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Nutritional Studies on *Chloroflexus*, a Filamentous Photosynthetic, Gliding Bacterium

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Abstract. Nutritional studies on four different strains of *Chloroflexus*, a new genus of filamentous, photosynthetic bacteria are described. This organism appears to be related to several different procaryotic groups, and in particular to the green sulfur bacteria and blue-green algae. Unlike these autotrophs, however, *Chloroflexus* is nutritionally diverse, being able to grow aerobically as a light-independent heterotroph, and anaerobically as a photoautotroph or photoheterotroph. Numerous organic carbon sources including hexoses, amino acids, short chain fatty acids, organic acids, and some alcohols are utilized under various growth conditions. These results suggest that this organism may be among the most nutritionally versatile organisms known.

Key words: Filamentous, Photosynthetic Bacteria — Nutritional Studies — Gliding Bacteria.

Filamentous, photosynthetic gliding bacteria of the newly proposed genus *Chloroflexus*[1] are found throughout the world in alkaline hot spring effluents, where they form mats with or without a close association with certain blue-green algae, at temperatures from 50—70°C (Pierson and Castenholz, 1971; Castenholz, 1973; Doemel and Brock, 1974). Pigment studies by Pierson and Castenholz (1971) have shown that both bacteriochlorophylls *c* and *a* are present in anaerobically grown cultures of *Chloroflexus*. The carotenoid pigments of *Chloroflexus* have been examined by Halfen *et al.* (1971), and they have identified at least 10 different carotenoid pigments, many of which are found primarily in the blue-green algae.

Although a taxonomically confusing organism, it now appears as if *Chloroflexus* is most closely related to the green sulfur bacteria. The G-C ratio of *Chloroflexus* DNA (as moles % Guanine plus Cytosine) is 53—55% (Pierson, 1973), which falls well within the range of G-C values characteristic of the green sulfur bacteria (Pfennig, 1967). The identification of the bacteriochlorophylls, and of structures very similar to the

[1] Previous publications concerning this organism have used the spelling *Chloroflexis* (Castenholz, 1973). Future publication of the official description of this organism will use the spelling *Chloroflexus* (Pierson and Castenholz, 1974).

"chlorobium vesicles" of other green bacteria in *Chloroflexus* by Pierson (1973), leaves little doubt that *Chloroflexus* shares many of the characteristics of this group. Nutritionally, however, *Chloroflexus* is capable of growing on complex media completely heterotrophically under aerobic conditions in the light or the dark (Pierson, 1973). Alternatively, the organism can grow photoheterotrophically on complex media under strictly anaerobic conditions (Pierson, 1973). It is known from the early work of Larsen (1953), and the more recent review by Pfennig (1967) that the established members of the green bacteria are not nutritionally versatile, in that complex substrates as well as most simple organic compounds other than acetate, are not significantly utilized. It was the purpose of this paper to examine several isolates of *Chloroflexus* for their ability to grow on a defined medium under a variety of nutritional conditions, and to develop cultural methods which would support autotrophic growth of these new and interesting organisms.

Materials and Methods

Cultures. Nutritional experiments were carried out on four strains of *Chloroflexus* designated strains OK-70-fl, J-10-fl, 254-2 and 396-1. Strain OK-70-fl was isolated by Pierson and Castenholz (1971) from effluent waters of the Ka-Nee-ta hot springs located on the Warm Springs Indian Reservation near Madras, Oregon. J-10-fl (Pierson and Castenholz, 1971) was cultured from mat material taken from a hot spring in the Hakone area of Japan. Strain 254-2 was isolated by Bauld from a mat in the thermal effluent waters of Grassland Spring, located in the Lower Geyser Basin of Yellowstone National Park, Wyoming (Bauld, 1973). Strain 396-1 was the strain of *Chloroflexus* isolated by Walter *et al.* (1973) from the siliceous bacterial stromatolites which form in the effluents of certain geysers in Yellowstone National Park.

Growth Procedures. Stock cultures of all strains of *Chloroflexus* were maintained on the mineral medium described by Castenholz (1969), supplemented with yeast extract at 0.05%, and the dipeptide glycyl-glycine (as a buffer) (Sigma Chemical Co., St. Louis, Mo.) at 0.05%. In addition 0.5 ml/l of the SL-6 trace metal solution of Pfennig (1965) was added. This medium was solidified with 1.5% agar for growth on plates, or used without agar as a liquid medium for growing cultures in flasks, bottles, or tubes. Defined media for aerobic growth experiments included the mineral salts of Castenholz (1969), 0.02% NH_4Cl, and the following 10 vitamins expressed in their final medium concentration: vitamin B_{12} (200 ng/ml), thiamine · HCl (60 ng/ml), nicotinic acid (100 ng/ml), para-amino benzoic acid (1 ng/ml), biotin (200 ng/ml), calcium pantothenate (100 ng/ml), pyridoxal · HCl (20 ng/ml), inositol (1 µg/ml), riboflavin (20 ng/ml), folic acid (200 ng/ml). Final pH of the medium was adjusted to 8.2–8.4. Growth experiments using defined media under aerobic conditions involved the inoculation of either 125 ml Delong culture flasks containing 40 ml medium, or 60 ml prescription bottles containing 20 ml of medium and one organic carbon source to a final concentration of 0.25% w/v. The flasks were covered with metal caps (Bellco Co., Vineland, N.J.), or loose fitting screw caps, and incubated at 50°C in a light cabinet (Controlled Environments, Pembina, North Dakota). Lighting was supplied by two 25 W incandescent bulbs providing incident illumination upon growth vessels of approximately 50 foot candles. Darkened con-

ditions, when required, were created by wrapping the flasks or bottles with two layers of aluminum foil.

Anaerobic media consisted of mineral salts (Castenholz, 1969) supplemented with 0.02% NH_4Cl, sulfide, bicarbonate, and the 10 vitamins and one organic carbon source as described above. Sulfide was supplied to the medium as an alkaline solution of analytical grade $Na_2S \cdot 9H_2O$ (Mallinckrodt Chemical Works, St. Louis, Mo.), to a final concentration of 0.05% (w/v). Bicarbonate preparations were made by aseptically bubbling sterile solutions of 0.03% $NaHCO_3$ (Fisher Scientific Co., Fair Lawn, N.J.) with pure CO_2 for $^1/_2$ hr, using a sterile bubbling apparatus fitted with stoppered glass cylinders packed with cotton to maintain sterility during gas transfer. All media components were sterilized by autoclaving for 18 min at 121°C and 17 psi.

After mixing all components the pH of anaerobic media was adjusted to 8.2—8.4, and was then dispensed into screw cap tubes, or large bottles with the containers being completely filled such that no gas space remained.

Due to the filamentous nature and frequent clumping behavior of *Chloroflexus*, the inocula used in these experiments were standardized by pooling cells from batch cultures, washing the cells three times with sterile mineral salts (Castenholz, 1969), then homogenizing the final suspension for 1 min using a flame-sterilized Teflon homogenizer (Arthur Thomas Co., Philadelphia, Pa.). Growth experiments under anaerobic conditions involved inoculation of 18.5 ml screw cap tubes of anaerobic media containing one organic carbon source at 0.25% (w/v). Incubation, illumination, and darkening procedures were as described for aerobically grown cultures. All carbon sources added were neutralized solutions sterilized by filtration.

Results

Nutritional Studies under Aerobic Conditions. Table 1 lists the results of nutritional experiments using defined media under aerobic conditions. These data demonstrate the fact that growth of these strains of *Chloroflexus* does occur in defined media under aerobic conditions. With some variability occurring between strains, tricarboxylic acid cycle intermediates, short chain alcohols, amino acids, hexoses and at least one pentose support the growth of these strains of *Chloroflexus* in the light. Many of these same substrates also support aerobic growth in the dark, although in several instances the yield was greater in the light.

Nutritional Studies under Anaerobic Conditions. Table 2 gives the results of nutritional experiments carried out under anaerobic conditions. As is the case for aerobically grown cells, a wide variety of substrates are utilized. However, it is readily apparent that *Chloroflexus* is dependent upon light for the ability to utilize these organic compounds under anaerobic conditions. Results such as these are in agreement with numerous studies summarized by Pfennig (1967), which conclusively show that the anaerobic growth of virtually every photosynthetic procaryote known is light dependent. The very slow growth of some members of the Rhodospirillaceae under dark, anaerobic conditions, however, has been demonstrated (Uffen and Wolfe, 1970).

Table 1. Utilization of organic compounds under aerobic conditions[a]

Carbon source	Strain							
	OK-70-fl		J-10-fl		254-2		396-1	
	Light	Dark	Light	Dark	Light	Dark	Light	Dark
1. 4% Formalin-killed control	−	−	−	−	−	−	−	−
2. Glutamate	+	+	++	+	++	+	+	+
3. Aspartate	++	+	+	+	+	+	+	+
4. Glycyl-glycine	−	−	−	−	−	−	−	−
5. Acetate	+	−	+	+	++	+	+	+
6. Pyruvate	++	+	+	+	+	+	++	+
7. Lactate	−	−	++	++	++	+	++	++
8. Succinate	+	+	+	−	+	+	++	+
9. Malate	+	+	++	++	++	+	+	+
10. Butyrate	++	−	++	+	++	+	+	+
11. Citrate	+	+	−	−	−	−	−	−
12. Ribose	++	+	+	+	+	+	+	+
13. Glucose	++	+	++	++	++	++	++	++
14. Galactose	++	+	++	++	++	+	++	++
15. Ethanol	+	+	+	−	+	−	+	−
16. Glycerol	+	−	++	++	+	+	++	++
17. Mannitol	++	++	+	+	+	+	+	+
18. Yeast extract	++	++	++	++	++	++	++	++
19. Casamino acids	++	++	++	+	++	++	++	+
20. HCO_3^-	−	−	−	−	−	−	−	−

[a] Relative growth values after 8 days incubation were assessed by determining whether the yield of cells was comparable to that of yeast extract (++), or whether the growth yield was significantly less than that derived from yeast extract-grown cells (+). A (−) value represents substrates or conditions not supporting growth of a particular strain of *Chloroflexus* when compared with a 4% formalin, killed-inoculum control.

It is not yet known if *Chloroflexus* is actively fixing CO_2 when organic compounds are present in the growth medium, although the observation has been made that sulfide, the presumed electron donor during photoautotrophic growth, is not required for growth on many of these same organic substrates anaerobically in the light. Sustained autotrophic growth in the light with bicarbonate and sulfide has been achieved with strain OK-70-fl.

Discussion

Most members of the Rhodospirillaceae are capable of aerobic respiratory growth, although members of the green and purple sulfur groups have previously been shown to be obligate anaerobes (Pfennig, 1967). Hansen and van Gemerden (1972) have shown that certain species of

Table 2. Utilization of organic compounds under anaerobic conditions[a]

Carbon source	Strain							
	OK-70-fl		J-10-fl		254-2		396-1	
	Light	Dark	Light	Dark	Light	Dark	Light	Dark
1. 4% Formalin-killed control	−	−	−	−	−	−	−	−
2. Glutamate	+	−	++	−	+	−	+	−
3. Aspartate	++	−	+	−	+	−	+	−
4. Glycyl-glycine	+	−	+	−	+	−	+	−
5. Acetate	+	−	+	−	+	−	+	−
6. Pyruvate	+	−	++	−	+	−	++	−
7. Lactate	−	−	+	−	+	−	−	−
8. Succinate	+	−	+	−	+	−	+	−
9. Malate	+	−	+	−	+	−	+	−
10. Butyrate	+	−	+	−	+	−	+	−
11. Citrate	+	−	+	−	+	−	−	−
12. Ribose	+	−	+	−	+	−	+	−
13. Glucose	++	±[b]	++	−	++	−	++	−
14. Galactose	+	−	+	−	+	−	+	−
15. Ethanol	+	−	+	−	+	−	−	−
16. Glycerol	++	−	+	−	+	−	+	−
17. Mannitol	+	−	+	−	+	−	+	−
18. Yeast extract	++	−	++	−	++	−	++	−
19. Yeast extract no sulfide	++	−	++	−	++	−	++	−
20. Casamino acids	++	−	++	−	++	−	++	−
21. HCO_3^-	+	−	−[c]	−	−[c]	−	−[c]	−

[a] Relative growth values after 8 days incubation were assessed by determining whether the yield of cells was comparable to that of yeast extract (++), or whether the growth yield was significantly less than that derived from yeast extract-grown cells (+). A (−) value represents substrates or conditions not supporting growth of a particular strain of *Chloroflexus* when compared with a 4% formalin, killed-inoculum control.

[b] Initial growth occurred, but sustained growth in the dark anaerobically was not achieved.

[c] Sustained autotrophic growth was not achieved in these strains.

Rhodopseudomonas and *Rhodospirillum* can utilize sulfide as an electron donor if the sulfide is present in very small amounts. *Chloroflexus* on the other hand, appears to be the first procaryotic phototroph which can, under the proper conditions, display metabolic diversity ranging from complete aerobic heterotrophy, to anaerobic photoautotrophic growth under high sulfide conditions. Whereas most of the strains examined by Hansen and van Gemerden, were found to tolerate only relatively low sulfide concentrations (Hansen and van Gemerden, 1972), *Chloroflexus* is routinely grown anaerobically in sulfide concentrations of 2—4 mM,

and certain strains can tolerate sulfide concentrations in excess of 6 mM (Bauld, 1973). In many ways *Chloroflexus* rivals the capabilities of a newly discovered species of *Rhodopseudomonas* by Hansen and Veldkamp (1973). These workers have isolated a highly sulfide tolerant organism which contains bacteriochlorophyll *a*. This isolate is capable of growing on a wide variety of organic compounds photoheterotrophically under anaerobic conditions. Organic compounds are also utilized in a respiratory fashion under aerobic conditions in the dark (Hansen and Veldkamp, 1973).

As the aerobic growth experiments demonstrate, heterotrophic growth of *Chloroflexus* on a wide variety of simple organic compounds is possible in both the light and the dark. The fact that *Chloroflexus* is capable of using a wide variety of different compounds anaerobically in the light, or aerobically in the light or the dark is a good indication that the algal component of the mat, which has been shown to excrete organic materials into the bacterial layers (Bauld and Brock, 1973, 1974), quite possibly releases a number of different small molecular weight compounds which can be used as carbon and energy sources for other members of the mat community including *Chloroflexus*. At night *Chloroflexus* probably depends on respiratory processes, whether of endogenous reserve material or of recently assimilated organic compounds. Doemel and Brock (1974) have demonstrated that *Chloroflexus* migrates upwards at night, moving on top of the algal component, and, presumably to a more oxygenated region where more active respiratory processes could occur.

It thus appears as if this new genus of photosynthetic bacteria may be one of the most nutritionally versatile organisms known. By possessing the machinery to enable itself to exist as a photoautotroph, photoheterotroph, or light-independent aerobic heterotroph, *Chloroflexus* most likely responds to local variations in the concentration of excreted organic materials, changes in light intensity, and variation in the redox potential of its microenvironment, by adjusting its energy generating mechanisms accordingly.

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References

Bauld, J.: Algal-bacterial interactions in alkaline hot spring effluents. Ph.D. dissertation, Department of Bacteriology, University of Wisconsin, Madison, Wisconsin (1973)

Bauld, J., Brock, T. D.: Ecological studies of *Chloroflexis*, a gliding, photosynthetic bacterium. Arch. Mikrobiol. **92**, 267—284 (1973)

Bauld, J., Brock, T. D.: Algal excretion and bacterial assimilation in mats of alkaline hot spring effluents. J. Phycol. **10**, 101—106 (1974)

Castenholz, R. W.: Thermophilic blue-green algae and the thermal environment. Bact. Rev. **33**, 476–504 (1969)

Castenholz, R. W.: The possible photosynthetic use of sulfide by the filamentous phototrophic bacteria of hot springs. Limnol. Oceanogr. **18**, 863–876 (1973)

Doemel, W. N., Brock, T. D.: Bacterial stromatolites: origin of laminations. Science **184**, 1083–1085 (1974)

Halfen, L. N., Pierson, B. K., Francis, G. W.: Carotenoids of a gliding organism containing bacteriochlorophylls. Arch. Mikrobiol. **82**, 240–246 (1971)

Hansen, T. A., van Gemerden, H.: Sulfide utilization by purple nonsulfur bacteria. Arch. Mikrobiol. **86**, 49–56 (1972)

Hansen, T. A., Veldkamp, H.: *Rhodopseudomonas sulfidophila*, nov. spec., a new species of the purple nonsulfur bacteria. Arch. Mikrobiol. **92**, 45–58 (1973)

Larsen, H.: On the microbiology and biochemistry of the photosynthetic green sulfur bacteria. Det. Kgl Norske Videnskabers Selskabs Skrifter NRI (1953)

Pfennig, N.: Anreicherungskulturen für rote und grüne Schwefelbakterien. Zbl. Bakt., I. Abt., Suppl. I, 179–189 (1965)

Pfennig, N.: Photosynthetic bacteria. Ann. Rev. Microbiol. **21**, 285–325 (1967)

Pierson, B. K.: The characterization of gliding filamentous phototrophic bacteria. Ph.D. dissertation, University of Oregon, Eugene, Oregon 1973

Pierson, B. K., Castenholz, R. W.: Bacteriochlorophylls in gliding filamentous prokaryotes from hot springs. Nature New Biol. **233**, 25–27 (1971)

Pierson, B. K., Castenholz, R. W.: A phototrophic gliding filamentous bacterium of hot springs, *Chloroflexus aurantiacus*, gen. and sp. nov. Arch. Microbiol. **100**, 5–24 (1974)

Uffen, R. L., Wolfe, R. S.: Anaerobic growth of purple nonsulfur bacteria under dark conditions. J. Bact. **104**, 462–472 (1970)

Walter, M. R., Bauld, J., Brock, T. D.: Siliceous algal and bacterial stromatolites in hot spring and geyser effluents of Yellowstone National Park. Science **178**, 402–405 (1973)

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