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Transcriptional Regulation of Several Genes for Bacteriochlorophyll Biosynthesis in *Rhodopseudomonas capsulata* in Response to Oxygen

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Although it has been shown that bacteriochlorophyll synthesis in *Rhodopseudomonas capsulata* is repressed by oxygen and high light intensity, few details of regulation by these environmental factors are known, primarily owing to a lack of assays for the biosynthetic enzymes. We have examined regulation at the transcriptional level by isolating and studying fusions between the Mu dl(Ap^r *lac*) phage and various *bch* genes. In these strains, the *lacZ* gene of the phage is under the control of *bch* gene promoters. We have found that atmospheric oxygen tension (20% O_2) reduces the expression of these fusions at least twofold compared with low oxygen tension (2% O_2). Therefore, transcription of the *bchA*, *bchB*, *bchC*, *bchG*, and *bchH* genes is regulated in response to oxygen.

In *Rhodopseudomonas capsulata*, the biosynthesis of protoheme and bacteriochlorophyll follows a common pathway from the condensation of succinyl coenzyme A with glycine to the formation of protoporphyrin IX. Biosynthesis of protoheme and bacteriochlorophyll proceeds from protoporphyrin IX by the chelation of iron and magnesium, respectively. The pathway from magnesium chelation to formation of bacteriochlorophyll is depicted in Fig. 1. The work of Cohen-Bazire et al. (6) was instrumental in demonstrating that oxygen represses bacteriochlorophyll synthesis in purple nonsulfur photosynthetic bacteria. However, study of the details of regulation by oxygen has proven difficult. One of the main reasons for this is the lack of assays for most of the enzymes involved in the conversion of protoporphyrin IX to bacteriochlorophyll. One enzyme of the common pathway that has been studied in some detail is δ-aminolevulinate synthase. This enzyme appears to be repressed in highly aerated cultures (10). The only other enzyme whose activity can be directly determined is magnesium protoporphyrin methyl transferase. Synthesis of this enzyme also appears to be repressed by oxygen (10). Gorchein (7, 8) was able to demonstrate the conversion of exogenous protoporphyrin IX to magnesium protoporphyrin monomethyl ester by whole-cell suspensions of *Rhodopseudomonas spheroides*. This conversion only occurred when the cells were grown under low oxygen tension. However, further work was hampered by the inability of cell extracts to catalyze the chelation of magnesium.

To elucidate the mechanism by which bacteriochlorophyll synthesis is regulated in response to oxygen, it is necessary to be able to measure the activity of each of the genes coding for bacteriochlorophyll biosynthetic enzymes. Because of the lack of assays for these enzymes, we have used an alternative method for measuring the transcription of the biosynthetic genes. This report details the use of the Mu dl(Ap^r *lac*) phage developed by Casadaban and Cohen (5) to create fusions between the *lacZ* (β-galactosidase) gene and various *bch* genes. By placing the *lacZ* gene under the control of various *bch* gene promoters, the activity of these promoters under different culture conditions can be determined by measuring the amount of β-galactosidase formed. Using these fusions, the regulation of many of the *bch* gene promoters in response to oxygen and light has been determined. We have found that growth in a low-oxygen environment increased the expression from the *bch* gene promoters two- to fourfold.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains used in this work are described in Table 1. pRPS404 is a derivative of RP1 containing all of the known *bch* and *crt* genes. The plasmid carries a gene for kanamycin resistance and the *crtD223* allele. The genes for ampi-

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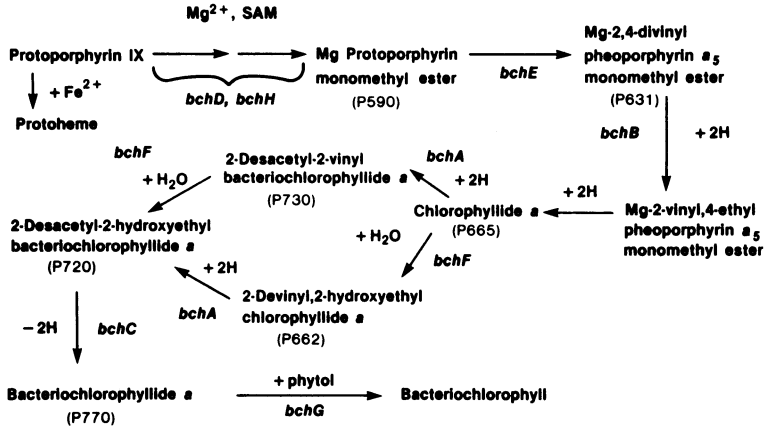

FIG. 1. Bacteriochlorophyll biosynthetic pathway (after references 15 and 16). The order of reactions has been inferred from consideration of the structures of accumulated intermediates. The red-most spectral peaks of the intermediates are indicated in parentheses. Genes have been positioned along the pathway on the basis of the bacteriochlorophyll intermediates accumulated in strains with lesions in those genes, but unambiguous assignments await further enzymological and genetic studies. SAM, *S*-Adenosylmethionine.

cillin and tetracycline resistance have been inactivated by amber mutations (11, 13).

Media. *R. capsulata* was routinely grown either in a malate-minimal salts medium (RCV) (19) or in 0.3% Difco Bacto-Peptone–0.3% Difco yeast extract (PYE). *Escherichia coli* was grown in minimal salts medium A1 (12) modified by the omission of sodium citrate and thiamine. The carbon source included in this medium was either 0.2% glucose or 0.2% sorbitol. L-broth (2) modified by omitting glucose and decreasing the sodium chloride to 0.5% was used as a rich medium. Antibiotics, when used, were added to yield the following final concentrations (μg/ml): ampicillin, 25; kanamycin, 10; streptomycin, 75; rifampin, 75. When solid media were employed, 1.5% agar (Difco) was added to the above media. Soft agar overlays contained 0.7% agar. 5-Bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal), used to determine the presence of β-galactosidase activity in colonies, was included in agar-containing media at a concentration of 40 μg/ml. Oxygen tensions, where indicated, were measured polarimetrically in the liquid phase of each culture with a Clark-type oxygen electrode (YSI model 53).

Mating procedures. Matings between *E. coli* donors and *R. capsulata* recipients were as described previously by Marrs (11). Matings between *E. coli* donors and recipients were accomplished by the liquid mating technique of Barth (1).

Induction of Mu dl(Ap^r *lac*) phage. Mu dl(Ap^r *lac*) lysates were obtained by thermal induction of strain MAL103 as described previously by Casadaban and Cohen (5).

Identification of precursors accumulated by Mu d insertion strains. Mu d insertion strains were grown for 2 to 3 days at 30°C in the dark without shaking in 30 ml of RCV containing 0.6% glucose, 0.5% pyruvate, and 0.5 M dimethyl sulfoxide (RCV$^+$) to obtain low oxygen tensions. Cultures were grown under high oxygen tension by inoculating 30 ml of RCV$^+$ in a 1-liter flask with the appropriate strain and incubating it for 1 day with vigorous shaking. Five milliliters of culture were extracted with acetone-methanol (7:2) as described previously by Cohen-Bazire et al. (6). The rest of the culture was harvested and suspended in 4 ml of Z-buffer (12). The cells, at 0°C, were disrupted by two 20-s bursts of sonication in a model 140 Branson Sonifier. The suspension was centrifuged for 20 min at 27,000 × *g*. Absorbances of the acetone-methanol extract, the sonic extract, and the culture fluid were recorded from 300 to 900 nm with a Beckman DU7 spectrophotometer and compared with spectra obtained from strains carrying known *bch* lesions.

Identification of porphyrins accumulated by *bchD*, *bchE*, and *bchH* strains. Strains containing mutations in *bchD*, *bchE*, and *bchH* were grown in 200 ml of RCV$^+$ for 2 to 3 days without shaking and extracted with 10 ml of acetone-methanol as described above. The emission spectra of these extracts, along with protoporphyrin IX, magnesium protoporphyrin IX, and protoporphyrin monomethyl ester standards (Porphyrin Products), were recorded by a Perkin-Elmer 512 fluorescence spectrophotometer with the excitation wavelength set at 402 nm. The extracted porphyrins and standards were also characterized by chromatography on Silica Gel G TLC plates (Fisher). The plates were developed in benzene-ethyl acetate-ethanol (4:1:1) and viewed under long-wave UV light while still wet.

Determination of β-galactosidase activity in *bch*::Mu d insertion strains. Overnight PYE broth cultures of the strains to be assayed were used to inoculate 10 ml of PYE in culture tubes (16 by 150 mm) to a Klett (red filter) value of 25. The tubes were fitted with rubber septa, each containing a glass tube as a gas inlet and a syringe needle as a gas outlet. The cultures were incubated with appropriate lighting and sparged with different gases as described below. The incubation was stopped when the cultures reached a density of ca. 100 Klett units, and the dissolved oxygen concentration of each culture was determined with a YSI model 53 dissolved oxygen monitor. Eight milliliters of culture were harvested by centrifugation at 27,000 × *g* for 10 min in a Sorvall SS-34 rotor. The cells were suspended

TABLE 1. Bacterial strains

Designation	Genotype[a]	Source or reference
E. coli K-12		
AJB361	*Δ(ilvGEDAYC)2049 leu-455 galT12 recA56 srlC300*::Tn*10*	Conjugation of CU505 with NK5304 as donor
AJB402	W3110[pRPS404]	pRPS404 moved by conjugation into W3110
AJB403	*recA56 srlC300*::Tn*10*[pRPS404]	Transduction of AJB402 with AJB361 as donor
AJB409	W3110[pRPS404 Φ(*bchH'-lacZ*$^+$)*700*]	This study
AJB417	W3110[pRPS404 Φ(*bchH'-lacZ*$^+$)*701*]	This study
AJB465	W3110[pRPS404 Φ(*bchG'-lacZ*$^+$)*702*]	This study
AJB469	W3110[pRPS404 Φ(*bchH'-lacZ*$^+$)*704*]	This study
AJB471	W3110[pRPS404 Φ(*bchC'-lacZ*$^+$)*705*]	This study
AJB477	W3110[pRPS404 Φ(*bchB'-lacZ*$^+$)*708*]	This study
AJB479	W3110[pRPS404 Φ(*bchB'-lacZ*$^+$)*709*]	This study
AJB496	W3110[pRPS404 Φ(*bchG'-lacZ*$^+$)*711*]	This study
AJB498	W3110[pRPS404 Φ(*bchC'-lacZ*$^+$)*710*]	This study
BEC404	HB101[pRPS404]	Marrs (11)
CU505	*Δ(ilvGEDAYC)2049 leu-455 galT12*	Watson et al. (18)
MAL103	(F$^-$) Mu cts d1(Ap^r *lac*) *araB*::Mu cts *araD139 Δ(proAB-lacIPOZYA)*XIII *strA*	Casadaban and Cohen (5)
NK5304	Hfr PO45 *srlC300*::Tn*10 recA56 ilv-318 thr-300 thi-1 rel-1 rpsE300*	N. Kleckner
W3100	(F$^-$) (λ$^-$)	H. Lozeron
R. capsulata		
AJB456	Φ(*bchH'-lacZ*$^+$)*700 hsd-1 str-2*	This study
AJB463	Φ(*bchH'-lacZ*$^+$)*701 crtD233 hsd-1 str-2*	This study
AJB466	Φ(*bchG'-lacZ*$^+$)*702 crtD233 hsd-1 str-2*	This study
AJB472	Φ(*bchC'-lacZ*$^+$)*705 crtD233 hsd-1 str-2*	This study
AJB478	Φ(*bchB'-lacZ*$^+$)*708 crtD233 hsd-1 str-2*	This study
AJB480	Φ(*bchB'-lacZ*$^+$)*709 hsd-1 str-2*	This study
AJB499	Φ(*bchC'-lacZ*$^+$)*710 crtD233 hsd-1 str-2*	This study
AJB500	Φ(*bchG'-lacZ*$^+$)*711 crtD233 hsd-1 str-2*	This study
BPY61	*bchD561 crtF129*	Taylor et al. (17)
BPY72	*bchF572 crtF129*	Taylor et al. (17)
BPY98	*bchC598 crtF129*	Derived from PY1291 by method described by Taylor et al. (17)
BRP3	*bchA603 crtF129 hsd-1 str-2*	Taylor et al. (17)
BRP4	*bchE604 crtF129 hsd-1 str-2*	Taylor et al. (17)
BRP33	*bchG633 crtF129 hsd-1 str-2*	Taylor et al. (17)
BRP50	*bchH650 crtF129 hsd-1 str-2*	Taylor et al. (17)
MB1007	*bchC1007*	Taylor et al. (17)
MB10072	*bchC1007 rif-10*	Transduction of MB1007 with gene transfer agent from SB1003
MB10081	*bchD1008 rif-10*	Marrs (11)
PAS100	*hsd-1 str-2*	Taylor et al. (17)
SB1003	*rif-10*	Yen and Marrs (20)
Y80	*bchB80 str-2*	Yen and Marrs (20)

[a] Genetic nomenclature is as suggested in Instructions to Authors, J. Bacteriol. **153**:vi–vii. For example, W3110[pRPS404 Φ(*bchH'-lacZ*$^+$)*700*] indicates that strain AJB409 is a derivative of *E. coli* W3110 carrying an R-prime plasmid in which the Mu d1 phage has inserted into the *bchH* gene of pRPS404 resulting in a fusion between the *lacZ* gene of the phage and the truncated *bchH* gene.

in 1.0 ml of Z-buffer and disrupted by sonication and centrifuged as described above. The assay mixture for β-galactosidase contained 1.0 ml of Z-buffer, 25 μl of sonic extract, and 0.2 ml of a solution of 4 mg of *o*-nitrophenyl-β-D-galactopyranoside per ml. The assay mixture was incubated at 37°C until a light-yellow color appeared. At that time, 0.5 ml of 1.0 M sodium carbonate was added to stop the reaction. The absorbance was determined at 420 nm. The protein concentration of the sonic extracts was determined by the dye binding method of Bradford (4) with crystallized bovine serum albumin (Pentex) as standard.

Extraction and restriction analysis of plasmid DNA. R-prime DNA was extracted from *E. coli* strains as

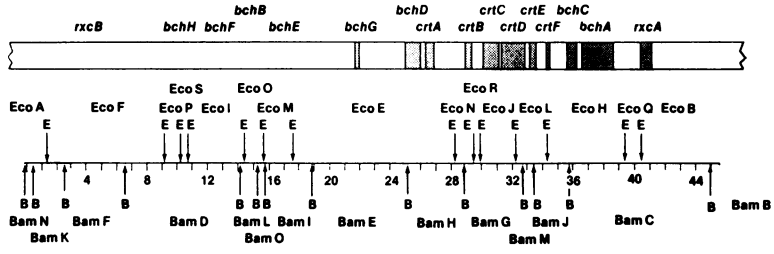

FIG. 2. Alignment of the genetic and restriction maps of the region of the *R. capsulata* chromosome coding for the photosynthetic apparatus. The *bch* genes affect bacteriochlorophyll synthesis, *crt* genes affect carotenoid synthesis, and *rxc* genes affect reaction center and antennae protein syntheses. The shaded areas indicate the genetically determined map positions of groups of mutations conferring the same phenotype. The positions of genes for which no shaded area is indicated were determined by marker rescue, but they have not yet been mapped by genetic techniques capable of precise positioning. Genetic linkage between *bchB* and *bchE* has been established. The 46 kilobases of *R. capsulata* DNA carried in pRPS404 are indicated on the restriction map. Arrows labeled E or B indicate the sites of digestion by *Eco*RI or *Bam*HI, respectively. The fragments produced by digestion with either enzyme are named alphabetically by size. The junction fragments, *Eco*RI-A, *Eco*RI-B, *Bam*HI-A, and *Bam*HI-B, carry regions of the R-factor that are not indicated on this map. *Eco*RI-C, *Eco*RI-G, and *Eco*RI-K are comprised entirely of DNA from the vector, pBLM2.

described previously by Birnboim and Doly (3). The DNA was suspended in a final volume of 20 μl, of which 10 μl was used in a 20-μl reaction mixture. Restriction endonucleases were from Bethesda Research Laboratories and from New England Biolabs. The restricted DNA samples were subjected to electrophoresis through 0.5% agarose gels (17).

RESULTS

Isolation of *E. coli* strains containing Mu d insertions in pRPS404. The initial step to isolate *E. coli* strains containing Mu d insertions in pRPS404 was to infect strain AJB403 with the Mu d phage (5). After allowing for expression of the *bla* gene, Ap^r transductants were selected by plating at 30°C on LB agar plates (12) containing 25 μg of ampicillin per ml. This resulted in many thousands of colonies, each carrying a Mu d phage inserted into either the chromosome or the R-prime plasmid. As a means of isolating R-prime plasmids with Mu d insertions, the colonies were washed off the plates and mated with strain W3110. Selection for W3110 strains containing pRPS404 with Mu d inserts was accomplished by plating the mating mixture on minimal A1 sorbitol agar plates containing 25 μg of ampicillin per ml. Since strain AJB403 will not grow on sorbitol, and strain W3110 will not grow in the presence of ampicillin unless it has received the Mu d phage, this selection procedure ensured that each of the resulting exconjugants contained a Mu d insert in pRPS404. Twenty colonies from each mating were purified by streaking them on minimal A1 sorbitol agar plates containing ampicillin.

Physical location of Mu d inserts in pRPS404. Plasmid DNA from each of these *E. coli* strains was isolated and digested with the restriction endonuclease *Bam*HI. The DNAs were then subjected to electrophoresis in 0.5% agarose gels. A restriction map of the *R. capsulata* DNA of pRPS404 is shown in Fig. 2. Presence of the Mu d phage in pRPS404 caused the appearance of three new *Bam*HI fragments. One of these fragments, composed entirely of Mu d DNA, migrated somewhat slower than the *Bam*HI C fragment. The positions of the other two fragments varied somewhat from plasmid to plasmid, but were usually located between the *Bam*HI A,B doublet and the *Bam*HI C fragment. These were presumably fusion fragments con-

TABLE 2. Physical and genetic location of Mu d1(Ap^r *lac*) insertions

E. coli strain[a]	Location of insert[b]	*R. capsulata* strain[c]	Phenotype[d]	Genotype[e]
AJB409	*Bam*HI-D	AJB456	BchH	*bchH*
AJB417	*Bam*HI-D	AJB463	BchH	*bchH*
AJB465	*Bam*HI-E	AJB466	BchG	*bchG*
AJB496	*Bam*HI-E	AJB500	BchG	*bchG*
AJB471	*Bam*HI-C	AJB472	BchA	*bchC*
AJB498	*Bam*HI-C	AJB499	BchA	*bchC*
AJB477	*Xho*I-I[f]	AJB478	BchB	*bchB*
AJB479	*Xho*I-F[f]	AJB480	BchB	*bchB*

[a] These strains each carry pRPS404 with an insertion described in the rest of the table.

[b] The restriction fragment that contains the site of insertion is indicated.

[c] These strains were formed by homologous recombination between the insert-bearing pRPS404 plasmids of column 1 and the corresponding region of PAS100.

[d] These phenotypes are expressed in the *R. capsulata* strains listed, each bearing a Mu d insert.

[e] The genetic location of the Mu d insert, deduced from columns 2 and 4.

[f] *Xho*I-I extends from kilobase 12 to 14 and *Xho*I-F from kilobase 15 to 21.

taining both plasmid and phage DNA. In approximately half of the DNA samples, a single *Bam*HI fragment of pRPS404 was missing, indicating the location of the insertion. Insertions in *Bam*HI fragments A, B, G, and H could not be identified because fragments A and B and fragments G and H migrate as doublets. Approximately half of the strains had a pRPS404 plasmid with a Mu d phage that could be shown to be in the *R. capsulata* DNA. Columns 1 and 2 of Table 2 list the *E. coli* strains containing the Mu d insertions in pRPS404 and the physical locations of those inserts as deduced by restriction endonuclease digestions. The plasmids from strains AJB477 and AJB479 were also digested with restriction endonuclease *Xho*I to further localize the sites of insertion in these strains. These two insertions are located between kilobase 12 and kilobase 16 (Fig. 2).

Recombination of Mu d inserts into the *R. capsulata* chromosome. We took advantage of the instability of pRPS404 in *R. capsulata* (21) to select for recombinants in which the Mu d phage-bearing gene from the R-prime plasmid replaced the homologous chromosomal gene.

Each *E. coli* strain bearing an R-prime plasmid with a Mu d phage insert was mated with *R. capsulata* strain PAS100, and exconjugants from each cross were plated with streptomycin and kanamycin on X-gal PYE agar. Colonies that showed some β-galactoside activity, as indicated by bluish coloring, were resuspended and streaked aerobically in darkness on PYE agar containing X-gal. Four different colored colonies arose in each streak: red, yellow, purple, and green. Under these conditions, red is the phenotype of wild-type (Lac^-) *R. capsulata*; yellow is the color of Lac^- colonies with the *crtD223* lesion, which causes the accumulation of neurosporene-like carotenoids; purple is the color seen when the red bacteria accumulate the blue X-gal cleavage product; and green is the result of yellow bacteria accumulating the blue product. The red and yellow colonies were stable upon restreaking, and they apparently arose from a loss of the Mu d phage DNA, with or without concommitant loss of the R-factor DNA. The yellow colonies probably represent the product of recombination between the *crtD* gene of the chromosome and the *crtD223* lesion on the R-prime plasmid before loss from the plasmid of the *R. capsulata* DNA with its Mu d insert. The red and yellow colonies were not studied further. The purple colonies were at first unstable, giving rise to red, yellow, green, and purple colonies. Further subculturing of the purple colonies gave rise to stable purple colonies. Green colonies were often unstable at first, giving rise to yellow and green colonies. Presumably, the *crtD223* lesion had recombined into the chromosome, but the strain was not stably Lac^+ until the Mu d phage recombined into the chromosome. Most, but not all, of the stable Lac^+ strains were unable to grow photosynthetically. Only the photosynthetically incompetent strains were studied further. These strains are listed in Table 2. For example, AJB409 is an *E. coli* strain carrying pRPS404 with a Mu d insert in the *Bam*HI D fragment. This R-factor was moved into *R. capsulata* PAS100 by conjugation, and the Mu d phage was recombined into the chromosome. The resulting *R. capsulata* strain was called AJB456.

Identification of bacteriochlorophyll precursors accumulated in Mu d insertion strains. Two methods were used to determine the gene into which the Mu d phage had inserted. The first was to grow the mutated *R. capsulata* strains in RCV^+ and extract the bacteriochlorophyll precursors. The spectra obtained from these extracts were compared with those of other strains whose *bch* lesions have been genetically mapped and that have had their bacteriochlorophyll precursors identified (20). While reviewing spectra from strains with well-characterized *bch* lesions, we observed that extracts from strains with *bchD*, *bchE*, and *bchH* mutations all gave spectra that were very similar to protoporphyrin IX. Because of the difficulty in distinguishing between the products accumulated by these mutants, the fluorescent emission spectrum of each product was obtained. The emission maxima, along with those of standard compounds, are in Table 3. The fluorescent emission spectra of extracts of strains with *bchD* and *bchH* lesions were identical to spectra of both protoporphyrin IX and its monomethyl ester and indicated that these strains accumulated porphyrins lacking magnesium, either protoporphyrin IX or protoporphyrin monomethyl ester. An extract of strain BRP4, a *bchE* mutant, however, yielded a spectrum with emission maxima at both 595 and 634 nm, indicating that a mixture of compounds

TABLE 3. Fluorescence spectroscopy and thin-layer chromatography of porphyrins

Compound or extract	Emission maximum (nm)	R_f[a]
Protoporphyrin IX	635	0.0
Mg-Protoporphyrin IX	595	0.0
Protoporphyrin monomethyl ester	634	0.65
bchD	635	0.05
bchH	635	0.05
bchE	595, 634	
bchE band A	594	0.68
bchE band B	632	0.65
bchE band C	628	0.61

[a] R_f determined on Silica Gel G TLC plates developed in benzene-ethyl acetate-ethanol (4:1:1).

accumulated in that strain, at least one of which contained magnesium. These extracts and standard compounds were also chromatographed on Silca Gel G TLC plates and developed in benzene-ethyl acetate-ethanol (4:1:1). The R_f values of these compounds are shown in Table 3. The fluorescent compound in both *bchD* and *bchH* extracts migrated very little, whereas the standard preparation of protoporphyrin IX did not migrate in this system. The compound accumulated by both *bchD* and *bchH* strains is most likely either protoporphyrin IX or some other dicarboxylic acid porphyrin, but is definitely not protoporphyrin monomethyl ester, which has an R_f of 0.65 in this system. Three fluorescent bands were detectable in the extract of the *bchE* mutant. The middle band ("*bchE* band B") comigrated with protoporphyrin monomethyl ester, with the other two bands migrating very close to the middle band. The three bands were eluted from the plate, and the fluorescent emission maxima were determined. The fastest migrating band ("*bchE* band A") had a fluorescence maximum of 594 nm, indicating that this compound is magnesium protoporphyrin monomethyl ester. Neither of the other bands ("*bchE* band B" and "*bchE* band C") contain magnesium. The middle band is probably protoporphyrin monomethyl ester. We have not identified the third band. Extracts from strains AJB456 and AJB463 yielded absorption spectra similar to that of protoporphyrin IX, indicating that the insertions were in *bchD*, *bchE*, or *bchH*. Fluorescent spectroscopy and thin-layer chromatography indicated that the lesions were in either *bchD* or *bchH*. Because both insertions were in *Bam*HI fragment D, we have listed both strains as having a BchH phenotype (Table 2). Strains AJB466 and AJB500 both accumulated bacteriochlorophyllide *a* and therefore had a BchG phenotype. Strains AJB472 and AJB499 both had a BchA phenotype, accumulating 2-devinyl,2-hydroxyethyl chlorophyllide *a*. Strains AJB478 and AJB480 accumulated magnesium 2,4-divinyl pheoporphyrin a_5 monomethyl ester, a BchB phenotype.

Accumulation of bacteriochlorophyll precursors by the mutant strains was much higher when they were grown under low oxygen tension than when they were grown under high oxygen tension. For example, a *bchH* mutant accumulated 48 nmol of protoporphyrin IX per mg of protein when grown under low oxygen tension, whereas there was no detectable formation of protoporphyrin IX when it was grown under high oxygen tension. Under similar conditions, the *bch*$^+$ parental strain, PAS100, produced 27 nmol of bacteriochlorophyll per mg of protein when grown under low oxygen tension and accumulated 1.1 nmol/mg of protein under high oxygen tension.

The locations of the inserts were also determined by complementation analysis. *E. coli* strains containing the Mu d insertions in pRPS404 were mated with recipient strains containing genetically mapped *bch* lesions. The mating mixtures were spotted on PYE agar with X-gal and overlaid with soft agar containing kanamycin and either streptomycin or rifampin, depending on the drug resistance phenotype of the recipient. When strain BPY72 was the recipient, the mating mixture was plated on RCV agar with X-gal and overlaid with soft agar containing kanamycin only, since BPY72 is sensitive to both streptomycin and rifampin. The plates were incubated aerobically in the dark until colonies developed. Both Lac$^+$ and Lac$^-$ colonies appeared on these plates. The Lac$^-$ colonies arose by deletion of the insert from pRPS404. The Lac$^+$ colonies were presumed to contain a large proportion of cells with an intact R-prime plasmid. Therefore, four Lac$^+$ colonies were selected from each plate and streaked onto PYE agar with X-gal. These plates were then incubated photosynthetically for 3 to 4 days. If the R-prime plasmid complemented the *bch* lesion on the chromosome, heavy purple Lac$^+$ growth occurred. If the R-prime plasmid did not complement the *bch* locus, either no growth occurred or red Lac$^-$ recombinants appeared. The results of the complementation analysis are shown in Table 4. The R-prime plasmids from most of the donor strains failed to complement only one class of recipient strains. For example, the R-prime plasmid from strain AJB409 complemented *bchF*, *bchB*, *bchE*, *bchD*, and *bchC* mutants, but not a *bchH* mutant. In this case, the complementation analysis indicates that the insertion is in *bchH* and agrees with the results from the physical analysis of the R-prime plasmid and the studies showing that the *R. capsulata* strain with this insertion, AJB456, accumulates protoporphyrin IX. However, the R-prime plasmids from strains AJB471 and AJB499 would not complement either *bchA* or *bchC* mutants, indicating that the insertions are in one of the two genes and are polar on the other. The last column of Table 2 shows the *bch* genotypes of the insertion strains, determined by identification of the precursors accumulated and by complementation analyses.

Regulation of β-galactosidase production by oxygen and light. *R. capsulata* strains with different *bch*::Mu d fusions were grown in PYE medium under different oxygen and light conditions to determine the amount of β-galactosidase produced under each of these conditions. High oxygen conditions were obtained by inoculating the culture tube to a density of 25 Klett units and sparging the culture with compressed air supple-

TABLE 4. Complementation assays between *bch* mutants and Mu d insertion strains[a]

Donor[b]	Recipient[c]							
	BRP50 (*bchH*)	BPY72 (*bchF*)	Y80 (*bchB*)	BRP4 (*bchE*)	BRP33 (*bchG*)	MB10081 (*bchD*)	MB10071 (*bchC*)	BRP3 (*bchA*)
AJB409 (BchH)	−	+	+	+	d	+	+	
AJB469 (BchH)	−	+		+				
AJB479 (BchB)	+	+	−	+				
AJB465 (BchG)			+		−	+	+	
AJB471 (BchA)			+			+	−	−
AJB499 (BchA)							−	−
BEC404 (+)	+	+	+	+	+	+	+	+

[a] Assays were performed by mating donors and recipients on aerobic plates and incubating transcipients under photosynthetic growth conditions. See text for details.

[b] Each donor is an *E. coli* strain bearing pRPS404 with a Mu d insert, except for BEC404, which carries pRPS404 with no insert. The phenotype expressed when each insert is recombined into the *R. capsulata* chromosome is indicated in parentheses for each donor.

[c] The recipients are each point mutants of *R. capsulata* that have been previously characterized. The mutational site in each strain is indicated in parentheses. No entry indicates not tested.

mented with oxygen to obtain an initial dissolved oxygen concentration of 23%. When the cultures were harvested at 100 Klett units, the dissolved oxygen concentration was approximately 20%. A mixture of 3% oxygen, 5% carbon dioxide, and 92% nitrogen was used to grow the strains under low oxygen conditions. The final dissolved oxygen concentrations varied from 1 to 2%. These cultures were grown in ambient light and received approximately 0.3 W m^{-2}. Growth in bright light was achieved by growing the cultures 1 in. (ca. 2.54 cm) away from a bank of three 60-W incandescent light bulbs so that the cultures received 158 W m^{-2}. The amounts of β-galactosidase produced by each strain under these conditions are recorded in Table 5. The parental strain, PAS100, had no measurable β-galactosidase activity under any condition. However, growth under low oxygen tensions increased the levels of β-galactosidase in all insertion strains by at least twofold. Growth under low oxygen tensions in bright light resulted in the same production of β-galactosidase as growth in dim light.

DISCUSSION

The study of the regulation of photopigment synthesis in purple nonsulfur photosynthetic bacteria has received considerable attention in many laboratories. Much of this work has been devoted to developing an understanding of the environmental factors influencing bacteriochlorophyll and carotenoid synthesis. It has, however, been difficult to obtain information on how this regulation occurs, primarily due to the lack of assays for the individual enzymes involved in photopigment synthesis.

The development of the Mu d1(Ap^r *lac*) phage by Casadaban and Cohen (5) and the isolation of R-prime plasmids bearing the genes specifying bacteriochlorophyll biosynthetic enzymes (11) has opened the way to study the details of photopigment synthesis. By fusing the *lacZ* gene to various *bch* genes, it has become possible to determine exactly how each gene responds to changes in the cellular environment.

In this paper, we have reported the isolation of Mu d fusions to the *bchB*, *bchC*, *bchG*, and *bchH* genes of *R. capsulata*. We chose to make the insertions in an *E. coli* strain carrying pRPS404 instead of directly into *R. capsulata* for two reasons. The first is that the *bla* gene of the Mu d phage is not expressed in *R. capsulata*, so there is no selection for insertion of the Mu d

TABLE 5. Effects of oxygen and light on β-galactosidase activity in fusion strains

Strain	Genotype	β-Galactosidase sp act[a]		
		High pO_2[b], dim light[c]	Low pO_2, dim light	Low pO_2, bright light
PAS100	*bch*$^+$	<0.01	<0.01	<0.01
AJB456	*bchH*	0.58	1.94	2.20
AJB463	*bchH*	0.06	0.10	0.08
AJB466	*bchG*	0.45	1.28	1.12
AJB500	*bchG*	1.12	2.99	2.18
AJB472	*bchC*	0.86	2.01	2.36
AJB499	*bchC*	1.17	3.45	3.14
AJB478	*bchB*	0.82	2.25	2.92
AJB480	*bchB*	0.58	1.28	1.38

[a] β-Galactosidase specific activity is expressed as micromoles of *o*-nitrophenol formed per minute per milligram of protein.

[b] "High" and "low" indicate an initial oxygen concentration of 23 and 3%, respectively. Growth conditions are described in the text.

[c] "Dim" and "bright" indicate an initial light intensity of 0.3 and 158 W m^{-2}, respectively.

phage (unpublished observation). The second reason is that introduction of the Mu d phage into pRPS404 allows us to physically locate the site of insertion. This ability to physically locate the insertions has provided some very useful information. The insertions in strains AJB477 and AJB479 are in *bchB* (Table 2). Before this study, the *bchB* gene had not been located, but the insertions have shown that this gene lies between *bchE* and *bchF*. This location is supported by data from gene transfer agent-mediated ratio test crosses that demonstrate linkage between some mutations in *bchB* and some in *bchE* (unpublished data).

To study the regulation of these fusions, it was necessary not only to move the R-prime plasmid into *R. capsulata* but also to recombine the insertions into the chromosome. Because of the possibility of the plasmid copy number varying with different growth conditions and the instability of the R-prime plasmid, it is much better to measure the expression of the fusions on the chromosome than on the R-prime plasmid. The instability of the R-prime plasmid in *R. capsulata* (21) made it very easy to detect recombination of the fusion into the chromosome. Upon recombination, the Lac^+ phenotype became stable. Instead of the strain giving rise to red, yellow, and green segregants, the strain became stable and remained either purple or green. The observation that yellow segregants arose at a much higher rate than green ones indicates that recombination of the *crtD223* lesion occurs more frequently than recombination of the Mu d fusion. It should be noted that colonies described as green in this work are a distinct leafy green when grown aerobically on PYE agar plates containing X-gal, and this is quite different from the color phenotype of classical "green" mutants, which form yellow colonies upon aerobic incubation and greenish colonies when grown photosynthetically.

Identification of *bch* lesions based on the accumulation of intermediates is relatively straightforward when dealing with blocks in the pathway beyond *bchE*. However, extracts of mutants with blocks in *bchD*, *bchE*, and *bchH* yield very similar spectra, and the absorbance peaks are often very small and difficult to observe. For this reason, we studied the compounds produced by these strains both by fluorescence emission spectroscopy and by thin-layer chromatography. Strains with lesions in *bchD* and *bchH* both accumulate a product with an emission maximum of 635 nm, indicating that neither strain can perform the magnesium chelation reaction (Table 3). Thin-layer chromatography shows that the compounds from these strains comigrate and that the compound has not been esterified. Thus, both *bchD* and *bchH* strains appear to accumulate either protoporphyrin IX or a compound very similar to it. Gorchein (7) was able to demonstrate the chelation of magnesium into protoporphyrin IX in whole cells of *R. spheroides*. Unexpectedly, the product of this reaction was not magnesium protoporphyrin IX, but magnesium protoporphyrin monomethyl ester. Subsequently, Gorchein (8) showed that magnesium protoporphyrin IX could be converted into the methyl ester in this sytem. This fact, together with the observation (7) that inhibition of methyl transferase resulted in complete inhibition of chelation, led Gorchein to propose that the chelation and methylation reactions are obligatorily coupled. It may therefore be possible that *bchD* and *bchH* code for two subunits of either the magnesium-chelatase or the methyltransferase or that each enzyme is represented by one of these two genes.

In contrast to *bchD* and *bchH* strains, *bchE* strains can methylate protoporphyrin, resulting in the accumulation of equal amounts of protoporphyrin monomethyl ester and magnesium protoporphyrin monomethyl ester, along with a lesser amount of a third compound (Table 3). It is not clear if methylation can occur without first having magnesium inserted or if the magnesium is artifactually removed after methylation.

In most instances, complementation analysis simply confirmed that the genotypes of the insertion strains, which we had determined on the basis of the phenotypes of the strains, were correct. For two strains however, the genotype was not the same as the phenotype. Strains AJB472 and AJB499 accumulated 2-devinyl,2-hydroxyethyl chlorophyllide *a*, indicating that the block is in *bchA* (Table 2). Complementation analysis (Table 4) showed that pRPS404 with either of these insertions did not complement *bchA* or *bchC* strains, indicating that the two genes form an operon. The direction of transcription of the operon, from *bchC* to *bchA*, was inferred from the observation that strain BPY98 has a BchA phenotype, although the lesion has been genetically mapped to the *bchC* gene.

Two environmental factors have been shown to influence the production of bacteriochlorophyll and carotenoids. Both high oxygen tension and high light intensity inhibit production of these photopigments (6). Regulation of the bacteriochlorophyll biosynthetic pathway at the level of enzyme activity is indicated by the fact that bacteriochlorophyll synthesis ceased immediately upon aeration (6). Information on the regulation of enzyme synthesis is available for only two enzymes. The synthesis of both δ-aminolevulinate synthase and magnesium protoporphyrin methyl transferase is inhibited by oxygen (8, 10). Use of the Mu d phage has allowed us to

determine if the synthesis of the enzymes involved in the conversion of protoporphyrin IX to bacteriochlorophyll is regulated in response to oxygen. In a *bch*::Mu d fusion, the *lacZ* gene is only transcribed when the *bch* gene promoter is switched on. Therefore, we can directly determine whether oxygen controls the transcription of *bch* genes by measuring the amount of β-galactosidase formed under different conditions. Insertions in genes *bchB*, *bchC*, *bchG*, and *bchH* are all regulated in response to oxygen (Table 5). This suggests that transcriptional regulation of the bacteriochlorophyll biosynthetic pathway is not limited to a few key steps but instead that many of the *bch* genes are regulated in response to oxygen.

Neither the parental strain nor the insertion strains will grow aerobically on RCV agar containing lactose instead of malate, despite the fact that the level of β-galactosidase activity found in the insertion strains would be enough to allow *E. coli* to grow on lactose. These observations were similar to those of Nano and Kaplan (14), who showed that strains of *R. spheroides* 2.4.1 containing the *lac* transposon Tn*951* could produce β-galactosidase but could not grow on lactose without additional genetic changes.

It might be expected that since the *bch* genes are clustered in one region of the chromosome, and since all of those tested are regulated in response to oxygen, that the genes would be grouped into operons. However, we can find no evidence to support this. The only operon we have found is *bchCA*. Since we do not have insertions in all of the genes, it is possible that other operons do exist.

It is interesting to note that insertions in *bchH* accumulate more protoporphyrin IX under low oxygen tension than under high oxygen tension. This indicates that one or more of the steps in protoporphyrin IX synthesis in *R. capsulata* is regulated by oxygen, as was shown to be the case for *R. spheroides* by Lascelles (10).

Although it is known that bacteriochlorophyll levels decrease with increasing light intensity (6), we have found that *bch*::Mu d fusion strains do not regulate their β-galactosidase levels in response to light intensity. This suggests that light does not directly regulate the transcription of the *bch* genes. It is likely, however, that regulation by light will become apparent only in strains that produce bacteriochlorophyll. We are presently exploring this possibility, and the possibility that bacteriochlorophyll itself may regulate the *bch* genes.

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