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Chloroflexus-like organisms from marine and hypersaline environments: Distribution and diversity

Beverly K. Pierson, Diane Valdez, Mark Larsen, Elizabeth Morgan & E. Erin Mack[1]
Biology Department, University of Puget Sound, Tacoma, WA 98416, USA; [1]*Microbiology Department, U.C. Davis, Davis, CA 95616-8665, USA*

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Abstract

We report the presence of a diverse number of *Chloroflexus*-like organisms in intertidal marine and submerged hypersaline microbial mats using light, infrared fluorescence, and electron microscopy. The intertidal organisms appear morphologically very similar to thermophilic *C. aurantiacus* while the 2 hypersaline strains are larger and have a more complex ultrastructure composed of chlorosome-bearing internal membranes that appear to arise as invaginations of the cell membrane. By comparing spectroradiometry of microbial mat layers with microscopic observations, we have confirmed that the *Chloroflexus*-like organisms are major constituents of the hypersaline microbial mat communities. In situ studies on mat layers dominated by *Chloroflexus*-like organisms showed that sulfide-dependent photoautotrophic activity sustained by near infrared radiation prevailed. Autoradiographic analyses revealed that autotrophy was sustained in the filaments by 750 nm radiation. Three morphologically distinct strains are now maintained in mixed culture. One of these appears to be growing photoautotrophically.

Abbreviations: MCLO – Marine *Chloroflexus*-Like Organism; ppt – parts per thousand (used to report salinities)

Introduction

Thermophilic *Chloroflexus aurantiacus* has been observed in alkaline hot springs throughout the world where it forms conspicuous mats usually in association with cyanobacteria (Pierson and Castenholz 1974, 1992). Many strains have been isolated from these mats and have been studied in pure culture (Pierson and Castenholz 1992). Modern microbial mats that appear similar to those that formed the ancient stromatolites are also found in extreme environments other than hot springs (Pierson 1992). Although the *C. aurantiacus* strains isolated from mats in hot springs are thermophilic, apparent mesophilic counter-parts have also been observed in mats or sediments from a variety of fresh-water, marine, and hypersaline non-thermal environments. The discovery of *Chloroflexus*-like organisms in non-thermal environments is important because many of the Precambrian stromatolites appear to be marine or lacustrine in origin (Walter 1983).

Microfossil records associated with Precambrian stromatolites have revealed the presence of filamentous bacteria that are morphologically very similar to *C. aurantiacus* in rock nearly 3,500 million years old (Schopf and Walter 1983; Walsh and Lowe 1985). On the basis of morphology and physiology, it appears that *Chloroflexus* may be more similar than other extant phototrophs to the early microfossils (Olson and Pierson 1987; Pierson and Olson 1989), although others have suggested these Precambrian filamentous microfossils could be cyanobacteria (Awramik 1992). Molecular analyses of 16S rRNA have revealed that *Chloroflexus* is only distantly related to other phototrophs (Oyaizu et al. 1987; Woese 1987). The antiquity of phototrophy and specifically of the *Chloroflexus*

group of bacteria might be clarified if more species and genera of related organisms were found to expand the 16S rRNA database. The deep branching of phototrophy in the eubacterial line of descent is ascribed to the one species of thermophilic *Chloroflexus*. More species are needed to add depth to our understanding of the origins and evolution of photosynthesis. Hence an understanding of the physiology, ecology, and phylogeny of all *Chloroflexus*-like organisms is particularly relevant to interpretations of fossil and molecular evidence for early photosynthetic life on earth.

Several mesophilic *Chloroflexus*-like organisms have been observed in a variety of fresh-water environments. A fresh-water strain of *C. aurantiacus* was isolated but the culture was subsequently lost (Gorlenko 1975). Other freshwater strains of potentially related organisms were previously described but not isolated or not maintained in culture. These include two other genera – *Chloronema* and *Oscillochloris*. The descriptions of these genera are published despite the lack of pure cultures (Gorlenko 1989a,b) and the status of these organisms has been summarized and reviewed (Pierson and Castenholz 1992, 1994). The ecology of these organisms was also reviewed (Gorlenko 1988). Recently, a new type species of *Oscillochloris trichoides* was successfully isolated and is currently maintained in pure culture (Keppen et al. 1993, 1994).

All other observations of mesophilic *Chloroflexus*-like organisms have been reported from marine or hypersaline marine environments. We have chosen to designate all of these diverse strains as Marine *Chloroflexus*-Like Organisms (MCLOs). We are using the designation MCLO to apply to all filamentous phototrophs containing chlorosomes and BChl *c*, *d*, or *e* and probably BChl *a* observed in marine or hypersaline marine habitats. We recognize, however, that future biochemical and phylogenetic analyses of these organisms might reveal that they are not actually closely related to *Chloroflexus*. Abundant MCLOs have been identified by transmission electron microscopy from hypersaline mats in Laguna Figueroa, Baja California, Mexico (Stolz 1983, 1984, 1990, 1991) and in Guerrero Negro, Baja California, Mexico, and Solar Lake, Sinai Peninsula, Egypt (D'Amelio et al. 1989). Venetskaya and Gerasimenko (1988) detected MCLOs in the hypersaline mats in lagoons near the Sea of Azov using electron microscopy. Evidence from distribution of pigments (bacteriochlorophylls and γ-carotene) in the hypersaline mats at Guerrero Negro correlates well with the EM evidence for the distribution of MCLOs in these mats (Palmisano et al. 1989). Since γ-carotene is restricted to *Chloroflexus* among phototrophs, this correlation supports the conclusion that the chlorosome-bearing filaments are *Chloroflexus*-like organisms rather than filamentous green sulfur bacteria.

MCLOs were observed in sandy sediments of normal salinity in the Great Sippewissett Salt Marsh, Falmouth, MA, in 1983 (R.W. Castenholz, personal communication). In 1984 we observed the MCLOs as part of a migrating consortium of phototrophs occupying the surface of the sandy sediments in a shallow intertidal pool at night and migrating deeper into the sediments during the day. We studied the ecology and ultrastucture of these MCLOs using field collections in 1986 and 1987, but did not succeed in culturing them. Some of these results were previously published (Mack and Pierson 1988). Subsequently a similar MCLO was successfully grown in mixed culture (by EEM) from intertidal sediments on Mellum Island, Germany. Although not purified, this MCLO has been successfully maintained as the sole phototroph in mixed culture since 1988, and its ultrastructure is reported here.

Our studies on the hypersaline microbial mats in the salt evaporation ponds at Exportadora de Sal in Guerrero Negro began in 1986 when we systematically surveyed the ponds to locate the mats with the highest densities of MCLOs. Although originally observed and studied most intensively in Pond 5 (D'Amelio et al. 1989; Palmisano et al. 1989; Des Marais et al. 1992), more massive accumulations of MCLOs were found in ponds at higher salinities (Ponds 6, 7, 8, and 9) and most of the work reported here concerns the field studies, initial culturing, and ultrastructural characterization of these hypersaline MCLOs.

Materials and methods

Light microscopy

Microscopic observations and photomicrographs were made with a Zeiss Standard 16 microscope equipped for epifluorescence. Previously described techniques for detecting bacteriochlorophyll fluorescence (Pierson and Howard 1972) were modified. Excitation of chlorophyll and bacteriochlorophyll autofluorescence was produced with a 200 W mercury lamp and Zeiss BG 12 and BG 38 filters. Red chlorophyll fluorescence was observed with a barrier system of a Zeiss 590 filter and a 510 dichroic mirror. Kodak Wratten

filters (88A or 87C) were inserted between the other barrier filters and the camera to exclude visible fluorescence for detection of NIR fluorescence from bacteriochlorophylls. Since NIR fluorescence is not visible, the microscope was equipped with a lead sulfide (IR VIDICON) video camera, Dage-MTI model LSC-67M (Michigan City, Indiana). Real time images of NIR-fluorescing living cells were then displayed on a monochrome monitor (Lenco PMM-925) (Jackson, Missouri). Chlorophyll *a*-containing cyanobacteria were readily recognized by their bright red fluorescence and very weak NIR fluorescence. BChl *a* and/or *c*-containing phototrophs were recognized by their total lack of visible fluorescence and very strong NIR fluorescence. BChl *d* or *e*-containing bacteria showed a very dim (barely detectable) dull red fluorescence and bright NIR fluorescence.

Field observations

Salinity of pond water was measured with a hand-held refractometer, Reichert Scientific Instruments Model 10419 (Buffalo, NY). Temperatures were measured with a YSI battery-operated Tele-thermometer, Model 42SC (Yellow Springs Instrument Co., Yellow Springs, Ohio). Total irradiance was measured with a pyranometer, Li-Cor 185B, (Lincoln, Nebraska). Spectral irradiance was measured with a Li-Cor spectroradiometer (Model LI-1800) connected to a fiber optic sensor as previously described (Pierson et al. 1990). pH was measured with ColorpHast narrow range indicator strips (EM Science, Gibbstown, NJ).

Pigment and protein analysis

Pigments were analyzed in methanolic extracts and in sonicated cell-free extracts in buffer as previously described (Pierson et al. 1987; Castenholz et al. 1992). All spectra were recorded using a Cary 2300 UV/Vis/NIR spectrophotometer (Varian-Techtron, Mulgrave, Victoria, Australia). Total protein was determined on the washed pellets used for the pigment determinations using the Folin-phenol method (Lowry et al. 1951) with lysozyme as the standard.

Uptake experiments

All uptake experiments were performed in situ at Guerrero Negro in November and April of 1987, 1988, and 1990, unless otherwise noted. The mat layers were separated from each other and the cells were homogenized with glass homogenizers in pond water or in modified MBL (Marine Biological Laboratory, Woods Hole, MA) artificial sea water containing: NaCl, 100 g/l; KCl, 0.67 g/l; $CaCl_2 \cdot H_2O$, 1.36 g/l; $MgCl_2 \cdot 6H_2O$, 4.66 g/l; $MgSO_4 \cdot 7H_2O$, 6.29 g/l; $NaHCO_3$, 0.18 g/l to form an even suspension. The pH was 7.8 to 8.2. ^{14}C-labelled $NaHCO_3$ (specific activity 8.4 mCi/mmol, New England Nuclear, Boston) was added to a final radioactive concentration of 0.5 μCi/ml. ^{14}C-labelled acetate (uniformly labeled, specific activity 56 mCi/mmol, Amersham International, Amersham, UK) was added to a final radioactive concentration of 0.05 μCi/ml. All of the cell suspensions contained 5×10^{-6} M 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and 0.5 to 2.0 mM sulfide (added as neutralized $Na_2S \cdot 9H_2O$) depending on the experiment and unless otherwise specified. The pH of the cell suspensions after all additions was around 8. The cell suspensions were mixed and dispensed in 2.0-ml aliquots into 2-ml capacity screw cap vials with narrow necks to be incubated under the appropriate conditions (described below) at 27 to 36 °C.

Sample vials were incubated in petri dishes blackened on the sides and bottom with electrical tape and maintained in a water bath for temperature regulation. Different filters were used to cover the dishes to obtain desired light conditions.

Variations in light intensity were obtained with different thicknesses of neutral density nylon mesh or vinyl film. Wratten gelatin filters (88A, 89B, 87A, B, C) (Eastman Kodak Co., Rochester, NY) were used to eliminate visible radiation for near infrared incubations. Narrow band interference filters (10 nm half band width, 750 nm) (Esco Products, Oak Ridge, NJ) were used to provide light absorbed exclusively by BChl *c*. Dark samples were incubated in dishes covered with blackened tops.

At the end of one hour of incubation, photosynthesis was stopped with the addition of 0.1 ml of 37% formaldehyde to each vial. The vials were stored in the dark at 4 °C until the samples were processed. One or two ml aliquots were filtered onto 25-mm membrane filters (Gelman GN-6: 0.45 μm; Metricel or Millipore GS: 0.22 μm) and counted in a liquid scintillation counter (Beckman LS-3133T or Packard Tri-Carb 2200 CA) using Biofluor Emulsifier Cocktail (New England Nuclear Research Products, Boston, MA). External standards were used to determine efficiency of counting in each sample and CPM were converted to DPM. Duplicate or triplicate samples were incubated in all experiments and data were reported as the aver-

Table 1. Light-dependent carbon metabolism pond 6 and 8 MCLO layers[a]

	Uptake ^{14}C-bicarbonate (DPM)			Uptake ^{14}C-acetate (DPM)		
	Site 1	Site 2	Site 3	Site 1	Site 2	Site 3
Dark + formalin[b]	224 ± 1	170 ± 3.5	246 ± 9	692 ± 86	7,159 ± 87	7,022 ± 188
Dark	480 ± 24	455 ± 5	1,036 ± 11	21,301 ± 1,274	24,585 ± 620	29,051 ± 902
40 Wm^{-2}	1,528 ± 59	2,260 ± 139	3,094 ± 77	28,130 ± 1,835	25,811 ± 738	30,863 ± 811
162 Wm^{-2}	1,928 ± 64	2,339 ± 20	3,334 ± 160	26,499 ± 959	26,688 ± 196	29,997 ± 175
349 Wm^{-2}	1,499 ± 9	2,740 ± 67	3,044 ± 28	28,292 ± 993	27,400 ± 993	29,835 ± 3,167
595 Wm^{-2} (full sun)	969 ± 2	1,609 ± 64	2,439 ± 534	24,065 ± 1,170	27,343 ± 300	31,519 ± 732
88A (NIR)[c]	1,684 ± 92	2,566 ± 66	2,615 ± 47	28,358 ± 398	27,755 ± 200	29,108 ± 2,808

[a]Cells from the lower olive-green layers in mats from Pond 8 (Site 1 and 2) and Pond 6 (Site 3) were suspended in pond water and incubated with ^{14}C-bicarbonate (0.5 μCi/ml) and 0.5 mM sulfide or ^{14}C-acetate (0.5 μCi/ml) under the conditions listed for one hour. All samples contained DCMU (5.0 μM) and were incubated at 24 °C. Salinity for Sites 1 and 2 was 123 ppt and Site 3 was 92 ppt. Samples (0.5 ml) were filtered and counts expressed as DPM. Results are presented as means with standard deviations.

[b]A sample was incubated in the dark with formalin to measure non-metabolic adsorption of labelled substrate.

[c]NIR radiation was supplied with a Kodak Wratten 88A filter. The irradiance was 170 Wm^{-2}.

age Disintegrations per Minute (DPM) with standard deviations.

The quantitative autoradiography experiment was done in the lab with enriched cultures obtained from Pond 6. The experimental conditions included the enrichment medium with 2.0 mM sulfide, DCMU (5.0 μM), and a high radioactive concentration of ^{14}C-bicarbonate (2.0 μCi/ml). Aliquots of cells were incubated in the dark, full spectrum radiation (10 Wm^{-2}), and under a 750 nm interference filter. Samples were processed for autoradiography as previously described (Pierson et al. 1984).

Electron microscopy

Cultured cells of the hypersaline strains were suspended in 8.5–13% NaCl in water (pH 7.5) and centrifuged at 7,000 rpm for 7 min. Pellets of cells were embedded in 1% agar in the same saline solution. The resulting 'beads' of agar-encased pellets were trimmed to 2-mm cubes and handled like animal tissue during fixation and embedding. The procedure described below has been modified from a procedure originally described by D'Amelio et al. (1987).

Primary fixation was done for 2 h on ice in a 0.2 M sodium cacodylate solution (final pH 7.3) containing 2% glutaraldehyde, 1% acrolein, approximately 50 mM $CaCl_2$, and 8.5–13% NaCl. Following the primary fixation, three 10 min rinses were carried out in 0.1 M sodium cacodylate buffer (pH 7.4) at room temperature. The first rinse contained NaCl. The secondary fixation was done for 2 h on ice in a 0.2 M sodium cacodylate solution (pH 7.4) containing 1–2% osmium tetroxide. The samples were rinsed as described above. Dehydration was carried out in a graded series of ethanol solutions for 10–15 min in 2 changes of each solution. The samples were washed in a 50:50 mixture of 100% ethanol and propylene oxide followed by 100% propylene oxide.

A soft epoxy resin consisting of LX112 (Epon) (5 g), Araldite 502 (3 g), and dodecenyl succinic anhydride (DDSA) (11 g) was used for embedding. Tri(Dimethylaminoethyl)phenol (DMP-30) was added to the above mixture (0.2 g/10 ml resin mixture). Samples were pre-embedded in propylene oxide/epoxy resin mixtures for several hours before pre-embedding in 100% epoxy resin for 3 h. Samples were then trimmed of excess agar, embedded in fresh resin and cured at 60 °C for 48 h.

Thin sections were cut with a diamond or glass knife and supported on 300 mesh copper grids. The sections were post-stained for 5–6 min in a saturated solution of uranyl acetate in 50% ethanol, rinsed with distilled water, and then stained for 30–37 min in 0.5 mg/ml alkaline bismuth subnitrate solution.

Electron micrographs were taken with a Zeiss 109 transmission electron microscope operating at 50 or 80 kV.

The Mellum Island cultures were prepared using the above procedure except the pH and salinity were adjusted to 8.0 and 3%, respectively. The Sippewissett field samples were fixed in seawater immediately

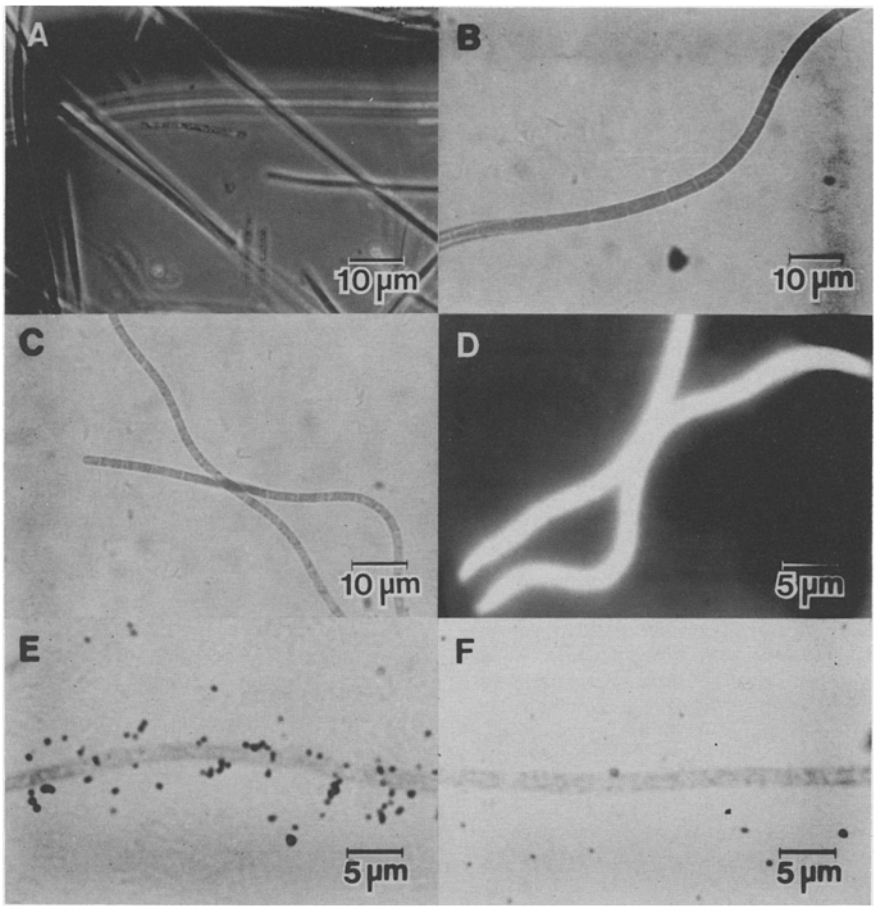

Fig. 1. **Photomicrographs of MCLOs. A. Phase contrast micrograph of MCLOs from Great Sippewissett Salt Marsh sometimes** found in association with ***Chloroherpeton*** **(long rod-shaped cells). B. Phase contrast micrograph of 3.0 µm diameter MCLO-2 from hypersaline mat. C.** Phase contrast micrograph of 1.5 µm diameter MCLO-1 from hypersaline mat. D. Photomicrograph of infrared fluorescence of filaments in C. E. Photomicrograph of autoradiogram of MCLO incubated with ^{14}C-bicarbonate in 750 nm light. F. Photomicrograph of autoradiogram of MCLO incubated with ^{14}C-bicarbonate in dark.

after collection with 2.5% glutaraldehyde and then processed as above except that they were post-stained with uranyl acetate and lead citrate.

Results and discussion

Habitat and distribution/marine intertidal environments

The distribution of MCLOs in the marine intertidal environment has been described (Mack and Pierson 1988). Unlike the relatively stable hypersaline ponds described below, the intertidal habitats were ephemeral, changing seasonally and yearly. *Chloroflexus*-like organisms were observed in association with may different bacteria at different times but most notably with species of *Thiocapsa*, *Chloroherpeton*, *Oscillatoria*, and *Beggiatoa* (Mack and Pierson 1988) (Fig. 1A). In the intertidal sandy sediments at Sippewissett Salt Marsh, mats containing MCLOs showed diel migrations of the MCLOs, *Beggiatoa*, and cyanobacteria. The MCLOs were found a few mm below the surface layer of *Thiocapsa* during the day. At night they migrated to the surface and could be induced to do so during the day when the mat surface was darkened or subjected to reduced light intensities (Mack and Pierson 1988).

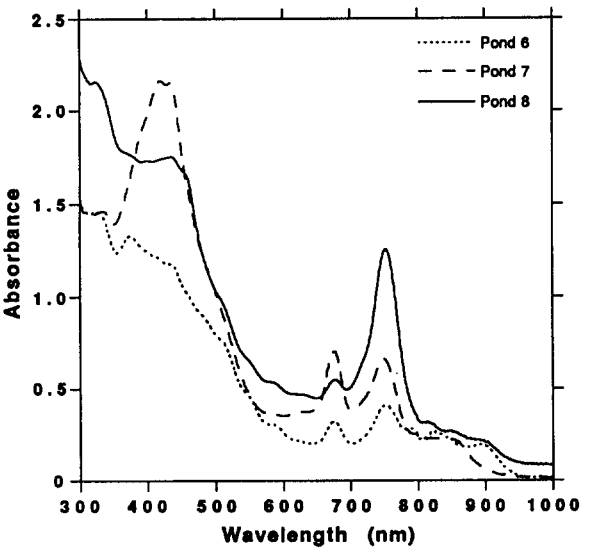

Fig. 2. Absorption spectra of pigments in MCLO mat layers from Ponds 6, 7, and 8. The in vivo absorption spectra (cells sonicated in buffer) of MCLO layers from three different sites reflect the different communities of phototrophs associated with MCLOs. In Pond 6 mat (. . .) the MCLOs (BChl *c* peak at 753 nm) are found along with cyanobacteria (Chl *a* at 676 nm) and very few purple sulfur bacteria (BChl *a* peaks from 790 to 900 nm). In Pond 7 mat (- - -) the MCLOs (BChl *c* at 750 nm) are found with the cyanobacterium *Spirulina* (Chl *a* at 676 nm). No purple sulfur bacteria were evident and the absorption from 800 to 900 nm may be due to the MCLO. In the Pond 8 mat (—) the MCLOs (BChl *c* at 753 nm) are found with very few cyanobacteria (Chl *a* at 678 nm). The source of the absorption maxima for BChl *a* (800 to 900 nm) remains undetermined. It could be the MCLOs or the few purple sulfur bacteria present.

Habitat and distribution/ hypersaline environments

General Habitat Description: MCLOs were observed in benthic microbial mats in several different salt evaporation ponds in Exportadora de Sal in Guerrero Negro, Baja California Sur, Mexico. A complete description of the pond system is included in Des Marais et al. (1992). The ponds were fairly shallow (rarely exceeding 1.0 m in depth), covered an area of several square km and were maintained within a limited range of salinities for periods of several months. Over the 4-year period of this study, salinities were measured in the following ranges: Pond 5 (75–95 ppt), Pond 6 (95–140 ppt), Pond 7 (124–160 ppt), Pond 8 (110–140 ppt), and Pond 9 (salinity greater than 130 ppt). The pH in all ponds ranged from 7.8–8.4. Water temperatures in the fall and spring when most experiments were conducted ranged from 18 to 21 °C. Temperatures in the mats themselves were frequently higher (28–30 °C), and mat temperatures in the summer could reach 40 °C or higher. Depths at which mats were found ranged from 40 cm in Pond 8 to 150 cm in Pond 7.

Microscopy and Pigment Analysis: The MCLO-containing layers within the mats were recognized as olive green layers frequently interlaced with white strands of *Beggiatoa* usually found below the cyanobacterial surface layer or below an intervening layer of purple sulfur bacteria. Although the olive-green layers contained abundant MCLOs, other microorganisms were also present. Both microscopic examination and pigment analysis were used to determine the identity of the major microorganisms in each mat layer. MCLOs in field collections were recognized microscopically as long yellow-green or olive-green filaments from 1.0 to 3.0 μm in diameter (Fig. 1B, C). The presence of bacteriochlorophylls in the filaments was confirmed with infrared fluorescence photomicrography (Fig. 1D). The absorption spectra of pigments in collected layers of MCLOs from Ponds 6, 7, and 8 (Fig. 2) show the range in relative proportions of Chl *a*-containing cyanobacteria and BChl *c*-containing MCLOs.

The MCLOs in Pond 5 were frequently found with *Beggiatoa* and some purple sulfur bacteria beneath the well-defined surface layer of cyanobacteria (primarily *Microcoleus chthonoplastes*). Some cyanobacteria were also found among the MCLOs deeper in the mat. The layer of MCLOs was not very conspicuous in the Pond 5 mats. The microbiology of Pond 5 mats dominated by *Microcoleus* was previously studied in detail (D'Amelio et al. 1989; Palmisano et al. 1989; Jørgensen 1989; Des Marais et al. 1992; Kühl and Jørgensen 1992).

Our studies were focussed on mats in Ponds 6, 7, and 8 which had more conspicuous layers dominated by MCLOs. The MCLOs in Pond 6 were also found in association with *Beggiatoa*, purple sulfur bacteria, and some cyanobacteria, primarily *Spirulina* and *Microcoleus*. The surface cyanobacterial layer was composed of both of these genera in Pond 6 and was less well-defined than in Pond 5. An intervening layer of purple sulfur bacteria was sometimes present. Layering was less well-defined in Pond 7 mats, and the MCLOs were well-mixed at the surface with the cyanobacterium *Spirulina* and with *Beggiatoa*, although the MCLOs were greatly enriched relative to the others just below the surface layer. The Pond 8 mats were very different from the other mats, being much less compact, more transparent, shallower, and 'chunky' with visible gypsum and carbonate deposition occurring. These mats were thick and high-

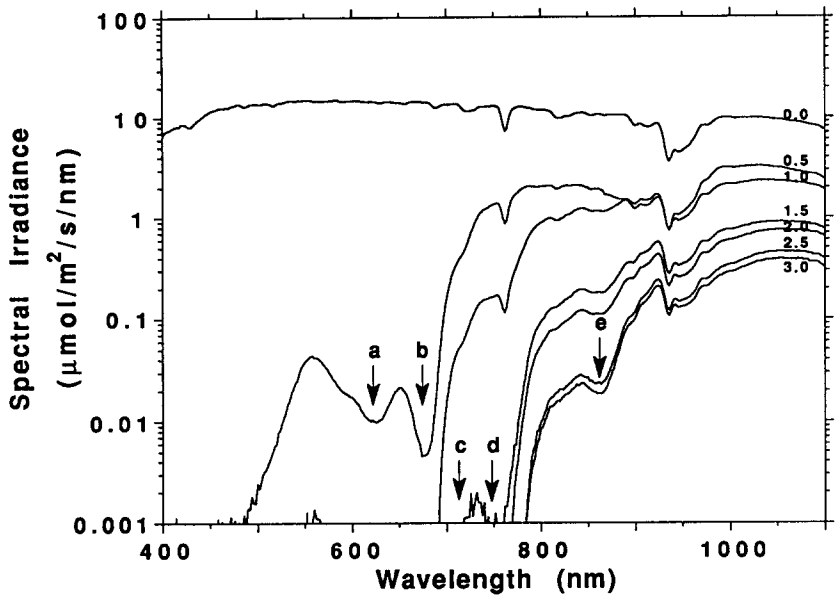

Fig. 3. Spectral irradiance as a function of depth in Pond 6 mat. Light penetration was measured at depth intervals of 0.5 mm in a mat removed from Pond 6 using a spectroradiometer with a fiber optic milliprobe positioned in the mat. This profile represents the spectral signatures of phototrophs within the mat in the absence of an overlying water column. The irradiance values therefore are not representative of actual in situ values. The troughs or valleys in each spectrum are due to the drop in irradiance at particular wavelengths caused by the absorption by pigments in the mat layers above the sensor. The top 1.0 mm of mat was dominated by cyanobacteria containing phycocyanin and chlorophyll *a* absorbing at 620 (a) and 670 (b) nm, respectively. The mat layer between 1.0 and 1.5 mm contained MCLOs attenuating the radiation at 710 nm (BChl *e*?) (c) and 750 nm (BChl *c*) (d). The attenuation by BChl *a* at 860 nm (e) was seen most strongly at depths greater than 2.0 mm.

ly layered. The surface was bright orange and gelatinous, composed of some filamentous cyanobacteria and abundant unicellular *Aphanothece.* The orange was due to carotenoid pigments probably synthesized in response to high solar irradiances experienced in these very shallow mats. Underneath this layer was a green layer of cyanobacteria and a dark pink layer of purple sulfur bacteria. Beneath this pink layer, the olive MCLO layer contained some *Aphanothece* as well as some filamentous cyanobacteria, some purple sulfur bacteria, and *Beggiatoa,* as well as very abundant MCLO filaments. Beneath the MCLO layer was a dark red layer of some filamentous and unicelluar bacteria that remain unidentified.

Distribution of MCLOs in Situ: Spectroradiometry with a fiber optic probe (see Pierson et al. 1990 for a complete description of this technique) was used to determine the distribution and relative abundances of MCLOs and other phototrophs within the mats. This technique allowed sensitive and immediate detection of the MCLOs at any depth in the mat without preselecting colored layers for laboratory microscopy and pigment extraction. The depth profiles of the different mats (Figs. 3, 4) are plots of spectral irradiance with depth measured in excised mat samples without the overlying water column, hence the absolute values of irradiance do not reflect the actual values in situ. The light fields within the mats in situ are greatly altered by the absorption and scattering properties of the overlying water (Pierson et al. 1990). Scattered light within the mats (not measured here) also alters the light fields (Kühl and Jørgensen 1992). These figures show relative spectral attenuation at each depth to identify the presence and relative abundance of particular pigments only. Since the troughs or valleys in each spectrum represent absorbance by all of the photosynthetic pigments present above the depth of the probe in the mat, the plots of spectral irradiance with depth provide a unique spectral signature for each mat which is representative of all the photosynthetic organisms present.

The mat from Pond 6 (Fig. 3) was collected from a depth of 1.0 to 1.5 m. At this depth water substantially attenuates near infrared radiation. The levels of light available to the MCLOs in situ would therefore be much lower than indicated on the depth plot (Fig. 3). This spectral signature (Fig. 3) reveals the presence of cyanobacteria containing phycocyanin and chloro-

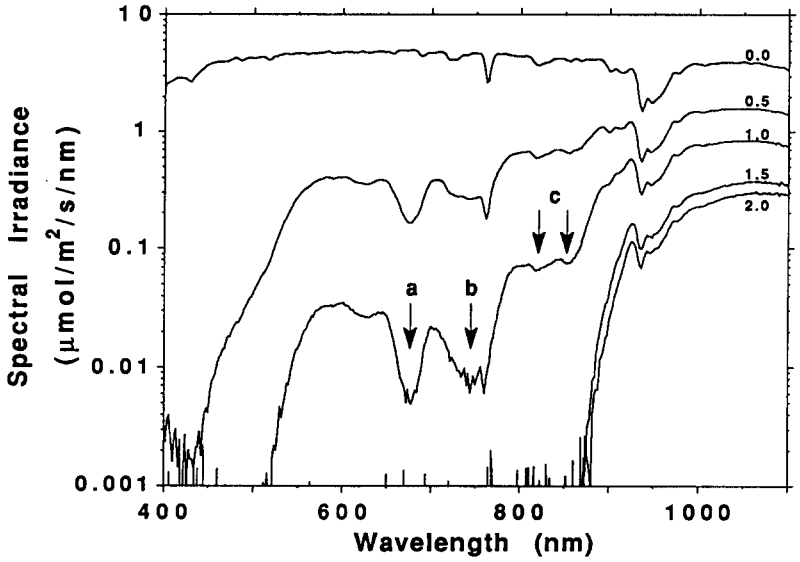

Fig. 4. Spectral irradiance as a function of depth in the Pond 7 mat. See Fig. 3 for explanation of method. In this mat the MCLO, cyanobacteria, and *Beggiatoa* exist together in the same layer within the top 1.0 mm of the mat. The MCLO is enriched in the layer from 0.5 to 1.0 mm. Chl *a* (a), BChl *c* (b), and BChl *a* (two arrows at c). Note that the irradiance values are relative only due to the absence of the overlying water column.

phyll *a* at a depth of 0.5 mm. Strong absorption by these pigments and carotenoids in cyanobacteria substantially attenuated the visible radiation (wavelengths less than 700 nm) at a depth of 1.0 mm. Evidence for the presence of BChl *a*-containing purple sulfur bacteria or BChl *a* in the MCLOs was seen as the slight trough at 860 nm at a depth of 1.5 mm in the mat. Also at this depth there was strong attenuation of radiation at 710 nm (possibly BChl *e* or perhaps a degradation product) and at 750 nm (BChl *c*) apparently from the MCLOs.

The spectral signature for the Pond 7 mat was different from that of Pond 6. The mat was a dark green color for the top 1 to 2 mm and orange beneath this. Evidence for both Chl *a* and BChl *c* was present in the top 0.5 mm of the mat (Fig. 4). The troughs were more pronounced in the spectrum at a depth of 1.0 mm showing the presence of BChl *a* attenuation near 810 and 860 nm, broad BChl *c* and perhaps *d* or *e* attenuation from 710 to 750 nm, Chl *a* attenuation at 670 nm and evidence for phycocyanin attenuation at 620 nm. The mat was comprised of a mixture of MCLOs, *Beggiatoa*, and *Spirulina*. Few if any purple sulfur bacteria were seen. Pond 7 was not in the regular concentrator series of ponds in the salt works. It was used as a holding pond that did not have a continuous flow pattern as in the other ponds. Consequently the salinity was very high (measured as high as 164 ppt) and was 148 ppt at the time these data were collected. The mats were found very deep in this pond under 1.5 to 2.0 m of water and must have been considerably light-limited. Because of its very restricted diversity and strong dominance of MCLOs we planned to focus our subsequent field and culture studies on these mats. However, in 1988 the retaining dike for Pond 7 broke, the Pond largely drained, the mats were destroyed, and our work here ceased except for one cultured organism described below.

The pond 8 mat (data not shown, see Pierson 1994) differed in several important ways from the other mats. It was a much thicker mat, very translucent, and was found in a depth of only 40 cm of pond water. Consequently light penetrated to much greater depths in this mat allowing for the growth of greater numbers of different phototrophs present as brightly colored layers to a depth of 10 mm. The substantial attenuation due to BChl *c* occurred in an olive layer 8.0 to 9.5 mm deep in the mat.

Light-dependence and autotrophy in situ: Marine intertidal environment

Uptake experiments analyzed by autoradiography were done with the MCLOs collected in the sandy mats

Table 2. Specific pigment contents of cell suspensions [a]

	Site 1	Site 2	Site 3
Total protein (mg/ml)	0.082	0.110	0.077
BChl *a* (μg/mg protein)	7.52	7.36	5.06
BChl *c* (μg/mg protein)	17.20	32.18	12.08
BChl *a* /BChl *c*	0.43	0.23	0.42

[a] The protein and pigment values reported here are for the cell suspensions used in the experiments described in Table 1.

of Great Sippewissett Salt Marsh. These experiments were done in normal salinity artificial seawater at pH 7.8. The data reported in Mack and Pierson (1988) showed only questionable evidence for autotrophy but did show significant photoincorporation of acetate in the presence of low (0.1 mM) and high (1.0 mM) sulfide. Photoinhibition occurred in full spectrum sunlight above 600 Wm^{-2} (Mack and Pierson 1988). The presence of photoheterotrophy in these marine populations is similar to the phototrophic activity of most *C. aurantiacus* populations in hot springs (Pierson and Castenholz 1992).

Light-dependence and autotrophy in situ: Hypersaline environments

Cell suspensions of MCLOs prepared in natural pond water were incubated in various conditions to measure photosynthetic activity. Such experiments were necessary to show the potentially significant impact of MCLOs on the mat ecosystem by measuring metabolic activities of freshly collected cells in situ. Initially the uptake of ^{14}C-bicarbonate was measured as a function of time. The uptake increased linearly with time up to at least 2 h (data not shown). Consequently all incubations were for 1 h.

The olive layers from Ponds 6 and 8 mats were separated in the field, homogenized to produce even suspensions of predominantly MCLOs, and were incubated with ^{14}C-bicarbonate to assess the photosynthetic activity of the filaments under various conditions (Table 1). Sites 1 and 2 were similar Pond 8 mats and Site 3 was in Pond 6. Initially uptake of ^{14}C-bicarbonate was compared with that of ^{14}C-acetate since it was not known whether the carbon metabolism of the hypersaline MCLOs in situ was primarily photoautotrophic (as observed in only a few *Chloroflexus* strains in hot springs) (Giovannoni et al. 1987) or photoheterotrophic as in most thermophilic *Chloroflexus* strains (Pierson and Castenholz 1992). A slight light-dependent uptake of ^{14}C-acetate was observed (Table 1), but the dark uptake was also very high. These data did not convincingly support a conclusion for photoheterotrophy, and autoradiographic analysis of the same samples revealed that the acetate uptake was confined to small unicellular bacteria present as contaminants in the suspension and was absent from the filamentous MCLOs (data not shown). The MCLOs were clearly photoautotrophic (Table 1) and autoradiography confirmed that the light-dependent uptake of ^{14}C-bicarbonate was due to the MCLOs (data not shown). In all cases there was significant photoinhibition at the highest irradiances used (Table 1). Such inhibition was not surprising given the low irradiances prevailing deep in these mats in situ.

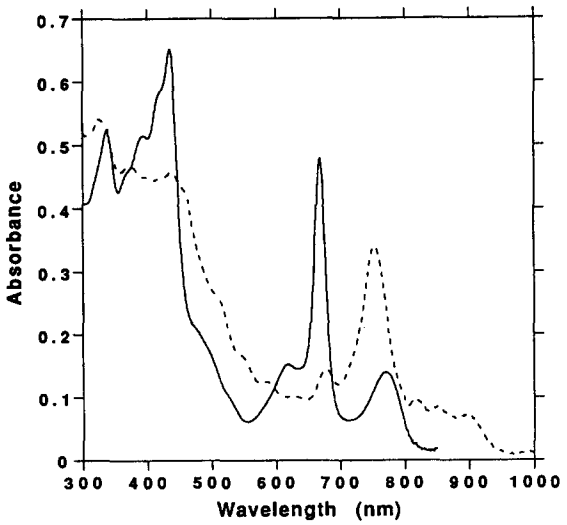

Fig. 5. Absorption spectra of pigments in mat layers used for uptake studies. The in vivo extract sonicated in buffer (- - -) and methanol extract (—) were prepared from the cell suspension of the MCLO layer in Pond 8 mat used to measure the light-dependent uptake of ^{14}C-bicarbonate in artificial sea water described in the text. The spectra of cell suspensions used in the other uptake experiments were very similar to these. The peaks at 753 nm in buffer and 668 nm in methanol are due to BChl *c* in the MCLOs. The complex of peaks in the buffer at 815, 850 and 894 nm are due to the BChl *a* (771 nm in methanol) which may be present in the MCLOs and other bacteria present in the suspension.

The MCLOs from deep within the mats were rarely if ever exposed to substantial visible radiation. In situ, the MCLOs were exposed to a light field of exclusively far red and NIR radiation (see Figs. 3, 4) of low total irradiance. There was substantial uptake in the presence of NIR radiation (Table 1). The presence of far

red and NIR-absorbing pigments in the incubation suspensions was verified with methanolic extracts and cell free extracts in buffer. All spectra were very similar to those in Fig. 5. BChl *c* with an absorption maximum at 753 nm in vivo and 668 nm in methanol (Fig. 5) was clearly the most abundant pigment. BChl *a* was also present with absorption maxima at 815, 850, and 900 nm in vivo and 771 nm in methanol (Fig. 5). Since the Wratten 88A filter used in this experiment (Table 1) transmitted radiation from 720 to 1100 nm, it is not clear whether one or both of these bacteriochlorophyll pigments sustained the autotrophic metabolism in these bacteria. The pigments were quantified in the methanolic extracts of each suspension used for uptake experiments and were expressed as μg pigment per mg protein (Table 2). The suspensions from the different sites had comparable biomasses as indicated by the protein concentrations and comparable amounts of BChl *a*. Site 2 had a much higher specific content of BChl *c* resulting in a BChl *a* to *c* ratio that was one-half that in Sites 1 and 3 (Table 2).

The photosynthetic activity of suspensions of Pond 8 MCLO layers was enhanced by the presence of added sulfide (data not shown). A light-dependent uptake of ^{14}C-bicarbonate occurred in the absence of sulfide but was considerably greater in the presence of 0.5 mM sulfide. Higher concentrations of sulfide (1.0 and 2.0 mM were tried) also enhanced the light-dependent uptake but not as much as 0.5 mM. The added sulfide actually depressed the ^{14}C-uptake rates in the dark while stimulating them in the light. From these observations it appears that chemoautotrophy (in *Beggiatoa*?) may be inhibited by sulfide as high as 0.5 mM while photoautotrophy in the MCLOs is stimulated. All experiments measuring autotrophic activity in MCLOs included 0.5 mM sulfide to avoid possible limitation of activity by low levels of endogenous reductant.

To verify that the observed light-dependent uptake of ^{14}C-bicarbonate represented true autotrophy and was not related to assimilation of unidentified organic substances in the pond water, one experiment was done with artificial seawater adjusted to a salinity of 100 ppt and totally lacking organic compounds. The pH was adjusted to 8.0 and sulfide was added (0.5 mM). Pond 8 MCLOs were used as in the other experiments described above, and the results were similar. High dark levels (1,421 ± 27 DPM) were obtained probably due to the activity of *Beggiatoa*. In the light, however, activity was greatly enhanced: 4,055 ± 39 DPM (irradiance of 480 Wm^{-2}) and 4,448 ± 177 DPM (irradiance of 250 Wm^{-2}). CO_2 uptake thus proceeded in

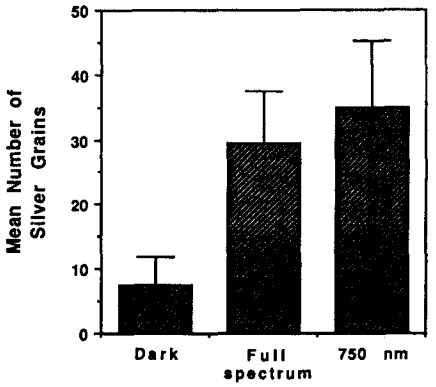

Fig. 6. Autoradiographic assay of ^{14}C-bicarbonate uptake by MCLO filaments in enrichment cultures. Filaments from a Pond 6 enrichment culture were incubated with ^{14}C-bicarbonate at high radioactive concentration in the presence of sulfide in the dark, full spectrum light (10 Wm^{-2}) and behind a 750 nm interference filter. Labelled filaments were deposited on slides and coated with emulsion. After development, silver grains were counted. Counts are expressed as mean number of grains per 50 μm length of filament with standard deviation. n = 20. See Fig. 1E, F for photomicrographs of representative filaments.

the absence of organic substrates. The spectra in Fig. 5 were from the cell suspension used in this experiment. All other experiments were incubated in the pond water collected at the site to maintain the microorganisms in the same salinities and ionic balances prevailing in situ at the time of collection.

All of the experiments discussed above indicated that the light-dependent autotrophy showed photoinhibition at high irradiances and that most of the uptake was sustained by NIR radiation (Table 1). Expanding the incubations to higher irradiances confirmed the presence of photoinhibition. The maximum uptake was at 102 Wm^{-2} (Table 3) decreasing to the lowest level at 920 Wm^{-2}. Even when maximally photoinhibited in this experiment at 920 Wm^{-2}, the MCLOs still showed twice the uptake rate as was observed in the dark. In the presence of most of the filters that transmitted only far red and NIR radiation, the uptake rates were equal to or greater than those occurring in the lowest full spectrum irradiance used in this experiment (102 Wm^{-2}) (Table 3). Only one filter (the Wratten 87A) sustained significantly lower rates of autotrophy which was probably because its transmission range (900–1100 nm) was beyond the absorption range of the bacteriochlorophylls in the MCLOs (Fig. 5). The very high uptake rates in the presence of infrared filters with total transmitted irradiances higher than the lowest full spectrum irradiance (Table 3) shows that

Table 3. Carbon uptake and irradiance in Pond 8 MCLO layer[a]

Incubation condition	Range (nm)	Irradiance(Wm^{-2})	DPM
Dark	–	0	1,000 ± 25
11% solar	400–1100	102	3,560 ± 68
26% solar	400–1100	238	3,435 ± 121
52% solar	400–1100	477	3,355 ± 157
70% solar	400–1100	648	2,905 ± 203
100% solar	400–1100	920	2,259 ± 179
87A filter	900–1100	16	1,796 ± 139
87B filter	800–1100	43	3,719 ± 211
87C filter	790–1100	59	4,004 ± 281
88A filter	720–1100	130	4,129 ± 390
89B filter	690–1100	146	3,523 ± 76

[a]Cells from the MCLO layer were suspended in pond water and incubated with ^{14}C-bicarbonate (0.5 μCi/ml), 0.5 mM sulfide, and DCMU (5.0 μM) for 1 hour at 31 °C. Salinity was 116 ppt. Results reported as means with standard deviations.

it is the NIR part of the spectrum that is sustaining photosynthesis in the MCLOs and that it is the shorter wavelength visible part of the spectrum that is causing photoinhibition.

To confirm that the light-dependent uptake of ^{14}C-bicarbonate was in the filaments of MCLOs and was dependent on BChl *c*, a quantitative autoradiographic analysis was done in the lab using MCLO filaments collected from a primary enrichment culture obtained from Pond 6 mat. The quantum flux under the filter (735–775 nm) was 31.1 μmol m^{-2} s^{-1}. The quantum flux over the same range of wavelengths in the full spectrum light was 6.0 μmol m^{-2} s^{-1}. The light-dependent uptake was clearly seen in the autoradiograms as a high density of silver grains associated with the filaments (Fig. 1E). The role of BChl *c* in sustaining this uptake was confirmed with the data for uptake under the 750 nm narrow band interference filter (Fig. 1E, 6).

Morphology

The MCLOs observed in this study in intertidal habitats of normal salinity at Great Sippewissett Salt Marsh were strikingly similar to *C. aurantiacus* in morphology. The flexible gliding filaments were indeterminate in length and 1.0–1.5 μm in diameter (Fig. 1A). The cross walls and thick cell wall (30–40 nm thick) were evident in the electron micrographs (Fig. 7A). Chlorosomes lined the cell membrane around the periphery of the cell and were similar in shape (roughly elliptical) to those in *C. aurantiacus*. The chlorosomes were similar in size to those of *C. aurantiacus*, measuring 60 by 100 to 170 nm. No internal membranes were observed, and abundant internal electron dense structures in the cytoplasm may be polyglucose storage granules although their identity remains uncertain. The cells from the Mellum Island cultures were very similar to the MCLOs from Sippewissett (Fig. 7B, C).

The hypersaline MCLO-1 cultured from Baja Pond 6 mat was ultrastructurally different from all previously described *Chloroflexus*-like organisms. The filaments were yellow-green with prominent cross-walls visible with light microscopy. They were flexible, gliding and measured 1.5–2.0 μm in diameter with sheath sometimes present (Fig. 1C). They stained Gram negative and showed no red autofluorescence but fluoresced strongly in the near infrared (Fig. 1D) confirming the presence of bacteriochlorophylls in the filaments.

Electron microscopy (Fig. 8) revealed thick cell walls and numerous unidentified inclusions. Some of the inclusions located centrally in the cytoplasm and of medium to light electron density were roughly polygonal in shape and bore some resemblance to carboxysomes (Fig. 8B, C). Their identity remains to be determined. Most striking, however, were the abundant membrane invaginations forming roughly spherical pockets or sacs 100–250 nm in diameter. These pockets often contained very electron dense inclusions similar to polyphosphate in appearence (Fig. 8). The invaginated pockets of membrane were lined on the cytoplasmic side with numerous chlorosomes. The chloro-

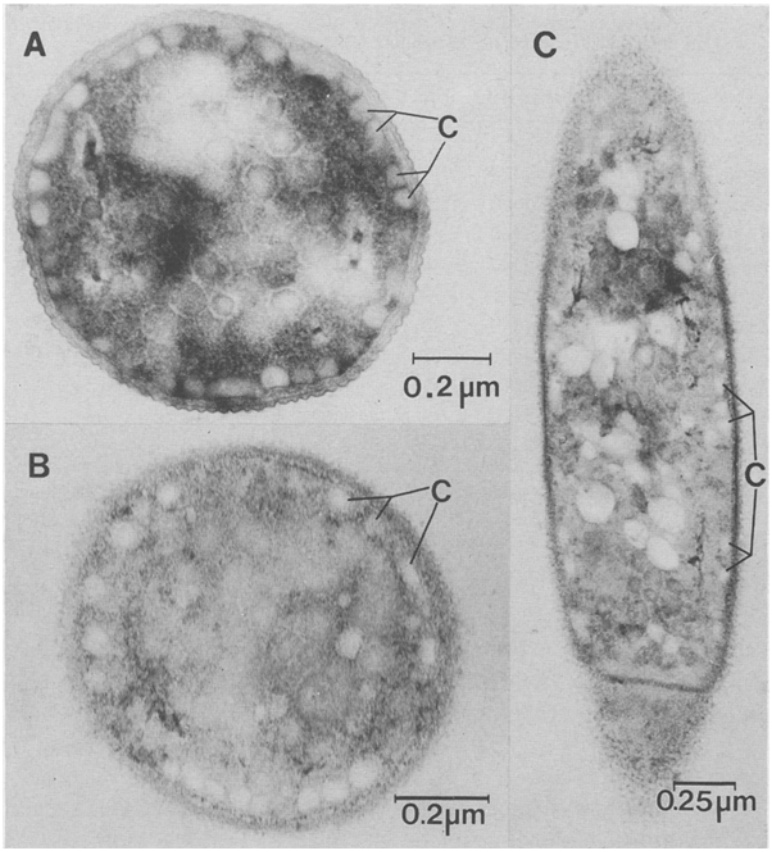

Fig. 7. **Electron micrographs of intertidal MCLOs. A. Cross section of Sippewissett MCLO (obtained from field collection)** containing no internal membranes and abundant peripheral chlorosomes (C) lining the cell membrane. B–C. Cross and longitudinal/oblique sections of Mellum Island MCLO (from a highly enriched culture) showing peripheral chlorosomes (C).

somes were also observed lining the cell membrane in regions between adjacent pockets. Chlorosomes were similar in general appearance to those described above although they appeared somewhat more flattened. The dimensions were in a similar range of 20 by 40–50 by 100–160 nm.

The hypersaline MCLO-2 cultured from Pond 7 mat was also quite different. The yellow-green, flexible, gliding filaments had readily visible crosswalls, were frequently sheathed, and were larger (2.5–3.0 μm in diameter) (Fig. 1B). They stained Gram positive, as does *O. trichoides* (Keppen et al. 1993), fluoresced very dimly red (barely detectable) and fluoresced strongly in the NIR.

Ultrastructurally they had a thick cell wall and often a very thick sheath (Fig. 9C). The cytoplasm contained apparent poly-B-hydroxybutyrate (PHB) inclusions and an extensive network of internal membranes that appeared to originate as invaginations of the cell membrane (Fig. 9C, D). Some of these membrane invaginations may have cell wall material included with them (Fig. 9C, D). The internal membranes occupied a high proportion of the internal cytoplasmic volume and were studded with chlorosomes although the peripheral cell membrane was mostly devoid of chlorosomes (Fig. 9A, E). These chlorosomes appeared smaller than the ones described above. They were 20–30 nm in diameter and about 15 nm in height. The length was difficult to obtain due to the absence of clear sections running parallel to the surface of the internal membranes for a sufficient distance to visualize the entire length of the chlorosomes. The length was estimated at 90–125 nm. All of the chlorosome dimensions reported here fall within or overlap closely with the range of dimensions reported for *C. aurantiacus* by various authors and reviewed by Sprague and Fuller (1991).

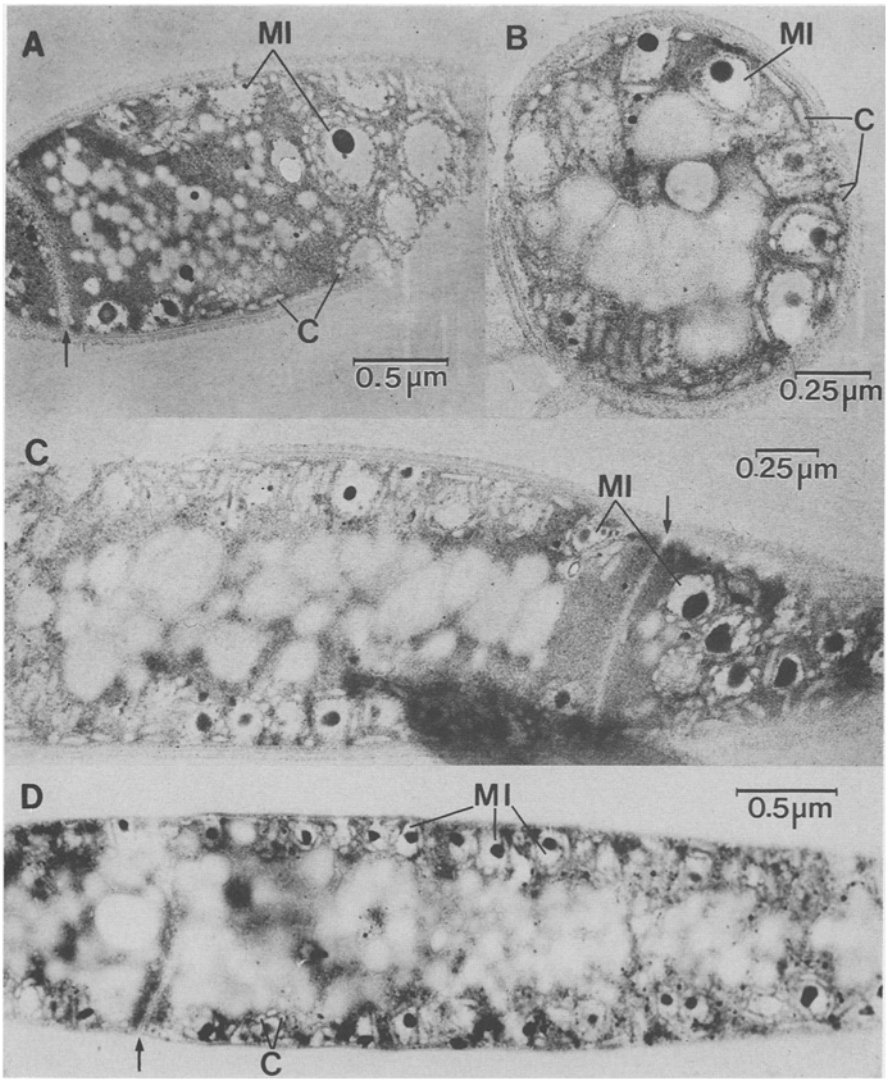

Fig. 8. **Electron micrographs of MCLO-1 from highly enriched cultures obtained from hypersaline microbial mats. A. Oblique** section showing septum (solid arrow) and pocket-like membrane invaginations (MI) lined with chlorosomes (C). B. Cross section showing MI pockets lined with chlorosomes and chlorosomes along cell membrane. C–D. Partially oblique and longitudinal sections showing abundant membrane invagination pockets (MI) and chlorosomes. Identity of dark-staining material in MI's remains undetermined but could be polyphosphate. Arrows show sites of septum formation.

Previous studies also revealed the presence of MCLOs in hypersaline microbial mats using TEM analysis of fixed mat material. Extensive electron microscopy by Stolz (1983, 1984) of the different layers observed in similar hypersaline microbial mats from Laguna Figueroa, Baja California, Mexico, showed the presence of MCLOs that were very similar in ultrastructure to the thermophilic *C. aurantiacus* and to the intertidal marine MCLOs described here having peripheral chlorosomes and no internal membranes. D'Amelio et al. (1989) described MCLOs found in the Pond 5 mats at Exportadora de Sal that likewise had an ultrastructure similar to *C. aurantiacus*. In addition to the MCLOs that looked similar to *C. aurantiacus*, Stolz reported the presence of MCLOs that he referred to as *Oscillochloris* because of the presence of chlorosome-lined internal membranes (1990 and 1991). Sprague and Fuller (1991) also included a micrograph from Stolz of this 'Oscillochloris'. These organisms are ultrastructually identical to the MCLO-2 described here (Fig. 9). Neither Stolz nor D'Amelio et al. (1989) reported an organism with the ultrastructure

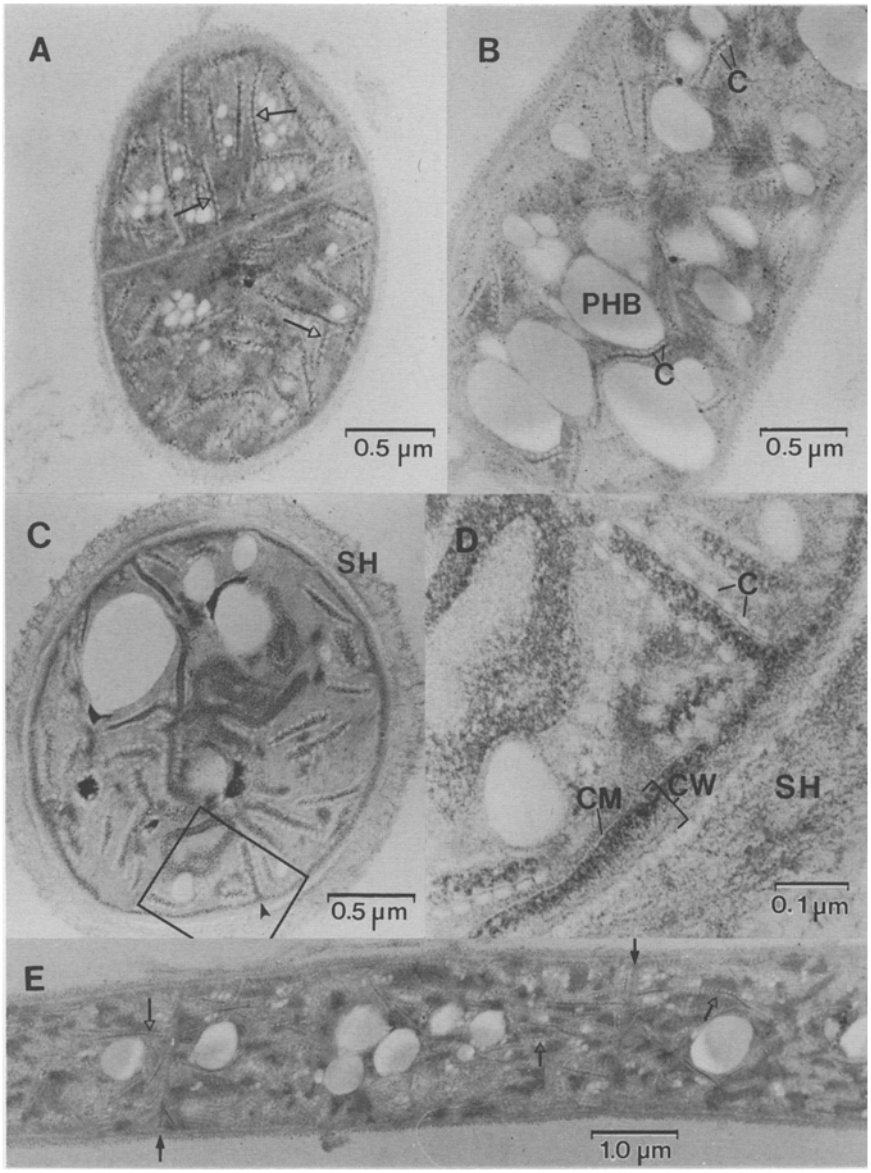

Fig. 9. **Electron micrographs of MCLO-2 from enrichment cultures obtained from hypersaline microbial mats. A. Oblique section** showing septum and abundant internal membranes lined with chlorosomes (open arrows). B. Longitudinal section showing PHB inclusions and internal membranes lined with chlorosomes (C). C. Cross section showing thick sheath (SH) and formation of internal membrane as invagination of cell membrane (inside box). D. Enlargement of boxed area in (C) showing invagination of cell membrane (CM), cell (CW), and chlorosomes (C). E. Longitudinal section showing sites of septum formation (closed arrows) and abundant internal membranes lined with chlorosomes (open arrows).

described here for MCLO-1, although D'Amelio had observed at least 4 ultrastructually distinct MCLOs in her unpublished electron micrographs of hypersaline microbial mats at Guerrero Negro (E.D. D'Amelio and D.J. Des Marais, personal communication). We are reluctant to refer to the MCLO-2 as *Oscillochloris* since the organization of the internal membranes is different from that of the type strains (Gorlenko 1989a) and neotype species (Keppen et al. 1993) of this genus.

Culture

Three MCLOs are currently maintained in culture in our labs. None of the cultures is pure, however, and

all three strains appear to be different in their growth requirements and are being maintained in quite different culture media. All strains have been maintained now for several years. Since all media are still being altered and growth conditions are being determined, it is not our intention to publish all of the details of the growth media or attempt to name the strains at this time. We have determined only a few of the growth characteristics. Most of the data reported here are on field studies and electron microscopy of these mixed cultures. The intertidal MCLOs from Sippewissett were never successfully cultured. The Mellum Island MCLO is maintained in a medium of normal seawater salinity modified from standard media for the enrichment and growth of purple and green sulfur bacteria (Pfennig and Trüper 1992). Modifications included the addition of acetate and yeast extract, the omission of sulfate, and the inclusion of an artificial sediment of aluminum phosphate for gliding bacteria (Widdel and Bak 1992).

The two hypersaline MCLOs were initially obtained as enrichment cultures in a similar medium with sodium chloride added to bring the salinity to 100 ppt. Successful enrichments were obtained by using 740 or 750 nm narrow band interference filters as the only light source to exclude growth of sulfide-tolerant cyanobacteria and purple sulfur bacteria. Enrichments were done at room temperature and included 2.0 mM sulfide. The enrichments also included low levels of yeast extract (250 mg/l). The MCLO-2, the larger filament, is currently maintained in liquid cultures containing yeast extract, acetate, sulfide, and an ionic balance similar to the Mellum Island culture medium except for the addition of extra NaCl (75 g/l). The pH is around 8 and cultures are grown at room temperature behind 740–750 nm interference filters. An aluminum phosphate sediment is included, and the filaments form a mat-like growth on the precipitate in the bottoms of the vials and spread over the glass walls of the vials. The cultures contain a large number of contaminants and may still contain other phototrophs as well. The electron micrographs reported here were obtained from these cultures.

The focus of our recent efforts has been cultures of the MCLO-1, the narrower filaments with the unique chlorosome-lined membrane sacs. It is apparently the only phototroph present in the crude cultures and the number of contaminants has been greatly reduced as the culture conditions have been improved. The cultures contain BChl *c* and *a* (Fig. 10A, B) but the BChl *a* is present in very low levels and is barely detectable

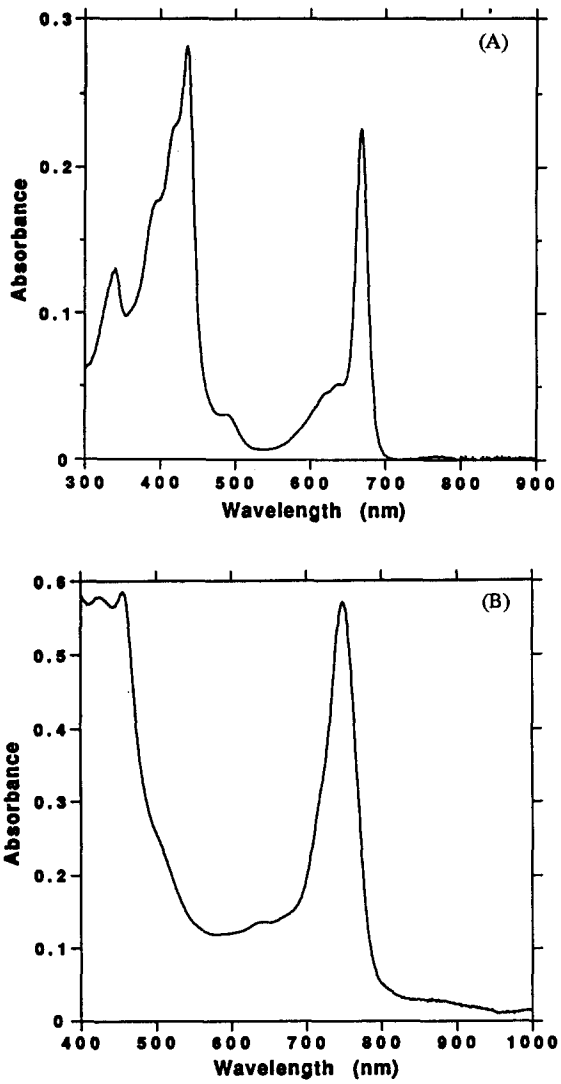

Fig. 10. Absorption spectra of MCLO-1 cultures. Cells were either extracted with methanol (A) or sonicated in buffer (B) to obtain absorption spectra of the photosynthetic pigments. The cultures were grown at low light intensity (10 Wm^{-2}) resulting in a very high BChl *c* content (absorption maxima at 750 nm in vivo and 668 nm in methanol).

even in organic extracts. The in vivo spectrum (Fig. 10B) is unlike that for *C. auantiacus* in this regard but similar to that for *Oscillochloris trichoides,* which also has a very low BChl *a* content (Keppen et al. 1993).

The current culture medium has enriched levels of calcium (1.45 g/l) and magnesium (5.78 g/l) similar to those estimated for Pond 6 water at a total salinity of 135 ppt from data provided by Javor (1989) for concentrator pond water at a salinity of 164 ppt. High NaCl

(100 g/l) is also included. The nitrogen source is ammonium. This medium contains no acetate and low levels of yeast extract (250 mg/l). The organisms appear to be growing photoautotrophically in the presence of sulfide (2.5 mM). The medium contains no added sulfate. We have not included the aluminum phosphate sediment in these cultures, and the bacteria do not grow well in liquid medium. For the growth studies summarized below, they have grown best forming high visible densities within 3 weeks when suspended in the medium containing 0.3% agar. The optimal temperature for growth was 35–40 °C with no growth occurring above 42 °C. The cultures appear to grow best under dim tungsten light (10 Wm^{-2}) although attempts to adapt them to higher intensities are being made.

Estimates of relative growth were made by eye using the soft agar cultures of MCLO-1 incubated under different growth conditions in the basic medium described above. The optimal pH was 7.5 with no growth at pH 6.0 and minimal growth at pH 9.0 Growth did not occur in the absence of added NaCl. The optimal salinity for growth was 100 ppt and reasonable growth was observed at 50, 70, and 120 ppt. No growth occurred at a salinity of 150 ppt although collections of mat were made in salinities above 140 ppt. It may be that the salinities prevailing in situ where the MCLOs are found exceed their optimal salinity for growth. If their competitors in situ are inhibted to a greater extent by high salinities, however, the MCLOs may attain significant biomass even though they are likely to be growing slowly under suboptimal conditions.

When sulfate was included in the medium, contaminating sulfate-reducing bacteria produced sufficient sulfide to sustain growth of MCLO-1. In the absence of sulfate, the sulfate-reducers no longer grew and the MCLO-1 could not grow unless sulfide was added. The MCLO-1 grew well with added sulfide over a wide range of concentrations from 0.25 to 10 mM. Best growth was obtained consistently in 2.5 mM sulfide. The good growth obtained with 10 mM sulfide was unexpected and attempts to find the upper limit of sulfide tolerated by this organism have revealed reasonable (half maximal) growth at concentrations as high as 50 and 100 mM. Chemical analysis of sulfide concentration in the medium after growth of the cultures showed no substantial loss of sulfide during incubation. Clearly the MCLO-1 not only survived exposure to but also grew in unusually high levels of sulfide. Such concentrations are not known to prevail in the natural habitats where these bacteria are found. The highest levels of sulfide measured in most microbial mats are in the range of 1 to 3 mM. Most other phototrophic bacteria do not grow well in sulfide levels exceeding this range (Pfennig 1975). We are aware of only one study of phototrophic green sulfur bacteria growing in exceptionally high levels of sulfide comparable to values indicated here (Bergstein-Ben Dan 1988).

Single filament isolations of MCLOs inoculated into standard growth medium have failed to grow. Future research will focus on improving the medium to enhance the likelihood of growth when single filaments are isolated so that pure cultures can be obtained.

Significance of marine and hypersaline Chloroflexus*-like organisms*

The family Chloroflexaceae includes a diverse group of bacteria that we are only just beginning to appreciate. The recent successful pure culture of *Oscillochloris trichoides* (Keppen et al. 1993) and the 3 marine and hypersaline strains reported here in crude culture are the beginning of a substantial expansion of our knowledge of these anoxygenic filamentous phototrophs.

The potential for physiological diversity is great. The MCLO-1 is growing best in our hands as a photoautotroph. The 3-OH propionate pathway for CO_2 fixation in thermophilic *C. aurantiacus* is unique among photoautotrophs (Strauss and Fuchs 1993) and is of particular interest in studying the evolution of autotrophy. The MCLOs described here may also be important in expanding our understanding of the evolution of autotrophy.

Most significantly the presence of a large diversity of organisms within the *Chloroflexus* group will enhance the database for molecular phylogeny of the phototrophs and for comparative studies on the evolution of reaction centers and photosynthesis in general. If the organisms described here are not related to the Chloroflexaceae and are in fact related to green sulfur bacteria or form an entirely new group of phototrophs, the expansion of the database of known photosynthetic bacteria will still be significant.

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