

Contemporaneous N₂ Fixation and Oxygenic Photosynthesis in the Nonheterocystous Mat-Forming Cyanobacterium *Lyngbya aestuarii*

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The nonheterocystous filamentous cyanobacterial genus *Lyngbya* is a widespread and frequently dominant component of marine microbial mats. It is suspected of contributing to relatively high rates of N₂ fixation associated with mats. The ability to contemporaneously conduct O₂-sensitive N₂ fixation and oxygenic photosynthesis was investigated in *Lyngbya aestuarii* isolates from a North Carolina intertidal mat. Short-term (<4-h) additions of the photosystem II (O₂ evolution) inhibitor 3(3,4-dichlorophenyl)-1,1-dimethylurea stimulated light-mediated N₂ fixation (nitrogenase activity), indicating potential inhibition of N₂ fixation by O₂ production. However, some degree of light-mediated N₂ fixation in the absence of 3(3,4-dichlorophenyl)-1,1-dimethylurea was observed. Electron microscopic immunocytochemical localization of nitrogenase, coupled to microautoradiographic studies of ¹⁴CO₂ fixation and cellular deposition of the tetrazolium salt 2,4,5-triphenyltetrazolium chloride, revealed that (i) nitrogenase was widely distributed throughout individual filaments during illuminated and dark periods, (ii) ¹⁴CO₂ fixation was most active in intercalary regions, and (iii) daylight 2,4,5-triphenyltetrazolium chloride reduction (formazan deposition) was most intense in terminal regions. Results suggest lateral partitioning of photosynthesis and N₂ fixation during illumination, with N₂ fixation being confined to terminal regions. During darkness, a larger share of the filament appears capable of N₂ fixation.

Microbial mats are ubiquitous in shallow, nitrogen-depleted marine habitats, including mud and sand flats, marshes, lagoons, reefs, and subtidal shelf regions (4). Cyanobacteria are dominant productive and structural components of mats (4, 20). Dominance may be ensured in part by the ability of certain taxa to fix N₂ and contribute significantly to nitrogen budgets (16). Cyanobacterial N₂ fixation relies on oxygenic photosynthesis as a carbon source (21, 25). Photosynthetically evolved O₂ can, however, be a formidable barrier to N₂ fixation, because the enzyme complex mediating the latter (nitrogenase) is readily inactivated by O₂ (25). This perplexing problem is circumvented in heterocystous cyanobacteria by confining nitrogenase to biochemically and structurally differentiated, O₂-deplete heterocysts (6, 24). Despite this obvious adaptation, heterocystous cyanobacteria are frequently absent from benthic microbial mat communities. Instead, mats are often dominated by filamentous nonheterocystous genera suspected of fixing N₂. The question therefore arises, How can N₂ fixation and oxygenic photosynthesis co-occur in these undifferentiated genera? This paradoxical problem was examined in the cosmopolitan nonheterocystous species *Lyngbya aestuarii*. Microautoradiographic studies of photosynthetic ¹⁴CO₂ incorporation coupled to cellular deposition of the tetrazolium salt 2,4,5-triphenyl-3-tetrazolium chloride (TTC) and electron microscopic immunocytochemistry of nitrogenase reveal a strategy facilitating contemporaneous N₂ fixation and photosynthesis in *L. aestuarii* filaments.

MATERIALS AND METHODS

Naturally occurring populations of *L. aestuarii* were obtained from laminated mats located in an intertidal lagoon on

Shackleford Banks, one of North Carolina's Atlantic coastal barrier islands (1, 7). Microscopic observations showed *L. aestuarii* to dominate the microalgal biomass, determined as cell numbers. Diverse diatoms and other cyanobacteria (*Oscillatoria*, *Microcoleus*, *Spirulina*, and *Synechocystis* spp.) accompanied *L. aestuarii*.

Purified populations were obtained by teasing individual *L. aestuarii* filaments from mats and transferring them to petri dishes containing a defined, combined nitrogen-free, soft marine agar (ASN-N) (18) plus cycloheximide (25 mg/liter). After several transfers in liquid ASN-N media, unialgal populations were obtained. Bacteria-free filaments were prepared by shearing them into small 10- to 20-cell-length pieces by mild sonication and placing them on ASN-N plates. Inoculated plates were irradiated for 15 min, 30 min, and 2 h with UV-B light. The 30-min treatment selectively killed bacteria associated with sheaths surrounding *L. aestuarii* filaments. The 2-h treatment killed both bacteria and *L. aestuarii*. It was crucial to obtain bacteria-free *L. aestuarii* filaments, because N₂-fixing heterotrophic bacteria have been isolated previously from these mats (11). Purified filaments were grown at 25°C on liquid ASN-N medium. Illumination was by a 1:1 mixture of cool white/Grolux fluorescent lamps at 200 microeinsteins m⁻² s⁻¹ on a 14-h light:10-h dark cycle.

To determine photosynthetic activity, triplicate transparent (illuminated) and opaque (dark) flasks were incubated on a slowly oscillating (80 rpm) shaker from 15 min to 4 h during the illuminated period. Photosynthesis was measured by incorporation of ¹⁴CO₂, added as NaH¹⁴CO₃ (2.5 μCi 20 ml⁻¹; specific activity, 58 mCi mmol⁻¹ (12)). ¹⁴CO₂ incorporation was assessed by liquid scintillation counting and microautoradiography (10). Details of microautoradiographic preparation techniques and analyses are provided by Paerl (10). Filaments examined by microautoradiography were

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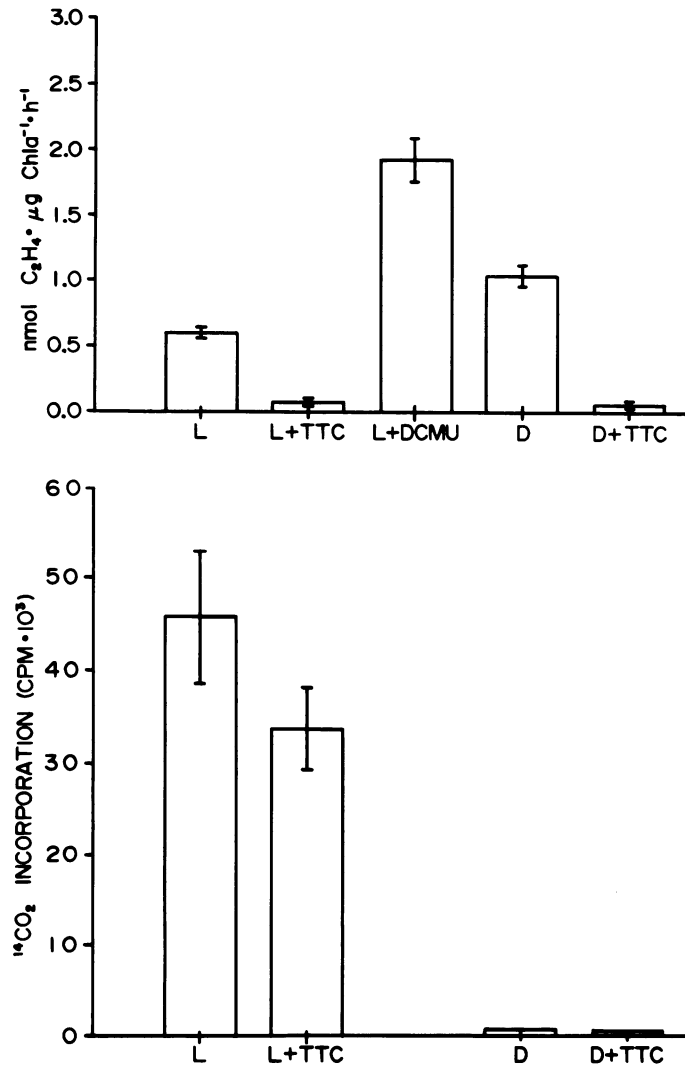


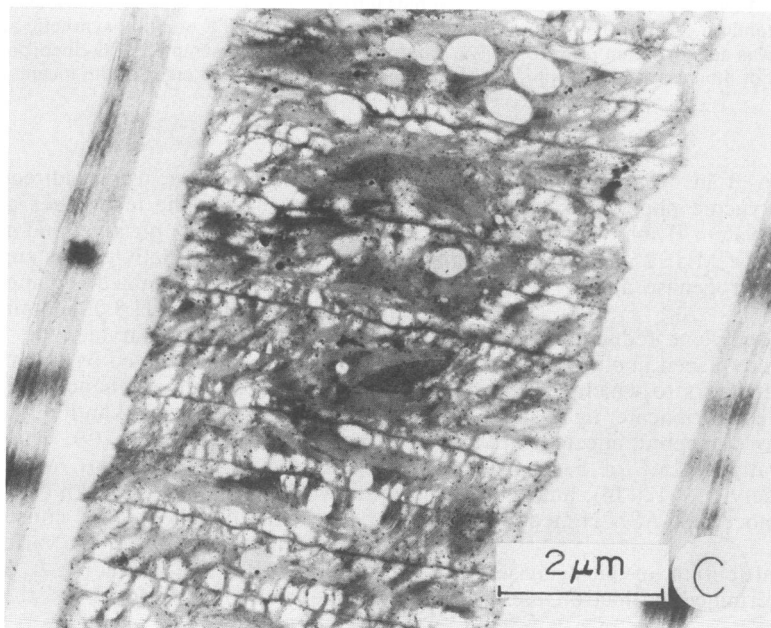
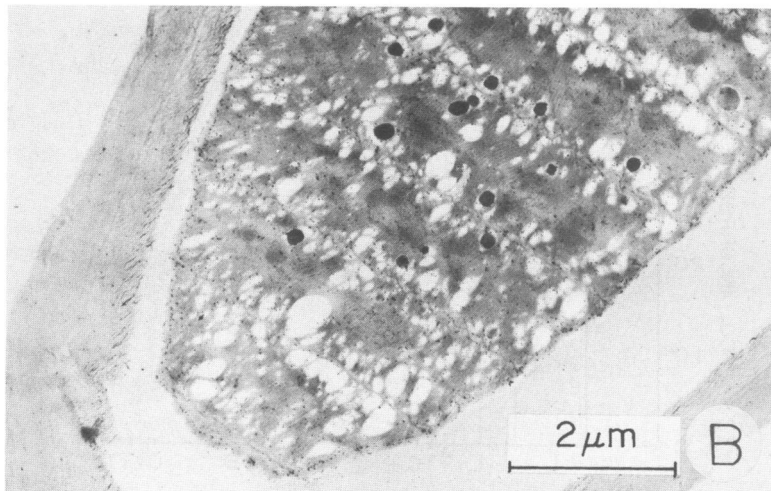
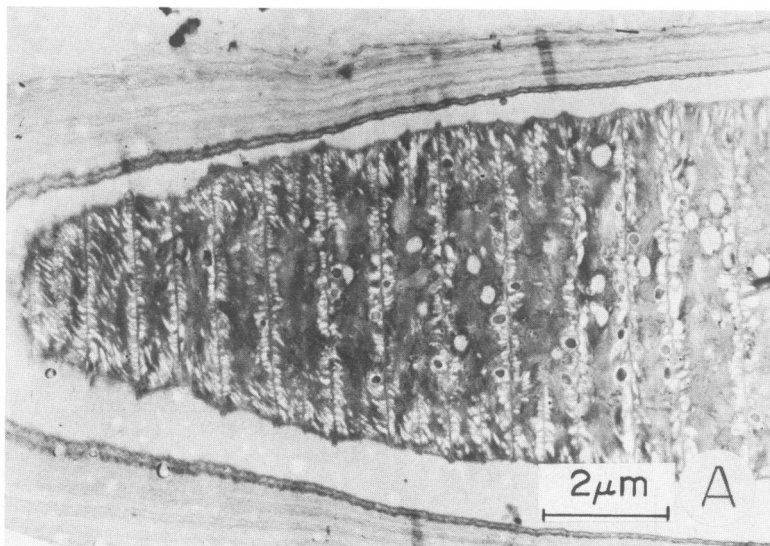
FIG. 1. (A) *L. aestuarii* chlorophyll *a*-specific NA assessed during a 4-h incubation period. Incubations were started at least 2 h after the initiation of illumination (200 microeinsteins of photosynthetically active radiation $m^{-2} s^{-1}$). NA was examined under continued illumination (L), illumination plus 2×10^{-5} M DCMU (L-DCMU), illumination plus 0.01% (wt/vol) TTC (L-TTC), darkness (D), and darkness plus TTC (D-TTC). Error bars indicate standard error of the mean. (B) Effect of 0.01% (wt/vol) TTC on photosynthetic and dark ¹⁴CO₂ incorporation in *L. aestuarii* filaments. TTC was added 10 min prior to assays. Control is an untreated sample. ¹⁴CO₂ incorporation is given as counts per minute per equivalent amounts of biomass (as chlorophyll *a*). Error bars indicate standard error of the mean.

stained with 2% erythrosin B in 5% phenol to enhance contrast. On occasions, oxygenic photosynthesis was arrested by adding the photosystem II inhibitor 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU; 2×10^{-5} M) 10 min before ¹⁴CO₂ fixation and nitrogenase activity (NA) measurements.

NA was determined by acetylene reduction assays (12, 22). *L. aestuarii* samples were placed in either 15-ml serum bottles or 25-ml Erlenmeyer flasks to which 7 or 15 ml of ASN-N medium was added. Triplicate light, light plus DCMU, and dark flasks were stoppered, injected with nitrogenase-saturating concentrations (15% of headspace volume) of CaC₂-generated acetylene (12, 14), and incubated alongside ¹⁴C samples. Uninoculated ASN-N medium flasks served as controls.

The occurrence and distribution of nitrogenase in *L. aestuarii* filaments were determined by electron microscopic

immunolocalization, using indirect immunogold labeling in accordance with the techniques given in Paerl et al. (15). Both N₂-fixing (NA positive) and non-N₂-fixing filaments, in which nitrogenase activity was suppressed by prior growth on NH₄⁺, were examined. Filaments were fixed in 0.2-μm filtered seawater at pH 8.0, containing 2% (vol/vol) electron microscope-grade glutaraldehyde. Fixation was overnight at 4°C, which was followed by several rinses of 0.05 M phosphate-buffered saline, ethanol dehydration, embedding, and sectioning. A polyclonal antibody to nitrogenase was kindly provided by P. Ludden (9). This antibody (Rr-II), raised against nitrogenase from *Rhodospirillum rubrum*, was shown previously by Western blot (immunoblot) analyses to be specific for the highly conserved Fe-protein subunit (dinitrogenase reductase) in a variety of N₂-fixing eubacteria and cyanobacteria, including *L. aestuarii* (5, 9, 15). Both active and inactive forms of the Fe-protein subunit are



recognized by this antibody (5, 9). Sectioned filaments were first exposed to Rr-II polyclonal (rabbit) antibodies (1:100 and 1:200 dilutions) or irrelevant antibodies (fetal bovine serum, used as controls for nonspecific binding). Sections were then exposed to colloidal gold (10-nm-diameter)-labeled goat anti-rabbit antibodies (E-Y Laboratories, San Mateo, Calif.). Sections were poststained with uranyl acetate and lead citrate, followed by electron microscopic (Philips CM12) examination (15).

To locate O₂-depleted regions in *L. aestuarii* filaments, reduction of the low-redox-potential ($E_0^1 = -0.4$ V) tetrazolium salt TTC was observed microscopically. Reduction of this salt at 0.01% (wt/vol) to red insoluble formazan crystals is most intense in heterocysts of N₂-fixing cyanobacterial genera (*Anabaena*, *Aphanizomenon*, and *Nostoc*) (7, 13) and in O₂-depleted microzones suspected of being the sites of N₂ fixation in the aggregated marine cyanobacterial genus *Trichodesmium* (3, 13).

RESULTS AND DISCUSSION

Among purified *L. aestuarii* isolates, NA occurred under illuminated and dark conditions. Maximum chlorophyll *a*-specific NA rates were often observed in dark and DCMU-amended samples incubated during the illuminated period and at the onset of darkness, following at least 2 h of prior illumination (Fig. 1). This N₂ fixation response has also been reported in laminated marine mats and aggregates dominated by the nonheterocystous filamentous cyanobacteria *Lyngbya*, *Microcoleus*, and *Oscillatoria* spp. It appears temporally and spatially linked to sudden decreases in mat O₂ tension, following cessation of photosynthesis at dusk (1, 12, 20). Interestingly, this response has not been observed in *L. majuscula*, a floating frond rather than mat-forming species (8).

We observed a substantial NA increase following inhibition of photosynthesis with DCMU (Fig. 1). This enhancement normally persisted through the first 4 h of DCMU addition. Longer incubations with DCMU yielded NA either close to or lower than that of untreated controls. Longer-term DCMU inhibition of NA may indicate exhaustion of photogenerated reductant, essential for supporting NA in cyanobacteria (12, 17, 21). No dark DCMU enhancement (above dark-incubated samples) of NA was observed. We interpret short-term NA enhancement to be alleviation of O₂ inhibition of NA or synthesis or both. The mechanism(s) responsible for short-term NA stimulation by DCMU remains unclear, beyond its direct effect of blocking photosystem II activity and intracellular O₂ production. It is possible that NA stimulation results from relief of direct O₂ inhibition of enzyme activity, production of new nitrogenase in cells previously downregulated by the presence of O₂, or through

possible changes in electron allocation once photosynthesis is stopped. It has been shown previously that O₂ enrichment leads to strong and immediate reduction of NA in this and other filamentous nonheterocystous cyanobacteria (11). This implicates photosynthetically produced O₂ as a key variable controlling N₂ fixation during daylight hours.

Immunolocalization by electron microscopy showed nitrogenase dispersed throughout *L. aestuarii* filaments (Fig. 2), in agreement with a recent study of the benthic cyanobacterium *Oscillatoria limosa* (19). Controls for nonspecific binding by the secondary gold anti-rabbit goat antibody and the use of irrelevant primary antibodies (in place of Rr-2) followed by secondary antibody applications both proved negative (Fig. 2). Repression of NA by growth on NH₄⁺-amended ASN media greatly reduced immunogold deposition throughout filaments and eliminated NA. Western blot analyses of cell lysate proteins from *Lyngbya* and other diazotrophic microorganisms probed with the Rr-2 antibody reveal a single band at 35 to 50 kDa (5, 15). This band corresponds to the Fe subunit of nitrogenase and only appeared in cultures grown under combined N-free conditions.

Microautoradiography indicated that approximately half of illuminated filaments had ¹⁴CO₂ uptake confined to intercalary regions within the first 20 min of incubation (Fig. 3). The remaining filaments were more uniformly labeled. After 30 min, the terminal 5 to 10 cells gradually became labeled (Fig. 3). Intercalary regions exhibited the highest degree of labeling among both types of filaments, even after 4 h of photosynthetic ¹⁴CO₂ incorporation. Illuminated filaments receiving DCMU and dark-incubated filaments remained free of labeling. In contrast, TTC formazan deposition was chiefly confined to terminal cells during the first 30 min of light incubation (Fig. 3). In darkness, formazan deposition was most intense in terminal cells, but also appeared among intercalary regions (Fig. 3). NA was more severely inhibited than ¹⁴CO₂ incorporation by TTC formazan deposition (Fig. 1).

Cellular ¹⁴CO₂ incorporation and TTC reduction patterns show that photosynthesis and concomitant O₂ evolution are greatly reduced or absent in many terminal regions of illuminated filaments. Photosynthetically fixed ¹⁴C does, however, accumulate in terminal cells during the course of a 2- to 4-h incubation period (Fig. 3). Furthermore, these cells reduce TTC far more quickly and extensively than intercalary cells, indicating that they are viable and capable of harboring highly reduced conditions. This sequence of events appears analogous to that observed in the heterocystous cyanobacterium *Anabaena cylindrica* (23). In *Anabaena* spp., CO₂ is exclusively fixed in oxygenic vegetative cells, while adjacent heterocysts lacking photosystem II activity fail to fix CO₂ and evolve O₂ (6, 23). Fixed carbon

FIG. 2. Transmission electron micrographs showing representative cellular patterns of immunogold deposition in illuminated *L. aestuarii* filaments. Filaments and segments of surrounding laminated sheaths are shown. Stacked lamellar thylakoids are dispersed throughout terminal and intercalary disk-shaped cells. Spherical to ovoid vacuoles (clear) most likely contain storage polymers (lipids). Small storage vacuoles are frequently concentrated near cell walls, while in some cells a significant portion of the cell volume is occupied by large vacuoles. Vacuoles are generally free of immunogold labeling. The heaviest concentrations of immunogold labeling are observed just inside the cytoplasmic membrane near the cell walls and in dense nucleoplasmic regions. Dark (black) granules frequently associated with storage vacuoles resemble cyanophycin granules. (A) Longitudinal section of control for nonspecific binding of irrelevant antibodies (fetal bovine serum added instead of Rr-II antibodies), followed by exposure to gold-labeled secondary antibodies. (B) Longitudinal sections of terminal region of illuminated filament positive for N₂ fixation. Immunogold deposition (as 10-nm electron-dense gold particles) was observed throughout terminal region. Note the absence of immunogold deposition on sheaths surrounding filaments. Dark-incubated filaments (not shown) exhibited immunogold deposition patterns similar to those shown in illuminated filaments. (C) Longitudinal section of internal region showing immunogold deposition among individual cells.

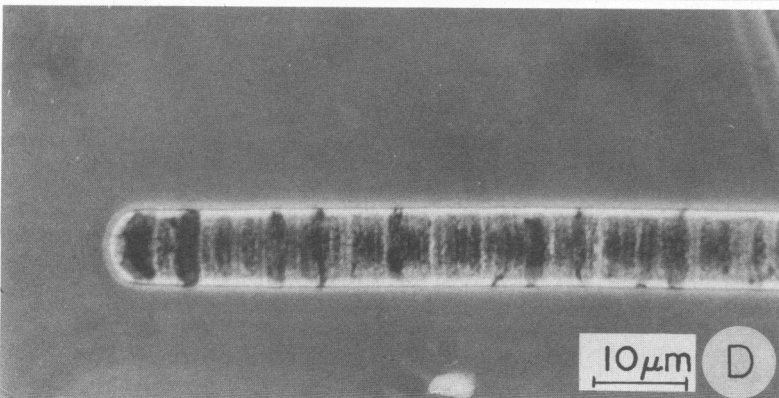
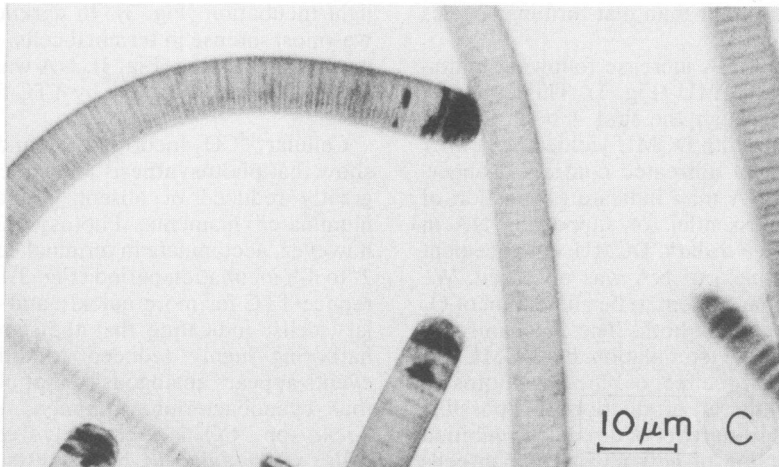
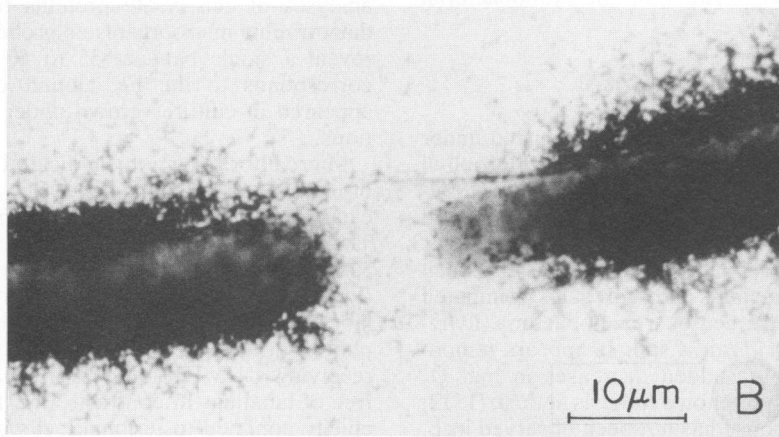
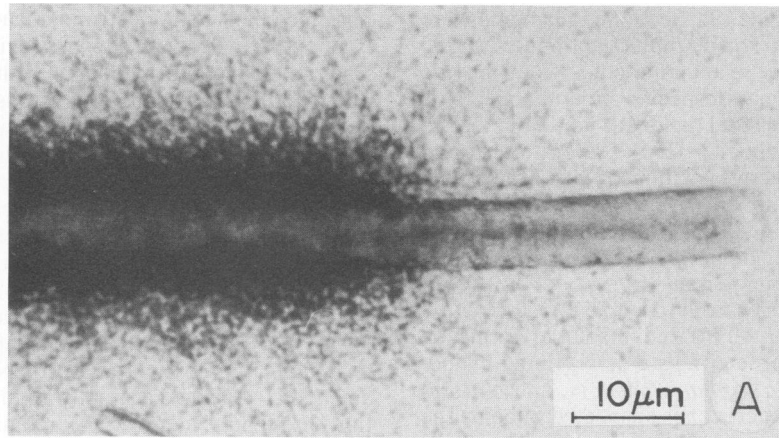


FIG. 3. Microautoradiographic detection of photosynthetic ¹⁴C₂ incorporation in conjunction with microscopic observations of cellular TTC reduction in illuminated and dark *L. aestuarii* filaments. (A) Representative view of heavily labeled internal versus unlabeled terminal region of filaments incubated with ¹⁴C-NaHCO₃ under 200 microeinsteins of photosynthetically active radiation m⁻² s⁻¹ for 20 min. (B) View of increased ¹⁴C movement to terminal regions (relative to panel A) of filament incubated for 1 h. (C) Photomicrograph of TTC formazan deposition exclusively in terminal regions of filaments incubated for 30 min under conditions described for panel A. (D) TTC formazan deposition in filament incubated in darkness for 30 min.

(reductant) is subsequently transported to the heterocysts. Such metabolic partitioning facilitates contemporaneous photosynthetic production and N₂ fixation, the latter having been shown to be localized in O₂-free heterocysts (2, 6).

As a functional alternative to heterocysts, *L. aestuarii* harbors nitrogenase in what appears to be undifferentiated terminal cells having reduced photosynthetic activities. Like intercalary cells, terminal cells reveal intact lamellar thylakoids, cell membranes, and walls, as well as diverse vacuoles. In contrast, however, terminal cells frequently appear more yellow than intercalary cells, indicating reduced concentrations of chlorophyll *a*. The reduction of photosynthesis in terminal regions facilitates NA during daylight hours. During these hours, NA may be strongly suppressed in photosynthetic O₂-evolving intercalary cells. These cells are, however, capable of synthesizing nitrogenase as witnessed by immunolocalization. Upon darkness, intercalary cells readily reduce TTC, indicating the potential for N₂-fixing conditions when O₂ evolution is inoperative. Intercalary cell nitrogenase may thus be largely inactive until oxygenic photosynthesis ceases, either in darkness or following inhibition by DCMU. The sudden dark or DCMU-mediated bursts of NA observed here could represent removal of O₂ inhibition of existing nitrogenase or rapid nitrogenase synthesis in intercalary regions. Further immunolocalization and immunoassay (Western blot) studies are under way to clarify the nature of this response.

With respect to localized O₂ dynamics on the scale of single and aggregated (mat) filaments, the strategy of optimizing NA in terminal regions appears advantageous. Supplementary, hitherto undescribed, cellular O₂-consuming mechanisms may further ensure localized O₂-depleted conditions essential for nitrogenase expression during daylight hours. In intact mats, O₂-scavenging heterotrophic bacteria attached to sheaths surrounding these filaments may provide an additional protective role. Beneficial impacts of actively respiring heterotrophic bacteria on N₂ fixation potentials in both nonheterocystous (*Microcoleus*) and heterocystous (*Anabaena*) cyanobacteria have been documented (11, 14).

Microscale autoradiographic, tetrazolium redox indicator, and immunochemical evidence reveals a lateral partitioning strategy which facilitates contemporaneous N₂ fixation and photosynthesis without necessitating heterocysts in the ubiquitous and ecologically important mat-building cyanobacterium *L. aestuarii*. This strategy appears confined to terminal cells and thus may only partly solve the problem of photosynthetic O₂ inhibition of NA for entire filaments. However, the ability to sustain N₂ fixation, even at suboptimal levels, is advantageous during daylight when N demands for biosynthesis and growth are high. The strategy supplements dark whole-filament N₂ fixation and assimilation of exogenous combined nitrogen, thereby maintaining a broad ability to satisfy N requirements. Such a lateral partitioning strategy may also operate among other nonheterocystous filamentous genera residing in oxic waters and soils. This strategy exemplifies ecophysiological and evolu-

tionary diversification among N₂-fixing cyanobacteria in habitats in which O₂ protection from nitrogenase is critically important.

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