Genetic and Biochemical Characterization of Carotenoid Biosynthesis Mutants of *Rhodobacter capsulatus*  

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We have used genetic and biochemical techniques to study carotenoid biosynthesis (*crt*) mutants of *Rhodobacter capsulatus*, a purple non-sulfur photosynthetic bacterium. All nine identified *crt* genes are located within the 46-kilobase pair photosynthesis gene cluster, and eight of the *crt* genes form a subcluster. We have studied the operon structure of the *crt* gene cluster using transposon Tn5.7 mutants. The Tn5.7 insertion sites in 10 mutants have been mapped to high resolution (25–267 base pairs) by Southern hybridization. Two insertions each map within the coding regions of the *crtA*, *crtC*, *crtE*, and *crtF* genes, and one insertion lies within the *crtI* gene. The insertion in *crtI* is not polar on the downstream *crtB* gene, suggesting that *crtI* and *crtB* may form two separate operons. Another insertion located in the 5' noncoding region between the divergent *crtA* and *crtI* genes has no effect on wild-type pigmentation and apparently lies between the promoters for these operons. A Tn5.7 mutation in the 3' region of *crtI* yields a bacteriochlorophyll-mutinus phenotype, while a 5' insertion affects only carotenoid biosynthesis. Regulatory signals for transcription of a downstream operon required for bacteriochlorophyll biosynthesis may thus overlap the coding region of *crtI*. We also present the first evidence for the functions of the *crtB*, *crtE*, and *crtJ* gene products using a new *in vitro* assay for the incorporation of [14C]isopentenyl pyrophosphate into carotenoid precursors and phytoene in cell-free extracts. Extracts from a *crtE* mutant accumulate [14C]prephytoene pyrophosphate, while those from *crtB* and *crtJ* mutants accumulate [14C]geranylgeranyl pyrophosphate. We therefore propose that CrtE is the phytoene synthetase and that CrtB, and possibly CrtJ, are components of the downstream operon required for bacteriochlorophyll biosynthesis (Fig. 1). Spheroidene is rapidly converted to spheroidenone upon the introduction of oxygen into the medium and the latter carotenoid accumulates in aerobically grown cultures of *Rhodobacter* (Schmidt, 1978). During photosynthetic growth the Bchl and carotenoid pigments are covalently associated with the structural polypeptides of the photosynthetic reaction center and two light-harvesting antenna complexes (Drews, 1985).

Nine carotenoid biosynthesis (*crt*) genes have been identified in *R. capsulatus* and all are contained within the 46-kb photosynthesis gene cluster of this organism (Yen and Marrs, 1976; Scolnik et al., 1980; Zsebo and Hearst, 1984; Armstrong et al., 1989). We have recently determined the nucleotide sequence of an 11-kb region encoding eight of the nine genes (*crtA*, *B*, *C*, *D*, *E*, *F*, *I*, and *K*), the first sequence of carotenoid genes from any organism (Armstrong et al., 1989). Identical nucleotide sequences for the *crtI* gene (Bartley and Scolnik, 1989) and the 3' portion of the *crtF* gene (Young et al., 1989) have subsequently been reported. The ninth carotenoid biosynthesis gene, *crtJ*, is separated from the other *crt* genes by about 12 kb (Zsebo and Hearst, 1984). The *crt* gene cluster is flanked by genetic loci denoted *bch* which are involved in Bchl biosynthesis (Zsebo and Hearst, 1984; Giuliano et al., 1988).

*R. capsulatus* carotenoid biosynthesis mutants are photosynthetically viable, and point, transposon, and interposon mutants are available for study (Yen and Marrs, 1976; Scolnik et al., 1980; Zsebo and Hearst, 1984; Giuliano et al., 1986, 1988). Genetic lesions in four loci within the *R. capsulatus*...
FIG. 1. The updated \textit{R. capsulatus} carotenoid biosynthetic pathway. Compounds from IPP to GGPP are used in other branches of isoprenoid metabolism, including the synthesis of chlorophyll in plants and algae, and Bchl in photosynthetic bacteria (Goodwin, 1980). Although the final carotenoids accumulated are usually all-trans (Bramley and Mackenzie, 1988), phytoene, the first $C_{15}$ carotenoid, is synthesized as either the 15-cis or the all-trans isomer depending on the particular organism (Goodwin, 1980). Trivial names for the carotenoids are used here and throughout the text. Semisystematic names have been given in Scolnik et al. (1986) and Giuliano et al. (1986). Assignments of genetic loci to biochemical functions in the pathway are based on \textit{in vivo} intermediate accumulation in \textit{crt} mutants or on \textit{in vitro} biochemical defects in cell-free carotenoid synthesizing extracts. Functions have been proposed for \textit{crtB} and \textit{crtE} (this work), \textit{crtI} (Giuliano et al., 1986), and \textit{crtC}, \textit{crtD}, \textit{crtF}, and \textit{crtA} (Scolnik et al., 1986). Tentative assignments have been made for \textit{crtK}, recently identified by DNA sequence analysis (Armstrong et al., 1989) and placed on the pathway based on our interpretation of mutational data (Giuliano et al., 1988) and for \textit{crtJ}, based on work described here. 7,8,11,12-Tetrahydrolycopene has been previously shown as an intermediate in the \textit{R. capsulatus} carotenoid pathway (Giuliano et al., 1986, 1988) by analogy to studies in related photosynthetic bacteria (Schmidt, 1978). \textit{β}-Carotene has been suggested as a possible alternative intermediate in mutants has been lacking.

The \textit{photosynthetic gene cluster}, \textit{crtB}, \textit{crtE}, \textit{crtI}, and \textit{crtJ}, produce blue-green color phenotypes rather than the wild-type redish-brown color. This pigmentation change results from the loss of visibly absorbing yellow and orange carotenoids (i.e. those with seven or more conjugated double bonds) and the continued synthesis of Bchl (Yen and Marrs, 1976; Zsebo and Hearst, 1984). No mutations specific to carotenoid biosynthesis would be expected to block the general isoprenoid biosynthetic pathway before the intermediate GGPP (Fig. 1), because this compound is also required for the synthesis of the phytyl or geranylgeranyl side chain of chlorophylls in bacteria and plants (Goodwin, 1980). The \textit{crtB} and \textit{crtE} loci were originally defined by point mutations which caused blue-green phenotypes (Yen and Marrs, 1976), while \textit{crtI} and \textit{crtJ} were similarly identified by Tn5.7 mutagenesis (Zsebo and Hearst, 1984). Giuliano \textit{et al.} (1986) have shown that \textit{crtI} point mutants accumulate phytoene, the first $C_{15}$ carotenoid, which can be converted \textit{in vitro} to later colored carotenoid intermediates by a mixture of crude cell-free extracts from \textit{crtI} and \textit{crtB} mutants. Their proposal that \textit{CrtI} is the phytoene dehydrogenase has recently been biochemically confirmed by the \textit{in vitro} inhibition of phytoene dehydrogenation using an antibody raised against an \textit{R. capsulatus} CrtI fusion protein (Schmidt \textit{et al.}, 1989). We have also recently shown that the deduced amino acid sequence of the \textit{R. capsulatus} \textit{crtD} gene product, which is required for the dehydrogenation of hydroxynorpropane and methoxynorpropane, shows two regions of strong amino acid similarity to the \textit{crtI} gene product (Armstrong \textit{et al.}, 1989). No functions for the \textit{crtB}, \textit{crtE}, and \textit{crtJ} gene products have been proposed, however, because information on the \textit{in vivo} or \textit{in vitro} accumulation of intermediates in mutants has been lacking.
The construction of several *R. capsulatus* Tn5.7 mutants blocked in carotenoid biosynthesis has been described previously (Zsebo and Hearst, 1984). We report here the construction and characterization of six new *R. capsulatus* strains containing Tn5.7 insertions within the *crt* gene cluster, as well as the mapping to high resolution of the Tn5.7 insertion sites in the four previously described mutants and the six new mutants. The phenotypes of the mutants have been correlated with the locations of the Tn5.7 insertion mutations. We also present the first evidence for the biochemical functions of the *crtB*, *crtE*, and *crtJ* gene products using a new cell-free in vitro system for incorporation of a ^14C^-labeled label into carotenoid pyrophosphate precursors and phytoene. The combination of genetic data from the Tn5.7 mapping results and biochemical data from the in vitro system allows us to present an updated *R. capsulatus* carotenoid biosynthetic pathway and scheme for the operon organization of the *crt* gene cluster. We have also localized regions containing the cis-acting elements required for transcription of the divergent *crtA* and *crtI* genes.

**MATERIALS AND METHODS**

**Media, Cultivation, and Manipulation of Bacterial Strains**—The bacterial strains and plasmids used are described in Table I. *Escherichia coli* strains were grown on LC medium (Marrs, 1981). *R. capsulatus* strains were grown in liquid culture aerobically on a rotary shaker at 200 rpm either on rich PYE medium (Marrs, 1981), or on rich *E. coli* strains were grown on LC medium (Marrs, 1981). *R. capsulatus* strains were inoculated into filled 15-ml screw top test tubes containing RCV, sodium pyruvate (Yen and Marrs, 1977). Procedures for conjugal matings between *E. coli* KZE strains (Table I) and *R. capsulatus* strain SB1003, selection for transfer of Tn5.7-mutated pRPS404 derivatives to SB1003 and the subsequent integration of Tn5.7 into the chromosome, and repurification of the resulting KZA Tn5.7 mutant strains have been described previously (Zsebo and Hearst, 1984). Strain PBR510 (courtesy of P. O'Brien, University of California, Delaware) was constructed by conjugation of *E. coli* strain BEC404 with SB1003, selection for kanamycin resistance to ensure the transfer of pRPS404 and subsequent repurification of green colonies arising from the recombination of the *crtD223* lesion carried on pRPS404 (Marrs, 1981) into the chromosomal copy of *crtD* in SB1003. Matings between KZE strains and SB1003 usually gave rise to two color classes of transconjugants, depending on whether both the Tn5.7 insertion and the *crtD223* point mutation or only Tn5.7 had recombined into the chromosome. Strains carrying the *crtD223* point mutation accumulate yellow carotenoids instead of the wild-type orange and red-orange carotenoid complement (Table I and Fig. 2). We selected transconjugants that had recombined only Tn5.7 based on the color phenotype of the colonies, although in the cases

**TABLE 1**

<table>
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<tr>
<th>Strain</th>
<th>Genotype*</th>
<th>Relevant phenotype†</th>
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<tr>
<td>E. coli*</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>NECO0100</td>
<td>leu thr thi trp recA lacY</td>
<td></td>
<td>Ditta et al., 1980</td>
</tr>
<tr>
<td>NEC0200</td>
<td>HB101 [pRK203]</td>
<td>Km'</td>
<td></td>
</tr>
<tr>
<td>NEC0205</td>
<td>HB101 [pLAFR1]</td>
<td>Tc'</td>
<td>Friedman et al., 1982</td>
</tr>
<tr>
<td>KZ8E9F2</td>
<td>NECO100 [pRPS404(33.4::Tn5.7)]</td>
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<td>Zsebo and Hearst, 1984</td>
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<tr>
<td>KZ8E9H5</td>
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</tr>
<tr>
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<td>Zsebo and Hearst, 1984</td>
</tr>
<tr>
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<td>Zsebo and Hearst, 1984</td>
</tr>
<tr>
<td>KZE capsulatus*</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>KZR8F2</td>
<td>crtFI(33.4::Tn5.7)</td>
<td>Green</td>
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</tr>
<tr>
<td>KZR8H5</td>
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<td>crtD223 rif-10</td>
<td>This work</td>
</tr>
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<td>This work</td>
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<td>crtF129</td>
<td>Green, SB1003 derivative</td>
<td>Sclonik et al., 1980</td>
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<td>rif-10</td>
<td>Muddy brown</td>
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<td>W1</td>
<td>crtB4</td>
<td>Wild-type pigments</td>
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</tr>
<tr>
<td>Y68</td>
<td>crtC08 str-2 trpA20</td>
<td>Blue-green</td>
<td></td>
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</table>

* Nomenclature for KZE and KZR strains was developed in Zsebo and Hearst (1984).

† Color phenotypes reflect aerobic growth on agar plates; wild-type pigmentation is reddish-brown under aerobic conditions (Fig. 2) and yellow-brown under photosynthetic conditions.

‡ Strains containing a pRPS404 derivative are Km'.

§ Strains containing Tn5.7 are Sp', Str', and Tm'; strains carrying the rif-10 allele are Rif'.

¶ These strains may carry the *crtD223* allele masked by an earlier blockage in carotenoid biosynthesis.

||
of Tn5.7 mutants blocked in carotenoid biosynthesis before crtD, it was not possible to distinguish whether transconjugants of the point mutation (see "Results"). The plasmid pLAFR1 (Friedman et al., 1982) was conjugated from E. coli strain NECO205 into some R. capsulatus Tn5.7-containing strains (Table I), using pRK2013 supplied by strain NECO200 as a helper plasmid (Ditta et al., 1980), as described previously (Zeo and Hearst, 1984). This procedure was used to cure strains of partially deleted pRPS404 derivatives remaining after the recombination of Tn5.7 into the chromosome. Antibiotics were obtained from Sigma and bacteriological supplies from Difco.

Pigment Extraction and Characterization—Total pigments were extracted from photosynthetically grown cells carrying out all manipulations in darkness at 4 °C. 15 ml of cells were pelleted by centrifugation and extracted three times with 1 ml of Me2SO. Extracts were centrifuged each time to pellet the cellular debris. Carotenoids and Bchl were fractionated by several extractions of the Me2SO phase with equal volumes of petroleum ether. The petroleum ether fraction containing the carotenoids was back-extracted with an equal volume of Me2SO to remove traces of Bchl and the petroleum ether then evaporated under a stream of dry nitrogen. Pigment extracts from previously uncharacterized R. capsulatus strains were analyzed by silica gel thin layer chromatography using a solvent system of acetone:petroleum ether (5:95). Standards for compounds of known structure (Scolnik et al., 1980) were obtained by extraction of carotenoids from characterized R. capsulatus crt mutants and the wild type (Table I).

Chromosomal DNA Isolation, Restriction Digestions, Preparation of M13 Probes, and Southern Hybridizations—Total cellular DNA from 15 ml cultures was isolated and purified as described in Cook et al. (1989). DNA was resuspended to a concentration of 67 ng/ml and digested with 5-5 units/μg DNA of one of the following restriction enzymes: FadI, BamHI, PstI plus BamHI, AcalI, or BsiHI. Restriction digestes were ethanol-precipitated, resuspended, and 10 μg of DNA/lane separated on agarose gels (0.8, 1.0, or 1.2%) run in 1 × TBE buffer (Maniatis et al., 1982). Southern blots were performed by transferring the DNA to nitrocellulose (Schleicher & Schuell) according to standard procedures (Maniatis et al., 1982). Single-stranded M13 phage generated during the sequencing of the R. capsulatus crt genes (Armstrong et al., 1989) were used as templates to synthesize radioactively labeled probes by primer extension, using a commercial oligonucleotide primer (P. L. Biochemicals). M13 probes were labeled as described in Zhu and Hearst (1986) using 50 μCi of 32P-ATP (400 Ci/mmol), except that the reaction was carried out for 15 min and the probes were digested with HpaII restriction endonuclease subsequent to separation of the unincorporated [α-32P]dATP from the labeled probe. Southern hybridizations to determine the transposon insertion sites (Table IV) were performed according to standard procedures (Maniatis et al., 1982). [α-32P]dATP was obtained from Amersham Corp. Restriction enzymes, Konow fragment of DNA polymerase I, and reagents for labeling of DNA were obtained and used according to protocols supplied by the manufacturers (Bethesda Research Laboratories, New England Biolabs, and P-L Biochemicals).

In Vitro Carotenoid Biosynthesis Assay and Analysis of the Products—Bacteria were grown aerobically in the dark in 200 ml of rich PYE medium until early stationary phase. Cells were harvested by centrifugation resuspended in 2 ml of 0.4 M Tris-HCl (pH 8.0), 5 mM dithiothreitol, and lysed in a French pressure cell at 8000 p.s.i. To 0.8 ml of the cell-free extract were added 50 μl of NADP+ (25 mM), 50 μl of ATP (150 mM), and 10 μl of MnCl2 (0.6 M); MgCl2 (0.4 M) solution. The reaction was started with 5 μl of [1,1-C]IPP (0.5 μCi), and the mixture was incubated in the dark for 2 h at 33 °C. The pyrophosphates were extracted according to the method of Qureshi et al. (1973). After addition of 9 ml of 1-butanol, the extract was centrifuged for 1 min in a Hettich centrifuge at 8000 × g. The butanol layer was dried by rotary evaporation and the residue redissolved in 2 ml of ethanol. The pyrophosphates were hydrolyzed for 15 min at 37 °C in the presence of 250 μl of HCl (25%). After the addition of 100 μl of ammonia (25%) and 200 μl of EDTA (0.25 M), the resulting alcohols were extracted into 5 ml of hexane and dried under a stream of dry nitrogen. The alcohols and pigments were redissolved in 25 ml of acetone and separated by silica gel thin layer chromatography using a solvent system of acetone:petroleum ether:hexane (3:1). Standards for compounds of known structure (Chromatographia) were obtained from Amersham Buchler (Frankfurt, Federal Republic of Germany). 14C-Labeled HPLC standards for prephytoene alcohol and phytoene were isolated from mutants of the fungi Neurospora crassa (al-2) (Bramley and Mackenzie, 1988) and Phycomyces blakesleeanus (C5) (Sandmann and Bramley, 1985), respectively. The identity of both standards was confirmed by mass spectroscopy. Pure [14C] geranylgeraniol was a kind gift of W. Rüdiger (University of Munich, Federal Republic of Germany).

RESULTS

Construction of Tn5.7-mutated Strains and Phenotypic Characterization of the Mutants—We constructed six new R. capsulatus strains carrying Tn5.7 insertions within the crt gene cluster by conjugation of Tn5.7-mutated pRPS404 derivatives (Zeo and Hearst, 1984) from E. coli to R. capsulatus, followed by selection for recombination of the transposon into the bacterial chromosome. All of the new Tn5.7 insertion mutants continued to produce Bchl and could be grown photosynthetically (data not shown). The color phenotypes (Fig. 2 and Table I) of the new strains (KZRS8B3, KZSR9D10, KZRS9F12, KZRS8F6, KZSR9H3, KZRS8F2) result from the combination of the Bchl and the carotenoids or carotenoid precursors accumulated. The carotenoid complement of the mutants was examined by organic extraction of pigments from photosynthetically grown cultures (Table II).

KZRS8B3 accumulates wild-type carotenoids and forms red-brown colonies on agar plates as does the wild-type strain (Fig. 2). Thus, the Tn5.7 insertion in KZRS8B3 does not inactivate any crt gene, although the two flanking insertions (Table I) in strains KZRS9A12 and KZRS9F4 cause yellow-brown and blue-green phenotypes, respectively (Fig. 2). Anaerobic photosynthetic cultures of KZRS9D10 and KZRS9F12 are green and accumulate neuropsorone as the sole colored carotenoid species (Table II), consistent with the inactivation of either crtC or crtK (Fig. 1) (Armstrong et al., 1989). KZRS9D10 and KZRS9F12 produce green-brown colonies on aerobically grown Petri plates (Fig. 2), however, although colonies of Y88, a crtC neuropsorone-accumulating point mu-
Table II

<table>
<thead>
<tr>
<th>Strain</th>
<th>Carotenoids accumulated in anaerobic photosynthetic cultures</th>
</tr>
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<tr>
<td>KZR8F2</td>
<td>Neurosporene, hydroxyneurosporene</td>
</tr>
<tr>
<td>HZ0113</td>
<td>Neurosporene, hydroxyneurosporene</td>
</tr>
<tr>
<td>KZR8F6</td>
<td>No visibly absorbing carotenoids</td>
</tr>
<tr>
<td>KZR9F12</td>
<td>Neurosporene</td>
</tr>
<tr>
<td>KZR9D10</td>
<td>Neurosporene</td>
</tr>
<tr>
<td>KZR8B3</td>
<td>Spheroidene, hydroxyspheroidene</td>
</tr>
<tr>
<td>SB1003</td>
<td>Spheroidene, hydroxyspheroidene</td>
</tr>
<tr>
<td>FY1291</td>
<td>Demethyl/spheroidene</td>
</tr>
<tr>
<td>PBR510</td>
<td>Neurosporene, hydroxyneurosporene, methoxynoeyrosporene</td>
</tr>
<tr>
<td>Y68</td>
<td>Neurosporene</td>
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</tbody>
</table>

*Genotypes are given in Table I.

We have taken advantage of the structure of Tn5.7 to map to high resolution (25-267 bp) the chromosomal insertion points. The previous alignment of Tn5.7 insertion sites with the genetic map (Zsebo and Hearst, 1984) and the instability of the color phenotypes suggested that the Tn5.7 point mutants are unstable and often accumulate stabilizing secondary mutations blocking carotenoid biosynthesis at an earlier stage or completely abolishing development of the photosynthetic apparatus.

To determine the biochemical intermediate accumulated in the Tn5.7 mutants compared with the wild-type and characterized point mutants.

Some Tn5.7 mutants may also carry the ctdD223 mutation masked by a color phenotype due to an earlier carotenoid biosynthetic blockage (Table I). We have observed no phenotypic differences, except for the change in carotenoid composition (Fig. 2), distinguishing strains PBR510 and SB1003 wild-type, which are isogenic except for the change in carotenoid composition (Fig. 2). This pattern of carotenoid accumulation indicates the simultaneous inactivation of both ctdF and ctdD in these strains (Fig. 1).

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The green derivatives of KZR8F2 and KZR3H3 have a slightly more green color. The basis of this difference in colony pigmentation is under investigation. KZR8F6 is blue-green and accumulates no colored carotenoids (Fig. 2).

Matings of E. coli strains KZE3H3 and KZE8F2 with R. capsulatus SB1003 produced green and muddy-brown transconjugants; the latter were unstable and gave rise to colony sectors with other color phenotypes. GlpF point mutants are unstable and often accumulate stabilizing secondary mutations blocking carotenoid biosynthesis at an earlier stage or completely abolishing development of the photosynthetic apparatus (i.e. mutations in Bchl biosynthesis genes) (Taylor et al., 1983). The previous alignment of Tn5.7 insertion sites with the genetic map (Zsebo and Hearst, 1984) and the instability of the color phenotypes suggested that the Tn5.7 point mutants are unstable and often accumulate stabilizing secondary mutations blocking carotenoid biosynthesis at an earlier stage or completely abolishing development of the photosynthetic apparatus.

We have detected this effect by the presence of a minor mutation in the ctd gene. Both KZR3H3 and KZR8F2 might be located within ctdF. In order to stabilize the mutant phenotypes, we therefore repurified only the green transconjugants which had recombined both the Tn5.7 insertion and the ctdD223 mutation, derived from pRPS404 (Marrs, 1981), into the chromosome (see "Materials and Methods" and "Discussion"). The green derivatives of KZR8F2 and KZR3H3 accumulate neurosporene and hydroxyneurosporene, but lack methoxynoeyrosporene (Table II) and maintain a stable phenotypic difference, except for the change in carotenoid composition (Fig. 1). The two hybrid restriction fragments in a mutant are composed of the interrupted wild-type fragment and portions of the IS50 elements of Tn5.7. The use of small DNA probes complementary to the region surrounding the Tn5.7 insertion site and various combinations of chromosomal restriction digests thus yield a high resolution physical map.

For the transposon mapping we used the enzymes PstI and BanII, which cut after bp 684 and 437, respectively, of the IS50 elements (Auerwald et al., 1980). AnaII, which was also used in the Tn5.7 mapping, cuts only within the portion of Tn5.7 derived from Tn7 not within the IS50 elements. BanII, which was used in some cases in combination with PstI to generate readily resolvable doubly digested chromosomal fragments, does not cut within Tn5.7 (Zsebo et al., 1984). Table III gives a detailed list of the M13 probes used to determine the Tn5.7 insertion sites and the results of Southern hybridizations. Fig. 3 shows representative Southern hybridizations used to localize the Tn5.7 insertion sites. Fig. 4 gives a summary of the locations of the Tn5.7 insertions on the physical map, relative to the positions of the ctd genes.

KZR8D7 (Bchl) carries a Tn5.7 insertion between bp 999 and 1244, within the 3' end of the ctdA gene. KZR9A12 (CrtA) carries a Tn5.7 insertion between bp 1651 and 1862, within the 5' coding region of ctdA. The phenotypes of these two ctdA insertion mutants are, however, dissimilar. KZR9A12 produces Bchl and accumulates spheroidene, while KZR8D7 lacks Bchl (Zsebo and Hearst, 1984). KZR8B3 contains an insertion in the 5' control region of the divergently transcribed ctdA and ctdF genes, between bp 2437 and 2481, but accumulates wild-type carotenoids (Table II). The insertion in KZR9F4 lies between bp 2755 and 2915, at the 5' end of the ctdF gene. Both KZR9D10 and KZR9F12 contain Tn5.7 insertions within the ctdC gene, between bp 6037 and 6037 and 6076 and 6773, respectively. The KZR9A11 Tn5.7 is inserted between bp 8461 and 8485, either within the 5' coding region of ctdE or less than five nucleotides 5' to the proposed ATG start (Armstrong et al., 1989). KZR8F6 bears a Tn5.7 insertion between bp 8948 and 9047, also within ctdE, which is consistent with the blue-green phenotype of this strain (Fig. 2). Both KZR3H3 and KZR8F2 carry Tn5.7 insertions within ctdF, between bp 9334 and 9600, and 9601 and 9835, respectively.

Determination of Carotenoid Precursors Accumulated in Blue-Green Mutants Using a New in Vitro System—We wanted to characterize the compounds accumulated in R. capsulatus ctdB, ctdC, and ctdJ blue-green mutants for a better understanding of the early steps in carotenoid biosynthesis. The carotenoid biosynthetic blockages in such strains had not been previously determined, and no functions had been assigned to the products of these genes. We also wished to determine the biochemical intermediate accumulated in the ctdF Tn5.7 insertion strain KZR9F4. If ctdF and ctdB were part
of the same operon, as has been suggested both on the basis of the DNA sequence of this region (Armstrong et al., 1989) and the phenotypes of interposon insertion mutants (Giuliano et al., 1988), one might expect the same intermediate to be accumulated in both a crtB mutant and the crtTn5.7 mutant.

In order to define the biochemical functions of the early crt gene products we developed an in vitro carotenoid biosynthesis system. Label is added to a cell-free extract in the form of [%]IPP, a five-carbon compound which is a basic building block of all isoprenoids, including phytoene and its pyrophosphate precursors (Fig. 1). Incorporation of label is monitored by HPLC analysis of the in vitro reaction products.

Using this in vitro assay, we observed comparable efficiencies of [%]IPP incorporation into phytoene and its pyrophosphate precursors in cell-free extracts from SB1003 wild-type and the crt mutant strains tested. Extracts of W4 (CrtB-) accumulate GGPP but neither PPPPP nor phytoene, while KZR8A1 (CrtE-) extracts accumulate PPPPP but not phytoene (Table IV). Extracts of KZR9E3 (CrtJ-) accumulate GGPP and small amounts of another unidentified compound\(^1\) which is not one of the intermediates shown in Fig. 1. The simplest interpretation of these data is that CrtE is required for the synthesis of phytoene from PPPPP, while both CrtB and CrtJ are required for the synthesis of PPPPP from GGPP. We propose that the crtE gene encodes phytoene synthetase, while CrtB encodes at least one component of PPPPP synthetase. CrtJ may have a direct enzymatic role in the conversion of GGPP to PPPPP, although this proposal should be considered tentative until the unknown compound can be identified. Alternatively, either CrtB or CrtJ may be required for the regulation or stabilization of PPPPP synthetase activity. The provisional assignment of the CrtE, CrtB, and CrtJ enzymatic functions is reflected in the updated R. capsulatus carotenoid biosynthetic pathway shown in Fig. 1.

The in vitro system described here does not convert phytoene into later carotenoids. This could be due to dissociation of the phytoene synthetase from membranes containing the later carotenoid biosynthetic enzymes under the conditions used. Phytoene synthetase is found in soluble or peripheral membrane fractions in other systems (Bramley and MacKenzie, 1988) while phytoene dehydrogenase is membrane-bound in R. capsulatus (Schmidt et al., 1989; Bartley and Scolnik, 1989). Alternatively, the isomer of phytoene produced in the in vitro system may not be recognized as a substrate by the phytoene dehydrogenase (see below; Fig. 1). The in vitro system did not allow us to unequivocally demonstrate that no carotenoids beyond phytoene were synthesized in KZR9F4 (CrtJ-). We therefore also examined the in vivo accumulation of phytoene in this strain (Table IV). KZR9F4 accumulates approximately 15-fold more phytoene than SB1003 wild type in vivo. Dark grown chemoheterotrophic and anaerobic photosynthetic cultures of R. capsulatus KZR9F4 accumulate all-trans and 15-cis phytoene in 58:42 and 50:50 ratios, respectively, as determined by HPLC separation of the extracted phytoene isomers.\(^2\) The distribution of the phytoene isomers produced in the in vivo system remains to be determined, however.

**Discussion**

**Tn5.7 Mapping and Operon Structure of the crt Genes—**

Tn5.7 insertions are polar, although about one-third of all insertions allow for low constitutive levels of expression of distal genes on the same operon (Berg et al., 1980). Even in these cases, however, residual transcription of distal genes seldom restores appreciable activity of their gene products (Berg and Berg, 1987). Tn5.7 contains the IS50 elements of Tn5 at its left and right borders (Zsebo et al., 1984). The polarity of Tn5.7 insertions has allowed us to study the operon structure of the crt gene cluster by the examination of mutant phenotypes. We have recently proposed that the crt genes form a minimum of four operons, crtA, crtIBK, crtDC, and crtEF, based on the complete nucleotide sequence and the organization of the crt gene cluster (Armstrong et al., 1989). Giuliano et al. (1988) have proposed that the crtA, C, D, E, and F genes each constitute separate operons and that crtI and crtB may be on the same operon. These conclusions were based on the carotenoid intermediates accumulated in interposon mutants and on complementation analyses.

Each of the Tn5.7 insertions in strains KZR9A1, KZR9F4, KZR9D10, KZR9F912, KZR8A1, KZR8F6, and KZR3H3, and

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\(^{a}\) A. Schmidt and G. Sandmann, unpublished data.

\(^{b}\) A. Connor and G. Britton, personal communication.
The Tn5.7 insertion in strain KZR8B3 lies in the crtA-crtl 5' flanking region, between bp 2437 and 2481 (Fig. 5, Table IV). This mutation causes no qualitative change in the wild-type carotenoid pigmentation (Fig. 2) and thus probably lies between the cis-acting elements required for the expression of the divergently transcribed crtA and crtl genes. A promoter for crtA is therefore located between bp 2391 and 2481, while a promoter for crtl is located between bp 2437 and 2551. Alternative interpretations of the KZR8B3 phenotype would be the fortuitous creation of a new promoter at the Tn5.7 insertion site or transcription initiated from a promoter within Tn5.7. We consider these explanations unlikely because an interposon insertion at a nearby NruI site (bp 2471) also has no qualitative effect on pigmentation (Giuliano et al., 1988; Bartley and Scolnik, 1989). The interposon insertion further defines the boundaries of the crtA and crtl cis-acting elements. A crtA promoter may lie between bp 2393 and 2471, while a promoter for crtl may lie between bp 2472 and 2551 (Fig. 5). crtl and crtA mRNAs accumulate differentially during the shift from aerobic respiratory growth to anaerobic photosynthetic growth, suggesting that the promoters for these two genes may have different structures. We have proposed that an E. coli-like σAP recognition sequence located from bp 2489 to 2516 may function as a promoter for crtl (Armstrong et al., 1989). Two palindromic sequences that may

KZR8F2 lies within the coding region of one of the crt genes (Fig. 4). The phenotypes of these mutants (Table II and Fig. 2) correlate directly with the proposed functions of the interrupted genes. The Tn5.7 insertions in strains KZR8D7 and KZR8B3 lead to unexpected phenotypes, however.

The transposon in KZR8D7 lies within the 3' coding region of crtA (bp 999-1244) although this mutant lacks Bchl. We have recently suggested that this phenotype is probably due to polar inactivation of a downstream gene, possibly ORF H, which is part of an operon required for Bchl synthesis (Armstrong et al., 1989) and whose transcription initiation signals overlap the 3' coding region of crtA. We do not know if KZR8D7 is also CrtA- because this phenotype would be masked by the earlier blockage in carotenoid biosynthesis due to the crtD223 point mutation (Table I). Taylor et al. (1983) have determined that the BamHI-H fragment of pRPS404 (bp 1-3908 in Fig. 4) complements the bchD1008 point mutation, suggesting that the closest restriction site the bchD gene is contained in this region. The mutation in KZR8D7 was originally assigned to a newly defined bchI locus on the basis of complementation analysis (Zsebo and Hearst, 1984), although Giuliano et al. (1988) have observed that a nearby interposon insertion at bp 1303 leads to a bchI+ phenotype (Fig. 4). The relationship between ORF H and the bchI and bchD genetic loci thus remains to be clarified. Because KZR9A12 produces Bchl while KZR8D7 and the interposon mutant do not, we suggest that crtA and ORF H are probably not on the same operon.

The relationship between ORF H and the bchI and bchD genetic loci thus remains to be clarified. Because KZR9A12 produces Bchl while KZR8D7 and the interposon mutant do not, we suggest that crtA and ORF H are probably not on the same operon.

G. Armstrong, unpublished data.
Fig. 4. Location of Tn5.7 insertions within the *crt* gene cluster. Vertical numbers indicate the nucleotide positions of translational starts and stops for the *crt* genes (shaded in gray) and the neighboring genes. Detached arrowheads on ORF H and *bchC* indicate their extension into the adjacent restriction fragments. Triangles and solid bars below denote the regions of Tn5.7 insertions in different *R. capsulatus* KZR mutants (see also Table III). Nucleotide locations of the insertion regions are given in the text. Solid circles show the locations of palindromic sequences proposed to be involved in the regulation of transcription (Armstrong et al., 1989). Restriction sites important to the mapping of Tn5.7 insertions are shown below (B, BamHI; P, PstI; A, AvaII; Bn, BanII). All BamHI and PstI sites are shown, while only those AvaII and BanII sites relevant for Tn5.7 mapping in strains KZR8B3 and KZRSAl, respectively, are shown. Flanking PstI sites not shown in this figure are located approximately 700 bp to the left of bp 1, and 1154 bp to the right of bp 11039.

We offer two possible explanations to account for the discrepancy in the phenotypes of the *crtI* interposon and Tn5.7 mutants. If one assumed that *crtI* and *crtB* are on the same operon, the mutant phenotypes can be explained by postulating that the Tn5.7 insertion in KZR9F4 fortuitously supplies a promoter, thus permitting the continued expression of the downstream *crtB* gene. If, however, *crtB* and *crtI* are contained on two separate operons, an alternative explanation might be that the cis-acting elements required for the transcription of *crtB* overlap with the 3' coding region of *crtI*. If the *crtB* cis-acting elements were located 3' to the Tn5.7 insertion but 5' to the interposon insertions (Fig. 4), only the *crtI* interposons would show a polar effect on *crtB* expression.

In support of this second alternative, we have previously identified a possible rho-independent transcription termination signal 3' to *crtI* (Armstrong et al., 1989). Additional experiments will be required to distinguish between these alternatives.

One interposon insertion described by Giuliano et al. (1988) lies within a region which we originally designated as ORF J (Armstrong et al., 1989), and which has subsequently been proposed to be the *bchC* Bchl biosynthesis gene (Fig. 4) (Young et al., 1989). The *crtF* Tn5.7 insertions described here do not qualitatively affect Bchl biosynthesis. We have previously identified a possible rho-independent transcription terminator 3' to *crtF* and a possible σ70 promoter sequence 5' to *bchC* (Armstrong et al., 1989). Recent experiments indicate that this sequence functions as a promoter when the *bchC* gene is introduced into *E. coli*. All of these data suggest that cotranscription of *crtF* and *bchC* is not obligatory, in agreement with the results of Young et al. (1989).

<TABLE IV>

<table>
<thead>
<tr>
<th>Strain</th>
<th>GPP made in vitro</th>
<th>GPPPP made in vitro</th>
<th>Phytoene made in vitro</th>
<th>Phytoene found in vivo</th>
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<td>7776</td>
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<td>7776</td>
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<tr>
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<td></td>
<td>7776</td>
<td>2149.1</td>
</tr>
</tbody>
</table>

* Genotypes are given in Table I.
* ["C"]IPP incorporated in dpm/ml cell-free extract; dashes indicate the compound was not detected.
* Pyrophosphates were converted to their corresponding alcohols for HPLC analysis (see "Materials and Methods").
* Accumulation in ng/ml cell-free extract; dashes indicate the compound was not detected.

<FIGURE>

FIG. 5. A detailed characterization of the sequences required for transcription of the downstream *crtB* gene. If, however, *crtB* and *crtI* are contained on two separate operons, an alternative explanation might be that the cis-acting elements required for the transcription of *crtB* overlap with the 3' coding region of *crtI*. If the *crtB* cis-acting elements were located 3' to the Tn5.7 insertion but 5' to the interposon insertions (Fig. 4), only the *crtI* interposons would show a polar effect on *crtB* expression.

In support of this second alternative, we have previously identified a possible rho-independent transcription termination signal 3' to *crtI* (Armstrong et al., 1989). Additional experiments will be required to distinguish between these alternatives.

One interposon insertion described by Giuliano et al. (1988) lies within a region which we originally designated as ORF J (Armstrong et al., 1989), and which has subsequently been proposed to be the *bchC* Bchl biosynthesis gene (Fig. 4) (Young et al., 1989). The *crtF* Tn5.7 insertions described here do not qualitatively affect Bchl biosynthesis. We have previously identified a possible rho-independent transcription terminator 3' to *crtF* and a possible σ70 promoter sequence 5' to *bchC* (Armstrong et al., 1989). Recent experiments indicate that this sequence functions as a promoter when the *bchC* gene is introduced into *E. coli*. All of these data suggest that cotranscription of *crtF* and *bchC* is not obligatory, in agreement with the results of Young et al. (1989).
Taylor et al. (1983) have proposed that crtF mutants accumulate secondary mutations as the result of selection rather than an enhanced mutation rate. crtI' mutants accumulate demethylspheroidene as the major carotenoid under anaerobic photosynthetic conditions (Seolnık et al., 1980). The stability of the phenotypes of the crtFred232 double mutants KZR3H3 and KZR8F2 suggests that the hydroxynoerosporene accumulated in the double mutants has no deleterious biological effect, and implicates demethylspheroidened as agents involved in the instability of crtF mutants. The basis of the selection against crtF mutants remains to be determined.

Biochemical Functions of CrtB, CrtE, and CrtJ in the R. capsulatus Carotenoid Biosynthetic Pathway—In R. capsulatus we propose that at least three gene products, CrtB, CrtE and CrtJ, are required for the conversion of GGPP to phytoene via PPPP. A bifunctional phytoene synthetase enzyme catalyzing both the conversions of GGPP to PPPP and the subsequent formation of phytoene from PPPP has recently been purified from Capsicum (red pepper) chromoplasts (Dogbo et al., 1988). This is the first reported purification to homogeneity of an enzyme involved in the synthesis of PPPP or phytoene. Both biosynthetic activities are kinetically coupled and are associated with a single polypeptide of apparent molecular mass 47.5 kDa. For comparison, the molecular masses of the R. capsulatus CrtB and CrtE polypeptides deduced from the DNA sequence are 37 and 30 kDa, respectively (Armstrong et al., 1989). No information is available about CrtJ, although the structure of the crtJ gene is currently under investigation. The contrasts between the prokaryotic and eukaryotic systems suggest that the PPPP synthetase and phytoene synthetase enzymatic activities may have been fused into a single polypeptide during the course of evolution from photosynthetic bacteria to higher plants.

The accumulation of GGPP in strain W4 (Table IV) and our proposal that CrtB is a component of the PPPP synthetase are consistent with earlier observations that a mixture of cell-free extracts from W4, which was known to be blocked before phytoene biosynthesis, and a crtI phytoene-accumulating point mutant complemented the biochemical defect of the crtI mutant in vitro (Giuliano et al., 1986). Recent evidence indicates that antibodies raised against an R. capsulatus CrtI fusion protein inhibit the dehydrogenation of phytoene in vitro using a solubilized membrane fraction from the cyanobacterium Aphanocapsa (Schmidt et al., 1989). The in vitro accumulation of phytoene in KZR9F4 confirms that the crtI gene product is required for phytoene dehydrogenation. KZR9F4 accumulates roughly equal amounts of all-trans and 15-cis phytoene in vitro, raising the question of whether the phytoene synthetase, CrtE, produces both isomers or whether one isomer is the primary product and is subsequently isomerized in vivo. Specific all-trans and 15-cis phytoene synthetases appear to exist in different carotenogenic organisms (reviewed in Bramley and Mackenzie, 1988). A more precise definition of the roles of CrtB, CrtJ, and CrtE in the early steps of carotenoid biosynthesis will require more detailed biochemical studies.

In summary, we have correlated the phenotypes and genotypes of 10 mutants of R. capsulatus carrying Tn5.7 insertions in the crt biosynthesis gene cluster by a combination of high resolution mapping of the mutation sites, along with characterization of carotenoid accumulated. Development of a new in vitro assay for the synthesis of phytoene and its pyrophosphate precursors has allowed us for the first time to examine the enzymatic blockages in crtB, crtE, and crtJ blue-green mutants. We propose that CrtB is one component of the PPPP synthetase and that CrtE is the phytoene synthetase based on the accumulation of phytoene precursors in the in vitro system, and present an updated R. capsulatus carotenoid biosynthetic pathway incorporating these results. The combination of the Tn5.7 mapping, phytoene accumulation in a crt1 Tn5.7 mutant, and GGPP accumulation in a crtB point mutant suggests that crt1 and crtB may not be on the same operon. Mapping results and precursor accumulation also suggest that neither crtA and ORF H nor crtF and bchC are on the same operons, although Young et al. (1989) have proposed the existence of transcriptional readthrough between the latter pair of genes. These authors, however, based a portion of their argument on the absence of potential transcription terminators 3' to crtF, although the existence of a possible terminator in this region has been previously reported (Armstrong et al., 1989). Giuliano et al. (1989) have shown that interposon insertions in crtD are not polar on crtC, indicating that these genes are on separate operons, while Young et al. (1989) demonstrated that crtEF form an operon. Thus, a minimal operon structure for the crt gene cluster of R. capsulatus, R. gelatinosus, R. capsulatus, and R. capsulatus now seems likely. Whether crtB and crtK are cotranscribed is uncertain, as mRNAs for these genes are expressed differentially during the shift from respiratory to photosynthetic growth. We have proposed possible overlaps between the cis-acting elements required for transcription of ORF H with the coding region of crtA and for cis-acting elements required for crtB transcription with crt coding sequences. Taken in conjunction with the results of Young et al. (1989), the overlap of cis-acting elements for a downstream gene with the coding region of an upstream gene may reflect a general feature of transcriptional organization in the R. capsulatus photosynthesis gene cluster.

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8 G. Armstrong and M. Alberti, unpublished data.
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