The Gene \textit{crtI} Mediates the Conversion of Phytoene into Colored Carotenoids in \textit{Rhodopseudomonas capsulata}\textsuperscript{*}

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Carotenoids are membrane pigments present in all photosynthetic organisms, providing essential photoprotective functions. The first carotenoid formed in the pathway is phytoene, a colorless compound which is then converted into colored carotenoids by a series of dehydrogenation reactions. In the photosynthetic bacterium \textit{Rhodopseudomonas capsulata} mutations that affect carotenoid biosynthesis before colored carotenoids are formed have a “blue-green” phenotype as opposed to the “red” of wild type cells. We have extracted carotenoids from several blue-green mutants and found that two strains (BPY69 and BPY102) accumulate phytoene and no colored carotenoids. These mutants failed to dehydrogenate phytoene in an \textit{in vitro} assay. However, dehydrogenation of this compound can be achieved \textit{in vitro} by adding a cell-free extract from another blue-green mutant blocked earlier in the pathway. Genetic complementation and deletion mapping indicate that the gene \textit{crtI} is responsible for the conversion of phytoene into colored carotenoids in these mutants.

All photosynthetic and many non-photosynthetic organisms can be sensitive to the combination of light and oxygen, which through the action of oxygen radicals, can start a series of potentially lethal oxidations. Protection against this process is provided by carotenoids, pigments that in their wild type form are colored membrane lipids. These compounds are synthesized from colorless precursors through a series of dehydrogenation reactions which create a set of conjugated double bonds in the center of the molecule (the chromophore) responsible for absorption of light in the visible region of the spectrum. Only colored carotenoids, that is compounds with at least nine double bonds in the chromophore, are effective in photoprotection. Other functions for carotenoids have been proposed, mainly as secondary antenna pigments and precursors of vitamin A in mammals (1).

In a previous study of the biosynthesis of colored carotenoids in the photosynthetic bacterium \textit{Rhodopseudomonas capsulata} (2) \textit{(R. capsulatus in the nomenclature of Imhoff et al. (3))} we showed that, as in other photosynthetic prokaryotes, there is an excellent correlation between the genetic and the biochemical information obtained from mutants affecting carotenoid biosynthesis. It is therefore possible to dissect the pathway by analyzing the precursors accumulated by different mutant strains.

The main carotenoids accumulated by \textit{R. capsulata} under anaerobic conditions are hydroxyxpheroidene and spheroidene; under aerobic conditions spheroidenone predominates (2) (see Fig. 5). The phenotypes of cells grown under these two conditions are brown and red, respectively. All genes affecting the synthesis of colored carotenoids have been mapped to a single genetic region of this bacterium (2, 4, 5). Mutations in four loci within this region \textit{(crtE, crtB, crtl, and \textit{crtJ})} results in a blue-green phenotype, due to the lack of accumulation of colored carotenoids and the presence of bacteriochlorophyll (4). In this paper we report that two mutations which cause accumulation of phytoene, the first carotenoid in the pathway, can be complemented by the \textit{crtI} gene, suggesting that it codes for the enzyme phytoene dehydrogenase. We have also designed an \textit{in vitro} assay to study carotenoid biosynthesis in this bacterium.

**MATERIALS AND METHODS**

\textbf{Strains and Media—}SB1003 was the wild type \textit{R. capsulatus} strain used. BPY69 and BPY102 are blue-green mutants derived from the brown mutant strain FY1291 (5), and they were kindly provided by B. Marrs (Du Pont). The strains and plasmids used in this paper are listed in Table I. The rich medium was FYE (5) supplemented with 2 mM MgCl\textsubscript{2} and 2 mM CaCl\textsubscript{2} (MPYE) and the minimal medium RCVB (6).

\textbf{Plasmids and Genetic Crosses—}Restriction enzymes were purchased from either New England Biolabs or Bethesda Research Laboratories. \textit{Escherichia coli} competent cells were prepared by method of Hanahan (7). The R-factor plasmid pRPS404 and the plasmid pRPSB4 and pRPSB105 were previously described (5). The plasmid pRGG102 was constructed by inserting the \textit{BglII D} fragment from pRPS404 (8) into the \textit{BglII} site of pRK290 (9). Mobilization was achieved by triparental mating with the helper pRK2013 (9). The plasmid pBG201 was constructed by ligating the \textit{BamHI H} fragment of pRPS404 into pBR22. The pBG202 series of plasmids contain a spectinomycin resistance gene cartridge derived from pRP455 (10). In this paper the helper plasmid was pDPT51 (5). Matings were performed on cored RCVB plates for 6–12 h. The bacteria were then spread and the plate was overlaid with 2.5 ml of RCVB top agar containing enough antibiotic to give a final concentration of 10 \textmu g/ml of either tetracycline (for pRK290), kanamycin, or spectinomycin. Plates were incubated photosynthetically at 35 °C for 3–6 days.

\textbf{Carotenoid Extraction—} \textit{R. capsulata} was grown photosynthetically in RCVB and the cells were extracted in the dark with a mixture of 50% (v/v) ethyl ether and methanol. The extract was cleared by centrifugation, dried under a stream of nitrogen, and resuspended in petroleum ether (b.p. 35–65 °C). Absorption spectra were obtained with a Perkin-Elmer 3840 spectrophotometer. Phytoene was purified on silicag gel thin layer chromatography plates using 10% acetone/petroleum ether (v/v) as solvent. The \textit{Rf} of different carotenoids in this solvent are: 0.87 for phytoene, phytol, pseudo- and neuropsycheroidene. When necessary, bands were scraped, extracted with acetone, dried under a stream of nitrogen, and resuspended in petroleum ether.

Trivial and semi-systematic names of the carotenoids mentioned

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The abbreviations used in this table are: Km, kanamycin; Ap, ampicillin; Tc, tetracycline; Sp, spectinomycin.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Relevant markers</th>
<th>References and comments</th>
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<tbody>
<tr>
<td>R. capsulata</td>
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<tr>
<td>SB1003</td>
<td>Wild type</td>
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<tr>
<td>BPY69</td>
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<td>This work, from B. Marrs</td>
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</tr>
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<td>pRPSB105</td>
<td>Km', Ap'</td>
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<tr>
<td>pRK290</td>
<td>Tc'</td>
<td>A vector (9)</td>
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<td>pRGG102</td>
<td>Tc'</td>
<td></td>
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<td>pBG201</td>
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<td>Ap', Sp'</td>
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TABLE II

Trivial and semi-systematic names (18) of the carotenoids mentioned in this publication

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<th>Carotenoid Name</th>
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<tr>
<td>Phytoene</td>
<td>15-cis-7,8,11,12,7',8',11',12'-Oc-tahydro-ψ,ψ-carotene</td>
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<td>Phytofluene</td>
<td>15-cis-7,8,11,12,7',8'-Hexahydro-ψ,ψ-carotene</td>
</tr>
<tr>
<td>7,8,11,12-Tetrahydrolycopene</td>
<td>7,8,11,12-Tetrahydro-ψ,ψ-carotene</td>
</tr>
<tr>
<td>ζ-Carotene</td>
<td>7,8,7',8'-Tetrahydro-ψ,ψ-carotene</td>
</tr>
<tr>
<td>Neusporone</td>
<td>7,8-Dihydro-ψ,ψ-carotene</td>
</tr>
<tr>
<td>Hydroxyspheroidene</td>
<td>1'-Methoxy-3',4'-didehydro-1,2,7,8,1',2'-hexahydro-ψ,ψ-carotene-1-ol</td>
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<tr>
<td>Spheroidene</td>
<td>1-Methoxy-3,4-didehydro-1,2,7,8'-tetrahydro-ψ,ψ-carotene</td>
</tr>
<tr>
<td>Spheroidenone</td>
<td>1-Methoxy-3,4-didehydro-1,2,7,8'-tetrahydro-ψ,ψ-caroten-2-one</td>
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</table>

in this paper are shown in Table II and an updated genetic-biochemical pathway is shown in Fig. 5.

In Vitro Dehydrogenation of Phytoene—Bacteria were grown photosynthetically in MYPYE to early stationary phase. 600 ml of culture were pelleted at 6000 x g for 10 min. The pellet was washed in cold buffer (100 mM Tris, pH 8, 4 mM dithiothreitol) and resuspended in 6 ml of the same buffer. Suspensions of the different blue-green strains were mixed in a 1:1 ratio and lysed by passage through a French pressure cell set at 18,000 psi. The controls were supplemented with an equal volume of buffer. The reaction was supplemented with ATP (10 mM), NADP (2 mM), MnCl₂ (6 mM), and MgCl₂ (4 mM) and allowed to proceed at 32 °C in the dark. Under these conditions the reaction, as judged by the formation of phytofluene, is linear for at least 4 h. At this time approximately 15% of the input phytoene is dehydrogenated. The reaction was stopped by adding EDTA (25 mM) and placing on ice. Membranes were pelleted at 48,000 rpm for 1 h in a Beckman SW 50.1 rotor. The pellet was extracted with 5 ml of acetone and then 5 ml of petroleum ether were added. The upper phase, containing the carotenoids, was transferred to a new tube and dried under a stream of nitrogen. This sample was resuspended in the appropriate solvent and fractionated by either column or thin layer chromatography. For column chromatography a magnesium oxide column (8 x 100 mm) equilibrated with hexane was used. Carotenoids were loaded in hexane and eluted with the solvents indicated in Table III.

RESULTS

The Mutants BPY69 and BPY102 Accumulate Phytoene—A mutant in the gene for phytoene dehydrogenase is expected to have a blue-green phenotype and to accumulate phytoene. We grew several R. capsulata blue-green strains under photosynthetic conditions, pelleted the cells by centrifugation, and extracted the pigments with organic solvents. Analysis of

![Absorption spectrum of purified phytoene](image-url)

Fig. 1. Absorption spectrum of purified phytoene. Phytoene was extracted from BPY69 and purified in silica gel plates as described under "Materials and Methods." The structure of the pigment is shown on top. A similar spectrum was obtained from BPY102.

![In vitro dehydrogenation of phytoene](image-url)

Fig. 2. In vitro dehydrogenation of phytoene. Cell homogenates from BPY69 (---), W4 (-----), and a 1:1 mixture of both strains (-----) were prepared according to "Materials and Methods" and incubated for 2 h. Carotenoids from 5-ml reactions were extracted and purified by thin layer chromatography. The region of the chromatogram with R₅ = 0.85–0.90 was eluted with acetone and resuspended in petroleum ether. Extracts from BPY102 show similar spectra in this reaction (not shown).
were extracted and analyzed by absorption spectroscopy. For the W4 strain. Interestingly, one of the carotenoids isolated from BPY102 mutants can be dehydrogenated by the extracts from it, suggesting that it is blocked in the pathway before phytoene. Mutant—We took advantage of the large amounts of phytoene accumulated by the BPY69 and BPY102 strains (BPY69 and BPY102) accumulate a carotenoid compound with absorption maxima at 275, 285, and 297 nm, consistent with the values obtained for phytoene (11, 13) (Fig. 1).

For reasons that are not clear at the present time, secondary mutations affecting pigment biosynthesis can be obtained from CrtF mutants of R. capsulata. Both BPY69 and BPY102 derived from FY1291, which carries a crtF 129 mutation (5), and thus they are double mutants. When cells are grown under aerobic conditions, CrtF mutants have a brown phenotype, easily distinguishable from the wild type red color.

In Vitro, Both Mutants Lack Phytoene Dehydrogenase Activity and Can Be Complemented by Extracts from a crtB Mutant—We took advantage of the large amounts of phytoene accumulated by both mutants to design an in vitro assay. Cell-free extracts (described under "Materials and Methods") were incubated in the dark and then carotenoids were extracted and analyzed by absorption spectroscopy. Formation of colored carotenoids in this system was only observed when an extract from the blue-green mutant W4 (crtB) was included (Fig. 2). W4 does not accumulate any carotenoid, indicating that the phytoene accumulated by the BPY69 and BPY102 mutants can be dehydrogenated by the extracts from the W4 strain. Interestingly, one of the carotenoids isolated seems to have absorption maxima that correspond more closely to ß-carotene than to 7,8,11,12-tetrahydrolycopene, the postulated bacterial compound (Ref. 12 and Fig. 5). The identity of this compound will have to be determined by mass spectroscopy.

**Both Point Mutations Are Complemented by the pRPS404 Plasmid—** Marrs and co-workers (5) have isolated a R-prime factor (pRPS404) containing a 50-kilobase pair insert with all the known genes affecting pigment biosynthesis in this organism. We mobilized this plasmid by conjugation into both mutants, selecting for kanamycin, the marker of pRPS404. Most of the exconjugants were red, indicating that pRPS404 complements both blue-green mutations, as well as the crtF 129 mutation present in both strains. Rare colonies with other color phenotypes, arising from recombination events between the insert of pRPS404 and the chromosome, were also observed.

**The Point Mutations Map in the BamHI H Fragment—** In order to further localize the gene we mobilized into the two blue-green mutants two plasmids (pRPSB4 and pRPSB105) which contain BamHI subfragments of the photosynthetic region. The plasmid pRPSB4, which carries the BamHI H fragment, complemented both blue-green mutations, as shown in Fig. 3, giving rise to brown colonies. The main carotenoid accumulated during photosynthetic growth of these mutants was demethylspheroidene, as determined by thin layer chromatography (2). This means that the BamHI H fragment was capable of complementing the mutations that result in the brown color phenotypes of the blue-green mutants.

### Table III

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rp</th>
<th>Column fraction</th>
<th>Absorption maxima</th>
<th>Identification</th>
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</thead>
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<tr>
<td>I</td>
<td>0.72</td>
<td>1-4</td>
<td>275, 285, 297</td>
<td>Phytoene</td>
</tr>
<tr>
<td>II</td>
<td>0.67</td>
<td>5-6</td>
<td>331, 348, 366</td>
<td>Phytofluene</td>
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<tr>
<td>III</td>
<td>0.62</td>
<td>9-11</td>
<td>377, 398, 423</td>
<td>ß-Carotene</td>
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<tr>
<td>IV</td>
<td>0.58</td>
<td>14-15</td>
<td>414, 437, 467</td>
<td>Neurosporene</td>
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</table>

*Separation by thin layer chromatography. Solvent used: 0.5% acetone/petroleum ether.

*Carotenoids loaded in a magnesium oxide column were eluted with the following proportions of acetone in hexane: fractions 1-3, 0%; fractions 4-6, 3%; fractions 7-10, 5%; fractions 11-15, 10%. Each fraction represents 1 column volume.

*Absorption maxima in petroleum ether.

*Tentative identification based on absorption maxima in petroleum ether.

The absorption spectra of these extracts showed that two strains (BPY69 and BPY102) accumulate a carotenoid compound with absorption maxima at 275, 285, and 297 nm, consistent with the values obtained for phytoene (11, 13) (Fig. 1).

For reasons that are not clear at the present time, secondary mutations affecting pigment biosynthesis can be obtained from CrtF mutants of R. capsulata (5). Both BPY69 and BPY102 derived from FY1291, which carries a crtF 129 mutation (5), and thus they are double mutants. When cells are grown under aerobic conditions, CrtF mutants have a brown phenotype, easily distinguishable from the wild type red color.

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![Fig. 3. Absorption spectra in petroleum ether of total carotenoids from different R. capsulata strains.](image-url)

Cells were grown photosynthetically in medium RCVB and pigments were extracted according to "Materials and Methods." Traces from BPY69 and BPY102 (---), SB1003 (-- --) and BPY69 (pRPSB4) (---) are shown. Wavelengths of absorption maxima are indicated on top.
accumulation of phytoene, but not the crtF mutation, as expected from the physical map of the region (4).
We also cloned the BgIII D fragment from pRPS404 into pRK290, a broad host range plasmid (9). The resulting construct (pRGG102) also complemented both blue-green mutations (not shown).

Deletion Analysis of the BamHI H Fragment—We used deletions in the BamHI H fragment to map both mutations. This segment was cloned in pBR322 and several deletions were constructed from the left and right ends by using different restriction enzymes (Fig. 4). We added spectinomycin' gene cartridges to this plasmids because the antibiotic markers of pBR322 are not expressed in R. capsulata (5). These constructs were mobilized by conjugation into the blue-green mutants BPY69 and BPY102, selecting for spectinomycin'. Fig. 4 shows that all the plasmids except pBGG202E rescue both mutations, which therefore have to map in the 930-base pair BamHI-EcoRI fragment.

An unusual situation was encountered in the conjugation experiments reported in Fig. 4. In general if a plasmid contains a complete gene with an active promoter, 100% of the exconjugants show a complemented phenotype. If, however, a fragment of a gene containing the wild type version of the mutated sequence is present in the episome, recombination with the chromosomal marker takes place at a low (less than 10% (14)) frequency, resulting in "rescue" of the chromosomal marker. The results obtained with all the plasmids described in Fig. 4 ranged between 10 and 50% of the colonies having a brown phenotype. These frequencies were also variable in different experiments. One would expect the larger fragments to show complementation of the crtI mutations but this was never observed, indicating an aberrant behavior of the plasmids that will have to be explored further.

DISCUSSION
Carotenoids are ubiquitous in nature, mainly because of their role in photoprotection. De novo synthesis has been shown to occur in both photosynthetic and non-photosynthetic bacteria, algae, fungi, red yeast, and higher plants (15). In all cases the first carotenoid formed is phytoene, which is then converted into colored carotenoids through a series of dehydrogenation reactions (12). The first step in this process is mediated by the enzyme phytoene dehydrogenase. There are a number of mutants in both plants and microbes that accumulate phytoene. It is therefore possible that the information we obtain from the analysis of crtI may help us understand the nature of mutations in other systems.
The chemistry of carotenoids has been extensively studied during the last 30 years (1). However, we have practically no information on the molecular biology of the process. This is partly due to the fact that the enzymes for carotenoid biosynthesis are membrane-bound proteins, which are difficult to purify to homogeneity in an active form (15).
In this paper we show that the mutants BPY69 and BPY102 lack phytoene dehydrogenase activity both in vitro and in vivo. The crtI gene is capable of restoring this activity, suggesting that it may code for the corresponding enzyme. Thus, we have now assigned putative functions for five of the genes affecting carotenoid biosynthesis in this organism (Fig. 5).
It could be argued that the gene cloned is actually a regulatory sequence, acting on an unlinked phytoene dehydrogenase gene. This is unlikely because it has been shown that the genes for carotenoid biosynthesis are not directly regulated in this organism (16). However, formal identification of the function of the protein product of the crtI gene will require the demonstration that it has enzymatic activity in vitro. At the present time this is very difficult because the enzymes for carotenoid biosynthesis are rapidly inactivated when separated from their membrane environment (15). The work reported in this paper may lead to alternative approaches, such as inhibition of the in vitro reaction with specific antibodies, that may facilitate the study of carotenoid biosynthesis in this organism.
It is interesting to note that none of the blue-green mutants...
we studied accumulates phytofluene or 7,8,11,12-tetrahydro-
lycopene. A similar situation was described in Rhodopseu-
domonas spheroides by Liaen-Jensen et al. (17) and led these
authors to postulate that only one enzyme mediates the de-
hydrogenation of phytoene into neurosporene. However, these
compounds are made in the in vitro reaction we described
here. Therefore, it should now be possible to use the in vitro
system for complementation studies that may help solve this
problem.

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