Regulation of carotenoid and bacteriochlorophyll biosynthesis genes and identification of an evolutionarily conserved gene required for bacteriochlorophyll accumulation

GREGORY A. ARMSTRONG,* DAVID N. COOK, DZWOKAI MA, MARIE ALBERTI,
DONALD H. BURKE and JOHN E. HEARST

Department of Chemistry and Laboratory of Chemical Biodynamics, University of California, Berkeley,
CA 94720, USA

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The temporal expression of ten clustered genes required for carotenoid (*crt*) and bacteriochlorophyll (*bch*) biosynthesis was examined during the transition from aerobic respiration to anaerobiosis requisite for the development of the photosynthetic membrane in the bacterium *Rhodobacter capsulatus*. Accumulation of *crtA*, *crtC*, *crtD*, *crtE*, *crtF*, *crtK*, *bchC* and *bchD* mRNAs increased transiently and coordinately, up to 12-fold following removal of oxygen from the growth medium, paralleling increases in mRNAs encoding pigment-binding polypeptides of the photosynthetic apparatus. The *crtB* and *crtI* genes, in contrast, were expressed similarly in the presence or absence of oxygen. The regulation patterns of promoters for the *crtA* and *crtI* genes and the *bchCXYZ* operon were characterized using *lacZ* transcriptional fusion and qualitatively reflected the corresponding mRNA accumulation patterns. We also report that the *bchl* gene product, encoded by a DNA sequence previously considered to be a portion of *crtA*, shares 49% sequence identity with the nuclear-encoded *Arabidopsis thaliana* Cs chloroplast protein required for normal pigmentation in plants.

Introduction

Carotenoids play a variety of biological roles in both carotenogenic and noncarotenogenic organisms. These pigments protect against the destructive effects of photosensitizing molecules, notably porphyrin derivatives, and against singlet oxygen (Krinsky, 1971). In photosynthetic organisms, all of which synthesize carotenoids, these pigments also supplement the light-harvesting capacity of chlorophyll (Chl) and bacteriochlorophyll (Bchl) (Cogdell & Frank, 1987). Recent findings implicate the carotenoid zeaxanthin in photosynthetic energy dissipation (Demmig-Adams, 1990). The general biosynthetic pathways of carotenoids and Chl/Bchl are described in detail elsewhere (Goodwin, 1980; Castelfranco & Beale, 1983).

The carotenoid and Bchl pigments are bound non-covalently to the polypeptides of the photosynthetic reaction centre (RC), and light-harvesting I and II (LH

I and LH II, or B875 and B800–850, respectively) antenna complexes of purple non-sulphur photosynthetic bacteria, such as *Rhodobacter* species (Kiley & Kaplan, 1988). The RC polypeptides are encoded by the *pufL*, *pufM* and *pufH* genes, the LH I polypeptides by the *pufA* and *pufB* genes, and the peripheral antenna LH II polypeptides by the *pucA* and *pucB* genes in *R. capsulatus* (Scolnik & Marrs, 1987). The *puf* and *pufH* operons and all identified genes required for carotenoid (*crt*) and Bchl (*bch*) biosynthesis comprise a 46 kb photosynthesis gene cluster in this organism (Marrs, 1981; Zsebo & Hearst, 1984; Burke *et al.*, 1991a) and in the closely related *R. sphaeroides* (Coomber *et al.*, 1990). The assignment of *crt* genes to specific biosynthetic reactions is summarized in Fig. 1, and a biosynthetic pathway linking *bch* genes with particular reactions in Bchl biosynthesis has been described (Biel & Marrs, 1983) and recently amended (Yang & Bauer, 1990; Burke *et al.*, 1991b).

Diverse factors, including oxygen, light and developmental state of the organism, govern the synthesis and accumulation of carotenoid pigments in plants, algae, fungi and bacteria (Bramley & Mackenzie, 1988), although the regulatory mechanisms remain largely obscure. The regulation of pigment synthesis in purple

*Author for correspondence. Present address: Institute for Plant Sciences, Department of Plant Genetics, ETH-Zentrum, CH-8092 Zürich, Switzerland. Tel. 41 1 256 3700; fax 41 1 252 0829; email armstrong@aeolus.ethz.ch

Fig. 1. The *R. capsulatus* carotenoid biosynthesis pathway. Genes encoding mRNAs that accumulate to higher levels (+) or remain at constant levels (=) during the onset of photosynthetic growth are indicated (see also Fig. 3). The regulation of *crtJ* was not tested (n.t.). The chemical structures and semisystematic nomenclature (Straub, 1987), and a more complete description of the pathway are presented elsewhere (Armstrong *et al.*, 1990c). IPP, FPP, GGPP and PPPP are isopentenyl pyrophosphate, farnesyl pyrophosphate, geranylgeranyl pyrophosphate, and prephytoene pyrophosphate, respectively. Phytoene is converted to neurosporene by three consecutive dehydrogenations, probably all mediated by CrtI (Bartley *et al.*, 1990). Spheroidene and spheroidenone (boxed) are the major wild-type carotenoids accumulated in the absence and presence of oxygen, respectively. The roles of the *crtJ* and *crtK* gene products in carotenoid biosynthesis are unclear. The mutation defining *crtJ* (Zsebo & Hearst, 1984) and data from an *in vitro* carotenoid biosynthesis system (Armstrong *et al.*, 1990c) suggest a blockage early in the pathway or, alternatively, a regulatory mutation. The involvement of CrtK in carotenoid biosynthesis was postulated on the basis of the neurosporene-accumulation phenotype resulting from a mutation (Giuliano *et al.*, 1988), later identified from the nucleotide sequence as defining a new gene (*crtK*) (Armstrong *et al.*, 1989). Interestingly, it has been recently been reported that CrtK is homologous to a mammalian mitochondrial peripheral-type benzodiazepine receptor capable of binding protoporphyrins with nanomolar affinities (Baker & Fanestil, 1991). Although we had tentatively proposed that CrtE was involved in the conversion of PPPP to phytoene (Armstrong *et al.*, 1990a) based on data from an *R. capsulatus* *in vitro* carotenoid biosynthesis system (Armstrong *et al.*, 1990c), recent evidence demonstrates that

photosynthetic bacteria has been a topic of research for almost 50 years (van Niel, 1944). Both oxygen and light intensity influence the synthesis of carotenoids, Bchl and the intracytoplasmic membrane harbouring the photosynthetic pigment-protein complexes in facultative photosynthetic bacteria such as *Rhodobacter* species (Cohen-Bazire *et al.*, 1957; Kiley & Kaplan, 1988). Either the elimination of oxygen from the growth medium accompanied by illumination of a previously dark-grown culture, or a downshift in light intensity during constant anaerobiosis, stimulates carotenoid accumulation in *R. capsulatus* (Golecki *et al.*, 1980; Kaufmann *et al.*, 1982) and *R. sphaeroides* (Cohen-Bazire *et al.*, 1957). Lowering the oxygen tension in dark-grown aerobic cultures of *R. capsulatus* produces a similar result (Schumacher & Drews, 1978; Biel & Marrs, 1985). Several studies report relatively little regulation of mRNA accumulation for the *R. capsulatus* *crt* genes during the transition from dark aerobic respiration to low oxygen conditions (Clark *et al.*, 1984; Klug *et al.*, 1985), or of *crt* promoter activities (Young *et al.*, 1989) and mRNA levels (Zhu & Hearst, 1986) in comparisons between steady-state dark aerobic and anaerobic photosynthetic cultures. Other experiments indicate differences in mRNA levels for some *crt* genes in comparisons of illuminated steady-state anaerobic and aerobic cultures (Giuliano *et al.*, 1988). These results contrast with the strong regulation of mRNA levels and promoter activities of genes (*puf*, *puh*, *puc*) encoding structural pigment-binding polypeptides of the photosynthetic membrane (Clark *et al.*, 1984; Klug *et al.*, 1985; Zhu & Hearst, 1986; Cook *et al.*, 1989; Young *et al.*, 1989).

The recent molecular characterization of carotenoid biosynthesis genes from *R. capsulatus* (Bartley & Scolnik, 1989; Armstrong *et al.*, 1989, 1990b, c) provides the basis for more detailed studies of the regulation of individual *crt* genes by environmental factors. We expect that this approach will lead to a more complete understanding of the mechanisms regulating the entire biochemical pathway.

We have determined the kinetics of mRNA accumulation for eight *R. capsulatus* *crt* and two flanking *bch* genes during the well-defined transition from aerobic respiration to anaerobic photosynthesis using, for the first time, gene-specific probes. These genes form a subcluster within the photosynthesis gene cluster. We have also examined oxygen regulation of promoters for

prokaryotic homologues of CrtE and CrtB function in GGPP synthesis and catalyse the conversion of GGPP to phytoene, respectively (Chamovitz *et al.*, 1992; Sandmann & Misawa, 1992; Math *et al.*, 1992).

the divergently transcribed *crtA* and *crtI* genes, and for the better characterized *bchCXYZ* operon (Wellington & Beatty, 1989, 1991; Burke *et al.*, 1991b) using plasmid-borne *lacZ* (β -galactosidase) transcriptional fusions.

Methods

Plasmid constructions. pZM400, a promoter probe vector suitable for stably maintaining *lacZ* transcriptional fusions in *R. capsulatus* (Ma *et al.*, 1993), was constructed by modification of pRK290, a broad host range low copy number plasmid containing an RK2 replicon (Ditta *et al.*, 1980). Briefly, the tetracycline resistance gene of pRK290 was inactivated by introduction of a kanamycin resistance gene from Tn903 (Oka *et al.*, 1981; Vieira & Messing, 1982), and a multiple cloning site was inserted 5' to a promoter-less *lacZ* gene from *Escherichia coli*. The translational start of *lacZ* was modified by inserting stop codons in all three reading frames upstream of a ribosome binding site, based on that found 5' to the translation initiation codon of the *R. capsulatus puhA* gene. A modified version of the *puc* transcription terminator containing four consecutive T residues at its 3' end (Chen *et al.*, 1988) was placed 5' to the multiple cloning site to minimize background expression of *lacZ* by preventing readthrough from vector sequences. A more detailed description of pZM400 will be presented elsewhere (Ma *et al.*, 1993). The common promoter region of the divergently transcribed *R. capsulatus crtA* and *crtI* genes was cloned as either a *BstEII*/*BalI* or as a *BstEII*/*SphI* restriction fragment (Fig. 3) into the pZM400 multiple cloning site. The resulting plasmids, pZM422 and pZM432, contain *R. capsulatus* DNA in the appropriate orientations to place *crtI* and *crtA* promoter sequences, respectively, 5' to *lacZ*. The *R. capsulatus bchCXYZ* promoter region was inserted into the pZM400 multiple cloning site as a *SmaI*/*Apal* fragment (Fig. 3), yielding pZM410. Molecular biological manipulations were carried out using standard protocols supplied by manufacturers.

Growth and manipulation of bacterial strains, isolation of RNA and measurement of LacZ activities. *R. capsulatus* strain SB1003, wild-type for photosynthetic functions (Yen & Marrs, 1976), was grown under the controlled conditions described by Cook *et al.* (1989) and below. Dark-grown aerobic cultures were sparged with a mixture of $N_2:O_2:CO_2$ (80:20:2, by vol.). Photosynthetic cultures were illuminated at 15 W m^{-2} by a bank of Lumiline lamps (General Electric) and sparged with a mixture of $N_2:CO_2$ (80:2, by vol.). At time zero of the transition from aerobic respiration to photosynthesis, the gas mixture was changed to eliminate O_2 from the growth medium and the cultures were exposed to light. To determine the effect of light on gene expression, duplicate dark-grown cultures were switched from the aerobic to the anaerobic gas mixture described above, with one culture simultaneously exposed to light.

Samples (6 ml) of the SB1003 cell cultures were removed at various times during aerobic respiratory growth or the transition to photosynthesis for RNA isolation. Samples were immediately mixed with an equal volume of ice cold buffer A (80 mM-Tris/HCl, pH 7.5, 10 mM-MgCl₂) supplemented with 200 μ g chloramphenicol ml⁻¹, 25 mM-sodium azide and 10 mM- β -mercaptoethanol (Zhu & Kaplan, 1985), cooled for 15 s in a dry ice/ethanol bath and harvested by centrifugation. The pellets were resuspended in lysis buffer, immediately frozen on dry ice and stored at -70°C until isolation of the RNA as described (Zhu & Kaplan, 1985).

pZM plasmids were introduced into *R. capsulatus* SB1003 by triparental matings with *E. coli* plasmid donor and helper strains as described (Zsebo & Hearst, 1984). For LacZ activity measurements, exponentially-growing *R. capsulatus* cells were shifted from aerobic to photosynthetic growth as described above. Samples (5 ml) were collected to monitor LacZ activities by the method of Miller (1972),

with 0.2 ml of cells assayed in 0.8 ml Z-buffer. All *R. capsulatus* cultures were grown at 32°C .

Preparation of RNA dot-blots, M13 probes and nucleic acid hybridizations. RNA dot-blots were prepared as previously described, using 5 μ g total RNA per dot (Cook *et al.*, 1989). RNA time courses were probed for specific mRNAs with single-stranded M13 subclones, labelled by primer extension across the insert region with the Klenow fragment of DNA polymerase I as previously described (Armstrong *et al.*, 1990c), except that 5'-[α -³²P]dATP was replaced by 50 μ Ci 5'-[γ -³²P]dCTP (3000 Ci mmol⁻¹; Amersham). Gene-specific M13 probes for the *crt* genes, *bchC* and *bchD* (Fig. 3) were generated during the sequencing of these regions (Armstrong *et al.*, 1989). The use of the gene-specific M13 probes for the *puf* operon (*pufL*) and the *puh* operon (*puhA*) (Zhu & Hearst, 1986) to obtain the data plotted in Fig. 2(b) has been described (Cook *et al.*, 1989), as has the gene-specific 15-mer oligonucleotide probe for the *puc* operon (*pucB*) (Zhu & Hearst, 1986). The *pucB* oligonucleotide (500 ng) was end-labelled with 50 μ Ci 5'-[γ -³²P]rATP (5000 Ci mmol⁻¹) and T4 polynucleotide kinase. To insure that equivalent amounts of RNA from each sample were applied to the membrane, we prepared dot-blots using about 3 ng total RNA per time point and hybridized these with either radiolabelled pRCI, containing cloned *R. capsulatus* rRNA genes (Yu *et al.*, 1982), or with radiolabelled total *R. capsulatus* chromosomal DNA isolated as described previously (Cook *et al.*, 1989). pRCI (1 μ g) or total chromosomal DNA (1 μ g) was labelled with 50 μ Ci 5'-[α -³²P]dCTP (3000 Ci mmol⁻¹) by nick-translation using a Bethesda Research Laboratories kit. RNA dot-blot hybridizations with Gene Screen nylon membranes (New England Nuclear), autoradiography, and quantification of the excised dots by scintillation counting (Fig. 2) were performed as described previously (Cook *et al.*, 1989), except that the RNAs to be hybridized with the *pucB* oligonucleotide probe were applied to a nitrocellulose membrane (Schleicher & Schuell). An autoradiogram of the *puc* hybridization was quantitated as above (Fig. 2b). Autoradiograms of membranes probed for *crtC*, *crtD* and *bchD* mRNAs were quantified by densitometry using a Hoefer Scientific Instruments scanning densitometer, Model GS 300 (data not shown).

Database searches and sequence alignments. Sequence similarity between the *R. capsulatus* Bchl and *A. thaliana* Cs proteins was detected by searching the NBRF database (release 28.0, 3/91) using the FASTA program from the GCG software package, version 6.2 (Devereux *et al.*, 1984). The BESTFIT program from this package was used for the initial sequence alignment, which was subsequently optimized by hand.

Results and Discussion

Accumulation of many crt and bch mRNAs increases during the transition to photosynthesis

During the transient cessation of cellular division that follows a rigorous transition from dark aerobic respiration to anaerobiosis in the light, *Rhodobacter* must adapt to photosynthetic growth through the synthesis of carotenoids, Bchl, pigment-binding polypeptides and an intracytoplasmic membrane system harbouring these components (Kiley & Kaplan, 1988). The rates of carotenoid and Bchl synthesis increase rapidly during this lag phase (Cohen-Bazire *et al.*, 1957; Schumacher & Drews, 1978).

We have previously demonstrated that *R. capsulatus* cell division ceases and rRNA levels remain constant during the first hour of such a transition (Cook *et al.*,

Fig. 2. Time courses and magnitudes of mRNA accumulation for *crt* and *bch* genes compared with the *puf*, *puh*, and *puc* mRNAs in the same experiment. Times in min are given relative to time zero when bacterial cultures were shifted from dark aerobic respiratory growth to anaerobic photosynthetic conditions. The quantification of bound radioactivity from RNA dot-blots hybridized with DNA probes is shown. (a) mRNA levels for *crt* and *bch* genes plotted relative to an arbitrary value of one at time zero for each mRNA. The probe for *crtI* mRNA was BSa6 (see Fig. 3 legend for exact locations of all probes used in this work). A similar hybridization pattern was also obtained using probe HH121 for *crtI* (data not shown). Standard deviations in the amounts of each mRNA, as determined based on the values at -20, -10 and 0 min, are as follows: *crtA* (□), 0.068; *crtB* (+), 0.350; *crtE* (●), 0.093; *crtF* (×), 0.320; *crtI* (■), 0.171; *crtK* (○), 0.379; *bchC* (▲), 0.151; rRNA (△), 0.061. (b) Comparison of *crtA* and *bchC* mRNA accumulation with the accumulation of *puf*, *puh*, and *puc* mRNAs. RNA dot-blots probed for *puf* and *puh* mRNAs (Cook *et al.*, 1989) and *puc* mRNA, with specific DNA probes for each of these sequences (Zhu & Hearst, 1986), were used for this quantitative presentation. Symbols and standard deviations in the amounts of specific mRNAs not given in (a), as determined based on the values at -20, -10 and 0 mins, are as follows: *pufL* (⊞), 0.086; *puhA* (◆), 0.076; *pucB* (◇), 0.198.

1989). In this experiment we also determined mRNA accumulation patterns for the *crt* and two *bch* genes during the adaptive period (Fig. 2). Probes derived from an rDNA sequence or total genomic DNA were used to demonstrate that equivalent amounts of RNA for each

time point had been applied to the membrane (Fig. 2 and data not shown). mRNA levels for all *crt* and *bch* genes remained constant prior to the simultaneous removal of oxygen from the growth medium and illumination of the cultures.

The *crt* and *bch* mRNAs fall into two groups depending on their response to this change in environmental conditions. The accumulation of mRNAs for *crtA*, *crtC* (not shown), *crtD* (not shown), *crtE*, *crtF*, *crtK*, *bchC* and *bchD* (not shown) increased 2- to 12-fold, while mRNA levels for *crtB* and *crtI* remained constant (Fig. 2a). In the same experiment, accumulation of *puf* and *puh* (LH I and RC) mRNAs increased comparably (6- to 8-fold; Cook *et al.*, 1989), while *puc* (LH II) mRNA increased 25-fold (Fig. 2b). The results of the *crt* and *bch* mRNA accumulation studies and the locations of the DNA probes used are summarized in Fig. 3.

Our results thus indicate that regulated *crt* and *bch* mRNA accumulation may play as significant a role in the expression of pigment biosynthesis enzymes as in the expression of pigment-binding polypeptides during the rigorous transition from dark aerobic respiration to anaerobic photosynthesis. Three important features combined in this study distinguish it from previous efforts, discussed below, in this area: (i) the use of gene-specific probes for each mRNA studied, including mRNAs representing both pigment biosynthetic enzymes and pigment-binding polypeptides, (ii) the measurement of both mRNA accumulation and promoter activities using identical and well-defined growth conditions to obtain two independent measures of gene regulation, (iii) the detailed sampling of time points during the early stages of the adaptation to photosynthetic growth.

Several earlier studies, including work from this laboratory, had indicated relatively small changes in *R. capsulatus crt* mRNA levels (Clark *et al.*, 1984; Klug *et al.*, 1985; Zhu & Hearst, 1986) or promoter activities (Young *et al.*, 1989) in response to low oxygen tensions or anaerobic photosynthetic conditions, in contrast to the *puf* operon. Discrepancies between our results and earlier comparative studies of mRNA accumulation probably result in large part from the previous unavailability of gene-specific probes for *crt* mRNAs and the absence, with one exception (Clark *et al.*, 1984), of detailed kinetic data. Differences in the experimental techniques, bacterial strains, or the growth conditions used could also be contributing factors. *puf* and *puc* mRNAs, for example, accumulate to significantly higher levels in dark-grown *R. capsulatus* cultures shifted from 20% to 3% oxygen tension (Leach *et al.*, 1991) than in cultures shifted to completely anaerobic conditions (Fig. 2b; Cook *et al.*, 1989).

Some evidence for the regulation of certain *crt* mRNAs was obtained by comparing anaerobic versus aerobic

Fig. 3. Genetic-physical map of the *R. capsulatus* carotenoid biosynthesis gene cluster. The scale at the bottom shows the locations of all *Bam*HI restriction sites. The *Bam*HI-H, -G, -M and -J restriction fragments of the 46 kb photosynthesis gene cluster carried on pRPS404 (Marrs, 1981; Burke *et al.*, 1991a) have been used as probes in several previous studies of *crt* gene expression (Clark *et al.*, 1984; Klug *et al.*, 1985; Zhu & Hearst, 1986). Orientations of the genes are as indicated. *bchI* (see Fig. 5 and text) was previously considered to be a portion of *crtA* (Armstrong *et al.*, 1989, 1990b, c). The detached arrowhead indicates that *bchD* extends into the flanking *Bam*HI-E restriction fragment. Other selected restriction sites used in the construction of *R. capsulatus* promoter-*lacZ* transcriptional fusion plasmids (pZM410, *bchCXYZ*; pZM422, *crtI*; pZM432, *crtA*) are indicated. The extents and orientations of the *R. capsulatus* DNA inserted 5' to the *lacZ* gene in these plasmids are indicated by rectangles with horizontal stripes. The nucleotide positions of the genes are given from the first nucleotide of the start codon to the last nucleotide of the stop codon (vertical numbers) (Burke *et al.*, 1991a). The numbering system used in the original publication describing this sequence (Armstrong *et al.*, 1989) can be obtained by subtracting 25243 from the nucleotide positions given here. Gene-specific single-stranded M13 probes used to detect mRNAs from different regions of the gene cluster are indicated above, with arrows showing the direction of the synthesized probe and the extent of the insert. The exact map locations of the M13 probes used are as follows: SpSa25 (*bchD*), 25470–25812; TT93 (*crtA*), 27105–27436; HH121 (*crtI*), 27997–28157; BSa6 (*crtI*), 28544–29150; XSp54 (*crtB*), 30250–30418; E'Sa90 (*crtK*), 30826–30892; SS110 (*crtC*), 31446–31651; EB96 (*crtD*), 32553–33018; TB4 (*crtE*), 33830–34060; SS41 (*crtF*), 34576–34842; XX6 (*bchC*), 35994–36085. mRNA accumulation during photosynthetic adaptation is indicated by shading of the genes: grey, 2- to 12-fold increase; white, no increase; vertical stripes, not tested. The black triangle indicates the position of a frameshift mutation in the previously reported *crtA* sequence (see Fig. 5 legend).

cultures under constant illumination (Giuliano *et al.*, 1988), a condition which allows *Rhodobacter* species to use both photosynthetic and respiratory growth modes simultaneously (Cohen-Bazire *et al.*, 1957; Keister, 1978). Our quantitative data on the temporal regulation of mRNA accumulation (Fig. 2a) do, despite the difference in growth conditions used, agree with the qualitative steady-state results previously reported (Giuliano *et al.*, 1988). The measured increases in *crtE* and *crtF* mRNAs during the transition to photosynthesis (Fig. 2a) also agree with the oxygen regulation attributed to an inducible promoter for the *crtEF* operon using plasmid-borne *LacZ* translational fusions (Young *et al.*, 1989).

The constitutive patterns of *crtI* and *crtB* mRNA expression detected using three different DNA probes (Fig. 3) are consistent with the proposed cotranscription of these genes (Giuliano *et al.*, 1988; Armstrong *et al.*,

1989). In contrast, *crtK* mRNA accumulation increased 8-fold during the shift from aerobic respiration to photosynthetic growth (Fig. 2a), suggesting that *crtK* may form a distinct operon. An alternative explanation would be the differential regulation of mRNA stability within the same transcript, as documented for the *R. capsulatus puf* operon (Belasco *et al.*, 1985; Chen *et al.*, 1988).

Although changes in light intensity during photosynthetic growth can influence carotenoid content in *Rhodobacter* species (Cohen-Bazire *et al.*, 1957; Golecki *et al.*, 1980; Kaufmann *et al.*, 1982), pigment accumulation can also be triggered solely by a downshift in oxygen tension in dark-grown cultures (Schumacher & Drews, 1978; Biel & Marrs, 1985). To address directly the possible regulation of *R. capsulatus crt* genes by light we examined the expression of *crtA*, a gene strongly regulated by oxygen tension, during the transition from

dark aerobic respiration to either dark anaerobic or anaerobic photosynthetic conditions. Cells shifted in the presence of light displayed a transient 10-fold increase in *crtA* mRNA, versus a 5-fold increase in the absence of light (data not shown). The patterns of mRNA accumulation versus time were equivalent in both of these experiments to the results presented in Fig. 2. Thus, the removal of oxygen from the growth medium suffices qualitatively for the induced accumulation of *crtA* mRNA, although the presence of light may play a modulating role.

mRNAs for different classes of genes required for photosynthesis accumulate coordinately

mRNAs for all regulated *crt* genes, and for *bchC* and *bchD*, accumulated coordinately during the first 45 min of the adaptation to photosynthetic growth (Fig. 2a). *puf*, *puh*, and *puc* mRNAs accumulated with time courses roughly similar to those of the regulated *crt*, and *bch* mRNAs, although their accumulation continued up to 60 min into the transition, in some cases (Fig. 2b). This temporal pattern of mRNA accumulation may reflect a coordinated programme of gene expression during the development of a functional photosynthetic membrane. The stable assembly of the pigment-protein complexes relies on the presence of the pigments in *R. capsulatus* (Zsebo & Hearst, 1984; Klug *et al.*, 1985), suggesting the biological significance of such an expression pattern.

One mechanism for a rapid and transient increase in carotenoid and Bchl production to meet the demands of the cell would be the accumulation of those mRNAs encoding enzymes governing rate-limiting reactions in the respective biosynthetic pathways (Fig. 1; see Biel & Marrs (1983) for an outline of the Bchl biosynthetic pathway). The intracellular levels of *CrtI* are apparently not a limiting factor in carotenoid biosynthesis (Bartley & Scolnik, 1989). Consistent with this, we did not observe regulation of *crtI* mRNA accumulation or transcriptional activity. Although the different *R. capsulatus* carotenoid biosynthesis enzymes have not yet been biochemically characterized and their relative enzymic activities have not been measured, the constitutive expression of *crtB* and *crtI* mRNAs indicates that biosynthetic capacity for the conversion of GGPP to neurosporene (Fig. 1) is not regulated at the level of mRNA. The regulated *crt* genes encode products required both early in carotenoid biosynthesis (before GGPP) and late in the pathway (after neurosporene). The increase in *crtE* expression could, for example, reflect an increased requirement for GGPP in the synthesis of both carotenoids and of the phytol moiety of Bchl in response to photosynthetic conditions. Expres-

Fig. 4. Time courses of *lacZ* expression driven by the *crtA*, *crtI* and *bchXYZ* promoters during the transition to photosynthesis. Growth conditions are as described in Fig. 2. The control ($\square$) is the promoterless construct, pZM400, while *bchXYZ* ($\blacktriangle$), *crtI* ($\blacksquare$) and *crtA* ($\square$) are the *lacZ* transcriptional fusion constructs pZM410, pZM422 and pZM432, respectively. Standard deviations in the LacZ activities, as determined based on the values at -10 and 0 min, are as follows: *crtA*, 12; *crtI*, 20.5; *bchXYZ*, 7; control, 3.6.

sion of a homologue of *crtE* from another bacterium significantly increases GGPP accumulation in *E. coli* (Sandmann & Misawa, 1992).

Oxygen regulation of crtI, crtA and bchXYZ promoter activities determined using lacZ transcriptional fusions

The oxygen regulation of *crtI*, *crtA*, and *bchXYZ* expression was further studied by fusion of promoter-containing restriction fragments (Fig. 3) 5' to a promoterless *E. coli lacZ* gene resident in the low copy number plasmid pZM400. The *crtI*, *crtA* and *bchXYZ* promoter constructs, pZM422, pZM432 and pZM410, respectively, were introduced into *R. capsulatus* strain SB1003. Cells were assayed for LacZ activity at different times during the adaptation to photosynthetic growth under the same conditions used to measure mRNA accumulation.

When *R. capsulatus* cultures were shifted from dark aerobic growth to anaerobic photosynthetic conditions, LacZ activity driven by the *crtA* and *bchXYZ* promoter regions increased 2- and 3.5-fold, respectively, reaching a plateau approximately 60 min after the maximal mRNA accumulation (Fig. 4). This lag may reflect the translation of the *lacZ* mRNA and the gradual accumulation of active LacZ enzyme. *crtI* promoter-driven LacZ activity remained roughly constant during the same time course, in agreement with the RNA data. The background level of LacZ activity from the promoterless control, pZM400, was an order of magnitude lower than that observed in the promoter-containing constructs under photosynthetic conditions. The difference between the increases in *crtA* promoter activity (two-fold; Fig. 4) and

Fig. 5. The revised nucleotide sequence of *R. capsulatus crtA* reveals a new gene, *bchI*, required for Bchl biosynthesis or accumulation. Nucleotide positions, indicated at the left, and the orientations of the protein coding sequences are given as in the original presentation of this nucleotide sequence in order to facilitate comparison (Armstrong *et al.*, 1989) (EMBL database accession number X52291). * indicates the position of a frameshift error caused by insertion of an extra nucleotide in the original sequence. The corrected nucleotide sequence shown here can be obtained from the EMBL database under accession number Z11165 (Burke *et al.*, 1991a), starting from position 26820. Single letter amino acid translations are given for portions of Bchl and CrtA (inclusive residues are shown in parentheses). Three dots indicate the new *crtA* stop codon. The putative *bchI* ribosome binding site is underlined. Doubly underlined amino acids in the CrtA sequence indicate new residues introduced between the frameshift and the revised stop codon.

mRNA accumulation (12-fold; Fig. 2) in response to anaerobiosis could reflect either post-transcriptional regulation, or differences in expression between the chromosomal and plasmid-borne copies of the *crtA* promoter due to DNA structure or plasmid copy number.

In agreement with our results, chromosomal (Biel & Marrs, 1983) and plasmid-borne (Wellington & Beatty, 1989, 1991; Young *et al.*, 1989) *lacZ* transcriptional and translational fusions in *R. capsulatus* have indicated that the steady-state activity of the *bchCXYZ* promoter (Burke *et al.*, 1991b) increases between 3- and 8-fold when comparing growth under high and low oxygen tensions or photosynthetic conditions with respiration. Although transcripts initiated upstream of the *bchCXYZ* promoter (Fig. 3) can read through into the *bchC* gene (Wellington & Beatty, 1991), the magnitude of *bchC* mRNA accumulation (3-fold) corresponds closely with the increase in transcription initiation from the *bchCXYZ* promoter in our experiments (compare Figs 2 and 4). A thorough characterization of the *bchCXYZ* promoter will be presented elsewhere (Ma *et al.*, 1993). The data presented here on the temporal expression of *bchCXYZ* promoter activity and *bchC* and *bchD* mRNAs, combined with measurements made by other researchers (Biel & Marrs, 1983; Hunter & Coomber, 1988; Young *et al.*, 1989; Yang & Bauer, 1990; Wellington & Beatty, 1991), demonstrate moderate regulation of many *bch* genes by oxygen tension.

Identification of the *bchI* gene and conservation of the predicted gene product in more evolved photosynthetic organisms

Previous experiments with *R. capsulatus* had established that the Tn5.7 insertion mutation defining the *bchI* locus as an entity distinct from *bchD* and *crtA* by

complementation (Zsebo & Hearst, 1984) mapped physically within the 3' portion of the ORF assigned to the *crtA* gene (Armstrong *et al.*, 1990c). This Tn5.7 mutation abolished the accumulation of Bchl or its visibly absorbing precursors (Zsebo & Hearst, 1984). In the course of the current study, we determined that the C-terminal half of the predicted *crtA* gene product displays a high degree of sequence similarity with the *Arabidopsis thaliana* Cs protein (Koncz *et al.*, 1990). *cs* (*ch-42*) is a light-regulated nuclear gene which encodes a protein destined for the chloroplast and required for normal pigmentation in plants. These data led us to consider the possibility that the published nucleotide sequence of *crtA* might contain an error (Armstrong *et al.*, 1989).

Redetermination of the nucleotide sequence of the entire *crtA* coding region revealed one change, the absence of a single nucleotide at position 1733 (Fig. 5), with respect to the original sequence. As a consequence, the long ORF of 591 amino acids thought to encode CrtA is split into two segments. 21 amino acids of the 5' ORF following the frameshift error (Thr-221 to Ala-241) differ from those originally reported for CrtA before an in-frame stop codon is reached. Overlapping the stop codon, an AUG start codon preceded by a potential ribosome binding site (Fig. 5) could be used to initiate translation of a 3' ORF of 350 amino acids, corresponding to residues 242 to 591 originally deduced for CrtA. In accordance with the above considerations, we propose that the 5' and 3' ORFs encode CrtA and Bchl, respectively. Based on the gene order in this region (Zsebo & Hearst, 1984; Burke *et al.*, 1991a), previous data from *in vivo* complementation studies (Taylor *et al.*, 1983; Zsebo & Hearst, 1984; Giuliano *et al.*, 1988), and the rough mapping of *bchD* transposon-induced mutations (Zsebo, 1984), we propose that the ORF downstream from *bchI* corresponds to *bchD*. Absolute confirmation of these proposals will, however, require

Fig. 6. The *R. capsulatus* BchI and *A. thaliana* Cs proteins are highly conserved and contain putative ATP-binding sites. Numbers at the left indicate the amino acid positions. Identities between the two sequences are shown by asterisks. Dashes indicate gaps inserted to maximize the sequence alignment. A putative ATP-binding site is shown in bold, compared to a consensus (Walker *et al.*, 1982; Husain *et al.*, 1986) in which h is a small hydrophobic residue and X is any residue. NBRF database accession numbers for BchI and Cs are S04401 (the former CrtA sequence, residues 242–591) and S08654, respectively.

characterization of the gene products derived from this region of the chromosome (Fig. 3).

Mutations in the *bchD* gene also disrupt the accumulation of Bchl or its immediate precursors (Zsebo & Hearst, 1984; Giuliano *et al.*, 1988). BchD is thought to be required either for insertion of magnesium or for the coupled methylation reaction required for the conversion of protoporphyrin IX into magnesium protoporphyrin IX monomethyl ester in *Rhodobacter* (Gorchein, 1973; Biel & Marrs, 1983). The similarity of BchI[−] and BchD[−] phenotypes (Zsebo & Hearst, 1984; Giuliano *et al.*, 1988) suggests that BchI may also be required for these coupled reactions.

Fig. 6 presents the alignment of the deduced amino acid sequences of BchI and the *A. thaliana* Cs proteins. The BchI protein displays 49% sequence identity with residues 70–424 of Cs, with similarity extending throughout the entire alignment. The predicted C-termini of the proteins align perfectly, while the N-terminal extension of Cs, encoding the putative transit peptide, has no counterpart in BchI. Considering the enormous evolutionary distance between photosynthetic bacteria and higher plants, the striking degree of similarity predicted between BchI and Cs indicates that these proteins are homologues, and likely perform crucial functions.

Mutations in the nuclear-encoded *A. thaliana* *cs* (*ch-42*) gene result in a pale pigmentation phenotype (Koncz *et al.*, 1990), although no qualitative changes in pigment content were initially reported in a leaky T-DNA mutant (unpublished data cited in Koncz *et al.*, 1990). The Cs protein appears to be involved in photosynthesis, based on the photosynthetically inviable phenotype of plants homozygous for the X-ray-induced *ch-42* mutant allele of the *cs* gene. A gene from the chloroplast genome of

Euglena gracilis, an alga, has also recently been found to encode a BchI/Cs-like product (Orsat *et al.*, 1992). This observation indicates the export of the *cs* (*ch-42*) gene from the chloroplast to the nucleus in the course of evolution from algae to higher plants.

The *R. capsulatus*, *E. gracilis*, and *A. thaliana* proteins all contain putative ATP-binding sites (Fig. 6). We note that chelation of magnesium into protoporphyrin IX is an ATP-requiring process in higher plants and that this reaction has been proposed to require at least two components, a soluble stromal protein and a membrane-associated protein (Castelfranco & Beale, 1983; Walker & Weinstein, 1991). It will be of interest to determine whether BchI/Cs-like gene products have been conserved in other photosynthetic organisms, and whether analogies exist between BchI and BchD from *Rhodobacter* and the two component system in higher plants.

Conclusions

The time courses of *R. capsulatus* *crt* and *bch* mRNA accumulation and promoter activities have been studied during a rigorous and well defined shift in growth conditions from aerobic respiration to anaerobic photosynthesis. The oxygen-regulated expression of many chromosomally clustered genes encoding pigment biosynthetic enzymes and pigment-binding polypeptides is temporally coordinated during the development of a photosynthetic membrane. A redetermination of the nucleotide sequence of *crtA* has revealed a frameshift, suggesting that the 3' portion of this gene actually corresponds to *bchI*. The predicted *bchI* gene product, required for Bchl synthesis or accumulation in *Rhodobacter*, is strongly conserved in more highly

evolved photosynthetic organisms, including *E. gracilis* and *A. thaliana*.

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