

RegA Control of Bacteriochlorophyll and Carotenoid Synthesis in *Rhodobacter capsulatus*^{▽†}

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We provide in vivo genetic and in vitro biochemical evidence that RegA directly regulates bacteriochlorophyll and carotenoid biosynthesis in *Rhodobacter capsulatus*. β -Galactosidase expression assays with a RegA-disrupted strain containing reporter plasmids for Mg-protoporphyrin IX monomethyl ester oxidative cyclase (*bchE*), Mg-protoporphyrin IX chelatase (*bchD*), and phytoene dehydrogenase (*crtI*) demonstrate RegA is responsible for fourfold anaerobic induction of *bchE*, threefold induction of *bchD*, and twofold induction of *crtI*. Promoter mapping studies, coupled with DNase I protection assays, map the region of RegA binding to three sites in the *bchE* promoter region. Similar studies at the *crtA* and *crtI* promoters indicate that RegA binds to a single region equidistant from these divergent promoters. These results demonstrate that RegA is directly responsible for anaerobic induction of bacteriochlorophyll biosynthesis genes *bchE*, *bchD*, *bchJ*, *bchI*, *bchG*, and *bchP* and carotenoid biosynthesis genes *crtI*, *crtB*, and *crtA*.

Studies have shown that anaerobic induction of numerous photosystem, cytochrome, respiratory, and tetrapyrrole biosynthesis genes in *Rhodobacter capsulatus* involves coordinate regulation by several transcription factors (5, 6, 7). The two-component sensor kinase-response regulator pair RegB-RegA (15, 20, 26) is involved in controlling anaerobic and aerobic expression of many cellular processes, including the synthesis of heme (encoded by *hem* genes) (27) and cytochrome apoproteins that bind heme as a cofactor (30, 31). Thus, RegB-RegA are partially responsible for controlling the stoichiometry of heme synthesis by regulating the expression of heme binding apoproteins. With regard to photosystem expression, RegB-RegA are known to control high-level anaerobic expression of light harvesting I, light harvesting II, and reaction center apoproteins encoded by the *puc*, *puf*, and *puh* operons (20, 26). However, direct involvement of RegB or RegA in controlling expression of bacteriochlorophyll and carotenoid photosystem pigments has not been firmly established. There is evidence from a microarray expression study that expression levels of numerous bacteriochlorophyll and carotenoid biosynthesis genes are reduced in a strain of *Rhodobacter sphaeroides* disrupted for a RegB-RegA homolog called PrrB-PrrA (17, 21). Putative RegA binding sites have also been tentatively identified within the *bch* and *crt* gene clusters by predictive hierarchical clustering analysis of the *R. sphaeroides* genome (18). There is also a transient reduction in *bchC* expression upon a shift to anaerobic conditions in a *regA*-disrupted strain of *R. capsulatus* (1). However, direct binding of RegA to bacteriochlorophyll and carotenoid promoter regions has not been previously established.

In this study we constructed and assayed the expression pattern of a reporter plasmid for the large *bchEJG-orf428-bchP-idi* operon, which encodes numerous enzymes in bacteriochlorophyll *a* biosynthesis (2, 10, 12, 29) and an enzyme involved in carotenoid biosynthesis (16). Analysis of the *bchE* expression patterns in wild-type strain SB1003 and in a RegA-disrupted strain of *R. capsulatus* indicate that the *bchEJG-orf428-bchP-idi* operon is indeed transcriptionally regulated by RegA.

In addition, we investigated the expression pattern of two divergent carotenoid biosynthesis promoters, *crtA* and *crtI*. The *crtA-bchIDO* operon is responsible for expressing spheroidene monooxygenase (*crtA*), an enzyme in the carotenoid biosynthesis pathway, and two subunits of Mg-chelatase (BchD and BchI), the first biosynthetic enzyme in the bacteriochlorophyll branch of the tetrapyrrole pathway (2). Divergently transcribed from the *crtA-bchIDO* operon is the *crtIBK* operon, which is responsible for expression of phytoene dehydrogenase (*crtI*) and phytoene synthase (*crtB*) (2, 3), which are the first committed enzymes in the carotenoid biosynthetic pathway. Analysis of the divergent *crtA-bchIDO* and *crtIBK* operons indicates they are coregulated by RegA, thereby providing a mechanism of coordinating bacteriochlorophyll *a* and spheroidenone biosynthesis.

DNase I protection analysis was performed using the constitutively active variant of RegA called RegA* (13) to identify binding sites of RegA to the *bchE*, *crtA*, and *crtI* promoters. The transcriptional start site of *bchE* was also mapped by primer extension analysis to delineate the location of RegA binding relative to the location of promoter recognition sequences. These results provide new insights into the mechanism of regulating the synthesis of pigmented and electron carrier components of the photosystem in *R. capsulatus*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The parent *R. capsulatus* strain SB1003 (33) and the *regA*-disrupted strain MS01 (26) were grown aerobically and

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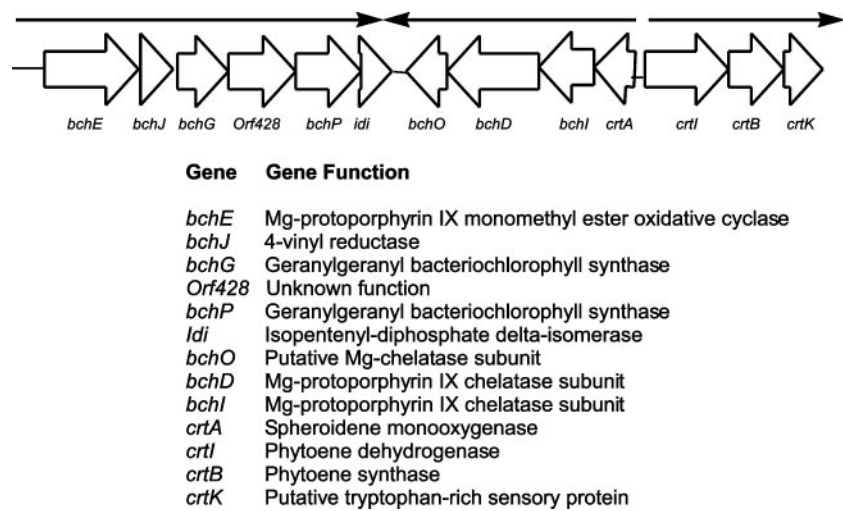


FIG. 1. Organization of the *bchEJG-orf428-bchP-idi*, *crtA-bchIDO*, and *crtIBK* operons. Large open arrows indicate genes with names of individual loci below. Thin solid arrows indicate transcripts. Putative functions of *bch*, *crt*, and *idi* genes have been studied elsewhere (4, 10, 11, 12). The *crtA-crtI* promoter region was defined by Elsen et al. (14), and the *bchE* promoter is as shown below in Fig. 3. The lengths of the transcripts are putative based on the absence of termination sites in the studied region (2).

photosynthetically at 34°C in PYS medium (34). *Escherichia coli* strain DH5α was grown aerobically in LB medium (24). Antibiotic concentrations used for *E. coli* were 200 μg/ml for ampicillin and 50 μg/ml for kanamycin, trimethoprim, and spectinomycin. Spectinomycin and kanamycin were both used for *R. capsulatus* at 10 μg/ml, while rifampin was used at 50 μg/ml.

Plasmid construction. Construction of a *bchE::lacZ* reporter plasmid involved PCR amplification of a 1.3-kb fragment using primers *bchESalI* (5'-GCCGCA GTCGTAGTTGTTCA-3') and *bchEEcoRI* (5'-CCGAATTCGGCCTGTTGT CGAAGGA-3') and standard procedures. The downstream *bchESalI* primer was designed such that it created an in-frame translational fusion of codon 242 of *bchE* to the *lacZ* reporter gene. The PCR-amplified fragment was digested with *EcoRI* and *SalI* and ligated into similarly digested pNM482 (19) and then transformed into DH5α. An *EcoRI*-digested spectinomycin-resistant omega cassette (23) was then introduced into the *EcoRI* site to produced the final *bchE::lacZ* reporter construct pJW10. The DNA sequence of the PCR-amplified fragment was confirmed by DNA sequence analysis.

Reporter plasmids pCrtI::ZΩ and pBchD::ZΩ used to determine the expression patterns of *crtI* and *bchD*, respectively, were previously described (22).

β-Galactosidase activity assays. Aerobic cultures used for determining β-galactosidase activity were grown in the dark as a 17-ml culture in a 250-ml flask that was shaken at 300 rpm. Photosynthetic cultures were grown in 15-ml screw-cap tubes that were completely filled and illuminated with a 60-W tungsten bulb

at 34°C as described by Young et al. (34). Inocula were from overnight cultures that were diluted 500-fold prior to incubation. Both aerobic and anaerobic photosynthetic cultures were grown to a cell density of 75 Klett units, chilled in an ice water bath, and harvested by centrifugation at 10,000 × g for 10 min at 4°C. Strains were disrupted by sonication and assayed for activity as described previously (34). β-Galactosidase activities reported represent averages of duplicate measurements of triplicate cultures, with error bars representing standard deviations. Units represent nanomoles of orthonitrophenyl-β-D-galactoside (ONPG) hydrolyzed per minute per milligram of cellular protein (20).

Primer extension analysis. Total RNA was isolated from log-phase cultures of *R. capsulatus* strain SB1003 grown photosynthetically in PYS medium. RNA was prepared from six preps containing 3 ml of culture each. Total RNA was isolated using an RNeasy mini kit (QIAGEN) and resuspended in RNase-free water to a final concentration of 3 mg/ml.

A commercially synthesized primer (5'-GCCGGAGTGGTAATTGGGG-3') was prepared complementary to the predicted mRNA transcript coding for the *bchE* gene. 5' ³²P labeling of the primer was carried out at 37°C for 1 hour in a 10-μl reaction mixture containing 50 pmol primer, 1× phage T4 kinase buffer (New England Biolabs), 10 units of phage T4 polynucleotide kinase (New England Biolabs), and 500 μCi of [γ-³²P]ATP (specific activity, 7,000 Ci/mmol; ICN Biomedicals). The enzyme was inactivated by heat treatment for 10 minutes at 80°C. Labeled primer was purified using QIAquick spin columns (QIAGEN) and

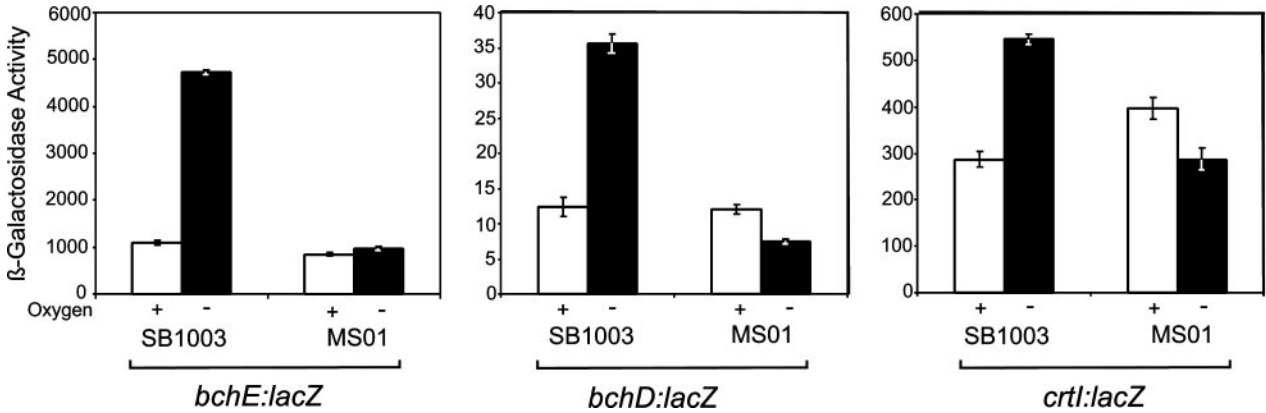


FIG. 2. β-Galactosidase assay of *bchE*, *crtI*, and *bchD*. White bars indicate aerobic expression, and black bars indicate anaerobic expression levels of activity. SB1003 is the parent strain, and MS01 is a *regA*-disrupted strain. Error bars represent standard deviations for triplicate assays. β-Galactosidase units are reported as nmol of ONPG hydrolyzed per minute per milligram of protein.

resuspended in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA buffer to a final concentration of 2 pmol/μl.

5'-³²P-labeled primer was annealed to target RNA in a 10-μl reaction volume comprised of 15 μg cellular RNA, 2.5 μl of ³²P-labeled primer, 10 mM Tris-acetate (pH 7.4), 60 mM NH₄Cl. The annealing reaction mixture was heated to 80°C for 10 minutes and then cooled slowly to 40°C and incubated at 40°C for a further 20 minutes. A 10-μl volume of premixed extension mix comprised of 20 mM Tris-acetate (pH 7.4), 20 mM magnesium acetate, 120 mM NH₄Cl, 80 μg/ml actinomycin D, 50 mM dithiothreitol, 750 μM deoxynucleoside triphosphates, and 10 units of murine leukemia virus SuperScript III reverse transcriptase (Invitrogen) was added to the annealing reaction mixture and incubated at 42°C for 2 hours. Unreacted primer and salt were then removed from the reaction mixture by using DyeExII spin columns (QIAGEN). Primer extension products were mixed with 10 μl of 3× formamide loading dye comprised of 20 mM EDTA, 0.05% (wt/vol) bromophenol blue, 0.05% (wt/vol) xylene cyanole in deionized formamide. A dideoxy sequencing ladder was generated from the same labeled primers by using the Sequitherm EXCEL II sequencing kit (Epicenter). Sequence ladders and primer extension products were heated to 95°C for 5 minutes and then placed on ice for 10 minutes prior to gel electrophoresis in an 8 M urea, 0.5× Tris-acetate EDTA, 6% (wt/vol) polyacrylamide gel.

DNase I footprint analysis. RegA* was purified as previously published (13). Amplification of the *crtI* and *bchD* promoter region using PCR was performed using fluorescently labeled primers, with either the top or bottom strand amplification primers labeled with 6-carboxyfluorescein phosphoramidate (FAM) (25, 32). The *crtI* and *bchD* promoter regions were amplified using primers *crtI*/AForward (5'-CGCATCGCTGCAGCAAGAC-3') and *crtI*/AReverse (5'-GGA CAGGATCATCTGGCTGAT-3'). Amplification of the top and bottom fluorescently labeled *bchE* promoter region was with primers *bchE*footForward (5'-CATGCCTGATTACCTTTG-3') and *bchE*footReverse (5'-GCCGGAGTGG TAATTGGG-3').

Individual footprint reactions were initiated in a 10-μl binding reaction mixture that contained 2 μl 5× footprint binding buffer composed of 125 mM HEPES (pH 7.8), 50 mM K-acetate (pH 8), 25 mM Mg-acetate, 10 mM CaCl₂, 5 mM dithiothreitol, and 125 μg/ml bovine serum albumin (31), 1 pM of FAM-labeled probe, and water to a final volume of 10 μl. The binding reaction mixture was added to a 10-μl protein solution composed of 1× footprint binding buffer (13) and various amounts of RegA*. Initial binding reaction mixtures were incubated for 30 min at 22°C followed by a 5-min DNase I digestion that was initiated by adding 2 μl of New England Biolabs DNase I at an approximately 1:6,000 dilution in 1× footprint binding buffer, which gave partial probe digestion. The digestion reactions were then stopped by adding 40 μl 0.5 M EDTA. DNA segments were purified using QIAGEN PCR cleanup columns using the manufacturer's recommended protocol and were concentrated in a Speed-Vac to a final volume of 10 μl, to which 10 μl of Sigma HiDi formamide was added. Samples were then transferred to a 96-well plate containing 0.5 μl of Applied Biosystems 500-LIZ size standards in each well (25, 32). The plate was then sealed using a septum, heated to 95°C for 5 min, and cooled quickly on ice. Fragment analysis was performed on an Applied Biosystems 3730 automated DNA sequencing machine with fragments detected using the GeneMapper-Generic protocol. Samples were analyzed using SoftGenetics GeneMarker 1.4 analysis software.

Sequencing ladders for use in defining a precise region of DNase I protection were generated using a Fermentas Life Sciences CycleReader DNA sequencing kit. The ladders were set up according to the manufacturer's protocol, with the exception that 6-FAM primers were used instead of radiolabeled primers. For template, 100 fmol of PCR product was used that was generated using the primers *bchE*footForward and *bchE*footReverse without the 6-FAM label.

RESULTS

RegA regulation of photopigment genes. Previous studies from our laboratory demonstrated that *regB* and *regA* null mutations had no effect on expression of the bacteriochlorophyll *bchH* or *bchC* promoters (20, 26). These findings, however, do not reflect the phenotypes of *regB* and *regA* null mutations that are known to be severely hampered in their ability to synthesize both bacteriochlorophyll and carotenoids (20, 26). In order to reconcile these differences, we constructed a translational fusion to the *bchE* promoter that drives expression of four enzymes involved in the Mg-tetra-

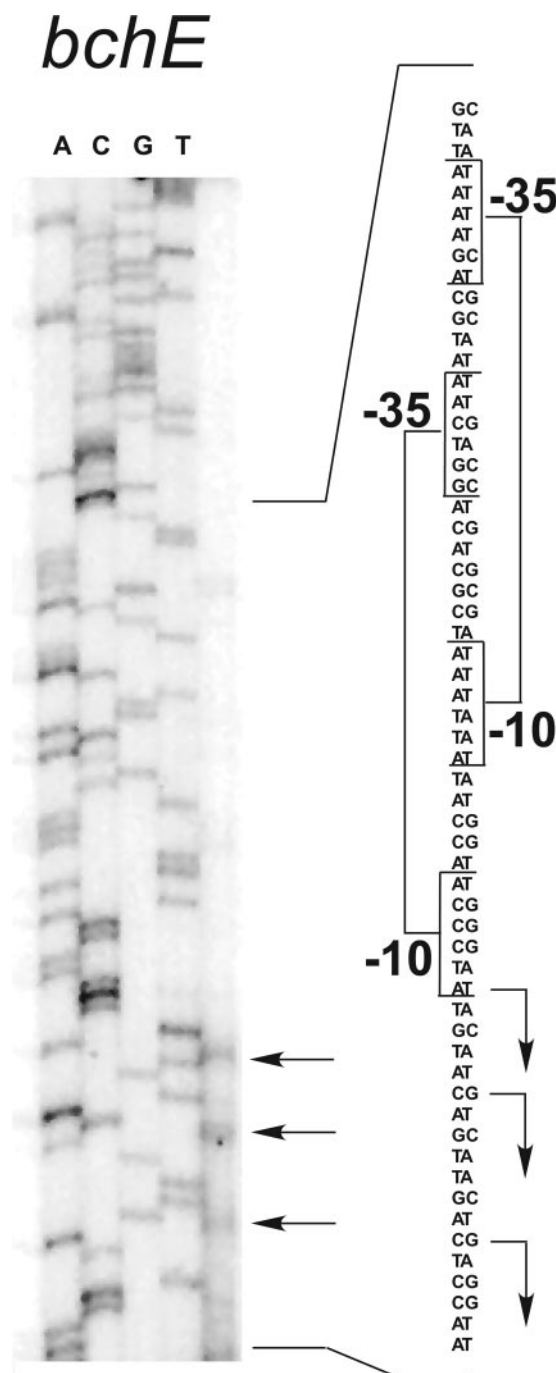


FIG. 3. Primer extension analysis of *bchE* RNA. Stable mRNA 5' ends are indicated by arrows next to a dideoxy sequencing ladder (indicated by G, C, A, and T). Arrows next to the sequence indicate the locations of the 5' end and the direction of transcription. Open boxes indicate putative σ -70-type -10 and -35 consensus promoter recognition sequences.

pyrrole branch of the bacteriochlorophyll biosynthesis pathway (Fig. 1). This reporter was assayed for expression in the wild type and in *regA*-disrupted strains of *R. capsulatus*. The results of β -galactosidase activity assays in Fig. 2 show that *bchE::lacZ* expression is between four- and fivefold higher in

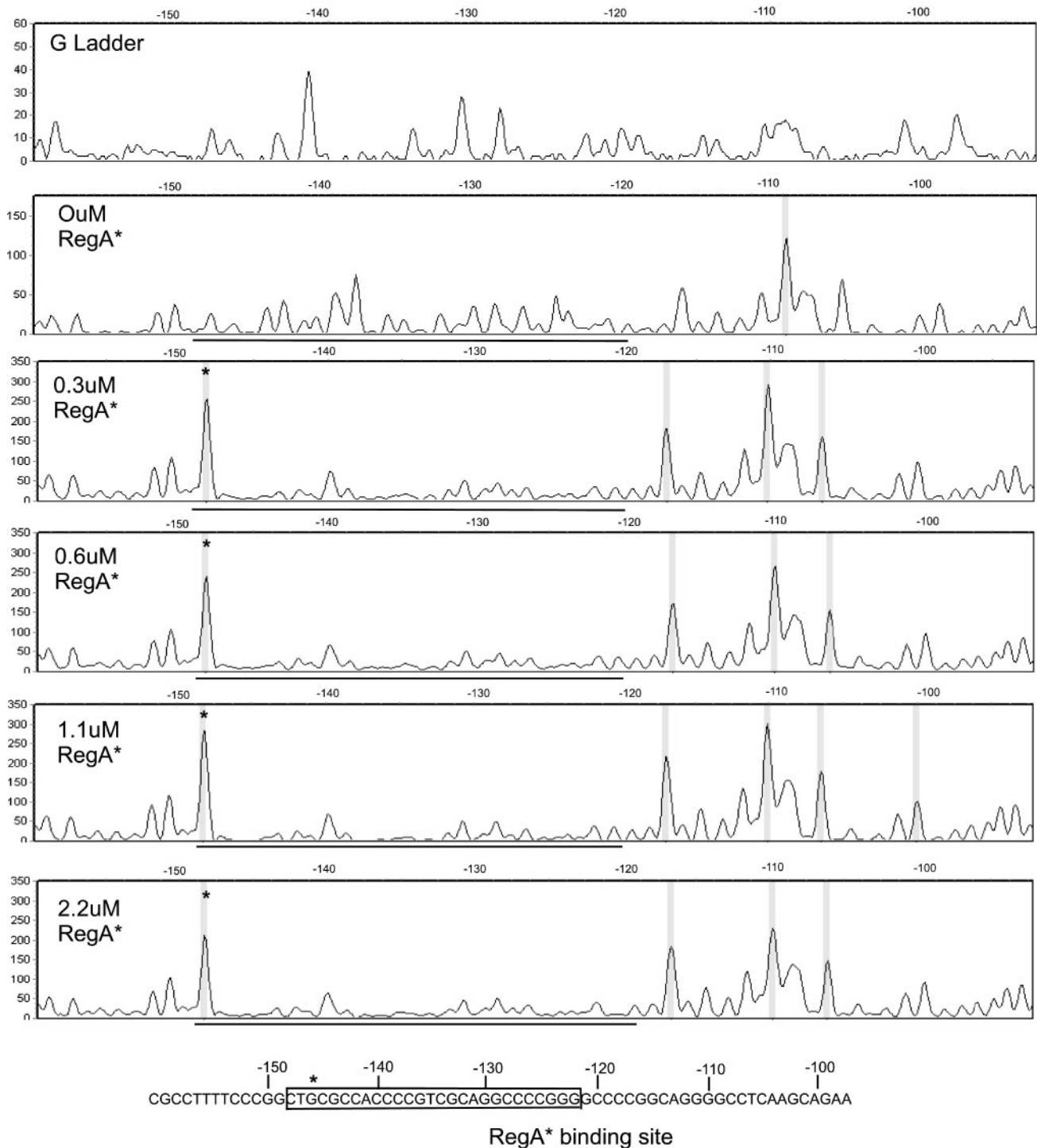
A *bchE1* Top

FIG. 4. DNase I footprint analysis of RegA* to the *bchE* and *crtA-crtI* promoter regions. Each footprint reaction was located to a distinct sequence by running parallel didoxy G, C, T, and A reactions using FAM-labeled DNA segments, with the G sequencing ladders shown as an example. Underlined regions correspond to areas of DNase I protection. Hypersensitive sites are indicated by an asterisk. RegA* concentrations are indicated in each line. Regions of protection corresponding to the sequence are indicated by a box on the bottom of the sequence. Numbering above the peak profiles and above the sequence is relative to the start of transcription for *bchE* (A) and *crtA* (B).

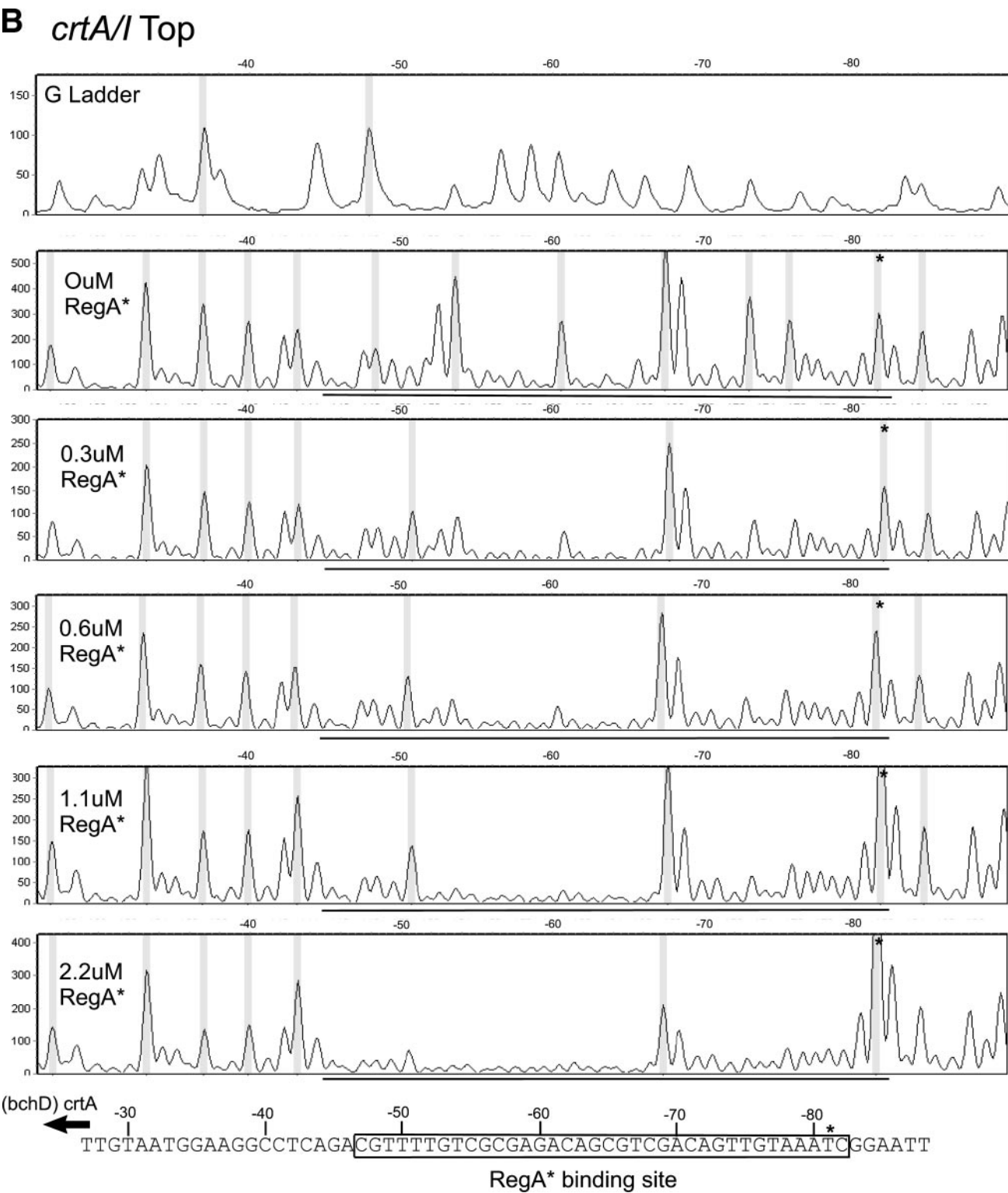


FIG. 4—Continued.

the parent strain, SB1003, when grown under steady-state anaerobic photosynthetic conditions than when grown under dark aerobic conditions. In contrast, there was no significant increase in expression of *bchE::lacZ* when the *regA*-dis-

rupted strain, MS01, was grown under anaerobic photosynthetic conditions.

We also undertook similar analysis of the *crtA* operon, which codes for spheroidene monooxygenase (CrtA), an enzyme late

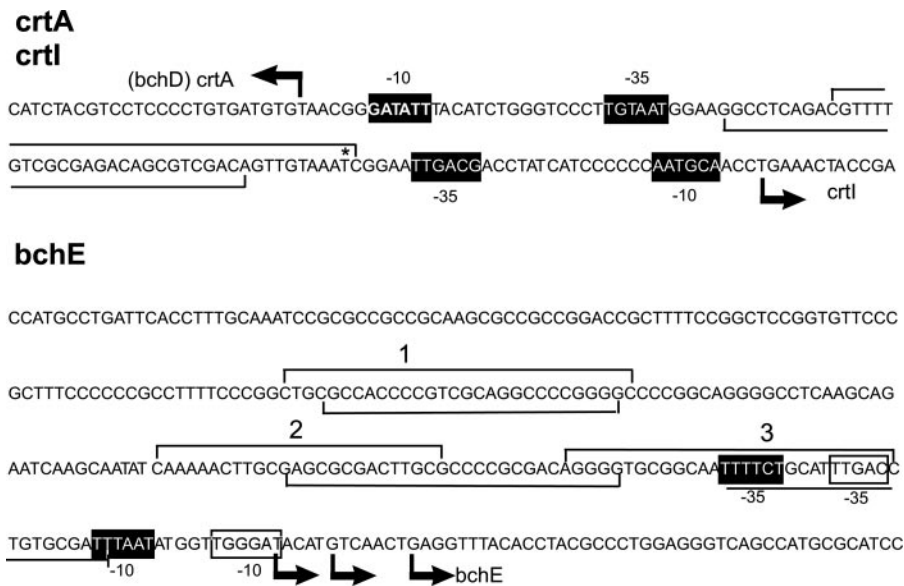


FIG. 5. Summary of identified RegA* regulatory binding sites. Arrows indicate start sites of transcription. Brackets indicate top and bottom strands of protection. Black and open boxes indicate RNA polymerase promoter recognition sequences.

in the carotenoid biosynthetic pathway (2, 3, 4) and two subunits of Mg-chelatase (*bchD* and *bchI*), the first enzyme in the Mg-tetrapyrrole branch of the bacteriochlorophyll *a* biosynthetic pathway (10). For this analysis, we used a *bchD::lacZ* translational expression vector that contains the *crtA* promoter region (22). Expression of *bchD::lacZ* (*crtA* operon) in the parent strain, SB1003, showed an approximate threefold increase in expression as these cells were shifted from aerobic to anaerobic growth conditions (Fig. 2). In contrast, there was no induction, and indeed a slight reduction, in *bchD::lacZ* expression when the *regA*-disrupted strain MS01 was shifted to anaerobiosis.

Divergently transcribed from the *crtA-bchI* operon is the *crtIBK* operon, which codes for phytoene dehydrogenase (*crtI*) and phytoene synthase (*crtB*), the first two enzymes in the biosynthetic pathway for the carotenoid spheroidenone (3, 4). Previous promoter mapping studies have demonstrated that the *crtA* and *crtI* promoters are both present in the *crtA-crtI* intergenic region, with the start sites of *crtA* and *crtI* separated by 66 bp (14). Analysis of *crtI::lacZ* expression in the parent strain, SB1003, showed that *crtI::lacZ* expression is approximately twofold higher under anaerobic conditions than under aerobic growth conditions (Fig. 2). As is the case for *bchD::lacZ* (*crtA* operon) expression, there is no anaerobic induction of *crtI::lacZ* expression in the *regA*-disrupted strain MS01.

RegA* binds to *bchE*, *crtI*, and *crtA* promoter regions. We first undertook primer extension analysis of the *bchE* transcription start site using a primer that was designed from the 5' region of *bchE*. Primer extension analysis resulted in three equally strong 5'-end products located 31, 38, and 43 bp upstream of the *bchE* start codon (Fig. 3). Analysis of the region upstream of the furthest upstream primer extension product showed the presence of two putative -10 and -35 σ -70 RNA polymerase binding sites that are similar to two previously noted putative σ -70 RNA polymerase binding

sites upstream of the *bchE* gene (2). These regions are indicated in Fig. 3.

We investigated whether the constitutively active variant of RegA called RegA* (13) could specifically bind to the *bchE*, *crtA*, and *crtI* promoter regions. Prior studies indicated that RegA* binds to identical sites as wild-type RegA does but with a signif-

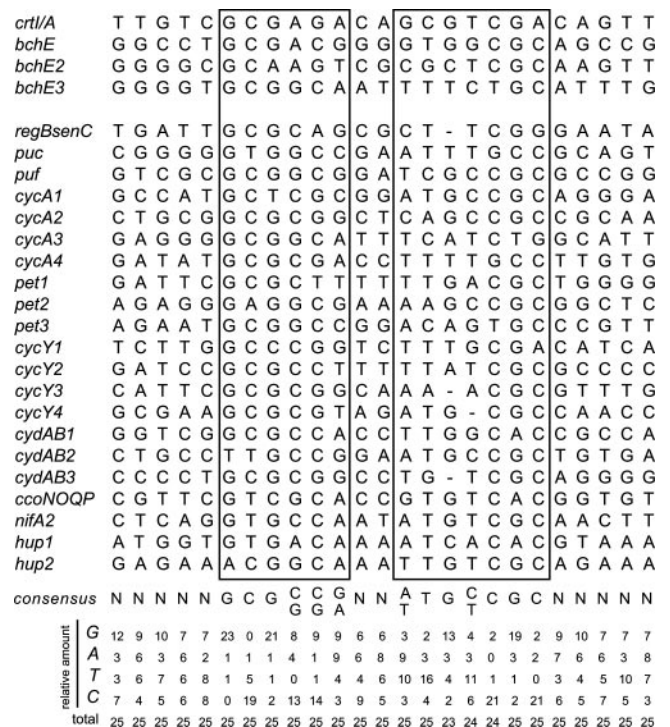


FIG. 6. Alignment of three *bchE* regions and the *crtA-crtI* region of DNase I protection by RegA* to previously identified RegA binding sites. The consensus pattern is shown below.

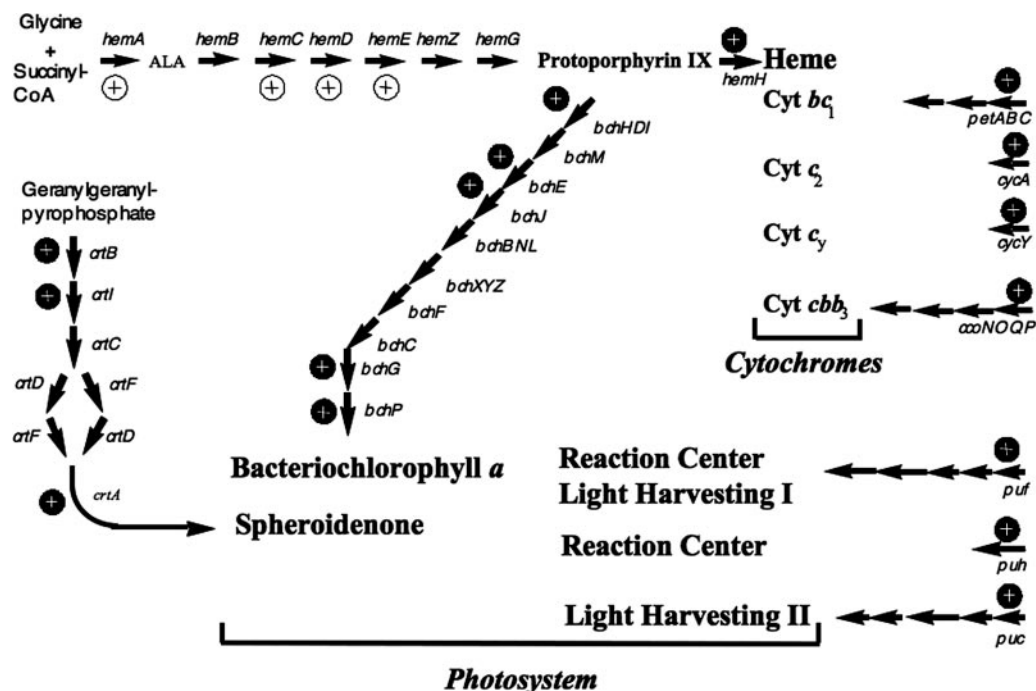


FIG. 7. Summary of genes regulated by RegA. The filled red circles with a + indicate genes directly under RegA control based on gene expression and DNase I protection studies. Open red circles with a + indicate genes under RegA control as assayed by gene expression studies.

icantly higher affinity (9, 13). For this analysis, we undertook DNase I protection assays using 6-FAM-fluorescently labeled DNA probes specific to the *bchE* and *crtA-crtI* promoter regions with the fluorescent label on the 5' end of either the top or bottom strands.

DNase I protection assays of RegA* binding to the *bchE* promoter region showed several areas that exhibited a reproducible reduction of peak height in the elution profile (Fig. 4a and b; see also Fig. S1a to e in the supplemental material). The top and bottom strands exhibited three regions of protection by RegA* with significant overlap in top and bottom strand protection (Fig. 5). Areas of observed top strand protection span from bp -148 to -121, from -86 to -62, and from -52 to -29 relative to the start site of *bchE* transcription (Fig. 5). Areas of bottom strand protection extend from bp -145 to -122, from -75 to -48, and from -38 to -14 relative to the start site of *bchE*. Each of the RegA* protected regions in the *bchE* promoter region contain putative RegA binding sequences, based on an alignment of protected sequences with known RegA/RegA* binding sites (Fig. 6) (31).

DNase I protection assays of RegA* binding to the divergent *crtA-crtI* promoter region show reproducible reduction of peak height in a discrete region of the elution profile (Fig. 4b; see also Fig. S2 in the supplemental material). Inspection of the protected sequence indicates that the area of top and bottom strand protection spans a region that is equidistant (37 and 35 bp, respectively) from the start of *crtA* and *crtI* transcription (Fig. 5). This area contains a single putative RegA binding sequence, based on an alignment with

RegA/RegA* protected areas that have been identified in other studies (Fig. 6) (31).

DISCUSSION

This study demonstrates that RegA* binds to the *bchE* and *crtA-crtI* promoter regions. There is also a decrease in bacteriochlorophyll and carotenoid gene expression that occurs upon disruption of RegA. The most straightforward interpretation is that the RegB-RegA regulon (Reg regulon) is directly involved in positive transcriptional activation of anaerobic expression of enzymes in the Mg-tetrapyrrole branch of the bacteriochlorophyll *a* biosynthetic pathway as well as enzymes in the spheroidenone pathway.

Expression of the *bchEJG-orf428-bchP-idi* operon is clearly directly controlled by RegA. This operon codes for Mg-protoporphyrin IX monomethyl ester oxidative cyclase (BchE), which is responsible for catalyzing the formation of the isocyclic (fifth) ring in bacteriochlorophyll (8, 10). This operon also codes for enzymes involved in later steps of the pathway, such as *bchI*, which encodes bacteriochlorophyll 4-vinyl reductase (28), and *bchG* and *bchP*, which encode enzymes involved in the attachment of geranylgeranyl and its subsequent reduction to phytol, respectively (Fig. 1 and 7) (11). The *idi* gene in this operon also codes for an enzyme that is crucial in the activation of isopentyl diphosphate as a substrate for the isoprenoid pathway (10, 11, 16). Anaerobic induction of expression of the *crtA-bchIDO* operon is also mediated by RegA. This operon codes for the BchI and BchD subunits of Mg-chelatase, which is involved in insertion of Mg^{2+} into protoporphyrin IX, which

is the first committed step of the bacteriochlorophyll *a* branch (2, 10). Thus, RegA has a significant role in regulating the partitioning of tetrapyrroles into the Mg-branch under anaerobiosis.

In addition to regulating bacteriochlorophyll production, we have also shown that RegA controls expression of the *crtIBK* operon, which codes for phytoene synthase (*crtI*) and phytoene dehydrogenase (*crtB*), enzymes that catalyze the first committed steps in the synthesis of spheroidenone (2, 3, 4). The divergent *crtA* and *crtI* promoters appear to contain a single RegA* binding site located approximately equidistant from the start site of transcription for both of these promoters. Thus, binding of RegA* to this single site allows coordinate expression of the *crtA-bchIDO* operon, which codes for the first enzymes in the Mg-tetrapyrrole branch of the bacteriochlorophyll pathway, and the *crtIBK* operon, which encodes the first two enzymes in the carotenoid biosynthetic pathway. Presumably, this allows RegA to control stoichiometric synthesis of these two photopigments.

Prior studies also indicated that RegA has a major role in controlling synthesis of the light harvesting and reaction center apoproteins that bind bacteriochlorophyll and carotenoid photopigments to form the pigmented components of the photosystem (20, 26). Indeed, inspection of gene expression assays from this and other studies (20, 26) indicates that RegA is responsible for significant increases in anaerobic transcription from the *puf*, *puc*, and *puh* promoters, which drive high-level photosystem apoprotein expression and anaerobic transcription from the *bchE*, *crtI*, and *crtA* promoters that express many enzymes involved in bacteriochlorophyll and carotenoid biosynthesis. Thus, the RegA regulon appears to have a major role in regulating synthesis of virtually all of the pigment and polypeptide components of the *Rhodobacter* photosystem (Fig. 7).

In addition to photosystem synthesis, the RegA regulon has a major role in controlling synthesis of heme and cytochrome apoproteins that bind heme (27, 30, 31). For example, expression of cytochromes *c_v* and *c₂* and the *bc₁* complex, which donate electrons from the ubiquinone pool to the reaction center, is regulated by RegA (31). Furthermore, RegA also controls both aerobic and anaerobic expression of various electron acceptors, such as ubiquinol oxidase, *cbb₃* oxidase, and dimethyl sulfoxide reductase, which in turn donate electrons derived from the quinone pool to the terminal electron acceptors O₂ and dimethyl sulfoxide, respectively (30). Thus, the RegA regulon controls expression of virtually all components of the photosystem and respiratory electron transfer system and is therefore a key regulator of these different growth modes.

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