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Early Steps in Carotenoid Biosynthesis: Sequences and Transcriptional Analysis of the *crtI* and *crtB* Genes of *Rhodobacter sphaeroides* and Overexpression and Reactivation of *crtI* in *Escherichia coli* and *R. sphaeroides*

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In the purple photosynthetic bacterium *Rhodobacter sphaeroides*, the desaturation of phytoene has already been implicated in the assembly of the light-harvesting 2 complex (H. P. Lang and C. N. Hunter, Biochem. J. 298:197–205, 1994). The phytoene synthase and desaturase enzymes mediate the first steps specific for carotenoid biosynthesis up to and including the synthesis of the colored carotenoid neurosporene. In this report, we present the DNA and deduced amino acid sequences of the genes encoding these proteins, namely, *crtB* and *crtI*, from *R. sphaeroides* and present evidence for the existence of a *crtIB* operon. Both genes have been shown to possess putative *puc* and *puf* operon-like promoter sequences, and oxygen regulation and the point of initiation of the *crtI* transcript have been demonstrated. The complete *crtI* gene has been overexpressed in *Escherichia coli* and *R. sphaeroides* and shown to catalyze three desaturations of phytoene to give neurosporene. This activity was shown to be ATP dependent, and the cofactor requirement was investigated by using a spectroscopic assay for in vitro carotenogenic activity. Although the *crtI* and *crtB* genes have been sequenced from a number of different organisms, the transcriptional organization and regulation of these genes have not been analyzed in detail. In this report, we have located the transcription initiation point and have shown that *R. sphaeroides* possesses an oxygen-regulated CrtI-type phytoene desaturase gene that forms a transcriptional operon with *crtB*.

Carotenoid pigments are unsaturated hydrocarbons that are produced in the general isoprenoid pathway. The first step that is specific for carotenoid biosynthesis is the condensation of two molecules of geranylgeranyl pyrophosphate to yield phytoene (10). This reaction is mediated by the CrtB, or phytoene synthase, enzyme (12, 21, 47, 55). Phytoene has only a short chromophore of three conjugated double bonds and is therefore colorless and incapable of photoprotection. Its conversion into colored carotenoids requires a series of desaturation reactions to extend the chromophore (10), and these are mediated by the phytoene desaturase (CrtI) enzyme (28).

Phytoene synthase and desaturase genes have been isolated and sequenced from a variety of bacteria and fungi (see reference 53 for a review; 46) as well as from cyanobacteria (12, 13) and higher plants (7, 8, 49). Comparisons of the deduced amino acid sequences of phytoene desaturases indicate that two distinct, evolutionarily unrelated types of phytoene desaturase enzyme exist in nature. Bacteria and fungi have a CrtI-type phytoene desaturase, while cyanobacteria, algae, and higher plants have a Pds-type enzyme (49). There is also a wide functional diversity in terms of their reaction products which reflects the number of desaturation steps performed by the enzyme (45).

In addition to acting as quenchers of potentially toxic oxygen radicals (11), carotenoids play an important role in photosynthesis as light-gathering pigments. This role is carried out efficiently as a result of specific attachment to reaction center and light-harvesting pigment proteins. A complete description of the role of carotenoids in the assembly and function of photosynthetic complexes and the coordination of pigment biosynthetic pathways with membrane protein insertion requires a high level of structural and spectroscopic information as well as the availability of molecular genetic techniques. *Rhodobacter sphaeroides* is an ideal model system for studies of light-harvesting processes since it is genetically well defined and there is a crystallographically determined structure for the reaction center (1, 14) as well as a great deal of spectroscopic information which has contributed to the proposed models for the light-harvesting complexes (33, 38, 39).

In *R. sphaeroides*, the carotenoid biosynthesis genes are clustered, as in the closely related bacterium *Rhodobacter capsulatus* (62, 66), and are flanked by the bacteriochlorophyll biosynthesis genes within a 45-kb region of photosynthesis-related genes (18) (Fig. 1A). The *crtI* and *crtB* genes, encoding phytoene desaturase and synthase respectively, lie next to each other on the genome of *R. sphaeroides* and have been proposed to form an operon (17).

In this report, we present the DNA and deduced amino acid sequence of *crtI* and *crtB* from *R. sphaeroides*. Oxygen- and light-regulated transcription have been investigated, and the possibility of a *crtIB* operon is discussed. The complete *crtI* gene has been cloned and overexpressed in both *Escherichia coli* and *R. sphaeroides*. In vitro desaturation of phytoene was observed in crude extracts of *E. coli*; this activity was shown to be ATP dependent, and the cofactor requirement was investigated. Complementation studies of the cloned *crtI* gene with *R.*

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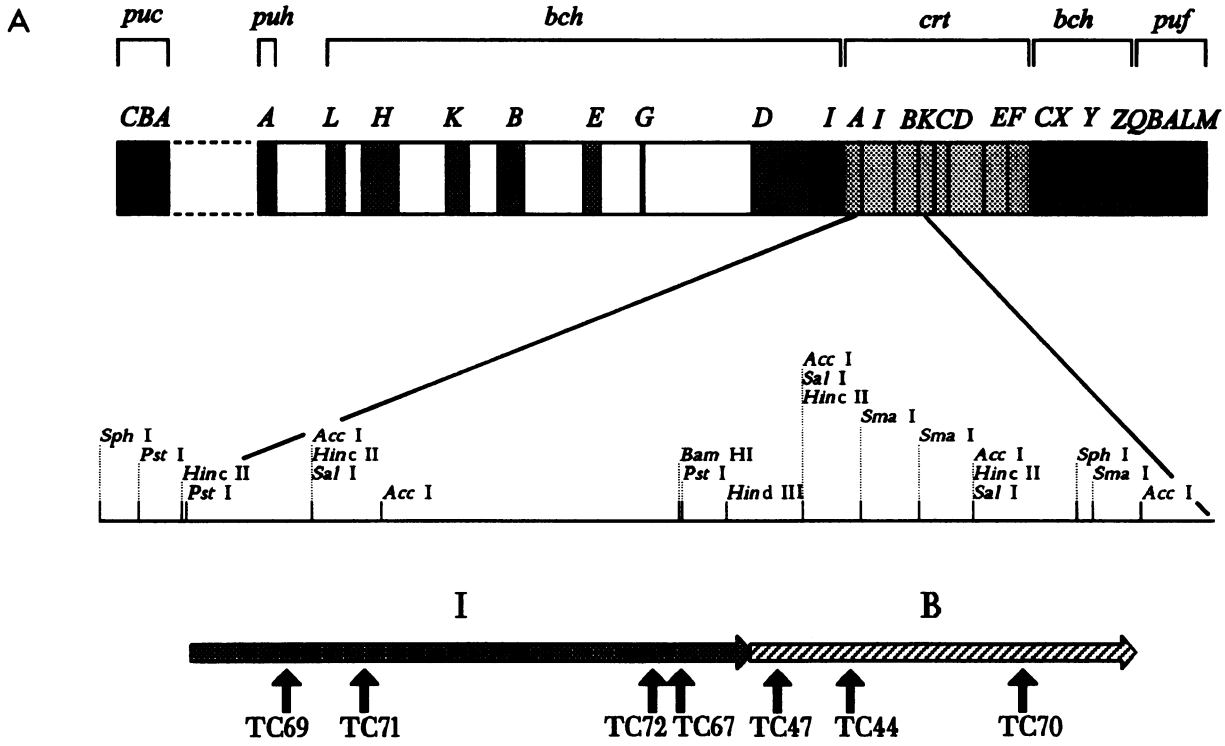

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mRNA
 -70 gccgcattgtaaacctgactagacagtctattgtatggggcatgttgacaagcactgcag -11

 -10 gagttcgcgccatgccctcgatctcgcccgcctccgacgccgaccgcgccctttgtgatcg 49
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                crtI

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       V  I  D  K  L  D  V  P  G  G  R  G  S  S  I  T  Q  E  G  H

                                                    TC69
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 230 agacctgcgggcgggacttcgacgccgatgtcgagctgaagccgatcgatccgttctacg 289
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       V  R  W  P  D  G  S  H  F  T  V  R  Q  S  T  E  A  M  K  A

 350 ccgaggtcgcgcgcctctcgcccggcgatgtggcgggatacgagaagttcctgaaggaca 409
       E  V  A  R  L  S  P  G  D  V  A  G  Y  E  K  F  L  K  D  S

 410 gcgaaaagcgctactggttcggttacgaggatctcggccgccgctcgatgcacaagctgt 469
       E  K  R  Y  W  F  G  Y  E  D  L  G  R  R  S  M  H  K  L  W

          TC71
 470 gggatctcatcaaggtgctgcccaccttcgggatgatgcgggccgaccgtacggtctacc 529
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       W  Y  F  G  T  K  G  T  K  G  M  W  P  D  V  G  H  H  T  I

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                                                    TC72
1190 tggactggcaggctgtggccgagccctaccgcgaaagcgtgctcgaggtgctcgaacagt 1249
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1250 cgatgccggggatcggggaacggatcgggccctcgctcgtcttcacccccgagaccttcc 1309
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                      TC67
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       E  *
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          crtB

                                                    TC47
1610 ttccttccatgccgcgtcccgcctgctgcccgagcgcgtgcgcgcgccgtcgctggcgct 1669
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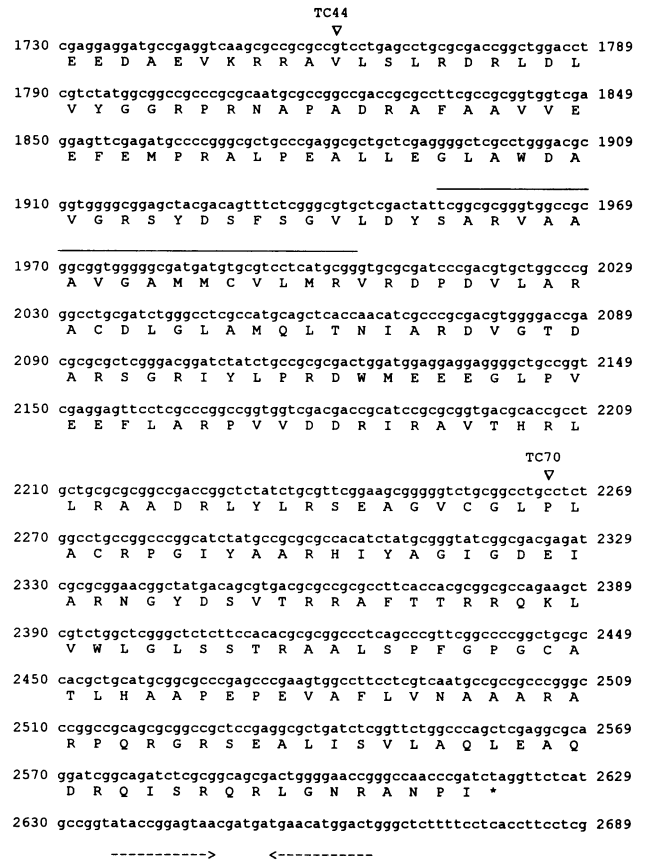

FIG. 1. Maps, nucleotide sequences, and deduced amino acid sequences of the *crtI* and *crtB* genes in *R. sphaeroides*. (A) The 45-kb photosynthetic cluster and *puc* operon adapted from Coomber et al. (17) and containing information from McGlynn and Hunter (48). Shaded regions represent the positions of the genes as mapped by insertion mutagenesis and subsequent sequencing studies. (B) Nucleotide and deduced amino acid sequences of the carotenoid gene cluster in *R. sphaeroides*. Arrows above the start codons indicate the direction of transcription. Putative ribosome binding sites preceding the start codons are underlined. Possible promoter sites are indicated by asterisks above the sequence. Putative palindromic regulatory sequences are indicated by pairs of solid converging arrows below the sequence. Possible factor-independent transcription terminators are indicated by pairs of dashed converging arrows above the sequence. Possible transmembrane domains, identified by using the Klein algorithm (36), are overlined. The nucleotide at the start of the *crtI* mRNA transcript is underlined and in boldface. The positions of the Tn*5* insertions are indicated.

sphaeroides crtI and *crtB* mutants confirm the identity of the cloned gene and show that *R. sphaeroides* possesses a CrtI-type phytoene desaturase, which catalyzes three desaturations of phytoene to give neurosporene.

MATERIALS AND METHODS

Media and antibiotics. *E. coli* strains were grown at 37°C in Luria-Bertani medium (52). *R. sphaeroides* strains were grown in M22+ medium (32); liquid cultures were supplemented with 0.1% Casamino Acids. These were grown at 34°C, aerobically or semiaerobically in the dark or photosynthetically in 30-ml screw-cap bottles at a light intensity of 10 W m^{-2}. Antibiotics were used at the following concentrations (micrograms per milliliter): for *E. coli*, 200 (ampicillin), 30 (kanamycin), and 10 (tetracycline); for *R. sphaeroides*, 20 (kanamycin) and 1 (tetracycline).

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1.

DNA sequencing, primer extension, and sequence analysis. Construction of sequencing clones and deletion constructs will be published elsewhere (41a). Sequencing reactions were carried out on double-stranded DNA template, prepared with the Promega Magic Miniprep kit. The dideoxynucleotide chain termination method of DNA sequencing was employed (56), using the United States Biochemical Corporation Sequenase version 2.0 7-deaza kit and [α-^{35}S]dATP (Amersham International Plc.). Primer extension was carried out as described by Sambrook et al. (52), and the primer was labelled with [γ-^{32}P]ATP by using T4 kinase (52). DNA and deduced amino acid sequences were analyzed with the University of Wisconsin Genetics Computer Group program package (20).

RNA preparation and Northern (RNA) blot analysis. RNA was prepared from aerobic, semiaerobic, and photosynthetic-cultures of wild-type *R. sphaeroides*, grown for 1 to 3 days until the A_{680} reached 0.3 to 1.0. Aerobic growth conditions were achieved by inoculating 200 ml of M22+ medium in a 2-liter conical flask and shaking overnight at 250 rpm and 34°C in the dark in an orbital incubator. Semiaerobic growth conditions were achieved by inoculating 70 ml of M22+ medium in a 100-ml conical flask and shaking at 180 rpm and 34°C in the dark until the cells became pigmented. Photosynthetic growth conditions were achieved by inoculating 30 ml of M22+ medium in a 30-ml universal and incubating at 34°C and 10 W m^{-2} until the cells became pigmented. Cells were resuspended in 150 μl of solution 1 (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl [pH 8.0]); 550 μl of extraction buffer (4 M guanidium isothiocyanate, 50 mM Tris-HCl [pH 7.5], 25 mM EDTA, 8% [wt/vol] β-mercaptoethanol) was added. Cells were sonicated with three 5-s bursts in a Soniprep sonicator; 70 μl of 3 M potassium acetate was added, and the RNA was extracted with phenol-chloroform and pelleted in a Beckman L2 JA20 rotor at 17,000 × *g* for 1 h at 4°C. The pellet was washed in 70% ethanol, vacuum desiccated for 20 min, and redissolved in 80 μl of H_2O.

Twenty micrograms of each RNA sample was denatured by glyoxylation and electrophoresed through a 1 to 2% agarose gel. Northern blotting was performed as described by Thurston et al. (63). DNA probes were excised from pSUP202-derived plasmids constructed by Coomber et al. (17) and labelled with [α-^{32}P]dCTP as described by Feinberg and Vogelstein (23).

Amplification and overexpression of the *R. sphaeroides crtI* gene in *E. coli*. The synthetic oligonucleotide primers 5′AGG GCATATGCCCTCGATCTCGCCC3′, containing the engineered *Nde*I site (underlined), and 5′ATGAAGATCTTCATC CGCGGCAAGCC3′, containing the engineered *Bgl*II site (underlined), were used in a PCR with pSCN6-20 in order to amplify *crtI*. Samples were subjected to 20 cycles of amplification (denaturation for 30 s at 98°C; annealing for 30 s at 50°C; extension for 1 min at 72°C) in a Techne PHC-3 Dri-Block cycler. The resulting DNA fragment was restricted with *Nde*I and *Bgl*II and ligated into *Nde*I-*Bam*HI-ended pET3a vector (51) to give pHLTPD1. pHLTPD1 was then digested with *Xba*I and *Eco*RV, filled in with the Klenow fragment of DNA polymerase to give blunt ends, and ligated into *Bam*HI-ended, blunted pRKSK1 (30) to give pHLKPD1. pHLTPD1 was transformed into *E. coli* BL21(DE3) (60), and overexpression

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Characteristics	Source or reference
E. coli		
DH5α	*supE44* δ*lacU*169 (ϕ80 *lacZ*δM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	
S17-1	RP4-2-Tc::Mu-Km::Tn*7* integrated into the chromosome; Tra^+ Tp^r Sm^r *recA pro res med*$^+$	
BL21(DE3)	(F^- *ompT* r_B^- m_B^-)$^+$ bacteriophage DE3	60
R. sphaeroides		
NCIB 8253	Wild type	R. A. Niederman (Rutgers University, Princeton, N.J.)
DD13/W1	Spontaneous white mutant of DD13 (genomic deletion of *pufBALMX*, insertion of Km^r gene; genomic deletion of *pucBA*, insertion of Sm^r gene	34
TC40	Tn*5* insertion into *crtC*	17
TC44	Tn*5* insertion into *crtB*	17
TC47	Tn*5* insertion into *crtB*	17
TC67	Tn*5* insertion into *crtI*	17
TC69	Tn*5* insertion into *crtI*	17
TC70	Tn*5* insertion into *crtB*	17
TC71	Tn*5* insertion into *crtI*	17
TC72	Tn*5* insertion into *crtI*	17
Plasmids		
pET3a	pBR322-derived translation vector; Amp^r Tc^r	51
pRKSK1	pRK415-derived expression vector with insertion of the 320-bp *StuI-KpnI* fragment of pRNSK1 containing the *puc* promoter	30
pHLTPD1	PCR-amplified *crtI* gene cloned into pET3a	This work
pHLKPD1	PCR-amplified *crtI* gene cloned into pRKSK1	This work
pSCN6-20	Ap^r Cm^r Tc^r pSUP202 derivative	18

was induced by addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM.

Transformation and conjugative crosses. pHLKPD1 was transformed into *E. coli* S17-1; matings into *R. sphaeroides* strains were then performed as described by Hunter and Turner (32). Transconjugants were grown aerobically in the dark on plates of M22+ medium supplemented with the appropriate antibiotics.

SDS-PAGE and intracytoplasmic membrane preparation. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed as described by Sambrook et al. (52), and gels were stained with Kenacid blue R. *R. sphaeroides* cultures were grown under semiaerobic conditions in the dark at 34°C. *E. coli* cultures were grown under aerobic conditions in the dark at 37°C. Cells were disrupted in a French pressure cell at 18,000 lb/in^2, and cell-free intracytoplasmic membrane fractions of *R. sphaeroides* strains were isolated by passage through a discontinuous (15%/40% [wt/wt]) sucrose gradient in a method adapted from that of Fraley et al. (24).

Assay of CrtI activity. The activity of CrtI in *E. coli* was measured by mixing cell extracts of *E. coli*(pHLTPD1) and *R. sphaeroides* DD13/W1, a mutant lacking light-harvesting and reaction center complexes which also accumulates phytoene (34). DD13/W1 was grown under semiaerobic conditions in the dark at 34°C for 2 days. *E. coli* BL21(DE3)(pHLTPD1) was grown overnight under aerobic conditions in the dark at 37°C. Cultures were harvested and resuspended in 100 mM Tris-HCl (pH 8.0)–4 mM dithiothreitol; cells were then disrupted in a French pressure cell at 18,000 lb/in^2. Cell extracts were mixed in equal ratios, and the reaction mixture was supplemented with ATP (10 mM), NADP (2 mM), $MnCl_2$ (6 mM), $MgCl_2$ (4 mM), phenylmethylsulfonyl fluoride (500 μM), and *N*-tosyl-L-phenylalanine chloromethyl ketone (50 μM). This mixture was then incubated at 34°C in the dark for 4 h, and the reaction was stopped by the addition of EDTA to a final concentration of 25 mM.

Absorption spectroscopy and carotenoid extraction. Room temperature absorption spectra were measured on isolated carotenoid pigments. Pigments were extracted from cell-free samples by the addition of 9 volumes of acetone-methanol (7:2 [vol/vol]) followed by 2 volumes of petroleum ether. The carotenoid pigments were extracted into the petroleum ether, and room temperature absorption spectra were obtained on a Guided Wave model 260 fiber-optic spectrophotometer (Guided Wave Inc.). Low-temperature (77 K) absorption spectra were measured on isolated intracytoplasmic membranes, using a liquid nitrogen cryostat (Oxford Instruments).

HPLC analysis. The carotenoid composition of whole cells and cell extracts was determined as described by Takaichi and Shimada (61). The pigments were extracted with chloroform-methanol (3:1 [vol/vol]) and filtered through a Hewlett-Packard membrane filter. They were then analyzed with a Hewlett-Packard high-pressure liquid chromatography (HPLC) system equipped with a Hewlett-Packard 1040A photo diode array detector and a Walters μ Bondapak G8 column. The elution was performed with a combination of gradient and isocratic elution with water and methanol. The pigments were identified by their retention times and absorption spectra.

RESULTS

The positions and approximate limits of the *crtI* and *crtB* genes within the photosynthetic gene cluster of *R. sphaeroides* have been determined previously by localized transposon Tn*5* mutagenesis (17). Seven of the resulting Tn*5* insertion mutants were used in this study (Table 1); however, the genotypes of two of these mutants were wrongly identified by Coomber et al. (17). TC69 and TC72, both previously designated *crtB* mutants, have been reassigned as *crtI* on the basis of subsequent sequencing studies (Fig. 1).

Nucleotide and deduced amino acid sequences of *crtI* and *crtB*. (i) Ribosome binding sites, translational starts, and

codon usage. A 3.7-kb region, known to include *crtI* and *crtB*, was sequenced on both strands; the nucleotide and deduced peptide sequences of the only two complete open reading frames (ORFs) that conform to a compiled codon usage table for *R. sphaeroides* photosynthesis genes (data not shown), and with a strong third-position GC bias (19), are shown in Fig. 1B. On the basis of codon usage, GC bias, and the presence of a Shine-Dalgarno ribosome binding site (58) homologous to the 3′ end of the *R. sphaeroides* 16S RNA (22), we locate the putative translation start for *crtI* at the ATG at bp 1 and that for *crtB* at the ATG at bp 1554 (Fig. 1B).

The terminator TGA for the first ORF, at bp 1555, overlaps the putative ATG translation start for *crtB*. Interestingly the same arrangement has been proposed in *R. capsulatus* (2, 6). This ORF terminates at bp 2619, and a third ORF appears to start at the ATG at either bp 2650 or 2653, and by comparison with *R. capsulatus*, this could correspond to the start of the *crtK* gene (2).

(ii) Properties of the *crtI* and *crtB* gene products. The predicted relative molecular weights of the *crtI* and *crtB* gene products are 57,244 and 39,000, respectively. Analysis of CrtI with the Klein algorithm (36) reveals a membrane-spanning region for amino acid residues 223 to 240 and three possible integral membrane regions for residues 12 to 28, 196 to 212, and 482 to 498 (Fig. 1B). Analysis of CrtB with the Klein algorithm reveals a short integral membrane region for residues 134 to 150 (Fig. 1B).

(iii) Transcription initiation and termination. To determine the precise location of the 5′ end of the *crtI* transcript, reverse transcriptase and the synthetic oligonucleotide 5′CGCATCG CAGCCGCAAGGCCCCCAGT3′, which binds at bp 57 to 83 in *crtI*, were used. As shown in Fig. 2, one distinct primer-extended product of 147 bp was observed. No other major bands were seen after extended exposure of the gel (data not shown). The position of the band in the primer extension track corresponds to the thymidine residue at bp −64 and is the proposed transcriptional start point for the *crtI* gene.

A search for factor-independent transcription terminators (see reference 50 for a review) revealed no such structures at the end of the *crtI* gene. However, two possible transcription terminators can be found downstream of the 3′ end of *crtB* (Fig. 1B). The putative transcription terminator, starting at bp 2696, has a ΔG (25°C) of −20.4 kcal (1 kcal = 4.184 kJ), and the one starting at bp 2761 has a ΔG (25°C) of −11 kcal, calculated by the rules of Tinoco et al. (64).

(iv) A putative promoter sequence with homology to the *puc* and *puf* operon promoters. Sequences upstream of the transcriptional start of *crtI* and *crtB* which show significant homology to the sequences of the promoters from the *R. sphaeroides puf* operon (31) have been located (Fig. 3). These homologies suggest that oxygen may be involved in regulation of *crtI* and possibly *crtB*, and therefore Northern blot analysis was performed.

Northern blot analysis of the *crtI* transcript. To investigate transcriptional regulation of *crtI* by oxygen, total RNA prepared from aerobic, semiaerobic, and photosynthetic cultures of wild-type *R. sphaeroides* was subjected to Northern blot analysis, using the 1.37-kb *Pst*I fragment of *crtI* as a probe (Fig. 4). When aerobic and semiaerobic cultures were grown in the dark, under aerobic conditions the transcript was undetectable; however, when the oxygen tension was lowered, levels of the *crtI* transcript increased dramatically. The *crtI* transcript was also visible in the photosynthetically grown culture, although it does not appear to be as abundant as in the semiaerobic culture. When the same RNA was analyzed with a *crtB* probe, the *crtB* transcript was undetectable in all three cultures

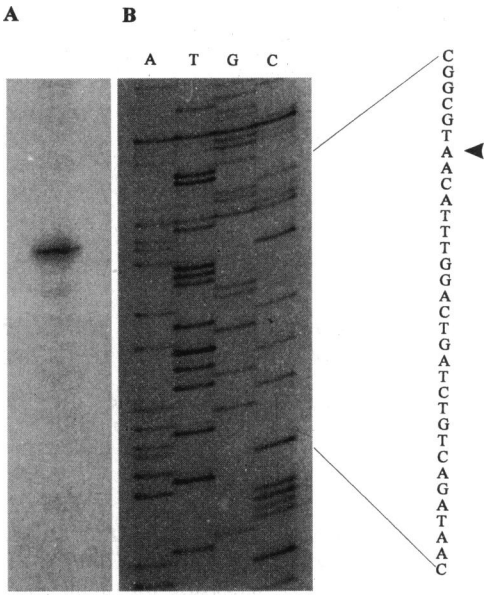

FIG. 2. Primer extension of *crtI*. (A) The ^{32}P-labelled primer, 83-C GCATCGCAGCCGCAAGCCCCCAGT-57, was hybridized to wild-type RNA and extended with murine reverse transcriptase. (B) Sequencing reactions carried out on a double-stranded DNA template by using the same primer, analyzed on a 6% acrylamide–7 M urea gel and autoradiographed. The nucleotide corresponding to the major primer extension band is indicated by an arrowhead.

(results not shown). Chamovitz et al. (12) were also unable to detect *pys* transcripts in Northern hybridization with *Synechococcus* RNA and suggested that *pys* mRNA is a low-abundance transcript.

Cloning and overexpression of *crtI* in *E. coli* and *R. sphaeroides*. The PCR-amplified *crtI* gene from *R. sphaeroides* was cloned into a pET3a overexpression vector to give plasmid pHLTPD1, in which the *crtI* gene is under control of the T7 promoter and is inducible by IPTG (Fig. 5A), and then cloned into the nonintegrative, self-replicating plasmid pRKSK1 (30) to give plasmid pHLKPD1. In this plasmid, the *crtI* gene is under control of the strong *R. sphaeroides puc* promoter (27, 43) (Fig. 5B). Overexpression of *crtI* from pHLTPD1 in *E. coli* was analyzed by SDS-PAGE. pHLKPD1 was introduced into *R. sphaeroides* transposon insertion mutants by conjugative transfer. Membranes were prepared from semiaerobic cultures of transconjugant strains and analyzed by SDS-PAGE (Fig. 5C).

The predicted M_r of *crtI* is 57,244; in *R. sphaeroides*, a strong band of M_r ~57,000 can be seen in the transconjugant strains TC70(pHLKPD1) and TC72(pHLKPD1). In TC70(pRKSK1) membranes, the same band can be seen accumulating. This is to be expected in TC70, since although the *crtB* gene is inactivated, *crtI* can still be expressed and the *crtI* gene product will accumulate; however, we have no explanation for the apparent enhancement of CrtI levels in TC70(pRKSK1). In *E. coli* cells containing pHLTPD1, a very strong band of M_r ~55,000 can be seen; this band is absent from cells containing pET3a only (Fig. 5C). The reason for the reduction in size of the CrtI protein in *E. coli* is not known, although it is possible that it is subjected to some posttranslational modification.

(i) Overexpression and activity of CrtI in *E. coli*. Having demonstrated that a protein of approximately the predicted size is expressed in *E. coli*, we wished to establish whether it

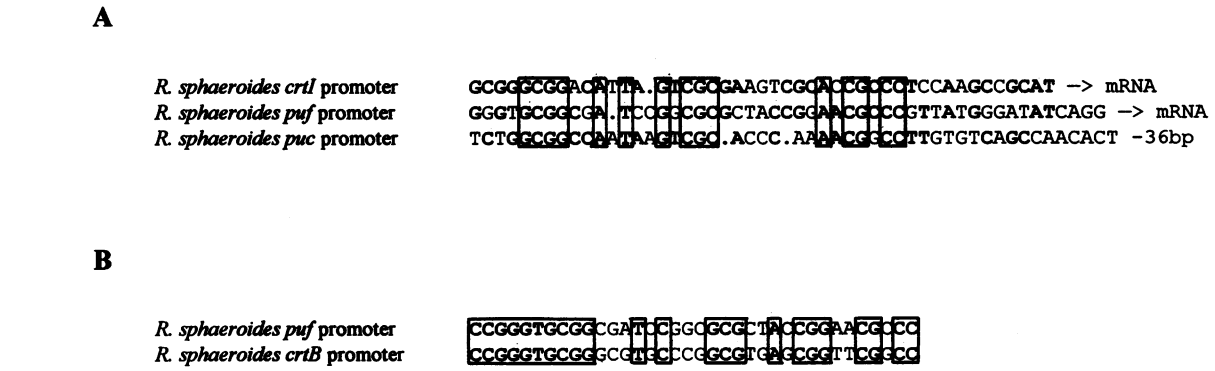

FIG. 3. Alignment of putative promoter sequences. Alignment of the nucleotide sequences of oxygen-regulated *puf* and *puc* operon promoters from *R. sphaeroides* with putative promoter sequences for the *R. sphaeroides crtI* and *crtB* genes. Conserved residues are in boldface. Conserved regions are boxed.

formed an active enzyme. Cell extracts of *E. coli* BL21(DE3) (pHLTPD1) and *R. sphaeroides* DD13/W1, a white mutant which accumulates the CrtI substrate phytoene and lacks all light-harvesting and reaction center complexes (34), were mixed in 1:1 ratios. The reaction mixture was supplemented with ATP, NADP, $MgCl_2$, and $MnCl_2$ and incubated at 34°C for 4 h in the dark. The carotenoids were extracted and analyzed by spectroscopy. Carotenoids were also extracted from TC40, a *crtC* transposon insertion mutant which accumulates neurosporene, the product of phytoene desaturation. The carotenoids extracted from the mixture of DD13/W1 and pHLTPD1 were green, and the spectra indicate that neurosporene was produced (Fig. 6A); this result was reproducible, and the identity of the product, neurosporene, was confirmed by HPLC analysis (results not shown). Thus, the *crtI* gene is producing an active enzyme in *E. coli* that is capable of desaturating phytoene to yield neurosporene. This finding confirms the role of the *crtI* gene in *R. sphaeroides*, a role first demonstrated in *R. capsulatus* by Giuliano et al. (28).

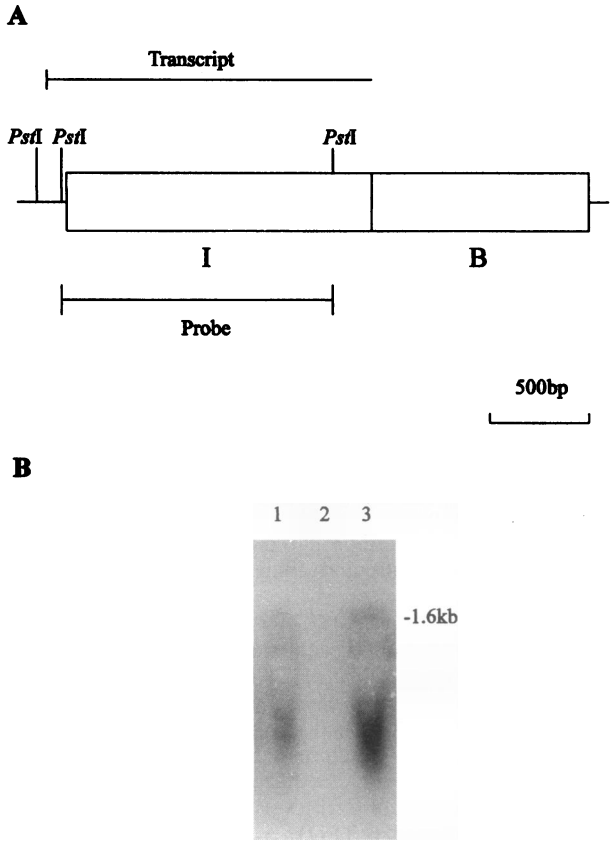

FIG. 4. Northern blot analysis of the *crtI* transcript. (A) Restriction map of the *crtIB* region showing the fragment used as a probe. (B) Autoradiograph of a Northern blot of RNA prepared from photosynthetically grown (lane 1), aerobically grown (lane 2), and semiaerobically grown (lane 3) wild-type *R. sphaeroides* and probed with *crtI* DNA. Twenty micrograms of RNA, denatured by glyoxylation, was loaded per lane on a 1.2% agarose gel.

(ii) ATP dependence and cofactor specificity of CrtI in *E. coli*. To prove that the activity of CrtI is ATP dependent and to investigate the cofactor requirement, the experiment with DD13/W1 was repeated, first omitting ATP and then substituting NAD or flavin adenine dinucleotide (FAD) for NADP (Fig. 6B). If ATP is omitted, no neurosporene is produced, thus proving the activity of CrtI in *E. coli* to be ATP dependent. The cofactor requirement is not so easy to determine since the presence of cofactors already present in the cells means that the reaction proceeds even if no cofactors are added to the reaction mixture, although the use of dialyzed extracts could minimize this problem of carryover. There does not appear to be a preference for either NAD or NADP, although any preference could be masked by the presence of sufficient quantities already in the cells. FAD, however, has a marked inhibitory effect on the activity of the *R. sphaeroides* CrtI enzyme.

Complementation of *crtI* and *crtB* transposon insertion mutants. pHLKPD1 was introduced into the following transposon insertion mutants by conjugative transfer: TC44, TC47, TC67, TC70, TC71, and TC72 (Table 1). TC44, TC47, and TC70 are *crtB* mutants, and TC67, TC71, and TC72 are *crtI* mutants; they are all blue-green in color. If the mutants were complemented by pHLKPD1, they should have produced the red/brown pigment spheroidene or spheroidenone, as in the wild type, so complementation was initially indicated by a change in pigmentation from blue-green to red. The *crtI* mutants were all complemented by the addition of pHLKPD1 and the colonies turned red, while the *crtB* mutants were not complemented and remained blue-green (Table 2). Membranes were isolated from semiaerobic cultures of transconjugant strains on discontinuous sucrose gradients, and low-

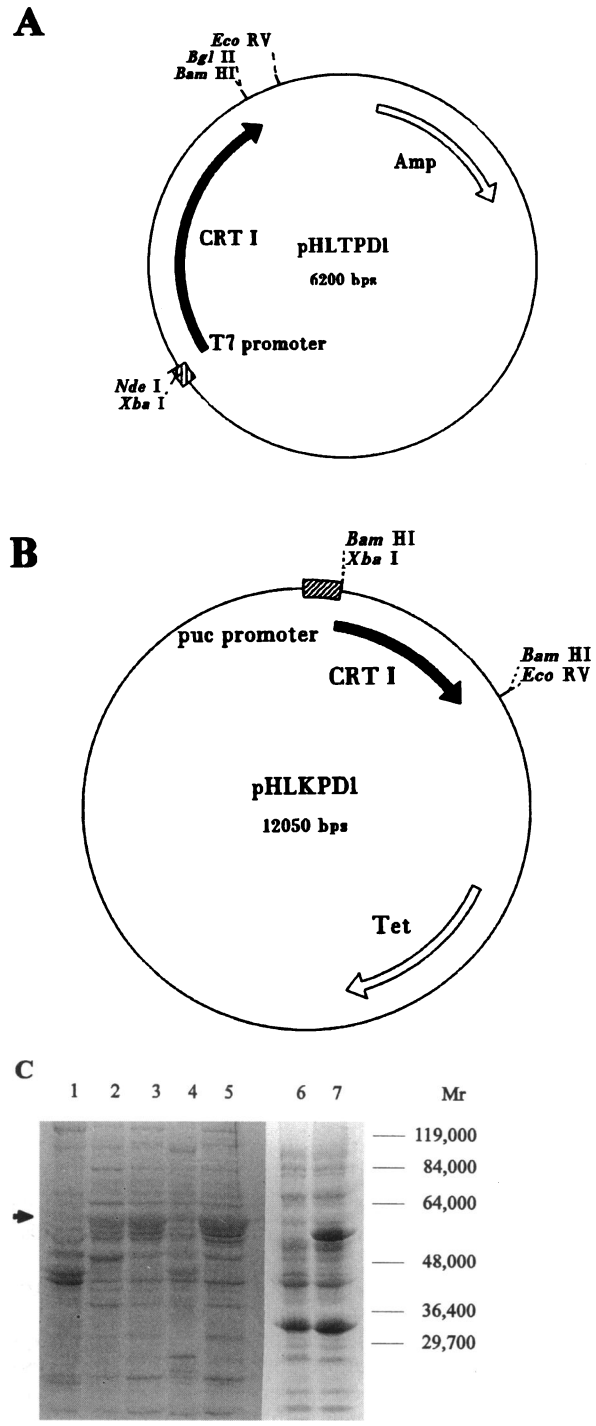

FIG. 5. CrtI polypeptide analysis. (A and B) Plasmids containing the PCR-amplified *crtI* gene, constructed for transformation of *E. coli* (A) and conjugative transfer into *R. sphaeroides* (B). The *crtI* gene is indicated by a solid arrow, ampicillin (Amp) and tetracycline (Tet) resistance genes are represented by open arrows, and T7 and *puc* promoters are indicated by shaded boxes. (C) Overexpression of *crtI* in *R. sphaeroides* and *E. coli*. Photosynthetic membranes were extracted from semiaerobic cultures of *R. sphaeroides*, and whole *E. coli* cells were used. Lanes: 1, wild type; 2, TC70; 3, TC70(pHLKPD1); 4, TC72; 5, TC72(pHLKPD1); 6, BL21(DE3)(pET3a); 7, BL21(DE3)(pHLTPD1). All samples were analyzed by electrophoresis through SDS–10% polyacrylamide vertical slab gels. An arrow indicates the position of the overexpressed *crtI* gene product.

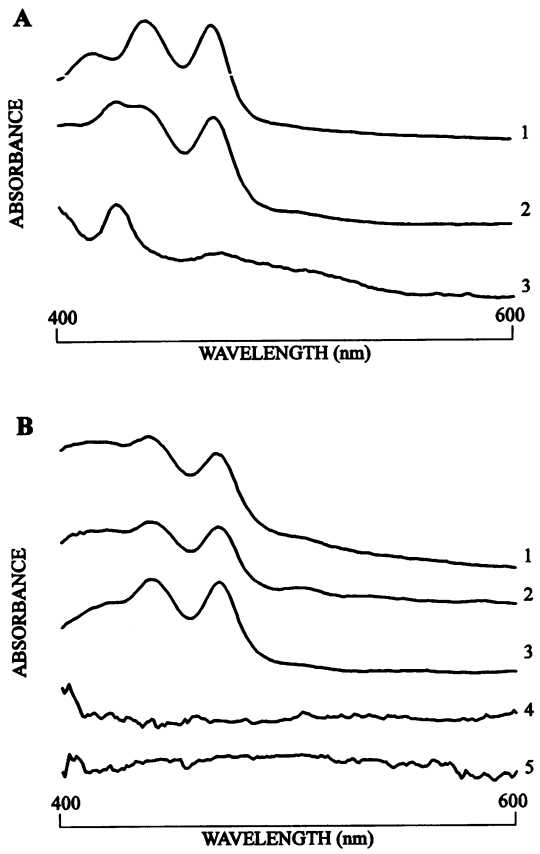

FIG. 6. Analysis of CrtI activity and cofactor requirement in *E. coli* by absorption spectroscopy. Activity of the CrtI enzyme was assessed by the production of neurosporene in cell extracts of BL21(DE3)(pHLTPD1). The phytoene substrate was provided by *R. sphaeroides* DD13/W1, and the reaction mixture was supplemented with ATP, NADP, $MnCl_2$, and $MgCl_2$. Carotenoids were extracted, and room temperature absorption spectra were recorded. (A) Production of neurosporene by CrtI. Spectra: 1, TC40 (a neurosporene accumulator); 2, BL21(DE3)(pHLTPD1) plus DD13/W1; 3, DD13/W1. (B) ATP dependence and cofactor specificity. In spectra 1 and 4, NADP is replaced by NAD and FAD, respectively. In spectrum 3, NADP is omitted. In spectrum 5, ATP is omitted and NADP is included.

temperature (77 K) absorption spectra were recorded for each strain (Fig. 7). Colored carotenoids absorb in the 450- to 570-nm region; a comparison with the wild-type spectrum shows that the production of colored carotenoids has been restored in the complemented *crtI* mutants but not in the *crtB* mutants. This was confirmed by HPLC analysis of transconjugant strains (Table 2), which showed that the complemented *crtI* transconjugants were now accumulating spheroidene and spheroidenone, as in the wild type, while the *crtB* transconjugants were still blocked, although they did contain traces of phytoene. The low-temperature absorption spectra also indicated that assembly of the light-harvesting 2 (LH2) complex was restored in the complemented *crtI* mutants, although the LH2/LH1 ratio had not been restored to wild-type levels, as demonstrated by the relative heights of the absorbance peaks at 800, 850, and 875 nm. Nevertheless, these levels of LH2 produce attendant effects on membrane morphology, as demonstrated recently (42); thus, the tubular membranes seen in *crtI* strains adopt the spherical morphology characteristic of the wild type.

TABLE 2. Complementation analysis of *crtI* and *crtB* transposon insertion mutants

Recipient strain	Complemented by pHLKPD1	Carotenoids produced[a]
TC44 (*crtB* mutant)	No	Trace of phytoene
TC47 (*crtB* mutant)	No	Trace of phytoene
TC67 (*crtI* mutant)	Yes	36% spheroidene, 64% phytoene
TC70 (*crtB* mutant)	No	Trace of phytoene
TC71 (*crtI* mutant)	Yes	53% spheroidenone, 17% spheroidene, 30% phytoene
TC72 (*crtI* mutant)	Yes	56% spheroidenone, 17% spheroidene, 27% phytoene

[a] Determined by HPLC analysis.

DISCUSSION

In this work, we have sequenced a 3.7-kb region of the *R. sphaeroides* genome which includes two complete ORFs. The first ORF is 1.6 kb long and encodes a protein with a deduced M_r of 57,244. Subsequent cloning and overexpression in *E. coli* have shown this to be the *crtI* gene encoding the *R. sphaeroides* phytoene desaturase enzyme. Analysis of the secondary structure of the deduced amino acid sequence, using the Klein algorithm and a hydropathy plot (41) (data not shown), suggests that this is a slightly hydrophobic protein with at least one membrane-spanning region and three other possible integral membrane regions (Fig. 1B). The transcription initiation point for *crtI* has been identified at bp −64 (Fig. 2), but the transcription termination point remains to be determined. No factor-independent transcription terminators could be found downstream of the 3′ end of *crtI* (Fig. 1B); however, Northern blot analysis of the *crtI* transcript (Fig. 4) reveals a band at approximately 1.6 kb. This finding implies that either transcription of *crtI* terminates close to the 3′ end of the gene or a longer transcript is synthesized and then subjected to posttranscriptional modification. The smeared signal below the transcript suggests that this mRNA undergoes rapid degradation.

The second ORF is 1.1 kb long and encodes a protein with a deduced M_r of 39,000. On the basis of phenotypic analysis (42a) and sequence alignments with other *crtB* genes, we propose that this is the *R. sphaeroides crtB* gene encoding the phytoene synthase enzyme. Analysis of the secondary structure of the deduced amino acid sequence suggests that this is a largely hydrophilic protein with one short highly conserved integral membrane region (Fig. 1B). Bartley et al. (7) showed that the phytoene synthase enzyme in tomatoes is membrane bound but easily solubilized by alkali treatment, and they suggest that it is a peripheral membrane protein. It is thus possible that the hydrophobic region identified in CrtB of *R. sphaeroides* represents the membrane attachment point for this enzyme. Two possible transcription terminators have been identified downstream of the 3′ end of *crtB* (Fig. 1B), but neither of these is a very strong terminator according to the specifications of Brendel and Trifanov (9), so it is possible that the transcript extends further downstream to include the next ORF, giving a *crtIBK* operon as postulated by Armstrong et al. (2) for *R. capsulatus*. An alignment of the deduced amino acid sequences of plant and bacterial phytoene desaturase and synthase enzymes showed the *R. sphaeroides* phytoene desaturase to be a CrtI-type enzyme (49). A lower level of conservation was found among phytoene synthase enzymes than desaturase enzymes. Also, there was a higher degree of sequence conservation among the plant and cyanobacterial Pds-type enzymes than among the bacterial and fungi CrtI-type enzymes so far studied. This finding is consistent with the established evolutionary trends.

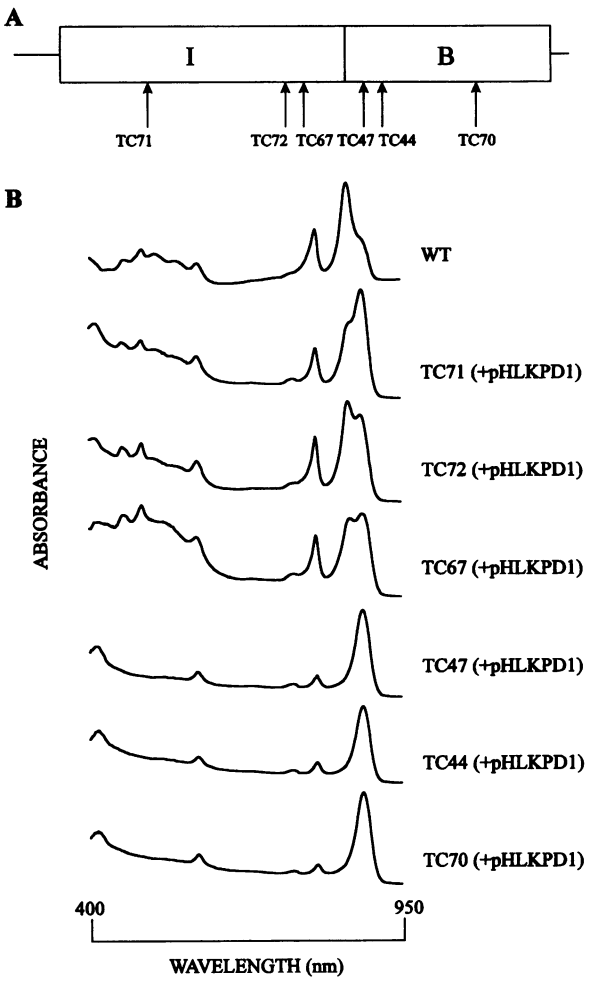

FIG. 7. Complementation of *crtI* and *crtB* transposon insertion mutants. (A) Diagram of the *crtIB* region showing the position of the transposon insertions. (B) Low-temperature (77 K) absorption spectra of photosynthetic membranes isolated from wild-type and transconjugant strains.

Overexpression of *crtI* and subsequent assay of CrtI enzyme activity in *E. coli* have established that the cloned gene does indeed encode the *R. sphaeroides* phytoene desaturase enzyme, which catalyzes the desaturation of phytoene, via three desaturation steps, to yield neurosporene. In *E. coli*, this activity seems to be associated largely with the membrane fraction, and the enzyme does not seem to function efficiently in the absence of membranes, despite the abundance of the CrtI protein in the cytoplasm of *E. coli* cells (data not shown). This is not unexpected, as in vivo, phytoene desaturase is membrane bound or membrane associated (53) and has only recently been purified in an active state (25, 26). Gold labelling studies suggest that phytoene desaturase might be exclusively localized in the photosynthetic membranes of prokaryotic organisms (57) and within the thylakoid membranes of higher plant chloroplasts (44). The presence of CrtI in the cytoplasm of *E. coli* may be due to the fact that there is so much of it in the cell that it cannot all become membrane associated.

In investigating the cofactor requirement of this enzyme, we have demonstrated the inhibitory effect of FAD, but it was not possible to show an absolute requirement for NAD(P) in the crude extracts (Fig. 6B). Numerous sequence alignments have demonstrated that carotenoid dehydrogenases have a highly conserved binding domain for FAD/NAD(P) (4), although it is interesting that the consensus FAD/NAD(P) binding motif proposed by Armstrong et al. (4) is correct only for CrtI-type enzymes and does not hold for Pds-type enzymes (data not shown). Although the NAD(P) binding site of *crtI* in *R. sphaeroides* appears to confer some specificity for NAD rather than NADP (65), it is not possible to determine the precise cofactor requirement on the basis of these motifs; indeed, Pecker et al. (49) suggest that these enzymes have a composite NAD(P) binding motif, and it would appear that the cofactor requirement of the phytoene desaturase enzyme varies depending on the organism (15, 26, 40, 54). The use of a qualitative spectroscopic assay for in vitro carotenogenic activity is unique and wholly reproducible. Valuable information on the activity of cloned genes can be obtained from such a coupled assay. However, it is intended simply to demonstrate the presence or absence of carotenogenic activity and is not intended for use in quantitative assays of enzyme activity.

The *puc* and *puf* operons, which encode polypeptides of the photosynthetic apparatus in *R. sphaeroides* and *R. capsulatus*, have been shown to be highly regulated by oxygen and light and are repressed under high-oxygen conditions (16, 31, 35). The alignment of the sequences of the putative promoters for *crtI* and *crtB* with the promoter sequences from the *R. sphaeroides puf* operon (31) and *puc* operon (43) shows a significant degree of homology between these sequences (Fig. 3). The positions of the putative *crtI* and *puf* promoter sequences are also virtually identical with respect to the proposed transcriptional starts for these operons (31), and it is therefore possible that these genes are subject to similar modes of regulation. It should be pointed out that these sequences cannot strictly be identified as promoters, i.e., the binding site for RNA polymerase, at the moment. However, they could influence transcription by binding *trans*-acting proteins. Cohen-Bazire et al. (16) showed that either (i) removal of oxygen from the growth medium accompanied by a shift from dark to light growth or (ii) a downshift in light intensity during constant anaerobic growth stimulates carotenoid accumulation in *R. sphaeroides*. However, a recent study by Armstrong et al. (3) showed that when *R. capsulatus* cells were shifted from dark aerobic respiratory growth to anaerobic photosynthetic conditions, the levels of *crtI* and *crtB* mRNAs remained constant while the levels of *crtA*, *-C*, *-D*, *-E*, *-F*, and *-K* increased 2- to 12-fold and the level of *puc* mRNA increased 25-fold, implying that *crtI* and *crtB* are not regulated by light and oxygen in *R. capsulatus*. Northern blot analysis (Fig. 4) shows that this is clearly not the case with the *R. sphaeroides crtI* gene. In *R. sphaeroides*, the levels of *crtI* mRNA are reduced under aerobic conditions, indicating an opposing form of regulation to that found in *R. capsulatus*.

It has been proposed by Coomber et al. (17) that *crtI* and *crtB* form an operon in *R. sphaeroides*, though two of the *crtIB* mutants used in that study were wrongly identified (Table 1) and the situation is far from clear. Chamovitz et al. (12) propose that there is transcriptional readthrough from *crtI* to *crtB* in *Synechocystis* sp. as a result of the lack of promoters between the two genes. Armstrong et al. (5) also looked at the possibility of a *crtIB* operon in *R. capsulatus*, but their results were inconclusive.

If *crtI* and *crtB* do form a tightly coupled transcriptional unit, such that mutations in *crtI* prevent transcription of *crtB*, it would not be possible to complement *crtI* mutants with pHLKPD1. pHLKPD1 is a nonintegrative plasmid, so the pathway would still be blocked at *crtB* on the genome and no phytoene would be synthesized. We have demonstrated complementation of *crtI* mutants with pHLKPD1 (Fig. 7), which would appear to suggest that *crtI* and *crtB* do not form such a unit. However, this is incomplete complementation, as seen when the levels of LH2 in the transconjugant strains are compared with the wild-type levels (Fig. 7). HPLC analysis of *crtI* mutants of *R. sphaeroides* shows that they accumulate only very small amounts of phytoene (42a), indicating that although there is a polar effect of *crtI* mutations on expression of *crtB*, it is not absolute. The lack of transcriptional terminators between *crtI* and *crtB* also implies transcriptional readthrough from *crtI*. There are two likely explanations for the incomplete complementation; one is that *crtI* and *crtB* do form a transcriptional unit, but that *crtB* can also be transcribed separately and at low levels from an internal promoter located in the 3′ end of *crtI*. A 20-bp region of dyad symmetry has been located in the 3′ end of *crtI*, from bp 1441 to 1461, which shows good homology to the region of dyad symmetry in the *puf* promoter (37) (Fig. 1B), and we propose that this is a putative promoter for *crtB*. The region of homology does not extend as far as with the putative *crtI* promoter sequences, so this may be a weaker promoter. The other explanation is that in the *crtI* Tn*5* insertion mutants, some transcription through *crtB* is initiated from the insertion sequence elements within Tn*5*. These explanations are not mutually exclusive, and either possibility or a combination of the two would further explain the abnormal phenotypes of the complemented *crtI* strains. If *crtB* is being transcribed only at low levels in such strains, this would limit normal carotenoid production despite the overexpression of *crtI*; the effect would be to reduce the level of assembly of the B800-850 LH2 complex, as observed in the low-temperature absorption spectra (Fig. 7). There may also be a small increase in the effect of the Tn*5* insertion sequence elements the nearer they are to the *crtB* gene, which would account for the minor differences in the LH2/LH1 ratios of the complemented strains.

The existence of transcription initiation from the Tn*5* insertion sequence elements does not diminish the case for a *crtIB* operon, since if there was a completely independent promoter site located between the point of Tn*5* insertion and the start of *crtB*, normal levels of phytoene would result. The fact that this does not occur is borne out by the low levels of phytoene detected in these mutants (42a) as well as the restricted carotenoid flux in complemented strains which lowers the levels of LH2. In further support of this last point, we have already demonstrated that LH2 assembly is dependent on carotenoid availability (42).

ACKNOWLEDGMENT

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