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Pigment-protein diversity in chlorosomes of green phototrophic bacteria

John F. Stolz, R. Clinton Fuller*, and Thomas E. Redlinger

Department of Biochemistry, University of Massachusetts, Amherst, MA 01003, USA

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Abstract. In order to compare and contrast the structure and function of the light-harvesting antennae (i.e. chlorosomes) of green bacteria, a procedure for isolating and characterizing them from green sulfur bacteria was developed. The chlorosomes from *Chlorobium* species with bacteriochlorophyll (Bchl) *c* or *e* were isolated by a two step sucrose density centrifugation in the presence of 2% miranol, a mild detergent, and 2 M sodium thiocyanate (NaSCN). Purified chlorosomes from two green sulfur bacteria, *Chlorobium phaeobacteroides* and *Chlorobium tepidum*, and the filamentous green bacterium *Chloroflexus aurantiacus* were analysed by spectrophotometry, SDS-polyacrylamide gel electrophoresis, and immunological procedures. Isolated chlorosomes from both *Chlorobium* species contain only two electrophoretically separable protein components with approximate molecular masses of 5–7.5 and 34.5 kDa. In addition, they have a major light-harvesting antenna pigment (Bchl *c* or *e*), a minor Bchl *a* species, and carotenoids. *Chloroflexus aurantiacus* antisera for the three major chlorosome proteins (5.6, 11, and 18 kDa), and the reaction center proteins (24 and 24.5 kDa) did not cross react with any *Chlorobium* proteins analyzed in this study. *Chlorobium limicola* f. *thiosulfatophilum* antisera against the 7.5 kDa chlorosome protein cross reacted strongly with the 5–7.5 kDa protein from *Cb. tepidum*, weakly with the *Cb. phaeobacteroides* protein, but not at all to the 5.6 kDa chlorosome protein from *Cf. aurantiacus*. These results provide further evidence for the evolutionary divergence of the chlorosomes from green phototrophic bacteria (e.g., *Chlorobium*-type and *Chloroflexus*-type).

Key words: Chlorosome – Green phototrophic bacteria – Bacteriochlorophylls a, c, and e – *Chlorobium phaeobacteroides* – *Chlorobium tepidum* – *Chlorobium limicola* – *Chloroflexus aurantiacus*

Offprint requests to: R. C. Fuller

Abbreviations: Cb.: *Chlorobium*; Cf.: *Chloroflexus*; Bchl.: bacteriochlorophyll; NaSCN: sodium thiocyanate; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The green phototrophic bacteria are a taxonomically diverse group of prokaryotes which have in common a similar extramembraneous light-harvesting antenna, the chlorosome (Staehlin et al. 1978, 1980). They have been divided into two families, the Chlorobiaceae or green sulfur bacteria, and the Chloroflexaceae (Trüper 1987). The Chlorobiaceae are obligate anaerobic photoautotrophs, while the Chloroflexaceae are facultative photoheterotrophs capable of growing autotrophically in the light (anaerobically) or heterotrophically in the dark (aerobically) (Castenholz and Pierson 1981). All contain bacteriochlorophyll (Bchl) *a* in the reaction center and as accessory pigments, and the major light harvesting pigment may be Bchl *c*, *d*, or *e* (Trüper and Pfennig 1981).

There is a marked difference in the pigment content, biochemistry and photochemistry of Bchl *c* containing chlorosomes from the Chlorobiaceae (Schmidt 1980; Gerola and Olson 1986; Wullick and van Bruggen 1988) and the Chloroflexaceae (Schmidt 1980; Feick and Fuller 1984). As a result, several models have been proposed for their molecular topology (Wechsler et al. 1985; Gerola et al. 1988; Brune et al. 1987; Blankenship et al. 1988). In the Feick and Fuller model (1984), it was suggested that chlorosomes of *Chloroflexus aurantiacus* contain two structural polypeptides (11 and 18 kDa) located in the chlorosome envelope and a 5.6 kDa protein situated internally and associated with the Bchl *c*. In their model, seven Bchl *c* molecules are arranged in an aggregated manner, wrapping around the outside of a single 5.6 kDa polypeptide, stabilized by hydrogen bonding between the porphyrin Mg and a charged amino acid. This is in contrast to the models proposed by Olson (1988) and Blankenship et al. (1988), where a pigment-pigment aggregation of Bchl (Mg-hydroxyl bond) forms the primary structure which is stabilized by protein. The chlorosome

is attached to the cytoplasmic membrane (containing the reaction center) by a structure (baseplate, Redlinger and Fuller 1985; attachment site, Olson 1988) which in *Cf. aurantiacus* contains a 5.8 kDa protein-Bchl *a* (792 nm) energy transfer protein (Betti et al. 1982; Redlinger and Fuller 1985).

The chlorosomes of *Chlorobium limicola* f. *thiosulfatophilum* have been reported to contain a Bchl *c* associated protein (4–5 kDa) and, in addition, small amounts of proteins with Mr 9, 20, 26, 27, 32, and 38 kDa (Gerola et al. 1988). More recently, the small molecular mass protein (4–5 kDa) has been resolved by high pressure liquid chromatography (HPLC) into at least two proteins, of molecular masses 6.3 kDa and 7.5 kDa (Wagner-Huber et al. 1988; Olson et al. 1990). The chlorosomes also contain Bchl *c* and Bchl *a* (794 nm; Gerola and Olson 1986) and are associated with the cell membrane by a water soluble Bchl *a*-protein complex which absorbs at 808 nm (Olson 1980; Gerola and Olson 1986). The water soluble Bchl *a* associated protein is present as a trimer, and has a monomer molecular mass of 40 kDa (Fenna and Matthews 1975; Matthews et al. 1979; Olson et al. 1976).

We report here on an improved technique for isolating and purifying chlorosomes from *Chlorobium* species and the results of our study of two species. We were interested in finding out if any of the *Chlorobium chlorosome* proteins were similar to those from *Chloroflexus*. Chlorosomes from *Cb. phaeobacteroides* and *Cb. tepidum* were isolated and compared to chlorosomes from *Cf. aurantiacus*, by spectral, SDS-PAGE, and western blot analyses. *Cb. phaeobacteroides* was chosen because of its unique absorption spectrum. It contains Bchl *e*, a formyl substituted bacteriochlorophyll (on ring II of the porphyrin; Gloe et al. 1975), and has an in vivo absorption maximum of 714–725 nm, the shortest found to date in green bacteria (Pfennig and Trüper 1981). *Cb. tepidum* was selected because it is a mild thermophile (42° C, Madigan et al., unpublished data) and intermediate between mesophilic *Chlorobium* species (22° C) and thermophilic *Cf. aurantiacus* (55° C). Neither strains have had their chlorosomes examined before.

Materials and methods

Organisms and culturing. The marine strain of *Chlorobium phaeobacteroides* was isolated from a mangrove swamp at Sugarloaf Key, Florida (USA) and grown at 22° C in Pfennig's medium (Trüper and Pfennig 1981), supplemented with sodium acetate (1 g/l), 2% NaCl and 7.6 mM sulfide. It is an obligate anaerobe which uses sulfide, but not thiosulfate. The strain of *Chlorobium tepidum* was obtained from M. T. Madigan (Southern Illinois University, Carbondale) and maintained on Pfennig's medium with 7.6 mM sulfide and 8 mM thiosulfate, at 42° C. *Chloroflexus aurantiacus*, strain J-10-fl, was grown at 55° C in medium D (Castenholz and Pierson 1981).

Chlorosome isolation. Chlorosomes were prepared by the method described in Feick et al. (1982) with modification. Harvested cells were resuspended in 10 mM *Tris* with 2 M NaSCN (Gerola and Olson 1986). The isolation buffer used for the marine strains also contained 2% NaCl. Cells were lysed by three passages through a French Pressure cell (16,000 Pa). DNase (1 mg/10 ml) was added after the first passage and phenylmethyl sulfonyl fluoride (0.2 M in 100% ethanol, 6 µl/ml) after the last. Whole cells and debris were removed by centrifugation at 18,000 × *g* for 20 min and the lysed cells were pelleted by centrifugation at 200,000 × *g* for 90 min. The supernatant from the 200,000 × *g* was decanted and placed on ice in the dark. The pellet was resuspended by homogenation in 10 mM *Tris* with 2 M NaSCN and the concentration was adjusted to an O.D. of 200 at 714 nm (Bchl *e*) for *Cb. phaeobacteroides*, 750 nm (Bchl *c*) for *Cb. limicola*, and 740 nm (Bchl *c*) for *Cf. aurantiacus*. The suspensions were treated with 2% miranol (final vol) for 30 min on ice, loaded onto a sucrose density gradient (15%, 23%, 34.5% w/v) and centrifuged at 200,000 × *g* for 3 h. Bands were collected, diluted in buffer, and pelleted by centrifugation at 200,000 × *g* for 1 h. The pellets were resuspended in buffer and stored at −10° C until analysis. For purification, a second sucrose density gradient was run after pretreatment with 2% miranol.

Pigment analysis. Absorption spectra for all fractions were recorded on a Cary 2300 spectrophotometer. Pigments were extracted using 100% methanol with the addition of 1% $MgCl_2$.

SDS-Polyacrylamide gel electrophoresis. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE), was carried out essentially according to the methods in Laemmli (1970); 1.5 mm or 0.5 mm (mini) thick gels with a 5% stacking gel and a 11.25%–16.88% gradient gel were used. Samples were first precipitated with trichloroacetic acid (10% final concentration), rinsed, and then heated for 30 min at 60° C in sample buffer containing 10% SDS, mercaptoethanol, glycerol and bromphenol blue in 0.5 M *Tris* (pH 6.8). Each lane was loaded with approximately 30 µg of total protein. Gels were run at constant voltage (200 V), and stained with either Coomassie blue or silver (RAPID-Ag-STAIN kit, ICN, Biomedical, Irvine, Calif., USA). Low molecular mass markers (Bio-Rad, Richmond, Calif., USA) were used (rabbit muscle phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; hen egg white ovalbumin, 42.7 kDa; bovine carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; hen egg white lysozyme, 14.4 kDa).

Western blot analysis. Western blot analyses of chlorosomal proteins were performed as previously described (Redlinger and Fuller 1985). Proteins were separated on 0.5 mm thick gradient (11.25–16.85%) minigels (7 × 8 cm, Mini Protean II, Bio-Rad) and blotted onto nitrocellulose (Mini Trans-blot, Bio-Rad). Primary antibodies used in this study were to the three chlorosome proteins (5.6, 11, 18 kDa) and the reaction center subunits (24 and 24.5 kDa) from *Cf. aurantiacus* and the 7.5 kDa chlorosome protein from *Cb. limicola* f. *thiosulfatophilum* (a kind gift from J. M. Olson, Odense University, Denmark).

Results

Chlorobium phaeobacteroides chlorosomes

Spectral analysis of *Cb. phaeobacteroides* cells indicated the presence of Bchl *e* (714 nm), Bchl *a* (808 nm), and carotenoids (Fig. 1A). Based on absorption spectra, the carotenoids are isorenieratene (513 nm) and chlorobactene (462) (Gloe et al. 1975). After cell lysis and differential centrifugation, a water soluble Bchl *a* (808 nm) was present in the supernatant of the first 200,000 × *g* spin (Fig. 1B). After electrophoresis of this fraction, several major proteins were observed, including a 40 kDa protein (Fig. 3A) which corresponded in molecular mass to the water soluble Bchl *a* protein reported for other green sulfur bacteria (Fenna and Matthews 1975; Matthews et al. 1979; Olson et al. 1976).

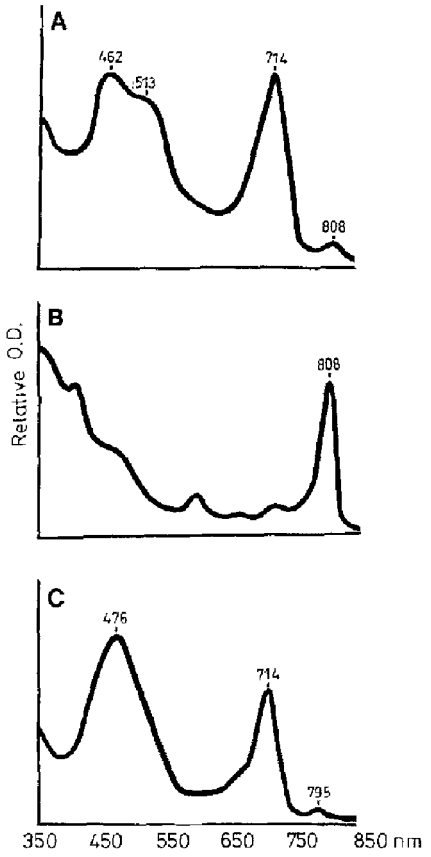

Fig. 1A–C. Absorption spectra of *Chlorobium phaeobacteroides*. **A** whole cells, showing Bchl *e* (714 nm), Bchl *a* (808 nm), and carotenoids chlorobactene (462 nm) and isoreneiratene (513 nm) **B** supernatant of the first 200,000 × *g* spin containing the water soluble Bchl *a* (808 nm) and **C**) purified chlorosomes with Bchl *e* (714 nm), energy transfer Bchl *a* (795 nm) and isoreneiratene (476 nm)

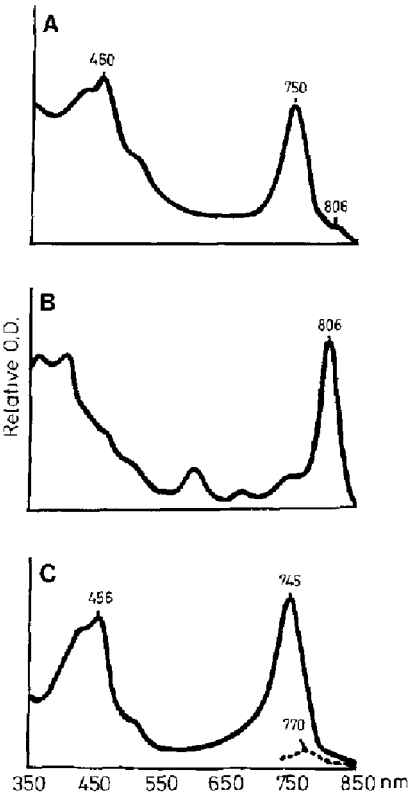

Fig. 2A–C. Absorption spectra of *Chlorobium tepidum*. **A** whole cells with Bchl *c* (750 nm), Bchl *a* (808 nm) and chlorobactene (460 nm), **B** supernatant of the first 200,000 × *g* spin containing the water soluble Bchl *a* (806 nm) and **C**) purified chlorosomes with Bchl *c* (745 nm) and carotenoid (456 nm). The *dotted line* in **B** shows the absorbance maximum at 770 nm of the Bchl *a* species in methanol

Cb. phaeobacteroides chlorosomes were purified by two sucrose gradient centrifugations. After the first sucrose density gradient, the partially purified chlorosomes examined by SDS-PAGE, consistently yielded eight proteins of molecular masses 55, 41, 34.5, 30, 27, 25, 21.5, and 5–7.5 kDa. After further purification on a second sucrose density gradient (after miranol treatment), only two electrophoretic bands, one at 34.5 kDa and one at 5–7.5 kDa, were detected (Fig. 3B). Spectral analyses of the purified chlorosomes showed major peaks at 714 nm (Bchl *e*) and 476 nm (carotenoid) and a minor peak at 795 nm (Fig. 1C).

Chlorobium tepidum chlorosomes

Spectral analysis of *Cb. tepidum* cells revealed a pigment composition consisting of Bchl *c* (750 nm), Bchl *a* (806 nm) and the carotenoid (460 nm) corresponding to chlorobactene (Fig. 2A). Cell fraction by differential centrifugation (as described above) resulted in the partial purification of a water soluble Bchl *a* (806 nm) in the supernatant (Fig. 2B). As in the case of *Cb. phaeobacteroides*, electrophoresis of this soluble fraction indicated several protein species, one of which was the 40 kDa protein.

Chlorobium tepidum chlorosomes were also purified by two sucrose density gradient centrifugations. The first sucrose density gradient was not sufficient to yield pure chlorosomes. When this fraction was analyzed by SDS-PAGE, it was found to contain proteins of molecular masses 34.5, 30, 21.5 and 5–7.5 kDa. After this fraction was subjected to the second sucrose density gradient centrifugation, electrophoresis revealed, as in the case of *Cb. phaeobacteroides*, that these chlorosomes contained only two protein bands, 34.5 kDa and 5–7.5 kDa (Fig. 3C). Spectral analysis indicated that the purified chlorosomes had absorption maxima at 745 nm (Bchl *c*) and 456 nm (carotenoid) (Fig. 2C). The presence of a minor Bchl *a* species in these chlorosomes is revealed only after methanol extraction. This converts both Bchl *c* and Bchl *a* to their monomeric forms which then can be distinguished by spectral analysis (Fig. 2C).

Western blot analyses

Purified chlorosomes of *Cb. phaeobacteroides* and *Cb. tepidum* were analyzed immunologically for relatedness to *Cf. aurantiacus* chlorosome proteins (5.6, 11, and

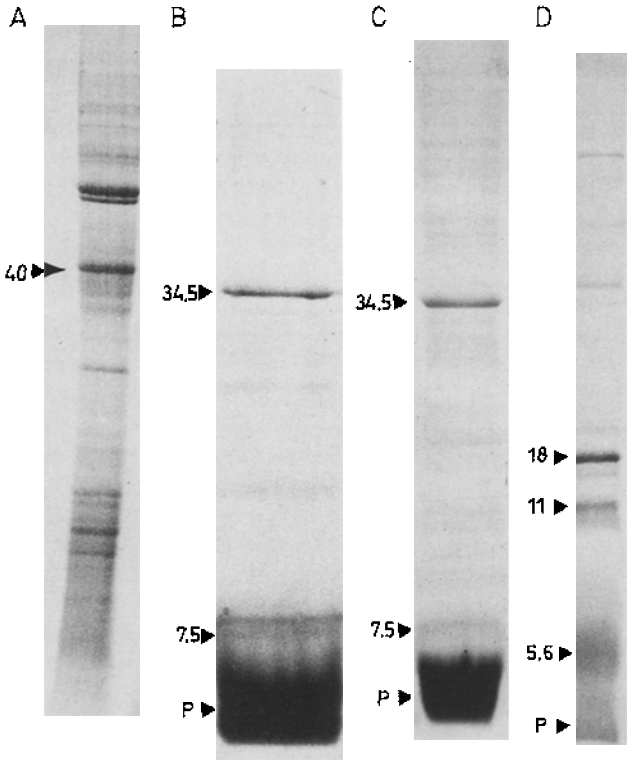

Fig. 3A – D. SDS-PAGE of **A** 40 kDa polypeptide, presumed to be the subunit of the water soluble Bchl *a* pigment-protein complex from *Chlorobium phaeobacteroides*, and purified chlorosomes from **B** *Cb. phaeobacteroides*, **C** *Chlorobium tepidum*, and **D** *Chloroflexus aurantiacus*

18 kDa) and the reaction center proteins (24, 24.5 kDa). Western blot analyses using *Chloroflexus* antisera showed no cross reaction (Fig. 4). Chlorosomes were also tested with antisera to the 7.5 kDa chlorosome protein from *Cb. limicola* f. *thiosulfatophilum*. Results of these western blots indicate strong cross reaction to the 5–7.5 kDa protein from *Cb. tepidum* but only weak cross reaction to *Cb. phaeobacteroides* (Fig. 4E). This antisera did not cross react with any chlorosome proteins from *Cf. aurantiacus*.

Discussion

By using an isolation buffer containing both Miranol (Feick et al. 1982) and 2 M NaSCN to stabilize the complex (Gerola and Olson 1986), we were able to purify *Chlorobium chlorosomes* after two sucrose density gradient centrifugation steps. In working with marine strains, it was also found that NaCl, although important for harvesting cells intact, was not neccessary after the cells were lysed and in fact deleterious to chlorosome isolation (i.e., it caused protein and pigment aggregation). We also discovered that a major advantage of using brown colored strains of *Chlorobium* is that both the water soluble Bchl *a* species (808 nm) and the energy transfer Bchl *a* species (795 nm) are readily detectable in whole cells and chlorosomes respectively.

All *Chlorobium* species that we analyzed electrophoretically (including a marine strain of *Chlorobium* with Bchl *c*, Stolz, data not shown) had only two major protein staining bands of molecular masses 34.5 kDa and 5–7.5 kDa. No other proteins were detected even after silver staining. The additional proteins seen with SDS-PAGE by Gerola and Olson (1986) when they isolated *Cb. limicola* f. *thiosulfatophilum* chlorosomes may be due to attachment site and reaction center complexes adhering to the chlorosome. Furthermore, the 5–7.5 kDa band could not be resolved into two bands (e.g., 6.3 and 7.5 kDa) even when gels designed to separate low molecular mass proteins (Burr and Burr 1983; Schägger and von Jagow 1987) were used (data not shown).

The strong antigenic reaction for *Cb. limicola* (J. M. Olson, personal communication) and *Cb. tepidum* indicates that these two 7.5 kDa proteins are antigenically similar. The weaker reaction observed for *Cb. phaeobacteroides* suggests greater evolutionary divergence at the primary amino acid level. The lack of cross reaction of the antisera to the 7.5 kDa protein with any chlorosome proteins from *Cf. aurantiacus* (including the 5.6 kDa protein) also suggests a greater evolutionary divergence in chlorosome proteins. These immunological results are supported by the sequence data determined for the 5.6 kDa (Wechsler et al. 1985), 7.5 kDa (Gerola et al. 1988) and 6.3 kDa (Wagner-Huber et al. 1988) proteins. The 7.5 kDa protein from *Cb. limicola f. thiosulfatophilum* has only a 17.6% similarity to the protein sequence of the 5.6 kDa protein from *Cf. aurantiacus* (Gerola et al. 1988). Wagner-Huber et al. (1988) have isolated and sequenced the 6.3 kDa Bchl-associated protein from *Cb. limicola* and three other species of green sulfur bacteria, *Pelodictyon luteum* (Bchl *c*), *Prosthecochloris aestuarii* (Bchl *c*), and *Chlorobium phaeovibrioides* (Bchl *e*). They reported that it has only a 30% amino acid sequence similarity to the 5.6 kDa protein from *Chloroflexus*. Furthermore, they showed a 98% sequence similarity between the Bchl *c* containing species of green sulfur bacteria, and an 84% sequence similarity to the Bchl *e* containing *Chlorobium* species. Although our results do not help to resolve whether the 7.5 or 6.3 kDa protein is the major pigment binding protein in *Chlorobium* species, it does indicate that the 7.5 kDa protein is present in chlorosomes isolated with miranol and that it is antigenically very different from the proposed pigment binding protein of *Chloroflexus*.

Immunological analysis of the 34.5 kDa *Chlorobium* chlorosome protein was not possible since no antisera exists for this protein. However, we were able to determine that this protein and the 5–7.5 kDa protein are not antigenically related to any of the *Cf. aurantiacus* chlorosome proteins. Neither the 34.5 kDa or the 5–7.5 kDa chlorosome proteins cross reacted to antibodies for the 5.6, 11, and 18 kDa polypeptides of *Chloroflexus*. With this formation we can now tentatively state that all three of the *Chloroflexus* proteins are not immunologically related to either the 5–7.5 or 34.5 kDa *Chlorobium* chlorosome proteins.

Although the reaction center of *Chlorobium* species has not been isolated, we can infer that its protein sub-

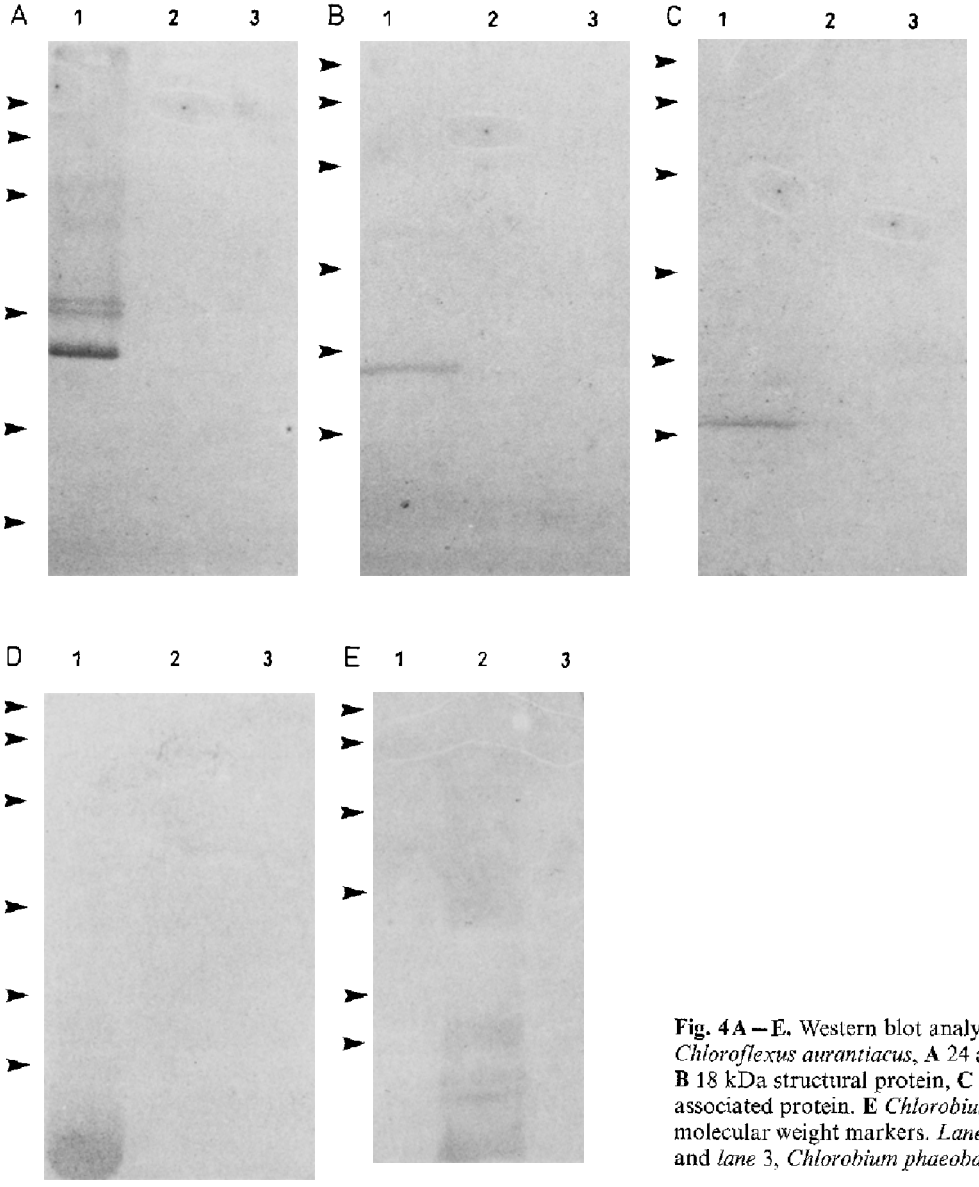

Fig. 4A–E. Western blot analyses for antibodies against the **A–D** *Chloroflexus aurantiacus*, **A** 24 and 24.5 kDa reaction center proteins, **B** 18 kDa structural protein, **C** 11 kDa protein, **D** 5.6 kDa Bchl *c* associated protein. **E** *Chlorobium limicola* 7.5 kDa protein. *Arrows* are molecular weight markers. *Lane* 1, *Cf. aurantiacus*, *lane* 2, *Cb. tepidum*, and *lane* 3, *Chlorobium phaeobacteroides*, in all figures

units will be different from those of *Cf. aurantiacus* based on our immunological results. The lack of cross reaction of any *Chlorobium* proteins with antisera to the reaction center subunits from *Cf. aurantiacus* (24 and 24.5 kDa) indicates no immunological relatedness. It has also been previously noted that this same antisera indicated a lack of immunological relatedness to the reaction center protein in *Rhodobacter sphaeroides* (Fuller and Redlinger 1985). Furthermore, the reaction center pigment-protein complex from green sulfur bacteria is spectrally different from *Cf. aurantiacus* (840 nm in *Prosthecochloris aestuarii*: Sybesma and Olson, 1964; 865 nm, in *Cf. aurantiacus*, Feick and Fuller 1984).

In addition to protein content, the chlorosomes of green sulfur bacteria are very similar, despite the differences in major antenna pigment and carotenoid content. Although they have different in vivo absorption maximum (Bchl *c*: 740–750 nm; Bchl *d*: 725–730 nm; and Bchl *e*: 714–720 nm; Gloe et al. 1975), these bacteriochlorophylls share a similar chemistry of a $CH(CH_3)$-OH group on ring 1 of the porphyrin. Purified chlorosomes from both species also contain a small amount of Bchl *a* species which is presumed to be the energy transfer protein described for *Cb. limicola* f. *thiosulfatophilum* (794 nm; Gerola and Olson 1986).

Differences between the Chlorobiaceae and the Chloroflexaceae are deeply rooted both at the physiological level, e.g., obligate anaerobic photoautotrophy vs. facultative photoheterotrophy (Pierson and Castenholz 1974; Castenholz and Pierson 1981) and at the molecular level, e.g., 16sRNA (Gibson et al. 1985; Oyaizu et al. 1987; Woese 1988). In this study we have shown that the chlorosome protein composition between these two families have diverged sufficiently that the *Chlorobium* proteins are not immunologically related to any of the three major proteins in *Chloroflexus*. In spite of this, the chlorosomes in both groups have as their central feature a large aggregation of light harvesting bacteriochlorophyll.

Thus, the essential feature of the chlorosome, the light harvesting bacteriochlorophyll antenna, has been preserved, while the structural components have been strikingly modified during the course of evolution.

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