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Molecular Genetic Analysis of Terminal Steps in Bacteriochlorophyll *a* Biosynthesis: Characterization of a *Rhodobacter capsulatus* Strain That Synthesizes Geranylgeraniol-Esterified Bacteriochlorophyll *a*†

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ABSTRACT: Site-directed mutational analysis of the *Rhodobacter capsulatus* photosynthesis gene cluster was undertaken in order to identify and characterize genetic loci involved in bacteriochlorophyll *a* biosynthesis. A mutant in *orf304* was shown to accumulate the tetrapyrrole intermediate "bacteriochlorophyllide *a*" which is a tetrapyrrole that has a bacteriochlorophyll *a* ring structure without the presence of an esterifying alcohol. A mutant in *orf391* is shown to synthesize bacteriochlorophyll *a* that is esterified with geranylgeraniol rather than the normal phytol. This latter result provides the first genetic confirmation that esterification of bacteriochlorophyllide *a* initially involves the addition of a geranylgeraniol group followed by sequential reduction of the geranylgeraniol moiety to phytol which is the end product of the pathway. An *R. capsulatus* strain synthesizing geranylgeraniol-esterified bacteriochlorophyll is shown to exhibit severely impaired photosynthetic growth capability. This is despite our observation that synthesis of geranylgeraniol-esterified bacteriochlorophyll does not affect the energy transfer rate from light harvesting to reaction center complexes nor the electron transfer function as measured by the yield of electron transfer to the primary and secondary quinones, the charge recombination rate from the quinones, and the rate of cytochrome c_2 oxidation. We conclude that the observed reduction of the photosynthetic growth rate observed for *R. capsulatus* strains that synthesize geranylgeraniol-esterified bacteriochlorophyll is primarily a consequence of the reduced steady-state level of the photosystem.

Anoxygenic photosynthetic eubacteria synthesize a wide variety of magnesium tetrapyrroles termed bacteriochlorophylls (BChl's)[1] that capture and convert light energy into a useful form of cellular energy. Structural analyses indicate that BChl's are related to chlorophylls that are synthesized by oxygen-evolving cyanobacteria, algae, and plants. Modifications of the ring structure that give rise to different magnesium tetrapyrroles involve changes in the hydration state of the ring structure as well as alterations of various side groups (Smith, 1991). These modifications allow different magnesium tetrapyrroles to absorb light over a wide range of wavelengths with the absorbance by BChl's occurring primarily in the near-infrared region of the spectrum.

In addition to modifications that occur within the conjugated ring structure, there is a less understood variation that occurs with the esterifying alcohol portion of magnesium tetrapyrroles (Beale & Weinstein, 1991; Rüdiger & Schoch, 1991). The majority of eubacterial species synthesize BChl *a* or BChl *b* with the ring-D propionate esterified with the C-20 isoprenoid compound phytol (phytol is also the predominant esterifying alcohol found in chlorophylls). However, diverse species of eubacteria, such as those in the family Chlorobiaceae, synthesize BChl's *c*, *d*, and *e* that contain a wide variety of esterifying alcohols such as geranylgeraniol, phytol, farnesol, a mixture of fatty acid linkages such as C-16, C-18, or C-18:1, or the unbranched alcohol sterol (Rüdiger & Schoch, 1991). The relative importance of synthesizing magnesium tetrapyrroles that are esterified with differing alcohols has remained unclear. However, there are several studies which indicate that the type of esterifying alcohol may indeed be an important component for the synthesis of a fully functional photosystem. For example, it has been reported that *Rhodospirillum rubrum* synthesizes a reaction center (RC) that has bacteriopheophytin (BChl lacking magnesium) that is esterified with phytol (Water et al., 1979). This is despite the observation that *R. rubrum* synthesizes BChl *a* which is predominantly esterified with geranylgeraniol rather than phytol (Katz et al., 1972). Photopigment exchange studies with RCs isolated from *R. rubrum* and *Rhodobacter sphaeroides* also indicate that there appears to be specificity for BChl that is esterified with specific hydration states of the alcohol moiety [discussed in Rüdiger and Schoch (1991), Scheer (1991), and Scheer and Struck (1993)]. A photosynthesis-deficient mutant of *Scenedesmus* has also been isolated that synthesizes chlorophyll *a* which is esterified with geranylgeraniol rather than the normal phytol (Henry et al., 1986). In addition, there are also several plant herbicides that cause the accumulation of chlorophyll *a* that is esterified with geranylgeraniol instead of phytol (Rüdiger & Schoch, 1991; Rüdiger et al., 1976). Collectively, these results suggest that the nature of the esterified alcohol portion of magnesium tetrapyrroles is indeed an important feature for proper synthesis and function of the photosystem.

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[1] Abbreviations: BChl, bacteriochlorophyll; BChl a_p, phytol-esterified bacteriochlorophyll; BCh a_{gg}, geranylgeraniol-esterified bacteriochlorophyll; BPhe, bacteriopheophytin; GTA, gene transfer agent; Km^R, kanamycin resistance; LDAO, lauryldimethylamine oxide; LH, light harvesting; ORF, open reading frame; PGC, photosynthesis gene cluster; RC, reaction center.

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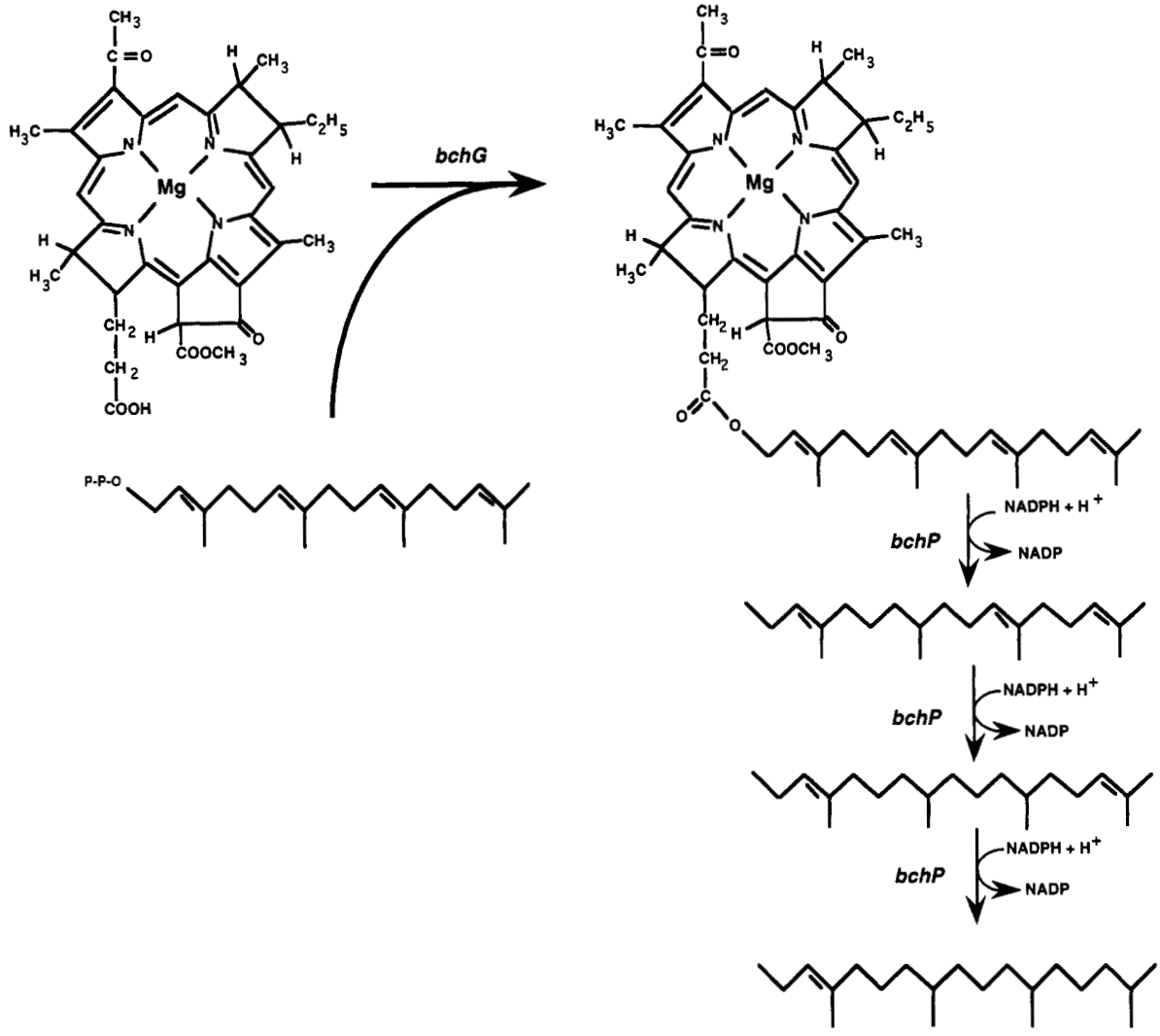

FIGURE 1: Esterification pathway and the genetic loci involved. The pathway for synthesis of bacteriochlorophyll from bacteriochlorophyllide is drawn according to the scheme that is thought to occur in greening plant chlorophyll synthesis (Beale & Weinstein, 1991). There is also an alternative pathway of hydration steps (not shown) which has also been proposed to occur in bacteria (Steiner et al., 1981).

Biosynthetic schemes for the esterification of magnesium tetrapyrroles have only recently been studied. Analysis of BChl *a* obtained from *R. sphaeroides* and *Rhodobacter capsulatus* indicates that approximately 96% of BChl *a* is esterified with phytol with the remainder being a mixture of BChl *a* esterified with geranylgeraniol, dihydrogeranylgeraniol, and tetrahydrogeranylgeraniol (Shioi & Sasa, 1984). This observation led to the conclusion that synthesis of BChl *a* most likely involves the initial esterification of bacteriochlorophyllide *a* with geranylgeranyl using a pyrophosphate-activated geranylgeraniol substrate (Figure 1) which is then followed by sequential hydration of the isoprenoid group in the sequence geranylgeranyl → dihydrogeranylgeranyl → tetrahydrogeranylgeranyl, and finally to phytyl which is the true end product of this complex biosynthetic pathway. There is also evidence that a similar pathway occurs during the initial greening of etiolated plant tissue where it has been reported that chlorophyllide *a* is initially esterified with geranylgeraniol (Schoch et al., 1977). However, the true nature of the esterification reaction in plants remains somewhat unclear since there is also evidence that phytol, rather than geranylgeraniol, may be the preferred substrate for esterification during chlorophyll synthesis that occurs in mature plant tissues (Rüdiger, 1987).

In this, and in related studies (Young et al., 1989; Yang & Bauer, 1990; Bollivar et al., 1994), we have undertaken systematic site-directed mutational analyses of the *R. capsulatus* photosynthesis gene cluster (PGC) in order to identify and characterize genetic loci involved in BChl *a* biosynthesis. The results of this study indicate that one open reading frame (ORF) in the PGC is involved in the initial esterification of BChl *a* with geranylgeraniol and that a second ORF is involved in the subsequent hydration of the esterified geranylgeraniol group to phytol. We also observed that strains that accumulate geranylgeraniol-esterified BChl *a* (BChl a_{gg}) exhibit reduced photosynthetic growth capability compared to that observed with the parent strain which synthesizes BChl *a* that is esterified with phytol (BChl a_p). We further show that this reduction in photosynthetic growth capability is not caused by an impairment of electron transfer capabilities of the photosystem which functions normally when assembled with geranylgeraniol-esterified BChl.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions. *R. capsulatus* strain SB1003 (*rif10*), the light harvesting (LH)-II deleted strain Δ-LHII (*rif10*, Δ*pucBACDE*::Ω), and the gene transfer agent (GTA) overproducing strain CB1127 (*rif10*, *crtG121*,

GTA-overproducer) have been described previously (Young et al., 1989; Yen & Marrs, 1976; LeBlanc & Beatty, 1993). Cells were routinely grown heterotropically or photosynthetically at 34 °C in PYS or RCV 2/3 PY medium as described (Young et al., 1989). Optimal production of tetrapyrrole intermediates was achieved by growth under low aeration conditions in RCV$^+$, a synthetic malate medium supplemented with glucose, pyruvate, and dimethyl sulfoxide (Young et al., 1989; Weaver et al., 1975). *Rhodospirillum rubrum* neotype strain 11170 was obtained from the American Type Culture Collection and grown in RCV 2/3 PY as described above for *R. capsulatus*. *Escherichia coli* strains NM522 (Gaugh & Murray, 1983) and Tec5 (Taylor et al., 1983) were grown at 37 °C in Luria broth or 1.25% agar plates as previously described (Ausubel et al., 1987).

DNA and Genetic Manipulations. DNA subcloning and transformation into NM522 were performed using standard procedures (Ausubel et al., 1987). Plasmid pDB26, which contains the entire *bchG* gene (*orf304*) as well as the 5′ portion of *orf428*, was constructed by subcloning of a 2036 bp *Bam*HI-(18791)–*Pst*I(20827) restriction fragment obtained from pRPSB31 (Taylor et al., 1983) into similar restriction sites in pUC19 (Yanisch-Perroin et al., 1985). [Numerical values of *R. capsulatus* restriction fragments represent the location of restriction sites on the recently sequenced photosynthetic gene cluster (EMBL Accession No. Z11165).] A disruption of *bchG* was then constructed by cloning a *Xho*I-digested kanamycin resistance (Km^R) cassette from pUC4-Kixx (Barany, 1985) into a unique *Sal*I(19432) site in pDB26. Plasmid pDB42, which contains the 3′ end of *orf428* and the 5′ end of *bchP* (*orf391*), was constructed by subcloning a 1321 bp *Pst*I(20827–22148) restriction fragment from pRPSB31 (Taylor et al., 1983) into a similar site of pUC19. The Km^R cassette was then inserted into *bchP* by cloning a *Sma*I restriction fragment from pUC4-Kixx into a unique *Sac*II(21535) restriction site present in pDB42.

Chromosomal Km^R insertion mutations of *bchG* and *bchP* were performed using GTA-mediated homologous recombination. For these strain constructions, plasmid derivatives containing the above-described Km^R disruptions of *bchG* and *bchP* were subsequently transformed into the plasmid-mobilizing strain Tec5 (Taylor et al., 1983) and mated with the GTA overproducing strain CB1127 (Young et al., 1989). Interposon mutagenesis of the chromosomal copy of *bchG* and *bchP* in *R. capsulatus* strains SB1003 and ΔLHII was then performed using GTA obtained from exconjugates as described by Scolnik and Haselkorn (1984). Correct insertion of the Km^R cassette was confirmed by performing Southern blot analysis of restriction fragment polymorphism on genomic DNA preparations that were obtained from mutated strains.

HPLC Analysis. HPLC analysis of esterified BChl was performed according to the method of Shioi et al. (1983). For this analysis, pigments were extracted from whole cells with cold (–20 °C) acetone/methanol (7:2), dried under a stream of nitrogen, and resuspended in 100% methanol. A 50 μL volume of sample containing 10 μmol of BChl was then loaded onto a Zorbax ODS column (250 × 46 mm; DuPont Co.) and eluted in 100% methanol at 40 °C. The retention time of BChl was followed by monitoring the absorption at 280 nm. Values of k' and α_2 for BChl a_{gg} and BChl a_p were calculated according to equations described by Shioi et al. (1983).

Spectral Analysis. Spectral analyses of whole cells and isolated membrane fractions were performed with a Beckman DU-50 spectrophotometer using techniques described previously (Young et al., 1989; Sojka et al., 1970). Spectral analyses of RCs were performed using RC preparations from DBL391 that were obtained by the protocol of Wang, Lin, Lin, Woodbury, and Allen (in preparation). Briefly, the cells were broken by French press, and crude intracytoplasmic membrane fractions were prepared by differential centrifugation. RCs were solubilized by the addition of 0.45% lauryldimethylamine oxide (LDAO) and purified by the use of ion exchange, diethylaminoethyl-Sephacel (Supelco), chromatography. For the described spectral studies, the protein was dialyzed against 10 mM potassium phosphate buffer, pH 7.4, and 0.05% LDAO. Steady-state absorption measurements of RCs were performed using a Cary 5 spectrophotometer (Varian) that was modified with an external 1000 W tungsten light source (Oriel) for light bleaching measurements. For transient absorption measurements, the samples were excited at 532 nm using 5 ns excitation flashes from a Surelite laser (Continuum) while monitoring the absorption changes at 860 nm using a system designed at Arizona State University. For measurement of the charge recombination rate from the primary quinone, 0.5 mM terbutryn was added to block transfer to the secondary quinone. Measurement of charge recombination from the secondary quinone was performed in the presence of 20 mM exogenous quinone with a RC concentration $A_{800} = 0.5$. For cytochrome turnover measurements, the RC concentration was set to $A_{800} = 0.3$ with exogenous ubiquinone-10 and cytochrome c_2 from *R. capsulatus* added at concentrations of 0.8 mM and 20 mM, respectively. The samples were excited at 532 nm using 5 ns excitation flashes as described above while monitoring the cytochrome c_2 absorption changes at 550 nm. The response time of the instrumentation was limited to ~1 ms by the detectors and amplifiers used in the system. This time is significantly longer than the characteristic rate of 1 μs for electron transfer from the cytochrome to the RC; thus, only a comparative study of the total amount of cytochrome c_2 oxidized was possible.

Fluorescence Analysis of Energy Transfer. Measurements of energy transfer from LH to the RC complex were performed using transient fluorescence spectroscopy. The fluorescence of intracytoplasmic membranes was measured using single photon counting in an apparatus previously described (Gust et al., 1990). Excitation was at 860 nm, and the emission at 900 nm was measured. Measurement of the fastest component was limited by the 10 ps time resolution of the apparatus.

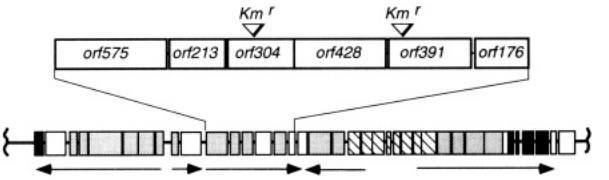

FIGURE 2: Open reading frames of the *R. capsulatus* photosynthesis gene cluster. Open reading frames involved in bacteriochlorophyll biosynthesis are shaded, whereas hatched boxes denote carotenoid biosynthesis genes and solid boxes denote light harvesting I and reaction center structural genes. Unfilled boxes are genes of undetermined function. The arrows indicate the direction of transcription. The expanded region is of the putative *bchE* (*orf575*) operon which contains the *bchG* (*orf304*) and *bchP* (*orf391*) open reading frames which were disrupted in this study by the insertion of a Km^R gene.

RESULTS

The expanded portion of Figure 2 shows the region of the photosynthesis gene cluster in which Km^R insertion mutations

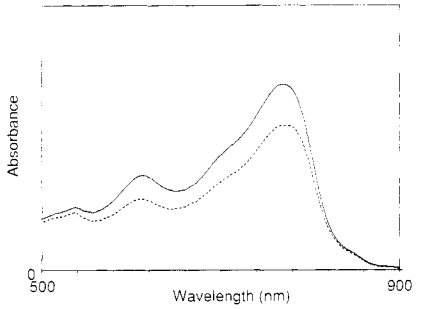

FIGURE 3: Spectral analysis of culture supernatants of the *bchG* strain BRP33 and the (*orf304*) disrupted strain DB304. Strains DB304 (dotted line) and the previously characterized *bchG* disrupted strains BRP33 (solid line) excrete similar magnesium tetrapyrrole intermediates into the culture supernatant. The absorbance maximum is 770 nm.

were constructed in this study. A disruption of *orf304* was created by GTA-mediated recombination of a Km^R cassette into the *R. capsulatus* chromosome which resulted in an insertion into the 77th amino acid codon of *orf304*. The resulting Km^R strain, DB304, is deficient in photosynthetic growth, and when grown under dark semiaerobic conditions in the medium RCV^+, this strain excretes copious amounts of a pigment absorbing at 770 nm. As shown in Figure 3, the spectrum of the culture supernatant from strain DB304 is identical to that described for strain BRP33 that contains a point mutation in the *bchG* gene. The pigment excreted by strain BRP33 has previously been shown to be bacteriochlorophyllide *a* that is in essence the BChl *a* tetrapyrrole ring structure lacking esterification with a long-chain alcohol (Taylor et al., 1983; Marrs, 1981; Brown et al., 1972). Thus, *orf304* (*bchG*) appears to code for a polypeptide that is responsible for catalyzing the initial esterification reaction.

A strain containing a mutation in *orf391* (strain DB391) was constructed by inserting the Km^R cassette into the 46th amino acid codon of this open reading frame (Figure 2). As discussed in more detail below, strain DB391 retains limited photosynthetic growth capability and thus has the ability to synthesize a functional photosystem. Spectral analysis indicated that DB391 accumulates a magnesium tetrapyrrole that is indistinguishable with that of BChl *a* that was obtained from the parent strain SB1003 (data not shown). To test the nature of the esterifying alcohol portion of the magnesium tetrapyrrole, which does not affect the absorption spectrum, the pigment was extracted from a DB391 cell paste with acetone/methanol and subsequently analyzed for its elution profile on a Zorbax octadecyl silica HPLC column. Previous studies have demonstrated that this chromatography system significantly separates BChl *a* structures that contain different hydrated types of esterifying alcohols (Shioi & Sasa, 1984; Shioi et al., 1983). The standards used for comparison were BChl *a* isolated from the wild-type *R. capsulatus* parent strain SB1003, which is known to be primarily esterified with phytol (Künzler & Pfennig, 1973), and BChl *a* that was extracted from *R. rubrum* neotype strain 11170, which has previously been demonstrated to be primary esterified with geranylgeraniol (Katz et al., 1972). It has also been noted that the HPLC retention times of BChl *a* esterified with various alcohols can vary between columns. Thus, a compensating mathematical formula has been described for calculating a value known as α_2 which is quite reproducible from column to column (Shioi et al., 1983). (α_2 is in essence a ratio of the retention time of the tested BChl over the retention time of BChl a_p; the formula for this calculation is given in the legend of Table 1.) As indicated by the values in Table 1, the majority

Table 1: HPLC Analysis of Esterified Bacteriochlorophyll[a]

strain	T_0	T_r	k'	α_2
R. rubrum				
11170	1.46	3.43	1.34	0.56
R. capsulatus				
SB1003	1.45	4.93	2.40	1.0
DB391	1.46	3.46	1.36	0.57

[a] T_0 denotes the elution time of material that is not retained by the Zorbax octadecyl silica column whereas T_r is the retention time of bacteriochlorophyll molecules. k' is calculated by the equation $k' = (T_r - T_0)/T_0$ whereas α_2 is calculated by the formula $\alpha_2 = k'_x/k'_p$ where k'_x is the experimental sample and k'_p is the k' value of phytol-esterified bacteriochlorophyll *a*.

of BChl *a* isolated from *R. rubrum* has an α_2 value of 0.56, a value that is identical to that previously reported for BChl a_{gg} (Shioi et al., 1983). Interestingly, strain DB391 also accumulates BChl *a* that has a similar α_2 value of 0.57, indicating that this mutant strain of *R. capsulatus* synthesizes BChl *a* that is esterified with geranylgeraniol rather than phytol as normally observed for this species. We conclude from these results that the polypeptide encoded by *orf391* is most likely involved in catalyzing the reductive maturation of BChl a_{gg} to BChl a_p. Since *orf391* is involved in BChl biosynthesis, we have by convention renamed this ORF *bchP*.

Physiological and Biophysical Consequences of Synthesizing BChl a_{gg}. In order to determine if synthesis of BChl a_{gg} has physiological consequences, we recorded growth curves with strain DB391 as well as with the wild-type parent strain SB1003. The results of this analysis indicate that when grown under nonphotosynthetic (dark aerobic) conditions, strain DB391 and its wild-type parent strain SB1003 both exhibit doubling times of approximately 1.5 h. However, when strain DB391 is grown under photosynthetic conditions (5000 lx light intensity), it exhibits a pronounced reduction in growth rate with a doubling time of 12 h compared with a 2 h doubling time observed for the parent strain SB1003.

The reduction in photosynthetic growth rate observed for the BChl a_{gg} synthesizing strain DB391 could either be a consequence of a lower steady-state level of a functional photosynthetic apparatus or be a consequence of the synthesis of a functionally impaired photosystem. To test these possibilities, the RC complex was isolated from strain DBL391 and subsequently analyzed for spectral and electron transfer properties. (DBL391 is a BChl a_{gg} synthesizing derivative of DB391 that also contains a deletion of the LH-II complex which facilitates RC purification.) The RC preparations isolated from DBL391 exhibited a spectrum that is similar to that obtained from wild-type cells with the curious exception that the 800 nm peak is shifted to 795 nm (data not shown). Since the phytol (or geranylgeranyl) chain is not part of the conjugated electron system of the tetrapyrrole, it is unlikely that the 5 nm peak shift is directly due to changes in esterification of the chain. Rather, the shift is probably due to an altered packing of the RC BChl, resulting in small changes in the interactions between BChl and other components of the RC. The dimer peak at 860 nm could be fully bleached by an external light source or by the addition of ferricyanide with a concomitant 5 nm shift in the 800 nm (wild type) or 795 nm (mutant) BChl peak upon oxidation. After bleaching, the 860 nm dimer peak recovered exponentially with time constants of 130 ms and 1.0 s for recombination from the primary and secondary quinones, respectively. Under similar conditions, the time constants were determined to be 130 ms and 0.8 s for the wild-type preparations. Since the recombination rates are not changed relative to wild type

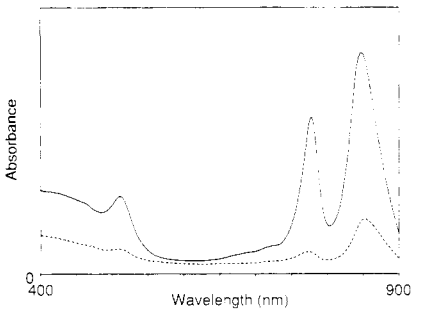

FIGURE 4: Spectral analysis of photopigment levels accumulated by strains SB1003 and DB391. Strain DB391 (dotted line) accumulates approximately 6-fold lower levels of photopigments than that observed for the parent strain SB1003 (solid line). The spectrum is of cell cultures containing the same density of cells.

[which is contrasted to the observed faster rates for RC polypeptide mutants that exhibit higher midpoint potentials (Bylina & Youvan, 1988; McDowell et al., 1991; Williams et al., 1992; Stocker et al., 1992; Murchison et al., 1993)], we can conclude that the presence of BChl a_{gg} does not affect the oxidation/reduction midpoint potential of the electron donor. Time-resolved transient fluorescence spectroscopy measurements of intracytoplasmic membrane fractions were also taken to measure the energy transfer rate from the LH complex to the RC. Fluorescence quenching measurements of wild-type (BChl a_p synthesizing parent) and mutant (DBL391) membrane fractions showed an identical dominant fast component of 50 ps and two smaller components with time constants of 625 and 5560 ps. It is thought that the fast fluorescence quenching component is a result of energy transfer from LH to RC complexes (Woodbury & Parson, 1984). Since this rate is the same in membrane preparations obtained from wild-type and geranylgeraniol-esterifying strains, there also appears to be no significant effect of the esterifying portion of BChl on LH to RC energy transfer. Finally, a cytochrome turnover measurement was also performed by using multiple laser flashes to measure the ability of RCs to perform multiple electron transfers. In this analysis, we observed that cytochrome c_2 was rapidly oxidized after each flash at a rate comparable to that of wild-type RC (data not shown), thereby indicating no effect of BChl a_{gg} on the RC turnover rate.

Since energy transfer from LH to RC as well as RC function appears normal, why is it that geranylgeraniol-esterifying strain DB391 exhibits such a severe reduction in photosynthetic growth rate? One clue to this puzzle is the surprising observation that RC preparations obtained from geranylgeraniol-esterifying cells are only stable for 1 day whereas similar preparations from wild-type cells were stable for several weeks at 4 °C (data not shown). This, in itself, would suggest that the stability of the *R. capsulatus* photosystem is impaired when it is assembled with geranylgeraniol-esterified BChl rather than with phytol-esterified BChl. This conclusion is further supported by whole cell spectral analysis which indicates that strain DB391 contains greatly reduced (~6-fold) steady-state levels of photopigments compared with that observed for the parent strain SB1003 (Figure 4). This would indicate that even though the nature of the esterification group may not affect electron transfer capabilities, it does affect the synthesis or stability of the photosystem.

DISCUSSION

Prior mutational analyses have indicated that all of the known essential genes involved in magnesium tetrapyrrole biosynthesis are located within a 45 kbp region of the *R. capsulatus* chromosome termed the "photosynthesis gene cluster" (PGC) (Young et al., 1989; Yang & Bauer, 1990; Yen & Marrs, 1976; Taylor et al., 1983; Marrs, 1981; Biel & Marrs, 1983; Zsebo & Hearst, 1984). This region of the chromosome has recently been sequenced in its entirety by Hearst and co-workers with the sequence information deposited in the EMBL data base (Accession No. Z11165). Near the central region of the PGC are several ORFs of unknown function which are highlighted in the expanded region in Figure 2. Prior complementation (Taylor et al., 1983) and transposition mutagenesis experiments (Biel & Marrs, 1983) indicated that this region encodes one or more genes involved in the initial esterification of bacteriochlorophyllide *a* with phytol. In order to unequivocally determine which ORFs are involved in different steps of the biosynthetic pathway, we have been performing directed interposon mutational analysis of each of the ORFs in the region of the PGC spanning from *puhA* to the carotenoid (crt) gene cluster (Figure 2) (Yang & Bauer 1990; Bollivar et al., 1994). The technology employed involves recombining a Km^R cassette into the chromosome via GTA-mediated homologous recombination (Yang & Bauer, 1990; Scolnik & Haselkorn, 1984). Constructed strains are subsequently assayed for the nature of the magnesium tetrapyrroles that they accumulate.

The observation that strain DB304, which has an insertion in *orf304*, accumulates bacteriochlorophyllide *a* indicates that *orf304* correlates with the previously described *bchG* locus (Taylor et al., 1983). Therefore, *orf304* appears to encode the bacteriochlorophyll synthase gene. It should be noted that none of the additional strains that we constructed which contained Km^R insertions in ORFs between *puhA* and *crtA* exhibit a similar phenotype (Yang & Bauer, 1990; Bollivar et al., 1994). Thus, *orf304* is most likely the only ORF involved in the esterification reaction. In addition, placement of the *bchG* locus within this region correlates well with previous mapping analysis which indicated that this locus was located within the *Bam*HI-E restriction fragment of the PGC (Taylor et al., 1983; Biel & Marrs, 1983). There are no known homologs of *bchG* as yet identified in plant systems, so it is unclear what relationship it has, if any, to chlorophyll synthase.

A mutation in *orf391* results in a strain that is defective in the ability to reduce BChl that is esterified with geranylgeraniol to BChl that is esterified with phytol. This phenotype provides the first genetic confirmation that BChl a_p biosynthesis involves a BChl a_{gg} intermediate. Insertion mutations in the downstream open reading frame *orf176*, as well as in other open reading frames of the PGC, did not lead to a similar defect in reduction of the esterification group [data not shown and Bollivar et al. (1994)]. This would indicate that *orf391* codes for a polypeptide that itself sequentially reduces the double bonds in the geranylgeraniol group to dihydrogeranylgeraniol → tetrahydrogeranylgeraniol → the final product of the biosynthetic pathway, phytol-esterified BChl *a*.

In X-ray diffraction studies of RCs from *R. sphaeroides* and *R. viridis* (Michel et al., 1986; Yeates et al., 1988), there are clear observations of electron density for the phytyl chains of the tetrapyrroles. These studies indicate that there may be specific interactions of phytyl groups with the protein environment that could be disrupted by the substitution of geranylgeranyl for phytyl. Chemical substitution studies have also shown that RCs can be assembled with tetrapyrroles containing various esterifying groups (Rüdiger & Schoch, 1991; Scheer, 1991; Scheer & Struck, 1993). However, the allowed substitutions are very specific; for example, although BChl esterified with a geranylgeraniol group can substitute

into RCs from *R. sphaeroides*, BChl with a phytyl chain cannot substitute into *R. rubrum* (Rüdiger & Schoch, 1991; Scheer, 1991; Scheer & Struck, 1993). Our biophysical measurements, however, indicate that synthesis of BChl a_{gg} does not per se impair function of the RC complex nor energy transfer from the LH complex. We have therefore concluded that the observed reduction of photosynthetic growth capability is most likely a result of decreased stability (or steady-state level) of the photosystem in strains synthesizing BChl a_{gg}. This conclusion is supported by the isolation of second-site suppressor mutations of DB391 that regain wild-type levels of photopigments concomitant with the wild-type growth rate (data not shown). Analysis of BChl synthesized by one such suppressor indicates that it continues to synthesize BChl a_{gg}, thereby strengthening the argument that electron transfer capabilities of the photosystem are not significantly affected by the presence of BChl a_{gg}.

Critical for the studies reported here is the observation that although the BChl monomers and BPhes of the reaction center can accept modified tetrapyrroles, there have been no reported substitutions of BChl's forming the primary donor. Thus, genetic modifications of the tetrapyrrole structure, as reported here, provide the first example where all of the tetrapyrroles in the RC are structurally altered. It should also be noted that RCs have been observed to retain function even after mutagenesis has altered amino acid residues involved in hydrogen bonding or Mg ligation to BChl's forming the primary donor (Bylina & Youvan, 1988; McDowell et al., 1991; Williams et al., 1992; Stocker et al., 1992; Murchison et al., 1993). Together, these results suggest that the RC is remarkably tolerant, in terms of function, to changes involving both the structural polypeptides and the tetrapyrroles.

It is also curious that RCs from *R. rubrum*, which is a species that synthesized BChl a_{gg}, are reported to contain BPhes that are esterified with phytol (Walter et al., 1979). Assuming that the *R. rubrum* tetrapyrrole biosynthetic pathway is similar to that of *R. capsulatus*, a specific mechanism must exist in *R. rubrum* that blocks an equivalent of the *orf391* gene product from reducing BChl a_{gg} to BChl a_p and yet still allows the synthesis of BPhe that is esterified with phytol. If so, it would implicate some interesting regulatory features at this step of the pathway.

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