'Chlorobium-type' Vesicles of Photosynthetically-grown *Chloroflexus aurantiacus* Observed Using Negative Staining Techniques

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Electron microscopic studies of several strains of *Chloroflexus aurantiacus*, a new genus of filamentous photosynthetic bacteria containing bacteriochlorophylls *a* and *c*, demonstrated that all strains contained vesicular structures very similar to the 'chlorobium vesicles' of the green bacteria when grown under anaerobic conditions in the light. The dimensions of these structures varied from strain to strain; they were 90 to 150 nm long and 25 to 70 nm wide. Photoautotrophically-grown *C. aurantiacus* as well as photoheterotrophically-grown organisms contained photosynthetic vesicles, while heterotrophically, dark-grown organisms contained no bacteriochlorophyll and no distinguishable 'chlorobium vesicles'. Filament diameter and length varied from strain to strain, although all strains examined were regularly septate.

INTRODUCTION

Chloroflexus aurantiacus has recently been described by Pierson & Castenholz (1974*a, b*) as a member of a new genus of bacteriochlorophyll *c*- and *a*-containing photosynthetic bacteria. The organism is filamentous, and inhabits alkaline hot spring effluents at temperatures from 50 to 70 °C (Pierson & Castenholz, 1971; Bauld & Brock, 1973; Doemel & Brock, 1974). *Chloroflexus aurantiacus* resembles the green and purple sulphur bacteria in that it is capable of photoautotrophic growth with sulphide (Madigan & Brock, 1975), and shows links with the Rhodospirillaceae by its ability to grow photoheterotrophically with or without sulphide, and heterotrophically in the dark (Pierson & Castenholz, 1974*a*; Madigan, Petersen & Brock, 1974). *Chloroflexus aurantiacus* also possesses characteristics which relate it to the blue-green algae (cyanobacteria) in that it is a gliding, filamentous organism and contains β -carotene (Halfen, Pierson & Francis, 1972).

The bacteriochlorophyll content of *C. aurantiacus* together with its deoxyribonucleic acid base ratio of 53 to 55% (as mol % GC) clearly indicate the strong links between this organism and the members of the green sulphur bacteria. In light of this it was interesting to note that Pierson & Castenholz (1974*a*) found vesicular structures in photosynthetically-grown *C. aurantiacus* very similar to the 'chlorobium vesicles' first described for green bacteria by Cohen-Bazire, Pfennig & Kunisawa (1964). The vesicles in *C. aurantiacus* lay at the periphery of the cell and appeared to be closely appressed to the cytoplasmic membrane, with their long axes oriented along the long axis of the filament (Pierson & Castenholz, 1974*a*).

Since the isolation and characterization of the type strain of *Chloroflexus aurantiacus* (strain J-10-fl; Pierson & Castenholz, 1971; 1974*a, b*) several new strains have been isolated from a variety of alkaline hot springs in Yellowstone National Park (Bauld, 1973). These strains have been nutritionally characterized (Madigan *et al.*, 1974), and ecological studies

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on the natural populations from which the strains were derived have been undertaken (Bauld, 1973; Bauld & Brock, 1973). Pierson & Castenholz (1974*a*) examined thin-sectioned preparations for the presence of 'chlorobium vesicles'. Negative staining provides a simpler and more clear cut way of showing these vesicular structures (Holt, Conti & Fuller, 1966) and we used this technique in our study of the Yellowstone isolates. We also examined the effect of growth conditions on the formation of vesicles, making use of cultures which we succeeded in adapting to photoautotrophic conditions (Madigan & Brock, 1975).

METHODS

Organisms. The cultures of the type strain (J-10-fl) and strain OK-70-fl were provided by R. W. Castenholz; they derive from Japanese and Oregon hot springs, respectively (Pierson & Castenholz, 1974*a*). Strain Y-400-fl was isolated by Castenholz from material provided by John Bauld (Department of Microbiology, University of Melbourne, Australia) which was taken from a thick mat at Octopus Spring (formerly referred to as Pool A), an alkaline hot spring located about 0.15 km SSE of Great Fountain Geyser, Yellowstone National Park, U.S.A. Strain 396-1 was isolated by Bauld from Conophyton Pool (unofficial name), a spring located in the south end of Fairy Creek Meadow, Lower Geyser Basin, Yellowstone National Park (Walter, Bauld & Brock, 1972). Strain 254-2 was isolated by Bauld & Brock (1973) from a thin mat in the outflow of Grassland Spring, Lower Geyser Basin. Methanol extracts of all the Yellowstone isolates showed absorption spectra typical of bacteriochlorophylls *a* and *c*. A main peak in the red region at 668 nm is characteristic of bacteriochlorophyll *c* (Stanier & Smith, 1960), and a smaller peak at 770 nm is due to bacteriochlorophyll *a*.

Cultivation. Cultures of all strains of *C. aurantiacus* were grown photosynthetically under anaerobic conditions as described by Madigan *et al.* (1974). Modified medium D at pH 8.0 was used as a basal medium, and this was supplemented with CO₂ and sulphide for photoautotrophic growth. For photoheterotrophic growth, single organic carbon sources or yeast extract were used, at concentrations from 0.01 to 0.2 % (w/v). Sulphide was added to all media at a final concentration of 2 mM. Heterotrophic growth occurred in rapidly stirred 250 or 500 ml culture flasks containing 25 or 50 ml of the complex medium described by Pierson & Castenholz (1974*a*) without sulphide, and capped with metal closures. All incubations were at 50 °C at a light intensity of 0.5 to 1 klx from a tungsten source.

Electron microscopy. Copper grids 200 mesh, covered with a film of 2.5 % (w/v) parlodion (in amyl acetate) strengthened by the addition of a thin film of evaporated carbon, were used in all studies. Negative staining was done according to Remsen, Valois & Watson (1967) except that 1.5 % (w/v) phosphotungstic acid pH 6.8 was used. Most cell suspensions were briefly homogenized with a Teflon homogenizer before being placed on the grids to break up any clumps. This procedure also disrupted some of the filaments, allowing stain to penetrate and enhance contrast.

For chemical fixation, cells were prefixed for 1 h at room temperature in 1 % (v/v) glutaraldehyde in 0.05 M-potassium phosphate pH 7.0, and postfixed for 1 h in 1 % (w/v) OsO₄ in phosphate buffer. Cells were washed, suspended in 2.5 % (w/v) ion agar, dehydrated in an acetone series and embedded in Durcupan (Fluka, 9470 Buchs, Switzerland). Sections were cut on a Porter-Blum ultramicrotome using glass knives, and double stained with 1.5 % (w/v) uranyl acetate followed by lead citrate (Reynolds, 1963).

All electron microscopy was done on a Zeiss EM-9S operating at 60 kV.

Photomicroscopy. Photomicrographs were taken on a Zeiss Universal phase-contrast microscope fitted with a Topcon 35 mm single-lens reflex camera. Kodak Plus X (ASA 125) or Tri-X (ASA 400) black-and-white films were used for all photomicrographs.

RESULTS AND DISCUSSION

Photomicroscopy

A photomicrograph of one of the Yellowstone strains is shown in Fig. 1. Morphologically, the Yellowstone strains appear similar to the type strain of *C. aurantiacus*, J-10-fl (Pierson & Castenholz, 1974*a*). Filament lengths vary considerably, ranging from 30 to 300 µm. Filament widths vary slightly from strain to strain, and also, to some extent, with growth conditions. Strains 254-2 and J-10-fl have average diameters of 0.5 to 0.6 µm, whereas strains Y-400-fl, 396-1 and OK-70-fl are wider (average diameters 0.7 to 0.8 µm).

Fig. 1. Photomicrograph of *Chloroflexus aurantiacus* strain 396-1. Bar marker represents 10 μm .

Fig. 2. Low magnification electron micrograph of negatively stained filaments of *Chloroflexus aurantiacus* strain 254-2. Bar marker represents 2 μm .

Fig. 3. Thin section of *C. aurantiacus* strain OK-70-fl. Bar marker represents 0.5 μm .

Note septa (arrows) in both figures, and prominent mesosomes in Fig. 2.

Electron microscopy

Although not evident in photomicrographs, filaments of all strains of *C. aurantiacus* examined with the electron microscope are regularly septate, with cell lengths from 1.5 to 6 μm . Figure 2 clearly demonstrates the septa in negatively stained whole cells, and Fig. 3 shows septa as seen in thin sections. Several densely stained inclusions, probably mesosomal elements, are also visible in Fig. 2. Mesosomes were detected in several of the strains of *C. aurantiacus* examined by Pierson & Castenholz (1974a), and they are known to be a prominent feature of the green bacteria (Cohen-Bazire *et al.*, 1964). A wavy granular substance can be seen in Fig. 3 which probably represents the remnants of a sheath. Pierson & Castenholz (1974a) noted that filaments of strains OK-70-fl and J-10-fl appeared to be sheathed and, although we have not obtained conclusive evidence using the electron microscope, phase-contrast microscopy has occasionally allowed observations of a sheath when an individual cell lyses leaving a clear area connecting intact cells along the filament.

The vesicles of photosynthetically-grown *C. aurantiacus* are most clearly observed in negatively stained preparations. Figures 4, 5 and 6 show filaments of strains OK-70-fl, 396-1 and 254-2 respectively; the cells were gently homogenized to break the filaments and allow penetration of the stain. The vesicular structures are clearly evident in these preparations as non-electron-dense ellipsoidal structures distributed throughout the interior of the cells. In Fig. 4 several vesicles can be seen aligned along the longitudinal axis in the cortical region of the cell, and this has been shown to be the location of the vesicles in chemically fixed preparations of *C. aurantiacus* not subjected to homogenization (Pierson & Castenholz, 1974a).

The vesicles from *C. aurantiacus* appear morphologically quite similar to the 'chlorobium vesicles' of *Chloropseudomonas ethylica* (*Chlorobium* sp., Gray *et al.*, 1973), first studied using negative staining techniques by Holt *et al.* (1966), and to the photosynthetic vesicles of *Chlorobium* which have been characterized in detail by Cruden & Stanier (1970) and Cruden, Cohen-Bazire & Stanier (1970). The vesicles of strain OK-70-fl measure 100 to 150 nm by 45 to 70 nm, whereas the vesicles of strain 396-1 are of a similar length but somewhat thinner, measuring 35 to 50 nm in width. Strain 254-2 possesses smaller vesicles which measure 90 to 95 nm by 25 to 35 nm, while the vesicles of strain Y-400-fl (Fig. 9) measure 100 to 150 nm by 40 to 60 nm. Thus, although the vesicles of these strains are morphologically similar, they appear to be of considerably different average size, with a general correlation between filament width and vesicle dimensions in agreement with the data of Pierson & Castenholz (1974a).

Figure 7 shows a group of vesicles which have been released by the homogenization process and remain associated with cell membrane material. It is apparent that the vesicles from any one pure culture are heterogeneous in morphology, varying slightly in physical dimensions yet still retaining the typical ellipsoidal shape characteristic of these structures as originally described for *Chlorobium* (Cohen-Bazire *et al.*, 1964). Although not demonstrable by negative staining techniques, Pierson & Castenholz (1974a) have determined from thin-sectioned material that the vesicles in *Chloroflexus*, as in *Chlorobium*, are bound by a 2 nm thick single-layered (non-unit) membrane. Since the vesicles from the strains examined in the present work retain their structural integrity upon release from the cell, it is likely that they possess an outer layer of a similar nature.

Pierson & Castenholz (1974b) have shown that bacteriochlorophyll synthesis in *Chloroflexus* is repressed under aerobic conditions. We have examined heterotrophically, dark-grown *C. aurantiacus* for the presence of photosynthetic vesicles and have found that cells grown under highly aerated conditions (see Methods) do not possess photosynthetic vesicles. In addition, anaerobic, photoautotrophically-grown cells of strain OK-70-fl contain a vesicular photosynthetic apparatus indistinguishable from cells grown anaerobically under photoheterotrophic conditions.

Figs 4, 5 and 6. Electron micrographs (at the same magnification) of negatively stained homogenized filaments of *Chloroflexus aurantiacus*: Fig. 4, strain OK-70-fl; Fig. 5, strain 396-1; Fig. 6, strain 254-2. Note photosynthetic vesicles distributed throughout the cells. Bar marker represents 0.5 μm .

General discussion

As has been pointed out by Trüper (1976), the isolation and characterization of *C. aurantiacus* has posed some taxonomic problems, in that this organism shares affinities with several different prokaryotic groups including the blue-green algae, the achlorophyllous flexibacteria, and the phototrophic bacteria.

Despite its widespread affinities, *Chloroflexus* is clearly a photosynthetic bacterium, in that its photosynthesis is of the anoxygenic type mediated by bacteriochlorophylls (Pierson & Castenholz, 1971, 1974*a, b*; Madigan & Brock, 1975). This characteristic distinguishes *Chloroflexus* from the chlorophyll *a*-containing blue-green algae (cyanobacteria). The bacteriochlorophylls of *C. aurantiacus* are bacteriochlorophylls *a* and *c*, a complement of bacteriochlorophylls unique to members of the Chlorobiaceae (Pfennig, 1967). Although

Figs 7 and 8. Electron micrographs of vesicles (negatively stained) released from *Chloroflexus aurantiacus* strain OK-70-fl after homogenization. Figure 8 is an enlargement of the marked area in Fig. 7. Bar markers represent 0.1 μm .

Fig. 9. Vesicles released from *C. aurantiacus* strain Y-400-fl. Bar marker represents 50 nm.

Pierson & Castenholz (1974a) did not provide evidence that the vesicular structure of photosynthetically-grown *Chloroflexus* actually contained the bacteriochlorophylls, it is likely that at least bacteriochlorophyll *c* is localized in this structure as is the case in *Chlorobium* (Cruden & Stanier, 1970).

Thus, although filamentous in morphology and capable of aerobic respiratory growth, *Chloroflexus aurantiacus* responds to anaerobic, light conditions by synthesizing bacteriochlorophylls *a* and *c* (Pierson & Castenholz, 1974b), and a vesicular structure very similar to the photosynthetic apparatus of *Chlorobium*. Due to its unique characteristics it is possible that *Chloroflexus* represents a transitional type of organism, intermediate in various ways between the photosynthetic bacteria and the oxygenic phototrophs. The blue-green alga (cyanobacterium) *Oscillatoria limnetica*, which is able to perform anoxygenic photosynthesis using sulphide instead of water as its electron donor (Cohen, Padan & Shilo, 1975), may represent a similar transitional form. These organisms may prove to be of significance in the development of a phylogenetic scheme leading from the anoxygenic photosynthetic bacteria to the first oxygen-evolving phototrophs.

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REFERENCES

- BAULD, J. (1973). *Algal-bacterial interactions in alkaline hot spring effluents*. Ph.D. dissertation, University of Wisconsin, U.S.A.
- BAULD, J. & BROCK, T. D. (1973). Ecological studies of *Chloroflexus*, a gliding photosynthetic bacterium. *Archiv für Mikrobiologie* **92**, 267-284.
- COHEN, Y., PADAN, E. & SHILO, M. (1975). Facultative anoxygenic photosynthesis in the cyanobacterium *Oscillatoria limnetica*. *Journal of Bacteriology* **123**, 855-861.
- COHEN-BAZIRE, G., PFENNIG, N. & KUNISAWA, R. (1964). The fine structure of green bacteria. *Journal of Cell Biology* **22**, 207-225.
- CRUDEN, D. L. & STANIER, R. Y. (1970). The characterization of chlorobium vesicles and membranes isolated from green bacteria. *Archiv für Mikrobiologie* **72**, 115-134.
- CRUDEN, D. L., COHEN-BAZIRE, G. & STANIER, R. Y. (1970). Chlorobium vesicles: the photosynthetic organelles of green bacteria. *Nature, London* **228**, 1345-1347.
- DOEMEL, W. N. & BROCK, T. D. (1974). Bacterial stromatolites: origin of laminations. *Science* **184**, 1083-1085.
- GRAY, B. H., FOWLER, C. F., NUGENT, N. A., RIGOPOULOS, N. & FULLER, R. C. (1973). Re-evaluation of *Chloropseudomonas ethylica* strain 2-K. *International Journal of Systematic Bacteriology* **23**, 256-264.
- HALFEN, L. N., PIERSON, B. K. & FRANCIS, G. W. (1972). Carotenoids of a gliding organism containing bacteriochlorophylls. *Archiv für Mikrobiologie* **82**, 240-246.
- HOLT, S. C., CONTI, S. F. & FULLER, R. C. (1966). Photosynthetic apparatus in the green bacterium *Chloropseudomonas ethylicum*. *Journal of Bacteriology* **91**, 311-323.
- MADIGAN, M. T. & BROCK, T. D. (1975). Photosynthetic sulfide oxidation by *Chloroflexus aurantiacus*, a filamentous photosynthetic gliding bacterium. *Journal of Bacteriology* **122**, 782-784.
- MADIGAN, M. T., PETERSEN, S. R. & BROCK, T. D. (1974). Nutritional studies on *Chloroflexus*, a filamentous photosynthetic gliding bacterium. *Archives of Microbiology* **100**, 97-103.
- PFENNIG, N. (1967). Photosynthetic bacteria. *Annual Review of Microbiology* **21**, 285-324.
- PIERSON, B. K. & CASTENHOLZ, R. W. (1971). Bacteriochlorophylls in gliding filamentous prokaryotes from hot springs. *Nature New Biology* **233**, 25-27.
- PIERSON, B. K. & CASTENHOLZ, R. W. (1974a). A phototrophic gliding filamentous bacterium of hot springs, *Chloroflexus aurantiacus*, gen. and sp. nov. *Archives of Microbiology* **100**, 5-24.
- PIERSON, B. K. & CASTENHOLZ, R. W. (1974b). Studies of pigments and growth in *Chloroflexus aurantiacus*, a phototrophic filamentous bacterium. *Archives of Microbiology* **100**, 283-305.
- REMSEN, C. C., VALOIS, F. W. & WATSON, S. W. (1967). Fine structure of the cytomembranes of *Nitrosocystis oceanus*. *Journal of Bacteriology* **94**, 422-433.
- REYNOLDS, E. S. (1963). The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *Journal of Cell Biology* **17**, 208-212.
- STANIER, R. Y. & SMITH, J. H. C. (1960). The chlorophylls of green bacteria. *Biochimica et biophysica acta* **41**, 478-484.
- TRÜPER, H. G. (1976). Higher taxa of the phototrophic bacteria: Chloroflexaceae fam. nov., a family for the gliding, filamentous, phototrophic 'green' bacteria. *International Journal of Systematic Bacteriology* **26**, 74-75.
- WALTER, M. R., BAULD, J. & BROCK, T. D. (1972). Siliceous algal and bacterial stromatolites in hot spring and geyser effluents of Yellowstone National Park. *Science* **178**, 402-405.