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Review

Chlorobium tepidum: insights into the structure, physiology, and metabolism of a green sulfur bacterium derived from the complete genome sequence

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Abstract

Green sulfur bacteria are obligate, anaerobic photolithoautotrophs that synthesize unique bacteriochlorophylls (BChls) and a unique light-harvesting antenna structure, the chlorosome. One organism, *Chlorobium tepidum*, has emerged as a model for this group of bacteria primarily due to its relative ease of cultivation and natural transformability. This review focuses on insights into the physiology and biochemistry of the green sulfur bacteria that have been derived from the recently completed analysis of the 2.15-Mb genome of *Chl. tepidum*. About 40 mutants of *Chl. tepidum* have been generated within the last 3 years, most of which have been made based on analyses of the genome. This has allowed a nearly complete elucidation of the biosynthetic pathways for the carotenoids and BChls in *Chl. tepidum*, which include several novel enzymes specific for BChl *c* biosynthesis. Facilitating these analyses, both BChl *c* and carotenoid biosynthesis can be completely eliminated in *Chl. tepidum*. Based particularly on analyses of mutants lacking chlorosome proteins and BChl *c*, progress has also been made in understanding the structure and biogenesis of chlorosomes. *In silico* analyses of the presence and absence of genes encoding components involved in electron transfer reactions and carbon assimilation have additionally revealed some of the potential physiological capabilities, limitations, and peculiarities of *Chl. tepidum*. Surprisingly, some structural components and biosynthetic pathways associated with photosynthesis and energy metabolism in *Chl. tepidum* are more similar to those in cyanobacteria and plants than to those in other groups of photosynthetic bacteria.

Abbreviations: BChl – bacteriochlorophyll; BChlide – bacteriochlorophyllide; Chl – chlorophyll; Chlide – chlorophyllide

Introduction

The green sulfur bacteria form a distinctive group, the phylum *Chlorobi*, of photosynthetic bacteria of limited diversity (Overmann 2000; Garrity and Holt 2001b). All members of the *Chlorobi* are characterized by their ability to perform anoxygenic photosynthesis, in which the oxidation of inorganic sulfur compounds (sulfide, polysulfide, or thiosulfate) or H_2 is coupled to the production of strongly reducing, soluble ferredoxins by way of a type I (iron–sulfur-type) reaction center. Carbon dioxide is assimilated by the reverse tricarboxylic acid cycle. All known green sulfur bacteria possess light-harvesting antennae known as chlorosomes, which are large, sac-like structures filled with BChl *c, d* or *e*, and which are connected to reaction centers in the cytoplasmic membrane through the BChl *a*-containing Fenna–Matthews–Olson (FMO)

protein (see Figure 1A). No green sulfur bacterium has yet been shown to be capable of dark, heterotrophic growth, and all studied strains are believed to be rather simple in their nutritional requirements and metabolic capabilities. This biochemical simplicity is reflected in the small size of green sulfur bacterial genomes, which range from about 2 to 3 Mb (Méndez-Alvarez et al. 1995; Eisen et al. 2002) and which thus potentially encode between 2000 and 3000 proteins.

Although originally described more than a century ago, research on the green sulfur bacteria has lagged behind that for most other photosynthetic organisms. This situation has remained true in spite of the fact that this group of organisms is as widespread and as ecologically significant as the other major groups of photoautotrophs (Overmann and Garcia-Pichel 2000). Possible reasons for this neglect could be that: (1) they are considered to be more difficult to cultivate due to their obligately anaerobic nature; (2) many of their proteins are inactivated by exposure to oxygen, making protein purification more challenging (especially proteins containing iron-sulfur clusters, such as the reaction center complex (Scott et al. 1997); the CO_2-fixing enzymes pyruvate synthase and 2-oxoglutarate synthase (F.R. Tabita, personal communication); and ferredoxins (Yoon et al. 2001)); (3) methods for genetic manipulation of these organisms were not available until very recently (Wahlund and Madigan 1995; Chung et al. 1998; Frigaard and Bryant 2001); and (4) their obligately photoautotrophic nature limits the degree of genetic manipulation possible for the photosynthetic apparatus.

As part of an effort to increase our understanding of bacterial diversity in general, and knowledge and interest in the green sulfur bacteria specifically, The Institute for Genome Research (TIGR; Rockville, Maryland) recently sequenced and annotated the *Chl. tepidum* genome as part of a program supported by the U.S. Department of Energy – Energy Biosciences (Eisen et al. 2002). In a related effort, the Joint Genome Institute (JGI; Walnut Creek, California) is now planning to sequence the combined genomes of '*Chlorochromatium aggregatum*', a bacterial consortium composed of a green sulfur bacterial epibiont and a non-photosynthetic, motile β-proteobacterium. A draft genome sequence of the green filamentous bacterium *Chloroflexus aurantiacus* J-10-fl has also been produced by JGI-DOE. *Cfx. aurantiacus* is a member of the only other group of organisms, the phylum *Chloroflexi*, which synthesize BChl *c* and use chlorosomes as light-harvesting antennae (Garrity and Holt 2001a; Hanada and Pierson 2002). Sequence information can be obtained and analysis of these genomes can be performed on-line using the web-based analysis tools and data at the sites of TIGR (www.tigr.com), JGI-DOE (www.jgi.doe.gov), and the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov). Additionally, the *Chl. tepidum* genome has recently been posted at Cyanobase, the cyanobacterial database of the Kazusa DNA Research Institute (Kazusa, Japan; www.kazusa.or.jp/cyano/Chlorobium).

Identification of genes encoding components of the photosynthetic apparatus

Sequence similarity

A large number of genes in the *Chl. tepidum* genome were readily annotated based upon previously published data from green sulfur bacteria and on the basis of sequence similarity to known genes encoding components of the photosynthetic apparatus in other organisms (Eisen et al. 2002). These included enzymes functioning in biosynthesis of BChl *a,* carotenoids, and menaquinone; proteins involved in sulfur metabolism; structural proteins of the reaction center, FMO protein, and chlorosomes; electron-transport proteins; proteins involved in CO_2 fixation; and a number of regulatory and protein-processing proteins.

Gene duplication and targeted gene inactivation

Assuming that a particular protein does not participate in a process required for the viability of the organism, the role of the protein may be identified by insertional inactivation of the corresponding gene. The natural transformation procedure available for *Chl. tepidum* (see below) is currently being used to study the role of known and unknown genes that have been identified by various approaches.

A significant number of genes encoding biosynthetic functions related to photosynthesis are duplicated in *Chl. tepidum* (Eisen et al. 2002), and gene inactivation studies have been essential in making correct functional assignments for these paralogous gene products. For example, several paralogous sequences for enzymes known to participate in BChl *a* and carotenoid biosynthesis were identified in the genome. Through gene inactivation analyses it has been possible to assign most of these genes to specific steps

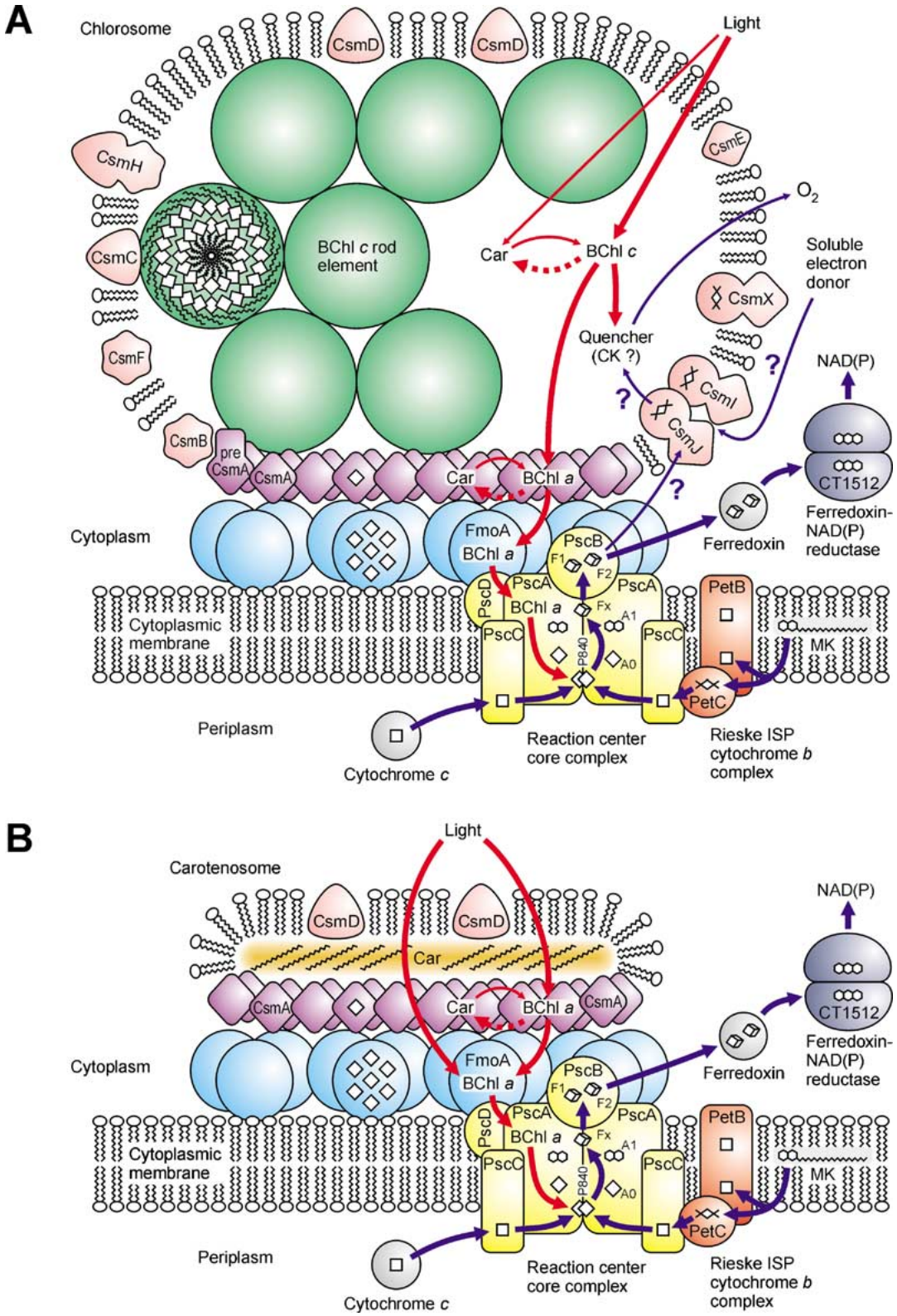

Figure 1. Model of the photosynthetic apparatus in (A) wild type *Chl. tepidum* and (B) a *bchK* mutant of *Chl. tepidum* which completely lacks BChl *c* and therefore only forms vestigial chlorosomes denoted carotenosomes (Frigaard et al. 2002). See text for details.

Table 1. Mutants of *Chl. tepidum* made by inactivation of a single locus

Gene	Locus	Gene product	Mutant phenotype
(Bacterio)chlorophyll biosynthesis			
bchH	*CT1957*	Mg chelatase subunit H	None detected
bchK	*CT1992*	BChl *c* synthase, (BchG paralog)	Contains no BChl *c* [a]
bchO	*CT1232*	Geranylgeraniol reductase II, (BchP paralog)	Contains modified BChl *a* and Chl *a* species
bchP	*CT2256*	Geranylgeraniol reductase	Contains modified BChl *a* and Chl *a* species
bchQ	*CT1777*	C-8^2 methyltransferase, (BchE paralog)	BChl *c* not methylated in the C-8^2 position
bchR	*CT1320*	C-12^1 methyltransferase, (BchE paralog)	BChl *c* not methylated in the C-12^1 position
bchS	*CT1955*	Mg chelatase subunit S, (BchH paralog)	BChl *c* content greatly reduced; decreased growth rate
bchT	*CT1295*	Mg chelatase subunit T, (BchH paralog)	BChl *c* content slightly reduced; decreased growth rate
bchU	*CT0028*	C-20 methyltransferase	Accumulates BChl *d*
bchV	*CT1776*	3-Vinyl hydratase, BChl *c*-specific (BchF paralog)	About 15% of BChl *c* has a 3-vinyl group
Carotenoid biosynthesis			
crtB	*CT1386*	Phytoene synthase	Carotenoids absent; decreased growth rate
crtC	*CT0301*	Carotene 1,2-hydratase	Contains no OH-carotene species
crtH	*CT0649*	Probable *cis-trans* isomerase	Accumulates lycopene and small amounts of other carotenoids
crtP	*CT0807*	Phytoene desaturase	Accumulates phytoene
crtQ	*CT1414*	ζ-Carotene desaturase	Accumulates ζ-carotene
crtU	*CT0323*	Chlorobactene synthase	Accumulates γ-carotene
Chlorosome proteins			
csmB	*CT2054*	Chlorosome envelope protein B	None detected
csmC	*CT1943*	Chlorosome envelope protein C	Blue-shifted BChl *c* absorption peak [b]
csmD	*CT2064*	Chlorosome envelope protein D	None detected
csmE	*CT2062*	Chlorosome envelope protein E	None detected
csmF	*CT1046*	Chlorosome envelope protein F	None detected
csmH	*CT1417*	Chlorosome envelope protein H	None detected
csmI	*CT1382*	Chlorosome envelope protein I	Altered chlorosome fluorescence quenching kinetics
csmJ	*CT0651*	Chlorosome envelope protein J	Altered chlorosome fluorescence quenching kinetics; slightly decreased growth rate
csmX	*CT0652*	Chlorosome envelope protein X	Altered chlorosome fluorescence quenching kinetics
Nitrogen fixation			
nifD	*CT1536*	Dinitrogenase subunit α	Incapable of diazotrophic growth [c]
Unknown function			
–	*CT0072*	BchE paralog	None detected
–	*CT0180*	CrtH homolog	None detected
–	*CT1502*	BchE paralog	None detected
–	*CT1697*	BchE paralog	None detected
–	*CT1763*	Tetrapyrrole methyltransferase paralog	None detected
–	*CT1771*	Oxidoreductase paralog	None detected [d]
–	*CT1772*	RuBisCO paralog	Defects in BChl *c*, sulfur, CO_2 metabolism; decreased growth rate [d]
–	*CT1903*	BchE paralog	None detected
–	*CT2010*	HemN paralog	None detected

[a] Frigaard et al. (2002).
[b] Chung et al. (1998).
[c] Frigaard and Bryant (2001).
[d] Hanson and Tabita (2001).

in carotenoid biosynthesis or to novel biosynthetic reactions in the biosynthetic pathway for BChl *c* (see below, Table 1 and Figure 3; Frigaard et al. 2003a).

Gene clustering

Another approach to identify novel genes has been to search for possible clustering of genes that could be involved in photosynthesis. In purple bacteria and other photosynthetic proteobacteria (Alberti et al. 1995; Igarashi et al. 2001; Béjà et al. 2002) and *Heliobacillus mobilis* (Xiong et al. 1998), most genes required for photosynthesis are clustered in regions of approximately 30–50 kb. These 'photosynthetic gene clusters' include the genes encoding antenna-complex subunits and reaction center polypeptides, the enzymes required for the biosynthesis of carotenoids and chlorophylls, and some electron transfer components.

Table 2. Mutants of *Chl. tepidum* made by inactivation of more than one loci

Genes	Mutant phenotype
(Bacterio)chlorophyll biosynthesis	
bchQ bchR	BChl *c* not methylated in the C-8^2 and C-12^1 positions
Chlorosome proteins	
csmD csmE	None detected
csmI csmJ	Altered chlorosome fluorescence quenching kinetics
csmI csmX	Altered chlorosome fluorescence quenching kinetics
csmJ csmX	Altered chlorosome fluorescence quenching kinetics
csmI csmJ csmX	Altered chlorosome fluorescence quenching kinetics

Unfortunately, the *Chl. tepidum* genome contains very few large operons for photosynthesis-related genes, and those operons that do exist are highly dispersed throughout the genome. However, it has been very useful to examine and compare carefully the context of genes known to be involved in photosynthetic functions in *Cfx. aurantiacus* with the arrangement of both orthologous and paralogous genes found in *Chl. tepidum* and vice versa. As noted above, *Cfx. aurantiacus* is the only other organism, for which genome sequence information is available, which also produces chlorosomes and BChl *c*. However, in contrast to the constitutive production of these cellular components in *Chl. tepidum*, BChl *c* biosynthesis and chlorosome biogenesis are induced upon transfer of *Cfx. aurantiacus* cells from oxic to anoxic conditions (Oelze and Golecki 1995). Thus, *Cfx. aurantiacus* might be expected to exhibit a higher degree of clustering of photosynthesis-related genes than *Chl. tepidum* in order to facilitate the coordination of gene expression under the anoxic growth conditions.

For example, one cluster of chlorosome-related genes in *Cfx. aurantiacus* encodes two chlorosome envelope proteins (CsmM and CsmN), BChl *c* synthase (BchK), and a methyltransferase (BchU) (Frigaard et al. 2003a). The position of a methyltransferase-encoding gene immediately upstream of *bchK* suggested to us that it might be involved in BChl *c* biosynthesis. Subsequent inactivation of the paralogous gene (*CT0028*) in *Chl. tepidum* showed that this gene, denoted *bchU*, indeed encodes a BChl *c*-specific C-20 methyltransferase (see below; Table 1 and Figure 3; J.A. Maresca, A. Gomez Maqueo Chew, N.-U. Frigaard, A.D. Jones, M. Ros Ponsatí, J.G. Ormerod, and D.A. Bryant, manuscript in preparation). The constitutively expressed *bchU* gene in *Chl. tepidum* does not cluster with any other genes encoding products that are obviously related to photosynthesis.

Another example involves CsmA, the BChl *a*-binding protein in chlorosomes (Figures 1A and B; Sakuragi et al. 1999; Montaño et al. 2001b; Bryant et al. 2002). Although the amino acid sequences of CsmA of *Chl. tepidum* and *Cfx. aurantiacus* have only low similarity, a similar gene arrangement is found surrounding the *csmA* genes of the two organisms (Frigaard et al. 2001). This conserved gene organization suggests that the products of these flanking genes probably play some role in chlorosome biogenesis (see below).

Proteomics

Another approach to identify genes that encode proteins involved in photosynthetic processes in *Chl. tepidum* is to search for novel proteins through proteomics approaches. The corresponding genes may then be identified from the genome sequence by determining the N-terminal sequence of the protein or by using mass spectroscopic sequencing and identification methods. For example, two proteins associated with the chlorosome envelope in a BChl *c*-less mutant were recently identified by N-terminal amino acid sequencing as the products of genes *CT0104* and *CT0105*, which may be involved in chlorosome biogenesis (see below).

Comparative genomics

The final approach that can be used to identify new photosynthesis genes is to perform comparative genome analyses (phylogenomics) to identify those genes that are only found in photosynthetic organisms. Thirty-eight such genes, encoding conserved hypothetic proteins with unknown functions, were originally identified in *Chl. tepidum* (Eisen et al. 2002).

Novel genes related to chlorosomes and BChl *c* biosynthesis may also be found by similar comparisons between the *Chl. tepidum* and *Cfx. aurantiacus* genomes. For example, a search of the *Cfx. aurantiacus* genome for proteins with similarity to the chlorosomal proteins CsmI, CsmJ, and CsmX in *Chl. tepidum* showed the presence of one gene encoding a protein with similarity to these three [2Fe–2S] proteins. N-terminal sequence analysis of proteins in a chlorosome fraction from *Cfx. aurantiacus* showed that the identified protein, now denoted CsmY, is a minor component of the chlorosome fraction (Frigaard et al. 2001). The CsmO protein, that has sequence similarity to both CsmB and CsmF of *Chl. tepidum*, was similarly identified in the *Cfx. aurantiacus* genome and chlorosomes (Frigaard et al. 2001; Vassilieva et al. 2001) as suggested by Lehmann et al. (1994).

Genes not present

It is also interesting to note genes that are (potentially) missing in the *Chl. tepidum* genome. For example, consistent with previous biochemical characterizations (Schütz et al. 2000; Hauska et al. 2001) and in contrast to the situation in other organisms, there appears to be no gene in the *Chl. tepidum* genome encoding a homolog of the cytochrome c_1 subunit of the quinol-oxidizing, cytochrome bc_1 complex. However, one must be aware of the shortcomings of searching based on sequence similarity. Sequence similarity searches of the genome suggested that *Chl. tepidum* does not contain a ferredoxin: $NAD(P)^+$ oxidoreductase (FNR), which in cyanobacteria and plants is important for generating NADPH from reduced ferredoxin. Nevertheless, recent biochemical characterization of cell extracts has identified a homodimeric 90-kDa protein (encoded by *CT1512*) that exhibits FNR activity but which has higher amino acid sequence similarity with thioredoxin reductases than with other known FNRs (Seo and Sakurai 2002).

Genetic transformation of green sulfur bacteria

To determine the identity of a proposed gene and verify its function, it is extremely useful to be able to inactivate the gene in the organism under investigation. This allows one to establish the phenotype of a null mutant lacking the product of the gene in question. This approach has been used to identify several novel genes involved in photosynthesis in *Chl. tepidum* as discussed in subsequent sections. A protocol for natural transformation has been established and optimized (Frigaard and Bryant 2001). Exogenous DNA is readily taken up by *Chl. tepidum* cells and through homologous recombination with the chromosomal DNA, targeted gene interruptions can readily be produced. Mutagenic recombinant DNA constructs can be made and amplified by *in vivo* methods (i.e., plasmids produced in *Escherichia coli* cells) or by strictly *in vitro*, PCR-based techniques (Frigaard et al. 2003b). The combined length of the flanking regions of homologous DNA required to direct the double homologous recombination events need not be greater than about 200 bp. However, additional (homologous or non-homologous) flanking DNA is necessary in practice, possibly because some of the flanking DNA is digested upon uptake. At least 500 bp of flanking DNA on both sides of the selection marker should be used in such experiments. The mesophilic *Chl. vibrioforme* strain 8327D is also naturally transformable, although the antibiotics and resistance markers that can be used are somewhat different from those for *Chl. tepidum* (Chung et al. 1998; Frigaard and Bryant 2001). Preliminary tests suggest that the brown-colored, BChl *e*-containing *Chl. phaeobacteroides* strain 1549 is not naturally transformable (M. Ros Ponsatí, N.-U. Frigaard, D.A. Bryant, unpublished data).

Chlorosome structure and biogenesis

The green sulfur bacteria are exquisitely adapted for growth at low light intensities. A green sulfur bacterium has been found growing at a depth of 100 m in the Black Sea, where the light intensity is only 0.0026 μmol photons m^{-2} s^{-1} (Overmann et al. 1992; J. Overmann, pers. comm.). Additionally, a green sulfur bacterium has recently been recovered from 2200 m below the surface of the Pacific Ocean, where it is believed to survive phototrophically on the black body radiation emitted by a black smoker (J.T. Beatty, R.E. Blankenship and J. Overmann, pers. commun.; see http://www.space.com/scienceastronomy/astrobio_extreme_030505.html). It is obvious that organisms that can grow under such low-light conditions must have very large and highly efficient light-harvesting antenna structures. Green sulfur bacteria indeed possess a remarkable and unique antenna structure, the chlorosome (Figure 1A; for reviews see Blankenship et al. 1995; Olson 1998; Vassilieva et al. 2000;

Frigaard et al. 2001; Blankenship and Matsuura 2003). As estimated from electron micrographs, an average *Chl. tepidum* cell probably contains about 200–250 chlorosomes. A typical chlorosome from *Chl. tepidum* is roughly 150 nm long, 50–60 nm wide, and 30 nm high (Wahlund et al. 1991; Bryant et al. 2002; Martinez-Planells et al. 2002) and contains about 200 000 BChl *c* molecules (Montaño et al. 2001a; Martinez-Planells et al. 2002). About 1% of the BChl in chlorosomes is BChl *a* (Francke and Amesz 1997; Bryant et al. 2002; N.-U. Frigaard and D.A. Bryant, unpublished data) which means that each chlorosome also contains about 2000 BChl *a* molecules. Each BChl *a* molecule is most likely bound to one CsmA protein molecule (Sakuragi et al. 1999; Montaño et al. 2001b; Bryant et al. 2002; N.-U. Frigaard, H. Li, K.J. Milks, and D.A. Bryant, manuscript in preparation). CsmA is the most abundant chlorosome protein and probably forms an oligomeric, paracrystalline CsmA–BChl *a* complex, known as the chlorosome baseplate, which transfers excitation energy out of the chlorosome to the FMO protein (Bryant et al. 2002). About 3% of the total (B)Chl in *Chl. tepidum* cells is BChl *a* (Frigaard et al. 1997; Borrego et al. 1999; Frigaard et al. 2002), which means that there are about 4000 extra-chlorosomal BChl *a* molecules per chlorosome. Most of this BChl *a* is contained in the trimeric FMO protein, and thus there are about 150–200 FMO trimers per chlorosome. When one considers the dimensions of the FMO trimer (Li et al. 1997; Rémigy et al. 1999), this corresponds roughly to the number that can fit under the chlorosome baseplate. Each chlorosome is probably associated with 25–40 reaction centers around the edge of the chlorosome. There are therefore roughly 4–8 FMO trimers per reaction center, of which only two FMO trimers bind tightly to one reaction center (Rémigy et al. 1999). Based upon these numbers, each reaction center on average receives excitation energy from roughly 5000 to 8000 antenna molecules (BChl *c*). This is the largest antenna pigment to reaction center ratio known among photosynthetic organisms. Antenna sizes for most other organisms are in the range of 50 to 300 pigment molecules per reaction center (Clayton 1980).

Chlorosomes differ from all other photosynthetic antennae in the organization of their BChl *c* molecules, which are not organized predominantly by interactions with protein molecules but rather are assembled through pigment-pigment interactions between neighboring BChl *c* molecules (Blankenship et al. 1995; Olson 1998; Blankenship and Matsuura 2003). It is possible that the green sulfur bacteria evolved this type of antenna structure because of powerful selection pressures associated with growth under very low light-intensity conditions, under which light energy, and not nutrients, is limiting for growth. The protein:pigment ratio of *Chl. tepidum* chlorosomes is approximately two amino acids per BChl (Vassilieva et al. 2002). The much higher protein:pigment ratios of plant antenna protein complexes (∼ 15 amino acids per Chl) or phycobiliproteins in cyanobacteria (60–160 amino acids per bilin) require a much greater energy expenditure per chromophore. Given the ATP cost of synthesizing peptide bonds as well as the typical protein content (∼55% of the dry weight) of typical bacterial cells like *E. coli*, a protein-based antenna system with the same number of chromophores would energetically bankrupt the green sulfur bacteria which contain such large antennae pigment numbers and which grow under such extraordinarily low light intensities.

Essentially nothing is known at present about how chlorosomes are assembled, except that their presence is constitutive in *Chl. tepidum* and inducible by anoxic growth conditions in *Cfx. aurantiacus*. Future research should be directed at obtaining information on the biogenesis of chlorosomes. The observation that the gene encoding BChl *c* synthase (*bchK*) in *Chl. tepidum* can be inactivated, and that BChl *c* thus is not essential for phototrophic growth of *Chl. tepidum* (Frigaard et al. 2002), implies that other genes specific for chlorosome formation may also be identified by gene inactivation.

Chlorosome proteins

Ten unique chlorosome proteins (CsmA, CsmB, CsmC, CsmD, CsmE, CsmF, CsmH, CsmI, CsmJ, CsmX), all of which are located in the protein-lipid envelope of the chlorosome, have been identified in *Chl. tepidum* (Chung et al. 1994; Chung and Bryant 1996a, b; Vassilieva et al. 2002). The genes encoding nine of these proteins were cloned and sequenced by traditional methods (Chung et al. 1994; Chung and Bryant 1996a, b; Vassilieva et al. 2002). CsmX had initially eluded detection in chlorosome preparations due to its very low abundance. However, analysis of the completed genome sequence revealed a gene, denoted *csmX*, immediately upstream from the paralogous *csmJ*. The *csmX* gene was predicted to encode a third [2Fe–2S] ferredoxin with high sequence similarity to CsmI and CsmJ (Vassilieva et al. 2001). It was subsequently confirmed by immunoblotting that

CsmX copurifies with other chlorosome proteins and thus truly is a chlorosome protein (Vassilieva et al. 2002). It has also been confirmed that CsmX contains a [2Fe–2S] cluster since EPR studies show a signal consistent with the presence of such a cluster in chlorosomes isolated from a *csmI csmJ* double mutant but which is absent in chlorosomes from a *csmI csmJ csmX* triple mutant (T.W. Johnson, H. Li, N.-U. Frigaard, J.H. Golbeck, and D.A. Bryant, unpublished data).

The genes encoding nine of the ten chlorosome proteins have been inactivated with surprisingly little phenotypic effect (Tables 1 and 2; N.-U. Frigaard, H. Li, K. J. Milks, and D.A. Bryant, manuscript in preparation). All nine *csm* mutant strains have growth rates similar to those of the wild type, and all of the mutants form functional chlorosomes which are similar to, or indistinguishable from, those found in the wild type with respect to absorption properties, and content of BChl *c*, BChl *a*, carotenoids, and isoprenoid quinones. In addition, chlorosomes from each mutant strain are missing only the protein whose corresponding gene is inactivated. These results establish that none of the known chlorosome proteins, with the possible exception of CsmA, are absolutely necessary for the biogenesis or light-energy-harvesting functions of chlorosomes. Similarly, none of these nine chlorosome proteins is apparently required for the normal assembly and organization of BChl *c* and BChl *a* within chlorosomes. These observations, as well as others, provide further strong evidence that the BChl *a* found in chlorosomes is associated with CsmA (Sakuragi et al. 1999; Montaño et al. 2001b; Bryant et al. 2002). Still unidentified proteins must therefore be responsible for the biogenesis of chlorosomes, and these are one of the targets of our current research.

Approaches to understand chlorosome biogenesis

A comparison of the genome sequences of *Chl. tepidum* and *Cfx. aurantiacus* reveals that the gene organizations surrounding their *csmA* genes share some striking similarities, which suggest that these flanking genes might be involved in chlorosome biogenesis (Chung et al. 1994; Frigaard et al. 2001). The *csmCA* locus in *Chl. tepidum* is flanked immediately upstream by an *arsA* homolog, *CT1945*, that was originally denoted ORFZ (Chung et al. 1994). A gene with strong sequence similarity to *arsA* and *CT1945*/ORFZ is similarly found upstream from the *csmA* locus in *Cfx. aurantiacus*. ArsA is an extrinsic component of a membrane-bound, ATP-dependent arsenite exporter (Zhou et al. 2000). Immediately downstream of *csmA* in *Chl. tepidum* is a gene, *CT1940*, that encodes a conserved, hypothetical protein originally denoted ORFX (Chung et al. 1994). In *Cfx. aurantiacus,* two *CT1940* homologs denoted ORFX and ORFX2 are also found downstream from the *csmA* gene (Frigaard et al. 2001). Finally, downstream from *CT1940*/ORFX in *Chl. tepidum* and from ORFX2 in *Cfx. aurantiacus,* additional homologs of *arsA* (*CT1939*/ORFZ2 in *Chl. tepidum* and ORFZ2 and ORFZ3 in *Cfx. aurantiacus*) are found (Frigaard et al. 2001). It is not known what functions the products of these genes might have in chlorosome formation nor where these proteins might be localized. Although they do not appear to be components of mature chlorosomes, these proteins could be involved in chlorosome biogenesis or the biosynthesis of a chlorosome component. Attempts to inactivate *CT1940*/ORFX and *CT1945*/ORFZ in *Chl. tepidum* have so far been unsuccessful. Both *Chl. tepidum* and *Cfx. aurantiacus* contain multiple other *arsA* homologs, whose genes are located elsewhere in these genomes (Frigaard et al. 2001; Eisen et al. 2002).

The *bchK* gene, encoding BChl *c* synthase, was insertionally inactivated recently, and the resulting *Chl. tepidum* mutant is unable to synthesize BChl *c* (Frigaard et al. 2002). Two proteins, ferritin (CT1740) and a small heat-shock protein (CT0644), are overproduced in cells of the *Chl. tepidum bchK* mutant (N.-U. Frigaard, J. Zhao, and D. A. Bryant, unpublished data). Although normal chlorosomes are not formed in this BChl *c*-less mutant, vestigial chlorosomes, denoted 'carotenosomes', can be isolated (Frigaard et al. 2002; N.-U. Frigaard and D.A. Bryant, unpublished data). Transmission-electron and atomic-force microscopic images show that carotenosomes have a shape resembling elongated pancakes; they have a width (25–60 nm) similar to chlorosomes; however, carotenosomes are shorter (50–70 nm) and have a thickness of only 3–6 nm (P. Martinsson, K.J. Milks, A. Stamouli, N.-U. Frigaard, D.A. Bryant, and T. Aartsma, unpublished data). Carotenosomes contain levels of carotenoids, BChl *a*, CsmA, and CsmD that are similar to those in wild-type chlorosomes. The BChl *a* has an absorption maximum at 798 nm, which probably results from an intact baseplate structure. Carotenosomes additionally contain small amounts of CsmB, CsmE, CsmF, and CsmI, whereas CsmC, CsmH, CsmJ, and CsmX are not found in detectable amounts. Figure 1B shows a model that depicts how carotenosomes may be organized in the *bchK* mutant cells. Considering the thickness of the carotenosomes and

that the CsmA baseplate is probably intact, we conclude that CsmD is probably located predominantly in that part of the envelope facing the cytoplasm (see Figure 1B).

It is possible that the carotenosomes in the *bchK* mutant mimic a situation in which the normal formation of chlorosomes has been blocked or slowed by the absence of BChl *c*. Thus, carotenosomes might retain components involved in chlorosome assembly that would normally detach after chlorosome maturation is completed in the wild type. Alternatively, some protein components involved in chlorosome biogenesis could exhibit altered expression levels in response to the absence of BChl *c* and/or mature chlorosomes. A comprehensive investigation of changes in the proteome of the *bchK* mutant and characterization of carotenosomes might thus be expected to provide new information concerning chlorosome biogenesis.

Biochemical characterization of carotenosomes has revealed the presence of small amounts of several proteins larger than 30 kDa not previously reported to occur in chlorosomes (N.-U. Frigaard, J. Zhao, and D.A. Bryant, unpublished data). Two of the minor large proteins in carotenosomes were identified as CT0104 and CT0105 by N-terminal sequencing. CT0104 may also be present in small amounts in preparations of chlorosomes from the wild type but CT0105 is not. CT0104 and CT0105 are homologs of EmrB and EmrA, respectively. The periplasmic EmrA and the cytoplasmic membrane-embedded EmrB are subunits of a transporter complex in *E. coli* that extrudes hydrophobic, xenobiotic compounds from the cytoplasm (Figure 2A; Lewis 2000). CT0104 and CT0105 could analogously form a complex that translocates the hydrophobic components of the chlorosomes (BChl *c*, carotenoids, and isoprenoid quinones) from the cytoplasmic membrane, in which they most likely are synthesized, to the chlorosome interior (Figure 2B).

In *E. coli* the EmrA-EmrB complex functions in conjunction with the universal channel-tunnel ('chunnel') protein TolC, which is anchored in the outer membrane by a β-barrel formed by the trimerization of TolC (Figure 2A; Lewis 2000; Andersen et al. 2001). We hypothesize that the chlorosome envelope in the cytoplasm, which has a galactolipid monolayer or asymmetric bilayer structure, could be analogous to the asymmetric bilayer that forms the outer membrane in Gram-negative bacteria. For the chlorosome transporter hypothesis to be valid, however, the *Chl. tepidum* homologs of EmrA and TolC should lack

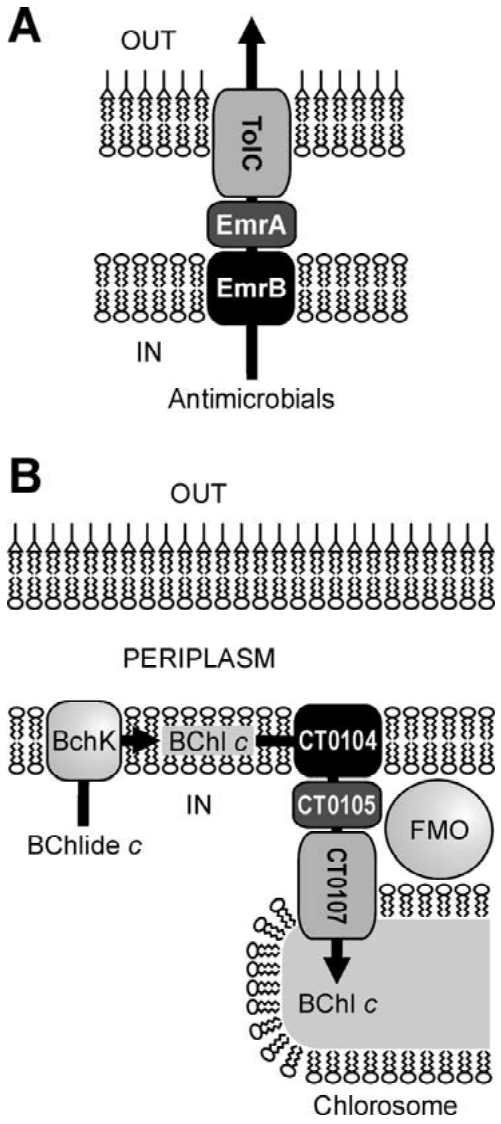

Figure 2. (A) Model of the EmrA-EmrB-TolC drug transporter complex in *E. coli*. (B) Model of the proposed transporter complex in *Chl. tepidum* that might transport BChl *c* (or other chlorosome components) from the cytoplasmic membrane into the chlorosome. See text for details.

signal peptides, since the proteins should be targeted to the cytoplasm rather than the periplasmic space. The *Chl. tepidum* genome encodes four homologs of *tolC*, one of which, *CT0107*, is encoded in an apparent operon, *CT0104–CT0105–CT0106–CT0107*, and predicts a 47-kDa protein. Analysis of the CT0107 sequence indicates that it does not have a signal sequence, in contrast to the three other *Chl. tepidum* TolC homologs (CT0758, CT1347, and CT2049) that do have signal peptides. The absence of a signal sequence is consistent with the hypothesis that CT0107

would not be translocated to the periplasm but would remain in the cytoplasm and form a channel-tunnel bridging the cytoplasmic membrane and the chlorosome envelope (Figure 2B). Although *E. coli* EmrA has a signal sequence which is cleaved during translocation to the periplasm, CT0105 does not appear to have a signal sequence and thus should remain in the cytoplasm as well. Further studies will be required to determine whether CT0107 can be detected in carotenosomes and/or chlorosomes preparations. The *CT0106* gene, found between *CT0105* and *CT0107* encodes an ATP-binding protein that is related to universal stress proteins and that might also form part of the hypothetical transporter complex. One additional observation suggests that the CT0104, CT0105, and CT0107 proteins are involved in chlorosome biogenesis. A homolog of *CT0104* occurs immediately downstream from *bchS* in *Cfx. aurantiacus*. BchS is a subunit of a protoporphyrin IX magnesium chelatase and uniquely affects BChl *c* synthesis when mutated in *Chl. tepidum* (see below; Table 1). As noted above, clustering of BChl *c* and chlorosome-related genes is somewhat more pronounced in *Cfx. aurantiacus*.

Bacteriochlorophyll and chlorophyll biosynthesis

Chl. tepidum synthesizes three types of chlorophyll: BChl *c* mainly esterified with farnesol (BChl c_F), BChl *a* esterified with phytol (BChl a_P), and Chl *a* esterified with Δ2,6-phytadienol (Chl a_{PD}). *Chl. tepidum* cells contain roughly 30 times more BChl *c* than BChl *a*, and roughly 10 times more BChl *a* than Chl a_{PD} (Frigaard et al. 2001). Since the genome sequence became available, the strategy for elucidating the biosynthetic pathways of these compounds has been a combination of (1) identifying homologs of genes known to be involved in BChl *a* and Chl *a* biosynthesis in other organisms and (2) identifying potential genes with novel functions by comparative genomics by inspection of the clustering of genes in both *Chl. tepidum* and *Cfx. aurantiacus* (Frigaard et al. 2003a). Apparent orthologs exist for all of the genes known to be required for oxygen-independent BChl *a* biosynthesis in *Rhodobacter* species (Eisen et al. 2002). Indeed, a few of these genes in *Chl. tepidum* (*bchF*, *bchG*, *bchM*) have been shown by Bauer and coworkers to complement *R. capsulatus* strains defective in specific steps of BChl *a* biosynthesis (Xiong et al. 2000). However, the *Chl. tepidum* genome also contain several paralogous genes for (B)Chl biosynthesis: three genes encoding (B)Chl synthases, seven *bchE* paralogs, three *bchH* paralogs, two *bchF* paralogs, two *bchP* paralogs, and two *hemN*-like genes. On the basis of these observations, a highly speculative pathway for (B)Chl biosynthesis was proposed (Eisen et al. 2002). Although it was plausible, the originally predicted pathway for BChl *c* biosynthesis was unfortunately almost entirely wrong! Fortunately, the availability of a transformation system allowed predictions to be tested experimentally. Prior to the identification of *bchK,* which encodes the BChl *c* synthase (Frigaard et al. 2002), no gene or enzyme specific for BChl *c* biosynthesis had been identified. A very successful utilization of the *Chl. tepidum* genome sequence has been the recent identification of at least nine new genes encoding enzymes involved in the biosynthesis of BChl *a* and BChl *c*, of which six are specific for BChl *c* biosynthesis (Table 1 and Figure 3; Frigaard et al. 2003a). This has also allowed general predictions to be made about the BChl *c* and BChl *a* biosynthesis in *Cfx. aurantiacus* (Frigaard et al. 2003a).

Figure 3 shows our current working model for the pathways for (B)Chl biosynthesis in *Chl. tepidum*. Although most of the BChl *c*-specific enzymes have now been identified, it is not yet clear how a key reaction in BChl *c* biosynthesis, removal of the C-13^2 carboxyl moiety, occurs. Two alternative pathways, which both lead to the proposed intermediate 3-vinyl BChlide *d* that lacks the C-13^2 carboxyl moiety, are shown in Figure 3. In one pathway, chlorophyllide (Chlide) *a*, is the last common intermediate in the biosynthesis of all three (B)Chls. Two unidentified enzymes would likely be required to convert Chlide *a* into 3-vinyl BChlide *d*: an esterase to hydrolyze the methyl ester on the C-13^2 carboxymethyl group and a decarboxylase to remove the C-13^2 carboxyl group. Interestingly, these proposed reactions have some similarities to reactions associated with Chl degradation in some algal and higher plant species (see Frigaard et al. 2003a for discussion). In another possible pathway, the magnesium chelatase containing the BchS subunit might channel its product (Mg Proto IX) directly to BchE and therefore bypass the methylation of the C-13^2 carboxyl group by BchM (see Figure 3). The subsequent reaction catalyzed by BchE might then cause decarboxylation of the unmethylated C-13^2 carboxyl group during the oxidative reactions leading to the formation of the isocyclic ring. Subsequent modifications by BchJ and BchNBL lead to 3-vinyl BChlide *d*. Current research is directed toward elucidating the actual pathway by investigating the effects of single and

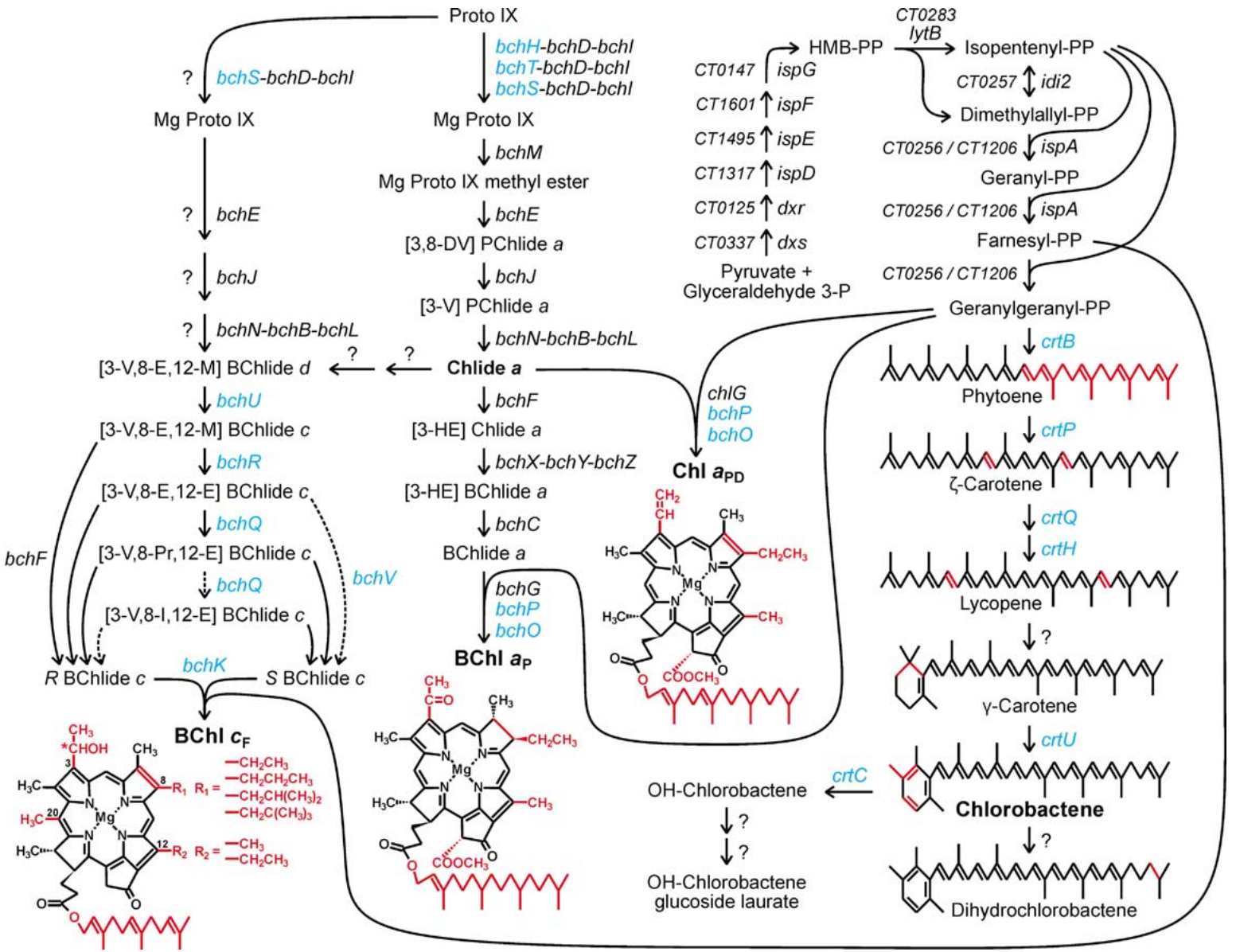

Figure 3. Biosynthetic pathways for BChl c_F, BChl a_P, Chl a_{PD} and carotenoids in *Chl. tepidum*. All carotenoid structures are shown in all-*trans* configuration although they may also appear in *cis* configurations *in vivo*. Genes that have been inactivated appear in blue. See text for details.

multiple gene inactivations and *in vitro* assays with recombinant enzymes (see below).

BChlide c *esterification*

The BChl *c* synthase (BchK) was identified by inactivation of one of the three homologs of (B)Chl synthases found in the genome (Table 1; Frigaard et al. 2002). The *bchK* mutant of *Chl. tepidum* is rusty-orange in color, grows about seven times slower than the wild type under limiting light, completely lacks BChl *c*, and forms vestigial chlorosomes denoted carotenosomes (see above). The *bchK* mutant should prove valuable for studies of the BChl *a* antennae and reaction centers in *Chl. tepidum*, which normally are optically masked in the wild type because of the strong BChl *c* absorption.

C-8^2 *and C-12*^{1} *methylation*

BChl *c*, *d*, and *e* in green sulfur bacteria differ from all other types of Chls and BChls by being variably methylated at the C-8^2 and C-12^1 positions (Senge and Smith 1995). As potential candidates responsible for these methylations, several BchE homologs were identified in the genome (Eisen et al. 2002). These homologs are members of a protein superfamily that includes P-methyltransferases, C-methyltransferases, and oxidative cyclases such as BchE (Gough et al. 2000). The C-methyltransferases are capable of adding a methyl group to an unactivated methyl or methylene carbon. They contain the motifs for binding vitamin B_{12} and an unusual [4Fe–4S] cluster, and they are postulated to proceed by the formation of an adenosyl radical (radical-SAM enzymes) as an obligate intermediate in the reaction mechanism. The ultimate methyl group donor is believed to be methyl-cobalamin, with that methyl group ultimately being derived from a second molecule of *S*-adenosyl-methionine. Six *bchE* homologs have been inactivated in *Chl. tepidum* (Table 1), of which one (*CT1777*) has been shown to encode the enzyme responsible for the C-8^2 methylation (now designated *bchQ*) and another (*CT1320*) to encode the enzyme responsible for the C-12^1 methylation (now designated *bchR*) (Frigaard et al. 2003a; A. Gomez Maqueo Chew, N.-U. Frigaard, A.D. Jones, and D.A. Bryant, manuscript in preparation). Inactivation of the other four *bchE* homologs produced no discernable phenotypic change. Inactivation of either the *bchQ* or the *bchR* gene seemingly has little effect on methylation by the other enzyme; this suggests that these two methylation events can take place independently and that the methylations reactions do not obligatorily occur in a specific order. Growth experiments show that cell cultures of the *bchR* mutant have a BChl *c* absorption peak similar to wild type cultures (around 750 nm) whereas cultures of the *bchQ* mutant have a BChl *c* absorption peak which is blue-shifted about 15 nm. In addition, in cultures of both the *bchR* and *bchQ* mutant, the bandwidth of the BChl *c* Q_y absorption peak is narrower than in wild type cultures. Growth rate measurements have also shown that at low light intensities, both the *bchR* and *bchQ* mutant grow somewhat slower than the wild type. From these results, it seems that the degree of BChl *c* methylation at C-8^2 and C-12^1 can be used by the cells to fine-tune the absorption properties of the BChl *c* in the chlorosomes to improve light-harvesting at low light intensities.

C-20 methylation

BChl *c* and BChl *e* in green bacteria differ from all other types of Chls and BChls by being methylated in the C-20 position (Senge and Smith 1995). The C-20 methyltransferase (now denoted BchU) is expected to belong to a different class of methyltransferases than the C-8^2 and C-12^1 methyltransferases, since it carries out the direct methylation of an aromatic carbon. Inactivation of a candidate gene (*CT1763*) selected on the basis of sequence homology with uroporphyrin-III C-methyltransferases proved futile. The C-20 methyltransferase was eventually identified by comparative genomics. A methyltransferase, originally identified as CrtF, a carotenoid *O*-methyltransferase, was observed immediately upstream from the *bchK* gene of *Cfx. aurantiacus* (Frigaard et al. 2003a). This same gene cluster includes other genes required for chlorosome biogenesis (*csmM*, *csmN*, and *csmP*), and it thus seemed possible that the product of this gene might play a role in BChl *c* biosynthesis. Supporting this hypothesis, *O*-methylation of carotenoids is not known to occur in either *Cfx. aurantiacus* or *Chl. tepidum* (Takaichi et al. 1995, 1997). Subsequent inactivation of the orthologous *bchU* gene in *Chl. tepidum*, produced a BChl *d*-containing mutant (Frigaard et al. 2003a; J.A. Maresca, A. Gomez Maqueo Chew, N.-U. Frigaard, A.D. Jones, M. Ros Ponsatí, J. G. Ormerod, and D.A. Bryant, manuscript in preparation). Unlike *bchU* in *Cfx. aurantiacus*, *bchU* in *Chl. tepidum* does not cluster with any other genes obviously related to photosynthesis. *Chl. vibrioforme* 8327D, which nat-

urally produces BChl *d*, can spontaneously revert to BChl *c* production when grown under low-light selection (Broch-Due and Ormerod 1978; Bobe et al. 1990). We have shown that the *bchU* gene of the 8327D strain has a one basepair insertion which causes a frameshift mutation that causes premature translation termination at an in-frame nonsense codon (J.A. Maresca, A. Gomez Maqueo Chew, N.-U. Frigaard, A.D. Jones, M. Ros Ponsatí, J.G. Ormerod, and D.A. Bryant, manuscript in preparation). The same gene from a spontaneous, BChl *c*-producing suppressor strain, *Chl. vibrioforme* 8327C, has the reading frame of *bchU* restored by a compensatory one basepair deletion. Experiments that compared the growth rates of the BChl *c*-containing strain with the BChl *d*-containing strain of both *Chl. vibrioforme* 8327 and *Chl. tepidum* have shown that at high light intensities the BChl *c* and BChl *d* strains grow with similar rates, whereas at low light intensities the BChl *c*-containing strain grows faster than the BChl *d*-containing strain of the same organism.

C-3^1 *hydration*

The C-3^1 carbon of BChl *c* is a chiral center that can exist in *R*- or *S*-stereochemistry (Figure 3). In green sulfur bacteria, BChl *c* homologs with low degrees of methylation at C-8^2 have predominantly *R*-stereochemistry at C-3^1 while BChl *c* homologs with high degrees of methylation at C-8^2 have predominantly *S*-stereochemistry at C-3^1 (Senge and Smith 1995). In total, approximately 10–15% of the BChl *c* typically has *S*-stereochemistry at C-3^1. In the BChl *a* biosynthetic pathway, hydration of the C-3 vinyl group is catalyzed by BchF. The *Chl. tepidum* genome contains two genes, which each predicts proteins with strong sequence similarity to BchF of purple bacteria. One of these genes occurs in an operon, *bchF-bchC-bchX*, with two other single-copy genes required for BChl *a* biosynthesis. Attempts to inactivate this *bchF* gene have failed thus far, and hence BchF may specifically be required for BChl *a* biosynthesis. The second *bchF* paralog, denoted *bchV*, is found upstream of, but divergently oriented from, *bchQ* (which encodes the BChl *c*-specific C-8^2 methyltransferase, see above). The *bchV* gene has been insertionally inactivated, and the resultant mutant strain has been biochemically characterized (Table 1; Frigaard et al. 2003a; A. Gomez Maqueo Chew, N.-U. Frigaard, A.D. Jones, and D.A. Bryant, manuscript in preparation). About 85% of the total BChl *c* in this mutant is normal, except that the most highly methylated species (8-*iso*-butyl, 12-ethyl BChl *c*) normally observed in the wild-type strain is absent. The remaining 15% of the total BChl *c* has mass and absorption properties which are consistent with the presence of a vinyl group at the C-3 position. These BChl *c* species are hypermethylated at C-8^2 and the least methylated species possible, 3-vinyl, 8-ethyl, 12-methyl BChl *c*, is notably absent. This pattern of methylation of the normal and aberrant BChl *c* molecules strongly implies that BchV catalyzes the hydration of the C-3 vinyl group of highly methylated BChlide species to produce *S*-stereochemistry, while BchF catalyzes the hydration of hypo-methylated BChlide species to produce *R*-stereochemistry at C-3^1 (Figure 3). An interesting corollary of these results is that the synthesis of BChl *a* appears to require *R*-stereochemistry at the C-3^1 position. Since the ensuing dehydrogenation reaction catalyzed by BchC would be expected to require a substrate of defined chirality at the C-3^1 carbon, it is not surprising that an intermediate of defined stereochemistry seems to be formed. Finally, although about 85% of the BChl *c* in the *bchV* mutant is normal, the absorption spectrum of the BChl *c* in the cells of this mutant is dramatically altered: the absorption maximum due to aggregated BChl *c* is decreased in intensity and blue-shifted about 10–15 nm and a shoulder that appears in the absorption spectrum around 670 nm is probably due to monomeric BChl *c* species. Since the C-3^1 hydroxyl group is essential for the interactions between the BChl *c* molecules in the chlorosomes (Blankenship et al. 1995; Olson 1998; Blankenship and Matsuura 2003), it is not surprising that its absence in a small fraction of the BChl *c* severely affects the properties of the BChl *c* aggregates that do form. As may be expected from the changes in BChl *c* aggregation and absorption properties, light harvesting and growth at low light intensities are seriously impaired in the *bchV* mutant.

Magnesium chelation

Three homologs (*CT1957*, *CT1955*, and *CT1295*) of the large subunit of the protoporphyrin IX magnesium chelatase are found in the *Chl. tepidum* genome, and these genes have been named *bchH*, *bchS*, and *bchT,* respectively (Table 1 and Figure 3). There is only one copy each of the genes encoding the other two subunits (*bchD* and *bchI*). Surprisingly, all three *bchH/bchS/bchT* paralogs can be insertionally inactivated (Frigaard et al. 2003a; A. Gomez Maqueo Chew,

N.-U. Frigaard, D. A. Bryant, unpublished data). This result seemingly indicates that no one gene product is specifically required for BChl *a* biosynthesis. Inactivation of *bchH* produces no obvious phenotype, and inactivation of *bchT* produces only a small but significant reduction in the amount of BChl *c* in cells. However, the *bchS* mutant has a more dramatic phenotype: only about 5% of the normal amount of BChl *c* is produced in this mutant. This observation could provide support for a substrate channeling mechanism in (B)Chl biosynthesis. Alternatively, this result may indicate that isoenzymes for the magnesium chelatase reaction exist to provide better feedback control of the entry of protoporphyrin IX into a pathway that leads to three required (B)Chl species in very different amounts [the ratio BChl *c*: BChl *a*: Chl *a* in *Chl. tepidum* cells is roughly 30:1:0.1 (Frigaard et al. 2001)]. Further biochemical studies will be required to establish the biochemical basis for these phenotypes.

Geranylgeraniol reduction

Two paralogous genes, denoted *bchP* (*CT2256*) and *bchO* (*CT1232*) are predicted to encode proteins with sequence similarity to geranylgeraniol reductase. CT2256 is most similar to the BchP proteins of purple bacteria. Both of these genes have been insertionally inactivated, and similar phenotypes were found (Table 1). The HPLC profiles for the BChl *a* and Chl *a* species were similar for the two mutants but were clearly different from those of the wild type. The HPLC profiles suggest that both the BChl *a* and Chl *a* species in the *bchP* and *bchO* mutants are esterified with alcohols less saturated than normal, probably mostly geranylgeraniol. Such a phenotype could be explained if a heterodimer of BchP and BchO is necessary for optimal reduction activity and if such a complex is responsible for forming both the phytol tail on BChl a_P and the Δ2,6-phytadienol tail on Chl a_{PD}. The carotenoid contents of wild type and the two mutants were similar, suggesting that the products of *bchP* and *bchO* are not involved in the synthesis of dihydro-derivatives of chlorobactene and γ-carotene (Takaichi et al. 1997).

Isoprene and carotenoid biosynthesis

Chl. tepidum utilizes the methylerythritol phosphate (MEP) pathway for synthesis of isoprene units, and it has homologs of all genes known to be involved in this pathway which only recently has been completely elucidated (Figure 3; Adam et al. 2002; Altincicek et al. 2002; Rodríguez-Concepción and Boronat 2002; Rohdich et al. 2002, 2003). Most eubacteria, plant chloroplasts, and the malarial parasite *Plasmodium falciparum* use the MEP pathway, which produces isopentenyl diphosphate and dimethylallyl diphosphate from pyruvate and glyceraldehyde 3-phosphate via the intermediates deoxyxylulose 5-phosphate and methylerythritol 4-phosphate (Rodríguez-Concepción and Boronat 2002). None of the enzymes specific for the alternative mevalonic acid (MVA) pathway found in Archaea, fungi, plants, and animals (Rodríguez-Concepción and Boronat 2002) are present in *Chl. tepidum*. The isopentenyl diphosphate isomerase (CT0257) in *Chl. tepidum* is a member of the unusual Type 2 class (Kaneda et al. 2001), examples of which are also found in many Gram-positive bacteria and Archaea. Two isoprenyl diphosphate synthases are found in the genome (CT0256 and CT1206). One probably functions as farnesyl diphosphate synthase (IspA) and the other as a polyprenyl diphosphate synthase, but an unambiguous assignment is not possible. The genes encoding isopentenyl diphosphate isomerase (*CT0257*) and one of the isoprenyl diphosphate synthases (*CT0256*) are organized in an apparent operon. All of the other genes functioning in the MEP pathway and the pathway leading to chlorobactene (see below; Figure 3) are not clustered with any other obvious carotenoid or photosynthesis related genes.

The major carotenoids of green sulfur bacteria contain aromatic rings at one or both ends (Imhoff 1995; Overmann 2000), a feature that occurs only rarely in the carotenoids of other organisms. The major carotenoid in *Chl. tepidum* is the monocyclic chlorobactene, which is characteristically found in green-colored, BChl *c*- or BChl *d*-containing green sulfur bacteria. Brown-colored, BChl *e*-containing green sulfur bacteria typically contain the dicyclic isorenieratene as their major carotenoid. In addition, *Chl. tepidum* contains γ-carotene, the 1′,2′-dihydro derivatives of both chlorobactene and γ-carotene, and smaller amounts of OH-chlorobactene, OH-γ-carotene, and the glucoside laurate ester of both OH-chlorobactene and OH-γ-carotene (Takaichi et al. 1997). The major carotenoid species in reaction center complexes isolated from *Chl. tepidum* are the glucoside laurate esters (Takaichi and Oh-oka 1999).

Surprisingly, it is possible to eliminate carotenoid biosynthesis completely in *Chl. tepidum* by inactivating *crtB* which encodes phytoene synthase (Table 1;

Figure 3; N.-U. Frigaard, C.E. Yunker, and D.A. Bryant, unpublished data). This mutant exhibits the most severely impaired growth rate of the *Chl. tepidum* carotenoid biosynthesis mutants that we have constructed so far, and this mutant should prove valuable for investigations of the function of carotenoids in green sulfur bacteria. By insertional inactivation of two genes paralogous to phytoene desaturase (*CT0807* and *CT1414*), it was also established that the desaturation of phytoene to lycopene occurs in two steps that are catalyzed by separate enzymes, as is the case in cyanobacteria and plants. Phytoene is first converted to ζ-carotene by CrtP (CT0807), and ζ-carotene is subsequently converted to lycopene by CrtQ (CT1414) (Table 1; Figure 3). In photosynthetic purple bacteria and most other eubacteria and fungi, a single enzyme, CrtI, desaturates phytoene to neurosporene or lycopene (Armstrong 1999). CrtI-type desaturases produce all-*trans* lycopene which can act as substrate for lycopene cyclases. However, CrtQ-type desaturases produce prolycopene (tetra-*cis* lycopene) which cannot act as substrate for lycopene cyclases (Giuliano et al. 2002). Cyanobacteria and plants therefore have a carotenoid *cis-trans* isomerase that converts prolycopene to all-*trans* lycopene [denoted CrtH in cyanobacteria and CRTISO in plants (Masamoto et al. 2001; Giuliano et al. 2002)]. The absence of this *cis-trans* isomerase causes accumulation of prolycopene in tomato (Isaacson et al. 2002) and *Arabidopsis thaliana* (Park et al. 2002) and various lycopene species in cyanobacteria (Masamoto et al. 2001). Inactivation of the *crtH* homolog (*CT0649*) in *Chl. tepidum* caused accumulation of lycopene, but small amounts of chlorobactene and γ-carotene were also observed (N.-U. Frigaard, A.D. Jones, and D.A. Bryant, unpublished data). Thus, *CT0649* most likely encodes a *cis-trans* isomerase. Photoisomerization caused by the presence of BChls probably converts a small fraction of prolycopene to all-*trans* lycopene, which then acts as substrate for lycopene cyclase. Inactivation of a more distantly related *crtH* homolog, *CT0180*, did not produce any obvious defect in carotenoid biosynthesis, and therefore the function of the product of this gene is not clear.

No obvious candidate for a lycopene cyclase has been identified in *Chl. tepidum*. Although three distinct classes of lycopene cyclase have been found in nature, at least a fourth type must exist (Krubasik and Sandmann 2000). Some cyanobacteria (e.g., *Synechococcus* sp. PCC 7942; Cunningham et al. 1994) are known to utilize a plant-type lycopene cyclase, but in many other cyanobacteria, including *Synechocystis* sp. PCC 6803, no lycopene cyclase has been identified thus far. It is an interesting possibility that these cyanobacteria and *Chl. tepidum* share a related lycopene cyclase and that comparative genomics may be able to identify candidate genes.

Finally, chlorobactene synthase (also denoted γ-carotene desaturase), CrtU, which converts the β-ring structure in γ-carotene to the aromatic ϕ ring in chlorobactene by a combined desaturation and methyltransfer mechanism (Krügel et al. 1999), has also been identified by gene inactivation (Table 1; Figure 3). CrtU (CT0323) contains a Rieske iron-sulfur domain inserted into the middle of a sequence that otherwise resembles phytoene desaturase. A possibility that cannot yet be excluded is that this protein acts as the lycopene cyclase as well as the γ-carotene desaturase. A carotene 1,2-hydratase, CrtC (CT0301), which acts on both chlorobactene and γ-carotene, has also been identified by gene inactivation (Table 1; Figure 3). The genes encoding the CrtC hydratase and the two subunits of the Rieske iron-sulfur protein/cytochrome *b* complex (PetB and PetC; Figure 1) form an apparent operon (*crtC-petC-petB*). At least three additional and presently unidentified enzymes are required to produce the known carotenoid species in *Chl. tepidum*: a 1,2-saturase, a glucosyl transferase, and an acyl transferase.

Isoprenoid quinone biosynthesis

Chl. tepidum contains three isoprenoid quinones: menaquinone-7, 1′-hydroxymenaquinone-7, and chlorobiumquinone (1′-oxomenaquinone-7) (Powls and Redfearn 1969; Frigaard et al. 1997; N.-U. Frigaard, S. Takaichi, and K. Matsuura, unpublished data). Menaquinone functions primarily as an electron carrier in the cytoplasmic membrane and possibly also in the reaction center. However, some of the menaquinone and 1′-hydroxymenaquinone, and almost all of the chlorobiumquinone, are located in the chlorosomes (Frigaard et al. 1997). Chlorobiumquinone almost certainly acts as a redox-sensitive quencher of excitation energy in the chlorosome antenna; this quencher is active under oxic conditions and inactive under anoxic conditions (Frigaard et al. 1997, 1998, 1999; van Noort et al. 1997; Frigaard and Matsuura 1999). Under aerobic conditions, this mechanism presumably allows for a reversible cessation of photosynthetic generation of reductants, which

might otherwise lead to oxidative damage to the cells.

Orthologs of all genes known to be involved in the biosynthesis of menaquinone in *E. coli* were identified in the *Chl. tepidum* genome (Meganathan 2001; Eisen et al. 2002). No biochemical studies concerning the biosynthesis of chlorobiumquinone or 1′-hydroxymenaquinone have been performed. However, it is likely that 1′-hydroxymenaquinone is a biosynthetic intermediate between menaquinone and chlorobiumquinone, since the cellular content of 1′-hydroxymenaquinone is highest in rapidly growing cells and decays in the stationary phase (N.-U. Frigaard, unpublished data). Good candidates for genes involved in chlorobiumquinone biosynthesis encode enzymes that can anaerobically introduce an oxo or hydroxyl group on the saturated α carbon of an aromatic ring substituent. Four potential candidates (CT0072, CT1502, CT1697, CT1903), related to the oxidative, isocyclic ring cyclase BchE and the C-methyltransferases BchR and BchQ of BChl biosynthesis, have been ruled out by gene inactivation studies (Table 1). Another candidate is related to the ethylbenzene dehydrogenase recently identified in *Azoarcus* sp. (Johnson et al. 2001; Rabus et al. 2002). Ethylbenzene dehydrogenase is a heterotrimer (EbdA-EbdB-EbdC) that presumably binds a molybdopterin cofactor (EbdA) and several iron-sulfur clusters (EbdB) and is related to the membrane-bound DMSO reductase family. This enzyme anaerobically oxidizes ethylbenzene to 1-phenylethanol, which subsequently is anaerobically oxidized to acetophenone by a short-chain dehydrogenase (Ped). *Chl. tepidum* has an apparent operon of genes (*CT0496-CT0495-CT0494*) that originally was annotated as a potential polysulfide reductase (Eisen et al. 2002) but which also is homologous to EbdA, EbdB, and EbdC. It is possible that the *CT0496-CT0495-CT0494* operon in *Chl. tepidum* encodes a menaquinone dehydrogenase that produces 1′-hydroxymenaquinone and this is currently being tested by gene inactivation. *Chl. tepidum* also has several homologs of short-chain dehydrogenases related to Ped which could convert 1′-hydroxymenaquinone to chlorobiumquinone. Genetic inactivation of chlorobiumquinone biosynthesis would not only establish the biosynthetic pathway for this novel quinone, but it would also permit the proposed role of this quinone in the redox-sensitive regulation of energy transfer in the chlorosome antenna to be critically evaluated.

Electron transfer components and sulfur oxidation

The metabolic strategy of the green sulfur bacteria is to convert the electrons derived from relatively weak reductants, such as sulfide, into more powerful reductants that can be used to reduce carbon dioxide by the reverse TCA cycle reactions. To accomplish this, the photosynthetic reaction center of green sulfur bacteria generates strongly reducing ferredoxins by photooxidation of soluble or membrane-bound *c*-cytochromes [Figure 1; (Sakurai et al. 1996; Rémigy et al. 1999; Hauska et al. 2001)]. The *Chl. tepidum* genome reveals only single copies of the four genes (*pscA*, *pscB*, *pscC*, *pscD*) encoding the reaction center subunits. No other gene paralogous to *pscA* (*CT2020*) is found, thus confirming the previous experimental evidence from studies in *Chl. limicola* that the reaction center is a homodimer of PscA (Büttner et al. 1992a, b). PscB (CT2019) has been shown to bind 2[4Fe–4S] clusters (Kjær et al. 1994); the extended N-terminal sequence of PscB has been suggested to resemble functionally the PsaD subunit of Photosystem I. The reaction center probably binds two identical cytochrome c_{551} subunits encoded by *pscC* (*CT1639*) (Oh-Oka et al. 1995a, b). There is no apparent ortholog of cytochrome c_1, which in most other organisms forms a complex with a Rieske iron-sulfur protein and cytochrome *b* (Figure 1; see Hauska et al. 2001). This raises the interesting possibility that PscC receives electrons directly from the Rieske iron-sulfur protein and delivers them to $P840^+$ (see Figure 1). The function of the fourth subunit, PscD (CT0641), is not known (Hager-Braun et al. 1995).

A number of sulfur sources can provide electrons for photosynthetic growth of green sulfur bacteria including sulfide, elemental sulfur, polysulfide, thiosulfate, and tetrathionate (Trüper et al. 1988; Brune et al. 1995; Overmann 2000). Green sulfur bacteria do not appear to be capable of assimilatory sulfate reduction and generally require a small exogenous supply of sulfide. This view is supported by genome analysis in *Chl. tepidum*. At present, little is known about the mechanisms by which the various sulfur sources are metabolized, although some speculations follow below (also see Eisen et al. 2002). This may be the subject of future research involving gene inactivation studies, since many of the genes involved may be dispensable because the organism can grow on multiple alternative sulfur sources. Such studies may

also reveal why so many of these genes are duplicated in the *Chl. tepidum* genome.

Eleven putative *c*-type cytochromes were identified in the genome (Eisen et al. 2002). Some of these can be assigned functions in photosynthesis and the oxidation of sulfur compounds. As noted above, the *pscC* gene (*CT1639*) encodes the reaction center-associated cytochrome c_{551}. A small, soluble cytochrome $c_{553/554}$ encoded by *CT0075* has been shown to donate electrons directly to PscC [Figure 1; (Okumura et al. 1994; Selvaraj et al. 1998; Itoh et al. 2002)]. The *CT0073* gene is found in an operon with *CT0075* and encodes a cytochrome *c* with a very similar sequence but with a putative C-terminal, cysteine-linked, diacylglycerol membrane anchor. It is possible that CT0073 represents the membrane-bound cytochrome c_{556} described by Oh-oka et al. 1998. SoxX (CT1016) and SoxA (CT1019) are the two subunits of a heterodimeric *c*-type cytochrome complex that probably is involved in thiosulfate oxidation and perhaps the oxidation of other sulfur compounds (Friedrich et al. 2001; Rother and Friedrich 2002). FccA (CT2080) is the cytochrome subunit of flavocytochrome *c*, which is believed to play a role in sulfide oxidation (Verte et al. 2002). DsrJ (CT2242) is a tri-heme cytochrome subunit associated with the membrane anchor of dissimilatory sulfite reductase. Four other genes, *CT0188, CT1704, CT1734*, and *CT2026,* encode proteins with sequence similarity to various *c*-type cytochromes in other organisms, but there are no good clues to possible functions for these proteins. It will probably be possible to assign the correct functions to some of these cytochromes by gene inactivation studies, since some of the electron transfer processes they participate in are likely to be dispensable under certain growth conditions.

Chl. tepidum contains several ferredoxin-encoding genes. Three soluble and highly similar 2[4Fe–4S] ferredoxins (CT1260, CT1261, CT1736) have been purified, and all have been shown to act as electron acceptors from purified reaction center preparations (Seo et al. 2001). CT1260 and CT1261 have also been shown to act as electron donors for pyruvate synthase (CT1628) which functions in CO_2 fixation (Yoon et al. 2001; see discussion of carbon assimilation below). The genome additionally predicts three similar 2[4Fe–4S] ferredoxins (CT0167, CT0168, CT0409) and two similar [2Fe–2S] ferredoxins (CT1541, CT1655). The *CT0409* gene is part of an apparent operon encoding a putative Fe(III) ABC transporter complex and thus might play a role in iron acquisition. Similarly, based on the clustering of genes, CT1541 may play a role in nitrogen fixation. *Chl. tepidum* also possesses three rubredoxins (CT1100, CT1101, CT2024) which may serve as electron acceptors for uptake hydrogenase (HupL; see below), pyruvate:ferredoxin/rubredoxin oxidoreductase (CT1628) (Yoon et al. 1999; this enzyme also functions in CO_2 fixation, see below) or rubredoxin:oxygen oxidoreductase (CT2285). In addition to these soluble electron transport proteins, a flavodoxin (CT1738) with high sequence similarity to the flavodoxins of cyanobacteria is also encoded in the genome. Three thioredoxins (CT0785, CT0841, and CT1215), a thioredoxin reductase (CT0842), a glutaredoxin-like protein (CT1727), and a ruberythrin (CT1327) are also encoded in the genome.

Electrons can be fed into and out of the menaquinol pool by various dehydrogenases and oxidoreductases. The *Chl. tepidum* genome contains an apparent operon, *ndhCKJHAIGEFDB* (*CT0766–CT0776*) encoding subunits with high sequence similarity to 11 subunits of a NADH:quinone oxidoreductase analogous to complex I. Complex I (also known as Type I NADH dehydrogenase or NADH:quinone oxidoreductase) is found in mitochondria, chloroplasts, and many bacteria and generally consists of 13 or 14 subunits in proteobacteria like *E. coli* (Weidner et al. 1993; Friedrich and Scheide 2000; Friedrich 2001). Eisen et al. (2002) suggested that the putative complex I in *Chl. tepidum* might provide reduced pyridine nucleotides for carbon fixation and gluconeogenesis by performing reverse electron transport as in purple bacteria. However, orthologs of *nuoE*, *nuoF*, and *nuoG,* which encode the NADH dehydrogenase/diaphorase module of complex I in *E. coli* (Friedrich 2001), are not found in the *Chl. tepidum* genome. The same three subunits (NuoEFG) are also not present in complex I in cyanobacteria and chloroplasts (Boison et al. 1998; Friedrich and Scheide 2000). Therefore, it is unlikely that complex I in *Chl. tepidum* oxidizes or reduces pyridine nucleotides. One possibility is that the complex receives electrons directly from reduced ferredoxin and participates in cyclic electron transport to produce protonmotive force for the generation of ATP. A similar role of complex I in cyanobacteria has been proposed (Mi et al. 1995). Another possibility is that an iron-sulfur flavoprotein (CT1300), which binds one FMN and one [4Fe–4S] cluster per subunit (Leartsakulpanich et al. 2000; Zhao et al. 2001) could

shuttle electrons between complex I and pyridine nucleotides. Alternatively, in combination with an uptake hydrogenase (see below), complex I could oxidize H_2 and produce protonmotive force and menaquinol.

Several other dehydrogenases and oxidoreductases can probably introduce or withdraw electrons from the menaquinone and pyridine nucleotide pools. These include a Type II NADH dehydrogenase (CT0369), three proteins related to sulfide:quinone oxidoreductase (CT1087, CT0876, and CT0117), a putative polysulfide reductase, a heterodisulfide reductase that may be coupled to the oxidation of sulfite to form adenylylsulfate, and dissimilatory sulfite reductase. Interestingly, genes for the latter appear to have been recently duplicated in the genome (Eisen et al. 2002). A surprising finding was the occurrence of the *cydABDC* operon (*CT1818–CT1822*), which encodes a putative cytochrome *bd* quinol oxidase (CydAB) and two related assembly proteins (CydC and CydD). This oxidase, which in *E. coli* has very high affinity for oxygen, might be used to reduce oxygen to water using menaquinol as the reductant, and it could thus protect *Chl. tepidum* from the toxic effects of brief exposures to oxygen. A rubredoxin:oxygen oxidoreductase (CT2285) is also encoded in the genome. This protein is also believed to provide protection against the toxic effects of oxygen by reducing oxygen directly to water (Frazão et al. 2000; Silva et al. 2001). Interestingly, an NADPH-dependent version of this enzyme has recently been characterized in cyanobacteria (Helman et al. 2003). Other oxygen-protection enzymes include superoxide dismutase (CT1211) and three putative proteins: thiol peroxidase (CT0754), thiol-specific antioxidant (CT1492), and peptide methionine sulfoxide reductase (CT1278). Finally, a recently identified ferredoxin:$NAD(P)^+$ oxidoreductase (FNR) is capable of producing NAD(P)H directly from photosynthetically produced ferredoxin (Seo and Sakurai 2002). The gene encoding this protein (*CT1512*) was originally annotated as a thioredoxin reductase, a point which illustrates how difficult it can be to identify proteins unambiguously by bioinformatics approaches alone.

Both green sulfur bacteria and green filamentous bacteria contain hydrogenases and can oxidize H_2; in fact, some green sulfur bacteria can use H_2 as the major or sole electron donor for photoautotrophic growth (Lippert and Pfennig 1969; Drutschmann and Klemme 1985; Gogotov et al. 1991; Heising et al. 1999). An active, unidirectional, monomeric, 66-kDa Ni-Fe hydrogenase, which can use rubredoxin as electron acceptor, has been isolated from *Chl. limicola* strain L (Gogotov 1988). As is the case in many cyanobacteria (Tamagnini et al. 2002; Schmitz et al. 2002), the *Cfx. aurantiacus* genome encodes two types of hydrogenases, an uptake hydrogenase (HupSL) and a heteropentameric, bidirectional hydrogenase (HoxEFUYH). It has been proposed that the bidirectional hydrogenase present in some cyanobacteria interacts with complex I to allow brief net H_2 production from low-potential electrons under certain light-induced stress conditions (Appel et al. 2000; Cournac et al. 2002). The enzyme would effectively act as a 'relief valve' to release transiently overly reduced pools of electron carriers during rapid increases in photosynthetic electron transport.

The *Chl. tepidum* genome encodes two proteins (CT0777 and CT0778) that are clearly related to Ni-Fe-type, uptake hydrogenase subunits. CT0777 has significant sequence similarity to the large subunit (HupL) of Ni-Fe hydrogenases but is missing about 110 amino acid residues at its N-terminus relative to HupL proteins from other organisms. Most importantly, two critical cysteine residues, which are ligands to the active-site Ni atom are missing from the N-terminal domain of CT0777. The predicted mass of CT0777 after C-terminal processing (see below) would be approximately 52 kDa, which is much smaller than the 66-kDa monomeric hydrogenase isolated from *Chl. limicola* strain L (Gogotov 1988). *CT0778* encodes a membrane-associated, *b*-type cytochrome subunit (HupC) that could presumably anchor CT0777 to the membrane and exchange electrons with the menaquinone pool. A third gene in this apparent transcriptional unit, *CT0779*, encodes a protein with similarity to the HupD protease that usually activates the HupL subunit by cleaving 15 C-terminal amino acid residues from the inactive precursor protein (Volbeda et al. 1995). No gene with sequence similarity to the smaller HupS subunit, which is typically encoded upstream from *hupL*, contains one or more iron-sulfur clusters, and exchanges electrons with the physiological acceptor (Vignais et al. 2001; Tamagnini et al. 2002), was found in the genome. Clearly, the functional role of these *hup* genes is far from clear and will require further biochemical studies.

Interestingly, the *Chl. tepidum*, *hupLCD* operon (*CT0777–CT0779*) seems to form an extended operon with the 11 genes encoding complex I (*CT0766–CT0776*; see above). This gene arrangement suggests that a functional interaction may exist between the putative HupL hydrogenase and complex I. HupL

may donate electrons directly to complex I via the NdhK subunit, which is distantly related to HupS (Vignais et al. 2001; Friedrich 2001). Interestingly, the NdhK homolog in *Chl. tepidum* (CT0767) contains additional cysteine residues that are not likely to be involved in ligating the 2[4Fe–4S] clusters typically present in this protein. It is an interesting possibility that the missing cysteine ligands to the Ni at the active site of CT0777 could be provided *in trans* by NdhK. Electrons from CT0777 might alternatively be transferred to menaquinone via HupC, which is a membrane-bound cytochrome *b* (Vignais et al. 2001).

In addition to the uptake hydrogenase described above, the genome encodes a putative heterotetrameric ($\alpha\beta\gamma\delta$), cytoplasmic bidirectional hydrogenase, which resembles *Pyrococcus furiosus* hydrogenase II and which catalyzes H_2 production and oxidation as well as the reduction of elemental sulfur and polysulfide to sulfide (Ma et al. 2000). The genes encoding this sulfhydrogenase form a putative operon, *CT1891–CT1894*. Another operon, *CT1245–CT1250*, includes genes encoding proteins with similarity to subunits A and C of heterodisulfide reductase and sulfhydrogenase subunits β, γ, δ, and an iron-sulfur cluster binding protein. The functional roles of these two hydrogenase-related electron transfer complexes is not known, but they could play a role in redox homoeostasis, sulfur and polysulfide reduction, or H_2 oxidation. The only reported H_2 production in green sulfur bacteria is nitrogenase-dependent and only occurs in the absence of ammonia (Warthman et al. 1992). Although *Chl. tepidum* probably primarily uses its hydrogenases for H_2 uptake, it cannot be excluded that, in analogy with cyanobacteria, these enzymes may become H_2 evolving under certain physiological conditions. It can be seen from the discussion above that there is much to discover about sulfur oxidation and other electron transfer processes in *Chl. tepidum*.

Carbon assimilation

Green sulfur bacteria fix CO_2 using the reductive tricarboxylic acid cycle (Buchanan and Arnon 1990; Sirevåg 1995; Wahlund and Tabita 1997; Atomi 2002). The pivotal enzyme of this cycle, ATP-citrate lyase, which converts citrate to oxaloacetate and acetyl-CoA using ATP, has been purified and characterized from both *Chl. tepidum* and *Chl. limicola* (Wahlund and Tabita 1997; Kanao et al. 2001, 2002a). Most bacterial citrate lyases are not ATP-dependent and function in citrate fermentation and amino acid biosynthesis. ATP-dependent citrate lyase has been found only in eukaryotes, in which it provides acetyl-CoA for biosynthetic purposes, and in those few types of prokaryotes which employ the reductive tricarboxylic acid cycle for CO_2 fixation: green sulfur bacteria, the H_2-oxidizing, chemolithoautotrophic *Aquifex pyrophilus* (Beh et al. 1993) and *Hydrogenobacter thermophilus* (Ishii et al. 1989), the sulfate-reducing *Desulfobacter hydrogenophilus* (Schauder et al. 1987), and the archaeon *Thermoproteus neutrophilus* (Beh et al. 1993). Both ATP-dependent and ATP-independent citrate lyases generally contain three domains, which are contained on a single gene product in animals, on two gene products in plants and fungi, and on three gene products in most bacteria. Interestingly, *Chl. tepidum* ATP-citrate lyase has high sequence similarity with eukaryotic ATP-citrate lyases (ACL) and not with common prokaryotic ATP-independent citrate lyases (CitDEF). In addition, the structure of *Chl. tepidum* ATP-citrate lyase resembles that found in plants and fungi by being encoded by two separate genes, *aclA* (*CT1088*) and *aclB* (*CT1089*). Currently there is little data available to judge whether this is typical for other prokaryotic ATP-citrate lyases functioning in the reductive tricarboxylic acid cycle, but evaluation of the genome sequence of *Aquifex aeolicus* and of the purified enzyme from *H. thermophilus* (Ishii et al. 1989) shows that these organisms most likely use a different type of ATP-citrate lyase. Like many bacteria, *Chl. tepidum* also encodes three additional proteins which are homologous to each of the three domains of eukaryotic ATP-dependent citrate lyases: CT0269, CT0380, and CT1835. These proteins have high sequence similarity to the α and β subunits of succinyl-CoA synthetase (SucCD) and citrate synthase (GltA), respectively. Succinyl-CoA synthetase functions in the reductive tricarboxylic acid cycle, but the actual activity and function of the citrate synthase-like protein in *Chl. tepidum* is unclear.

Two key CO_2-fixing enzymes, both ferredoxin-dependent, are also found in *Chl. tepidum*: a homodimeric pyruvate synthase (pyruvate:ferredoxin oxidoreductase) encoded by *CT1628* and a heterodimeric 2-oxoglutarate synthase encoded by *CT0162* and *CT0163* (Yoon et al. 2001). Interestingly, the CT1628 protein has also been shown to catalyze decarboxylation of pyruvate with rubredoxin as electron acceptor (Yoon et al. 1999, 2001); this finding may reflect an important regulatory role for this enzyme in carbon metabolism. The only other

CO_2-fixing enzyme in the reductive tricarboxylic acid cycle, NAD(P)H-dependent isocitrate dehydrogenase (CT0351), has been purified and characterized from *Chl. limicola* (Kanao et al. 2002b; Lebedeva et al. 2002). Isocitrate dehydrogenase isolated from *Rhodopseudomonas palustris* (which uses the tricarboxylic acid cycle) and from *Chl. limicola* show virtually no difference in enzymatic and kinetic properties (Lebedeva et al. 2002).

The reduction of CO_2 to biomass in plants, cyanobacteria, and most autotrophic bacteria occurs via the Calvin cycle (Tabita 1994; Atomi 2002). Surprisingly, *Chl. tepidum* contains a homolog, *CT1772*, of the large subunit of the key CO_2-fixing enzyme in this pathway, ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO) (Hanson and Tabita 2001). However, CT1772 lacks several active site residues which are universally conserved in RuBisCO enzymes from autotrophs that utilize the Calvin cycle, and the purified recombinant CT1772 protein has no CO_2 fixation activity. A *CT1772* mutant of *Chl. tepidum* that lacks this 'RuBisCO-like-protein' showed phenotypic effects on oxidative stress response and sulfur metabolism; these observations, when fully understood, may provide an evolutionary clue to the origin and function of early RuBisCO-like enzymes (Hanson and Tabita 2001). No homologs of the RuBisCO small subunit or of another key enzyme of the Calvin cycle, phosphoribulokinase, are found in *Chl. tepidum*.

Physiological characterization of *Chl. tepidum* and other green sulfur bacteria has shown that these organisms have a very limited capacity for assimilating exogenously supplied, organic carbon sources (Wahlund et al. 1991). Out of a large number of organic substrates tested including various carboxylic acids, carbohydrates, and amino acids, only acetate and pyruvate stimulated growth of *Chl. tepidum*. These findings are consistent with genome sequence analyses that showed a very low number of potential transporters that could allow assimilation of exogenous carbon sources (Eisen et al. 2002).

Are there alternatives to the photosynthetic life style?

Genome analysis provides few clues for alternative modes of energy generation. Only a few genes encoding transporters are found (see Eisen et al. 2002 for a more complete discussion); their presence suggests that *Chl. tepidum* may take up and utilize a few organic compounds, including amino acids (e.g., arginine and lysine) and possibly sugars. However, consistent with the inability of *Chl. tepidum* to utilize glucose, the genome does not appear to encode either glucose kinase or a glucose transporter. Several genes related to the phosphoenolpyruvate-sugar phosphotransferase system were found, although it seems likely that these gene products are more likely to be involved in a signal transduction/regulatory pathway for balancing carbon and nitrogen metabolism than in sugar uptake. All known green sulfur bacteria are strictly photoautotrophic, and attempts by us to grow *Chl. tepidum* chemoheterotrophically have so far failed.

Discovery of conditions under which *Chl. tepidum* could be grown chemoheterotrophically would greatly facilitate the genetic manipulation of the photosynthetic apparatus of this organism. This would obviously be most important with respect to the manipulation of genes encoding essential components for photosynthesis, such as enzymes of BChl *a* and Chl a_{PD} biosynthesis, the FMO protein, and the reaction center subunits. If the current strains of *Chl. tepidum* and other transformable green sulfur bacteria are incapable of heterotrophic growth, future research goals might be to isolate new strains from natural sources rich in organic molecules or to genetically engineer strains capable of heterotrophic growth.

Concluding remarks

Computational analyses of the *Chl. tepidum* genome have allowed many predictions to be made about the metabolic capabilities of *Chl. tepidum* and for green sulfur bacteria more generally. Combined with gene inactivation studies, which have produced about 40 mutant strains so far (Tables 1 and 2), many of these predictions have been tested and either confirmed or reformulated. For example, all gene inactivations presented in Table 1 (except the chlorosome protein mutations) were based on analyses of the genome sequence and allowed a relatively rapid identification of novel genes in BChl *c* biosynthesis (Frigaard et al. 2003a) and correct functional assignment of several genes in carotenoid biosynthesis.

It is notable that many components of photosynthesis and energy metabolism in *Chl. tepidum*, including some electron transfer complexes and metabolic pathways, are more similar to their counterparts in plants and cyanobacteria than they are to their counterparts in purple bacteria and other photosynthetic bacteria. Examples include: (1) complex I with 11 subunits, (2) carotenoid biosynthesis which involves two-step desaturation of phytoene using CrtP, CrtQ,

and a *cis-trans* isomerase, (3) protoporphyrin biosynthesis using the glutamate C_5 pathway for making the precursor 5-aminolevulinate, and (4) the structure of the ATP-citrate lyase. It is also notable that (5) *Chl. tepidum* synthesizes Chl *a*; no organisms other than green sulfur bacteria, cyanobacteria, and eukaryotic phototrophs (algae and plants) synthesize Chl *a*.

The availability of genomic information from representatives of the five eubacterial taxa that are photosynthetic (purple bacteria, green sulfur bacteria, green filamentous bacteria, heliobacteria, and cyanobacteria) have led to several recent papers discussing the evolutionary origins of photosynthesis (Xiong et al. 2000; Xiong and Bauer 2002a, b; Raymond et al. 2002). Xiong and coworkers have argued that purple bacteria are the earliest emerging photosynthetic lineage and that bacteriochlorophyll biosynthesis evolved before chlorophyll biosynthesis. Raymond et al. (2002) found plurality support for trees that grouped *Synechocystis* sp. PCC 6803, *Heliobacillus mobilis* and *Chloroflexus aurantiacus* together and separate from a distinct cluster containing *Rhodobacter capsulatus* and *Chl. tepidum*. However, on the basis of whole-genome comparisons, these authors also conclude that the components of photosynthesis have been subject to extensive horizontal gene transfer. As more examples of photosynthetic taxa are subjected to whole-genome analysis, and as more is learned about the metabolism and biochemistry of phototrophs, it may become possible to develop a more refined and coherent picture of the early evolution of photosynthesis.

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