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# Pleiotropic Effects of *puf* Interposon Mutagenesis on Carotenoid Biosynthesis in *Rubrivivax gelatinosus*

## A NEW GENE ORGANIZATION IN PURPLE BACTERIA*

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***Rubrivivax gelatinosus*** **mutants affected in the carotenoid biosynthesis pathways were created by interposon mutagenesis within the *puf* operon. Genetic and biochemical analysis of several constructed mutants suggest that at least *crtC* is localized downstream of the *puf* operon and that it is cotranscribed with this operon. Sequence analysis confirmed the genetic data and showed the presence of *crtD* and *crtC* genes downstream of the *puf* operon, a localization different from that known for other purple bacteria. Inactivation of the *crtD* gene indicated that the two *crt* genes are cotranscribed and that they are involved not only in the hydroxyspheroidene biosynthesis pathway as in *Rhodobacter sphaeroides* and *R. capsulatus*, but also in the spirilloxanthin biosynthesis pathway. Carotenoid genes implicated in the spirilloxanthin biosynthesis pathway were thus identified for the first time. Furthermore, analysis of carotenoid synthesis in the mutants gave genetic evidence that *crtD* and *crtC* genes are cotranscribed with the *puf* operon using the oxygen-regulated *puf* promoter.**

The photosynthetic apparatus in many purple bacteria contains three types of pigment-protein complexes. The two light-harvesting LHII[1] and LHI antenna capture light and transfer energy to the third complex, the reaction center (RC) in which charge separation occurs. The three complexes contain pigments, bacteriochlorophyll and carotenoids, which play an important role in the photosynthetic process.

Carotenoids have three functions in bacterial photosynthesis. They act in photoprotection, they function as accessory light-harvesting pigments by absorbing light in the 450–570 nm region (1, 2), and, in addition, they participate in the assembly of the light-harvesting antenna (3, 4).

Biosynthetic pathways of carotenoids in some purple bacteria are now well known (5–7). Spectroscopic and chemical studies of the processes of carotenoid biosynthesis have been performed in different mutants, in particular in *R. sphaeroides* and *R. capsulatus* (8–11). In these bacteria the end products of the biosynthetic pathway are spheroidene and spheroidenone; in other bacteria such as *Rhodospirillum rubrum* and *Rubrivivax gelatinosus*, in addition to the spheroidene pathway, another pathway leading to biosynthesis of spirilloxanthin and derivatives was described (5) (Fig. 1).

The genes encoding many carotenoid biosynthetic enzymes have now been mapped (12). The arrangement of carotenoid genes was first described for *R. capsulatus* (13). As in *R. sphaeroides*, the carotenoid genes are clustered and flanked by bacteriochlorophyll genes within a 45-kb region of the chromosome (14, 15). In these bacteria, the first enzyme assigned to carotenoid biosynthesis is the geranylgeranyl-pyrophosphate synthase encoded by *crtE* gene (16, 17). The condensation of two geranylgeranyl pyrophosphate by an enzyme encoded by *crtB* lead to the synthesis of phytoene (16, 17), which is transformed to neurosporene by sequential desaturations which involved *crtI* (18, 19). In *R. sphaeroides* and *R. capsulatus*, a hydratase encoded by *crtC* catalyzes hydration of neurosporene to hydroxyneurosporene (8), which is then transformed to spheroidene by the products of *crtF* and *crtD* (8). The *crtA* and *crtC* products catalyze the transformation of spheroidene to spheroidenone and hydroxyspheroidene, respectively (8), and finally *crtA* product catalyzes oxygenation of hydroxyspheroidene to hydroxyspheroidenone (11). In *R. rubrum* and *R. gelatinosus*, neurosporene is also transformed to lycopene. A hydratase catalyzes the formation of rhodopin from lycopene, and subsequent reactions (desaturations and methylations) lead to the formation of spirilloxanthin, the end product of this second pathway. The genes involved at each step of this second pathway have not yet been identified.

The expression of pigment biosynthesis genes has been studied mainly in *R. sphaeroides* and *R. capsulatus.* Bacteriochlorophyll and carotenoid genes are proposed to be organized in transcription units (operons) (20). The existence of such an organization is supported by the results of RNA mapping (20) and interposon mutagenesis experiments (21). In particular, *crtEF*, *bchCXYZ*, and *puf* operons are organized in a "super-operon" (22–24). In these bacteria, the cotranscription of the three operons has been found to be phenotypically significant for adaptation to changes in environmental conditions (25). Up to now, nothing is known about the relevance of the genes downstream of the *puf* operon.

*R. gelatinosus* is a facultative phototrophic non-sulfur bacterium belonging to the β subclass of purple bacteria. This strain, as *R. sphaeroides* and *R. capsulatus* and unlike *Rhodopseudomonas viridis*, can grow very easily under aerobic conditions in the dark as well as under photosynthetic conditions. These trophic characteristics are very useful for studies of the photosynthetic processes using mutants. The genes coding for the subunits of LHI and RC and the polypeptides of LHII are organized in operons. The nucleotide sequence of the *puf* operon has been determined for two strains of *R. gelatinosus*, strain IL144 (26, 27) and strain S1 (28, 29); it contains two open

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[1] The abbreviations used are: LH, light harvesting complex; kb, kilobase(s); bp, base pair(s); RC, reaction center.

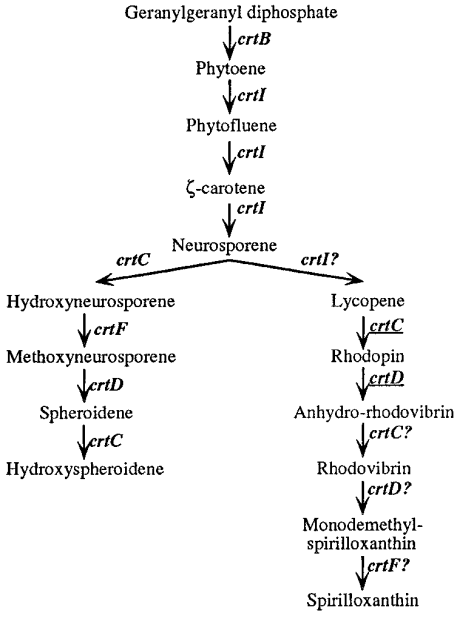


FIG. 1. **Pathways of carotenoid biosynthesis in *R. gelatinosus*.** *crt* genes and product functions are as follows: *crtB*, prenylpyrophosphate condensation; *crtC*, hydration; *crtD*, 3,4-dehydrogenation; *crtF*, *O*-methylation; *crtI*, dehydrogenation. *Underlined* genes are those which have been demonstrated in this work to be involved in the biosynthesis pathway of spirilloxanthin. Genes followed by "?" are postulated to be involved in this pathway.

reading frames (ORFs) in addition to five photosynthetic genes which have been reported in species belonging to the $\alpha$ subclass. These genes include *pufB*, *-A*, *-L*, *-M*, and *-C* coding for the $\beta$ and $\alpha$ subunits of the B875 light-harvesting protein and for the L, M, and cytochrome subunits of the RC complex, respectively. The localization and the organization of the pigment genes are unknown. Recently, gene transfer systems in *R. gelatinosus* have been established (29), and genetic evidence for an organization of the *puf* operon and upstream *bch* genes in a superoperon has been obtained (29).

In this study we report the construction and the analysis of interposon and insertion *R. gelatinosus* mutants, in which carotenoid biosynthesis has been affected. The accumulation of neurosporene and lycopene in some mutants suggested that at least the *crtC* gene could be localized downstream of the *puf* operon. Sequence analysis of the downstream region of the *puf* operon revealed the presence of *crtD* and *crtC* genes. Inactivation of *crtD* gene indicated that the two carotenoid genes are cotranscribed. Analysis of mutants gave evidence of the involvement of both genes in the hydroxyspheroidene and the spirilloxanthin carotenoid biosynthesis pathways. Furthermore, analysis of the accumulation of carotenoid intermediates in the different constructed mutants showed that *crtD* and *crtC* genes are cotranscribed with *puf* operon using the oxygen-regulated *puf* promoter.

## EXPERIMENTAL PROCEDURES

*Bacterial Strains, Plasmids, and Growth Media—Escherichia coli* strains were grown at 37 °C on LB medium (30). *R. gelatinosus* strain S1 (31) and the constructed strains were grown anaerobically (photoheterotrophic conditions) or aerobically (nonphotosynthetic conditions) at 32 °C in malate (ML) medium (32). Antibiotics were used at the following concentrations for *E. coli* and *R. gelatinosus*: spectinomycin, 50 $\mu$g/ml; streptomycin, 50 $\mu$g/ml; ampicillin, 100 $\mu$g/ml; kanamycin, 50 $\mu$g/ml; tetracycline, 10 $\mu$g/ml. Bacterial strains and plasmids used in this work are listed in Table I.

*Gene Transfer*—Plasmid DNA was introduced into *R. gelatinosus* cells using the electroporation system described in Ref. 29. Following the electric pulse, cells were diluted in 10 ml of ML medium. After incubation at 32 °C for 6 h in darkness, serial cell dilutions were plated on nonselective ML plates to assess cell survival and on selective ML plates to select transformants. Two different antibiotic resistance markers were used to distinguish a double crossover event from a single crossover event, the first one being located on the vector and the second one as the cartridge inserted in the gene to be inactivated.

*Chromatophore Isolation and Carotenoid Extraction; Spectrophotometric Measurements*—Chromatophores from *R. gelatinosus* were prepared by differential centrifugation after disruption of cells with a French press and were resuspended in 10 mM Tris HCl pH 8 buffer. Carotenoids were extracted from chromatophores with acetone/methanol (7:2, v/v) and separated by thin-layer chromatography in a acetone/petroleum ether (1:9) solvent, as described in Ref. 3. Each carotenoid spot was recovered, eluted from silica by a small volume of acetone, and analyzed by spectroscopy. Spectral analysis was carried out on a Cary 2300 spectrophotometer interfaced with a computer.

*Molecular Biology Techniques*—Standard methods were performed, if not otherwise indicated, according to Ref. 30. Plasmid DNAs were purified using Qiagen columns (Diagen). DNA was treated with restriction enzymes and other nucleic acid modifying enzymes (Klenow fragment, alkaline phosphatase, T4 DNA ligase) according to manufacturer specifications. DNA fragments were analyzed on agarose gel, and different restriction fragments were purified using a Geneclean kit (Bio 101). Genomic DNA was purified as described in Ref. 29. Southern hybridization analysis of genomic DNA was performed as indicated by Amersham; probes were labeled with [$\alpha$-$^{32}$P]CTP by nick translation.

*Construction of Plasmids*—Construction of pMP8 and pSO8 has been described previously in Ref. 29. Plasmid pMP8 was linearized with *Bst*BI restriction enzyme and treated with the Klenow fragment to create blunt ends. The resulting plasmid was ligated with the *Sma*I Ω or Km cartridge to construct pSM2 and PSO6, respectively. For constructing pSO14 and pSO15, the *Bam*HI Ω or Km cartridge was inserted in the unique *Bgl*II site of *pufL* in pMP8 plasmid. To inactivate the *crtD* gene, pSOX plasmid was linearized with *Bgl*II restriction enzyme and ligated with the *Bam*HI Ω or Km cartridge to create pSO20 and pSO21, respectively.

*Cloning of crt Genes*—To clone the region downstream of the *puf* operon, genomic DNA from the SIC strain was digested with different restriction endonucleases and ligated. The ligation product was used to transform *E. coli*. Transformants were analyzed and several plasmids were obtained. The *crt* genes were subcloned in plasmid pSOX and pSO24. Sequencing was performed on both strands using the dideoxy chain termination method of Sanger with the Sequenase version 2.0 kit, Amersham.

## RESULTS

*Polar Effect of Interposon Mutagenesis within puf Operon*—A *puf* operon deletion strain called SΔP was described previously (29). A pleiotropic effect of the deletion was a change in the color of the cells which became green (the wild type being purple). Absorption spectra of chromatophores of this strain showed that the three main absorption peaks of carotenoids in the wild type (455, 481, and 512 nm) were shifted to the blue, giving a shoulder at about 425 nm and two peaks at 458 and 487 nm in the mutant. This phenotype was due neither to spontaneous mutations nor to the Ω cartridge itself, but to the disruption of the *puf* operon. Indeed, transformants with pSO8, a replicative plasmid bearing the Ω cartridge, were purple, but the complemented strain CSΔP, bearing the *puf* operon on a replicative plasmid, was also green. Furthermore, we isolated revertants from the complemented strain CSΔP which became purple and in which the correct chromosome structure was restored as a result of recombination between the chromosome and the complementing plasmid (29).

Other interposon strains, SIL1 and SΔC1 (Table I), in which the Ω cartridge was inserted in *pufL* and in *pufC* genes, respectively, were constructed (Fig. 2*A*). Sp$^r$ and Ap$^s$ transformants resulting from double crossover events were selected. The inactivation of the genes with the drug cartridges was confirmed by Southern blot analyses. Fig. 2*B* shows an example of Southern analysis. Genomic DNAs from wild type and two different clones of SΔC1 strains were digested either by *Sac*I or by *Sal*I and probed with the *Sal*I fragment containing a large part of *pufC*. The 4.9-kb *Sac*I band seen in the wild type was replaced,

TABLE I
*Bacterial strains and plasmids*

The abbreviations used are: Ap$^r$, ampicillin-resistant; Km$^r$, kanamycin-resistant; Sm$^r$, streptomycin-resistant; Sp$^r$, spectinomycin-resistant; Tc$^r$, tetracycline-resistant.

| Strains and plasmids | Relevant characteristics | Source or reference |
|---|---|---|
| *E. coli* | | |
| C600 | F$^-$, *thi*-1, *thr*-1, *leu*B6, *lac*Y1, *ton*A21, *sup*E44 | Stratagene |
| XL1-Blue | *sup*E44, *hsd*R17, *rec*A1, *end*A1, *gyr*A46, *thi*-1, *rel*A1, *lac*$^-$F′ [*pro*AB$^+$, *lac*I$^q$, *lac*ZΔM15 Tn10 (Tc$^r$)] | Stratagene |
| *R. gelatinosus* | | |
| S1 | Wild type | (31) |
| SΔP | *puf* deletion strain (*puf*::Ω) | (29) |
| SIC | Insertion strain: plasmid pSM1 integrated into the downstream region of *puf* operon on the chromosome | (29) |
| SIO | Insertion strain: plasmid pSM1 integrated into the upstream region of *puf* operon on the chromosome | (29) |
| SIL1 | Interposon strain (*pufL*::Ω) | This work |
| SIL2 | Interposon strain (*pufL*::Km) | This work |
| SIL3 | Insertion strain: plasmid pSO15 integrated into the downstream region of *pufL* on the chromosome | This work |
| SΔC1 | Interposon strain (*pufC*::Ω) | This work |
| SΔC2 | Interposon strain (*pufC*::Km) | This work |
| SIC2 | Insertion strain: plasmid pSO6 integrated into the downstream region of *pufC* on the chromosome | This work |
| SID1 | Interposon strain (*crtD*::Ω) | This work |
| SID2 | Interposon strain (*crtD*::Km) | This work |
| SID3 | Insertion strain: plasmid pSO21 integrated into *crtD* on the chromosome | This work |
| Plasmids | | |
| Bluescript KS$^+$ | Cloning vector (Ap$^r$) | Pharmacia |
| pBBR1MCS-2 | (bom$^+$, Km$^r$) | (38) |
| pBR322 | Cloning vector (bom$^+$, Ap$^r$, Tc$^r$) | (39) |
| pDW9 | Plasmid with Ω cartridge (Sp, Sm)$^r$ | (40) |
| pMP8 | pUC18 + 5-kb *Sac*I *puf* operon | (29) |
| pSM1 | pUC18 + *puf*::Ω at *Mun*I-*Bst*BI sites within *puf* | (29) |
| pSM2 | (pUC18 + *pufC*::Ω). Ω cartridge cloned in *Bst*BI site within *pufC* | This work |
| pSO6 | (pUC18 + *pufC*::Km). Km cartridge cloned in *Bst*BI site within *pufC* | This work |
| pSO8 | pBBR1MCS-2 + 2-kb *Eco*RI Ω cartridge | (29) |
| pSO14 | (pUC18 + *pufL*::Ω). Ω cartridge cloned in *Bgl*II site within *pufL* | This work |
| pSO15 | (pUC18 + *pufL*::Km). Km cartridge cloned in *Bgl*II site within *pufL* | This work |
| pSO20 | (Bluescript KS$^+$ + *crtD*::Ω). Ω cartridge cloned in *Bgl*II site within *crtD* | This work |
| pSO21 | (Bluescript KS$^+$ + *crtD*::Km). Km cartridge cloned in *Bgl*II site within *crtD* | This work |
| pSOX | Bluescript KS$^+$ + 1.2-kb *Sac*I fragment of *crtD* | This work |
| pSO24 | Bluescript KS$^+$ + 1.8-kb *Sac*I fragment of *crtD-crtC* | This work |
| pUC18 | Cloning vector (Ap$^r$) | Pharmacia |
| pUC4K | Plasmid with Km cartridge | Pharmacia |

as expected, by a 6.9-kb band resulting from the 2-kb Ω cartridge insertion in the gene. The 1.1-kb *Sal*I band seen in the wild type was replaced, as expected, by two small 0.7- and 0.4-kb bands because of the two *Sal*I sites of the polylinkers flanking the Ω cartridge. The same type of analysis, using other restriction enzymes, was performed with the SIL1 strain (data not shown) to confirm the inactivation of the *pufL* gene. The SΔC1 and SIL1 strains were also green, substantiating the polar effect of the *puf* deletion. Spectroscopic analysis carried out on chromatophores indicated a modification in the carotenoid absorption peaks. It was assumed that the green phenotype reflected a lesion in the carotenoid biosynthesis.

*Analysis of the Constructed Strains: Carotenoid Contents*—Pigment modifications in the mutants SIL1 and SΔC1 were checked by recording absorption spectra of acetone/methanol extracts from chromatophores of these strains and comparing them with that of the wild type (Fig. 3). In the wild type, the three main carotenoid absorption peaks are located at 430, 455, and 485 nm, and there is a minor peak at 530 nm. In both mutants, the three major peaks were shifted toward the blue giving maxima at 415, 440, and 470 nm, with a minor peak at 500 nm. This indicated that the green color was due to a change in the carotenoid composition of the cells and that the mutants were blocked presumably at the same step in the carotenoid biosynthesis pathway. To confirm this result, the carotenoid extracts of the mutants and of the wild type were analyzed by thin layer chromatography (3), and the major carotenoids were identified by their relative mobilities and by their absorption spectra (Table II). In the wild type, the major carotenoids were identified as hydroxyspheroidene, spheroidene, and spirilloxanthin, as in Ref. 5. In the mutants, two major carotenoids were identified as neurosporene and lycopene (Table III). Since the next steps in the carotenoid biosynthesis pathway for transforming, respectively, neurosporene to hydroxyneurosporene and lycopene to rhodopin are hydrations (Fig. 1), our results suggested that in the mutants the synthesis of a hydratase encoded by *crtC* gene was blocked and that at least this gene could be located downstream of the *puf* operon.

*Nucleotide and Deduced Amino Acid Sequence of the crtC and crtD Genes*—To check the localization of the *crtC* gene and to see whether it is the only *crt* gene localized downstream of the *puf* operon, cloning of this region was achieved by insertional cloning using SIC and SID3 strains in which recombinant plasmids were inserted downstream of the *puf* operon (Table I). Two plasmids pSOX and pSO24 were obtained, and the cloned DNA was sequenced on both strands. The complete nucleotide sequence of a 3-kb *Sac*I fragment downstream of the *puf* operon (Fig. 2*A*) was determined. This fragment was found to contain two ORFs immediately downstream of the *pufC* gene. The initial assignment of the ORFs was based on the alignment with *R. capsulatus* and *R. sphaeroides* photosynthetic gene cluster, which showed that they correspond to *crtD* and *crtC* carotenoid genes. The *crtD* and *crtC* genes were in the same direction as the *puf* operon. The nucleotide and deduced

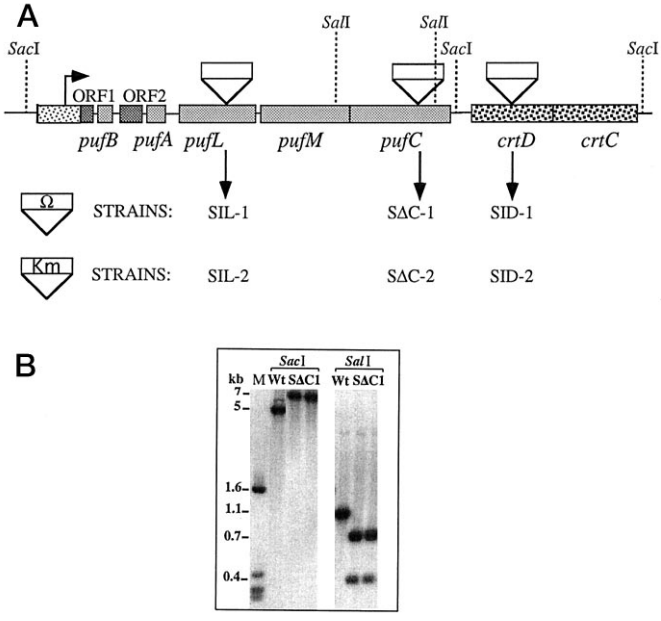


FIG. 2. *A*, SIL1, SIL2, SΔC1, SΔC2, SID1, and SID2 constructions in *R. gelatinosus*. Phenotypes of the strains are indicated in Table III. *B*, Southern blot analysis of genomic DNAs of wild type (*Wt*) and SΔC1 (two different clones) strains digested by *Sac*I and *Sal*I. The *Sal*I-*Sal*I fragment was labeled with [$\alpha$-$^{32}$P]CTP by nick translation and used as a probe. *M*, molecular weight marker.

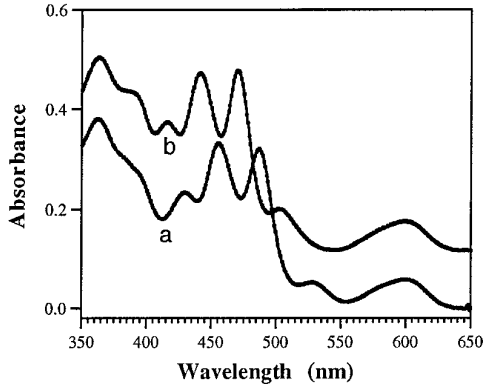


FIG. 3. **Absorption spectra of carotenoid extracts from *R. gelatinosus* wild type (*a*) and SIL1 mutant (*b*).** SΔC1 carotenoid extract presented the same spectrum as SIL1 (not shown). The spectra were normalized in the bacteriochlorophyll band at 600 nm. The zero level of the upper spectrum was shifted for better viewing.

amino acid sequences were deposited in the GenBank™ Data Library under the accession number U73944.

*Characteristics of the Nucleotide Sequence*—The *crtD* and *crtC* genes have the same transcriptional orientation, and no transcriptional terminator was found between them, suggesting that they could be cotranscribed. For both genes, an ATG initiating codon is proposed. The *crtC* gene initiation codon was found within the *crtD* gene (overlap of 372 nucleotides). No alternative initiation codon could be found. Putative translation starts of these genes were based on the position of a Shine-Dalgarno ribosome binding site evaluated by comparison with other sequenced genes from *R. gelatinosus* known to encode proteins and comparison with genes from *R. capsulatus* (10). The first third of the *crtC* gene shows atypical codon usage. In *R. gelatinosus*, the codon usage deduced from the *pufBALMC* genes is characterized by the presence of a majority (89%) of C and G at the third position. The first 126 codons of *crtC* have only 64% CG at the third position, whereas the rest

TABLE II
*Spectral characteristics and mobilities in HTLC-silica gel of the different carotenoids, relative to neurosporene*
For details, see "Experimental Procedures."

| Carotenoid | Absorption maxima in acetone (nm) | Relative mobility |
|---|---|---|
| Hydroxyspheroidene | 429–454–484 | 0.2 |
| Spheroidene | 429–454–484 | 0.8 |
| Spirilloxanthin | 465–495–528 | 0.5 |
| Neurosporene | 416–440–468 | 1.0 |
| Methoxyneurosporene | 416–440–468 | 0.83 |
| Hydroxyneurosporene | 416–440–468 | 0.25 |
| Lycopene | 442–470–500 | 0.97 |
| Rhodopin | 442–470–500 | 0.18 |

of the sequence has a value of 93% comparable with the mean value. No putative promoter sequence with significant homology to the known *puf* promoter or to the *crt* promoters from *R. capsulatus* and *R. sphaeroides* was found for the two genes; this is in agreement with genetic data concerning their expression (see below).

*Cotranscription of crtD and crtC Genes*—We disrupted *crtD* gene either with an Ω cartridge which contains a strong transcriptional terminator or with a Km cartridge devoid of the transcriptional terminator (Fig. 2*A*). If the two genes were cotranscribed, inactivation of *crtD* with an Ω cartridge would lead to a *crtC*-less phenotype, and cells would accumulate neurosporene and lycopene since CrtC operates upstream of CrtD in the carotenoid biosynthetic pathway, whereas inactivation with a Km cartridge leads to a *crtD*-less phenotype, and cells accumulate other carotenoid intermediates.

Plasmids pSO20 and pSO21 were used to transform *R. gelatinosus* leading to SID1 and SID2 strains, in which the *crtD* was inactivated with the Ω and the Km cartridge, respectively (Table I, Fig. 2*A*). All the obtained transformants were green. Southern blot analyses were performed and confirmed the inactivation of *crtD* by a double crossover event in the transformants (data not shown). Carotenoids were extracted and identified by their relative mobilities in thin layer chromatography and their visible absorption spectra (Table III). In SID1, the major carotenoids corresponded to neurosporene and lycopene, a phenotype of a *crtC*$^-$ genotype, whereas in SID2 the carotenoid intermediates corresponded to neurosporene, methoxyneurosporene, hydroxyneurosporene, lycopene, and rhodopin, a phenotype of a *crtD*$^-$ genotype. When the whole plasmid pSO21 was inserted into the chromosome, the resulting strain SID3 was green and had the same phenotype and pigment composition as SID1. These results indicated that *crtD* and *crtC* are cotranscribed.

*Characteristics and Similarities of Deduced Amino Acid Sequences*—The *crtD* gene is 1572 bp long and encodes a protein with a predicted molecular mass of about 56 kDa. The CrtD protein from *R. gelatinosus* is highly similar (53%) to the corresponding protein from *R. capsulatus* (Fig. 4*A*). Particularly, a stretch of residues in the C-terminal region which is highly conserved in the carotenoid dehydrogenases (CrtD and CrtI) from *R. capsulatus* (10) is also found in *R. gelatinosus* (*underlined* in Fig. 4*A*).

The *crtC* gene is 1218 bp long and encodes a protein with a predicted molecular mass of about 44 kDa. The CrtC protein from *R. gelatinosus* presents less similarity (42%) than *crtD* to the corresponding protein from *R. capsulatus* (Fig. 4*B*). This is owing to the extension of 120 residues in the N-terminal region of the *R. gelatinosus* protein.

It is noteworthy that in *R. gelatinosus,* the first part of the CrtC protein, which corresponds to the region with atypical codon usage, was enriched in proline (10.5%) whereas the rest

TABLE III
*Major carotenoids identified in the different strains*

P, purple; G, green; *, strain SIO remains green when grown in darkness and aerobiosis, but becomes purple when grown in light and anaerobiosis.

| | WT | SΔP | SIC | SIO* | | SIL1 | SIL2 | SΔC1 | SΔC2 | SID1 | SID2 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | P | G | G | G | P | G | P | G | P | G | G |
| Hydroxyspheroidene | + | | | | + | | + | | + | | |
| Spirilloxanthin | + | | | | + | | + | | + | | |
| Spheroidene | + | | | | + | | + | | + | | |
| Neurosporene | | + | + | + | | + | | + | | + | + |
| Lycopene | | + | + | + | | + | | + | | + | + |
| Methoxyneurosporene | | | | | | | | | | | + |
| Hydroxyneurosporene | | | | | | | | | | | + |
| Rhodopin | | | | | | | | | | | + |

of the sequence has the usual percentage of proline, *i.e.* about 6–7%. The hydropathy plots of both proteins were determined according to Kyte and Doolittle (33), and they did not reveal any putative transmembrane domain.

*Involvement of CrtC and CrtD in the Spirilloxanthin Biosynthesis Pathway*—Evidence of the involvement of the CrtC and CrtD proteins in the spirilloxanthin biosynthesis pathway arises from the carotenoid content of SID1 and SID2 mutants. In this biosynthesis pathway, lycopene is converted to rhodopin by a hydration reaction. In SID1 strain (*crtC*$^-$), this conversion does not occur, implying that the *crtC* gene product catalyzes the transformation of lycopene to rhodopin in *R. gelatinosus.*

The formation of anhydrorhodovibrin from rhodopin needs a dehydrogenase. Inactivation of *crtD* gene in SID2 strain (*crtD*$^-$) results in the accumulation of rhodopin. Thus, in *R. gelatinosus*, the *crtD* gene product catalyzes the formation of anhydrorhodovibrin at the expense of rhodopin.

*Evidences of Read-through puf Operon for the Expression of crt Genes*—Analysis of the interposon strains indicated that there is no promoter for carotenoid genes within the *puf* operon (see above). One possible promoter could be the *puf* operon promoter itself. To examine this hypothesis, two insertion strains called SIO and SIC (Table I) were used (29). In the SIO strain, a recombinant plasmid has been inserted between the *bch* genes and the *puf* operon and thus the *puf* operon remained linked to the carotenoid genes. In the SIC strain, the recombinant plasmid has been inserted between the *puf* operon and the carotenoid genes (29). Because of this chromosome organization, if the *crt* genes are transcribed using the *puf* promoter (which had been shown to be repressed under aerobic conditions), we expected the SIC strain to be green independently of the growth conditions. On the contrary, the SIO strain would be green in darkness under aerobiosis, but would become purple when grown under photosynthetic conditions (light and anaerobiosis).

In darkness under aerobiosis, the SIC and SIO strains were green. Pigment analysis indicated that both strains accumulated neurosporene and lycopene (Table III). When transferred in photosynthetic conditions, the SIC cells remained green, whereas the SIO cells became progressively purple. Carotenoid were extracted from cells after 2, 5, and 7 days of growth in photosynthetic conditions. For the SIC strain, no difference was observed (not shown). For SIO, the spectra of the carotenoid extracts from cells grown during 2 days and during 7 days in photosynthetic conditions were different (Fig. 5) and the difference spectrum, computed between these two culture extracts, showed the same carotenoid absorption peaks as in the wild type. Analysis of carotenoids by thin layer chromatography showed the progressive disappearance of neurosporene and lycopene and the progressive appearance of spheroidene, hydroxyspheroidene, and spirilloxanthin (Table III). These results indicated that the carotenoid genes are cotranscribed with the *puf* operon using the oxygen-regulated *puf* promoter. To confirm these results, we constructed two other interposon strains SIL2 and SΔC2 in which the *pufL* or *pufC*, respectively, were disrupted by the Km cartridge which is devoid of a transcriptional terminator (Table I, Fig. 2*A*). The resulting strains were purple, and they had the same pigment composition as the wild type. However, when the whole plasmids (pSO15 and pSO6) were inserted into the chromosome, the resulting strains SIL3 and SIC2 were green and had the same phenotype and pigment composition as SIL1 and SΔC1.

## DISCUSSION

The Ω cartridge has been proved to be an efficient polar mutagen (34). We have used it to construct *puf* inactivations and deletions. The resulting strains exhibited a green phenotype, suggesting a localization of carotenoid genes downstream from the *puf* operon (29). In this work we demonstrate that the localization of the *crtD* and *crtC* carotenoid genes in *R. gelatinosus* is different from that described up to now in the other purple bacteria. Indeed, *crtD* and *crtC* were found downstream of the *puf* operon in *R. gelatinosus,* whereas in *R. capsulatus* and *R. sphaeroides* the two genes are located in a carotenoid cluster including all the *crt* genes and flanked by the *bch* genes, upstream of the *puf* operon.

The polarity effects arising from the interposon mutagenesis within *crtD* showed that *crtD* and *crtC* are cotranscribed and in the *crtD-crtC* order. More interestingly, interposon mutagenesis within *puf* operon also had polar effects on the carotenoid genes; this indicated that the *crt* genes are cotranscribed with the *puf* operon. Unlike the situation in *R. capsulatus* and *R. sphaeroides*, no promoter sequence was found for the transcription of the *crtD* and *crtC* genes in *R. gelatinosus*, and we have genetic evidences that under photosynthetic conditions the *crtD* and *crtC* genes are cotranscribed with *puf* using its oxygen-regulated promoter. Thus, one may wonder how the *crt* genes are transcribed under nonphotosynthetic conditions. Since in darkness and under aerobiosis *R. gelatinosus* is still pigmented, we suppose that these genes are transcribed from an oxygen-insensitive promoter. We have already shown that under nonphotosynthetic conditions the *puf* operon is transcribed from an upstream promoter insensitive to oxygen regulation, and that cells under these conditions produced a reduced amount of LHI and RC proteins (29). We propose that the carotenoid genes localized downstream of the *puf* operon could be also transcribed from this oxygen-insensitive promoter. In *R. capsulatus*, it was proposed that *crtEF*, *bchCXYZ*, and *puf* operons were cotranscribed under nonphotosynthetic conditions using the *crt* promoter (35) and that *bchFBNHLM-F1696* and *puhA* are cotranscribed using the *bch* promoter (22). In *R. gelatinosus,* we have to assume that *bch*, *puf*, and *crt* genes could be cotranscribed under the nonphotosynthetic conditions using the *bch* oxygen-insensitive promoter; this hypothesis will

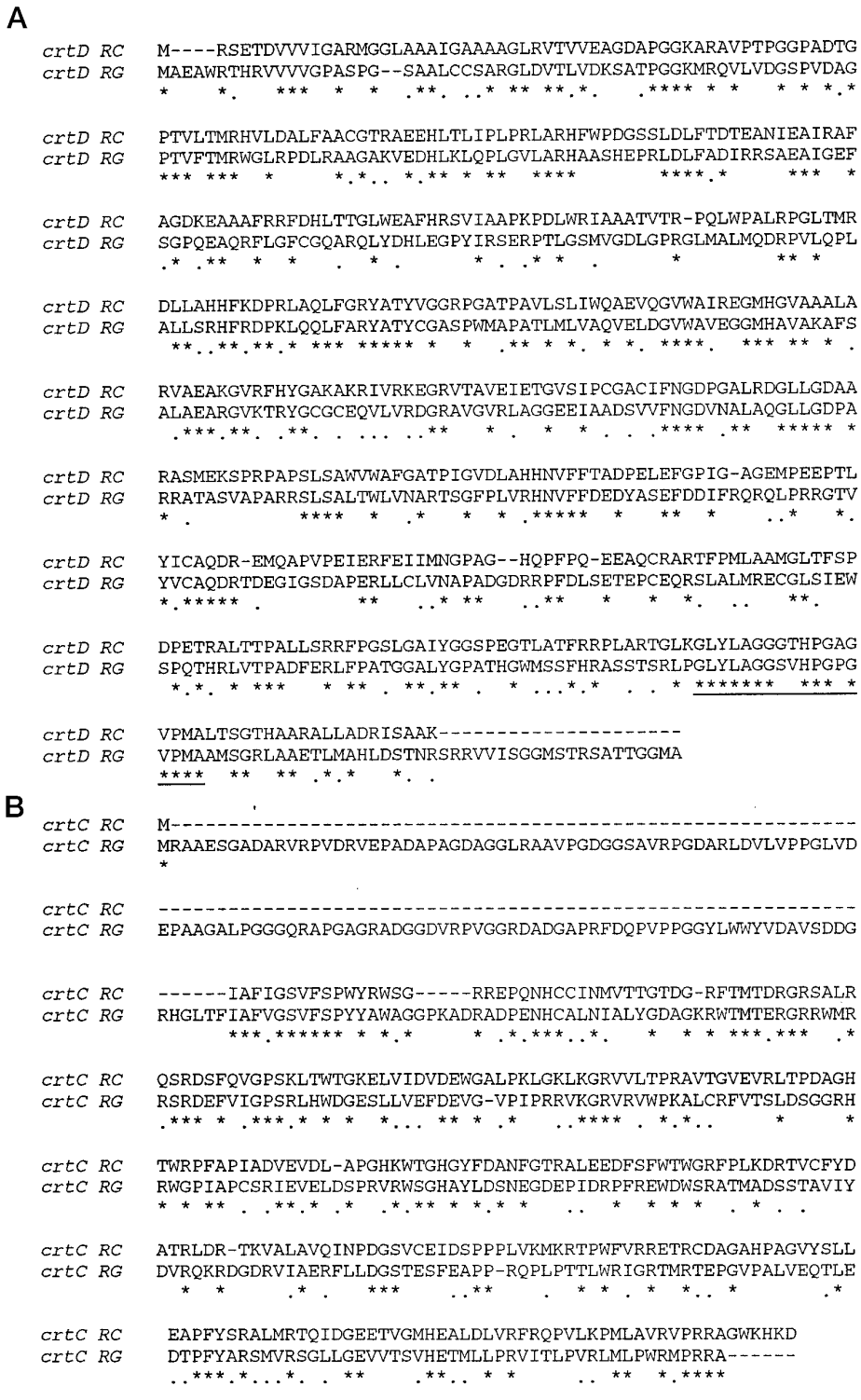


FIG. 4. **Comparison of deduced *crtD* (*A*) and *crtC* (*B*) gene products of *R. capsulatus* (*RC*) and *R. gelatinosus* (*RG*).** The symbols * and . indicate identical and similar amino acids, respectively (using the Clustal W program). In *A*, the *underlined* stretch of residues in the C-terminal region corresponds to a highly conserved region in the carotenoid dehydrogenases (CrtD and CrtI) from *R. capsulatus* (10) which is also found in *R. gelatinosus*.

be confirmed by mRNA analysis (under investigation).

A ρ-independent terminator-like structure was found between *pufC* and *crtD* genes of *R. gelatinosus*. Similar structures were found in *R. capsulatus*, *R. sphaeroides*, *R. rubrum*, and *R. viridis* (36). We would suggest that these structures could have a regulatory function and serve to control the stability of the transcript. The carotenoid genes which encode enzymatic proteins would be required in low amounts, in contrast to *puf* genes encoding structural polypeptides which require a higher level of expression, and, accordingly, the hairpin structures could protect *puf* mRNA from exonuclease digestion to increase its lifetime. Beatty (20) speculated that in spite of the two ρ-independent terminator-like structures found downstream of *pufX* in *R. capsulatus*, a read-through transcription downstream of the *puf* operon was possible; here we confirm this hypothesis for *R. gelatinosus*.

In *R. capsulatus* and *R. sphaeroides*, only one carotenoid biosynthesis pathway has been identified, leading to the synthesis of spheroiden(on)e; the reactions and the involved genes are now well known (6). In *R. gelatinosus*, the diversity of carotenoids is greater and two carotenoid biosynthesis pathways lead, respectively, to synthesis of hydroxyspheroidene and spirilloxanthin (5). The reaction steps of both pathways are known (5), but the involved genes have not been identified.

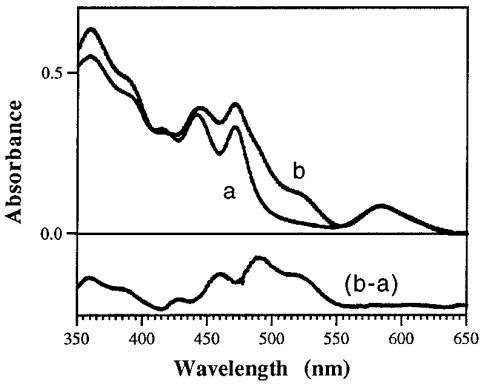


FIG. 5. **Absorption spectra of carotenoid extracts from *R. gelatinosus* SIO cells.** After growth in aerobiosis and darkness conditions, the cells were transferred over 2 days (*spectrum a*) and 7 days (*spectrum b*) in photosynthetic conditions (light and anaerobiosis). The spectra were normalized in the bacteriochlorophyll band at 600 nm. The difference spectrum ($b - a$) was shifted for better viewing.

The biosynthesis of hydroxyspheroidene, in *R. gelatinosus*, might involve the same enzymatic reactions as in *R. capsulatus* and *R. sphaeroides*. Here we report the identification of the *crtD* and *crtC* genes, and we confirm the functions of their products in the spheroidene branch.

The biosynthesis of spirilloxanthin via rhodopin in *R. gelatinosus* and in *R. rubrum* includes several known reactions (5). In this work, we propose another function to *crtC* and *crtD* gene products in *R. gelatinosus*. The *crtC* gene, encoding a hydratase, and the *crtD* gene, encoding a dehydrogenase, are, respectively, involved in the formation of rhodopin from lycopene and the formation of anhydrorhodovibrin from rhodopin (Fig. 1). It implies that the *crt* genes involved in the hydroxyspheroidene pathway are also involved in the spirilloxanthin one.

To complete the scheme, we propose the participation of *crtI, crtC*, *crtD*, and *crtF* in the final steps of spirilloxanthin synthesis (Fig. 1). Indeed, transformation of neurosporene to lycopene requires a "specific" desaturation catalyzed by a dehydrogenase which could be encoded by *crtI*. It is interesting to note that in *R. capsulatus* and *R. sphaeroides*, this CrtI enzyme is present but its catalytic function stops at the level of neurosporene (7). This difference in the functioning of the dehydrogenase suggests that in *R. gelatinosus*, the *crtI* gene could be different from the corresponding gene in *R. capsulatus* and *R. sphaeroides*. Conversion of anhydrorhodovibrin to rhodovibrin requires a hydration which could be catalyzed by *crtC* gene product. The *crtD* gene encodes a dehydrogenase which could catalyze the formation of monodemethyl spirilloxanthin from rhodovibrin. Finally, formation of the spirilloxanthin requires the methylation of the monodemethyl spirilloxanthin which is catalyzed by an *O*-methyltransferase. In the hydroxyspheroidene pathway, the *O*-methyltransferase is encoded by the *crtF* gene; thus, *crtF* might also be implicated in the spirilloxanthin pathway. This has been also suggested by Yildiz *et al.* (37) as a *crtF* mutant of *R. capsulatus* has been complemented by DNA from *R. centenum* which synthesizes spirilloxanthin rather than spheroidene. Further genetic experiments have to be performed to confirm these hypotheses.

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