APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1989, p. 2965–2975
0099-2240/89/112965-11$02.00/0

Vol. 55, No. 11

Immunochemical Localization of Nitrogenase in Marine *Trichodesmium* Aggregates: Relationship to N_2 Fixation Potential

HANS W. PAERL,[1]* JOHN C. PRISCU,[2] AND DIANE L. BRAWNER[3]

Institute of Marine Sciences, University of North Carolina at Chapel Hill, Morehead City, North Carolina 28557,[1] and Departments of Biology[2] and Microbiology,[3] Montana State University, Bozeman, Montana 59717

Received 22 May 1989/Accepted 16 August 1989

Colonial aggregation among nonheterocystous filaments of the planktonic marine cyanobacterium *Trichodesmium* is known to enhance N_2 fixation, mediated by the O_2-sensitive enzyme complex nitrogenase. Expression of nitrogenase appears linked to the formation of O_2-depleted microzones within aggregated bacterium-associated colonies. While this implies a mechanism by which nonheterocystous N_2 fixation can take place in an oxygenated water column, both the location and regulation of the N_2-fixing apparatus remain unknown. We used an antinitrogenase polyclonal antibody together with postsection immunocolloidal gold staining and transmission electron microscopy to show that (i) virtually all *Trichodesmium* cells within a colony possessed nitrogenase, (ii) nitrogenase showed no clear intracellular localization, and (iii) certain associated bacteria contained nitrogenase. Our findings emphasize the critical role coloniality plays in regulating nitrogenase expression in nature. We interpret the potential for a large share of *Trichodesmium* cells to fix N_2 as an opportunistic response to the dynamic nature of the sea state; during quiescent conditions, aggregation and consequent expression of nitrogenase can proceed rapidly.

The ubiquitous and ecologically significant marine cyanobacterial genus *Oscillatoria* (*Trichodesmium*) supports relatively high rates of light-mediated N_2 fixation in the absence of morphologically differentiated cells termed heterocysts (5, 6, 11). The physiological means by which oxygen-sensitive N_2 fixation occurs contemporaneously with oxygenic photosynthesis in this filamentous genus remains elusive (19, 20). However, field (in situ) and short-term (<1-day) laboratory studies on freshly collected populations have pointed to the importance of a colonial existence, as either fusiform tufts or spherical puffs of aggregated filaments, for N_2 fixation to proceed and be optimized (3, 7, 18). Despite a recent report on the successful isolation and culture of a marine *Oscillatoria* strain which apparently can fix N_2 as single filaments under oxic conditions (16), naturally occurring photosynthetically active populations consistently reveal either a complete lack or severe inhibition of N_2 fixation when aggregates are disrupted (with oxygenic photosynthesis remaining uninhibited) (3, 7, 18). After extensive examinations of natural populations, Carpenter and McCarthy (6) and, later, Carpenter and Price (7) suggested that aggregation of filaments allowed for compartmentalization of O_2-evolving photosynthesis and O_2-inhibited N_2 fixation. Indeed, autoradiographic examinations of photosynthetic $^{14}CO_2$ incorporation reveal this process to be largely confined to terminal regions of filaments (7); hence, internal regions of aggregated filaments could be a likely place for N_2 fixation to occur. Subsequent studies, utilizing the reduction of a low-redox-potential tetrazolium salt, 2,3,5-triphenyl-3-tetrazolium chloride, strongly hinted of highly reduced conditions in internal regions of photosynthetically active aggregates (3, 18). More recently, aggregates have been probed with O_2 microelectrodes, and the results directly demonstrate the existence of O_2-depleted internal microzones within photosynthetically active aggregates (17).

While these studies have implied a mechanism by which N_2 fixation can accompany photosynthesis in aggregates, key questions remain as to the location and regulation of the N_2-fixing apparatus in *Trichodesmium*. Thus far, it has not been conclusively shown that *Trichodesmium* is in fact the site of N_2 fixation as opposed to the diverse array of microflora (both bacteria and microalgae) with which N_2 fixation is commonly found in nature. The question follows that, if N_2 fixation is indeed confined to *Trichodesmium*, what are the intra- and intercellular distributions of the enzyme complex (nitrogenase) responsible for this process? Also, is the distribution of nitrogenase related to its environmental regulation and colonial aggregate formation?

The nitrogenase enzyme complex consists of two distinct polypeptides forming dinitrogenase (Mo-Fe protein) and the dimeric polypeptides of dinitrogenase reductase (Fe protein). To address the above-mentioned questions, polyclonal antibodies against dinitrogenase reductase were used in postsection immuno electron microscopy (IEM) (1) studies to observe intra- and intercellular localization of nitrogenase in *Trichodesmium* aggregates recently obtained from: (i) coastal Atlantic Ocean (Gulf Stream) waters during a late summer 1988 bloom near Beaufort, N.C., and (ii) several open-ocean locations in the western Caribbean Sea.

MATERIALS AND METHODS

Sampling locations and procedures. Samples were collected from a variety of pelagic (offshore) locations in the western Caribbean Sea during a cruise of the R/V *Columbus Iselin* conducted between 4 and 23 November 1988. Samples for immunolocalization of nitrogenase were obtained from a station at 11°20′ N, 82°22′ W during calm sunny conditions. Additional samples were collected in coastal Atlantic Ocean waters 6 km southwest of Beaufort Inlet, N.C., during a late summer bloom in 1988. In both cases, individual *Trichodesmium* aggregates from the same sample as used for immunolocalization studies were examined for N_2-fixing activity

* Corresponding author.

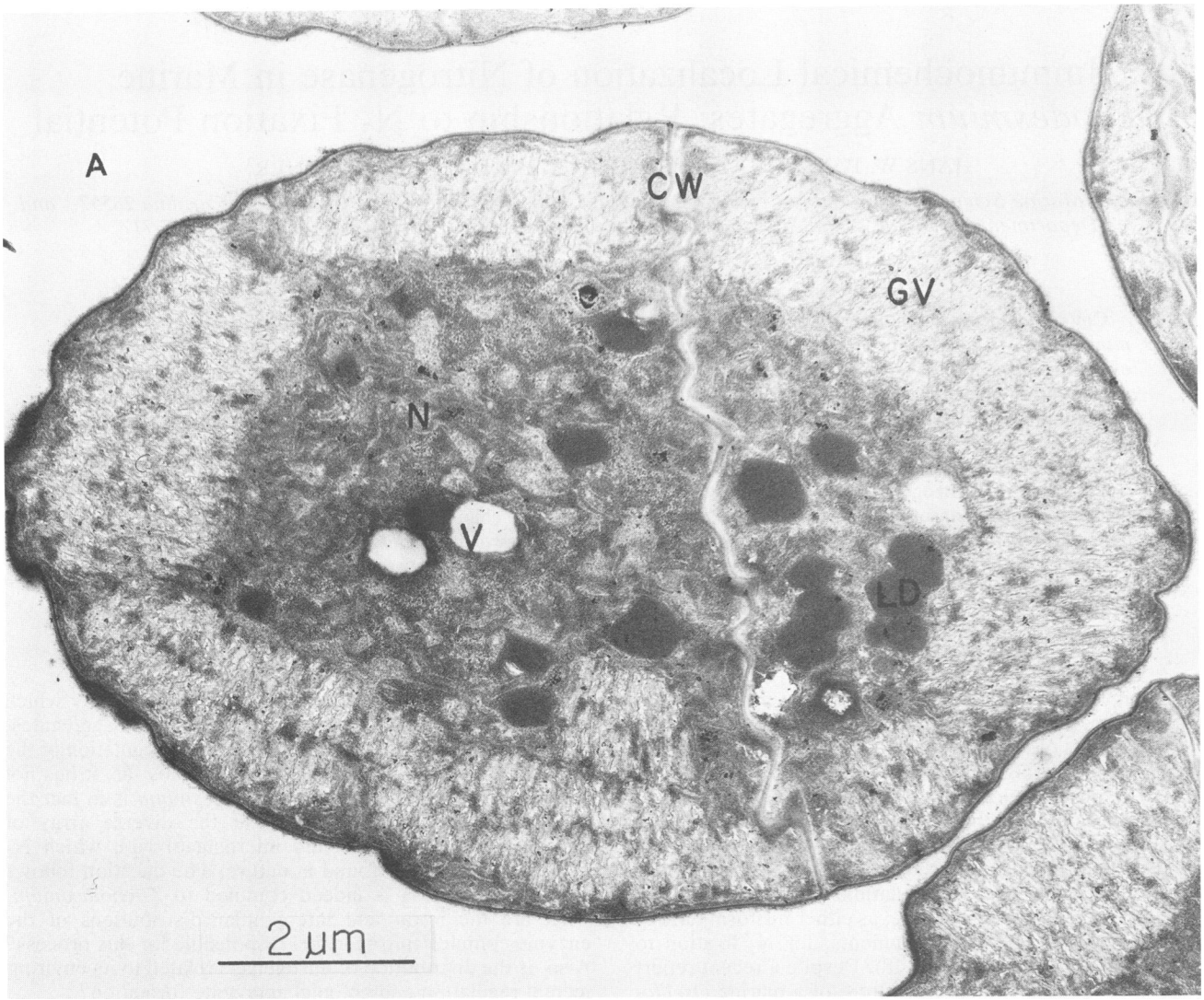

FIG. 1. IEM examinations of CG-labeled *Trichodesmium* spp. filaments. Sample obtained from North Carolina coastal Atlantic waters. (A) Oblique cross-sectional view showing representative morphology of two cells intersected by a cell wall (CW). The nucleoplasmic (N) region, gas vacuoles (GV), other vacuoles (V), and lipid droplets (LD) are indicated. Detailed ultrastructural descriptions are provided by Van Baalen and Brown (21), Gantt et al. (10), and Haxo et al. (12). Electron-dense, spherical CG particles are distributed throughout both nucleoplasmic and gas vacuolate regions, but are difficult to discern at this magnification owing to their small size (10-nm diameter). (B) High-magnification cross-sectional view revealing CG particles (arrows) dispersed throughout nucleoplasmic (N) regions, vacuoles (V), and gas vacuole (GV) regions. Intracellular CG labeling did not appear confined to specific ultrastructural components. However, note that the outer cell wall (OCW) and associated membrane revealed relatively little CG labeling. (C) Longitudinal section. In support of cross-sectional views, the longitudinal section exhibited CG deposition throughout both nucleoplasmic (N) and gas vacuole (GV) regions. In this view, photosynthetic lamellae (P) are evident; generally, little CG was associated with these structures. (D) High magnification longitudinal view of CG deposition (arrows). Note general absence of CG labeling in either the photosynthetic lamellar or outer cell.

by the acetylene reduction assay (5, 17). Relatively high cellular acetylene reduction rates were observed under illuminated conditions in both instances (17; D. G. Capone, unpublished data), indicating the presence of nitrogenase associated with aggregates. Nitrogen-fixing aggregates were gently collected from near the sea surface (0 to 2 m) with a 200-µm mesh plankton net and immediately fixed in 2% (vol/vol) glutaraldehyde for 20 min, washed with phosphate-buffered saline (pH 7.8), and transferred to fresh phosphate-buffered saline for refrigerated (4°C) storage until analysis.

Immunochemical studies. Upon return to the laboratory (within 3 weeks), 5 to 15 aggregated filaments were washed in 0.1 M phosphate-buffered saline at pH 7.2, transferred to BEEM capsule chamber pipettes (8), postfixed for 1 h in aqueous OsO_4, and dehydrated. The dehydrated filaments were then embedded (with care given to orientation of the filaments) and thin sectioned (2). Sections were immunostained with rabbit anti-dinitrogenase reductase polyclonal antibodies, either Rr-2 or universal. Both types of antibodies were kindly provided by P. Ludden, University of Wisconsin, Madison. Rr-2 denotes the anti-rabbit polyclonal anti-

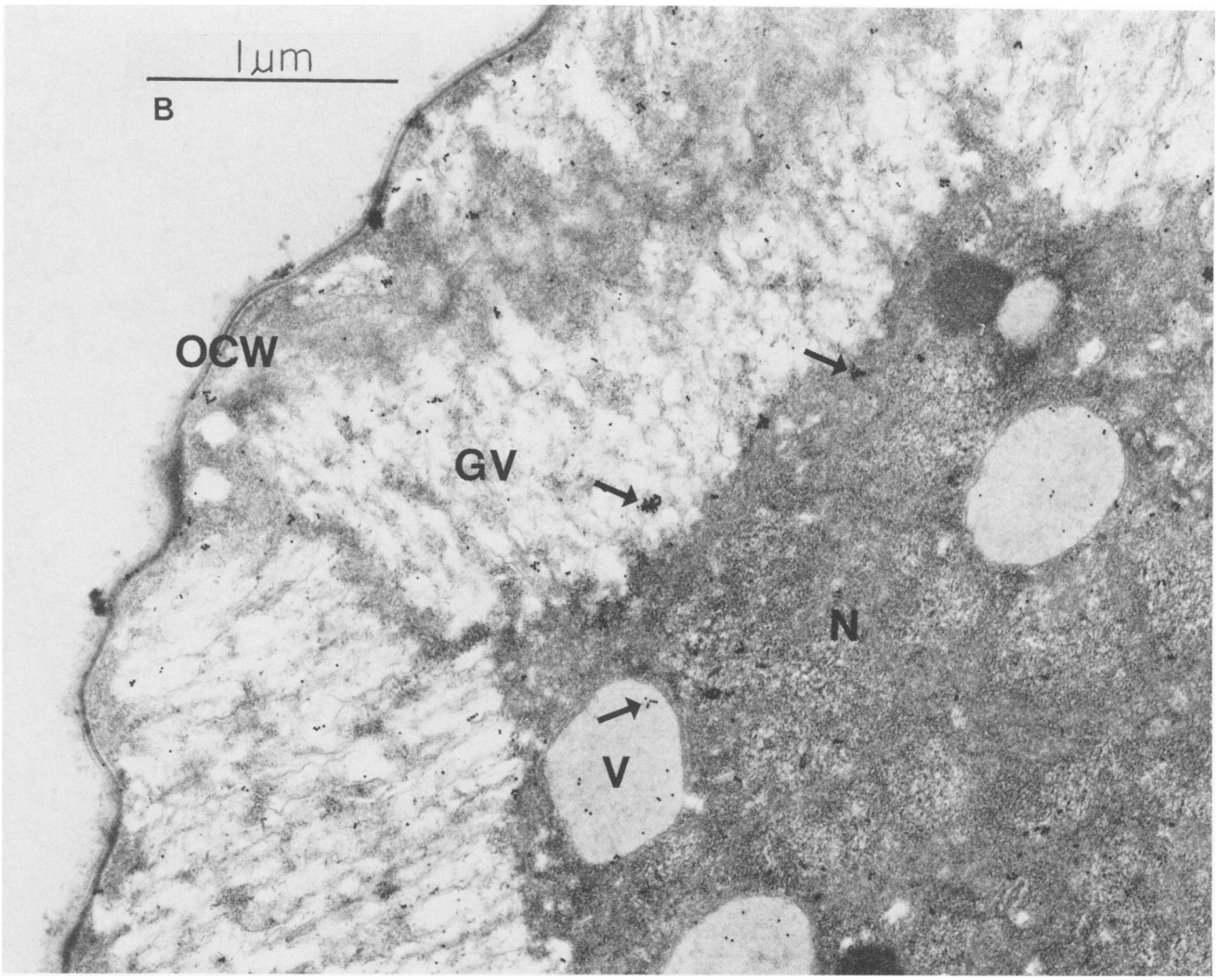

FIG. 1—*Continued*

bodies against dinitrogenase reductase (subunit II or Fe protein) of the nitrogenase complex of *Rhodospirillum rubrum*, a diazotrophic bacterium (14). Universal Fe protein denotes the polyclonal antibodies against the dinitrogenase reductase from a variety of N_2-fixing bacteria, including *R. rubrum*. Previous studies by Ludden et al. (14) critically evaluated the specificity of these polyclonal antibodies toward crude and highly purified Fe protein. Using both Western blotting (immunoblotting) following electrophoretic separation of proteins and Ouchterlony immunodiffusion assays, a highly specific reaction with the Fe protein was observed, with no significant cross-reactions with irrelevant proteins. In addition, these investigators were able to discriminate between active and inactive forms of the Fe protein, based on very small differences in molecular weight, as detected by Western blot assays. We have recently observed similar specificities and small molecular-weight differences in a variety of marine and freshwater N_2-fixing isolates (C. A. Currin, H. W. Paerl, G. Suba, and R. S. Alberte, Limnol. Oceanogr., in press; J. C. Priscu, unpublished data).

Antibodies were applied at dilutions of 1:200 (Rr-2; Caribbean samples), 1:500 (Rr-2; N.C. samples), or 1:50 (universal; Caribbean samples). Antibody dilutions were based on individual antibody titers. Following reaction of the primary antibody with nitrogenase, sections were reacted with 10% (in blocking buffer) goat anti-rabbit gold-conjugated antibody (10-nm particle size; Janssen Life Science Products, Piscataway, N.J.), as described by Brawner and Cutler (2), except that blocking buffer (TTBS) consisted of 7% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.), 400 mM $(NH_4)_2SO_4$, 1.0 mM $MgCl_2$, 0.1 mM $ZnCl_2$ (J. T. Baker Chemical Co., Phillipsburg, N.J.), and 0.1% Tween 80 (Sigma) in 0.05 M Tris buffer, pH 7.5. A minimum of 50 cells per grid was observed with either a JEOL 100-CX or a Zeiss model EM 10C/CR electron microscope.

Controls to test for nonspecific binding of the goat anti-rabbit gold-conjugated antibody consisted of fetal bovine serum (N.C. samples) and an irrelevant antibody (polyclonal rabbit anti-*Candida albicans* 9938; Caribbean samples) in dilutions matching and replacing that of primary polyclonal serum.

Western blots done by Laemmli's protocol (13) were conducted on lyophilized *Trichodesmium* to determine the

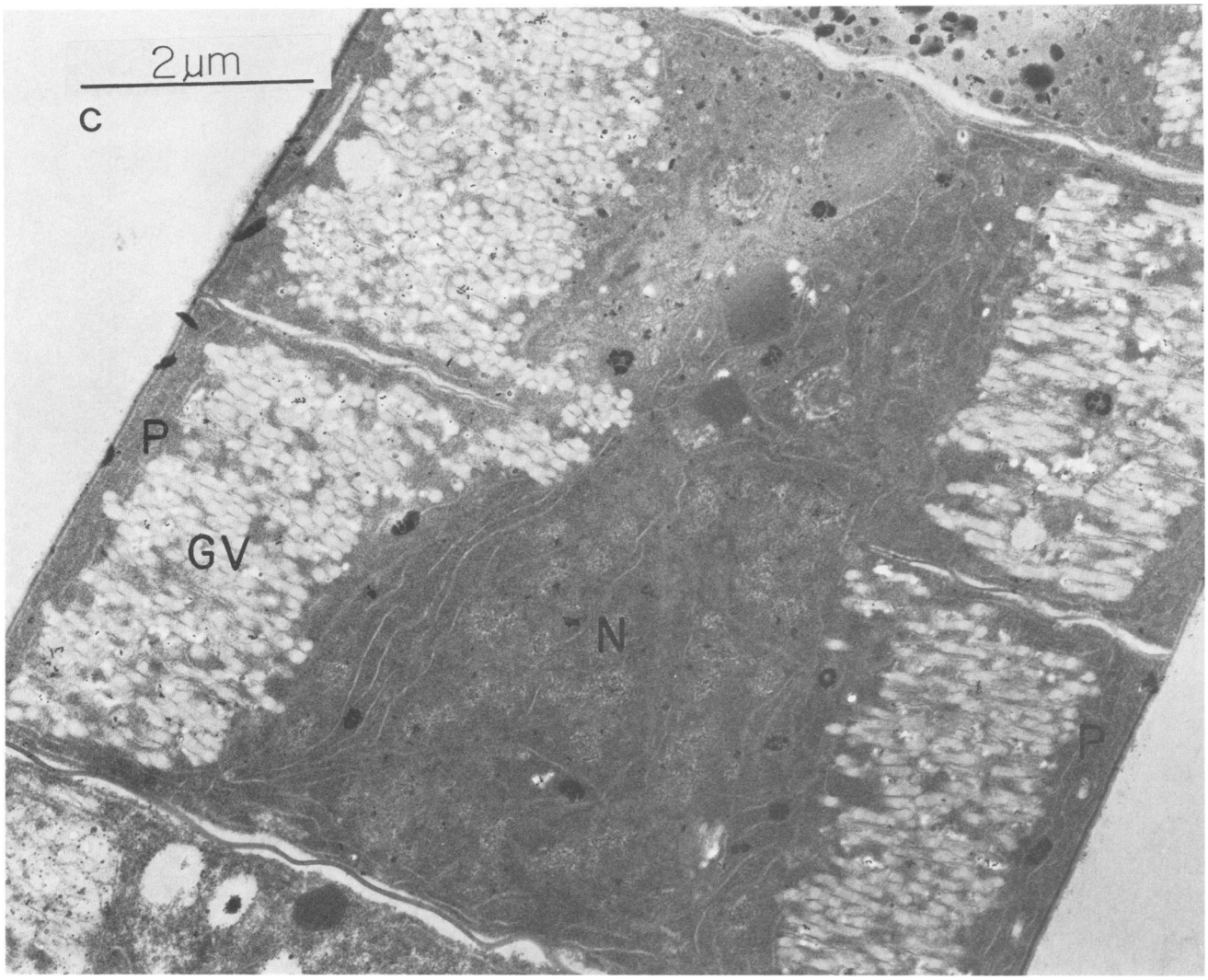

FIG. 1—*Continued*

specific cross-reactivity of the primary antibody. Lyophilized samples were kept at −20°C until use. We suspended 10 to 12 mg of freeze-dried sample in 1.0 ml of Laemmli sample buffer, sonicated twice at low power for 30-s intervals, and heated at 100°C for 5 min. Samples and molecular mass markers (prestained markers, 17 to 130 kilodaltons [kDa]; Bio-Rad Laboratories, Richmond, Calif.) were then loaded onto 7.0-cm 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels at 10 μl per well. Gels were electrophoresed for 40 min in a Bio-Rad Mini-PROTEAN II unit supplied with a constant 200 V. Following electrophoresis, gels were equilibrated for 15 min in a methanol transfer buffer (25 mM Tris, 150 mM glycine, 20%, vol/vol, methanol, pH 8.3). Proteins were then electroeluted onto nitrocellulose membranes in a Bio-Rad Mini Trans-Blot transfer cell supplied with a constant 100 V for 1 h. Blocked (1% bovine serum albumin) membranes were then probed for the presence of the nitrogenase enzyme, using either Rr-2 or universal antibodies. Membranes were incubated for 1 h in a 1:10,000 working dilution (in TTBS) of either of these antibodies. Primary antibody was replaced with a conjugated secondary antibody solution (a 1:7,500 dilution of goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase in TTBS) following several short washes in TTBS, pH 7.5, to remove excess primary probe. Following a 1-h incubation in secondary antibody, membranes were washed in Tris-buffered saline (0.5 M NaCl, 20 nM Tris) at pH 7.5 and placed in an alkaline phosphatase color development system containing Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate ptoluidine salt.

Bacterial culturing studies. Maruyama et al. (15) reported on N_2-fixing eubacteria associated with *Trichodesmium* aggregates in the Pacific Ocean. We made parallel efforts at isolating and characterizing such eubacteria in this study. Freshly collected (November 1988) Caribbean *Trichodesmium* tuft and puff aggregates were individually picked from water samples, using sterile plastic inoculating loops (10 μl), and given two washes of 0.1-μm filter (Nuclepore Corp., Pleasanton, Calif.)-sterilized seawater. Aggregates were then transferred with loops to sterile culture tubes containing a mannitol (0.8%)–phosphate (100 μM)–Fe-EDTA (30 μM)-amended seawater soft agar. Purified (Difco purified grade; Difco Laboratories, Detroit, Mich.) nitrogen-free agar (3.0 g/liter) was used. This medium was shown previously to

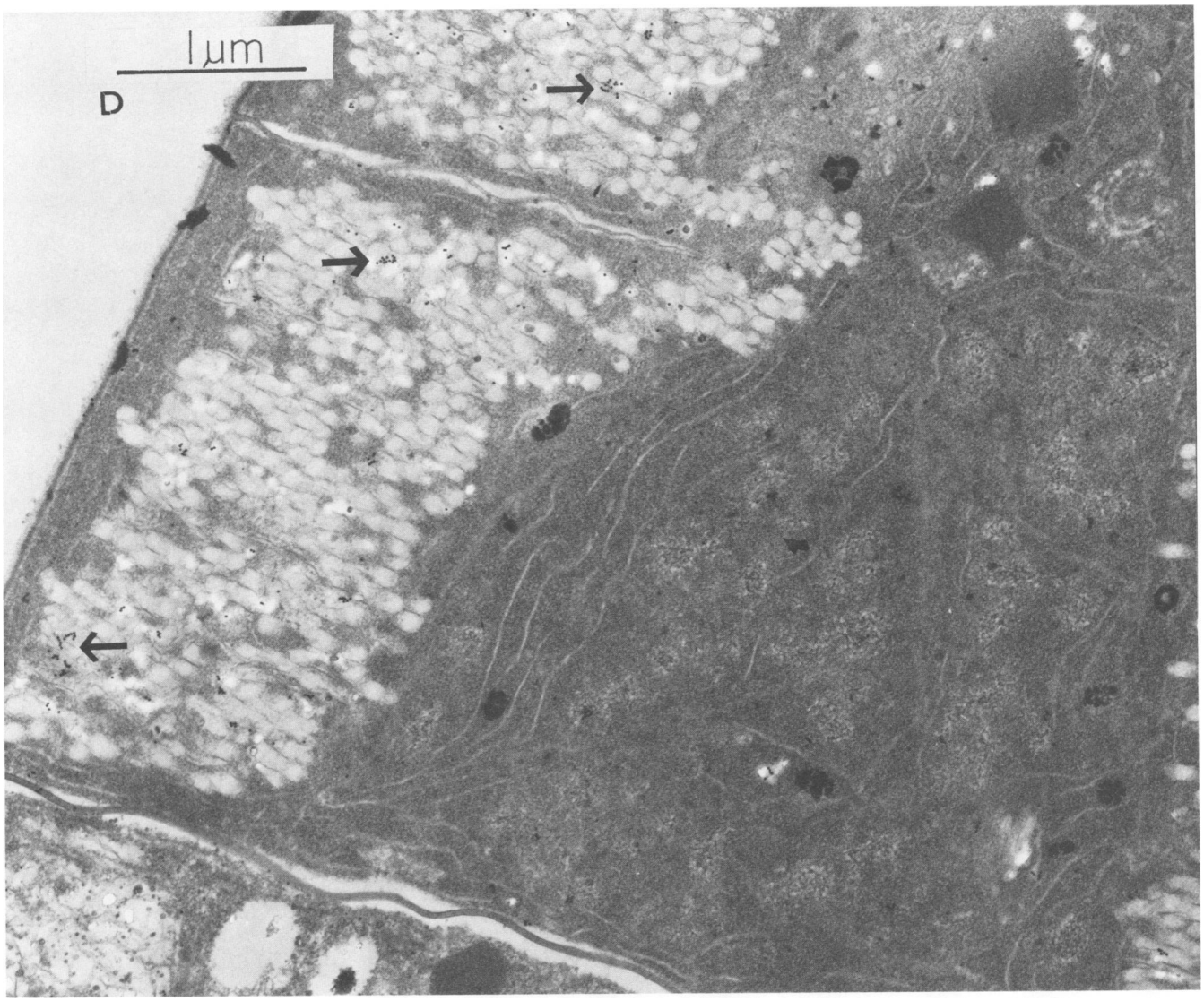

FIG. 1—*Continued*

select for N_2-fixing microheterotrophs in a variety of North Carolina coastal Atlantic and Caribbean habitats (H. W. Paerl, B. M. Bebout, and L. E. Prufert, J. Phycol., in press). Washed *Trichodesmium* aggregates were both placed on the surface and stabbed into the agar. Cultures were incubated for 2 to 7 days in darkness at 25°C.

RESULTS AND DISCUSSION

Immunostained cells viewed by IEM showed deposition of colloidal gold (CG) throughout individual cells collected from both North Carolina coastal Atlantic waters (Fig. 1) and the Caribbean Sea (Fig. 2). High-magnification examinations of samples from both locations revealed no clear intracellular localization of CG deposition. Enumeration of CG deposition in Caribbean samples yielded mean (10 replicate counts) densities of 75 ± 15 gold particles per μm^2 following treatment with primary and secondary antibodies. Examination of over 50 cells in cross section treated in this manner (which encompassed filaments on both the inside and the outside of aggregates) revealed similar total densities of CG in all cells, indicating that nitrogenase was not concentrated or isolated within specific portions of the aggregate. Occasional deposition of CG particles outside the cell wall is presumably an artifact of cell sectioning, rather than nonspecific immunochemical reactions; no evidence exists for nonspecific binding in either the irrelevant antibody or fetal bovine serum controls or in the Western blots (see below).

We conducted additional tests to ensure that CG distribution represented specific binding. Fetal bovine serum and irrelevant antibody controls were consistently negative (less than two CG particles per μm^2 throughout cells), verifying that the secondary antibody was specific for the primary antibody to nitrogenase (Fig. 3). The specificity of the primary antibody is shown in the Western blots (Fig. 4), which indicated a single band occurring at approximately 40 kDa for each sample when calibrated against Bio-Rad prestained markers. We observed prestained markers to migrate further down the gel than comparable molecular-weight

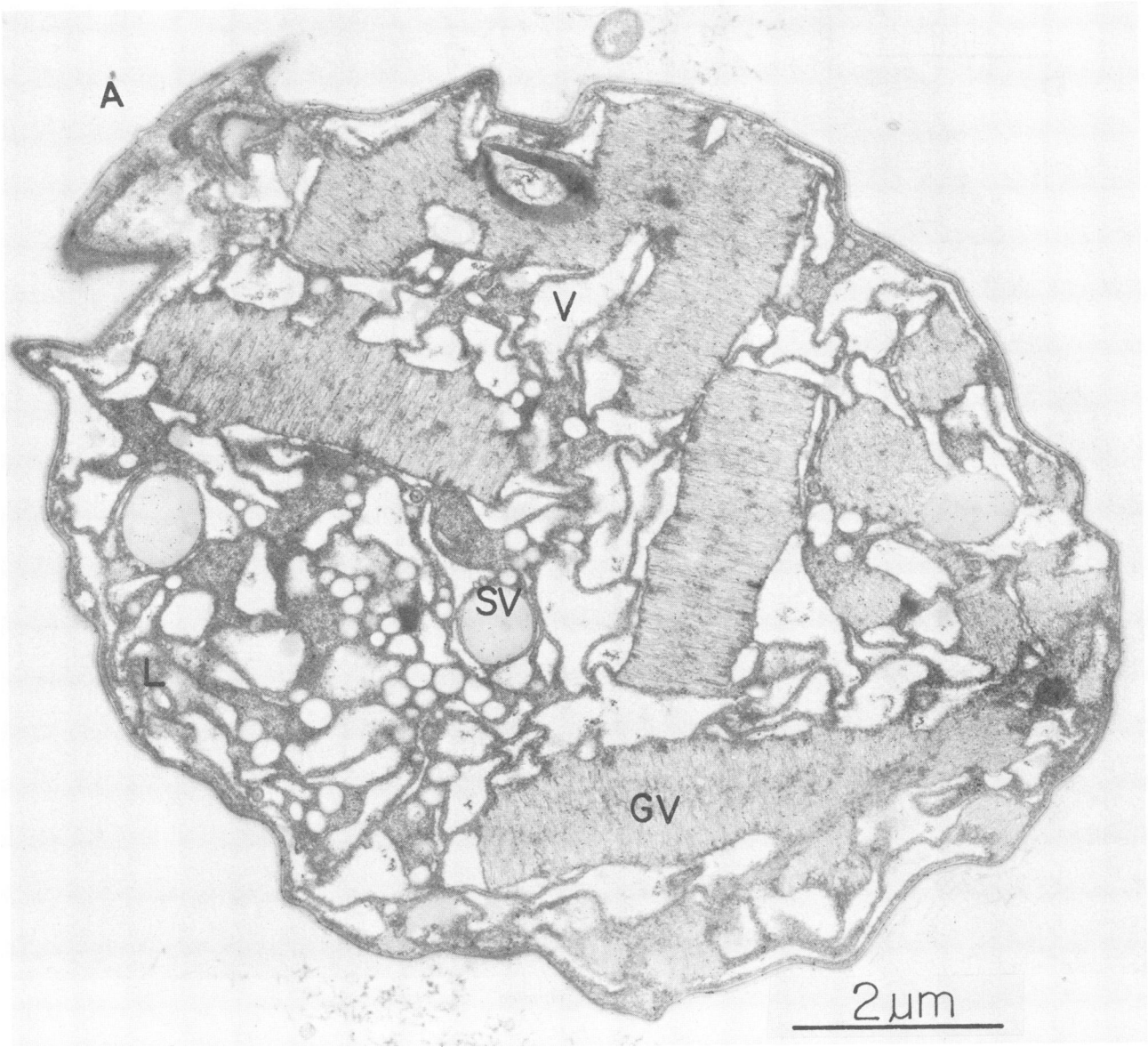

FIG. 2. TEM views of CG-labeled *Trichodesmium* spp. filaments. Filaments were obtained from the Caribbean Sea. (A) Low-magnification cross-sectional view showing CG deposition patterns. Deposition is closely associated with cylindrical, fully inflated gas vacuoles (GV) and spherical vacuoles (SV). We observed additional, although less dense, CG deposition associated with convoluted lamellae (L), while electron-transparent vacuolated (V) regions appeared free of CG particles. (B) High-magnification region of panel A. CG deposition patterns in both gas vacuoles and spherical vacuoles were readily seen at this magnification.

markers stained after electrophoresis. This finding confirms Bio-Rad's precautions (provided with the Mini-PROTEAN II unit) regarding rigorous interpretations of molecular masses solely based on prestained markers. Our cross-calibration of pre- and poststained protein standards reveals a sizable discrepancy (Fig. 4). When calibrated against poststained markers, the Fe protein-specific band appears at approximately 35 kDa. It is known that the dimeric dinitrogenase reductase dissociates in the presence of sodium dodecyl sulfate before electrophoresis, resulting in a monomeric form with a reported molecular mass of ca. 30 to 40 kDa (4, 9). Our results fall within this range.

Additional verifications of antibody specificity have been conducted on Western blots of electrophoresed extracts from other well-documented diazotrophs, including *Azotobacter*, *Klebsiella*, *Vibrio*, and *Anabaena* (Currin et al., in press). When probed with either Rr-2 or universal antinitrogenase antibodies, a single band appeared at approximately 30 to 40 kDa (Currin et al., in press). This band was absent when the same microorganisms were grown on combined nitrogen (nitrogenase activity repressed). In concert, current and previous results consistently showed antibodies to be highly specific for nitrogenase among diverse N_2-fixing genera.

Stab cultures consistently showed the presence of N_2-fixing bacteria, confirmed by positive acetylene reduction assays and serial transfers into N-free mannitol agar tubes which yielded heavy growth. Characteristically, noticeable

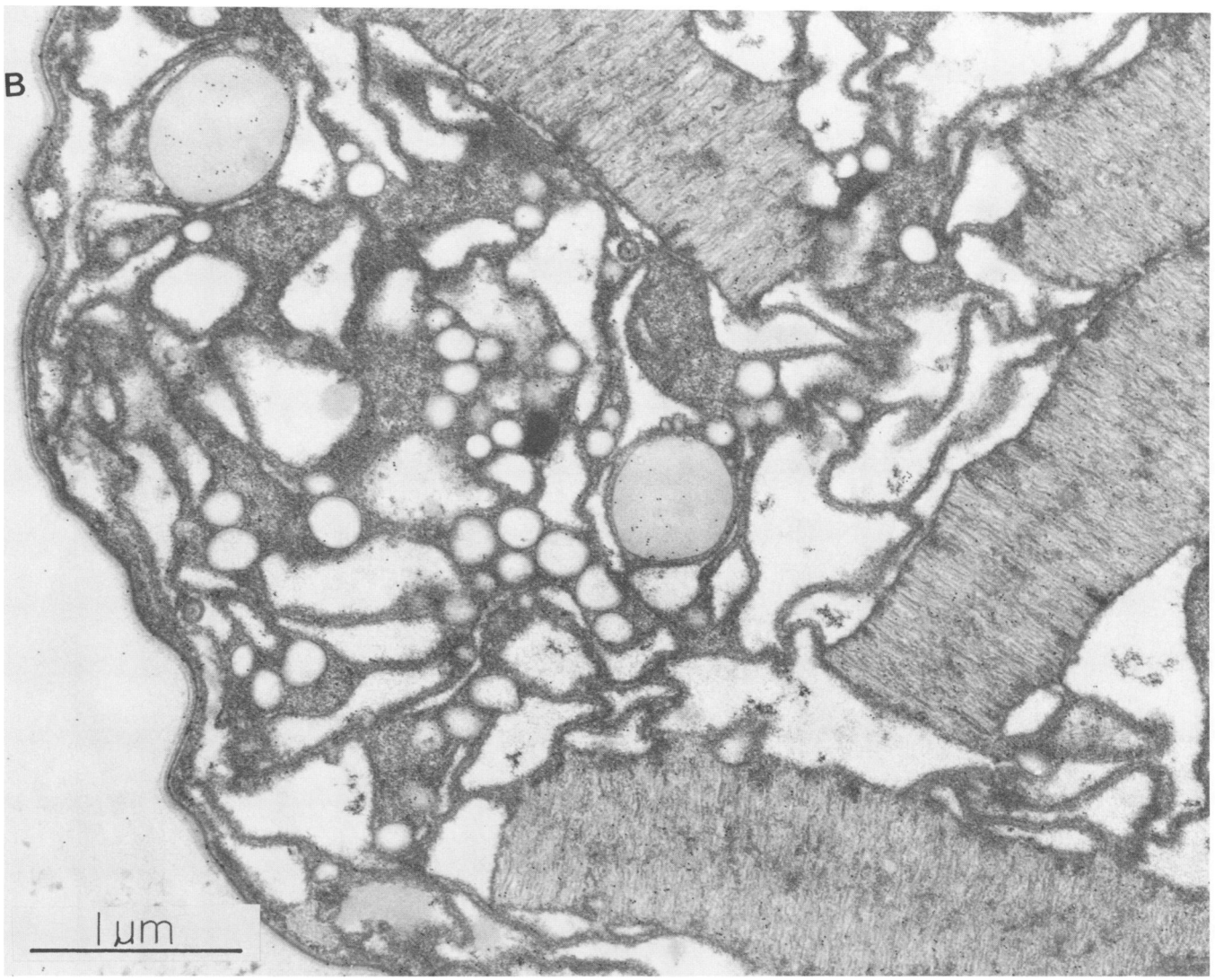

FIG. 2—*Continued*

bacterial growth occurred within 2 to 3 days as white bands or plates situated 0.5 to 1 cm below the agar surface. Oxygen microelectrode profiles revealed that plates represented the oxic-anoxic interface of tubes (Paerl et al., in press). This indicated that N_2-fixing bacterial isolates required microaerophilic or anaerobic conditions or both for expression of nitrogenase.

It was suggested previously that spatial segregation of N_2 fixation and O_2-evolving photosynthesis exists within *Trichodesmium* aggregates (3, 7, 18) and that regions of low oxygen tension within the aggregate may allow N_2 fixation to be localized in cells within the aggregate (i.e., not exposed to the bulk medium) (17). Our present findings lead us to conclude that nitrogenase is present throughout all cells of the aggregate, but that catalytic expression of the nitrogenase is closely regulated by microscale features such as biotically induced low O_2 tension within the aggregate (17).

Several types of eubacteria were associated with aggregates of the Caribbean Sea *Trichodesmium*. Some of these bacteria contained CG, indicating that certain associated bacteria have the potential to fix N_2. By using microaerophilic stab culturing techniques, we were able to isolate N_2-fixing heterotrophic bacteria associated with Caribbean *Trichodesmium* aggregates, confirming the previous observation of Maruyama et al. (15). While we note the presence of such bacteria, they account for <1% of total CG deposition among *Trichodesmium* cells. Furthermore, microaerophilic conditions were apparently required for active N_2 fixation to take place among these bacteria. Such requirements greatly restrict N_2 fixation potentials among bacteria associated with the periphery of *Trichodesmium* aggregates, a region known to be well oxygenated during daytime photosynthetic periods (17). Hence, bacterial contributions to aggregate N_2 fixation are likely to be small. However, these heterotrophic bacteria provide respiratory O_2 consumption which may promote the development and maintenance of low O_2 microzones within aggregates (17). In turn, these microzones facilitate expression of nitrogenase in both

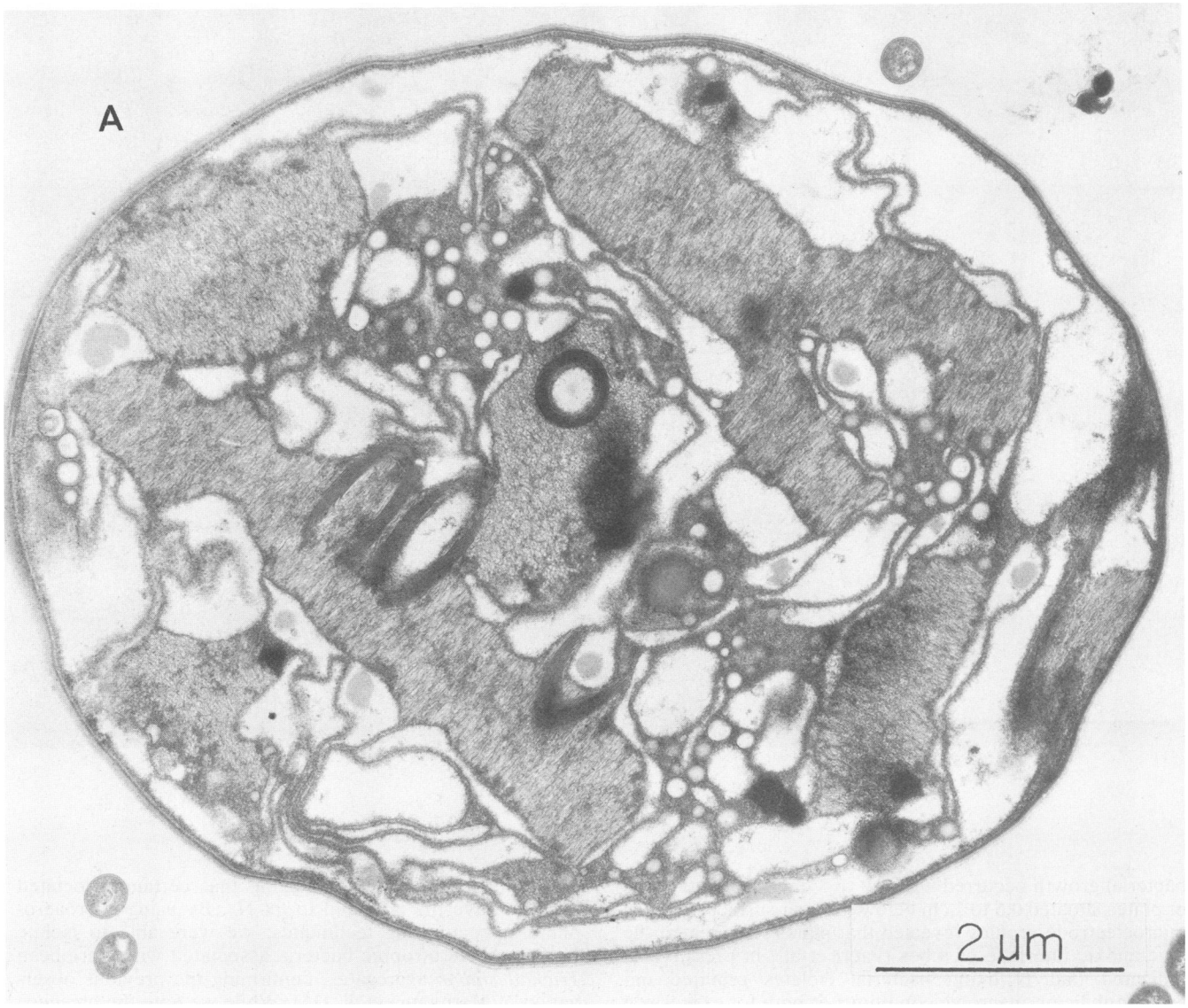

FIG. 3. IEM cross-sectional views of an irrelevant antibody control (conducted on Caribbean *Trichodesmium* spp. filaments) illustrating that the CG-conjugated secondary antibody was specific for the primary antibody to nitrogenase. The use of either fetal bovine serum or anti-*C. albicans* 9938 antiserum in place of the antinitrogenase primary antibodies yielded similar results, namely, no detectable CG labeling of *Trichodesmium* sections. (A) Low-magnification view; (B) high-magnification view.

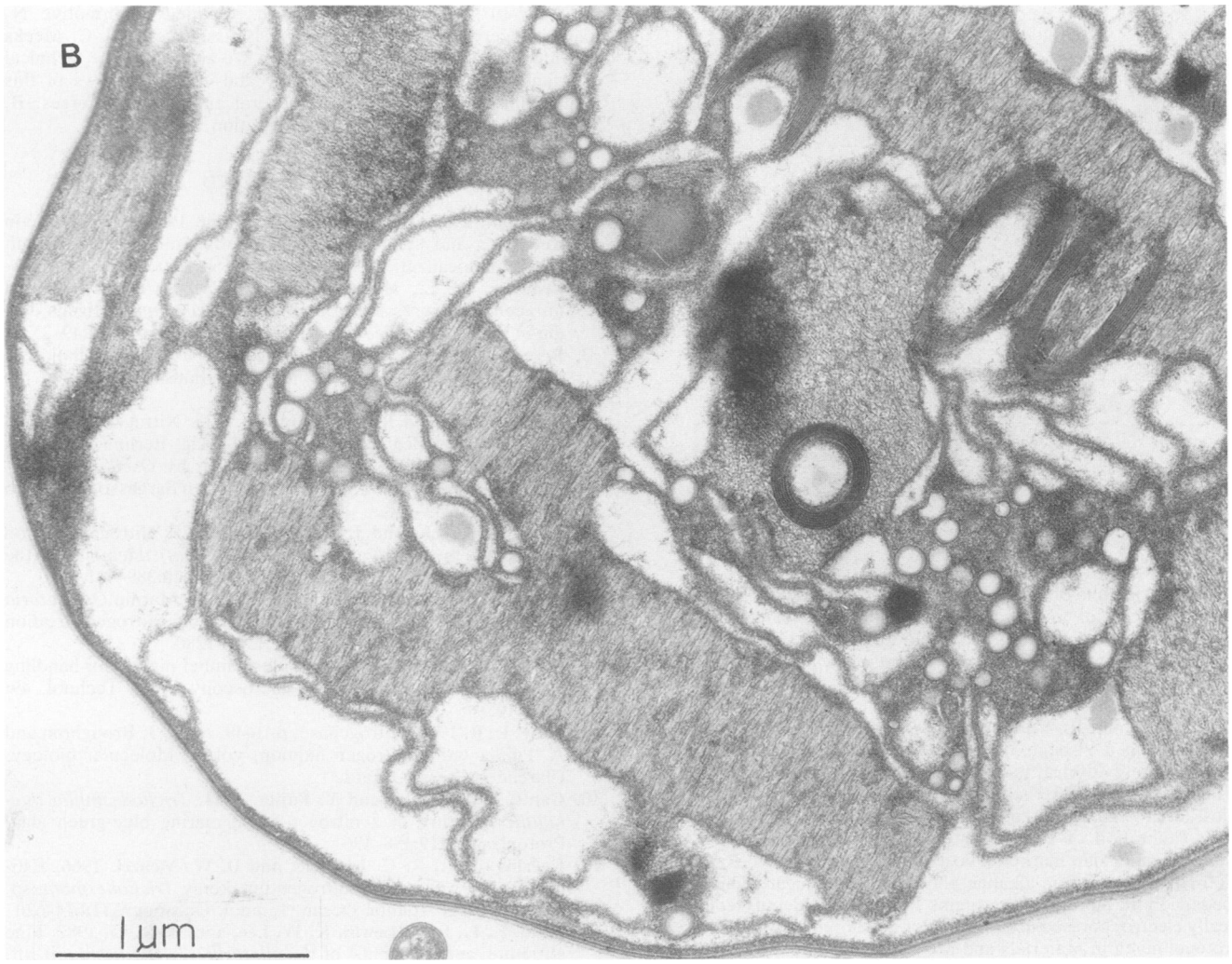

FIG. 3—*Continued*

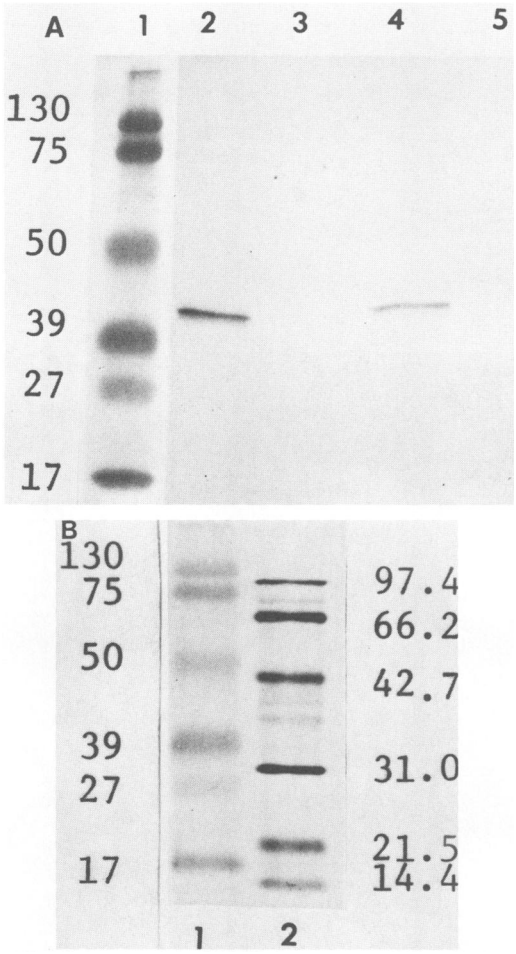

FIG. 4. Results of Western blots. (A) Western blot following gel electrophoresis of a well-documented, actively fixing diazotroph (*Azotobacter chroococcum*, lane 2), a non-N_2-fixing (negative control) marine diatom (*Thalassiosira* spp., lane 3), actively fixing North Carolina *Trichodesmium* spp. (lane 4), and non-N_2-fixing North Carolina *Trichodesmium* spp. (lane 5). Western blot was probed with anti-Rr-2 antibody (the same antibody used in TEM shown in Fig. 1 through 3). A single band consistently appeared at approximately 40 kDa, indicating specificity of the antinitrogenase primary antibody in these samples. Molecular masses (10^3) of Bio-Rad prestained markers (lane 1) are indicated. (B) Western blot cross-calibration of Bio-Rad prestained (lane 1) and poststained (lane 2) molecular markers. Poststained markers (Bio-Rad) were biotinylated; an avidin-alkaline phosphatase conjugate which binds specifically to biotinylated proteins was used to identify colorimetrically electrophoresed proteins. Color development was identical to protocol given in Materials and Methods.

Trichodesmium and internally associated bacteria. How such consortial microzones are established and organized (i.e., from physiological and ecological perspectives) remains unknown.

Our findings underscore the crucial role that low O_2 tension induced by colonial growth plays in regulating nitrogenase expression and resultant contributions of fixed nitrogen to ambient nitrogen-depleted planktonic habitats by *Trichodesmium*. On a global scale, potential N_2 fixation inputs by *Trichodesmium* are strongly controlled by the sea state; frequently, becalmed tropical and subtropical waters yield optimal conditions for aggregation and high biomass-specific rates of N_2 fixation (3, 7, 17, 18). Possession of N_2 fixation potential in virtually all cells throughout filaments appears to be an opportunistic response to the dynamic nature of shifting sea states. When calm conditions arise, both aggregation and consequent expression of nitrogenase can rapidly proceed, thereby optimizing N_2 fixation inputs even during short-term periods of low hydraulic turbulence.

ACKNOWLEDGMENTS

This work was supported by the Soap and Detergent Association and by National Science Foundation grants OCE 85-00740, OCE 88-20036, and BSR 86-14951.

E. J. Carpenter and D. G. Capone kindly shared cruise support (OCE 87-10798; OCE 87-16907) and provided confirmative N_2 fixation (acetylene reduction) data. Discussions with J. C. Meeks provided initial impetus for this study. We appreciate the technical assistance of G. Suba and S. Zasky and critical reviews of this manuscript by J. C. Meeks and several anonymous referees. B. Bright assisted with manuscript preparation.

LITERATURE CITED

1. **Bergman, B., P. Lindblad, and A. N. Rai.** 1986. Nitrogenase in free-living and symbiotic cyanobacteria: immunoelectron microscopic localization. FEMS Microbiol. Lett. **35**:75–78.
2. **Brawner, D. L., and J. E. Cutler.** 1987. Cell surface and intracellular expression of two *Candida albicans* antigens during *in vitro* and *in vivo* growth. Microb. Pathogen. **2**:249–257.
3. **Bryceson, I., and P. Fay.** 1981. Nitrogen fixation in relation to bundle formation and trichome differentiation. Mar. Biol. **61**: 159–166.
4. **Burns, R. C., and R. W. F. Hardy.** 1975. Nitrogen fixation in bacteria and higher plants. Springer-Verlag, Berlin.
5. **Carpenter, E. J.** 1973. Nitrogen fixation by *Oscillatoria* (*Trichodesmium*) *thiebautii* in the southwestern Sargasso Sea. Deep Sea Res. **20**:285–288.
6. **Carpenter, E. J., and J. J. McCarthy.** 1975. Nitrogen fixation and uptake by *Oscillatoria* (*Trichodesmium*) *thiebautii* in the western Sargasso Sea. Limnol. Oceanogr. **20**:389–401.
7. **Carpenter, E. J., and C. C. Price IV.** 1976. Marine *Oscillatoria* (*Trichodesmium*): explanation for aerobic nitrogen fixation without heterocysts. Science **191**:1278–1280.
8. **Day, J. W.** 1974. A BEEM capsule chamber pipette for handling small specimens for electron microscopy. Stain Technol. **49**: 408–410.
9. **Eady, R. R.** 1986. Nitrogenase, p. 1–49. *In* W. J. Broughton and A. Puhler (ed.), Nitrogen fixation, vol. 4. Molecular biology. Clarendon Press, Oxford.
10. **Gantt, E., K. Ohki, and Y. Fujita.** 1984. *Trichodesmium thiebautii*; structure of a nitrogen-fixing marine blue-green alga. Protoplasma **119**:188–196.
11. **Goering, J. J., R. C. Dugdale, and D. W. Menzel.** 1966. Estimates of *in situ* rates of nitrogen uptake by *Trichodesmium* sp. in the tropical Atlantic Ocean. Limnol. Oceanogr. **11**:614–620.
12. **Haxo, F. T., R. A. Lewin, K. W. Lee, and M.-R. Li.** 1987. Fine structure and pigments of *Oscillatoria* (*Trichodesmium*) aff. *thiebautii* (Cyanophyta) in culture. Phycologia **26**:443–456.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
14. **Ludden, P. W., G. G. Preston, and T. E. Dowling.** 1982. Comparison of active and inactive forms of iron protein from *Rhodospirillum rubrum*. Biochem. J. **203**:663–668.
15. **Maruyama, Y., N. Taga, and O. Matsuda.** 1970. Distribution of nitrogen-fixing bacteria in the central Pacific Ocean. J. Oceanogr. Soc. Jpn. **26**:360–366.
16. **Ohki, K., and Y. Fujita.** 1988. Aerobic nitrogenase activity measured as acetylene reduction in the marine non-heterocystous cyanobacterium *Trichodesmium* spp. grown under artificial conditions. Mar. Biol. **98**:111–114.
17. **Paerl, H. W., and B. M. Bebout.** 1988. Direct measurements of

O_2-depleted microzones in marine *Oscillatoria* (*Trichodesmium*): relation to N_2-fixing capabilities. Science **241**:441–445.
18. **Paerl, H. W., and P. T. Bland.** 1982. Localized tetrazolium reduction in relation to N_2 fixation, CO_2 fixation, and H_2 uptake in aquatic filamentous cyanobacteria. Appl. Environ. Microbiol. **43**:218–226.
19. **Postgate, J. R.** 1978. Nitrogen fixation. Studies in biology, no. 92. E. Arnold, London.
20. **Stewart, W. D. P. (ed.).** 1975. Nitrogen fixation by free-living microorganisms. Cambridge University Press, London.
21. **Van Baalen, C., and R. M. Brown, Jr.** 1969. The ultrastructure of the marine blue-green alga, *Trichodesmium erythraeum*, with special reference to the cell wall, gas vacuoles, and cylindrical bodies. Arch. Mikrobiol. **69**:79–91.