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Genes Involved in the Biosynthesis of Photosynthetic Pigments in the Purple Sulfur Photosynthetic Bacterium *Thiocapsa roseopersicina*

Ákos T. Kovács, Gábor Rákhely, and Kornél L. Kovács*

Institute of Biophysics, Biological Research Center, Hungarian Academy of Sciences, and Department of Biotechnology, University of Szeged, H-6726 Szeged, Hungary

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A pigment mutant strain of the purple sulfur photosynthetic bacterium *Thiocapsa roseopersicina* BBS was isolated by plasposon mutagenesis. Nineteen open reading frame, most of which are thought to be genes involved in the biosynthesis of carotenoids, bacteriochlorophyll, and the photosynthetic reaction center, were identified surrounding the plasposon in a 22-kb-long chromosomal locus. The general arrangement of the photosynthetic genes was similar to that in other purple photosynthetic bacteria; however, the locations of a few genes occurring in this region were unusual. Most of the gene products showed the highest similarity to the corresponding proteins in *Rubrivivax gelatinosus*. The plasposon was inserted into the *crtD* gene, likely inactivating *crtC* as well, and the carotenoid composition of the mutant strain corresponded to the aborted spirilloxanthin pathway. Homologous and heterologous complementation experiments indicated a conserved function of CrtC and CrtD in the purple photosynthetic bacteria. The *crtDC* and *crtE* genes were shown to be regulated by oxygen, and a role of CrtJ in aerobic repression was suggested.

The photosynthetic apparatus in many purple bacteria consists of three kinds of pigment-protein complexes: two types of the light-harvesting antenna complex (LHI and LHII) absorb light and transfer the energy to the third complex, the reaction center (17). The pigment components of these complexes are essential for efficient photosynthetic performance.

In bacterial photosynthesis, carotenoids absorb light energy, participate in the assembly of the light-harvesting antenna complex (13), and protect the cells from photodamage (3).

Numerous pathways have been described for the biosynthesis of more than 100 known carotenoids in photosynthetic anoxygenic bacteria (30). The operons coding for the enzymes of specific carotenoid pathways in photosynthetic bacteria have been studied mainly in nonsulfur bacteria belonging to the α and β subclasses of proteobacteria (1, 10, 14, 16). Little is known about the genes of carotenoid biosynthesis in purple sulfur γ proteobacteria.

Oxygen and light are the main environmental factors affecting the transcription and assembly of the photosynthetic apparatus in *Rhodobacter capsulatus* and in *Rhodobacter sphaeroides* (19). In *Rhodobacter sphaeroides* the *crt* operons are repressed under aerobic conditions by PpsR (CrtJ is a counterpart in *Rhodobacter capsulatus*) (7, 20, 23). *Thiocapsa roseopersicina* BBS is a purple sulfur photosynthetic γ proteobacterium belonging to the family *Chromatiaceae*. It can be cultivated under photosynthetic anaerobic conditions and requires reduced sulfur compounds for growth. It also grows chemolithoautotrophically under dark, aerobic conditions. The members of the family *Chromatiaceae* have either spirilloxanthin (normal, unusual spirilloxanthin, and carotenal) or okenone carotenoid biosynthetic pathways (30). Spirilloxanthin was reported to be the major carotenoid in *T. roseopersicina* 1711 (DSM 217) (29). Here we describe the isolation and genetic analysis of a *T. roseopersicina* mutant strain with altered carotenoid content and the characterization of a 22-kb locus containing genes involved in pigment biosynthesis. The regulation of carotenoid biosynthesis genes in this purple sulfur bacterium is also discussed.

* Corresponding author. Mailing address: Department of Biotechnology, University of Szeged, Temesvári krt. 62, H-6726 Szeged, Hungary. Phone: 36 62 544 351. Fax: 36 62 544 352. E-mail: kornel@nucleus.szbk.u-szeged.hu.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used are listed in Table 1. *T. roseopersicina* strains were maintained in Pfennig's mineral medium (containing, per 1,000 ml, 20 g of NaCl, 1 g of KH_2PO_4, 1 g of $MgCl_2$, 1 g of KCl, 1 g of NH_4Cl, 2 g of $Na_2S_2O_3$, 200 μl of vitamin B_{12} [100 μg/ml]), 1 ml of Fe-EDTA [3.3 g/liter], and 1 ml of microelement solution [2,975 mg of Na$_2$-EDTA, 300 mg of H_3BO_4, 200 mg of $CaCl_2 \cdot 6H_2O$, 100 mg of $ZnSO_4 \cdot 7H_2O$, 30 mg of $MnCl_2 \cdot 4H_2O$, 30 mg of $Na_2MoO_4 \cdot 2H_2O$, 20 mg of $NiCl_2 \cdot 6H_2O$, and 10 mg of $CuCl_2 \cdot 2H_2O$ in 1,000 ml of H_2O]); they were grown photoautotrophically and anaerobically in liquid cultures for 3 to 4 days (22). Plates were solidified with 7 g of Phytagel (Sigma) per liter, which was supplemented with acetate (2 g/liter) when selecting for transconjugants, and incubated for 2 weeks in anaerobic jars by using the GasPak (BBL) or AnaeroCult (Merck) systems. Cultures were illuminated with continuous light at 27 to 30°C (5). In the presence of oxygen the culture was supplemented with 5 g of D-glucose per liter and cultivated in dark under air. *Escherichia coli* strains were maintained on Luria-Bertani medium (27). Antibiotics were used at the following concentrations (micrograms per milliliter): for *E. coli*, streptomycin (50), ampicillin (100), kanamycin (50), and tetracycline (20); for *T. roseopersicina*, kanamycin (20) and streptomycin (5).

Plasposon mutagenesis. Plasmid pTn*Mod*-OKm (4) was introduced into a *T. roseopersicina* BBS recipient by conjugation (5), and mutants were selected for kanamycin resistance. From the kanamycin-resistant colonies, which were expected to contain the plasposon, a pigment mutant pale colony was chosen for further work.

Molecular biology techniques. Standard recombinant DNA techniques were carried out as described previously (27) or according to the specifications of the manufacturers.

Isolation and analysis of the locus surrounding the plasposon. DNA fragments were isolated from genomic DNA digested with *Bam*HI, *Kpn*I, and *Xba*I enzymes, self-ligated, and transformed into XL1-Blue MRF′ competent cells. A 21.7-kb region was subcloned and sequenced on both strands by primer walking with an automated Applied Biosystems 373 Stretch DNA sequencer.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Reference or source
T. roseopersicina strains		
BBS	Wild type	2
RM26	BBS *crtD*::Km	This work
E. coli strains		
XL1-Blue MRF′	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endAL supE44 thi-1 recA1 gyrA96*, *relA1 lac* [F′ *proAB lacI*^{q}*Z*Δ*M15* Tn10 (Tet^r)]	Stratagene
S17-1(λpir)	294 (*recA pro res mod*) Tp^r Sm^r (pRP4-2-Tc::Mu-Km::Tn7) λ*pir*	8
BL21(DE3)	*E. coli* B F$^-$ *dcm ompT hsd*S(r_B^- m_B^-) *gal* λ(DE3)	Novagen
Plasmids		
pTn*Mod*-OKm	Km^r; Tn5-based plasposon delivery plasmid with Km^r	4
pGEM T-Easy	Amp^r; cloning vector	Promega
pBluescript SK(+)	Amp^r; cloning vector	Stratagene
pBBRexSm2	Sm^r; broad host range vector	B. Fodor unpublished
pRM261	3.5-kb *Bam*HI fragment harboring the plasposon from RM26	This work
pRM265	4.9-kb *Kpn*I fragment harboring the plasposon from RM26	This work
pRM268	18.8-kb *Xba*I fragment harboring the plasposon from RM26	This work
pSOX	pBluescript KS(+); carries 1.2-kb *Sac*I fragment of *crtD* from *Rubrivivax gelatinosus*	18
pSO24	pBluescript KS(+); carries 1.8-kb *Sac*I fragment of *crtD-crtC* from *Rubrivivax gelatinosus*	18
pRcrt3	pBluescript SK(+); carries the *Apa*I-*Sac*I fragment of promoterless *crtD-crtC* from *Rubrivivax gelatinosus*	This work
pRcrt4	Derivative of pRcrt3; contains the promoter of *crtD* from *T. roseopersicina*	This work
pRcrt5	pBBRexSm2 containing *Bam*HI-*Kpn*I fragment of pRcrt4	This work
pTcrt3	pBluescript SK(+); carries the wild-type *Bam*HI-*Sac*I fragment of the *crtDC* operon of *T. roseopersicina*	This work
pTcrt4	pBBRexSm2 containing *Bam*HI-*Sac*I fragment of pTcrt4	This work
pPHU235	Broad-host-range *lacZ* fusion vector	9
pK18*mobsacB*	Km^r; *sacB*; RP4 *oriT*; ColE1 *ori*	28
pKlac2	*Eco*RI-*Sal*I fragment from pPHU235 in pK18*mobsacB*	This work
pCrtlac4	pKlac2 with 1,067-bp *Pst*I-*Xho*I fragment from pRM265	This work
pCrtlac9	pKlac2 with 1,067-bp *Pst*I-*Xho*I fragment from pRM265	This work
pET28::CrtJ	pET28 overexpression plasmid with *crtJ* gene	23
pCRTI	pGEM T-Easy; contains 444-bp fragment of *crtI*	This work
pPPSR	pGEM T-Easy; contains 929-bp fragment of *ppsR*	This work

Identification of the *crtI* and *ppsR* genes. Multiple alignment of the known CrtI and PpsR proteins was performed, and conserved domains were chosen for designing PCR primers corresponding to the selected amino acid sequences as follows: MGLFVWY (amino acids [aa] 312 to 318) and AWFRPHN (aa 457 to 464) in the *Rhodobacter capsulatus* CrtI protein and ETRYRVL (aa 154 to 160) and LYVKLRR (aa 454 to 460) in the *Rhodobacter capsulatus* CrtJ (PpsR) enzyme. The presence of *crtI* and *ppsR* in the genome of *T. roseopersicina* was demonstrated by using PCR with the following primers: for *crtI*, crtio1 (5′ATG GGIYTITTYGTSTGGTA3′) and crtio2 (5′TTRTGSGGIGCRAACCASGC 3′); for *ppsR* (*crtJ*), ppso1 (5′GAIACICGITAYCGNGTSCT3′) and ppso2 (5′C GICGIAGYTT SACRTASAG3′) (where S is C or G, R is A or G, and Y is C or T). The PCR products (444 bp for *crtI* and 929 bp for *ppsR* [*crtJ*]) were cloned into pGEM T-Easy vector and sequenced.

Bioinformatics tools. Comparisons of DNA and protein sequences with the various databases were done with the FASTA and BLAST (N, P, and X) programs (www.ncbi.nih.nlm.gov). Multiple alignments were performed with the CLUSTALX program.

Constructions for complementation. The plasmid for the homologous complementation of *crtDC* mutant strains was generated as follows. The 4.9-kbp *Bam*HI-*Sac*I fragment from the pRM261 clone (Table 1) containing the *crtDC* genes was cloned into the pBluescript SK(+) *Bam*HI-*Sac*I sites (pTcrt2). This region contained the plasposon inserted into the *crtD* gene (at nucleotide [nt] 16812 on the whole sequence). To restore the genomic sequence, a 526-bp region was amplified from the wild-type genome by using the following primers upstream and downstream from the plasposon insertion site: caro4 (5′GGACCG ACGG TCTTCACGAT 3′; nt 17300 to 17325, reverse), and caro5 (5′GTCTG ATGCA TGCCGCCTTC 3′; nt 16799 to 16818, forward). The PCR fragment was cloned and sequenced, and the 439-bp *Xho*I-*Sph*I fragment of this clone replaced the corresponding region of the pTcrt2 construct, restoring the wild-type sequence (pTcrt3). The pBBRexSm2 vector was generated by cloning the polished 2,019-bp *Hin*dIII fragment of the pHP45Ω (24) vector harboring the streptomycin resistance cassette into the blunted *Sph*I-*Eco*RV site of pBBR1ex vector. The pBBR1ex construct contained the *Eco*RV-*Sph*I fragment of pET15b (Novagen) in pBBR1-MCS5 *Pvu*I (polished)-*Sph*I sites (12). The relevant features of pBBRexSm2, which will be a component of a vector set, are that it is a small, broad-host-range, mobilizable vector conferring streptomycin resistance to the host cells (B. Fodor et al., personal communication). The pTcrt4 construct was produced by cloning the 2.9-kbp *Bam*HI-*Sac*I fragment of pTcrt3 into *Bgl*II-*Ssp*I-digested pBBRexSm2. The plasmid for heterologous complementation of *crtDC* mutant strains, a 2,850-bp *Apa*I-*Sac*I fragment carrying the promoterless *crtDC* genes of *Rubrivivax gelatinosus*, was assembled from the *Sac*I fragment of the pSOX vector and the *Sac*I-*Apa*I fragment of the pSO24 plasmid (18) in pBluescript SK(+) (pRcrt3) (Table 1). The 116-bp *Bam*HI-*Hae*III fragment of pRM261, containing the *crtDC* promoter from *T. roseopersicina*, was cloned into the *Bam*HI-*Eco*RV sites of the pRcrt3 vector (pRcrt4). The whole operon was transferred into the pBBRexSm2 *Bgl*II-*Ssp*I sites (pRcrt5) as a *Bam*HI-*Kpn*I fragment after polishing of the noncompatible ends.

Construction of the *crtD*::*lacZ* and *crtE*::*lacZ* fusion strains. The promoterless and slightly truncated *lacZ* gene coding for active enzyme was cloned from pPHU235 as an *Eco*RI-*Sal*I fragment (9) into the *Eco*RI-*Sal*I sites of the mobilizable suicide vector pK18*mobsacB* (pK18lac2). The blunted 1,071-bp *Pst*I-*Xho*I fragment from pRM265 (containing a 247-bp region of the *crtD* gene, a 703-bp section of *crtE* gene, and the intergenic region of these genes) was inserted into the unique *Sca*I site of pK18lac2. Two plasmids containing the insert in different orientations were chosen: in one orientation (pCrtlac4), the *crtD* promoter drove the expression of the *crtD*::*lacZ* fusion gene, while in the other (pCrtlac9), the *crtE* promoter was active in producing the *crtE*::*lacZ* fused transcript. These plasmids were conjugated into *T. roseopersicina* BBS. The site of recombination was verified by PCR on genomic DNA with primers specific for the vector (reverse primer) and the *crt* genes (for the *crtD* fusion, caro5 [see above]; for *crtE*::*lacZ*, caro17 [5′TGCGAACCGACGCGACCTAA3′]). In both cases, frag-

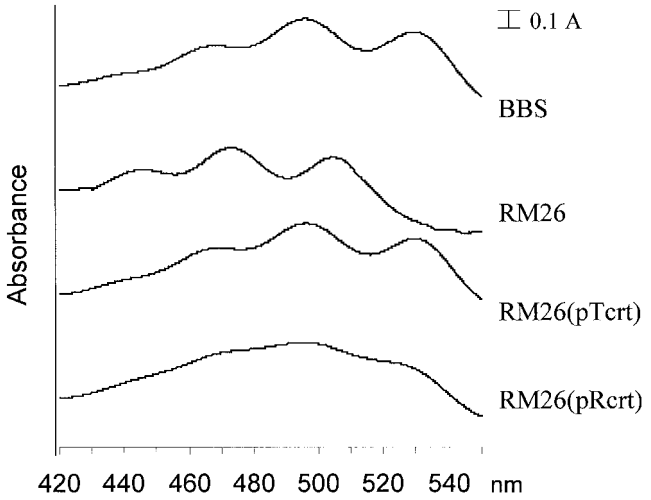

FIG. 1. Absorption spectra of carotenoid extracts from wild-type *T. roseopersicina*, a *crtD* mutant, and complemented strains. BBS, wild type; RM26, *crtD* mutant; RM26(pTcrt) and RM26(pRcrt), RM26 complemented with *crtDC* of *T. roseopersicina* and *Rubrivivax gelatinosus*, respectively. The zero lines of the spectrums were shifted for better viewing. For details, see text.

ments of the expected size were obtained, i.e., 1,282 bp for the *crtD*::*lacZ* fusion and 1,505 bp for the *crtE*::*lacZ* fusion.

Spectrophotometric analysis of the pigments. Carotenoids were extracted from the cells (and from the dots in the thin-layer chromatography [TLC] plates) with acetone-methanol (7:2, vol/vol) as described previously (18). Spectral analysis was carried out with a UV2 Unicam spectrophotometer interfaced with a computer.

β-Galactosidase assay. The β-galactosidase activities of the toluene-permeabilized cell extracts were assayed as described earlier (15). One Miller unit corresponded to 1 mmol of *o*-nitrophenyl-β-galactoside (Sigma-Aldrich) hydrolyzed per min, normalized to the optical density at 650 nm.

Overexpression and purification of CrtJ. Plasmid pET28::CrtJ (23) harboring the *Rhodobacter capsulatus crtJ* gene was transformed into *E. coli* strain BL21(DE3) (Novagen), and CrtJ was expressed and purified as described previously (23).

Gel mobility retardation assay. The 120-bp *Bam*HI-*Hin*dIII fragment from pRcrt4 (see above), containing the putative CrtJ recognition sequence elements, was isolated and labeled with α-^{35}S-dATP. The binding mixture contained 1 ng of radiolabeled DNA, 1 μg of poly(dI-dC), and various amounts of proteins in the binding buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 50 mM potassium acetate, 20% [vol/vol] glycerol). Each reaction mixture was then incubated for 30 min at 30°C and loaded onto a 6% nondenaturing polyacrylamide gel. The gel was electrophoresed at 70 V for 2 h, dried, and analyzed in a PhosphorImager (Molecular Dynamics).

Nucleotide sequence accession number. The 21,710-bp sequence determined in this study has been deposited in GenBank under accession number AF528191.

RESULTS

Isolation of pigment mutant strains of *T. roseopersicina*. Plasposon-mediated mutagenesis was used to isolate colonies with altered pigmentation. A pale mutant colony (RM26) was chosen from a library of 4,000 kanamycin-resistant colonies. Spectral analysis of the extracted pigments showed that the absorbance peaks at 468, 496, and 528 nm (Fig. 1, BBS) disappeared and that new maxima appeared at 445, 472, and 504 nm (Fig. 1, RM26). These values coincided well with the published data for spirilloxanthin (465, 495, and 528 nm) and lycopene (442, 470, and 500 nm), respectively (18) (the actual values depend slightly on the solvent used). Separation of the extracted carotenoids by TLC on a silica gel revealed a single spot for the wild type and four to five spots with distinct mobilities for the mutant (data not shown). Each spot was cut out from the TLC plate, and their UV spectra were recorded after extraction. In each case, only peaks characteristic of lycopene could be observed (data not shown), indicating that these compounds were likely lycopene derivatives. Therefore, we concluded that wild-type *T. roseopersicina* synthesizes spirilloxanthin as the main carotenoid but that the carotenoid biosynthesis is aborted at lycopene in the RM26 mutant. The pathway previously described for spirilloxanthin biosynthesis is shown in Fig. 2 (11, 18, 30).

Isolation and sequence analysis of genes involved in pigment biosynthesis. Overlapping restriction fragments of the genome of the RM26 containing the plasposon were isolated and cloned, and an almost-22-kb DNA region was sequenced. The in silico analysis of this contig resulted in the identification of genes coding for putative enzymes participating in bacteriochlorophyll and carotenoid biosynthesis (Fig. 3). The annotated open reading frames (ORFs) are listed in Table 2. Nineteen ORFs were identified; nine were involved in bacteriochlorophyll (two of them, *bchB* and *bchX*, were partial), four were involved in carotenoid biosynthesis, and the remaining six coded for putative proteins of the photosynthetic reaction center or heme biosynthesis or their function was not clear.

The orientation of all ORFs was the same, with the exception of the *crtC* and *crtD* genes (Fig. 3). Several ORFs overlapped, and in few cases the genes were separated by gaps (Table 2.). Generally, the ORFs were preceded by more-or-less conserved ribosomal binding sites; four ORFs started with GTG, and one (*bchC*) probably started with TTG. Local arrangements of some photosynthetic genes, such as *bchBHLM* and *puhA-orf218-orf138*, were similar to that in *Rhodobacter* and *Rubrivivax* strains (Fig. 3). The arrangement of the *crtCDEF-bchCX* genes was the same in the case of *Rhodobacter* species but distinct from that in *Rubrivivax gelatinosus*, where the *crtCD* and *crtEF* genes are separated by many genes involved in the biosynthesis of bacteriochlorophyll and the photosynthetic center. Moreover, a few genes, like *hemN* and *orf543*/*orf495* ("extra genes" in Fig. 3) inserted into this region; their locations in the other three photosynthetic bacteria were completely different. Most of the gene products showed the highest similarity to the corresponding proteins of *Rubrivivax gelatinosus* (10) (Table 2). In addition to CrtC, CrtD, CrtE, and CrtF, which could be identified in this locus, the spirilloxanthin pathway needed two additional enzymes, CrtB and CrtI (Fig. 2) (11, 30), but the corresponding genes were not found on this fragment. Hence degenerate primers were produced on the basis of the conserved regions of CrtB and CrtI of *Rubrivivax* and *Rhodobacter* species (see Materials and Methods). In the case of *crtI* the expected 444-bp fragment could be amplified and the deduced sequence showed the highest similarity (74%) to that of *Rubrivivax gelatinosus* CrtI (10). For CrtB, this approach did not succeed (data not shown).

The plasposon was inserted into the middle of the *crtD* gene (at nt 16812) (Table 2), coding for the methoxyneurosporene dehydrogenase. Downstream from the plasposon insertion site, the *crtC* gene was found in the same direction as *crtD*, and the two genes had a 304-bp overlap, so they are likely cotranscribed. Moreover, the orientation of the kanamycin resistance

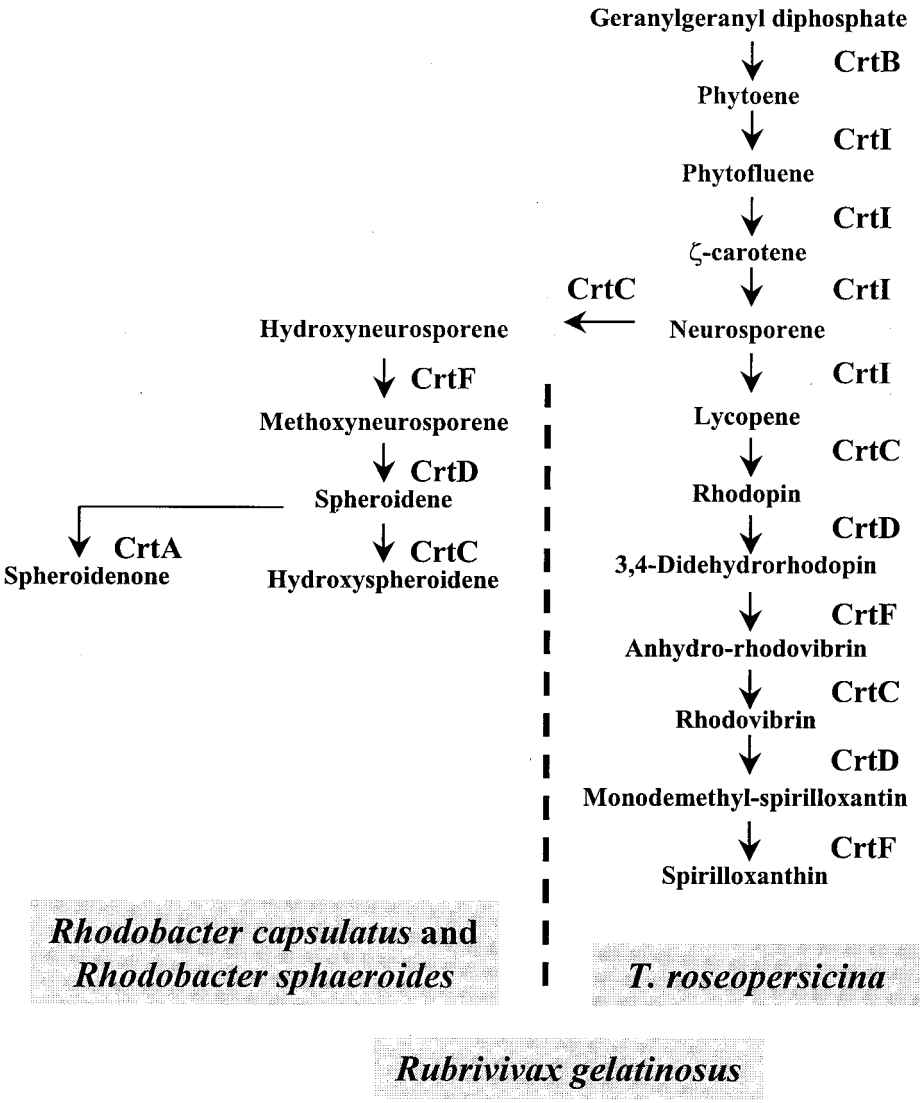

FIG. 2. Biosynthesis of speroidene and spirilloxanthin in photosynthetic bacteria. *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* produce spheroidene (1, 14). In *T. roseopersicina* only spirilloxanthin can be detected, while in *Rubrivivax gelatinosus* both spheroidene and spirilloxanthin are synthesized (18).

gene in the inserted plasposon is opposite to that in the *crtDC* genes, so the promoter of this gene cannot drive the expression of the *crtC* gene. Consequently, the mutation should have a polar effect as well, and we consider RM26 to be a *crtDC* mutant.

Complementation with *crtDC* genes. Homologous complementation of the mutated *crtDC* genes restored the wild-type carotenoid composition [Fig. 1, RM26(pTcrt) spectrum]. CrtC was shown to be involved in the synthesis of hydroxyneurosporene from neurosporene in the spheroidene branch of carotenoid biosynthesis in *Rhodobacter* species (1, 14) and in the synthesis of both spheroidene and spirilloxanthin in *Rubrivivax gelatinosus* (18) (Fig. 2). CrtI has to catalyze three and four consecutive steps in the spheroidene and spirilloxanthin pathways, respectively (three-step and four-step phytoene desaturase) (Fig. 2). The spheroidene and spirilloxanthin pathways have a common origin, and they branch after the synthesis of ξ-carotene (Fig. 2). The next step is catalyzed by CrtC in the spheroidene pathway and by CrtI in the spirilloxanthin pathway. Downstream from this branching point the same enzyme set is used in both pathways, except for an additional step catalyzed by CrtA in the spheroidene pathway. Thus, the special properties of CrtC and or CrtI, which may be distinct in various species, determine the actual pathway taking place in the cells. In the *crtIC* mutant strain of *Rhodobacter sphaeroides*, the native four-step phytoene desaturase (CrtI) in *trans* was able to produce significant amount (13%) of lycopene in a *crtC* background (6). Lycopene is an intermediate of the spirilloxanthin route, which is normally not present in *Rhodobacter sphaeroides*. This suggested that CrtC might have a key role in determining the selection of the various carotenoid biosynthetic pathways. The isolated *T. roseopersicina crtDC* mutant also contained lycopene and its derivatives (Fig. 1, RM26). We addressed the question of whether the CrtC enzyme of *Rubrivivax gelatinosus* (in which both the spirilloxanthin and spheroidene pathways exist

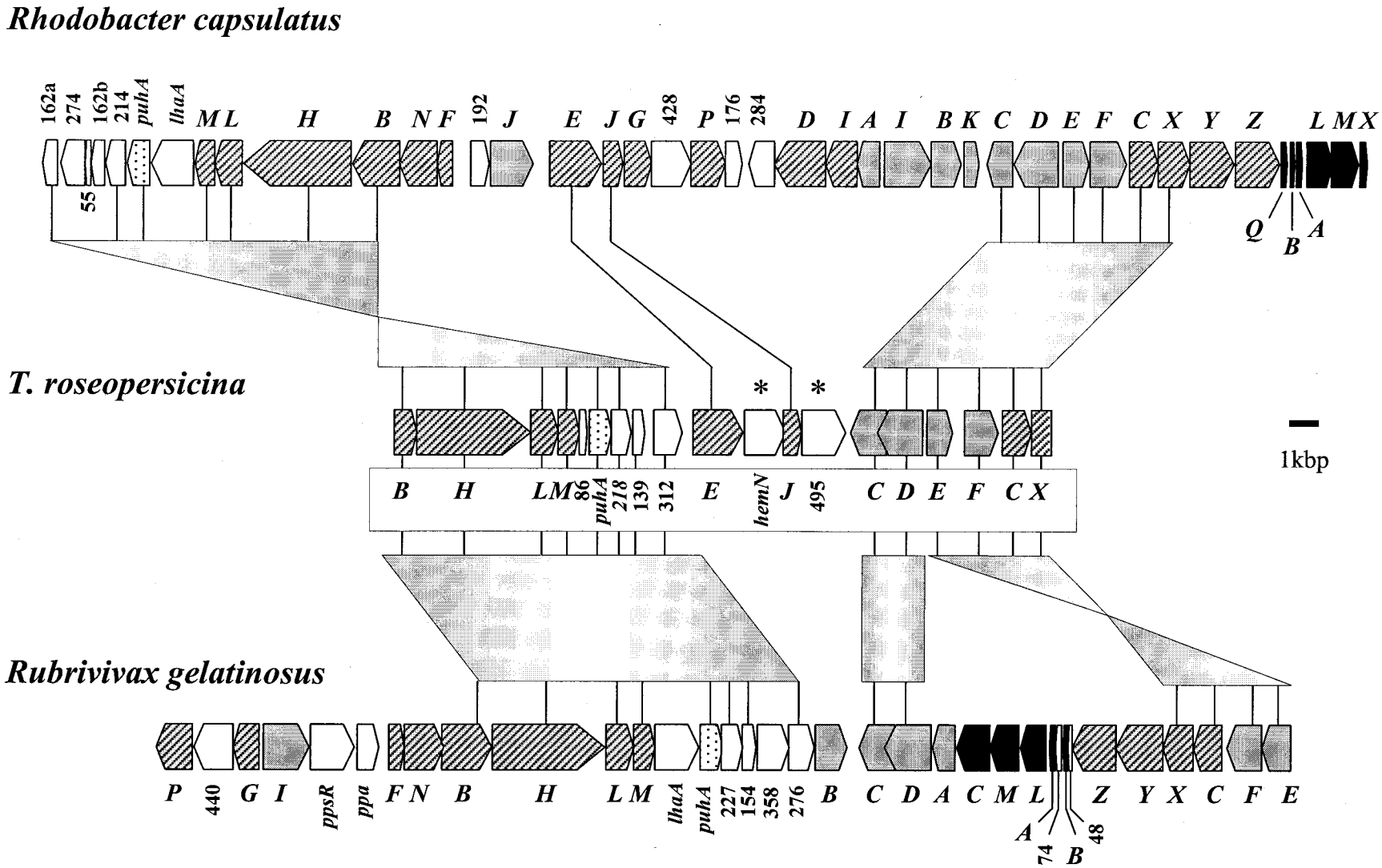

FIG. 3. The structure of the photosynthetic gene cluster of *T. roseopersicina* compared to those of *Rhodobacter capsulatus* (1) and *Rubrivivax gelatinosus* (10). Genes and ORFs are represented as arrows pointing in the direction of their transcription. Solid arrows indicate the *puf* genes, coding for the reaction center and light-harvesting center; stippled arrow symbolize the gene of the reaction center H subunit (*puhA*). The hatched and gray arrows stand for ORFs assigned to bacteriochlorophyll and carotenoid biosynthesis, respectively. Genes showing unusual locations in *T. roseopersicina* are labeled with asterisks. Open arrows indicate ORFs with unknown functions.

TABLE 2. Description of the ORFs identified

Gene[a]	Known or putative function of product	Length (aa)	nt (start-stop)	Top BLAST hits	
				Gene product	% Identity/E value
bchB	Light-independent prochlorophyllide reductase *b* subunit	258	1–777	*Rubrivivax gelatinusus* BchB	72/e-107
bchH	Mg-protoporphyrin IX chelatase H subunit	1,245	752–4489[b]	*Rubrivivax gelatinusus* BchH	66/0.0
bchL	Light-independent prochlorophyllide reductase iron-sulfur ATP binding subunit	294	4513–5397	*Rhodospirillum rubrum* BchL	66/e-105
				Rubrivivax gelatinusus BchL	64/e-105
bchM	Mg-protoporphyrin methyltranserase	233	5397–6098	*Rubrivivax gelatinusus* BchM	60/9e-76
orf86	Hypothetical protein	86	6095–6355		
puhA	Photosynthetic reaction center H subunit	255	6381–7148	*Thermochromatium tepidum* PuhA	72/e-108
				Rubrivivax gelatinusus PuhA	49/e-63
orf218(G)	Hypothetical membrane protein	218	7145–7801	*Rubrivivax gelatinusus* ORF227	46/e-43
orf139	Hypothetical protein	139	7853–8272	*Rubrivivax gelatinusus* ORF154	39/9e-18
orf312	Hypothetical membrane protein	312	8535–9473[c]	*Rubrivivax gelatinusus* ORF276	43/6e-58
bchE	Mg-protoporphyrin IX monomethylester oxidative cyclase subunit	551	9864–11519[c]	*Heliobacillus mobilis* BchE	70/0.0
hemN	O_2-independent coproporphyrinogen III oxidase	453	11533–12894	*Aquifex aeolicus* HemN	38/6e-77
				Rubrivavax gelatinusus HemN	40/2e-76
bchJ (G)	4-Vinyl reductase	208	12870–13496[b]	*Rhodobacter sphaeroides* BchJ	42/e-35
orf543	Long-chain fatty acid CoA ligase	543	13330–14961[b]	*Halobacterius sp.* strain Lfl1	38/3e-52
orf495 (G)	*o*-Succinyl-benzoic acid CoA ligase	495	13474–14961[b]	MenE *Listeria innocua*	34/4e-40
crtC ⇐	Hydroxyneurosporene dehydrogenase	405	16294–15077[b]	*Rubrivivax gelatinusus* CrtC	55/e-92
crtD ⇐	Methoxyneurosporene dehydrogenase	498	17487–15991	*Rubrivivax gelatinusus* CrtD	54/e-150
crtE	Geranylgeranyl pyrophosphate synthase	288	17607–18473[c]	*Rubrivivax gelatinusus* CrtE	55/6e-88
crtF (G)	Hydroxyneurosporene methyltransferase	371	18868–19982[c]	*Rubrivivax gelatinusus* CrtF	49/e-89
bchC (T)	2-α-Hydroxyethyl bacteriochlorophyllide oxidase	317	20092–21045	*Rubrivivax gelatinusus* BchC	61/e-106
bchX	Bacteriochlorophyllide reductase subunit	282	20871–21710	*Bradyrhizobium* sp. BchX	74/6e-79
		225	21042–21710	*Rubrivivax gelatinusus* BchX	73/2e-76

[a] G and T, GTG and TTG start codons, respectively. The arrows indicate the inverse orientation of the ORFs.
[b] Overlaps with preceding ORF by more than 10 bp.
[c] Gap longer than 50 bp.

[Fig. 2]) can supplement the carotenoid pathway with the spheroidene branch in purple sulfur bacteria, since *T. roseopersicina* is able to synthesize spirilloxanthin only. The *crtD* gene from *Rubrivivax gelatinosus* S1 (18) was fused to the promoter of *T. roseopersicina crtD* and introduced into the RM26 mutant. In this construct the *crtC* gene was located downstream from the *crtD* gene, and they were thought to be cotranscribed (18), so in our construct the expression of both the *crtD* and *crtC* genes of *Rubrivivax gelatinosus* was driven by the *T. roseopersicina crtD* promoter. The spectral and TLC analyses of the pigments indicated the synthesis of spirilloxanthin (and the lycopene derivatives as in the case of the RM26 mutant), but intermediates of the spheroidene lineage could not be detected [Fig. 1, RM26(pRcrt) spectrum]. Moreover, the complementation was not as effective as with the homologous *crtDC* genes, and the spectrum is broadened, which might be caused by accumulated intermediates appearing in the spirilloxanthin biosynthesis as a consequence of reduced activity of the heterologous enzymes (S. Takaichi, personal communication).

Regulation of the *crtD* and *crtE* genes. *T. roseopersicina* growing under oxygenic conditions has as pale a color as the RM26 mutant, suggesting that the carotenoid biosynthesis is

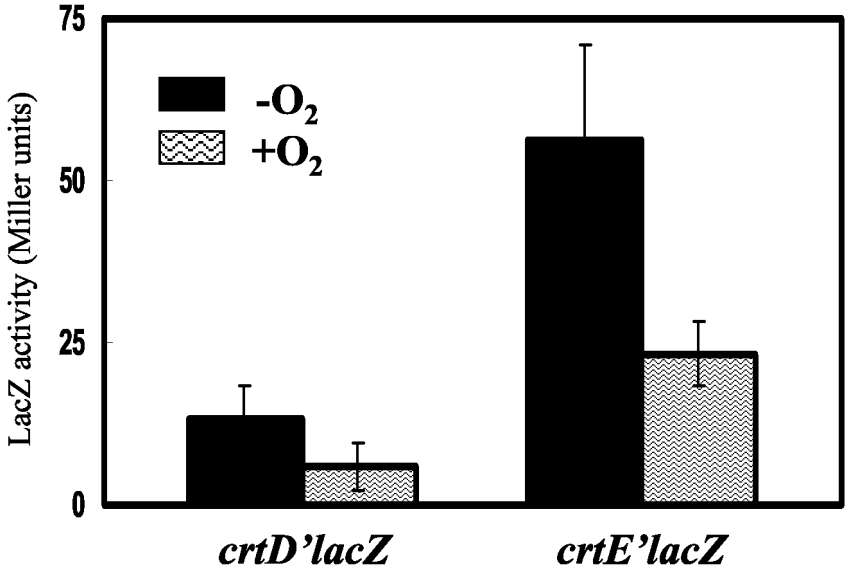

FIG. 4. Activity of LacZ expressed from the *crtD* and *crtE* promoters in *T. roseopersicina* BBS grown under aerobic and anaerobic conditions. For details, see Materials and Methods. Error bars indicate standard deviations.

repressed by molecular oxygen. To test this hypothesis, the regulation of the *crtD* and *crtE* genes was monitored with the aid of translational *lacZ* reporter gene fusions. The activity of LacZ produced from either the *crtD* or *crtE* promoter was measured in *T. roseopersicina* cells grown in the presence and absence of oxygen. The expression of both *crt* genes was repressed in the presence of oxygen (Fig. 4). The extent of the repression was the same in both cases (around 43%), but the promoter of the *crtE* gene seemed to be almost five times stronger. However, it could not be excluded that this effect derived from the fact that different sequences were fused to the *lacZ* gene (see Materials and Methods), resulting in dissimilar mRNA stabilities and consequently different LacZ activities (21). Since the *crtC* gene is believed to be cotranscribed with the *crtD* gene, this aerobic repression should regulate the expression of the *crtC* gene as well. The distance between the *crtE* and *crtF* genes is too large (395 bp) for such a conclusion to be made in this case.

The regions upstream from the *crtD* and *crtE* genes have sequences similar to consensus σ^{70} promoters, which are typical of photosynthetic operons (Fig. 5A) (10). In *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*, oxygen affected the expression of the *crt* and *bch* genes via a complex cascade to a repressor protein, named CrtJ in *Rhodobacter capsulatus* and PpsR in *Rhodobacter sphaeroides* (7, 20, 23). This factor (CrtJ) in *Rhodobacter capsulatus* recognized a palindrome TGT-N12-ACA sequence motif (23) which overlapped with the putative promoter. The consensus sequence could be found in two copies between the *crtD* and *crtE* genes of *T. roseopersicina* (Fig. 5A). In addition, a consensus sequence within the *bchH* gene was recognized, which might be irrelevant. To test whether these elements were really CrtJ (PpsR) binding motifs, CrtJ from *Rhodobacter capsulatus* (23) was overexpressed in *E. coli* and examined in a gel mobility retardation assay. The purified CrtJ protein bound strongly to the intergenic region of the *crtD* and *crtE* genes (Fig. 5B). The specificity of the interaction was confirmed by experiments in which specific and nonspecific cold competitors were added to the binding mixture. The disappearance of the band corresponding to the CrtJ-DNA complex required at least 1,000 times more nonspecific [poly(dI-dC)] molecules than specific competitor (data not shown), indicating the specific interaction of the labeled DNA probe and CrtJ. The presence of the repressor, PpsR, in *T. roseopersicina* was demonstrated by amplification and sequencing of an almost-1-kb region of the *ppsR* gene with degenerate primers, which were planned on the basis of the conserved regions of the known PpsR (CrtJ) proteins. The deduced amino acid sequence had 42% identity to the corresponding region of the PpsR protein in *Bradyrhizobium* sp. strain ORS278 (data not shown).

DISCUSSION

In accordance with the only literature source (29), spirilloxanthin was determined as the main carotenoid in the wild-type *T. roseopersicina* BBS. A mutant strain was produced, in which a plasposon was inserted into the *crtD* gene, disrupting the methoxyneurosporene dehydrogenase, and likely the expression of the CrtC was also affected. The carotenoid extract of this mutant revealed an absorption spectrum characteristic of lycopene (Fig. 1), but by TLC on a silica gel, four to five distinct bands could be separated (data not shown). It is possible that the functional CrtF in the RM26 mutant strain converted the lycopene to its nonnatural derivatives, since the expression of *crtF* was not influenced by the mutation. This is supported by the fact that the spectra of the isolated spots had absorption maxima characteristic of lycopene (data not shown). A similar situation has been described for *Rhodospirillum rubrum*, where a strain having a mutation in the rhodopin 3,4-desaturase gene was shown to contain not only rhodopin but its nonnatural derivatives produced by CrtF (11).

On a 22-kb locus surrounding the plasposon, 19 ORFs were

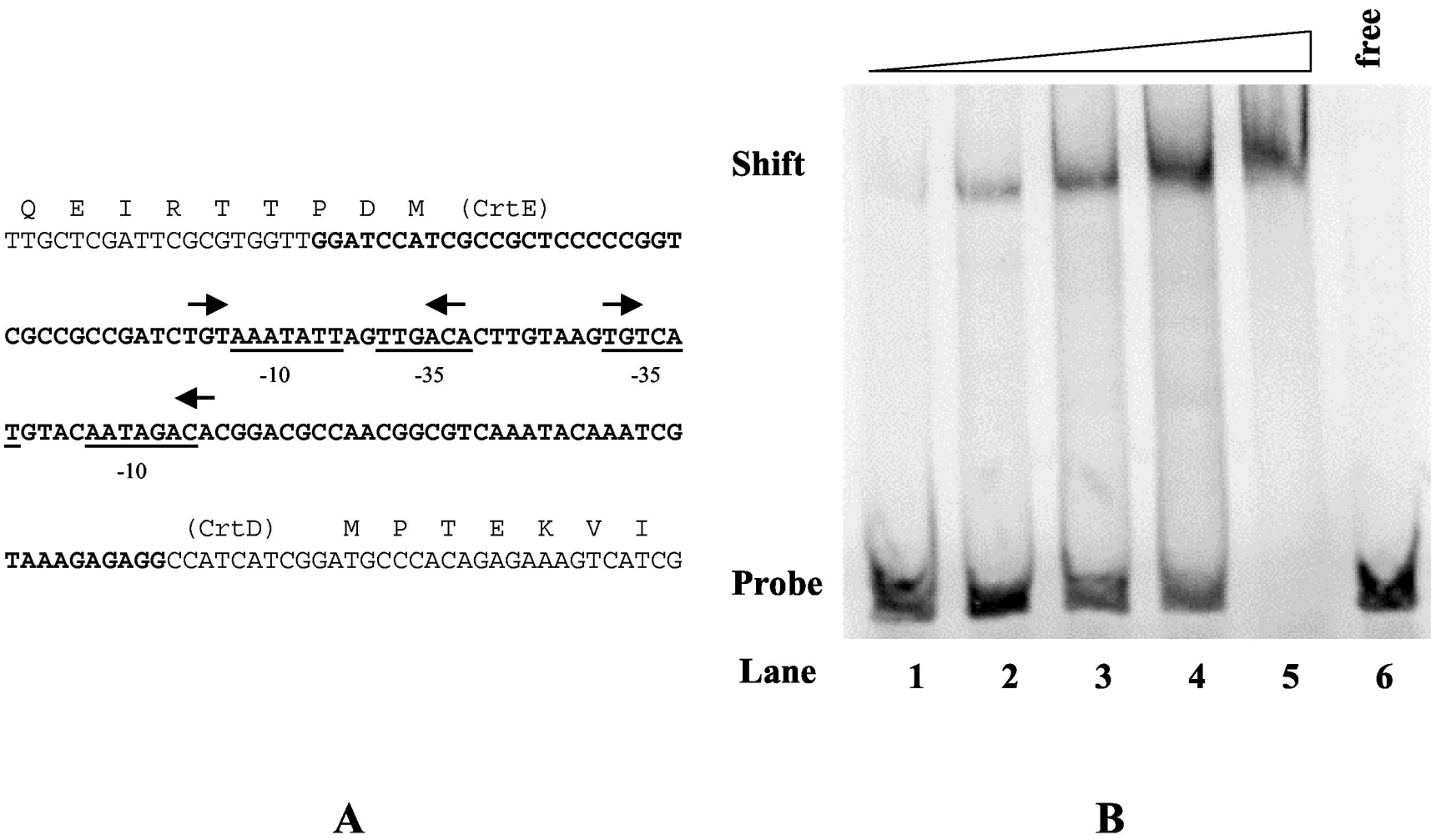

FIG. 5. Binding of CrtJ to the promoter region of the *crtD* and *crtE* genes. (A) The intergenic region between the divergent *crtE* and *crtD* genes, where the relevant region of this fragment is displayed. The putative −10 and −35 promoter regions are underlined. The arrows indicate putative CrtJ palindrome recognition sites. (B) Gel retardation assay with the recombinant CrtJ. A 120-bp *Bam*HI-*Hin*dIII fragment containing the *crtE-crtD* intergenic region (the region used is in boldface in panel A) was isolated and labeled with α-^{35}S-dATP (see Materials and Methods). This labeled fragment was incubated with various amounts of *Rhodobacter capsulatus* CrtJ protein overexpressed and purified in *E. coli* and loaded onto a 6% native polyacrylamide gel (see Materials and Methods). Lane 6, control lane containing only the free DNA probe. Lanes 1 to 5, the DNA probe was incubated with increasing amounts (0.05, 0.11, 0.23, 0.45, and 0.9 μg, respectively) of pure CrtJ.

identified (Fig. 3). Most of them code for putative proteins involved in bacteriochlorophyll and carotenoid biosynthesis. The majority of the putative gene products have higher identity to their counterparts in *Rubrivivax gelatinosus* than to those in *Rhodobacter capsulatus* or *Rhodobacter sphaeroides* (Table 2). This coincides with the relationship established from the 16S RNA analysis (16) and with the fact that *Rubrivivax gelatinosus* produces spirilloxanthin (18). However, the contig organization resembles that of the *Rhodobacter* species (10): the order of the *crtCD-crtEF-bchCX* genes is the same (Fig. 3). The arrangement of the pigment biosynthesis gene cluster has a few unusual features in *T. roseopersicina*. Most importantly, the *crtB* and *crtI* genes are missing from this region, although *crtI* is detected elsewhere in the genome. In *T. roseopersicina*, the scattering of functionally related genes appears to be a characteristic feature (26). Other differences between the species exist, such as the fact that *hemN*, involved in the heme biosynthesis, or the hypothetical coenzyme A (CoA) ligase gene (*menE* or *lfl*1) is located between *bchE*/*bchJ* and *bchJ*/*crtC*, respectively. These genes are localized outside the photosynthetic operon in *Rubrivivax gelatinosus* (10).

Introduction of the homologous *crtDC* genes into *T. roseopersicina* could restore the wild-type spirilloxanthin biosynthetic route in the mutant RM26. It has been reported that the CrtC and CrtD enzymes of *Rubrivivax gelatinosus* are involved in the biosynthesis of both spirilloxanthin and spheroidene (18). The two pathways branch after the synthesis of neurosporene: CrtC synthesizes hydroxyneurosporene, while CrtI produces lycopene (Fig. 2). Although these two genes are apparently present in *T. roseopersicina*, no carotenoid corresponding to the spheroidene pathway was detectable. The intriguing question that remained to be answered was what determines the branching selection of the carotenoid biosynthesis in bacteria having the enzymes for both pathways. In order to address this puzzle, heterologous complementation of the *crtDC* mutant *T. roseopersicina* strain with the *crtDC* genes of *Rubrivivax gelatinosus* was carried out. This apparently did not switch the carotenogenesis of *T. roseopersicina* toward the spheroidene pathway; absorption peaks corresponding to the spirilloxanthin pathway could be observed. TLC analysis of the pigment composition revealed only thee spots, which were attributed either to the wild type (spirilloxanthin) or to the RM26 mutant (lycopene and its derivatives) (data not shown). One possible explanation of the results is that in *T. roseopersicina* the CrtI protein, belonging to the four-step desaturases, may have very strong affinity to neurosporene and no free neurosporene remains in the cells for CrtC. Alternatively, it is also conceivable that in *T. roseopersicina* the spheroidene pathway is not functionally active.

The regulation of the *crtD* and *crtE* genes in *T. roseopersicina* was studied with promoter fusion constructs and gel mobility retardation assay. The expression of the operons is similarly affected by oxygen, which seems to be mediated by the repressor CrtJ (PpsR), which is known in purple nonsulfur bacteria (7, 20, 23). The consensus binding regions for CrtJ (PpsR) overlapping with the σ^{70} promoter-like sequences were identified upstream from the carotenoid biosynthesis genes *crtD* and *crtE* in both directions. Heterologously overexpressed CrtJ of *Rhodobacter capsulatus* binds to the *crtD-crtE* intergenic region of *T. roseopersicina*, suggesting an evolutionarily conserved mechanism for the anaerobic regulation. Remarkably, we could not detect any other consensus binding site of CrtJ in the 22-kb locus, although this was expected in the case of *bchC*, *hemN*, or *bchE* (19). Fnr is another redox regulator controlling the expression of the photosynthetic genes (19), but its consensus binding site was not found in this contig. The organization of the genes, gaps, overlapping regions, potential loops, and rare start codons might have a role in posttranscriptional events such as mRNA degradation (25) or translation, where the usage of rare start codons leads to reduced translational efficacy. These might result in altered expression levels of the various components, even with linked functions.

The similarity of the proposed genes and carotenogenesis and the regulation through PpsR (or CrtJ) of the *crtDC* and *crtE* genes suggest a strong relationship between the photosynthetic gene clusters of the purple sulfur bacteria from the γ subdivision and the purple nonsulfur bacteria from the β subdivision. The putative proteins of *T. roseopersicina* that are involved in pigment biosynthesis show higher identity to the corresponding enzymes of *Rubrivivax gelatinosus* than to those of the *Rhodobacter* species, although the arrangement of their genes suggests otherwise. One may thus speculate on the possibility of horizontal gene transfer from the *Rhodobacter* species to *Rubrivivax gelatinosus* through *T. roseopersicina* followed by genome rearrangements induced by environmental factors, such as oxidative stress (10, 17).

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