Chapter 15

Bacteriochlorophyll Biosynthesis in Green Bacteria

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Summary

The photosynthetic green sulfur bacteria synthesize a complex mixture of bacteriochlorophylls and chlorophylls. Depending on the strain, the dominant species is bacteriochlorophyll (BChl) *c*, *d*, or *e*, which serves as the major light-harvesting pigment in the chlorosome antenna. Each of these BChl species occurs as a mixture of homologs differing in stereochemistry, methylation, and esterifying alcohol. In addition, BChl *a* is present in various protein-based antenna complexes and in the reaction centers. A third chlorophyll (Chl) species, Chl *a* esterified with Δ2,6-phytadienol, functions as the primary electron acceptor in the reaction center. Until recently, relatively little was known about the biosynthesis of the so-called 'chlorosome Chl' (formerly '*Chlorobium*

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Chl'; Smith, 1994), BChl *c*, *d*, and *e*. However, it is now clear that these species are not derived from BChl *a* but that their biosynthesis involves some enzymes shared with BChl *a* biosynthesis as well as several novel enzymes. This chapter summarizes the current knowledge of BChl *c* biosynthesis in the green sulfur bacterium *Chlorobium* (*Chl.*) *tepidum*, for which the complete genome has been sequenced and analyzed. Because BChl *c* is not required for the viability of *Chl. tepidum*, it has been possible to inactivate genes postulated to play roles in BChl *c* biosynthesis by interposon mutagenesis. This genomics-enabled, biochemical genetics approach has led to the identification of several BChl *c*-specific gene products and has allowed the general features of BChl *c* biosynthesis to be established. Comparative genomics has also allowed some general conclusions concerning BChl synthesis in *Chloroflexus aurantiacus* to be drawn.

I. Introduction

The green sulfur bacteria and the green filamentous bacteria (collectively called green bacteria) are unique among photosynthetic organisms in synthesizing BChl *c*, *d*, and *e* (Fig. 1). These pigments differ from other BChls and Chls by having a chiral C-3[1] hydroxyl group and by lacking a C-13[2] methylcarboxylate group (Holt et al., 1966; Smith et al., 1982, 1983a; Smith, 2003). Moreover, BChl *c* and *e* have a C-20 methyl group, and in green sulfur bacteria BChl *c*, *d*, and *e* may have additional methyl groups at the C-8[2] and C-12[1] positions. BChl *c*, *d*, and *e* exclusively occur in light-harvesting antennas called chlorosomes, which are large, light-harvesting antenna structures appressed to the cytoplasmic surface of the cytoplasmic membrane (Blankenship et al., 1995; Oelze and Golecki, 1995; Frigaard et al., 2001, 2003; Blankenship and Matsuura, 2003). The characteristic structural features of the BChl *c*, *d* and *e* molecules are undoubtedly related to their organization within the chlorosome, in which they form rod-like aggregates stabilized by pigment-pigment interactions and not by pigment-protein interactions as is commonly found in other antenna systems (Holzwarth et al., 1992; van Rossum et al., 2001). The chlorosome antenna and the organization of the BChls within it have been extensively investigated (for reviews, see Holzwarth et al., 1992; Blankenship et al., 1995; Blankenship and Matsuura, 2003; Chapter 20, DeBoer and DeGroot).

The second most abundant (B)Chl species in green bacteria is BChl a_P (BChl *a* esterified with phytol), which occurs in the reaction center, in the CsmA antenna protein of the chlorosome envelope (Sakuragi et al., 1999; Bryant et al., 2002; Montaño et al., 2003), in the FMO antenna protein in green sulfur bacteria, and in the LH1-like, B808-B866 antenna protein in green filamentous bacteria (Feick and Fuller, 1984; Wechsler et al., 1987, 1991). The least abundant (B)Chl species in green sulfur bacteria is Chl $a_{\Delta 2,6}$ (Chl *a* esterified with Δ2,6-phytadienol), which is found in the Type 1 reaction centers where it acts as the primary electron acceptor (Kobayashi et al., 2000).

The biosynthesis of Chl *a* and BChl *a* is reasonably well understood from genetic and biochemical analyses (for reviews, see Senge and Smith, 1995; Porra, 1997; Suzuki et al., 1997; Willows, 2003; also Chapters 10–14). However, in spite of numerous speculations (Senge and Smith, 1995; Porra, 1997; Smith, 2003), this was not the case for BChl *c* biosynthesis until very recently. Present knowledge of BChl *c* biosynthesis derives from comparative analyses of the genome sequence of *Chlorobium* (*Chl.*) *tepidum* (Eisen et al., 2002; Frigaard et al., 2003) with data derived from the genomes of other photosynthetic organisms. Using information derived from gene duplications in *Chl. tepidum* and sequence similarity comparisons, predictions were made concerning the genes likely to be involved in BChl *c* biosynthesis, and a general scheme was proposed (Eisen et al., 2002). Because BChl *c* is not required for the viability of *Chl. tepidum* (Frigaard et al., 2002) and because *Chl. tepidum* is naturally transformable (Frigaard and Bryant, 2001), it has been possible to inactivate many of these genes (Frigaard

Abbreviations: ALA – 5-aminolevulinic acid; (B)Chl – bacteriochlorophyll or chlorophyll, prefixes in square brackets indicate the specific substituents at sites with variable substituents; BChl – bacteriochlorophyll; BChlide – bacteriochlorophyllide; BPhe – bacteriopheophytin; *Cfx*. – *Chloroflexus*; Chl – chlorophyll; *Chl*. – *Chlorobium*; Chlide – chlorophyllide; DV – divinyl; E – ethyl; *E.* – *Escherichia*; F – farnesyl, this and other esterifying alcohols are indicated as subscripts to the respective (B)Chls; FMO protein – Fenna-Matthews-Olson protein; GG – geranylgeranyl; *Hba.* – *heliobacillus*; HE – 1-hydroxyethyl; I – *iso*-butyl; M – methyl; P – phytyl; PChlide – protochlorophyllide; Pr – *n*-propyl; Proto – protoporphyrin IX; *Rba*. – *Rhodobacter*; *Rsp.* – *Rhodospirillum*; S – stearyl; V – vinyl; SAM – s-adenosylmethionine; Δ2,6 – Δ2,6-phytadienyl

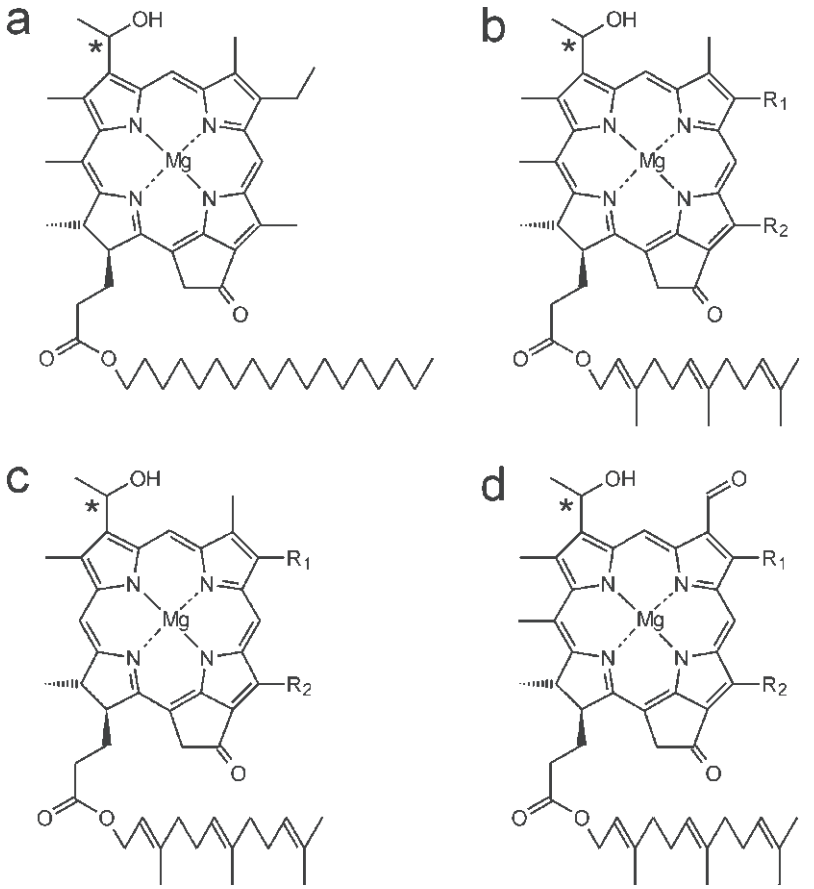

Fig. 1. (a) Structure of [8-E,12-M]-BChl *c* esterified with stearol, which is the predominant BChl *c* homolog in the green filamentous bacterium *Cfx. aurantiacus*. (b, c, d) Structures of the [8-R_1,12-R_2] homologs of BChl *c*, BChl *d*, and BChl *e*, respectively, esterified with farnesol as they typically occur in green sulfur bacteria. (R_1 = ethyl, *n*-propyl, *iso*-butyl, or *neo*-pentyl; R_2 = methyl or ethyl; a star denotes the chiral carbon C-3[1], which can be either R or S configured).

et al., 2002, 2003). The resulting mutants that have been generated by this approach have been characterized and their pigment contents analyzed by HPLC and mass spectroscopic analyses. Although BChl *a* is required for the viability of *Chl. tepidum*, it has nevertheless been possible to generate mutations in some genes thought to be involved in the synthesis of this BChl. These findings can be used to probe those portions of BChl *a* that are most critically involved in its functions in vivo.

II. Approach to Elucidating Bacteriochlorophyll Biosynthesis in Green Bacteria

When the photosynthesis gene cluster, a region of approximately 46 kb that includes all genes necessary for photosynthesis, was identified and completely sequenced in the purple bacterium *Rhodobacter* (*Rba.*) *capsulatus*, new approaches became possible for the elucidation of the biosynthetic pathway for BChl *a* (Alberti et al., 1995; Naylor et al., 1999). A similar organization has been found in other proteobacteria (Béjà et al., 2002) and *Heliobacillus* (*Hba.*) *mobilis* (Xiong et al., 1998). The facile identification of the genes for BChl *a* biosynthesis in purple bacteria facilitated extensive studies by genetic complementation of mutants deficient in BChl *a* biosynthesis, by the inactivation of genes in *Rhodobacter* species (Coomber et al., 1990; Bollivar et al., 1994b; Naylor et al., 1999), and by the characterization of heterologously expressed enzymes (Bollivar et al., 1994a; Porra, 1997; Suzuki et al., 1997; Willows, 2003; Chapters 10–14). Unfortunately, no extensive clustering of photosynthesis genes is observed in the green bacteria. This drawback and the lack of suit-

able genetic methods meant that the study of BChl biosynthesis in green bacteria lagged significantly behind that in purple bacteria. An approach to the elucidation of the BChl *c* biosynthetic pathway in the green bacteria has been to identify genes encoding potential BChl biosynthetic enzymes in the genome sequences of *Chl. tepidum* and *Chloroflexus* (*Cfx.*) *aurantiacus* by sequence similarity to genes encoding known enzymes involved in the synthesis of Chl *a* and BChl *a* in other organisms. As candidates for novel activities, ancillary genes, which are found in putative operons and other gene clusters that contain genes encoding BChl biosynthesis or other photosynthesis genes in both *Chl. tepidum* and *Cfx. aurantiacus*, have additionally been targeted for mutagenesis.

A. Bacteriochlorophyll Biosynthesis Genes in Chlorobium tepidum

Orthologs of all genes known to be involved in the oxygen-independent, anaerobic synthesis of BChl *a* are found in the genome of *Chl. tepidum* (Eisen et al. 2002; see Fig. 2 and Table 1). Additionally, the *Chl. tepidum* genome contains numerous duplications of related genes: two paralogs (*CT0372/hemN* and *CT2010/hemN-II*) of the oxygen-independent coproporphyrinogen oxidase (HemN); two paralogs (*CT1421/bchF* and *CT1776/bchV*) of the 3-vinyl hydratase (BchF); two paralogs (*CT2256/bchP* and *CT1232/bchO*) of a BchP-like geranylgeranyl reductase; three paralogs (*CT1992/bchK*, *CT1610/bchG*, and *CT1270/chlG*) of (B)Chl synthases; three paralogs (*CT1957/bchH*, *CT1955/bchS*, and *CT1295/bchT*) of the large subunit of magnesium chelatase (BchH); and seven paralogs (*CT1959/bchE*, *CT1777/bchQ*, *CT1320/bchR*, *CT0072*, *CT1502*, *CT1697*, and *CT1903*) of enzymes related to the oxidative cyclase (BchE) and C- and P-methyltransferases. Fig. 2a shows the organization of operons encoding some of these genes. Only a few (B)Chl biosynthesis genes in *Chl. tepidum* are encoded in operons, e.g., *bchNBL*, *bchFCX*, and *bchID* (Fig. 2a), and these are

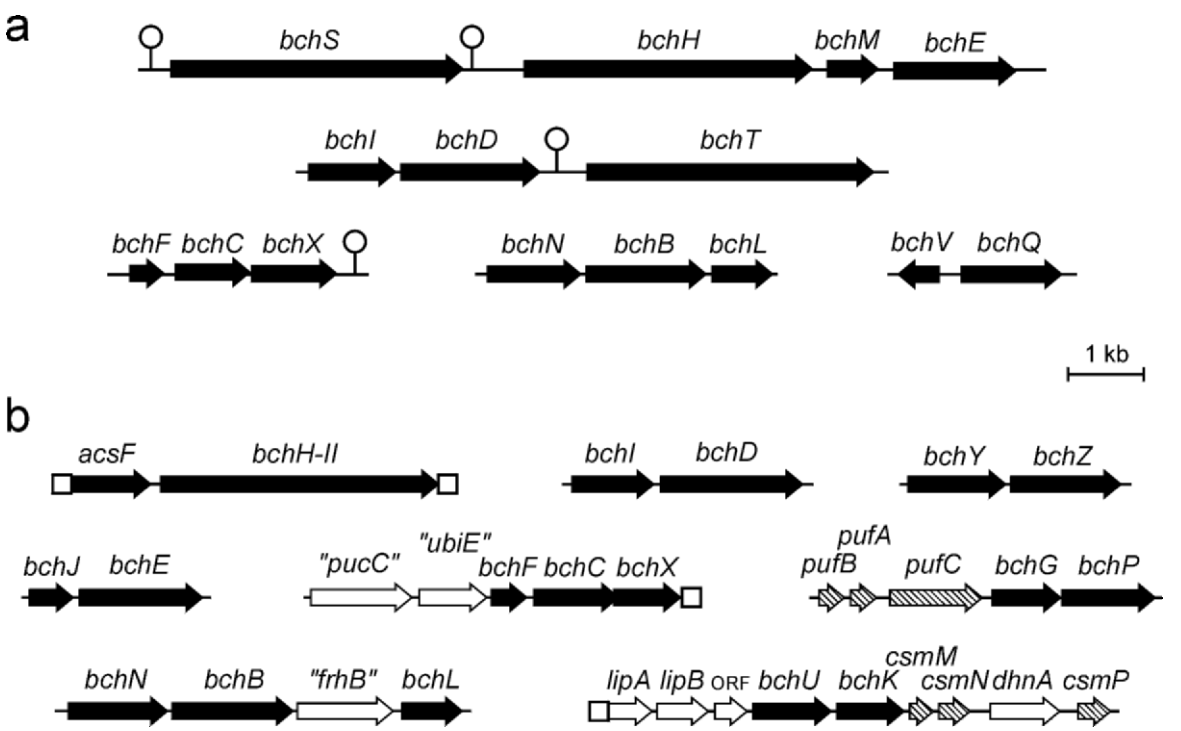

Fig. 2. Organization of genes involved in BChl biosynthesis in (a) *Chl. tepidum* (Eisen et al., 2002) and (*b*) *Cfx. aurantiacus* (DOE Joint Genome Institute, http://www.jgi.doe.gov). BChl biosynthesis-related genes are shown in black, other known photosynthesis-related genes in stripes, and other genes in white. Circles indicate potential Rho-independent transcription termination sequences (no analysis of such sequences was carried out on the *Cfx. aurantiacus* data) and squares indicate end of available DNA sequence. Only genes that cluster with other photosynthesis-related genes are shown. The following genes do not cluster with other known photosynthesis-related genes in *Chl. tepidum*: *bchG, bchJ, bchK, bchP, bchU, bchY, bchZ*, and *chlG* and in *Cfx. aurantiacus*: *bchH-I, bchM* and *bchS*.

Table 1. Confirmed and predicted genes functioning in BChl and Chl biosynthesis in *Chl. tepidum*

Gene	Locus	Gene product	Comments
bchB	*CT2151*	Protochlorophyllide reductase subunit B	
bchC	*CT1422*	[3-Hydroxyethyl]-bacteriochlorophyllide *a* dehydrogenase	BChl *a*-specific
bchD	*CT1296*	Mg chelatase subunit D	
bchE	*CT1959*	Mg-protoporphyrin IX methyl ester cyclase	Possibly also [13-vinyl] Mg-protoporphyrin IX cyclase
bchF	*CT1421*	3-Vinyl hydratase	Probably produces C-3^1 R-stereochemistry
bchG	*CT1610*	BChl *a* synthase	BChl *a*-specific
bchH	*CT1957*	Mg-chelatase subunit H	Possibly BChl *a* and Chl *a* specific; gene inactivated
bchI	*CT1297*	Mg-chelatase subunit I	
bchJ	*CT2014*	8-Vinyl reductase	
bchK	*CT1992*	BChl *c* synthase	BChl *c*-specific; gene inactivated (mutant lacks BChl *c*)
bchL	*CT2150*	Protochlorophyllide reductase subunit L	
bchM	*CT1958*	SAM:Mg-protoporphyrin IX methyl transferase	Possibly BChl *a* and Chl *a* specific
bchN	*CT2152*	Protochlorophyllide reductase subunit N	
bchO	*CT1232*	Geranylgeranyl reductase II	Gene inactivated (altered tailing for BChl *a* and Chl *a*); BchP paralog
bchP	*CT2256*	Geranylgeranyl reductase I	Gene inactivated (altered tailing for BChl *a* and Chl *a*)
bchQ	*CT1777*	C-8^2 methyltransferase	BChl *c*-specific; gene inactivated
bchR	*CT1320*	C-12^1 methyltransferase	BChl *c*-specific; gene inactivated
bchS	*CT1955*	Mg-chelatase subunit S	BChl *c*-specific; gene inactivated; BchH paralog
bchT	*CT1295*	Mg-chelatase subunit T	Probably BChl *a*-specific; gene inactivated; BchH paralog
bchU	*CT0028*	C-20 methyltransferase	BChl *c*-specific; gene inactivated; CrtF homolog
bchV	*CT1776*	3-Vinyl hydratase	BChl *c*-specific; gene inactivated; probably produces C-3^1 S-stereochemistry; BchF paralog
bchX	*CT1423*	Chlorophyllide *a* reductase subunit X	BChl *a*-specific
bchY	*CT1826*	Chlorophyllide *a* reductase subunit Y	BChl *a*-specific
bchZ	*CT2125*	Chlorophyllide *a* reductase subunit Z	BChl *a*-specific
chlG	*CT1270*	Chl *a* synthase	Chl *a*-specific

similar to operons found in *Cfx. aurantiacus* (Fig. 2b; see below). Interestingly, analysis of potential Rho-independent transcription terminator sequences suggests that, although the *bchS* and *bchT* genes are clustered with other genes encoding proteins with functions related to BChl biosynthesis, these genes are probably transcribed independently of the other genes in their respective clusters (Fig. 2a).

B. Bacteriochlorophyll Biosynthesis Genes in Chloroflexus aurantiacus

Orthologs of all known genes involved in BChl *a* biosynthesis can also be found in the genome of *Cfx. aurantiacus* (DOE Joint Genome Institute, http://www.jgi.doe.gov). In contrast to *Chl. tepidum*, obvious paralogs of genes involved in BChl biosynthesis are more limited in number. They include two paralogs of the oxygen-independent coproporphyrinogen oxidase (HemN); two paralogs (BchG and BchK) of BChl synthases; three paralogs of the gene encoding the large subunit of magnesium chelatase (BchH); and two paralogs of the oxidative cyclase (BchE). The *bchU* gene, encoding the BChl C-20 methyltransferase (Section VII.D), is found upstream of *bchK* encoding BChl *c* synthase and genes encoding chlorosome proteins CsmM, CsmN, and CsmP. Homologs of the BchQ and BchR C-methyltransferases are not found, since unlike green sulfur bacteria, *Cfx. aurantiacus* does not synthesize BChl *c* with additional methylations at the C-8^2 and C-12^1 positions (Section VII.C).

III. Overview of Proposed Pathways

Several BChl *c*-specific genes have been identified by interposon mutagenesis. This group includes genes that encode a Mg-chelatase subunit (*bchS*), a C-20 methyltransferase (*bchU*), a C-8^2 methyltransferase (*bchQ*), a C-12^1 methyltransferase (*bchR*), a C-3^1 hydratase (*bchV*), and a BChl *c* synthase (*bchK*) (see Section VII and Table 1). All of these genes, except *bchU*, encode proteins that are paralogous to enzymes that function in BChl *a* biosynthesis. Some genes encoding enzymes with activities required in both BChl *a* and BChl *c* biosynthesis are obviously not duplicated in the *Chl. tepidum* genome, and the enzymes encoded by these genes probably function in both pathways. This category includes genes encoding two Mg-chelatase subunits (*bchD* and *bchI*), Mg-Proto monomethyl ester oxidative cyclase (*bchE*), C-8 vinyl reductase (*bchJ*), and PChlide reductase (*bchN*, *bchB*, and *bchL*) (Section V.A).

It is not yet clear at which step the BChl *c* and BChl *a* pathways bifurcate, but the bifurcation is most likely related to removal of the C-13^2 carboxylate moiety. The removal of this C-13^2 carboxylate or methylcarboxylate group could conceivably involve any intermediate from Proto up to Chlide *a*, and it is not clear whether decarboxylation occurs before or after formation of the isocyclic ring. In any case, it appears likely that *Chl. tepidum* uses different subunits of the Mg-chelatase to regulate the relative yields of BChl *c* and BChl *a* (and possibly Chl $a_{\Delta 2,6}$). Since green sulfur bacteria typically synthesize about 30 times more BChl *c* than BChl *a* and about 10 times more BChl *a* than Chl $a_{\Delta 2,6}$, such regulation is essential (Frigaard et al., 2002, 2003). If all three Mg-chelatases accept Proto as substrate, substrate destiny could be controlled if the BchH, BchS, and BchT subunits bind the substrate until several enzymes have acted upon it (a phenomenon known as 'substrate channeling'). If removal of the C-13^2 carboxylate moiety occurs after chelation, then Chlide *a* would seem to be the most logical substrate and would lead to the production of [3-vinyl]-BChlide *d*. An attractive aspect of this postulated pathway is that only BChl *c*-specific enzymes are required to produce BChlide *c* from Chlide *a* (except C-3^1 hydration which most likely involves BchF). Pathway A in Figs. 3 and 4 illustrates this possibility. If removal of the C-13^2 carboxylate group occurs prior to chelation, the substrate for chelation would likely be [13-vinyl]-Proto and this could explain the need for a BChl *c*-specific chelatase (BchSDI). Pathway C in Figs. 3 and 4 illustrates this possibility. The proposed Pathways A and C require unidentified enzyme(s) to remove the C-13^2 group. If the BchSID enzyme prevents methylation by BchM, the subsequent oxidative cyclization by BchE could lead to spontaneous decarboxylation. This possibility, which does not require enzymes other than those currently known, is illustrated in Pathway B in Figs. 3 and 4.

Anesthetic gases, such as ethylene, acetylene or N_2O, have been found to inhibit BChl *d* synthesis but not BChl *a* formation in *Chl. vibrioforme,* and they cause the accumulation of Mg-Proto monomethyl ester (Ormerod et al., 1990; Frigaard and Ormerod, 1995). One interpretation of this observation is that the anesthetic gases inhibit an enzyme that is specific for BChl *d* biosynthesis, most likely the one involved

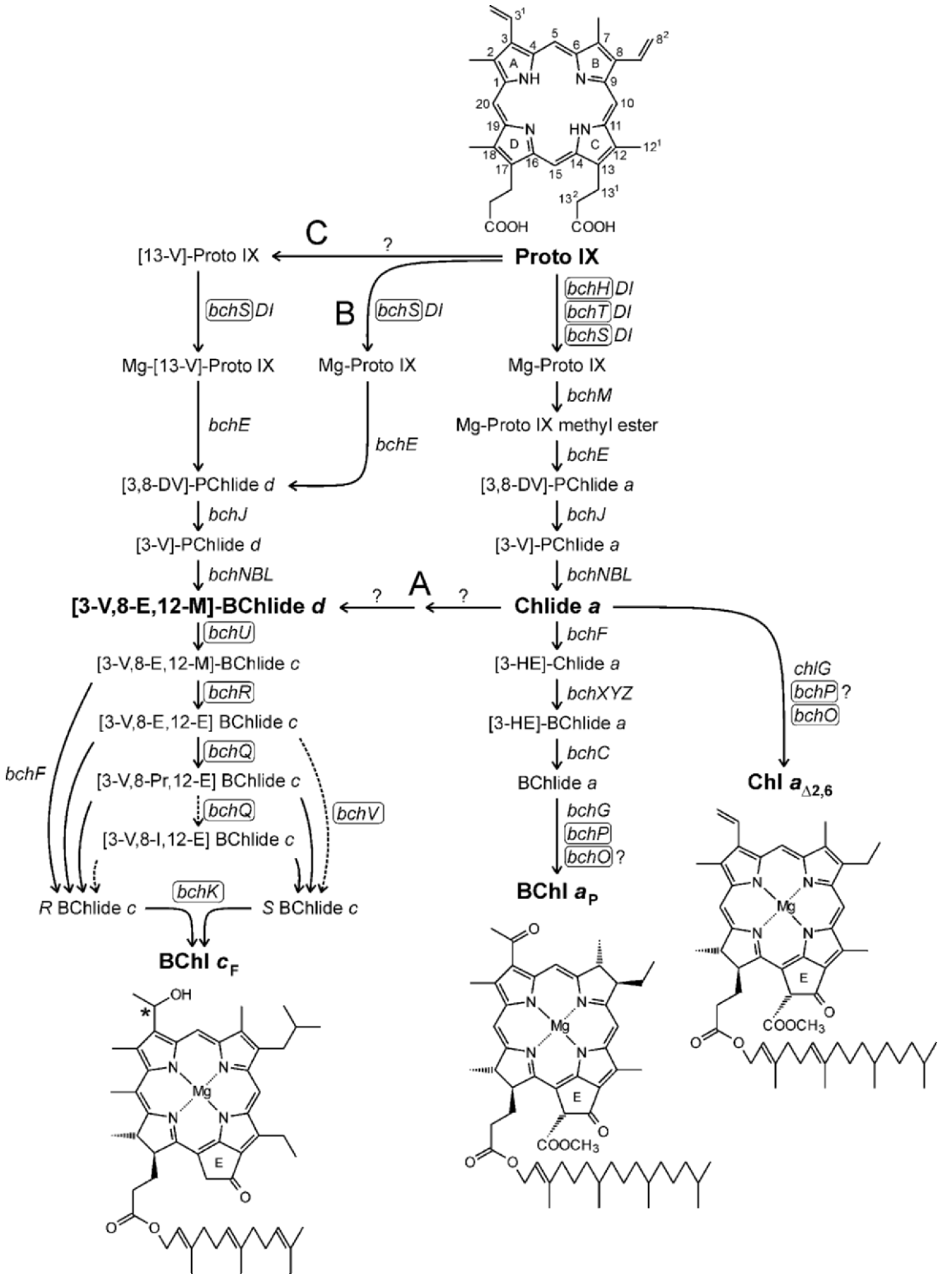

Fig. 3. Proposed biosynthetic pathways of BChl c_P, BChl a_P, and Chl $a_{\Delta 2,6}$ in *Chl. tepidum*. Genes that have been inactivated are boxed. The three alternative pathways A, B, and C leading to [3-vinyl]-BChlide *d* are indicated. See text for details. Only the maximally methylated BChl *c* is shown as the final product.

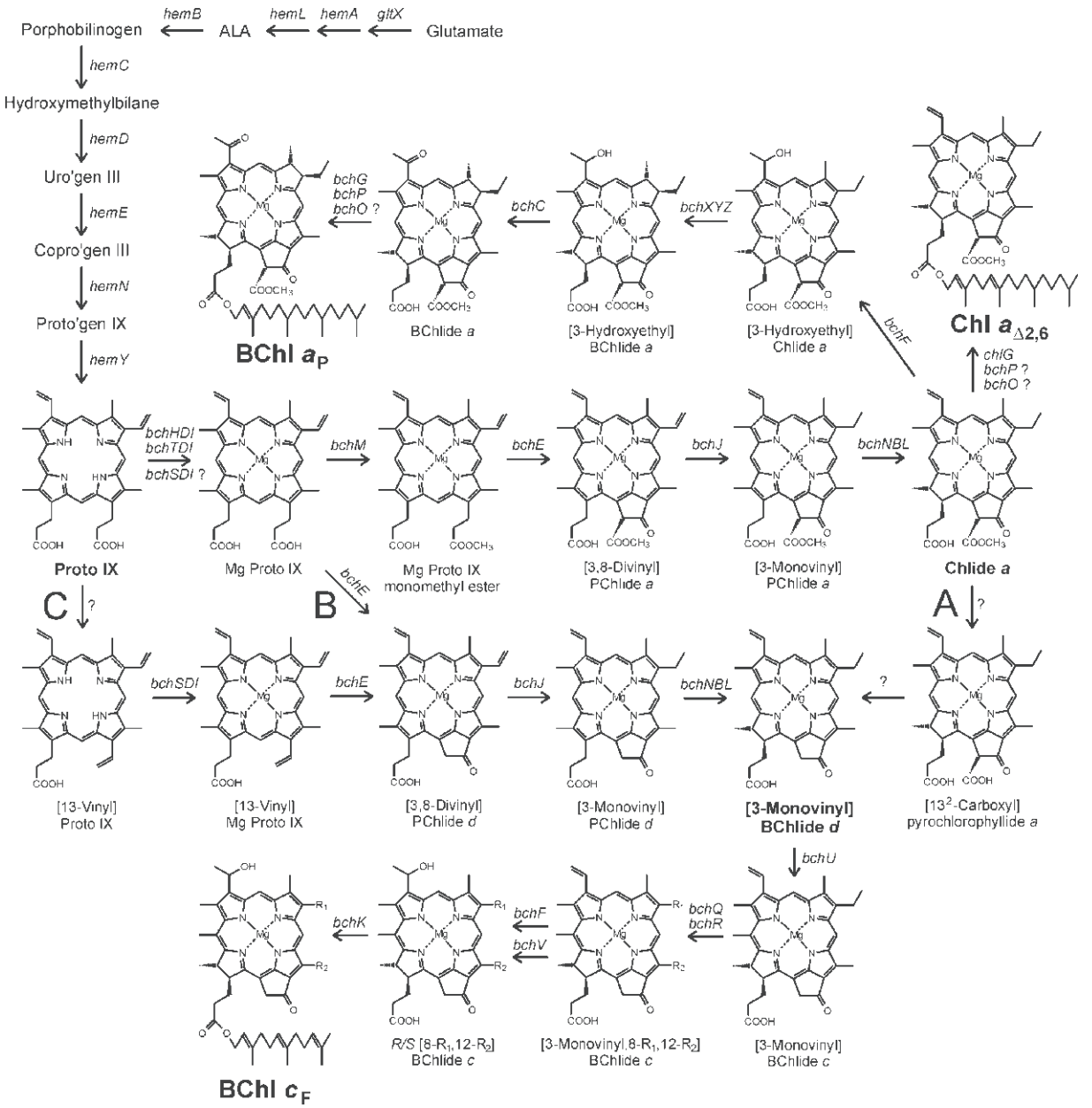

Fig. 4. Proposed biosynthetic pathways of BChl c_F, BChl a_P, and Chl $a_{\Delta 2,6}$ in *Chl. tepidum* showing structures of the intermediates. The three alternative pathways A, B, and C leading to [3-monovinyl]-BChlide *d* are indicated. See text for details

in removal of the C-13^2 carboxylate or methylcarboxylate group. Pathway A proposed above for BChl *c* biosynthesis seems to be most consistent with this observation, since a block in this pathway could potentially cause the accumulation and excretion of Mg-Proto monomethyl ester.

Although the relationship may not be immediately apparent, the synthesis of BChl *c*, which is a pyrochlorophyll (i.e., a Chl that has no carbon substituents at C-13^2), could be related evolutionarily, or at least mechanistically, to Chl degradation in eukaryotic algae and higher plants. Pyropheophytins and pyropheophorbides have been detected in various organisms, including photosynthetic bacteria (Haidl et al., 1985), eukaryotic algae (Schoch et al., 1981; Ziegler et al., 1988), and plants (Shimokawa et al.,

1990). Similar to the proposed reactions in Figs. 3 and 4, specific enzymatic reactions eliminate the C-13[2] methylcarboxylate group as early steps in the Chl *a* degradation pathways of some organisms (Matile et al., 1996, 1999; Hörstensteiner, 1999; Matile, 2000; Takamiya et al., 2000; Suzuki et al., 2002). Three possible mechanisms have been proposed: 1) a methyl esterase and a decarboxylase may act in succession; 2) a methyl esterase acts alone and is followed by spontaneous decarboxylation; and 3) a single unidentified enzyme removes the methylcarboxylate group in a concerted fashion. Kräutler and Hörtensteiner (Chapter 17) provide a detailed description of Chl biodegradation.

IV. Early Steps in Porphyrin Biosynthesis

The first universal, committed precursor in tetrapyrrole biosynthesis is 5-aminolevulinate (ALA) (Beale, 1995; Chapter 11, Beale). Plants and most bacteria synthesize ALA from glutamate, whereas purple bacteria, other α-proteobacteria, and eukaryotes other than plants synthesize ALA from glycine and succinyl-CoA. In both *Chlorobium* and *Chloroflexus* species, ALA is synthesized via the C_5 glutamate pathway as shown by isotope labeling studies (Anderson et al., 1983; Smith and Huster, 1987; Avissar et al., 1989; Rieble et al., 1989; Swanson and Smith, 1990; Smith, 1991; Beale, 1995). Beale and coworkers (Majumdar et al., 1991) showed that *Chl. vibrioforme hemA* could complement an *Escherichia* (*E.*) *coli hemA* mutant, thereby establishing that *hemA* encodes the glutamyl-tRNA reductase. In agreement with these studies, the *gltX*, *hemA*, and *hemL* genes, encoding glutamyl-tRNA synthetase, glutamyl-tRNA reductase, and glutamate-1-semialdehyde 2,1-aminomutase, respectively, of the glutamate C_5 pathway are found in the genome sequence of *Chl. tepidum* and *Cfx. aurantiacus* (Fig. 4). No gene encoding 5-aminolevulinate synthase, the enzyme responsible for the synthesis of 5-aminolevulinate in the alternative succinyl-CoA pathway (Beale, 1995), has been found in the genomes of these green bacteria (Eisen et al., 2002; DOE Joint Genome Institute, http://www.jgi.doe.gov).

The genes encoding the remaining enzymes leading to the key tetrapyrrole intermediate, Proto, have also been identified in the *Chl. tepidum* genome sequence by their sequence similarity to the known enzymes of *Chl. vibrioforme* (Rhie et al., 1996) and other bacteria such as *E. coli* (Eisen et al., 2002). These include *hemB* (5-aminolevulinate dehydratase), *hemC* (porphobilinogen deaminase), *hemD* (uroporphyrinogen III synthase), *hemE* (uroporphyrinogen III decarboxylase), *hemN* (oxygen-independent coproporphyrinogen III oxidase), and *hemY* (protoporphyrinogen oxidase) (Fig. 4). A single *hemH* gene, encoding ferrochelatase, which converts Proto into protoheme used in cytochrome biogenesis, is also found in *Chl. tepidum*. The *hemA*, *hemC* and *hemD* genes appear to form an operon in *Chl. tepidum*, and are additionally located near the *hemB* gene, as is the case in *Chl. vibrioforme* (Majumdar et al., 1991; Rhie et al., 1996). The *hemE*, *hemH*, *hemN*, and *hemY* genes of *Chl. tepidum* are not clustered with other genes that encode enzymes for tetrapyrrole biosynthesis.

In *Cfx. aurantiacus*, the genes encoding *hemC*, *hemD*, and *hemH* have not yet been sequenced or are too dissimilar to be identified by sequence similarity search methods (DOE Joint Genome Institute, http://www.jgi.doe.gov). However, most of the remaining genes are found in two apparent operons: *hemN-hemY* and *hemB-hemE-hemL-hemF*. Interestingly, the *Cfx. aurantiacus* genome has genes encoding enzymes for both oxygen-independent and oxygen-dependent enzymes coproporphyrinogen III oxidase (HemN and HemF, respectively) and protoporphyrinogen IX oxidase (HemY and HemK, respectively). These observations are consistent with the ability of *Cfx. aurantiacus* to grow heterotrophically under oxic conditions or photoautotrophically or photoheterotrophically under anoxic conditions.

V. Bacteriochlorophyll *a* Biosynthesis

A. Bacteriochlorophyllide *a* Biosynthesis

Three genes, *bchF*, *bchG*, and *bchM*, involved in BChl *a* biosynthesis in *Chl. tepidum* have been identified by complementation of *Rba. capsulatus* mutants (Xiong et al., 2000). Orthologs of all other genes that are known to be involved in oxygen-independent BChl *a* biosynthesis in purple bacteria have been identified in the *Chl. tepidum* genome by sequence similarity (Eisen et al., 2002). Thus, the biosynthetic pathway for BChlide *a* in *Chl. tepidum* is likely to be identical to that in *Rhodobacter* spp. (Suzuki et al., 1997; Porra, 1997; Willows, 2003; Chapters 10–14) and can briefly be described as follows (see also

Figs. 3 and 4): A heterotrimeric Proto Mg-chelatase (BchHDI) converts Proto to Mg-Proto (Gibson et al., 1995; Willows et al., 1996; Willows and Beale, 1998; Fodje et al., 2001). The C-13^2 carboxylate group of Mg-Proto is then methylated by BchM (Bollivar et al., 1994a; Gibson and Hunter, 1994), possibly while the substrate is still bound to the BchHDI Mg-chelatase (Hinchigeri et al., 1997; Karger et al., 2001). This methylation reaction is followed by the oxidative cyclization reaction, catalyzed by BchE, that forms ring E of [3,8-divinyl]-PChlide *a* (Bollivar et al., 1994b; Gough et al., 2000). The C-8 vinyl group is reduced by BchJ to form [3-monovinyl]-PChlide *a* (Suzuki and Bauer, 1995), and the double bond between C-17 and C-18 of ring D is reduced by PChlide reductase (BchNBL) to form Chlide *a* (Burke et al., 1993b; Fujita and Bauer, 2000). Reduction of the double bond between C-7 and C-8 by BchXYZ produces [3-vinyl]-BChlide *a* (Burke et al., 1993a; McGlynn and Hunter, 1993). The C-3 vinyl group is hydrated by BchF (Burke et al., 1993b), and the resulting hydroxyl is oxidized by BchC to form the C-3^1 oxo group of BChlide *a* (Wellington and Beatty, 1989; McGlynn and Hunter, 1993).

B. Esterification and Reduction

The esterifying isoprenoid tail is added to BChlide *a* and reduced by the combined action of BChl *a* synthase (BchG) and geranylgeranyl reductase (BchP) (Bollivar et al., 1994b,c; Oster et al., 1997; Addlesee and Hunter, 1999, 2002; Addlesee et al., 2000). It is still uncertain whether the reduction of the geranylgeranyl moiety to a phytyl moiety takes place before or after the esterification with BChlide *a* (Oster et al., 1997; Addlesee and Hunter, 1999; Addlesee et al., 2000). In vitro studies with recombinant BChl synthase from *Rba. capsulatus* suggest that the preferred substrate for the esterification reaction is phytyl (P) diphosphate rather than geranylgeranyl (GG) diphosphate, although both substrates are utilized by the BchG enzyme (Oster et al., 1997). This could imply that reduction by BchP takes place prior to esterification. However, Addlesee and coworkers (Addlesee and Hunter, 1999, 2002; Addlesee et al., 2000) have reached the opposite conclusion based on studies with enzymes from *Rba. sphaeroides* and *Rhodospirillum* (*Rsp.*) *rubrum*. They find that BchP can act after esterification of BChlide *a*, and have additionally identified a homolog of BchP that can reduce the double bonds of BPhe a_{GG} to produce BPhe a_P in *Rsp. rubrum* (Addlesee and Hunter, 2002).

The esterifying alcohol in Chl $a_{\Delta 2,6}$, Δ2,6-phytadienol, is also most likely derived from geranylgeranyl diphosphate by reduction of two double bonds, but again it is unclear whether this reduction takes place before or after esterification and whether the reductase is the same that reduces three double bonds in the geranylgeranyl moiety in BChl *a* to phytyl. Biochemical characterization of the BChl *a* synthase (BchG) and Chl *a* synthase (ChlG) will be required to resolve this issue, although even this may not adequately account for possible substrate channeling effects and the relative in vivo concentrations of phytyl diphosphate and geranylgeranyl diphosphate.

Two paralogous *bchP* genes, *CT2256* and *CT1232*, are present in the *Chl. tepidum* genome. The product of *CT2256* is more similar to the BchP proteins of purple bacteria, while the product of *CT1232*, denoted BchO (Table 1), is more distantly related to the BchP proteins of purple bacteria. Because the esterifying alcohols of BChl *a* and Chl $a_{\Delta 2,6}$ are different, it was initially hypothesized (Eisen et al., 2002) that these different gene products would catalyze the reduction of these two different esterifying alcohols in a fashion similar to that suggested by Addlesee and Hunter (2002) recently for BChl *a* and bacteriopheophytin (BPhe) *a*. However, the HPLC profiles of pigments extracted from *Chl. tepidum* mutants lacking either BchP or BchO were virtually identical, although clearly different from wild type (A. Gomez Maqueo Chew, N.-U. Frigaard, and D. A. Bryant, unpublished). Both mutants contain BChl *a* and Chl *a* species with esterifying alcohols that appear to be less reduced than in the wild-type strain. Further studies will be required to establish the roles of these two enzymes.

VI. Chlorophyll *a* Biosynthesis

It is possible that Chl $a_{\Delta 2,6}$ is derived from the same pool of Chlide *a* that is used for BChl *a* biosynthesis (Section III; Figs. 3 and 4). However, since the *Chl. tepidum* genome encodes three homologs of the large subunit of Mg-chelatase, and since one of these homologs clearly is nearly specific for BChl *c* biosynthesis, it is also possibly that Chl $a_{\Delta 2,6}$ is synthesized from Proto via intermediates sequestered from those of BChl *a* biosynthesis by a substrate channeling mechanism (Sections III and VII).

The *Chl. tepidum* genome encodes a polyprenyl transferase (CT1270) that is apparently orthologous to

cyanobacterial and plant-like Chl *a* synthases (Eisen et al., 2002; Frigaard et al., 2002), and Chl $a_{\Delta 2,6}$ is probably synthesized from Chlide *a* by this synthase. Attempts to inactivate this gene have not yet been successful (Frigaard et al., 2002). These results suggest that Chl $a_{\Delta 2,6}$ is essential in *Chl. tepidum* and cannot easily be substituted by another (B)Chl species.

VII. Bacteriochlorophyll *c* Biosynthesis

A. Magnesium Chelation

The first committed step in Chl *a* and BChl *a* biosynthesis is the Mg insertion into Proto by magnesium chelatase (Bollivar et al., 1994b; Gibson et al., 1995; Jensen et al., 1996; Willows et al., 1996; Porra, 1997; Suzuki et al., 1997; Willows and Beale, 1998; Willows, 2003). This enzyme consists of a large, Proto-binding subunit of about 140 kDa (BchH) and two smaller subunits with masses of approximately 70 kDa (BchD) and 40 kDa (BchI) (Bollivar et al., 1994; Gibson et al., 1995; Petersen et al., 1998a,*b*). The structure of the BchI ATPase was recently determined by X-ray crystallography (Fodje et al., 2001). The *Chl. tepidum* genome has only single genes encoding each of the smaller subunits, *bchD* and *bchI* (Table 1) but has three paralogous genes, *CT1295*, *CT1955*, and *CT1957,* that encode the largest subunit (Eisen et al., 2002). These have been designated *bchT*, *bchS*, and *bchH*, respectively (Table 1), and the phylogenetic relationship of these proteins to one another and to related proteins in *Cfx. aurantiacus* and other organisms is shown in Fig. 5. One simple hypothesis for the presence of multiple BchH-like proteins in *Chl. tepidum* is that each performs a specialized function. Inactivation of the *bchS* gene (*CT1955*) caused the nearly complete elimination of BChl *c* biosynthesis (A. Gomez Maqueo Chew, N.-U. Frigaard, D. A. Bryant, unpublished), although small amounts of BChl *c* are still synthesized in this mutant. This result suggests that BChl *c* cannot readily be synthesized from the tetrapyrrole intermediates produced by the BchHID and BchTID enzymes that presumably participate in BChl *a* and Chl $a_{\Delta 2,6}$ biosynthesis. Inactivation of the *bchH* gene had little effect on the (B)Chl content of the cells, but a *bchT* mutant produced somewhat less BChl *c* than the wild type.

One hypothesis to explain these observations is that the BchH, BchS, and BchT proteins do not release their Mg-chelated products but instead channel these

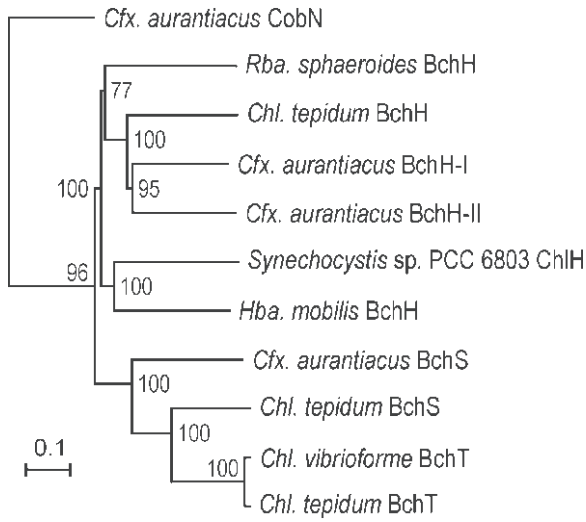

Fig. 5. Phylogenetic neighbor-joining distance tree showing the relationships among the largest subunit of Proto Mg-chelatase (BchH, BchS, BchT, and ChlH) from various photosynthetic organisms. CobN, a subunit of cobalt chelatase for cobalamin biosynthesis, was used as an outgroup. The scale bar relates the branch lengths to the relative number of amino acid substitutions. Bootstrap values (in percentage) based on 1000 replications are also shown. Abbreviations: *Rba*., *Rhodobacter*; *Chl*., *Chlorobium*; *Cfx*., *Chloroflexus*; *Hba*., *Heliobacillus*.

products, possibly through several enzymatic steps, to specific product endpoints ('substrate channeling,' Pathway A in Fig. 3). Several lines of evidence suggest that this could be the case. Hinchigeri et al. (1997) found that exogenously added BchH stimulated the conversion of Mg-Proto to Mg-Proto monomethyl ester by BchM. Jensen et al. (1996) showed that, when ChlH, ChlI, and ChlD were expressed in *E. coli*, Mg-Proto accumulated, but when ChlM was coexpressed in the same cells, only Mg-Proto monomethyl ester was detected. In these in vitro experiments, the ratio of ChlH to ChlM determined whether Mg-Proto or only its monomethyl ester accumulated. At substoichiometric ratios of these proteins, Mg-Proto was detected, but only the monomethyl ester was detected when the ratio of these proteins was 1:1. Since *Chl. tepidum* produces three classes of (B)Chls (BChl *a*, Chl *a*, and BChl *c*), each BchH homolog might channel Mg-Proto towards a specific (B)Chl end product. Finally, Karger et al. (2001) recently reported that the K_d for Mg-Proto of BchH is actually lower than the K_d for the substrate, Proto (i.e., the product is more tightly bound by BchH than the substrate). This observation is consistent with the idea that a BchH-Mg-Proto complex is the substrate for

BchM, the next enzyme in the biosynthetic pathway for BChl *a* and Chl *a*.

Yet unidentified enzymes, possibly an esterase and a decarboxylase, would be required to remove the C-13^2 methylcarboxylate moiety (Pathway A in Figs. 3 and 4). Another possibility is that the BchSID Mg-chelatase prevents subsequent methylation by the BchM methyltransferase. During the subsequent oxidative cyclization reaction catalyzed by BchE, decarboxylation of the C-13^2 carboxyl group might occur (Pathway B in Figs. 3 and 4). Finally, it is possible that the substrate for the BchSID Mg-chelatase is different from those for the BchHID and BchTID enzymes. Since the major structural feature that distinguishes BChl *c*, *d*, and *e* from BChl *a* and Chl *a* is the absence of the C-13^2 methylcarboxylate moiety, it is possible that an intervening enzymatic reaction leads to decarboxylation of the C-13 propionate of Proto prior to Mg-chelation by BchSID (Pathway C in Figs. 3 and 4).

B. Isocyclic Ring Formation

In BChl *a* biosynthesis, the C-13 propionate group of Mg-Proto is converted into ring E of PChlide by a complex oxidation that is catalyzed by BchE (Bollivar et al., 1994b; Gough et al., 2000). This oxidation occurs after the carboxylate group is protected by methylation by BchM (Bollivar et al., 1994a; Gibson and Hunter, 1994). The exact substrate for the formation of ring E in BChl *c* biosynthesis is not yet known; it would be determined by when decarboxylation or demethylcarboxylation at C-13^2 occurs (see Figs. 3 and 4, pathways A, B, and C).

The *Chl. tepidum* genome contains seven *bchE* paralogs and all except *CT1959* have been insertionally inactivated (A. Gomez Maqueo Chew, N.-U. Frigaard, D. A. Bryant, unpublished). Since CT1959 is most similar in sequence to the BchE product of *Rba. capsulatus*, and since the *CT1959* gene lies downstream from *bchM*, it is likely that this gene encodes the oxidative cyclase in *Chl. tepidum* and would thus be required for viability. Consistent with this interpretation, none of the six mutants, each lacking a single *bchE* paralog, is devoid of BChl *c*, BChl *a*, or Chl *a*. These results provide further evidence that the product of *CT1959* probably participates in the biosynthesis of BChl *a*, Chl $a_{\Delta 2,6}$ and BChl *c*.

In contrast to BChl *a* and Chl $a_{\Delta 2,6}$ (see Figs. 1 and 3), BChl *c* has no C-13^2 methylcarboxylate group. BchE could play a role in the removal of this group in BChl *c* biosynthesis. If the BchSID chelatase prevents methylation of the Mg-proto IX by BchM, then BchE could cause oxidative decarboxylation during the cyclization reaction (Pathway B in Figs. 3 and 4). Alternatively, if decarboxylation occurs prior to Mg chelation in BChl *c* biosynthesis, then BchE would have to convert [13-vinyl]-Mg-proto IX to [3,8-divinyl]-PChlide *d* (Pathway C in Figs. 3 and 4). Resolution of this issue will probably require in-depth biochemical studies with BchE and the predicted substrates described above, although it is possible that mass spectroscopic analyses of intermediates that accumulate in various mutants will also help to resolve this issue.

C. C-8^2 and C-12^1 Methylation

The typical distribution of BChl *c* homologs in *Chl. tepidum* obtained under laboratory growth conditions is about 5% [8-E,12-M]-BChl *c*, 60% [8-E,12-E]-BChl *c*, 30% [8-Pr,12-E]-BChl *c*, and 5% [8-I,12-E]-BChl *c* but the exact composition varies with growth conditions (Borrego et al., 1999). The 8-*neo*-pentyl homolog is not routinely detected in *Chl. tepidum*, although this molecule is found in small amounts in some other green sulfur bacteria containing BChl *c*, *d*, or *e* (Smith et al., 1983a; Bobe et al., 1990). In general, at least 90% of the BChl *c*, *d*, or *e* in green sulfur bacteria is methylated to the ethyl species in the C-12^1 position, and between 30 and 60% is methylated in the C-8^2 position (Borrego et al., 1999; Airs et al., 2001; Glaeser et al., 2002). It is not known how this methylation is regulated, but the observation that the degree of methylation increases slightly at low light intensity indicates that light intensity or growth rate may influence the regulation (Bobe et al., 1990; Huster and Smith, 1990; Borrego and Garcia-Gil, 1995; Borrego et al., 1998, 1999; Bañeras et al., 1999; Airs et al., 2001; Guyoneaud et al., 2001). The major esterifying alcohol is farnesol, although subsets of homologous BChl *c* molecules with other long-chain, esterifying alcohols are usually detected (Section VII.F). However, the methylation pattern on C-8^2 and C-12^1 is similar in all subsets regardless of the esterifying alcohol (Steensgaard et al., 1996; Airs et al., 2001; Glaeser et al., 2002), which indicates that the methylation reactions occur before the esterifying alcohol is added. Methylation on the C-8^2 and C-12^1 carbons does not occur in *Cfx. aurantiacus*.

As noted above, the *Chl. tepidum* genome contains seven paralogs of *bchE*, the gene that encodes

the oxidative cyclase that forms ring E in PChlide (Section VII.B). This group of enzymes belongs to the P-methyltransferase/oxidative cyclase superfamily, and since BchE acts on a porphyrin substrate, it was suggested that some of the BchE homologs probably function as C-methyltransferases in BChl *c* biosynthesis (Eisen et al., 2002). Indeed, two of the *bchE* homologs in *Chl. tepidum* have been shown to encode C-methyltransferases: inactivation of *CT1777*, now designated *bchQ*, causes the accumulation of [8-E,12-M]-BChl *c* and [8-E,12-E]-BChl *c* and inactivation of *CT1320*, now designated *bchR*, causes the accumulation of three BChl *c* homologs, tentatively identified as [8-E,12-M]-BChl *c*, [8-Pr,12-M]-BChl *c*, and [8-I,12-M]-BChl *c* (A. Gomez Maqueo Chew, N.-U. Frigaard, D.A. Bryant, unpublished). Thus, BchQ is the C-8^2 methyltransferase and BchR is the C-12^1 methyltransferase. A double mutant in both *bchQ* and *bchR* accumulates only [8-E,12-M] BChl *c*. Inactivation of other *bchE* homologs did not cause any detectable changes in BChl *c* biosynthesis, and thus BchQ is able to catalyze the introduction of multiple methyl groups on the C-8^2 carbon. Since the *bchR* mutant still produces homologs that differ in the degree of methylation at C-8^2, it is clear that the introduction of these additional methyl groups at C-8^2 does not depend upon prior methylation at C-12^1.

All BchE homologs in *Chl. tepidum* are proteins with masses of 50 to 60 kDa, and they share sequence similarity to biotin synthase, lipoic acid synthase and other enzymes that contain a Cys-X_3-Cys-X_2-Cys-X_n-Cys [4Fe-4S] cluster and a sequence signature for binding cobalamin (Gough et al., 2000). The methyl groups on the C-8^2 and C-12^1 carbons have been shown to originate from *S*-adenosylmethionine (SAM) (Huster and Smith, 1990). The methylation reactions catalyzed by BchQ and BchR probably involve hydrogen abstraction from the C-8^2 or C-12^1 position followed by transfer of a methyl group from methylcobalamin to the carbon radical at C-8^2 or C-12^1. By analogy to other enzymes of this family, it is presumed that SAM binds to the $[4Fe\text{-}4S]^{2+}$ cluster of BchQ or BchR, which is reduced to the +1 state; this is accompanied by the reductive cleavage of SAM into methionine and a 5′-deoxyadenosyl radical. This radical would then abstract a hydrogen atom from the C-8^2 or C-12^1 position of the substrate to yield 5′-deoxyadenosine and a substrate radical. This substrate radical is proposed to abstract a methyl radical from methylcobalamin to yield the methylated substrate and cob(II)alamin. Reduction of cob(II)alamin and a subsequent reaction with a second molecule of SAM would regenerate the methylcobalamin moiety with adenosylhomocysteine as the byproduct. Thus, two SAM molecules would be consumed during the addition of one methyl group to the substrate: one would act to generate a hydrogen-abstracting radical and one would serve as the methyl donor.

The C-8^2 and C-12^1 methylations most likely occur on intermediates lacking a long-chain esterifying alcohol, since the tetrapyrroles that accumulate in a *bchK* mutant of *Chl. tepidum* (Section VII.F) appear to be a complex mixture of species carrying from none to three methyl groups (N.-U. Frigaard, D. A. Bryant, unpublished data). The C-8^2 and C-12^1 methylations probably take place on 3-vinyl BChlide precursors because a C-3^1 hydratase *bchV* mutant of *Chl. tepidum* accumulates 3-vinyl BChl *c* species that are methylated in both C-8^2 and C-12^1 (Section VII.E).

D. C-20 Methylation

A single enzyme is apparently responsible for the methylation of the C-20 methine carbon in the BChl *c* and BChl *e* biosynthetic pathways. This methyltransferase enzyme is expected to be of a different class than the C-8^2 and C-12^1 methyltransferases discussed above, since it acts on an activated carbon atom that is part of an aromatic system. As for the C-8^2 and C-12^1 methylations, the methyl group at C-20 is derived from SAM (Huster and Smith, 1990).

In an effort to identify the C-20 methyltransferase, the *Chl. tepidum* genome was initially surveyed for paralogs of *cbiL* (*CT0388*), which encodes a C-20 methyltransferase involved in cobalamin biosynthesis. Only one gene (*CT1763*) was found that predicted a product with some sequence similarity to CbiL. However, a mutant of *Chl. tepidum* in which *CT1763* was insertionally inactivated retained the ability to synthesize BChl *c*, and thus it was concluded that *CT1763* could not encode a BChl C-20 methyltransferase (Maresca et al., 2004).

In a different approach, it was noticed that, in *Cfx. aurantiacus*, a *crtF* paralog occurs immediately upstream of *bchK*, encoding BChl *c* synthase, and genes encoding chlorosome envelope proteins CsmM, CsmN, and CsmP (Fig. 2b). This operon structure suggested that the *crtF* paralogous gene might also play some role in BChl *c* synthesis and/or in chlorosome formation or function. Although this *Cfx. aurantiacus* gene was originally annotated *crtF* by Niedermeyer et al. (1994), the protein product is in fact not very

similar to the CrtF proteins of purple bacteria; however, the predicted protein is unusually similar in sequence to a homolog in *Chl. tepidum* (CT0028). Since neither *Chl. tepidum* nor *Cfx. aurantiacus* are known to synthesize O-methylated carotenoids (Takaichi et al., 1995, 1997; Takaichi, 1999), it seemed unlikely that these proteins could be true orthologs of the CrtF enzymes of purple bacteria. Subsequent insertional inactivation of *CT0028* in *Chl. tepidum* produced a BChl *d*-producing mutant; this confirms that CT0028 is a C-20 methyltransferase, and this gene has been named *bchU* (Table 1; Maresca et al., 2004).

Other experimental observations are consistent with CT0028 being the C-20 methyltransferase. *Chl. vibrioforme* strain 8327d synthesizes BChl *d* and therefore lacks the C-20 methyltransferase activity (Broch-Due and Ormerod, 1978; Bobe et al., 1990). However, when cultured under very low light intensities, this strain has been reported to produce revertants that have regained the ability to synthesize BChl *c* (Broch-Due and Ormerod, 1978). This observation indicates that *Chl. vibrioforme* strain 8327d is a mutant that produces a defective C-20 methyltransferase, but that this mutant can regain its C-20 methyltransferase activity through reversion or a second-site suppressor mutation. The open reading frame corresponding to *bchU* has been amplified by PCR from three *Chlorobium* strains: *Chl. tepidum*, *Chl. vibrioforme* strain 8327d, and *Chl. phaeobacteroides* strain 1549 (Maresca et al., 2004). When compared to the same sequence from *Chl. tepidum* and *Chl. phaeobacteroides* 1549, the DNA fragment amplified from *Chl. vibrioforme* 8327d contained a single nucleotide insertion that causes a frameshift mutation in this gene. The protein produced from this mutated gene would be about half the normal predicted size due to premature termination at a stop codon. The *bchU* gene amplified from a BChl *c*-producing revertant of *Chl. vibrioforme* 8327d, denoted strain 8327c, showed a second mutation that restored the reading frame necessary to produce a full-length BchU product.

The actual substrate(s) for the C-20 methyltransferase is not clear. Concentrated cell suspensions of BChl *c*-producing *Chlorobium* sp. fed with various tetrapyrrole precursors have been found to excrete uroporphyrins, coproporphyrin III, Mg-Proto monomethyl ester, and other compounds, none of which are methylated in the C-20 position (Richards and Rapoport, 1966; 1967). A similar examination of the non-esterified tetrapyrroles excreted by the *bchK* mutant of *Chl. tepidum* may reveal those substrates on which C-20 methylation can take place (Frigaard et al., 2002). The observation that BChl *d* apparently is produced in a similar amount in the *bchU* mutant as BChl *c* in the wild type either suggests that C-20 methylation occurs late in the pathway or suggests that, if the methylation occurs early in the pathway, the subsequent enzymes do not strongly discriminate against the presence or absence of a methyl group in the C-20 position.

E. C-3^1 Hydration

BChl *c* has a chiral carbon at the C-3^1 position, which is produced by hydration of the C-3 vinyl group (Figs. 3 and 4). Both the R and S epimers at C-3^1 have been identified in BChl *c*, *d*, and *e* from *Chlorobium* species as well as in BChl *c* from *Cfx. aurantiacus* (Smith et al., 1983b; Smith and Simpson, 1986; Fages et al., 1990; Senge and Smith, 1995). In *Chlorobium* species the ratio of the R and S epimers is highly correlated with the degree of methylation at the C-8^2 and C-12^1 positions, such that highly methylated BChl species are more likely to have S-stereochemistry at C-3^1 and a similar distribution is found in BChl *c*, *d*, and *e* from all *Chlorobium* species investigated (Senge and Smith, 1995). All [8-E,12-M] homologs have R stereochemistry at C-3^1, and most of the [8-E,12-E] homologs also have R stereochemistry. The [8-Pr,12-E] homologs are roughly equally divided into R and S stereochemistry; however, nearly all [8-I,12-E] homologs have S stereochemistry.

The *Chl. tepidum* genome contains two paralogs of the *Rhodobacter* sp. *bchF* gene, which encodes the C-3^1 vinyl hydratase of the BChl *a* pathway (Burke et al., 1993b). One of these paralogs in *Chl. tepidum*, *CT1421*, encodes a protein which is most closely related to the BchF proteins found in purple bacteria, while the second, *CT1776*, has been denoted *bchV* (Table 1) and encodes a more distantly related paralog. BchF presumably functions as the C-3^1-vinyl hydratase in the BChl *a* biosynthetic pathway (Figs. 3 and 4), and the presence of the paralogous *bchV* gene initially suggested that this gene product might be specific for BChl *c* biosynthesis (Eisen et al., 2002). Insertional inactivation of *bchV* in *Chl. tepidum* causes the accumulation of abnormal BChl *c* species which have tentatively been identified through a combination of absorption and mass spectrometry as [3-vinyl]-BChl c_F (A. Gomez Maqueo Chew, N.-U. Frigaard, D. A. Bryant, unpublished). However, only

about 15% of the BChl *c* is affected, which suggests that *bchV* indeed is specific, but not essential, for BChl *c* biosynthesis. The dominant BChl *c* species in the *bchV* mutant is [8-E,12-E]-BChl c_F, and smaller amounts of the [8-E,12-M] and [8-Pr,12-E] homologs are present, but no detectable [8-I,12-E] homolog is found. In the *bchV* mutant the most abundant homolog of [3-vinyl]-BChl c_F is [8-Pr,12-E]; smaller amounts of [8-E,12-E] and [8-I,12-E] were also found, but no [8-E,12-M] homolog was detected. These data suggest that BchF inefficiently hydrates the more highly methylated species and that BchV thus functions to hydrate the more highly methylated BChl homologs. Since the more highly methylated species of BChl *c* are predominantly S epimers, it seems reasonable to suggest that BchV is a hydratase that specifically produces S stereochemistry at C-3[1] and that BchF is a hydratase that produces R stereochemistry at C-3[1]. Assuming these conclusions are correct, a corollary would be that the [3-hydroxyethyl]-BChlide *a* intermediate in BChl *a* biosynthesis should have predominantly if not exclusively R stereochemistry at C-3[1].

F. Esterification

When green sulfur bacteria are grown under optimal laboratory conditions, the predominant esterifying alcohol for BChl *c*, *d*, and *e* is farnesol (Steensgaard et al., 1996; Borrego et al., 1999). However, in contrast to other classes of (B)Chls, other long-chain alcohols are also found in varying amounts including phytol, Δ2,6-phytadienol, geranylgeraniol, hexadecanol, hexadecenol, and tetradecanol (Caple et al., 1978; Airs et al., 2001; Glaeser et al., 2002). *Chl. tepidum* can also readily incorporate alcohols such as phytol, geranylgeraniol, and dodecanol into BChl *c* if these alcohols are supplied in the growth medium (Steensgaard et al., 1996). The synthase incorporating alcohols into BChl *c* seems to be quite promiscuous in its choice of the esterifying alcohol, and thus appears to have less stringently defined specificity for the esterifying alcohol than other (B)Chl synthases.

Insertional inactivation of open reading frame *CT1992* in *Chl. tepidum*, which has strong sequence similarity to Chl *a* and BChl *a* synthases from other organisms, resulted in a BChl *c*-less mutant (Frigaard et al., 2002). Thus, this gene encodes BChl *c* synthase and was subsequently designated *bchK*. Although the *bchK* mutant does not form chlorosomes, it does synthesize vestigial chlorosomes that contain carotenoids and BChl *a* (Frigaard et al., 2002, 2003). These structures, denoted carotenosomes, have a simplified protein composition relative to mature chlorosomes, and they may provide clues to how chlorosomes are formed in vivo. These structures are easily isolated because of their very low density, and they provide an ideal starting material for characterizing the organization of the chlorosome-associated BChl *a*. Finally, a surprising finding is that the *bchK* mutant grows nearly as fast as the wild-type at light intensities above 300 μE m^{-2} s^{-1}; this is mostly due to photoinhibition of the wild type at high light intensities (Frigaard et al., 2002).

VIII. Bacteriochlorophyll *d* Biosynthesis

BChl *d* differs from BChl *c* only by the absence of the C-20 methyl group. BChl *d* biosynthesis is thus expected to be identical to BChl *c* biosynthesis except that C-20 methyl transferase activity would be lacking. *Chl. vibrioforme* strain 8327d, which naturally produces BChl *d* but readily mutates under low-light growth conditions to produce BChl *c* (Broch-Due and Ormerod, 1978; Bobe et al., 1990), is a natural frameshift mutant that encodes a *bchU* gene carrying a single nucleotide insertion (Maresca et al., 2004). Although some reports suggest that some strains exhibit a regulation of the relative amounts of BChl *c* and BChl *d* (Bañeras et al., 1999), BChl *c* and BChl *d* rarely coexist in a green sulfur bacterium in comparable amounts. Since the C-20 methyltransferase in *Chl. tepidum* has now been identified by insertional inactivation (Section VII.D), it will now be possible to use this information to examine the structure and regulation of the C-20 methyltransferase in such species.

IX. Bacteriochlorophyll *e* Biosynthesis

BChl *e* differs from BChl *c* by the presence of a formyl group rather than a methyl group at position C-7. Thus, BChl *e* biosynthesis probably differs from BChl *c* biosynthesis only by the addition of an enzyme to oxidize the C-7 methyl group to a formyl moiety. This enzyme may also belong to the BchE/P-methyltransferase/C-methyltransferase superfamily, since BchE can introduce an oxo group anaerobically (Section VII.B). Alternatively, an enzyme similar to ethylbenzene dehydrogenase of *Azoarcus* sp. could

introduce an oxo group into this position (Johnson et al., 2001). Chl *b* of prochlorophytes, green algae and higher plants also has a C-7 formyl group, but its formation occurs by a different mechanism, since the activity of Chl *a* oxygenase is dependent upon the availability of oxygen (Tanaka et al., 1998).

BChl *e* biosynthesis also requires a C-20 methyltransferase activity similar to BChl *c* biosynthesis (Section VII.D). Polymerase chain reaction has been employed to amplify most of the coding sequence for a *bchU* ortholog from the BChl *e*-containing strain *Chl. phaeobacteroides* 1549, and sequence analysis confirms that this strain has a C-20 methyltransferase with strong sequence similarity to BchU of *Chl. tepidum* (Maresca et al., 2004; Section VII.D). Inactivation of the *bchU* paralog in a strain that synthesizes BChl *e* would presumably cause production of BChl *f*, the structure of which has been proposed (Smith, 2003) but has not been identified from natural sources.

X. Bacteriochlorophyll *c* Biosynthesis in Green Filamentous Bacteria

The photosynthetic green filamentous bacteria are the only organisms other than green sulfur bacteria that contain BChl *c* and chlorosomes, but these eubacteria are otherwise not closely related either phylogenetically or physiologically to the green sulfur bacteria (Pierson and Castenholz, 1995). *Cfx. aurantiacus* contains both BChl *a* and BChl *c* and while the BChl *a* is esterified with phytol as in green sulfur bacteria, the BChl *c* occurs exclusively as the [8-E,12-M] homolog and is predominantly esterified with stearol (Gloe and Risch, 1978; Bobe et al., 1990). Similar to green sulfur bacteria, *Cfx. aurantiacus* can vary its homolog composition through changes in the esterifying alcohol (Larsen et al., 1994) and can incorporate exogenously added long-chain alcohols into BChl *c* (Larsen et al., 1995). Both BChl *a* synthase (BchG) and BChl *c* synthase (BchK) from *Cfx. aurantiacus* have been overexpressed in *E. coli* and tested for substrate specificity (Schoch et al., 1999). It can be anticipated that BChl *c* biosynthesis in *Cfx. aurantiacus* will occur by the same pathway that is employed in *Chl. tepidum*.

Homologs of all genes discussed above as playing a role in BChl *c* biosynthesis in *Chl. tepidum* are found in the currently available genome sequence data of *Cfx. aurantiacus*, with the exception of *bchQ*, *bchR*, and *bchV* (DOE Joint Genome Institute, http://www.jgi.doe.gov). The absence of *bchQ* and *bchR* is expected, since *Cfx. aurantiacus* synthesizes only [8-E,12-M]-BChl *c* (Gloe and Risch, 1978; Bobe et al., 1990; Section VII.C). The absence of *bchV* may also be explained if the product of this gene alone is specifically involved in hydration of BChl *c* precursors that have increased methylation in the C-8 and C-12 positions (Section VII.E). The chirality of the C-3[1] carbon in BChl *c* in *Cfx. aurantiacus* is about 67% R and 33% S isomers (Fages et al., 1990). Although one BchF hydratase might produce this mixture of R and S stereoisomers, it is also possible that a *bchV* ortholog, which would encode an S-specific hydratase as suggested in Section VII.E, can not yet be identified since the genome data is incomplete. Alternatively, if BchF produces only one BChl *c* epimer, an unidentified epimerase could account for the observed mixture of epimers.

Cfx. aurantiacus can grow under both oxic and anoxic conditions. Consistent with this ability, the genome of this bacterium encodes some enzymes of tetrapyrrole biosynthesis as both the oxygen-independent and oxygen-dependent forms. Examples include the Mg-Proto monomethylester oxidative cyclase (anoxic, BchE; oxic, AcsF), coproporphyrinogen III oxidase (anoxic, HemN; oxic, HemF), and protoporphyrinogen IX oxidase (anoxic, HemY; oxic, HemK). *Cfx. aurantiacus* also contains three BchH paralogs: two BChl *a*-type (BchH-I and BchH-II) and one BChl *c*-type (BchS) (see Figs. 2b and 5). The latter is most closely related in sequence to the BchS and BchT proteins of *Chl. tepidum*, of which BchS appears to be specifically involved in BChl *c* biosynthesis under anoxic growth conditions (Section VII.A). BchH-I is probably used for BChl *a* biosynthesis under anoxic conditions and BchH-II for BChl *a* biosynthesis under micro-oxic conditions, since *bchH-II* clusters with *acsF*, which encodes a binuclear iron protein that catalyzes the oxygen-dependent, oxidative cyclization of Mg-Proto monomethylester in *Rubrivivax gelatinosus* (Pinta et al., 2002). Transcription in *Cfx. aurantiacus* is modulated by oxygen tension (Gruber and Bryant, 1998), and this gene arrangement could compensate for the oxygen-mediated repression of *bchH-I*.

Recent research suggests that the distinction between BChl *c* biosynthesis in green sulfur bacteria and green filamentous bacteria may not be so clearly delineated as described above. A recently characterized strain of green filamentous bacterium, a *Chloronema* sp., has chlorosomes that have characteristics

of both green filamentous bacteria (BChl *c* esterified with stearol and little redox-dependent fluorescence quenching) and green sulfur bacteria (C-8[2] and C-12[1] methylation, chlorobactene) (C. M. Borrego, personal communication).

XI. Future Directions

Although many new details of BChl *c* biosynthesis have been identified in the past two years, the precise order of all reaction steps is still not certain. Of critical importance will be a demonstration of when and how the removal of the C-13[2] carboxylate or methylcarboxylate moiety occurs. Clues to resolve this and other issues may be obtained from more careful analyses of the intermediates that accumulate in the various mutant strains that have been constructed. Now that most of the genes encoding enzymes of BChl *c* biosynthesis have been identified, heterologously expressed enzymes functioning in BChl *c* biosynthesis can be prepared and characterized with respect to substrate specificity and reaction mechanism. Interesting proteins include the three BchH homologs of magnesium chelatase from *Chl. tepidum*, the C-8[2] and C-12[1] methyltransferases BchQ and BchR, the C-20 methyltransferase BchU, the C-3[1] hydratases BchF and BchV, and the BChl *c* synthase BchK (the latter from both *Chl. tepidum* and *Cfx. aurantiacus*). Important questions to answer are whether Mg-Proto, when it is bound to Mg-chelatase, can be methylated by BchM, and if this methylation is specifically affected by binding to a different chelatase subunit (BchH, BchS, or BchT). It should also be possible to verify the biosynthetic pathway in *Cfx. aurantiacus* by complementing *Chl. tepidum* BChl *c* biosynthesis mutants with the appropriate genes. An interesting question here is whether the BchK of *Cfx. aurantiacus* would exhibit relaxed substrate specificity when expressed in the *Chl. tepidum bchK* mutant background. It should be possible to purify various substrates for in vitro characterization of specific enzymatic reactions from the appropriate mutant strains of *Chl. tepidum*.

Note added in Proof

Nagata et al. (2005) recently identified the [3,8-divinyl]-protochlorophyllide 8-vinyl reductase of higher plants and *Synechococcus* sp. WH8102, and surprisingly they identified a close homolog (*CT1063*) of these genes in the genome of *Chl. tepidum*. Inactivation of *CT1063* in *Chl. tepidum* leads to the accumulation of the 8-vinyl derivatives of BChl c_F (with methyl or predominantly ethyl substituents at the C-12 position), Chl $a_{\Delta 2,6}$, and BChl a_P (A. Gomez Maqueo Chew and D. A. Bryant, unpublished). A small amount of BChl a_P with an ethyl substituent at the C-8 position (i. e., normal BChl a_P) was also detected. These results indicate that the predominant 8-vinyl reductase activity in *Chl. tepidum* is the product of *CT1063* and that the *bchJ* gene (*CT2014*) in the genome is at most responsible for a weak, residual activity in the synthesis of BChl a_P. Efforts to inactivate the *bchJ* gene and to produce a *CT1063 bchJ* double mutant of *Chl. tepidum* are in progress. No homologs of *CT1063* have yet been identified in the incompletely sequenced genomes of *Chl. limicola* DSMZ 245, *Chl. phaeobacteroides* DSMZ 266, *Chl. phaeobacteroides* BS-1 (Black Sea Strain 1), and *Cfx. aurantiacus*. In contrast, proteins with strong sequence similarity to BchJ have been found in all eight green sulfur bacterial genomes that have been sequenced as well as in the incomplete genome of *Cfx. aurantiacus*. These results suggest that some green sulfur bacteria may use a BchJ-like reductase while others may use a plant-type (CT1063-like) 8-vinyl reductase. Recent progress in the understanding of (B)Chl biosynthesis in green bacteria has been reviewed in Frigaard and Bryant (2004).

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