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# A genetic-physical map of the *Rhodobacter capsulatus* carotenoid biosynthesis gene cluster

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**Summary.** We used the interposon mutagenesis technique to map a cluster of *Rhodobacter capsulatus* genes that are responsible for the biosynthesis of carotenoids, membrane pigments that protect living organisms from photooxidations. Fifteen interposons define 7 genes (*crtA, crtI, crtB, crtC, crtD, crtE* and *crtF*) in an approximately 8 kb of the chromosome. This cluster is flanked by genes affecting bacteriochlorophyll biosynthesis. Based on the analysis of the phenotypes of the insertional mutants and the results of complementation analyses, we present a genetic and physical map of this region. Our data indicate that, with the possible exception of *crtI* and *crtB*, all genes analyzed are organized as single transcriptional units. We used nuclease S1 protection analysis to study the influence of oxygen on the regulation of expression of 4 genes (*crtA, crtI, crtC* and *crtE*). Under anaerobic conditions, mRNA levels for *crtA, crtC* and *crtE* are elevated and a new *crtE* transcript is detected.

**Key words:** Carotenoids – Photosynthetic bacteria – Interposon mutagenesis – Transcription

## Introduction

Carotenoids are pigments that are essential to protect photosynthetic organisms against photooxidation (Krinsky 1971). Although the chemistry of carotenoids has been extensively studied during the last 50 years (Goodwin 1971), little information exists on the nature of the enzymes that mediate the biosynthesis of these pigments. This paucity of information if mostly due to the fact that the enzymes for carotenoid biosynthesis are membrane-bound, and thus difficult to purify. An analysis of the genes that code for these proteins could provide useful information about their structure and regulation.

Mutations in genes affecting carotenoid biosynthesis have been isolated in many photosynthetic organisms, including the purple photosynthetic bacterium *Rhodobacter capsulatus*. Because this organism is very amenable to genetic manipulations (Scolnik and Marrs 1987), we are currently using it to characterize genes for carotenoid biosynthesis.

Genetic maps for six genes (*crtA, crtB, crtC, crtD, crtE* and *crtF*) in the *R. capsulatus* carotenoid gene cluster were constructed by Yen and Marrs (1976) and Scolnik et al. (1980). After the genes responsible for the synthesis of these compounds were cloned in the R-prime factor pRPS404 by Marrs (1981), physical maps were obtained for four genes: *crtB* by Taylor et al. (1983) and *crtI, crtE* and *crtA* by Zsebo and Hearst (1984). With the identification of *crtI* by Zsebo and Hearst (1984), the cluster appears to contain seven genes. Putative functions have been assigned to five of these genes by Scolnik et al. (1980) and Giuliano et al. (1986).

Here we report the results of combining the techniques of interposon mutagenesis (Scolnik and Haselkorn 1984; Scolnik and Marrs 1987), conjugation-mediated marker rescue (Taylor et al. 1983) and biochemical analysis of carotenoid pigments (Scolnik et al. 1980; Giuliano et al. 1986) to construct a complete physical, genetic and biochemical map of the *R. capsulatus* carotenoid gene cluster.

## Materials and methods

*Strains and growth conditions.* The strains used in this work are shown in Table 1. All the interposon insertions were constructed on the wild-type strain SB1003. *R. capsulatus* cells were grown in MPYE (Giuliano et al. 1986). For aerobic growth 10 ml of the cell suspension was incubated in a 50 ml flask in an environmental chamber at 200 rpm. For photosynthetic growth, cells were grown in 15 ml screw-cap tubes in water tanks with incandescent lights providing a fluence of 40 J/m$^2$ sec. Temperature for both growth modes was 35° C. For RNA extractions, the same light fluence was used for aerobic and anaerobic growth. Genetic crosses were performed according to Giuliano et al. (1986).

*Interposon mutagenesis.* The spectinomycin resistance Ω (Prentki and Krisch 1984) or the kanamycin resistance KM117 (unpublished) interposons were cloned into appropriate DNA fragments by standard techniques (Maniatis et al. 1982) and recombined into the *R. capsulatus* SB1003 chromosome as previously described (Scolnik and Haselkorn 1984).

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**Table 1.** Strains and plasmids

| Strain | Description[a] | References |
|---|---|---|
| *Escherichia coli* | | |
| BGG216 | TEC5 (pBGG216) | This work |
| BGG237 | TEC5 (pBGG237) | This work |
| DGG240 | TEC5 (pDGG240) | This work |
| HB101 | *recA*, $r_B^-$, $m_B^-$ | Boyer and Roulland-Dussoix (1969) |
| RGG105 | HB101 (pRGG105) | Boyer and Roulland-Dussoix (1969) |
| TEC5 | C600 (pDPT51) | Taylor et al. (1983) |
| TEC5-11 | TEC 5 (pRPSB4) | Taylor et al. (1983) |
| TEC5-22 | TEC 5 (pRPSB105) | Taylor et al. (1983) |
| *Rhodobacter capsulatus* | | |
| BPY69 | *crtI*$^-$ | Giuliano et al. (1986) |
| BW604 | *crtE*$^-$ | Yen and Marrs (1976) |
| MB1008 | *bchD* 1008 | Marrs (1981) |
| MB10110 | *bchA* 305 | Marrs (1981) |
| R121 | *crtD*$^-$ | Scolnik et al. (1980) |
| S10DSP | Ω::*crtC* 10 | This work |
| SB4 | KM117::*crtI* 4 | This work |
| SB11 | Ω::*crtC* 11 | This work |
| SB203E | Ω::*crtI* 203 | This work |
| SB203N | Ω::203 | This work |
| SB203S | Ω::*crtA* 203 | This work |
| SB218 | KM117::*crtI* 218 | This work |
| SB221 | Ω::*crtD* 221 | This work |
| SB226 | Ω::*BchlI* 226 | This work |
| SB227 | Ω::*crtD* 227 | This work |
| SB228 | Ω::*crtE* 228 | This work |
| SB229 | W::*bchlC* 229 | This work |
| SB236 | KM117::*crtF* 236 | This work |
| SB238 | Ω::*crtE* 238 | This work |
| SB1003 | Wild-type photopigments | Marrs (1981) |
| SG103 | Ω::*crtB* 103 | This work |
| W4 | *crtB*$^-$ | Yen and Marrs (1976) |
| Y68 | *crtC*$^-$ | Yen and Marrs (1976) |
| Y126 | *crtC*$^-$ | Yen and Marrs (1976) |
| Y262 | GTA overproducer | Yen et al. (1979) |
| Plasmids[b] | | |
| pBGG216 | Sp$^r$ | This work |
| pBGG237 | Km$^r$ | This work |
| pBR322 | Cloning vector | Bolivar et al. (1977) |
| pDGG240 | Km$^r$ | This work |
| pDPT42 | Km$^r$ cloning vector | Taylor et al. (1983) |
| pDPT51 | Conjugation helper | Taylor et al. (1983) |
| pRGG105 | Tet$^r$ | This work |
| pRK290 | Tet$^r$ cloning vector | Ditta et al. (1980) |
| pRPS404 | R′ plasmid | Marrs (1981) |
| pRPSB4 | Km$^r$, carries *Bam*HI H | Taylor et al. (1983) |
| pRPSB105 | Km$^r$, carries *Bam*HI G | Taylor et al. (1983) |

[a] GTA, gene transfer agent; Ω and KM 117 denote, respectively, spectinomycin (Sp; Prentki and Krisch 1984) and kanamycin (Km; P.A. Scolnik, unpublished data) resistance interposons

[b] pBGG216 and pBGG237 are drivatives of pBR322 in which antibiotic resistance is conferred by either Ω or KM117. pRGG105 is a derivative of pRK290, a tetracycline (Tet) resistance vector. The cloning vector for pDGG240 is pDPT42. Inserts for pBGG216, pBGG237, pDGG240 and pRGG105 were obtained from a cosmid library (Scolnik and Haselkorn 1984)

*RNA isolation and nuclease S1 protection analyses.* RNA was isolated from mid-logarithmic SB1003 cells by the method of Belasco et al. (1985). Fifteen micrograms of RNA were co-precipitated with 10000 to 20000 cpm of double-stranded DNA probe, 5′ end-labelled with $\gamma$-[$^{32}$P] ATP and polynucleotide kinase (Maniatis et al. 1982). This amount was previously determined to be in excess with respect to the transcript to be mapped by increasing the probe/RNA ratio until the signal from the protected band showed no increment. The pellet was resuspended in 20 µl of 40 mM PIPES (pH 6.4), 1 mM EDTA, 0.4 M NaCl, 80% (v/v) formamide, heated at 100° C for 2 min, and hybridized overnight at 52° C. One hundred and eighty microliters of ice-cold S1 buffer (50 mM sodium acetate pH 4.6, 0.28 M NaCl, 4.5 mM $ZnSO_4$, 20 ng/µl denatured carrier salmon sperm DNA, 1 unit/µl nuclease S1) was added and the mixture was incubated at room temperature for 1 h. After ethanol precipitation, the reaction was run on 4% urea-acrylamide gels cast in 0.5 × TBE (Maniatis et al. 1982).

*General methods.* Chromatophores were prepared by a sonication procedure (Youvan et al. 1983). Carotenoids were extracted and analyzed by thin layer chromatography as described (Scolnik et al. 1980; Giuliano et al. 1986). *R. capsulatus* chromosomal DNA was extracted using a minipreparation technique: 1 ml of cells was grown aerobically in MPYE, pelleted in a microcentrifuge and the pellet was frozen in dry ice. Cells can be stored at this stage. The pellet was resuspended in 1 ml hot (65° C) lysis buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA, 1% SDS) and incubated at 45° C in the presence of proteinase K (100 µg/ml) for 1 h. The suspension was extracted twice with phenol-chloroform (1:1 v/v) and ethanol precipitated. This procedure yields 20 µg DNA/ml culture. RNAse A (0.5 µg/µg DNA) was added during restriction endonuclease digestions. To obtain a wild-type copy of the *crtD* gene, the *R. capsulatus* cosmid library in pDPT5Cm (Scolnik and Haselkorn 1984) was screened by colony hybridization (Maniatis et al. 1982) with a *Bam*HI G probe derived from pRPS404 (Taylor et al. 1983).

## Results

We constructed 15 insertions into the carotenoid region of the *R. capsulatus* chromosome (Fig. 1). Insertions were constructed using appropriate subclones of pRPS404. However, since this R-prime contains a *crtD223* mutation (Taylor et al. 1983), 4 insertions (S10DSP, SB221, SB227 and SB228) were constructed in wild-type DNA fragments cloned from *R. capsulatus* cosmid library (Scolnik and Haselkorn 1984). Insertions were recombined into the chromosome of the wild-type strain SB1003 using the GTA-mediated technique previously described (Scolnik and Haselkorn 1984) and the correct localization was confirmed by Southern blotting (not shown). All the Ω (Frey and Krisch 1985) and most of the KM117 insertions are polar, thus possible polarity effects on adjacent genes were checked by examining the carotenoids accumulated by the various mutants and by complementation analyses.

Two insertions that affect bacteriochlorophyll biosynthesis (SB226 and SB229) flank a region of approximately 8 kb that contains genes for carotenoid biosynthesis. The phenotypes, as determined by comparison with spectra obtained from known point mutations, are BchlD$^-$ and BchlA$^-$ for SB226 and SB229, respectively (Fig. 1; Fig. 3). These results will be further evaluated in the Discussion.

The first mutant (from left to right as drawn in Fig. 1) affecting carotenoid biosynthesis, SB203S, blocks the for-

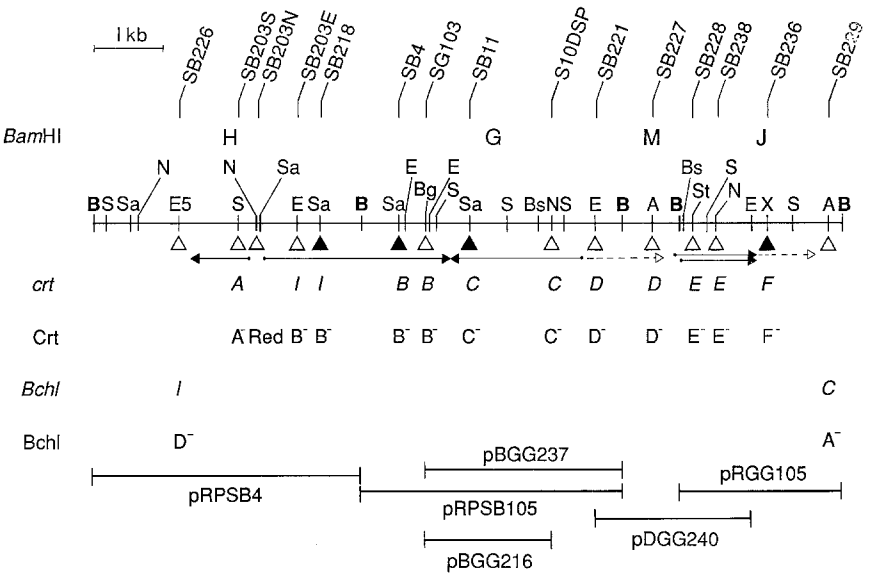


**Fig. 1.** Genetic-physical map of the *Rhodobacter capsulatus* carotenoid gene cluster. Name of the strains derived from interposon insertions are listed on top. *crt* and CRT describe, respectively, genotypes and phenotypes of carotenoid mutants. *Bchl* and Bchl refer, respectively, to genotypes and phenotypes of bacteriochlorophyll mutations. *Open* and *closed triangles* refer, respectively, to Ω and KM117 insertions. *Bam*HI fragments are listed according to the nomenclature of Zsebo and Hearst (1984). Inserts of plasmids used for complementation analyses are shown at the bottom. Restriction enzyme sites: A, *Apa*I; B, *Bam*HI; Bg, *Bgl*II; Bs, *Bst*EII; E, *Eco*RI; E5, *Eco*RV; N, *Nru*I; S, *Sph*I; Sa, *Sal*I; St, *Stu*I; X, *Xho*I. Only relevant markers are shown for A, Bs, E5 and St. *Solid arrows* indicate start point and direction of transcription. *Dashed arrows:* direction of transcription deduced from preliminary DNA sequence information (not shown)

**Table 2.** Main carotenoids accumulated by the different *Rhodobacter capsulatus* strains

| Strain | Aerobic growth | Anaerobic growth |
|---|---|---|
| SB 1003, SB203N | Spheroidenone, Demethylspheroidenone (Red) | Spheroidene Hydroxysheroidene (Brown) |
| SB203S | Spheroidene Hydroxyspheroidene (Brown) | Spheroidene, Hydroxyspheroidene (Brown) |
| SB4, SB203E, SB218, SB228, SB238, SG103 | – (Blue-green) | – (Blue-green) |
| SB11, S10DSP | Neurosporene (Green) | Neurosporene (Green) |
| SB221, SB227 | Neurosporene, Hydroxyneurosporene Methoxyneurosporene (Green) | Neurosporene Hydroxyneurosporene, Methoxyneurosporene (Green) |
| SB236 | Demethylspheroidene, Demethylspheroidenone (Muddy brown) | Demethylspheroidene (Brown) |

For semi-systematic names for carotenoids refer to Giuliano et al. (1986). Color of cells are shown in parenthesis

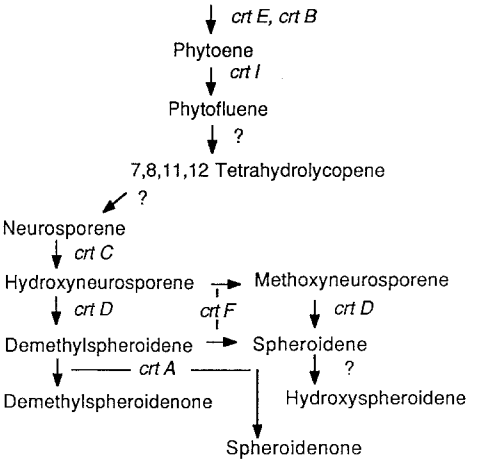


**Fig. 2.** A genetic-biochemical map of carotenoid biosynthesis in *Rhodobacter capsulatus*; adapted from Giuliano et al. (1986). The products of the *crtA* gene (spheroidenone and demethylspheroidenone) form only under aerobic conditions. *Question marks* are shown in biosynthetic steps for which genes have not been identified. Semi-systematic names for these carotenoids were previously published (Giuliano et al. 1986). Point mutations in *crtE* and *crtB* accumulate no carotenoids (B. Marrs, personal communication)

mation of spheroidenone under aerobic conditions (Table 2), indicating that the *crtA* gene has been mutated (Fig. 2). S1 analysis indicates that transcription starts at approximately 120 bp to the left of the *Nru*I site used to construct SB203N and proceeds towards the left (Fig. 4). SB203S is complemented by the pRPSB4 plasmid (Table 3), thus *crtA* is entirely contained within the *Bam*HI H fragment.

Wild-type carotenoids are found in SB203N (Table 2). The next two insertions (SB203E and SB218) map to the region of the *crtI* gene (Giuliano et al. 1986), but exhibit a carotenoidless phenotype (Table 2), characteristic of the adjacent *crtB* gene (Fig. 2). Also, we observed that although *crtI* point mutations, which accumulate phytoene, can be complemented by pRPSB4, this plasmid does not complement SB203E (Table 3). The discrepancy between the phenotypes of interposon insertions and point mutations in *crtI* as well as the results of the conjugational analysis,

**Table 3.** Complementation analysis of point mutations

| Donor strain | Plasmid | Recipient strain | Complementation | Marker rescue |
|---|---|---|---|---|
| TEC 5–11 | pRPSB4 | BPY69 | + | |
| | | SB203E | – | |
| | | SB203S | + | |
| | | SB226 | + | |
| TEC 5–22 | pRPSB105 | BPY69 | – | – |
| | | SB203E | – | – |
| | | SG103 | + | |
| | | W4 | + | |
| BGG237 | pBGG237 | R121 | – | + |
| | | S10DSP | + | |
| | | Y68 | + | |
| | | Y126 | + | |
| BGG216 | pBGG216 | Y68 | – | + |
| | | Y126 | – | + |
| DGG240 | pDGG240 | BW604 | + | |
| | | R121 | – | – |
| | | SB227 | – | + |
| | | SB228 | + | |
| RGG105 | pRGG105 | BW604 | – | + |
| | | SB228 | – | + |

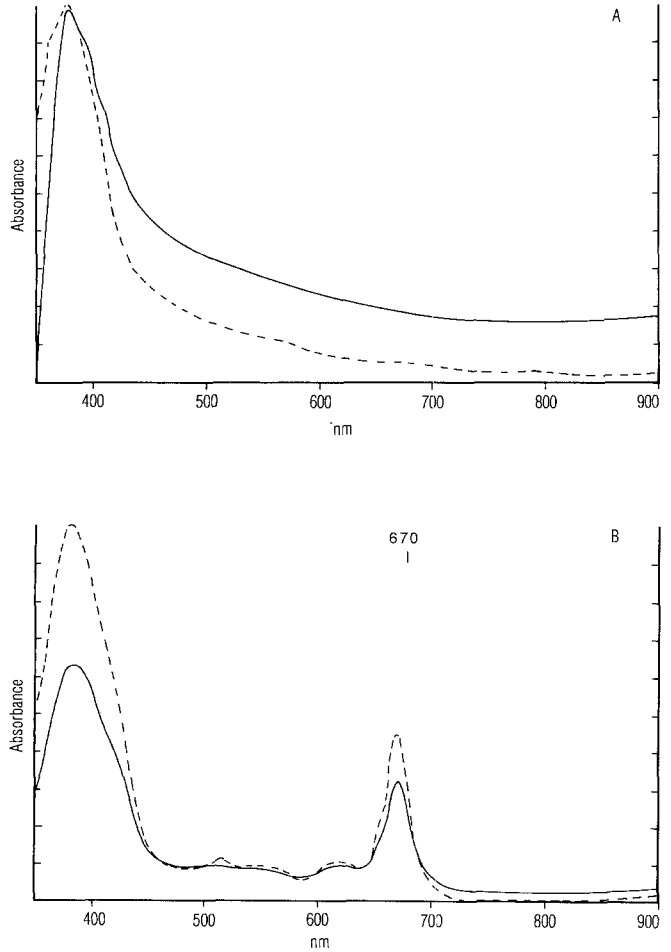


**Fig. 3A, B.** Absorption spectra of membranes of bacteriochlorophyll mutants. **A** SB226 (upper trace) and MB1008 (lower trace). **B** SB229 (upper trace) and MB10110 (lower trace)

strongly suggest a polar effect of the interposon insertion in *crtI* on the adjacent *crtB* gene. Transcription of *crtI* starts at approximately 200 bp to the right of the *Nru*I site of SB203N and proceeds towards the right (Fig. 4). This is compatible with the existence of a *crtI-crtB* operon.

The next five insertions are in the *Bam*HI G region. SB4 and SG103 accumulate no carotenoids (Table 2), a phenotype corresponding to the *crtB* gene, which has been previously mapped to this region (Taylor et al. 1983). SG103 and the *crtB* point mutation W4 are complemented by the plasmid pRPSB105 (Table 3). The carotenoid composition of SB11 and S10DSP (Table 2) indicates that the *crtC* gene has been mutated (Fig. 2). Transcription of *crtC* starts approximately 250 bp to the left of the *Eco*RI site that was used to construct SB221 and proceeds towards the left (Fig. 3). The plasmid pBGG237 complements both *crtC* point mutations and insertions (Table 3), indicating that this gene is entirely contained within the large *Bgl*II-*Bam*HI fragment of *Bam*HI G. SB221 and SB227 accumulate carotenoids corresponding to a *crtD* mutation (Table 2; Fig. 2), indicating that this gene spans the boundary between *Bam*HI G and *Bam*HI M. This is confirmed by the results of the complementation analyses that indicate that the *crtD*121 mutation (strain R121) and the SB227 insertion are not complemented but are marker-rescued, respectively, by pBGG237 and pDGG240 (Table 3).

The strains SB228 and SB238 accumulate no carotenoids and are assigned to *crtE* (Taylor et al. 1983). Under aerobic conditions transcription of this gene starts at approximately 80 bp to the left of *Bam*HI J and proceeds rightward (Fig. 4). Another transcript, approximately 20 bp longer, can be detected under anaerobic conditions (Fig. 4). Consistently with the S1 mapping, SB228 and BW604 are complemented by pDGG240, but only rescued by pRGG105 (Table 3).

Finally, analysis of the carotenoid content of SB236 indicates that the *crtF* gene is affected (Table 2; Scolnik et al. 1980).

Three of the four genes tested with S1 protection (*crtA*, *crtC* and *crtE*) show an increase in mRNA levels under anaerobic conditions (Fig. 4). In the case of *crtE*, most of the increase is due to the appearance of a transcript that is almost undetectable under aerobic conditions (Fig. 4).

## Discussion

We used the interposon mutagenesis technique to construct a detailed map of the *R. capsulatus* carotenoid gene cluster which, based on the distance between SB203S and SB236, has a minimum size of 7.6 kb.

With the data presented here we can now calculate the minimum and maximum sizes for individual genes. The largest is *crtC*, with a minimum size of 1.2 kb (distance between SB11 and S10DSP) and a maximum of 2.2 kb (distance between the starting point of transcription and the right *Bam*HI site of *Bam*HI G). This gene has to be entirely contained within *Bam*HI G as demonstrated by the complementation results with pBGG237. The smallest gene is *crtE*, with a minimum size of 0.3 kb (distance between SB228 and SB238) and a maximum of 1.1 kb, the distance from the starting point of transcription of the *Eco*RI site in *Bam*HI J at which the insert of pDGG240 ends. *crtA* has to be smaller than 1.2 kb, the distance between the start of transcription and the *Eco*RV site used to construct

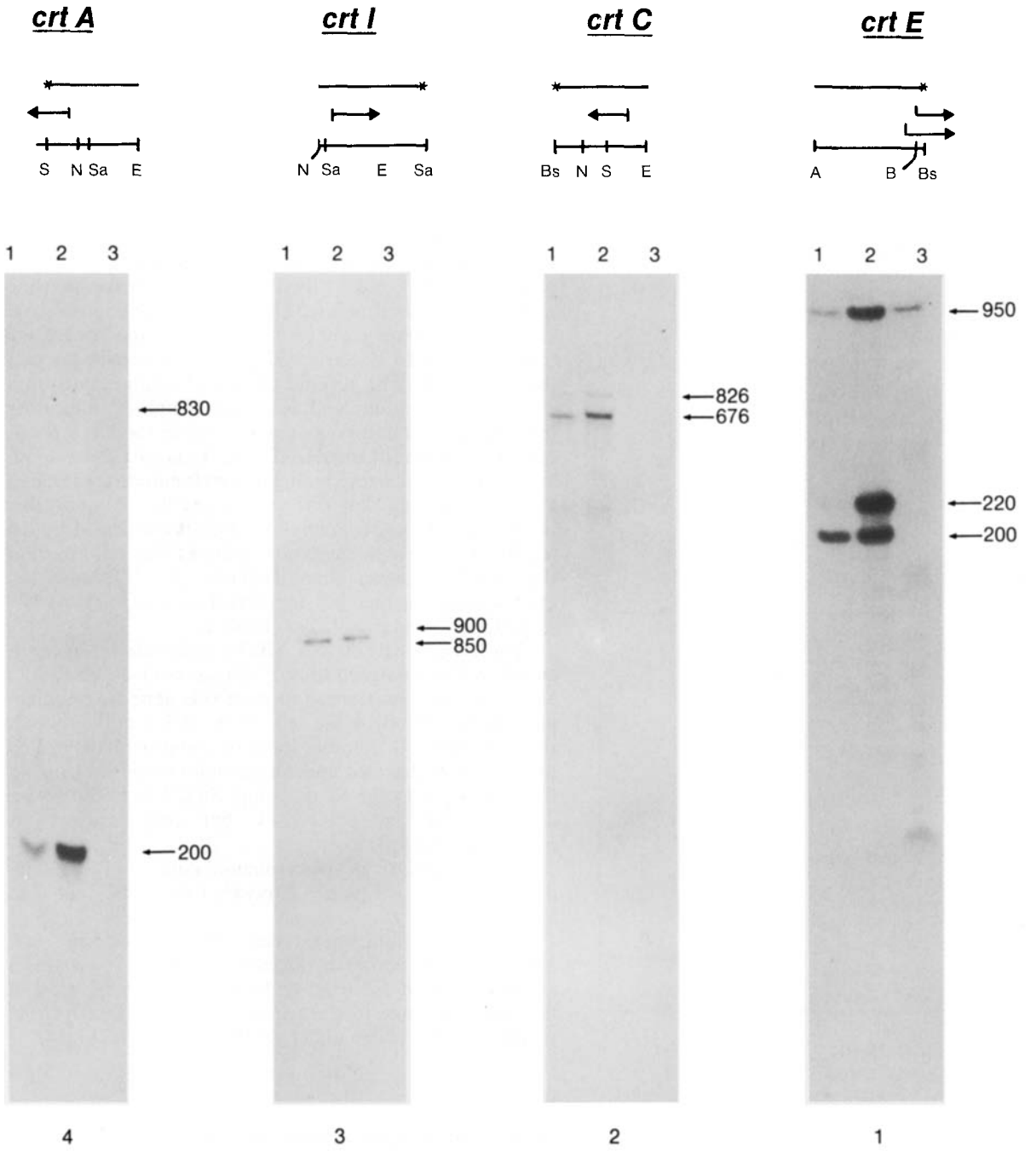


**Fig. 4.** S1 protection analyses. Genes mapped are shown on top. Restriction enzymes: S, *Sph*I; N, *Nru*I; Sa, *Sal*I, E, *Eco*RI; Bs, *Bst*EII; A, *Apa*I. *Asterisks* mark labelled end. *Arrows* indicate start point and direction of transcription. Lanes 1 and 2: *Rhodobacter capsulatus* RNA extracted under aerobic and anaerobic conditions respectively. Lane 3, *R. capsulatus* RNA was replaced by an equal amount of yeast tRNA. Size of probes (top) and protected fragment(s) in basepairs are indicated on the side of each panel

SB226. For *crtI* the minimum is 0.35 kb (distance between SB203E and SB218) and the maximum 1.4 kb, the distance between the start of transcription and the right *Bam*HI site of *Bam*HI H. The minimum for *crtB* is 0.64 kb, distance between SB4 and SG103. The maximum is 1.4 kb, distance between the left *Bam*HI site of *Bam*HI G and the *Sal*I site at which SB11 was constructed. For *crtD* the minimum is 0.68 kb (distance between SB221 and SB227) and the maximum 1.7 kb (distance between S10DSP and SB228). *crtF* has to be smaller than 1.5 kb, the distance between SB238 and SB229.

In light of our results, the previous maps of Taylor et al. (1983) and Zsebo and Hearst (1984) have to be revised. Using complementation analysis of *crtB*, coupled with previously published gene transfer agent (GTA) mapping results, Taylor et al. (1983) mapped *crtD* and *crtE* to, respectively, the *Bam*HI G and the *Bam*HI M fragments. However, our data indicate that these genes map approximately 0.7 kb to the right of that location. In the map of Zsebo and Hearst (1984), the *Bgl*II site corresponding to SG103 is actually in *crtB* and the *Xho*I site corresponding to SB236 is actually in *crtF*. Regarding the size of genes, values had

been previously obtained by mapping with GTA. If one GTA map unit is equivalent to 4.5 kb (Taylor et al. 1983), then the results of Yen and Marrs (1976) and Scolnik et al. (1980) indicate minimum sizes of 0.36 kb for *crtB*, 0.76 kb for *crtC*, 0.54 kb for *crtD* and 0.4 kb for *crtE*. Although these measurements are underestimates, it is interesting to note that the overall size of the carotenoid cluster (1.73 GTA map units or 7.8 kb), is in good agreement with our measurements. The remaining discrepancies are to be expected due to the limitations of both GTA mapping of point mutations and transposon mutagenesis.

Clark et al. (1984) determined that during the shift from 20% to less than 1% oxygen, total carotenoid content increases about twofold. Our results with *crtE, crtA* and *crtC* suggest that this induction is, at least partially, mediated at the mRNA level. The fact that a new transcript is detected under anaerobic conditons in *crtE* opens the possibility that this gene may be transcribed from both a constitutive and an anaerobiosis-inducible promoter, a situation also described for the *R. capsulatus puf* operon (Bauer et al. 1988). The role of *crtE* in the carotenoid pathway is not currently known. No carotenoids are found in *crtE*$^-$ mutants, indicating that this gene affects early steps in carotenoid biosynthesis. Using probes encompassing several genes, Zhu and Hearst (1986) concluded that the levels of *crtA, crtC* and *crtD* transcripts increase under aerobic conditions. The conclusions regarding the first two genes are not supported by our work with gene-specific probes. These authors also report that the *Bam*HI J fragment codes for a transcript(s) that increases under anaerobic induction. This is consistent with our results with *crtE*.

Insertions in both *crtA* and *crtF* result in mutants that are able to grow photosynthetically, indicating that both genes are transcriptionally separated from the adjacent *Bchl* genes. The Bchl D$^-$ and Bchl A$^-$ phenotypes of SB226 and SB229 are puzzling for the *bchl* genes reported to flank the carotenoid cluster are *bchI* and *bchC* (Zsebo and Hearst 1984). We have to conclude that either these genes are very small or that, in contrast with the model of Zsebo and Hearst (1984), there are two *bchl* operons, pointing outwards from the carotenoid cluster. A more careful study of the organization of the *bchl* genes in this region is needed.

We also examined the polypeptide composition of several mutants (not shown) and concluded that the blue-green strains SB203E, SB4 and SG103 are missing the $\gamma$ subunit of the light-harvesting II system (LH-II), which is in agreement with the results of Zsebo and Hearst (1984). Thus, when the biosynthesis of colored carotenoids is blocked by either transposon (Zsebo and Hearst 1984) or interposon (this work) mutagenesis, LH-II does not form. However, Dörge et al. (1987) claimed to have observed variable levels of LH-II in the absence of carotenoids.

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