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MINI-REVIEW

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Seeing green bacteria in a new light: genomics-enabled studies of the photosynthetic apparatus in green sulfur bacteria and filamentous anoxygenic phototrophic bacteria

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Abstract Based upon their photosynthetic nature and the presence of a unique light-harvesting antenna structure, the chlorosome, the photosynthetic green bacteria are defined as a distinctive group in the Bacteria. However, members of the two taxa that comprise this group, the green sulfur bacteria (Chlorobi) and the filamentous anoxygenic phototrophic bacteria (''Chloroflexales''), are otherwise quite different, both physiologically and phylogenetically. This review summarizes how genome sequence information facilitated studies of the biosynthesis and function of the photosynthetic apparatus and the oxidation of inorganic sulfur compounds in two model organisms that represent these taxa, *Chlorobium tepidum* and *Chloroflexus aurantiacus*. The genes involved in bacteriochlorophyll (BChl) *c* and carotenoid biosynthesis in these two organisms were identified by sequence homology with known BChl *a* and carotenoid biosynthesis enzymes, gene cluster analysis in *Cfx. aurantiacus*, and gene inactivation studies in *Chl. tepidum*. Based on these results, BChl *a* and BChl *c* biosynthesis is similar in the two organisms, whereas carotenoid biosynthesis differs significantly. In agreement with its facultative anaerobic nature, *Cfx. aurantiacus* in some cases apparently produces structurally different enzymes for heme and BChl biosynthesis, in which one enzyme functions under anoxic conditions and the other performs the same reaction under oxic conditions. The *Chl. tepidum* mutants produced with modified BChl *c* and carotenoid species also allow the functions of these pigments to be studied in vivo.

Keywords Bacteriochlorophyll *a* · Bacteriochlorophyll biosynthesis · Bacteriochlorophyll *c* · Carotenoid biosynthesis · *Chlorobium* · *Chloroflexus* · Chlorosome · Functional genomics · Inorganic sulfur metabolism

Introduction

Members of the phylum Chlorobi, or green sulfur bacteria, are strictly anaerobic and obligately photoautotrophic Bacteria, which form a distinct phylogenetic group that shares a common root with the Bacteroidetes (Garrity and Holt 2001a). The light-harvesting antennae, or chlorosomes, of these bacteria are large structures and mostly consist of bacteriochlorophyll (BChl) *c*, *d*, or *e* in addition to smaller amounts of BChl *a* and carotenoids with aromatic end groups (Blankenship et al. 1995; Blankenship and Matsuura 2003; Fig. 1)]. *Chlorobium tepidum* (Wahlund et al. 1991) has emerged as the model organism of choice for the green sulfur bacteria, because

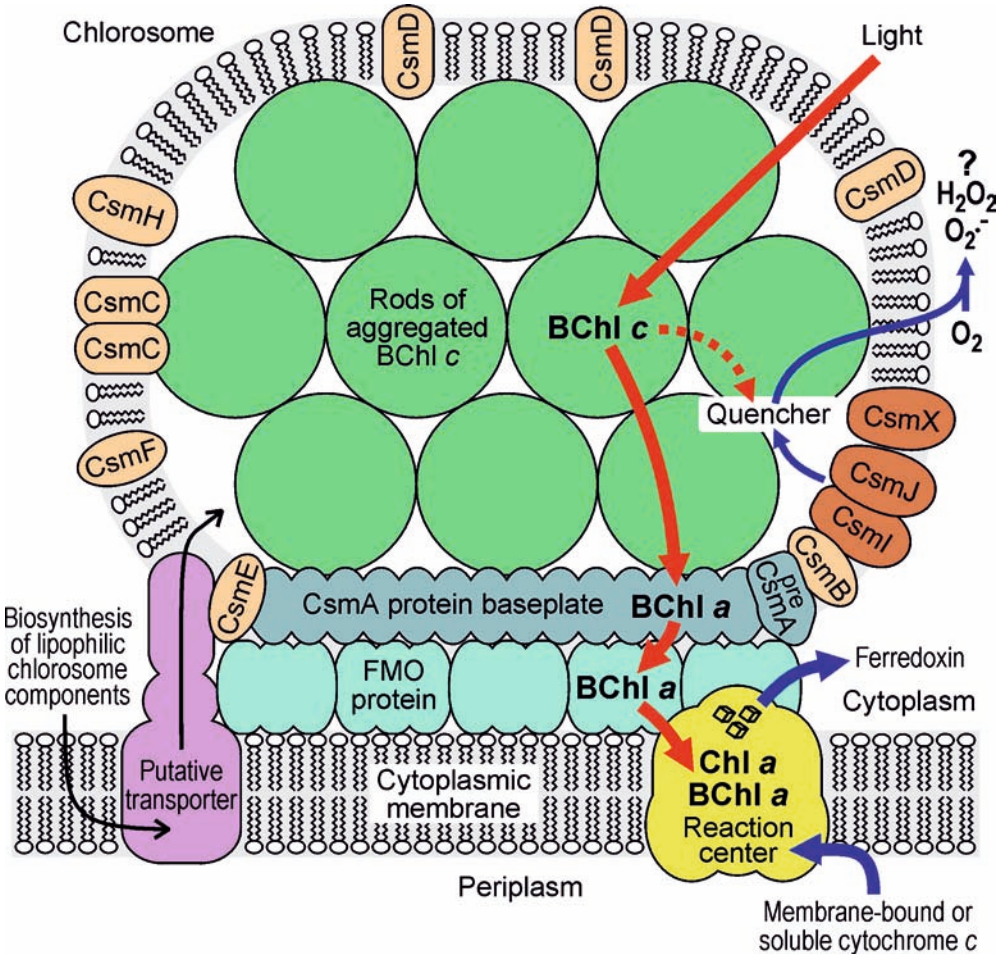

Fig. 1 Simplified model of the photosynthetic apparatus in *Chl. tepidum*. Carotenoids (not shown) are associated with all complexes containing BChls, except the Fenna–Matthews–Olson (FMO) protein. Under oxic conditions (which are detrimental to the bacteria), the quencher in the chlorosome prevents excitation transfer from BChl *c* to the reaction center and thus prevents photosynthetic electron transfer. The quencher is activated by oxidation, with the electrons probably being delivered directly or indirectly to O_2; and it is inactivated by reduction by the chlorosome (Csm) proteins CsmI and CsmJ. Excitation transfer is shown by *red lines* and electron transfer is shown by *blue lines*

it is naturally transformable (Frigaard and Bryant 2001; Frigaard et al. 2004d) and because its genome has been completely sequenced (Eisen et al. 2002). Much has already been learned about the biosynthesis of carotenoids, BChls, and other structures and processes in this organism by a combination of bioinformatic and gene inactivation approaches (Frigaard et al. 2002, 2003, 2004a, b, c; Hanson and Tabita 2001, 2003; Maresca et al. 2004). A new nomenclature of the green sulfur bacteria was recently proposed, in which *Chl. tepidum* is renamed *Chlorobaculum tepidum* (Imhoff 2003).

The phylum Chloroflexi, which is divided into two orders, the ''Chloroflexales'' and the ''Herpetosiphonales,'' is a deep-branching lineage of the Bacteria (Garrity and Holt 2001b). Members of the former order synthesize BChls and are obligately or facultatively phototrophic, while members of the latter order do not synthesize BChls and are not phototrophs. Although members of both groups are designated as Gram-negative, they do not synthesize lipopolysaccharide and thus do not possess outer membranes. Like all members of the Chlorobi, some but not all of the ''Chloroflexales'' synthesize BChl *c* and/or *d* and contain chlorosomes. *Chloroflexus aurantiacus* is the best-studied member of the Chloroflexi (Pierson and Castenholz 1974a). This bacterium is facultatively phototrophic and only induces the synthesis of its photosynthetic apparatus, including chlorosomes, under anoxic conditions. The synthesis of BChl *c* and BChl *a* is inhibited under oxic conditions, whereas carotenoid biosynthesis persists. However, the carotenoid composition is different under oxic and anoxic conditions (see below). As *Cfx. aurantiacus* synthesizes BChl *c*, it is commonly known as a green filamentous bacterium or a green ''non-sulfur'' bacterium. However, in recognition of the fact that some members of the ''Chloroflexales'' are not ''green'' and do not synthesize BChl *c*/*d* and chlorosomes, members of this group are now commonly referred to as ''filamentous anoxygenic phototrophic bacteria'' or ''photosynthetic flexibacteria.'' The genome of *Cfx. aurantiacus* has been partially sequenced (see http://www.jgi.doe.gov and below) and currently is being sequenced to completion (R.E. Blankenship and J. Raymond, personal communication). At present, this is the only genome

information available for the phylum Chloroflexi, and to date there has been no demonstration that *Cfx. aurantiacus* is amenable to genetic manipulation.

Green sulfur bacteria are typically found in anoxic aquatic or terrestrial environments where both sulfide and light occur. Green filamentous bacteria are often found in the anoxic layer of microbial mats but also occur in some aquatic environments. The light intensities are exceedingly low in the anoxic layers of stratified lakes or microbial mats in which these organisms typically occur. For example, a layer of *Chl. phaeobacteroides* occurs at the chemocline of the Black Sea at a depth of approx. 100 m. The light intensity at this depth is nearly 10^6 times less than at the surface and has been determined to be ca. 3 nmol photons m^{-2} s^{-1} (Overmann et al. 1992). Under these conditions, a single chlorophyll molecule will absorb one photon in a period of ca. 6 h. In order to survive under such low light intensities, green bacteria evolved highly specialized light-harvesting antennae, known as chlorosomes, which contain the largest numbers of chromophores known in nature. In *Chl. tepidum*, each chlorosome can contain more than 200×10^3 BChl *c* molecules (Martinez-Planells et al. 2002; Montaño et al. 2003a) and a cell can contain about 200–250 chlorosomes. Thus, a *Chl. tepidum* cell can contain up to 50×10^6 BChl *c* molecules, or ten times the estimated number of protein molecules per cell.

Overview of the *Chl. tepidum* and *Cfx. aurantiacus* genomes

The Institute for Genomic Research (Rockville, Md.) sequenced the genome of *Chl. tepidum* (Table 1; Eisen et al. 2002). The 2.15-Mbp *Chl. tepidum* genome comprises a single, circular DNA molecule and contains 2,288 assigned coding sequences, two rRNA operons (16S-23S-5S), 50 tRNAs, and two small stable RNAs. More than half of the proteins (1,217) were similar to proteins of known function and role category and another 98 proteins were similar to proteins of known function but unknown role category. Of the remaining 973 proteins of unknown function, the majority (680 proteins) encoded purely hypothetical proteins. Phylogenetic analyses of the genomic data support the hypothesis that the Chlorobi are closely related to the Bacteroidetes (Cytophaga–Flavobacteria–Bacteroides) grouping. Interestingly, a rather high percentage (12%) of the predicted proteins are most similar to proteins from archaeal species.

The Joint Genome Institute (JGI; Walnut Creek, Calif., USA) of the United States Department of Energy (DOE) has determined a draft genome sequence for *Cfx. aurantiacus* strain J-10-fl (Table 1). The genomic information presently resides on 1,142 contigs containing a total of 4.9 Mbp. Of these contigs, 541 are larger than 2 kb and contain a total of 4.3 Mbp. Based on this information, the total genome size is probably roughly 5 Mbp and perhaps even larger. The data are available from JGI (http://genome.jgi-psf.org/draft_microbes/chlau/chlau.download.html) or GenBank (accession number NZ_AAAH00000000). Because of the incomplete nature of the *Cfx. aurantiacus* data and the imprecise size-prediction for its genome, it is not possible to estimate reliably the number and nature of the coding sequences and stable RNA genes. Although the current genomic sequence information is sufficient for identifying many genes of interest, the fragmented nature of the data is a serious limitation in whole-organism analyses and comparative genomics. Efforts are therefore

Table 1 Comparison of *Chl. tepidum* and *Cfx. aurantiacus* and their genomes. Data are taken from Eisen et al. (2002), Frigaard et al. (2004c), and Garrity and Holt (2001b)

	Chl. tepidum strain TLS	*Cfx. aurantiacus* strain J-10-fl
Genome size (bp)	2,154,946	Estimated at ca. 5,000,000
Number of predicted coding sequences	2,288	Estimated at ca. 5,000
Number of rRNA operons	2	Estimated at 2
G + C content (mol%)	56.5	57
Optimum growth temperature	48°C	ca. 55°C
Growth mode	Photolithoautotrophic, strictly anaerobic	Photoheterotrophic or photoautotrophic under anoxic conditions; chemoheterotrophic under oxic conditions
Morphology	Rods (ca. 0.7×2.0 μm)	Filaments of indefinite length; individual cells are ca. 1×3 μm
Motility	None	Gliding
CO_2 fixation	Reductive tricarboxylic acid cycle	3-Hydroxypropionate cycle
Photosynthetic reaction center	Iron–sulfur type (photosystem I-like)	Quinone type (photosystem II-like)
Photosynthetic antennae	Chlorosomes, FMO protein	Chlorosomes, B800–B866 complex (LHI-like)
Major pigments under phototrophic conditions	BChl *c*, BChl *a*, chlorobactene	BChl *c*, BChl *a*, β-carotene, γ-carotene
Carotenoid biosynthetic pathway	CrtP/CrtQ/CrtH-dependent	CrtI-dependent
Gram-type; outer membrane (OM)	Gram-negative; OM present	Gram-negative; OM absent

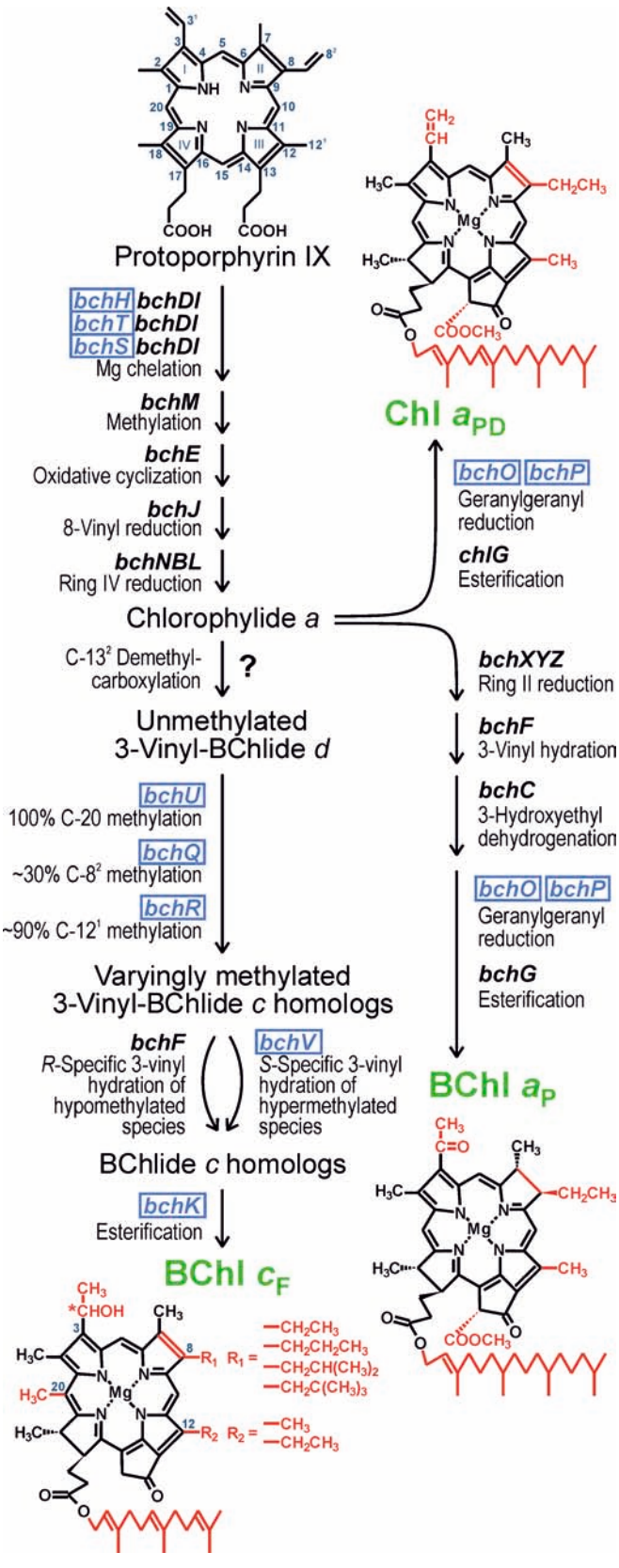

Fig. 2 Proposed biosynthetic pathways for BChl *c*, BChl *a*, and Chl *a* in *Chl. tepidum*. The structural features that differ between the three end products are indicated in *red*. Genes that have been inactivated are *boxed* and indicated in *blue*. See text for details

underway to produce a completely sequenced genome (R.E. Blankenship and J. Raymond, personal communication).

Chlorosome BChls are specialized antenna chlorophylls

Chlorosomes contain specialized chlorophyll derivatives not found elsewhere in nature: BChl *c* or *d* in green filamentous bacteria and BChl *c*, *d*, or *e* in green sulfur bacteria (Blankenship et al. 1995; Blankenship and Matsuura 2003). These BChls are unique by the presence of a C-3^1 hydroxyl group and by the absence of a C-13^2 methylcarboxyl group. These features are essential for the formation of the large BChl aggregates that form inside chlorosomes (Blankenship et al. 1995; Blankenship and Matsuura 2003). The chlorosome BChls are also unique by having methyl groups not found in other chlorophyll species. BChl *c* and *e* have a methyl group at the C-20 methine bridge of the tetrapyrrole ring. Additionally, the chlorosome BChls in green sulfur bacteria have one or more methyl groups in the C-8^2 and C-12^1 positions; and these methylations do not occur in *Cfx. aurantiacus*. The function of these methylations is not fully clear, but it is suspected that they help to control the physical size and to optimize the absorption properties of the BChl aggregates (see below). The protein-independent, self-aggregation properties of the chlorosome BChls probably enable the cells to accommodate physically and to afford energetically the extremely high BChl contents needed for growth at very low light intensities.

A recently characterized member of the "Chloroflexales," *Chloronema* sp. strain UdG9001, contains chlorosomes that contain both BChl *c* and *d* (Gich et al. 2003). Interestingly, the chlorosomes and the chlorosome BChls from this organism have features characteristic of both green sulfur bacteria (C-8^2, C-12^1 methylation of BChl *c*, *d*) and *Cfx. aurantiacus* (BChl *c*, *d* esterified with various alcohols, and an excitation energy transfer that is not very sensitive to the ambient redox potential).

Elucidation of the BChl *c* biosynthetic pathway

Chl. tepidum synthesizes three chlorophyll species: most is BChl *c* (about 97% of the total chlorophyll), mainly esterified with farnesol (BChl c_F). Also produced are

smaller amounts of BChl *a* (about 3%), esterified with phytol (BChl a_P), and an even smaller amount of Chl *a* (about 0.3%), esterified with Δ2,6-phytadienol (Chl a_{PD}; see distribution in Fig. 1). A large fraction, perhaps as much as 30%, of the carbon in these cells flows through the BChl and Chl biosynthetic pathways.

The determination of the genome sequence of *Chl. tepidum* revealed duplications of several genes known to be involved in Chl *a* and BChl *a* biosynthesis (Eisen et al. 2002). Inactivation of most of these genes in *Chl. tepidum* and subsequent pigment analyses of the resulting mutants successfully led to the identification of genes encoding enzymes involved in specific steps of BChl *c* biosynthesis (Frigaard et al. 2003, 2004a). Five of the identified genes specific for BChl *c* biosynthesis (*bchK*/*CT1992*, *bchQ*/*CT1777*, *bchR*/*CT1320*, *bchS*/*CT1955*, *bchV*/*CT1776*) are paralogous to enzymes functioning in BChl *a* biosynthesis; and one gene that is specific for BChl *c* biosynthesis (*bchU*/*CT0028*) is distantly related to an enzyme functioning in carotenoid biosynthesis in purple bacteria (Maresca et al. 2004). The *bchU* gene was initially recognized as a putative methyltransferase located upstream of *bchK* in *Cfx. aurantiacus* and, because of this location, was suggested to be involved in BChl *c* biosynthesis.

Based on the intermediates that accumulate in the various *bch* mutants of *Chl. tepidum*, a tentative BChl *c* biosynthetic pathway has been proposed (Frigaard et al. 2003, 2004a; Fig. 2). Like Chl *a*, Chl *b*, and BChl *a* biosynthesis, the proposed BChl *c* biosynthetic pathway branches at the level of chlorophyllide *a*. The first committed reaction is probably elimination of the C-13^2 methylcarboxyl moiety. However, no genes or enzymes involved in the proposed reaction(s) have yet been identified. This putative reaction(s) would convert chlorophyllide *a* to 3-vinyl-8-ethyl-12-methyl-BChlide *d*, which probably is the first intermediate specific to BChl *c* and *d* biosynthesis. This reaction could be performed by yet unidentified enzymes or be the consequence of a substrate channeling mechanism involving a BChl *c*-specific subunit of magnesium chelatase (BchS), which is the first devoted enzyme of any chlorophyll derivate biosynthesis (see below). Methylation at the C-20 position by the BchU methyltransferase of this (or a later) intermediate results in BChl *c* biosynthesis. A single methylation at the C-12^1 position by the BchR methyltransferase and one or more methylations at the C-8^2 position by the BchQ methyltransferase result in a mixture of homologues varying in their degree of methylation. Stereospecific hydration of the vinyl group at the C-3 position probably follows the C-8^2 methylations. One hydratase, BchF/CT1421, hydrates hypomethylated species (and the vinyl intermediate in BChl *a* biosynthesis) and produces *R*-specific stereochemistry at C-3^1. A paralogous hydratase, BchV/CT1776, which is BChl *c*-specific, hydrates hypermethylated species and produces *S*-stereochemistry at C-3^1. It is interesting to note that the *bchQ* and *bchV* genes are located next to each other but are divergently transcribed, since the substrate affinity of the BchV hydratase appears to correlate with the degree to which the substrates have been methylated by the BchQ methyltransferase. The final step is esterification with a long-chain alcohol by the BChl *c* synthase, BchK/CT1992.

Homologues of BchK (AAG15233), BchS (ZP_00017875), and BchU (ZP_00019713) are found in *Cfx. aurantiacus*. (The *bchK* gene in *Cfx. aurantiacus* was previously called *bchG2* or *bchGc* due to its homology with *bchG*; Niedermeier et al. 1994.) BChl *c* biosynthesis in *Cfx. aurantiacus* is thus likely to be identical to that in *Chl. tepidum* except for the absence of the BchR and BchQ methyltransferases. Unlike *Chl. tepidum*, *Cfx. aurantiacus* has two homologues of BchH, denoted BchH-I (AGG15206) and BchH-II (fusion of ZP_00017290, ZP_00020346), both of which share high sequence similarity with the BChl *a*-specific BchH homologues from other bacteria (Frigaard et al. 2004a). It is possible that this is a consequence of the different cyclases utilized in BChl *a* biosynthesis under anoxic and microoxic conditions (BchE, AcsF, respectively; see below). Like *Chl. tepidum*, *Cfx. aurantiacus* only has one homologue of each of the two other chelatase subunits, BchI (AAG15216/ZP_00020971) and BchD (ZP_00020972). No BchV homologue has been found in *Cfx. aurantiacus*, but this could be due to the incompleteness of the available genome sequence. Alternatively, BchF (ZP_00018459) in *Cfx. aurantiacus* could give rise to both *R*- and *S*-stereochemistry, or *Cfx. aurantiacus* could possess an unidentified C-3^1 epimerase. Finally, since the esterifying alcohols of BChl *c* in *Cfx. aurantiacus* are different and more diverse than in *Chl. tepidum*, the BChl *c* synthase in *Cfx. aurantiacus* may have a different affinity for long-chain alcohols.

Lessons from *Chl. tepidum* mutants deficient in specific steps of BChl *c* biosynthesis

Mutants deficient in BChl *c* biosynthesis have not only provided information on BChl *c* biosynthesis but have also provided information on the function and organization of BChl *c* in green sulfur bacteria. An extreme case is the *bchK* mutant of *Chl. tepidum* that completely lacks BChl *c* (Frigaard et al. 2002). This mutant is rusty-orange in color due to its carotenoids, has a growth rate about seven-fold slower than the wild type under limiting light, and forms vestigial chlorosome structures that we denote as carotenosomes. Carotenosomes can be isolated by sucrose gradient centrifugation and have been shown to contain carotenoids and BChl *a*, but are almost completely devoid of all usual chlorosome

proteins except CsmA (N.-U. Frigaard, H. Li, D.A. Bryant, unpublished data). 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.

Mutants lacking the normal C-8^2 (*bchQ*), C-12^1 (*bchR*), and C-20 (*bchU*) methylations of BChl *c* exhibit several interesting phenotypes. All combinations of single, double, and triple mutants have been made (Maresca et al. 2004; A. Gomez Maqueo Chew, J.A. Maresca, N.-U. Frigaard, D.A. Bryant, unpublished data). Under low light conditions, all mutants contain less BChl *c* or BChl *d* per cell than the wild type; and a *bchQ bchR* mutant produces chlorosomes that are much smaller than those of the wild type. The mutants also exhibit changes in the absorption properties of BChl *c* or BChl *d* aggregates in vivo, including a decreased molar absorptivity and a blue-shift and narrowing of the Q_y absorbance maximum. This is especially pronounced in the *bchQ* and *bchU* single mutants and most pronounced in the *bchQ bchR bchU* triple mutant. It appears that the methylation mutants are deficient in their ability to increase their cellular BChl *c* content under low light conditions. A possible explanation for this observation is that the altered intermediates in the pathway prevent an up-regulation of BChl *c* biosynthesis under low light conditions. Alternatively, the lack of methylation could prevent the formation of large BChl *c* aggregates, which in turn could prevent the accumulation of large amounts of BChl *c* in the cells. Whatever the reason, it appears that the methylation reactions greatly increase the efficiency of light harvesting under low light conditions. This is especially important for green sulfur bacteria, since they are most often found in extremely low light environments.

The first committed step in the biosynthesis of any BChl or Chl is the chelation of protoporphyrin IX with magnesium, a reaction that is catalyzed by a heterotrimeric magnesium chelatase. Whereas the genes (*bchD*/*CT1296*, *bchI*/*CT1297*) encoding the two smallest subunits of magnesium chelatase are present only in single copies, three paralogous genes (*bchH*/*CT1957*, *bchS*/*CT1955*, *bchT*/*CT1295*) are found to encode the largest, protoporphyrin-binding subunit. All three single mutants have been made and efforts to make all possible double mutants are in progress (A. Gomez Maqueo Chew, N.-U. Frigaard, D.A. Bryant, unpublished data). Mutants lacking *bchS* are severely deficient in BChl *c* and the BchS subunit thus appears to be fairly specific for BChl *c* biosynthesis. One interpretation of these results is that a substrate-channeling effect, controlled by the large subunit (BchH, BchS, or BchT) of the magnesium chelatase, causes the formation of a specific end-product (BChl *c*, BChl a_P, or Chl a_{PD}, which are synthesized in very different amounts). However, if this is the case, a small amount of spillover apparently occurs to allow a small amount of the other (B)Chls to be made in the absence of their committed large subunit. Alternatively, the observed specificity effects could result from differential inhibition of the activities of the three magnesium chelatases by downstream products of the three pathways.

Finally, a significant fraction (ca. 15%) of the BChl *c* in the *bchV* mutant of *Chl. tepidum* is the C-3 vinyl derivative of the highly methylated BChl *c* homologues. The C-3^1 hydroxy group in normal BChl *c* is essential for formation of the BChl *c* aggregates by its coordination to the central magnesium in neighboring BChl *c* molecules. As may thus be expected, the absorption spectrum of the pigments in the *bchV* mutant suggests a severe disruption of the BChl *c* aggregates and the presence of a large (more than 10%) fraction of monomeric BChl *c* species.

Carotenoid biosynthesis and function

Most of the carotenoids in *Chl. tepidum* are located in the chlorosomes, which contain about one carotenoid molecule per ten BChl *c* molecules (Frigaard et al. 1997). The major carotenoid in *Chl. tepidum* is chlorobactene, which is unusual in having an aromatic ϕ end group. Minor carotenoids are γ-carotene, 1′,2′-dihydrochlorobactene, 1′,2′-dihydro-γ-carotene, OH-chlorobactene, and the laurate esters of both OH-chlorobactene glucoside and OH-γ-carotene glucoside (Takaichi et al. 1997). Carotenoids have been shown to contribute both to light harvesting and to quenching BChl *a* and BChl *c* triplets efficiently in both *Chl. tepidum* and *Cfx. aurantiacus* (Melø et al. 2000; Carbonera et al. 2001). In brown-colored green sulfur bacteria, carotenoids were once thought to be responsible for the broad absorption band around 500 nm, but this absorption band is mainly due to the special properties of aggregated BChl *e* (Steensgaard et al. 2000).

Gene inactivation studies in *Chl. tepidum* have identified all of the biosynthetic steps leading to chlorobactene and OH-chlorobactene (Frigaard et al. 2004c; J.A. Maresca and D.A. Bryant, unpublished data). The first step in the pathway is catalyzed by phytoene synthase (CrtB/CT1386). The *crtB* mutant is thus completely devoid of carotenoids. The intermediate lycopene is synthesized from phytoene by the combined actions of two desaturases (CrtP/CT0807, CrtQ/CT1414) and a *cis–trans* isomerase (CrtH/CT0649). This CrtP/CrtQ/CrtH-dependent pathway has otherwise only been found in oxygenic phototrophs (cyanobacteria, algae, plants). A lycopene cyclase (CT0456), apparently similar to that found in some cyanobacteria, then forms γ-carotene (J.A. Maresca and D.A. Bryant, unpublished data). A

γ-carotene desaturase (CrtU/CT0323), similar to the CrtU enzymes found in actinomycetes, next forms chlorobactene. *Chl. tepidum* also possesses a carotenoid hydratase (CrtC/CT0301), an enzyme which forms OH-chlorobactene and OH-γ-carotene and is similar to the carotenoid hydratase found in purple bacteria. Lastly, it appears that chlorobactene and γ-carotene are saturated to their 1′,2′-dihydro derivatives by the concerted action of the same two enzymes (BchP/CT2256, BchO/CT1232) that reduce the esterifying isoprenoid alcohols of BChl a_P and Chl a_{PD} (A. Gomez Maqueo Chew, N.-U. Frigaard, D.A. Bryant, unpublished data). Further characterization of mutants lacking BchP and BchO is in progress.

Until recently, the functions of carotenoids in green bacteria could only be probed by using chemical inhibitors of carotenoid biosynthesis. This approach has the disadvantages that not all carotenoid biosynthesis is inhibited and the inhibitor may have secondary effects on cell physiology and morphology. A better approach is to analyze the various *crt* mutants of *Chl. tepidum* now available (*crtB*, *crtP*, *crtQ*, *crtH*, *crtU*, *crtC*; Frigaard et al. 2004c). Such analyses have shown that mutants lacking colored carotenoids (*crtB*, *crtP*, *crtQ*) have a significantly decreased BChl *a* content and decreased growth rates under both limiting and saturating light intensities (Frigaard et al. 2004c). Since only colored carotenoids can quench BChl triplets, it is possible that this phenotype is somehow due to excess formation of BChl triplets in the *crtB*, *crtP*, and *crtQ* mutants. Alternatively, colored carotenoids may serve a structural role in the assembly and stability of certain pigment–protein complexes in *Chl. tepidum*, as colored carotenoids appear to do in some pigment–protein complexes from purple bacteria and plants (Paulsen 1999).

The major carotenoids in *Cfx. aurantiacus* grown photosynthetically under anoxic conditions are β-carotene, γ-carotene, and fatty acid esters of OH-γ-carotene glucoside (Halfen et al. 1972; Takaichi 1999). BChl *c* synthesis and chlorosome biogenesis are inhibited under oxic conditions; and the carotenoids present are oxygenated to echinenone (4-oxo-β-carotene) and myxobactone (4-oxo-OH-γ-carotene glucoside) (Halfen et al. 1972; Pierson and Castenholz 1974b). A detailed in silico analysis of the putative carotenogenic enzymes encoded by the *Cfx. aurantiacus* genome has been performed (Frigaard et al. 2004c). Phytoene desaturation to all-*trans*-lycopene probably occurs by the classic bacterial CrtI-dependent reaction, since at least two CrtI homologues are present (ZP_00019255, ZP_00020402). Alternatively, *Cfx. aurantiacus* may employ a chimeric pathway similar to that found in the cyanobacterium *Nostoc* sp. PCC 7120, in which a CrtP phytoene desaturase (ZP_00019755), a CrtI-related ζ-carotene desaturase, and a *cis–trans* isomerase form all-*trans*-lycopene (Linden et al. 1994). However, no carotenoid *cis–trans* isomerase has yet been found in the incomplete *Cfx. aurantiacus* genome. Lycopene cyclization is catalyzed by a classic bacterial CrtY-type enzyme (ZP_00019083), which probably forms both γ-carotene (monocyclic) and β-carotene (dicyclic). Under oxic conditions, the 4-oxo-β end groups of the carotenoids are probably introduced by a cyanobacterium-like CrtO ketolase (fusion of ZP_00017980, ZP_00017981), rather than by the unrelated CrtW ketolase known from non-photosynthetic bacteria.

Chlorosome structure and biogenesis

Although their exact size varies with growth conditions, chlorosomes are rather large cellular organelles. A typical chlorosome from *Chl. tepidum* is about 100–200 nm long, 50 nm wide, and 20–30 nm high and contains about 200,000 BChl *c* molecules, 2,500 BChl *a* molecules, 20,000 carotenoid molecules, 15,000 chlorobium-quinone molecules, 3,000 menaquinone-7 molecules, and about 5,000 protein molecules (Bryant et al. 2002; Martinez-Planells et al. 2002; Montaño et al. 2003a; Frigaard et al. 2004b). With an estimated content of about 0.1 mol of total lipid per BChl *c*, a chlorosome also contains about 20,000 lipid molecules, which are roughly evenly distributed between polar lipids (predominantly galactosyldiacylglycerols) and wax esters (M. Miller, P.G. Sørensen, R.P. Cox, personal communication). The 6.2-kDa CsmA/CT1942, which binds one BChl *a* molecule and probably one or two carotenoid molecules per monomer (Sakuragi et al. 1999; Bryant et al. 2002; Montaño et al. 2003b), accounts for nearly half of the protein. In *Chl. tepidum*, nine other proteins are present in smaller amounts (CsmB/CT2054, CsmC/CT1943, CsmD/CT2064, CsmE/CT2062, CsmF/CT1046, CsmH/CT1417, CsmI/CT1382, CsmJ/CT0651, CsmX/CT0652; Fig. 1), but their roles are less well characterized. Only four structural motifs exist in chlorosome proteins. Single-locus mutants of *Chl. tepidum*, each of which lacks a single chlorosome protein except for CsmA, have been made (Frigaard et al. 2004b). Surprisingly, these mutants show very little or no phenotypic effects; this leads to the inevitable conclusion that chlorosomes are remarkably robust. The *csmC* mutant makes chlorosomes that are a significantly smaller than those of the wild type. In addition, the absorption maximum of the aggregated BChl *c* of chlorosomes of the *csmC* mutant is slightly blue-shifted, which is consistent with the presence of smaller BChl *c* aggregates.

Using chlorosomes isolated from wild-type and *csm* mutant strains, studies of chlorosome protein organization by chemical cross-linking provide insights into how these proteins interact in *Chl. tepidum* (Bryant et al.

2002; H. Li, N.-U. Frigaard, D.A. Bryant, manuscript in preparation). CsmA forms multimers and appears to form the paracrystalline structure that constitutes the baseplate (Bryant et al. 2002). CsmB interacts with preCsmA but not with the predominant, processed form of CsmA found in the paracrystalline structures; CsmB can also be cross-linked to CsmJ and CsmI. CsmC forms oligomers at least up to tetramers.

Three of the chlorosomes proteins in *Chl. tepidum* (CsmI, CsmJ, CsmX) are iron–sulfur proteins (Vassilieva et al. 2001). Cross-linking studies show that CsmI and CsmJ form homodimers and heterodimers, and thus probably form $CsmI_2CsmJ_2$ heterotetramers. The content of CsmX is about ten-fold lower than that of either CsmI or CsmJ. The redox midpoint potentials of each cluster have been determined in chlorosomes from wild type and mutants lacking one or two of these proteins (T.W. Johnson, H. Li, N.-U. Frigaard, D.A. Bryant, J.H. Golbeck, manuscript in preparation). In the wild type, CsmI and CsmJ have midpoint potentials of about −346 mV and +90 mV, respectively. However, in mutants lacking CsmJ or CsmX, the CsmI cluster has a midpoint potential of about −200 mV, and in mutants lacking CsmI or CsmX, the CsmJ cluster has a midpoint potential of about 0 mV. Thus, it appears that the interaction of CsmI and CsmJ affects the midpoint potential of their iron–sulfur clusters in a manner that is dependent on CsmX. A *csmI csmJ csmX* triple mutant is clearly defective in inactivating the redox-dependent BChl *c* fluorescence quencher in chlorosomes exposed to oxygen (H. Li, N.-U. Frigaard, D.A. Bryant, manuscript in preparation). The iron–sulfur proteins of chlorosomes may thus function in restoring energy transfer from the chlorosome to the reaction center after an oxic-to-anoxic transition by reducing the chlorosome-associated quencher, most likely chlorobiumquinone (Frigaard et al. 1997; Blankenship and Matsuura 2003; Fig. 1).

Chlorosomes from *Cfx. aurantiacus* are smaller than chlorosomes from *Chl. tepidum* and vary greatly in their dimensions and composition, depending on growth conditions. However, a typical chlorosome has about 50,000 BChl *c* molecules, 2,000 BChl *a* molecules, 15,000 carotenoid molecules, and 6,000 menaquinone-10 molecules (Frigaard et al. 1997). The 5.7-kDa protein CsmA (P09928) is the most abundant chlorosome protein and is present in a ratio ca. 1:1 with BChl *a* (Montaño et al. 2003b). Two additional proteins related to CsmC and CsmD in *Chl. tepidum* with unknown function are also present, 11-kDa CsmM (AAG15234) and 18-kDa CsmN (AAG15235). Protein analysis of isolated chlorosomes by SDS-PAGE and N-terminal sequencing of distinct protein bands combined with genome sequence information has potentially identified three additional chlorosome proteins: CsmO (AAN85641), CsmP (AAG15237), and CsmY (ZP_00017757; Frigaard et al. 2001). CsmO is similar to CsmB and CsmF of *Chl. tepidum*; and CsmY has a putative iron–sulfur cluster binding motif and strong sequence similarity to CsmX from *Chl. tepidum*. Based on its sequence similarity to CsmM, another potential chlorosome protein, CsmQ (ZP_00018889), has also been tentatively identified in the genome. Some genes encoding chlorosome proteins and enzymes involved in BChl *c* biosynthesis are clustered in the *Cfx. aurantiacus* genome: *csmMN* is located immediately downstream of *bchUK* and *csmP* is located further downstream in the operon that includes *csmMN* (Frigaard et al. 2001).

Essentially nothing is known about chlorosome biogenesis. Interestingly, similar genes are adjacent to the *csmA* genes of both *Chl. tepidum* and *Cfx. aurantiacus*; and these flanking genes may be involved in chlorosome biogenesis (Frigaard et al. 2001). A TolC-EmrA-EmrB-like transport system may also be involved in chlorosome biogenesis because traces of EmrA-like and EmrB-like proteins (CT0105, CT0104) were identified in carotenosomes (chlorosomes devoid of BChl *c*) from *Chl. tepidum* (Frigaard et al. 2003; depicted as a putative transporter in Fig. 1). In *Cfx. aurantiacus*, an *emrB* homologue (ZP_00017873) is located immediately downstream of *bchS*. As *bchS* appears to be specific for BChl *c* biosynthesis in *Chl. tepidum* (see above) and thus essential for biogenesis of mature chlorosomes, this gene clustering in *Cfx. aurantiacus* may imply that the *emrB* homologue is involved in chlorosome biogenesis.

Adaptations in *Cfx. aurantiacus* to both oxic and anoxic life

Cfx. aurantiacus can grow under both oxic and anoxic conditions and it has been demonstrated that transcription in this organism is modified by changes in sigma factor expression that are modulated by O_2 tension (Gruber and Bryant 1998). For example, production of the photosynthetic apparatus is only induced under anoxic or microoxic conditions, whereas the respiratory system is presumably largely or completely repressed under anoxic conditions.

In agreement with the ability to grow under different O_2 tensions, the *Cfx. aurantiacus* genome in some cases encodes distinctly different enzymes to catalyze the same reaction in the presence or absence of O_2. For example, both O_2-dependent (HemF/ZP_00017864) and O_2-independent (HemN/ZP_00017183) coproporphyrinogen III oxidases are encoded in the genome for tetrapyrrole biosynthesis. Among the enzymes that are specific for BChl biosynthesis, both an O_2-dependent (AcsF/ZP_00017291; Ouchane et al. 2004) and an O_2-independent (BchE/AAG15204) magnesium protoporphyrinogen IX monomethyl ester cyclase are encoded. HemF and AcsF are both O_2-dependent enzymes containing

di-iron centers; and HemN and BchE are both O_2-independent (and probably O_2-sensitive) radical-SAM enzymes containing iron–sulfur clusters. Although both O_2-dependent and O_2-independent protoporphyrinogen IX oxidases are known to occur, *Cfx. aurantiacus* apparently possesses only a single type of protoporphyrinogen IX oxidase (HemY; probably a fusion of ZP_00017182, ZP_00018422). There are two possible explanations for this observation. One possibility is that the HemY protein of *Cfx. aurantiacus* is both O_2-independent and O_2-insensitive and thus functions under both anoxic and oxic conditions. The second possibility is that HemY only functions under anoxic conditions, and that under oxic conditions, the HemF coproporphyrinogen III oxidase is responsible for the oxidation of both coproporphyrinogen III and protoporphyrinogen IX, as reported for *Escherichia coli* (Narita et al. 1999). The *hemY* gene of *Cfx. aurantiacus* is adjacent to and probably cotranscribed with the *hemN* gene, which should only be expressed under anoxic conditions because of its O_2-sensitivity. Thus, the second possibility, that HemF has dual oxidase activities, seems most probable.

The presence of both O_2-dependent and O_2-independent oxidative cyclases is consistent with the observation that, although BChl *c* biosynthesis is completely inhibited under oxic conditions, a very low level of BChl *a* biosynthesis persists under microoxic conditions (Pierson and Castenholz 1974b; Garrity and Holt 2001b). This may allow the organism to more rapidly adapt to phototrophic growth during an oxic to anoxic transition. Interestingly, the genome also encodes two homologues of the large subunit of magnesium chelatase, BchH-I and BchH-II, which are similar to BchH homologues from BChl *a*-producing organisms. (*Cfx. aurantiacus* actually encodes a total of three homologues of the large subunit of magnesium chelatase; and the third is BchS, which in *Chl. tepidum* is BChl *c*-specific. See above.) BchH-I is probably used for BChl *a* biosynthesis under anoxic conditions and BchH-II for BChl *a* biosynthesis under microoxic conditions, since the *bchH-II* gene appears to be part of an operon that includes *acsF*. The co-expression of the *acsF–bchH-II* genes under oxic conditions could compensate for O_2-mediated repression of *bchH-I*. Alternative, although magnesium chelatase probably is not O_2-sensitive, different large subunits of magnesium chelatase may be needed under oxic and anoxic conditions if the oxidative cyclases are different and the chelatase and the cyclase interact as a result of substrate channeling involving the magnesium chelatase (see above).

Recently, Blankenship and co-workers identified two multi-subunit molybdopterin oxidoreductase complexes containing cytochrome *c* in *Cfx. aurantiacus*: one of these is expressed under oxic conditions and the other under anoxic conditions (Yanyushin et al. 2004). Since *Cfx. aurantiacus* lacks a cytochrome bc_1-like complex, these researchers proposed that one complex functions in photosynthetic electron transfer under anoxic conditions and that the other functions in respiratory electron transfer under oxic conditions.

Evolution of the photosynthetic apparatus in green bacteria

The observations that all identified enzymes specific to BChl *c* biosynthesis, except *bchU*, are related to enzymes in BChl *a* biosynthesis and that BChl *c* is dispensable in *Chl. tepidum*, strongly suggest that BChl *c* biosynthesis evolved from BChl *a* biosynthesis by gene duplication and subsequent divergence. This may have happened in an organism that initially had BChl *a*-based photosynthesis and then evolved under strong selective pressure to grow in a low light environment.

BChl *c* biosynthesis in *Chl. tepidum* and *Cfx. aurantiacus* is obviously related (see above). The protein compositions of the chlorosomes in these two organisms are also distantly related (see above). As most other cellular components in these bacteria are very different, it seems quite likely that the ability to produce chlorosomes was horizontally transferred between ancestors of present-day green sulfur bacteria and filamentous anoxygenic bacteria. In phylogenetic analyses of the BChl *a* biosynthesis enzymes from all known groups of photosynthetic organisms, the enzymes from *Chl. tepidum* and *Cfx. aurantiacus* also group together (Xiong et al. 2000), although such analyses can be difficult to evaluate (Green and Gantt 2000). Other components of the photosynthetic apparatus (reaction centers, accessory light-harvesting antennae, carotenoid biosynthesis enzymes; see Table 1) are clearly not related and are sometimes more related to components in other groups of photosynthetic organisms. This illustrates the mosaic evolutionary nature of the photosynthetic apparatus in photosynthetic organisms, as discussed in detail by others (Raymond et al. 2002).

Inorganic sulfur metabolism

Reduced inorganic sulfur compounds are the primary electron donors for photosynthetic carbon fixation in green sulfur bacteria (Garrity and Holt 2001a). *Chl. tepidum* grows on sulfide, thiosulfate, or elemental sulfur and is thus a good model for studying the inorganic sulfur metabolism in green sulfur bacteria. As no genome sequence is yet available for any purple sulfur bacterium, *Chl. tepidum* may serve as a model for the anoxygenic sulfur-oxidizing phototrophs in general. Little information is available on the initial oxidation

reactions and virtually nothing is known about how *Chl. tepidum* produces and then subsequently consumes the sulfur globules that form when excess sulfur sources are available.

At least two pathways appear to exist for the oxidation of sulfide in the periplasm: flavocytochrome *c* sulfide dehydrogenase (SoxEF/CT2080–2081) and sulfide–quinone reductase (SQR). A total of three paralogues of the gene encoding the sulfide-binding flavoprotein subunit of flavocytochrome *c* (SoxF) occur in *Chl. tepidum* (*CT1015*, *CT1025*, *CT2081*); and the two extra SoxF homologues may function in other sulfane-transfer reactions including the Sox system for thiosulfate oxidation (see below). Three paralogues of the *sqr* gene also occur in *Chl. tepidum* (*CT0117*, *CT0876*, *CT1087*). Interestingly, none of the encoded proteins contains all three cysteine residues currently thought to be conserved and essential for sulfide oxidation by SQR (Griesbeck et al. 2002).

Some of the *sox* genes involved in thiosulfate oxidation in *Paracoccus pantotrophus* are also present in *Chl. tepidum*: important exceptions include *soxC* and *soxD*, which in *P. pantotrophus* are essential for thiosulfate oxidation, probably by regenerating the sulfane-carrying SoxYZ (CT1017–1018) protein (Bamford et al. 2002). Thiosulfate oxidation in *Chl. tepidum* must thus occur by an alternative mechanism possibly involving a SoxF homologue and with a presumed transfer of the sulfane sulfur to a polysulfide intermediate. Preliminary experiments have shown that a mutant lacking soluble cytochrome c_{554} (CT0075) is capable of, but impaired in, growth on thiosulfate as the sole electron donor, thus suggesting a role of this cytochrome as electron acceptor in thiosulfate oxidation (Y. Tsukatani and H. Oh-oka, personal communication). The RuBisCO-like protein (RLP) of *Chl. tepidum* (CT1772) has also been implicated in thiosulfate metabolism, since a mutant lacking this protein cannot oxidize thiosulfate (Hanson and Tabita 2001, 2003).

Several enzymes may be involved in sulfur globule formation and degradation, since more or less complete duplications exist of genes encoding putative polysulfide reductases, heterodisulfide reductases, and sulfur reductases. At least one of the polysulfide reductases should have its active site positioned in the periplasm because its subunits carry signal peptides. However, whether these proteins are involved in inorganic sulfur metabolism remains to be established. In any case, there clearly must be more and novel enzymes involved in sulfur globule metabolism.

Some members of the "Chloroflexales" can utilize sulfide for growth but, although this may be important in some natural environments, sulfide is usually not the preferred substrate under laboratory conditions (Garrity and Holt 2001b). *Cfx. aurantiacus* oxidizes sulfide for phototrophic growth and deposits elemental sulfur outside the cell as do green sulfur bacteria (Madigan and Brock 1975). No sulfide-utilizing enzyme has been identified in *Cfx. aurantiacus*. However, the genome encodes homologues of two putative sulfide-binding flavoproteins related to SQR (ZP_00018906) and SoxF (ZP_00020895) that may be involved in sulfide oxidation. No close homologues of the SoxE (subunit of flavocytochrome *c* sulfide dehydrogenase) and SoxA cytochromes related to sulfide utilization in other organisms are found in the genome.

Future directions: more genomes to come

Although much progress has been achieved in a short time period with the limited genome sequence data available, a deluge of data for the Chlorobi will soon become available, thanks to JGI/DOE. The sequence of the epibiont of the phototrophic consortium "*Chlorochromatium aggregatum*" was recently completely determined (see http://genome.jgi-psf.org/draft_microbes/chlag/chlag.home.html) and annotation of the data is now in progress (T. Li, D.A. Bryant, J. Overmann, unpublished data). The genome of the free-living epibiont, provisionally named *Chl. chlorochromatii*, is a single circular DNA molecule of 2.57 Mbp with a G + C content of 44.3 mol%. A preliminary analysis of the sequence data indicates that approximately 1,500 genes are shared between *Chl. tepidum* and *Chl. chlorochromatii*. Assuming that sufficient DNA can be obtained from the central, rod-shaped β-proteobacterium of the consortium, the genome sequence of this partner organism will also be determined. Efforts to obtain sufficient DNA for library construction are in progress. It can be anticipated that this information will provide both an insight into the nature of the symbiosis and the means by which these two organisms communicate to coordinate their cell division and achieve phototaxis.

In order to survey the genetic diversity of the green sulfur bacteria even more broadly, draft sequences of the genomes of eight additional green sulfur bacteria will be determined by JGI/DOE in the coming year. The organisms selected for sequence analysis include seven well described type strains that span the known physiological diversity within the Chlorobi. The strains to be sequenced are *Chl. limicola* DSM 245^T (the type strain for the entire group), *Prosthecochloris aestuarii* DSM 271^T; *Chl. vibrioforme* subsp. *thiosulfatophilum* DSM 265, *Pelodictyon luteolum* DSM 273^T, *Chl. phaeobacteroides* DSM 266^T, *Pel. phaeoclathratiforme* DSM 5477^T, and *Chloroherpeton thalassium* ATCC 35110^T. The genome of one environmental isolate, a *Chl. phaeobacteroides* strain that was recently isolated for a second time by J. Overmann from 100 m below the surface of the Black Sea (Overmann et al. 1992), will also be

sequenced. These data will provide an unparalleled and in-depth view of the genetic diversity (or lack thereof) in the green sulfur bacteria and should provide the data necessary for detailed comparative genomics studies that should definitively answer questions about the taxonomic and phylogenetic relationships of these organisms. It can be anticipated that this information will also provide the necessary background information for the development of approaches to characterize thoroughly the green sulfur bacteria in their natural habitats. The availability of all of this data should help to provide answers to many fundamental questions concerning the physiology and metabolism of the green sulfur bacteria. This data set may also provide novel insights into broader issues such as genome evolution in organisms routinely subjected to energy limitation.

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