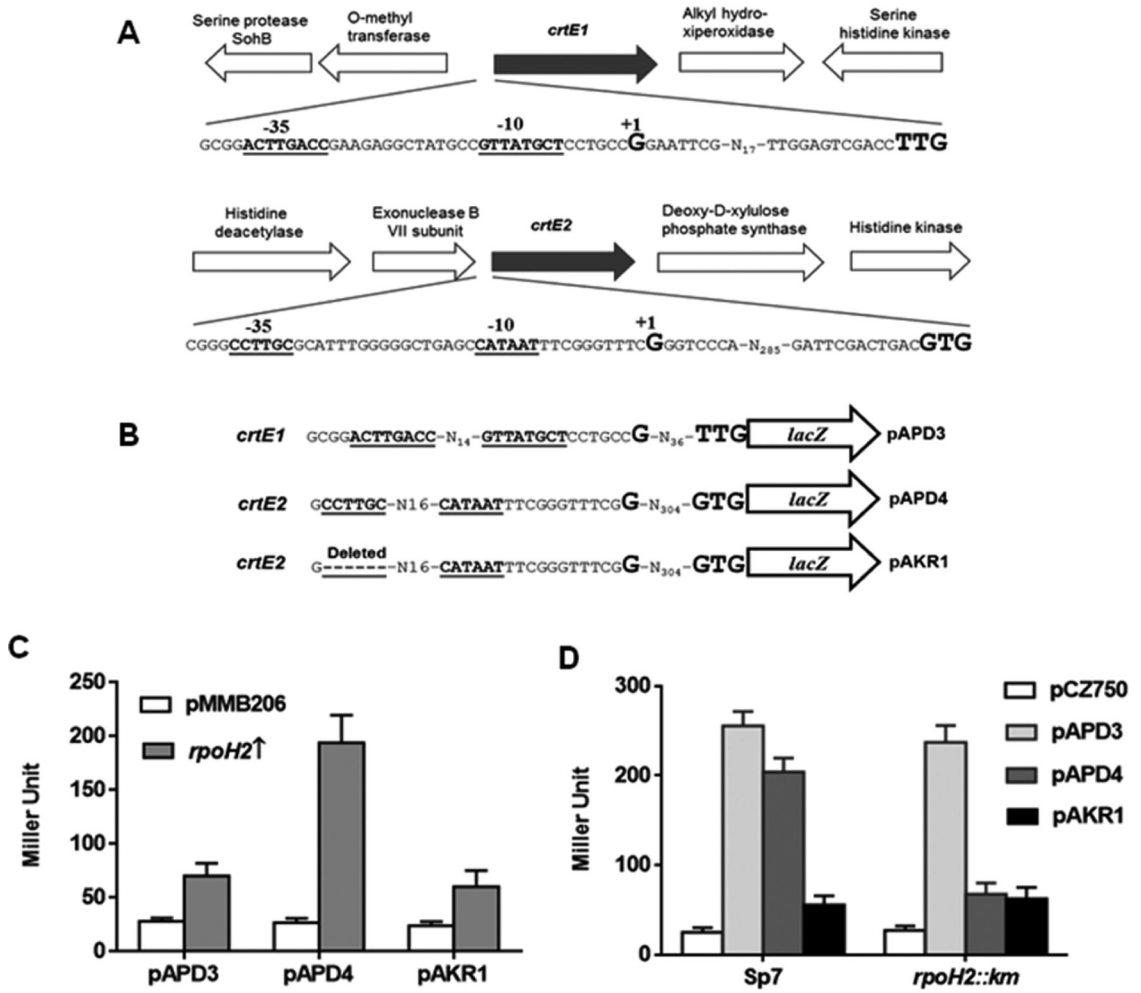

FIG 3 (A) Organization of *A. brasilense crtE1* and *crtE2* loci and possible −35 and −10 elements (boldface and underlined) of the *crtE1* and *crtE2* genes predicted on the basis of the identified TSS (shown as "+1" and in a larger and boldface font); start codons are shown in a larger and boldface font. (B) Representation of the promoter-*lacZ* fusions showing promoter elements of *crtE1*(pAPD3) and *crtE2*(pAPD4) as well as a derivative of the *crtE2* promoter with a deletion of the −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 the two-plasmid system in *E. coli* DH5α. Error bars show standard deviations from three replicates. (D) β-Galactosidase activity of the *lacZ* fusions fused with the promoters of *crtE1*(pAPD3), *crtE2*(pAPD4), and the −35 element deletion derivative of the *crtE2* promoter (pAKR1) in *A. brasilense* Sp7 and *rpoH2*::Km mutants.

CA**TA**AT sequences, respectively, which showed strong resemblance to the RpoH-dependent promoter consensus. (Conserved residues are in boldface.) To validate this prediction, we determined the TSS to identify promoter elements of both *crtE* genes, which revealed that −35 (CCTTGC) and −10 (C**TA**TGC) hexamers of only *crtE2* match the RpoH-dependent consensus, while possible −35 (TTGACC) and −10 (TTATGC) hexamers of *crtE1* did not match with 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 the *crtE2* 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 the *rpoH* gene of *E. coli* is expressed under a heat shock condition, expression of β-galactosidase in *E. coli* DH5α can be observed only if an *A. brasilense* RpoH protein is able to drive the expression of *lacZ* from the *crtE1* or *crtE2* promoters. Each RpoH paralog was expressed in *E. coli* DH5α harboring *crtE1*::*lacZ* or *crtE2*::*lacZ* to examine their involvement in the transcription of *crtE* genes. Promoter activity showed that only the *crtE2*::*lacZ* fusion was activated by both RpoH1 and RpoH2 (Fig. 2B); the *crtE1* promoter does not seem to be activated by any of the RpoH paralogs. When we examined the ability of *A. brasilense* RpoH2 to activate a derivative of the promoter of the *crtE2*::*lacZ* fusion in which the −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 compared to 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 car-

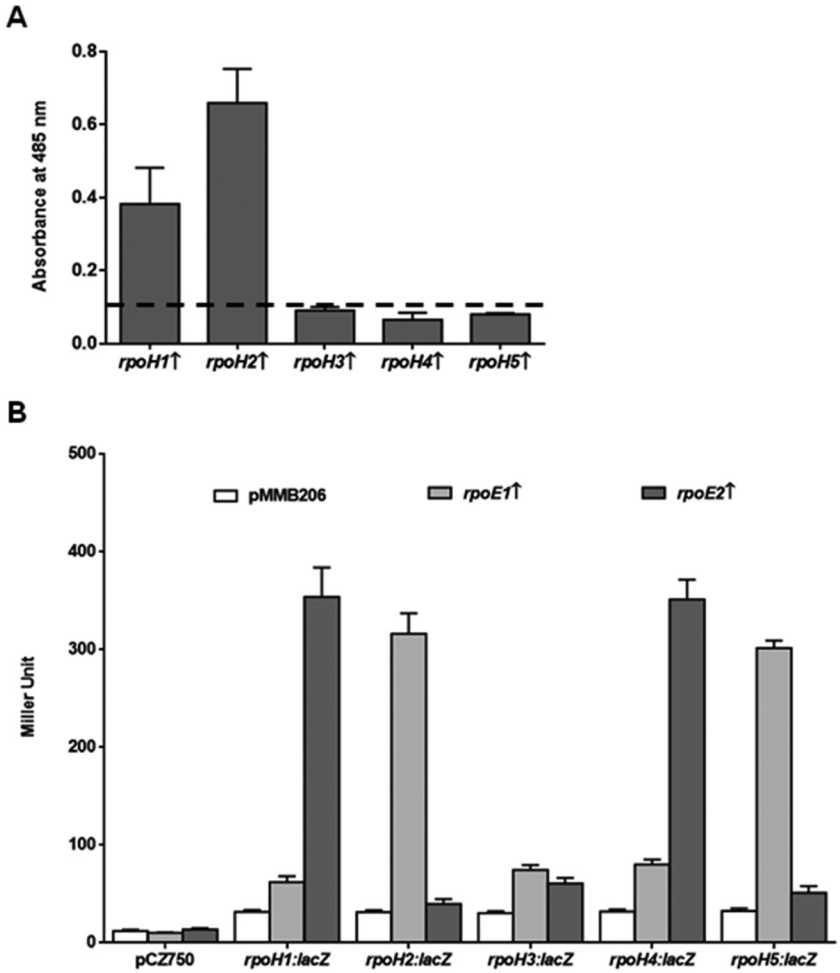

FIG 4 (A) Effect of overexpression of the *rpoH* paralogs (indicated by upward arrows) on the carotenoid content of *A. brasilense* Sp7. Carotenoid content was compared by measuring absorption maxima at 485 nm of the methanolic extracts of the strains. The dashed baseline shows the carotenoid content in *A. brasilense* Sp7 harboring 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 the two-plasmid system in *E. coli* DH5α. pCZ750 and pMMB206 are the vectors used to construct *lacZ* fusions and express sigma factor genes, respectively. Error bars show standard deviations from three replicates.

otenoid 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. Figure 4A shows that the overexpression of *rpoH1* and *rpoH2* caused very significant increases in carotenoid content in *A. brasilense* Sp7; RpoH2 caused maximum synthesis of carotenoids. The carotenoid contents in the strain derivatives 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 RpoH2 as an intermediate regulator. To confirm this hypothesis, we used the two-plasmid system once again to examine whether RpoE1 and RpoE2 directly regulate the *rpoH* paralogs. Figure 4B shows that the activities of the *rpoH1*::*lacZ* and the *rpoH4*::*lacZ* fusions, respectively, were upregulated about 3-fold by RpoE2. Similarly, the activities of *rpoH2*::*lacZ* and *rpoH5*::*lacZ* fusions were 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 the *rpoH3*::*lacZ* fusion, however, does not seem to be affected by the expression of RpoE1 or RpoE2.

***rpoH2* has an RpoE1-dependent promoter.** Since RpoH2 directly regulates *crtE2*, and the expression of *rpoH2* seems to be directly regulated by RpoE1, we attempted to examine whether *rpoH2* harbors an RpoE1-dependent promoter. Determination of the *rpoH2* TSS revealed a "G" located 163 nucleotides upstream of the start codon and TGATCC and CGTATG as possible −35 and −10 elements, respectively, with a space of 16 nucleotides (Fig. 5A). The promoter elements of *rpoH2* predicted on the basis of TSS matched well with the earlier reported promoter sequence recognized by the RpoE1 sigma factor (25). In order to prove further that the predicted −35 and −10 elements are indeed utilized by the RpoE1 sigma factor to drive the transcription of *rpoH2*, we generated three site-directed mutants of the *rpoH2* pro-

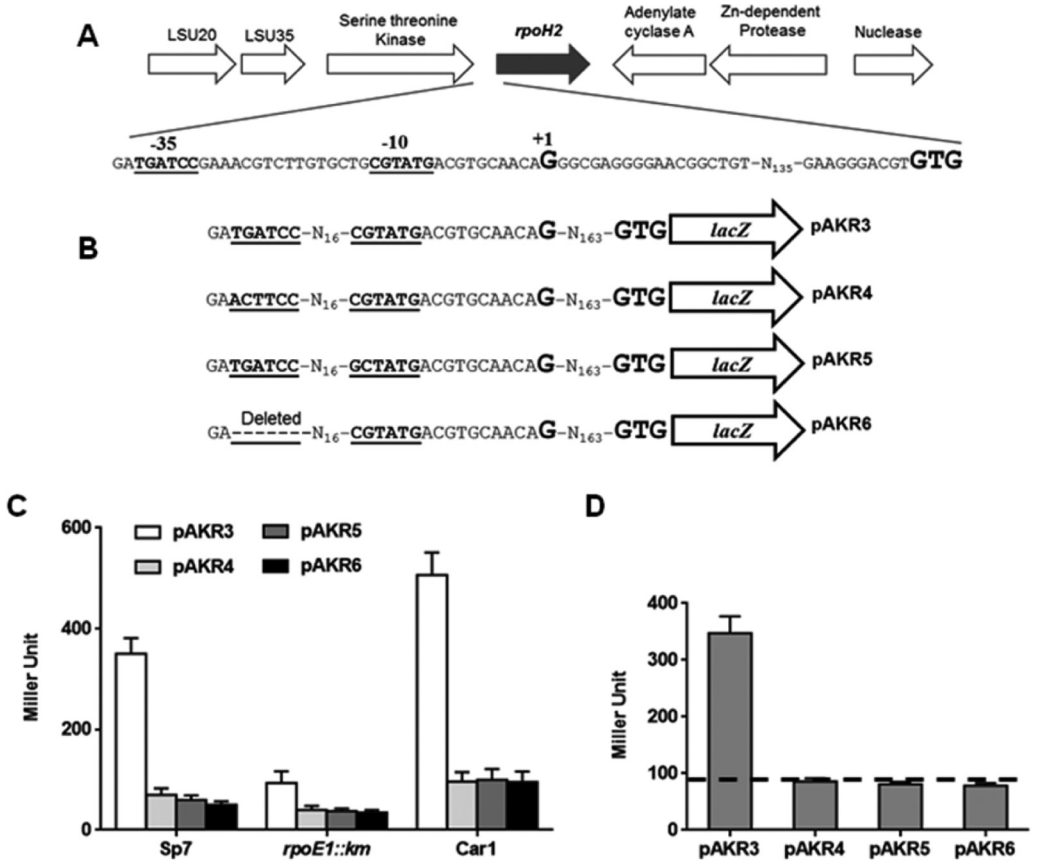

FIG 5 (A) Organization of the *A. brasilense rpoH2* locus. Shown are the −35 and −10 elements (in boldface and underlined) of the *rpoH2* gene predicted after determination of the transcription start site (shown as "+1" in boldface and larger font). The *rpoH2* start codon (GTG) is shown in boldface and larger font. (B) Schematic diagram showing the *rpoH2*::*lacZ* fusion (pAKR3) and its derivatives with the mutated −35 element (pAKR4) and mutated −10 element (pAKR5), as well as with deletion of the −35 element (pAKR5). (C) β-Galactosidase activity due to the *rpoH2*::*lacZ* fusion and its derivatives in *A. brasilense* Sp7 and the *rpoE1*::Km and Car-1 (*chrR1*::Tn*5*) mutants. (D) Effect of overexpression of *A. brasilense rpoE1* on the β-galactosidase activity from the *A. brasilense rpoH2* promoter-*lacZ* fusion and its mutant derivatives using the two-plasmid system in *E. coli* DH5α. The dashed baseline shows the β-galactosidase activity in *E. coli* DH5α expressing *A. brasilense rpoE1* and the promoterless *lacZ* gene on pCZ750.

moter. In one, TGA of the −35 element was replaced with ACT. In the second, CG of the −10 element was replaced by GC. Finally, in the third, the −35 element was deleted completely (Fig. 5B). The *lacZ* fusion with the *rpoH2* native promoter gave the maximum and minimum activities in the Car-1 and *rpoE1*::Km mutants, respectively, indicating dependence of *rpoH2* promoter on RpoE1. The derivatives of the *rpoH2*::*lacZ* fusion carrying three different mutant versions of the promoters showed very low activity even in the Car-1 mutant, clearly indicating that the identified −35 and −10 elements were the essential promoter elements of the RpoE1-dependent regulation of *rpoH2*.

To prove the RpoE1-dependent direct regulation of *rpoH2* promoter and that the −35 and −10 elements are real promoter elements, we used the two-plasmid system to examine the ability of RpoE1 to directly activate the *lacZ*-fused native *rpoH2* promoter and any of the *rpoH2* promoter mutant versions. Figure 5D shows that RpoE1 was able to directly activate *lacZ*-fused *rpoH2* native promoter, whereas it failed to activate any of the mutant versions of the *rpoH2* promoter. These observations further established that TGATCC and CGTATG are the −35 and −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 and the *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 (see Fig. S2 in the supplemental material). This result further corroborated our inference about an RpoE1-dependent regulation of *rpoH2*, as the *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 (16–18, 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.

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

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

On the basis of these as well as our previous observations (16, 18), it can be hypothesized that the RpoEs may indirectly regulate *crtE2* via RpoH1/RpoH2 to control carotenoid synthesis 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 the 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 the −35 element of the *crtE2* promoter abolishes regulation by RpoH2.

By demonstrating a major role of *crtE1* in carotenoid biosynthesis in *A. brasilense* and strong upregulation of the *crtE1* promoter in the Car-1 mutant, in which RpoE1 is constitutively overexpressed, our observations clearly indicate the 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 the *crtE1* promoter in the *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 enterica* serovar 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).

Although alternative sigma factors can act independently, a considerable level of overlap between the genes controlled by alternative sigma factors was demonstrated in *Borrelia burgdorferi* (37, 38). Our previous study showed that *A. brasilense* RpoE1 and RpoE2 are highly homologous, and hence, they not only regulate carotenoid synthesis but also regulate the expression of several common genes in *A. brasilense* (18). Data from this work show that although RpoE1 and RpoE2 both regulate carotenoid biosynthesis in *A. brasilense*, they bring about this regulation through different RpoH sigma factors (RpoE1 regulating RpoH2/5 and RpoE2 regulating RpoH1/4), indicating recognition of different promoters by the two RpoEs. An overlapping function of these RpoEs is manifested by RpoH1- and RpoH2-mediated regulation of a common gene (*crtE2*). A similar overlap of functions between RpoH1 and RpoH2 has also been demonstrated in *R. sphaeroides* (39) and *Sinorhizobium meliloti* (40). Furthermore, RpoE1- and -2-mediated regulation of RpoH4 and -5, which are not involved in carotenoid regulation, provides an insight into the utilization of RpoE1→RpoH4 and RpoE2→RpoH5 regulatory cascades for responding to other stresses, such as salinity and organic stresses, in which involvement of RpoE has been shown (25).

Since *A. brasilense* is a nonphotosynthetic bacterium, it may not be as vulnerable to photooxidative stress/damage as photosynthetic bacteria like *R. sphaeroides*. However, being a soil bacterium, it may be exposed to the damaging effects of light while swimming under submerged soil conditions. Inducibility of carotenoid synthesis in response to light might thus be a desirable and beneficial trait in *A. brasilense*. On the basis of this study and our earlier observations, we propose a model of regulation of carotenoid biosynthesis in *A. brasilense* (Fig. 6). In the absence of light, RpoE sigma factor remains bound to its cognate anti-sigma factor (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 geranylgeranyl pyrophosphate synthetase to catalyze the first step of the carotenoid biosynthesis. However, identification of the RpoE-regulated alternative sigma factor that directly regulates the expression of *crtE1* is needed to develop a better understanding of carotenoid biosynthesis in *A. brasilense*.

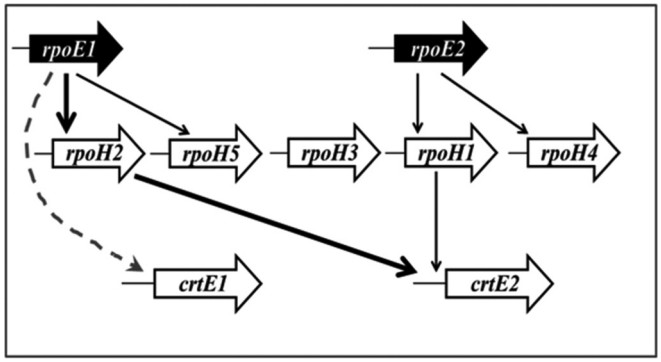

FIG 6 Proposed network of alternative sigma factors regulating the expression of the *crtE2* gene of the carotenoid biosynthetic pathway in *A. brasilense* Sp7. In response to appropriate stimuli, RpoE1 regulates RpoH2 and RpoH5, while RpoE2 regulates RpoH1 and RpoH4. RpoH1 and RpoH2 initiate the transcription of a common gene, *crtE2*, to regulate carotenoid biosynthesis in *A. brasilense*. The *crtE1* promoter is also activated in an RpoE1-dependent manner but via an unknown intermediate regulator or regulators. 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 an unknown intermediate sigma factor or factors.

ACKNOWLEDGMENTS

This work was supported by a grant from the Department of Biotechnology, New Delhi, to A.K.T. A.K.R., A.P.D., S.K., and M.N.M. were supported by fellowships from ICMR and CSIR.

We thank Lacy Daniels (Texas) for reading the manuscript.

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