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Unicellular, Aerobic Nitrogen-Fixing Cyanobacteria of the Genus *Cyanothece*

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Two marine, unicellular aerobic nitrogen-fixing cyanobacteria, *Cyanothece* strain BH63 and *Cyanothece* strain BH68, were isolated from the intertidal sands of the Texas Gulf coast in enrichment conditions designed to favor rapid growth. By cell morphology, ultrastructure, a GC content of 40%, and aerobic nitrogen fixation ability, these strains were assigned to the genus *Cyanothece*. These strains can use molecular nitrogen as the sole nitrogen source and are capable of photoheterotrophic growth in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea and glycerol. The strains demonstrated a doubling time of 10 to 14 h in the presence of nitrate and 16 to 20 h under nitrogen-fixing conditions. Rapid growth of nitrogen-fixing cultures can be obtained in continuous light even when the cultures are continuously shaken or bubbled with air. Under 12-h alternating light and dark cycles, the aerobic nitrogenase activity was confined to the dark phase. The typical rates of aerobic nitrogenase activity in *Cyanothece* strains BH63 and BH68 were 1,140 and 1,097 nmol of C_2H_2 reduced per mg (dry weight) per h, respectively, and nitrogenase activity was stimulated twofold by light. Ultrastructural observations revealed that numerous inclusion granules formed between the photosynthetic membranes in cells grown under nitrogen-fixing conditions. These *Cyanothece* strains possess many characteristics that make them particularly attractive for a detailed analysis of the interaction of nitrogen fixation and photosynthesis in an aerobic diazotroph.

In all nitrogen-fixing organisms, the nitrogen fixation process is carried out by nitrogenase, an extremely oxygen-sensitive enzyme. Microorganisms have developed various strategies to protect their nitrogenase from oxygen inhibition (2, 5, 6). Among the nitrogen-fixing microorganisms, the cyanobacteria occupy a unique position because these are the only oxygenic photosynthetic organisms capable of nitrogen fixation under aerobic conditions (13). Nitrogen fixation has been reported for all three major morphological groups of cyanobacteria: heterocystous filamentous, nonheterocystous filamentous, and unicellular forms (5, 13). The oxygen protection mechanisms employed by these organisms vary considerably. In the heterocystous filamentous strains, about 5 to 10% of the cells undergo morphological differentiation into specialized cells called heterocysts under nitrogen-fixing conditions (13, 49). In this arrangement, nitrogen fixation and photosynthetic oxygen evolution are spatially separated, so that oxygenic photosynthesis takes place in vegetative cells and nitrogen fixation occurs in heterocysts. The fixed nitrogen from these cells is exported to the neighboring vegetative cells, and the reductant for nitrogen fixation is imported from the vegetative cells (5, 49).

There are many genera of nonheterocystous cyanobacteria, both filamentous and unicellular, that are capable of nitrogen fixation. The nonheterocystous filamentous forms have been placed in the genera *Trichodesmium*, *Oscillatoria*, and *Plectonema*, and the unicellular forms have been placed in the genera *Gloeothece*, *Aphanothece*, and *Synechococcus* (3, 7, 20, 28, 42). In a survey, Rippka et al. (35) found that among 133 nonheterocystous strains surveyed, 46 were capable of nitrogen fixation. Of these, only five were capable of true aerobic nitrogen fixation. The first reported unicellular cyanobacterium capable of aerobic nitrogen fixation was a *Gloeothece* sp. (50), although several other marine and freshwater strains have since been added to this group. All of the freshwater strains were isolated from rice paddy fields in India, southern Taiwan, and Vietnam (16, 27, 37). The isolates from India and Vietnam were placed in the genus *Aphanothece* (27, 37). Two of the four strains isolated from Taiwan, RF-1 and RF-2, belong to the genus *Synechococcus*; the other two strains, RF-6 and RF-7, fall in the genus *Gloeothece* (16, 17).

Several fast-growing and slow-growing unicellular, aerobic nitrogen-fixing cyanobacteria have been isolated from the marine environment and characterized (47, 48). One interesting strain, *Synechococcus* strain BG043511, was isolated near the Bahama islands in the Atlantic Ocean (23). This cyanobacterium fixes nitrogen aerobically and shows cell cycle-dependent nitrogen fixation activity (26). Aerobic nitrogen-fixing cultures of this bacterium, when placed under an argon atmosphere, simultaneously produce molecular hydrogen and oxygen in a light-dependent manner (32). This hydrogen production is mediated exclusively by nitrogenase (31). Another strain, *Synechococcus* strain SF-1, was isolated from the blade of a pelagic brown alga (40). This isolate is relatively slow growing but is capable of aerobic nitrogen fixation. Both freshwater and marine strains are being used to study aerobic nitrogen fixation and oxygen protection under alternating light-dark regimens and in synchronous cultures.

The number of strains added to the aerobic nitrogen-fixing unicellular group is increasing. The earliest-isolated strains of this type were given the genus name *Synechococcus* (16, 23, 40) even though they were larger than most strains in the genus and were aerobic diazotrophs. The main criteria used to assign them to this genus were the unicellular nature, cell division in one plane perpendicular to the long axis of the

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cell, and the lack of a sheath. The recent classification of *Chroococcales* proposed by Waterbury and Rippka (46) clearly separates the nitrogen-fixing unicellular cyanobacteria from traditional *Synechococcus* spp. and makes provision to place these organisms in a separate genus, *Cyanothece.*

There is still much to be learned about the strategies used by unicellular cyanobacteria to fix N_2 under aerobic conditions (8, 29). It would be helpful to obtain fast-growing, nitrogen-fixing unicellular strains that are transformable so that the oxygen protection mechanisms of nitrogenase can be studied by genetic and molecular techniques. In this report, we describe the isolation and preliminary characterization of two fast-growing, marine unicellular cyanobacteria capable of aerobic nitrogen fixation. We present the relevant physiological properties and discuss the development of numerous intracellular inclusion bodies under N_2-fixing conditions. From the properties of these strains, we assigned both of them to the genus *Cyanothece.*

MATERIALS AND METHODS

Isolation and growth. The *Cyanothece* strains were cultured in medium ASP2 (30), as modified by Van Baalen (45; see also references 21 and 44), or modified ASNIII medium (35). In addition, the iron and chelating system of ASNIII was replaced by that of ASP2. Both media were buffered with 1 g of TAPS [*N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid] per liter preadjusted to pH 8.2 or 1 g of TAPS plus 1 g of TAPSO {3-[*N*-tris(hydroxymethyl)methylamino]-2-hydroxypropanesulfonic acid} preadjusted to pH 7.7. Vitamin B_{12} supplementation was not required. Cultures with combined nitrogen contained either 1 or 1.5 g of $NaNO_3$ per liter. All stock solutions and media were prepared with high-quality nanopure water (Barnstead). Photoheterotrophic cultures were grown in the presence of 20 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and 10 mM glycerol. For growth measurements, 100-ml cultures were grown in 250-ml Erlenmeyer flasks at 30 or 38°C and shaken at 110 rpm on an orbital shaker. The 1% inoculum was taken from a 1-week-old culture that was grown without combined nitrogen. Culture growth in a 4-ml aliquot was monitored at each time interval by measuring the optical density at 750 nm or by counting cells in a Petroff-Hauser counter. These techniques yielded very similar results for cell growth, as demonstrated in Fig. 1.

Plating of our *Cyanothece* isolates can be difficult. The medium is solidified with either 0.5% DNA grade high-melting-point agarose or 1% washed agar; unwashed agar is inhibitory. In the presence or absence of nitrate, colony formation can be seen within 7 to 10 and 10 to 14 days, respectively. Plating is enhanced with pH 7.7 buffer, fresh plates, high humidity, and a light intensity of between 30 and 80 microeinsteins/m^2/s.

Several samples were collected from intertidal areas near Port Aransas, Texas. These were incubated under conditions similar to those used to select rapidly growing *Anabaena* strains (10, 41). The culture tubes were incubated at 39°C under continuous light and bubbled with 1 to 2% CO_2. Greening was observed in 4 to 6 days, and growth under N_2-fixing conditions was repeated two or three more times. The cultures were then made axenic by repeated streakings on plates that contained combined nitrogen. The two strains described in this article have been deposited with the American Type Culture Collection (Rockville, Md.) and given accession numbers ATCC 51141 (strain BH63) and ATCC 51142 (strain BH68).

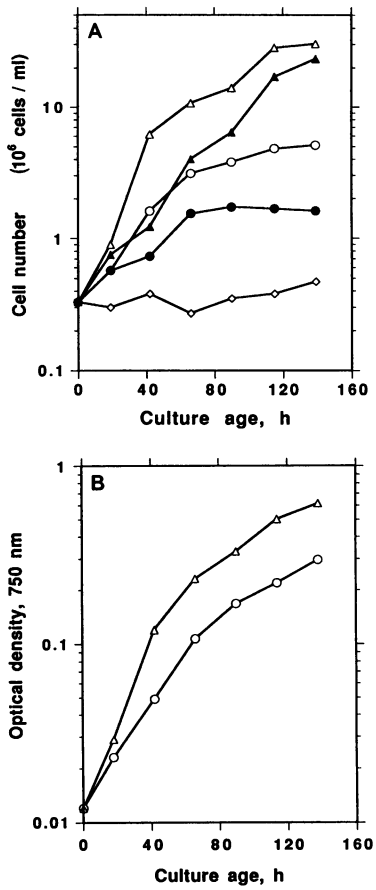

FIG. 1. Growth curves of (A) *Cyanothece* strain BH63 and (B) *Cyanothece* strain BH68 grown in saltwater-based medium with illumination (70 microeinsteins/m^2/s). (A) *Cyanothece* strain BH63 grown at 38°C under autotrophic and photoheterotrophic conditions in the presence and absence of nitrate. Cell density was monitored in a Petroff-Hauser counter for cells grown with NO_3 (△), with NO_3 plus DCMU plus glycerol (▲), without NO_3 (○), without NO_3 but with DCMU plus glycerol (●), and without NO_3 but with DCMU (◇). (B) Growth of *Cyanothece* strain BH68 in the presence (△) and absence (○) of NO_3. Cells were grown at 30°C in modified ASNIII medium, and cell growth was monitored by measuring the optical density at 750 nm.

For acetylene reduction measurements, *Cyanothece* strain BH63 and *Cyanothece* strain BH68 were cultured in 1-liter aspirator bottles containing 800 ml of modified ASNIII medium lacking combined nitrogen. The cultures were grown at 30°C, subjected to 12-h alternating light and dark cycles, and bubbled continuously with air. After three successive light and dark cycles, 2-ml culture samples were taken at 3-h intervals for acetylene reduction measurements.

Acetylene reduction. Acetylene reduction assays were carried out in 6.5-ml Becton Dickinson Vacutainer tubes. The tubes contained 2.0 ml of culture with 10% acetylene in the gas phase and were incubated at 30°C. After 1 h of incubation, 0.2 ml of 5 M NaOH was injected into each tube to stop the reaction. An aliquot of the gas phase (0.5 ml) was injected into a Varian gas chromatograph fitted with a Porapak N column and flame ionization detector. Gas flow

rates of 30 ml of nitrogen, 30 ml of hydrogen, and 340 ml of air per min were used. The column and injector port temperatures were set at 100 and 150°C, respectively.

Absorption spectra, dry weight measurement, and determination of DNA base composition. The absorption spectra of whole cells were measured with a Beckman model DU-7HS spectrophotometer. Dry weight was measured by filtering 25 to 30 ml of culture onto a glass fiber filter, rinsing twice with water, and drying at 70°C until a constant weight was achieved.

DNA was isolated from the *Cyanothece* strains and the thermal melting profile (T_m) was determined by standard methods (12). The DNA was resuspended in 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and the melting profile was determined with a computer-controlled spectrophotometer. *Escherichia coli* DNA was used as a standard.

Freeze-substitution preparation for transmission electron microscopy. Cells grown in the presence and absence of nitrate at a light intensity of 60 microeinsteins/m^2/s were concentrated 50- to 100-fold by centrifugation at 5,000 × *g* and resuspended in 2 ml of medium A. The cells were incubated for 10 to 30 min, pelleted, and resuspended in 0.5 ml of 1.5% aqueous agarose (Sigma type VII low-temperature-gelling agarose) at 45°C. The cells were then thinly spread on glass slides and allowed to cool until the agarose solidified (2 to 5 min). The cell-agarose sheet was cut into squares (9 to 16 mm^2), and the squares were plunged into baskets suspended in liquid propane (cooled to −190°C in a liquid nitrogen bath). The freezing apparatus has been described previously (14). Frozen samples were transferred to 4-oz (ca. 113 g) screw-top specimen jars that contained 20-ml beakers of substitution fluid (precooled to −85°C) to begin the gradual substitution of organic solvent for H_2O in the cells. Ethanol was used for substitution when specimen material was to be used for immunocytochemical localization, whereas acetone followed by acetone-OsO_4 was used for general morphological examination. The dryness of the 100% ethanol or acetone was ensured by the addition of anhydrous sodium sulfate crystals to 1-pint (ca. 0.5 liter) stock bottles at least 48 h prior to use.

The ethanol substitution fluid was changed three times at 24-h intervals prior to removal of the specimens from the −85°C freezer. The beaker of substitution fluid was surrounded by silica gel to form a desiccator bottle for the final change. About 8 h after the final transfer, the jars were placed in a cooled styrofoam chest, covered with a 1- to 2-cm layer of crushed dry ice, and moved to a −20°C freezer. The lid was removed, and the dry ice evaporated over the next 15 h, allowing the samples to warm up gradually. The final warm-up took place when the bottles were transferred to a refrigerator (4°C) for 2 h. Specimens were transferred from the freezing baskets into vials and infiltrated for 6 h with Lowicryl HM20 low-temperature resin (3:1, 1:1, and 1:3 ethanol-resin mixtures for 2 h each) at 4°C. After final infiltration overnight in 100% resin, specimens were transferred to fresh resin in Beem capsules for photopolymerization by UV light for 48 h at 4°C, followed by an additional 48 h at room temperature.

When acetone was used as the primary substitution fluid, a similar schedule was followed with the following exceptions. The last change consisted of cooled 1% (wt/vol) OsO_4 dissolved in dry acetone. Since the OsO_4 does not penetrate the specimen material well at low temperature, the specimens were allowed to sit at room temperature for 2 h before transfer from the OsO_4-acetone fixative to 100% acetone. The specimens were then infiltrated with acetone-Spurr's resin mixtures at room temperature on a rotator (3:1 and 1:1 for 2 h each, followed by 1:3 with accelerator overnight) before embedding in final resin mix. These specimens were polymerized at 60°C for 48 h.

Thin sections were cut, picked up on Formvar- and carbon-coated grids, stained with uranyl acetate and lead citrate, and examined in a Philips EM-400 transmission electron microscope at an accelerating voltage of 80 kV.

RESULTS

Strain characteristics and growth. The original independent isolates collected from three different sites near Port Aransas, Texas, were given the names *Cyanothece* strains BH63, BH68, and BH93. The first two strains were used in the present study. These strains showed differences in their phenotypic characteristics, especially in slime secretion and phototactic properties. Strains BH63 and BH93 did not produce slime and tended to settle under nonshaking conditions, whereas strain BH68 produced slime and never settled in liquid medium. Under settled conditions, strains BH63 and BH93 showed positive phototaxis when side illuminated, a behavior not demonstrated by strain BH68.

Figure 1 shows typical growth curves for *Cyanothece* strains BH63 and BH68 obtained under nitrogen-fixing and non-nitrogen-fixing conditions. Both strains grew up to twice as fast in the presence of nitrate as in N_2-fixing conditions, and the diazotrophic cultures rarely reached the same cell density as those grown with nitrate. Exponential growth lasted for approximately 3 days under these growth conditions, with cultures reaching an A_{750} of ~0.3 to 0.6 and 2 × 10^7 cells per ml in a 5-day period. The doubling times in the presence and absence of a combined nitrogen source were 10 to 14 h and 16 to 20 h, respectively.

DNA base composition. The T_m of *Cyanothece* DNA was used to determine the DNA base composition. The T_ms for two isolates of *Cyanothece* strain BH68 (BH68F and BH68K) and one isolate of *Cyanothece* strain BH63 (BH63E) were virtually identical, resulting in a calculated GC content of 40% ± 1.5% (data not shown). This value is consistent with the initial reason for establishing the genus *Cyanothece*, which was to better classify the rather large (4 to 6 μm) cells of low GC composition from the genus *Synechococcus* (34). The two strains initially included in the genus *Cyanothece* (strains PCC7424 and PCC7418) have a GC content of 42% (34). Therefore, our two isolates have the size, morphology, physiology, and GC content appropriate for classification within the genus *Cyanothece*.

Photoheterotrophic growth. Preliminary experiments were conducted to see whether these strains could grow under photoheterotrophic conditions with glucose, fructose, sucrose, and glycerol. Of these compounds, only glycerol supported photoheterotrophic growth of strains BH63 and BH68. Figure 1 shows the ability of *Cyanothece* strain BH63 (strain BH68 yielded similar results) to grow under photoheterotrophic conditions with glycerol. The photosystem II inhibitor DCMU at 20 μM completely abolished the photoautotrophic growth of both nitrogen-fixing and non-nitrogen-fixing cultures. There was a significant difference in growth rate between nitrogen-fixing and non-nitrogen-fixing cultures in the presence of DCMU and glycerol. Nitrate cultures grew about twice as fast as nitrogen-fixing cultures, with doubling times of about 14 and 22 h, respectively. The nitrogen-fixing photoheterotrophic cultures stopped growing after approximately 3 days and seldom exceeded 2 × 10^6

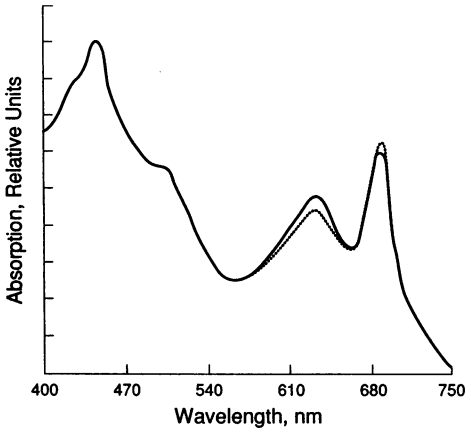

FIG. 2. Absorption spectra of *Cyanothece* strain BH63 grown in the presence (——) and absence (– – –) of nitrate.

cells per ml. Nitrate-grown, photoheterotrophic cultures grew extremely well and often approached 2×10^7 cells per ml, the cell density of the photoautrophic cultures.

Absorption spectra. The absorption spectra of whole cells of *Cyanothece* strain BH63, grown in the presence and absence of nitrate, are presented in Fig. 2 (the findings for strain BH68 were virtually identical). The two spectra are qualitatively quite similar, and the absorbance levels are proportional to the cell density. The discrete absorption peak near 620 nm indicates that the nitrogen-fixing cells retain high levels of phycocyanin. The A_{620}/A_{680} ratios were in the range of 0.92 to 0.96 for cells grown under either condition, which indicates that the ratio of phycocyanin to chlorophyll concentrations remains relatively constant. This suggests that phycobilisomes are not significantly degraded and used for a nitrogen reserve. We have determined that *Cyanothece* strains contain high levels of cyanophycin, which may represent an important nitrogen reserve in these strains (36a). Much of the cyanophycin is in cyanophycin granules, which are distinct from the inclusion granules discussed below.

Morphology and fine structure. Individual cells of *Cyanothece* strain BH63 and *Cyanothece* strain BH68 were spherical to oval in shape, depending on the stage of the cell division cycle. Cell division occurred by binary fission, and there was no sheath material present around the cells. The cells were 4 to 5 μm wide and 7 to 8 μm long. Immediately after cell division, the cells were spherical and 4 to 5 μm in diameter. They later become elongated, reaching an oblong shape just prior to cell division.

It was evident from phase and fluorescence microscopy that the nitrogen-fixing cells differed from the NO_3-grown cells in that they contained refractile granules. Preparation for electron microscopy by conventional fixation methods (e.g., aldehyde-OsO_4 fixation) resulted in cells that contained empty spaces or "holes," presumably representing lost granules. A similar ultrastructural appearance was described by Chou and Huang (4) in their study of the strain designated *Synechococcus* strain RF-1. In order to visualize the intact granules, alternative preparation procedures had to be developed. Since we also intended to use immunocytochemistry in future experiments, freeze-substitution techniques were investigated. These procedures utilize ultrarapid plunge-freezing in a liquid nitrogen-cooled liquid propane bath followed by substitution of water by organic solvents at −85°C. Freeze-substitution preserves cellular morphology extremely well, such as the relationship of the cell wall to the plasma membrane, with much less distortion than traditional chemical fixation methods (43). In addition, the material was embedded in Lowicryl HM20, a low-viscosity, nonpolar resin that was polymerized by UV light at 4°C. The combination of freeze-substitution and appropriate embedding resins gave improved preservation of ultrastructural integrity and antigenicity. Experimentation with different solvents determined that substitution with ethanol provided the best visualization of the thylakoids and preservation of antigenicity, whereas acetone (with 1% OsO_4) yielded the best preservation of inclusion granules in these species.

Figure 3 shows the ultrastructural features of *Cyanothece* strains BH63 and BH68 grown under continuous illumination in the presence and absence of NO_3. The NO_3-grown cells have an extensive thylakoid membrane system that radiates from various areas around the cell periphery. The thylakoids are particularly prominent in cells that were prepared for electron microscopy with ethanol during freeze-substitution (Fig. 4A and B). These cells show structures between the thylakoids that resemble phycobilisomes. There are often light-staining structures between the cytoplasmic membrane and the proximal ends of the thylakoid, as shown in Fig. 4A for *Cyanothece* strain BH68. The NO_3-grown cells are otherwise typical unicellular cyanobacteria, with a well-defined cell wall, carboxysomes, and phosphate granules.

Growth under N_2-fixing conditions results in major ultrastructural changes (Fig. 3B and D). The cells become larger, and nitrogen-fixing cells are generally more ellipsoid, although this is still cell cycle dependent. However, the most noticeable difference is the presence of numerous inclusion bodies in nitrogen-fixing cells. These inclusions form within the intermembrane spaces between thylakoids, as shown in Fig. 4C and D. The inclusion bodies generally range from 150 to 250 nm in length, although they can reach 500 nm in length. The inclusion bodies differ substantially in density and often appear to be surrounded by a proteinaceous shell. Figure 4D shows the organization of the granules between the membranes and suggests that granules are in different stages of formation or degradation. As indicated in Fig. 4D, a single cell can have inclusion bodies of different sizes and densities. This suggests that they are in a constant state of flux and likely have some functional significance. It is evident that the granule periphery has a substructure and that this structure may interact with the adjoining thylakoid. Cells grown under photoheterotrophic conditions can possess a few very large inclusion bodies, which occupy about 50% of the cross-sectional area (data not shown).

Nitrogen fixation under alternating light and dark cycles. In other unicellular cyanobacteria, nitrogenase activity is temporally separated when cells are subjected to 12-h alternating light and dark cycles. In order to study the pattern of nitrogenase activity in these *Cyanothece* strains, the nitrogen-fixing cultures were subjected to alternating 12-h light and dark periods and acetylene reduction activity was measured at 3-h intervals. In both strains, nitrogenase activity occurred exclusively during the dark period, and no activity occurred in the light phase (Fig. 5). Nitrogenase activity began to appear 3 h after initiation of the dark period, reached a peak after 6 h of dark, and later declined sharply, reaching almost zero within a 3-h period. The appearance and disappearance of nitrogenase activity in the second dark period was nearly identical to the pattern in the first dark period. Nitrogenase activity assayed in the dark showed about 40 to 60% of the activity of a similar sample assayed in

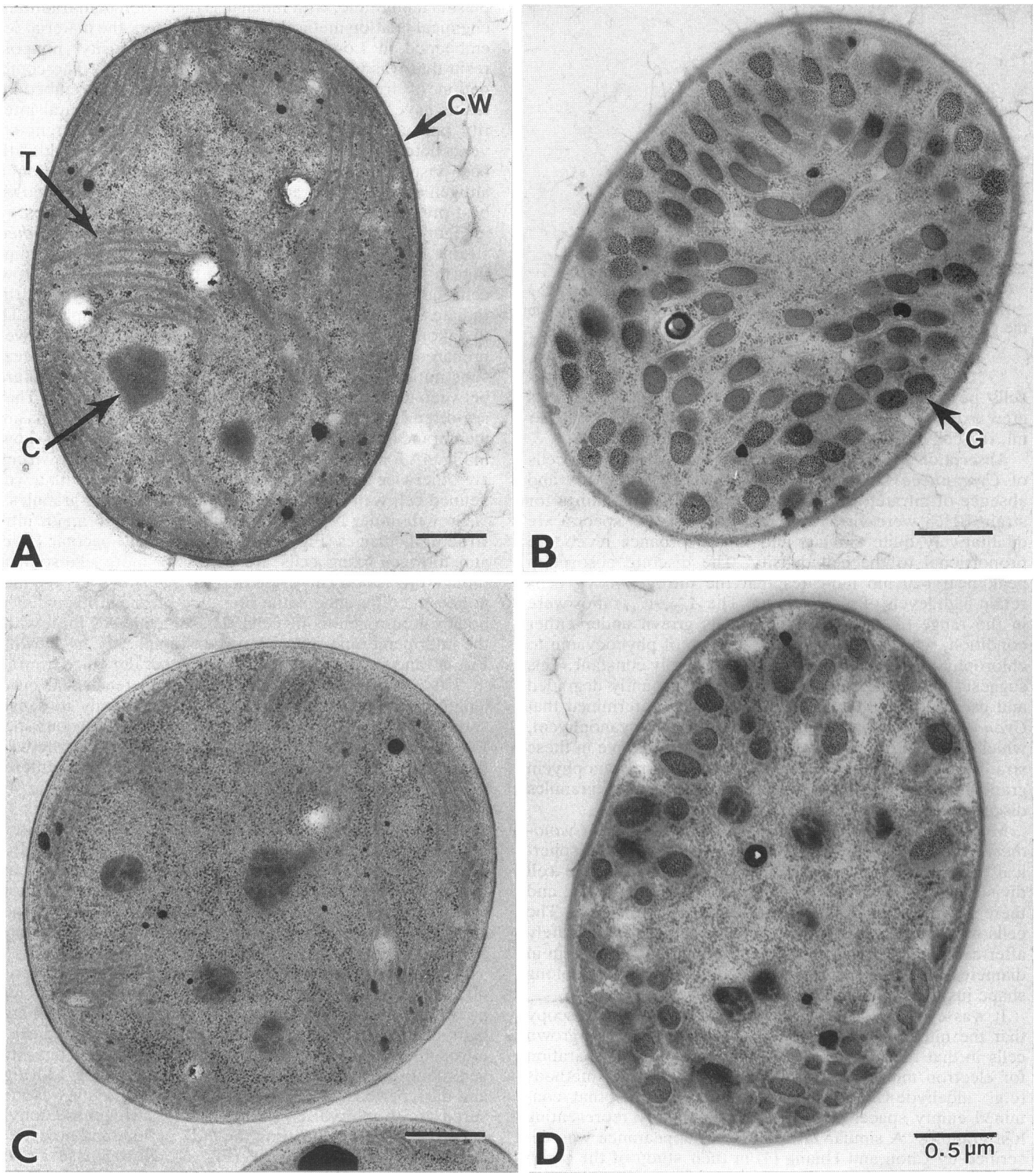

FIG. 3. Electron micrographs of *Cyanothece* strain BH63 and *Cyanothece* strain BH68 grown in the presence and absence of NO_3. The cultures were freeze-substituted with acetone plus OsO_4. (A and B) *Cyanothece* strain BH63 grown with (A) and without (B) NO_3; (C and D) *Cyanothece* strain BH68 grown with (C) and without (D) NO_3. The inclusion granules in the N_2-fixing cells (B and D) can be seen aligned between the thylakoids. CW, cell wall; C, carboxysome; G, inclusion granule; T, thylakoid. Bars, 0.5 μm.

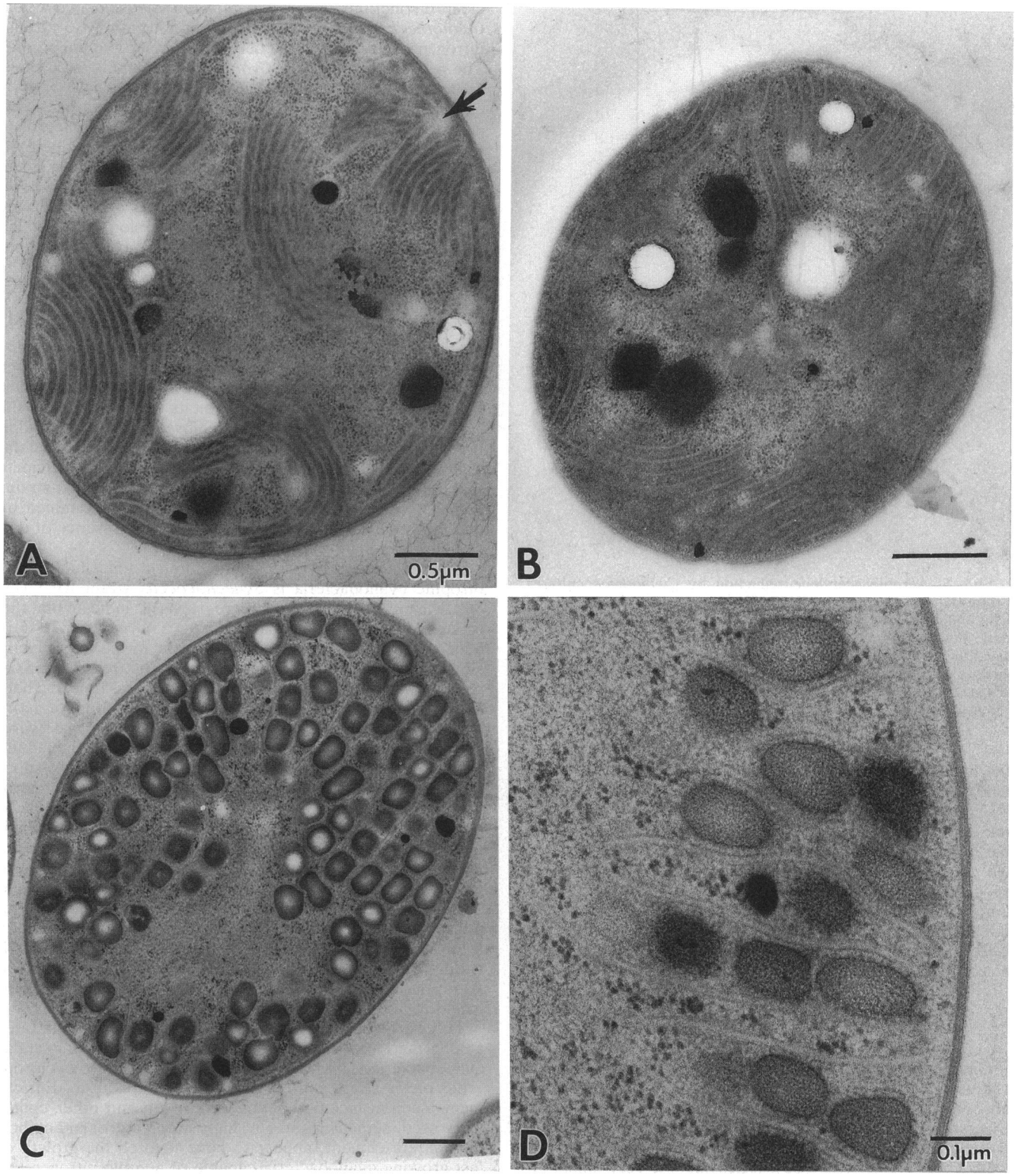

FIG. 4. Electron micrographs of *Cyanothece* strains BH63 and BH68 that emphasize the thylakoids (A and B) or the inclusion granules (C and D). *Cyanothece* strain BH68 (A) and *Cyanothece* strain BH63 (B) were grown in the presence of NO_3 and freeze-substituted with ethanol. The thylakoids are radially oriented from two or three locations along the cell periphery and often appear associated with light-staining, proteinaceous structures (A, arrow). (C and D) *Cyanothece* strain BH63 grown in the absence of NO_3 and freeze-substituted with acetone and OsO_4. These cells demonstrate that the inclusion granules form and are arrayed between the thylakoids. The different densities of the granules are evident. Higher magnification (D) indicates that the granules are surrounded by a limiting structure which may also interact with the thylakoid membrane. Bars: (A, B, and C) 0.5 μm; (D) 0.1 μm.

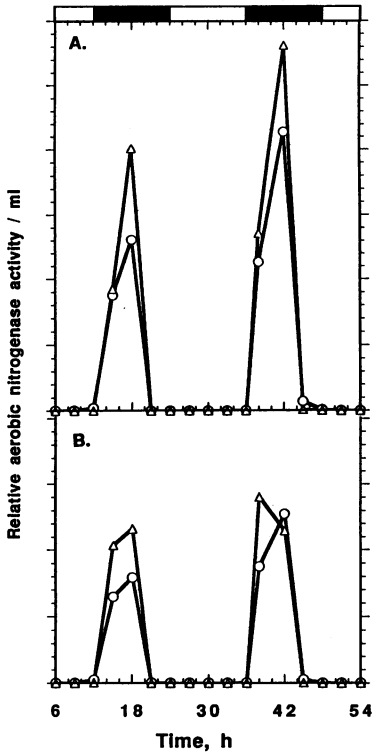

FIG. 5. Aerobic acetylene reduction by *Cyanothece* cultures. Strain BH63 (○) and strain BH68 (△) were grown in alternating 12-h light and dark periods. The solid and open bars at the top represent dark and light periods, respectively. Acetylene reduction was assayed in the light (A) and in the dark (B). The acetylene reduction peaks assayed in the light of *Cyanothece* strain BH63 and *Cyanothece* strain BH68 at 42 h correspond to 1,140 and 1,097 nmol of C_2H_2 reduced per mg per h, respectively.

the light but followed a similar pattern. The specific acetylene reduction rates were similar in the two strains. The peak acetylene reduction activities of *Cyanothece* strain BH63 in the light were 734 and 1,140 nmol of C_2H_2 reduced per mg per h during the first and second dark periods, respectively. On the other hand, *Cyanothece* strain BH68 expressed specific activities of 690 nmol/mg/h during the first and 1,197 nmol/mg/h during the second dark phase. The cycling was not dependent on alternating light and dark phases, since cells grown under continuous light demonstrated similar peaks of nitrogenase activity at 24-h intervals (data not shown).

DISCUSSION

On the basis of their morphological and physiological characteristics, the two new strains of unicellular, aerobic nitrogen-fixing cyanobacteria were classified in the genus *Cyanothece*. The cyanobacteria are classified into five groups, and the modern classification is based on bacteriological nomenclature (34, 35). It has become very difficult to classify unicellular cyanobacteria at the genus level because of the lack of adequate structural and developmental characteristics. In the 1989 edition of *Bergey's Manual of Systematic Bacteriology*, initial attempts were made to classify the order *Chroococcales* into provisional genera or groups based on reproduction, thylakoids, cell division, cell diameter, and the presence of a sheath (46). In this grouping, a clear distinction was made between *Synechococcus* and *Cyanothece* based on cell diameter and the ability to express nitrogenase activity under aerobic or anaerobic conditions. The newly created genus *Cyanothece* accommodated those unicellular cyanobacteria that express nitrogenase activity and are larger than 3 μm in diameter. All traditional *Synechococcus* strains which are less than 3 μm in diameter and incapable of nitrogen fixation are retained under the *Synechococcus* name. The two unicellular cyanobacteria described in this article possess the properties of the *Cyanothece* group in the following respects: (i) cell size; (ii) aerobic nitrogen fixation capability; (iii) binary fission with division in one plane; (iv) no sheath material; and (v) GC content. From these characteristic features, we propose that the strains described in this article be referred to as strains of the genus *Cyanothece*.

The genus name *Cyanothece* will allow easy distinction of such unicellular strains based simply on nitrogen-fixing ability and the dispersed arrangement of thylakoids. The nitrogen-fixing unicellular cyanobacteria isolated in 1986 from marine and freshwater habitats were placed in the genus *Synechococcus* (16, 23). Subsequently, some additional strains were added to this group, and such unicellular organisms now appear to be quite widespread. We anticipate that the isolation of aerobic nitrogen-fixing unicellular strains will increase as more and more habitats are examined by appropriate enrichment procedures for the presence of unicellular cyanobacteria.

One of the most intensively studied unicellular diazotrophic cyanobacteria is *Synechococcus* strain RF-1 (11, 16). The published electron micrographs (4) indicate that this isolate should also be classified as a *Cyanothece* strain because of its morphology, including the formation of inclusion bodies under N_2-fixing conditions. The freshwater *Synechococcus* strain RF-1 differs from the strains reported here in its slower doubling time and the production of more extracellular slime. These cells also adhere to glassware, which can be prevented by replacing the normal medium with conditioned medium (15). This strain is also more difficult to plate and does not appear to grow photoheterotrophically. Nonetheless, the strain has been shown to possess some important properties. It is reported to exhibit a circadian rhythm for N_2 fixation when grown under a normal light-dark regimen (15, 18, 19). When cultures were grown with alternating light and dark periods of 14 h of each, 12 h of each, or 10 of each, they demonstrated an endogenous N_2-fixing rhythm with a free-running period of about 24 h for at least four cycles (15). We are interested in investigating this phenomenon further and believe that *Cyanothece* strains BH63 and BH68 represent better experimental organisms for such studies.

There is a considerable degree of variation in growth rates among marine and freshwater aerobic N_2-fixing unicellular cyanobacteria. This variation depends on the intrinsic properties of the strain and the growth conditions used. Enrichment medium lacking combined nitrogen and other conditions used, such as high light, high temperature, and carbon dioxide enrichment, appear to have favored the growth of fast-growing cyanobacteria capable of aerobic nitrogen fixation. The *Cyanothece* strains reported here had doubling times of 10 to 20 h, but we have not yet determined the optimal growth conditions. These doubling times are similar to those of two diazotrophic marine strains, *Synechococcus* strain Miami BG043511 and *Synechococcus* strain SF-1 (40). On the other hand, the *Cyanothece* strain growth rates were much faster than those of the freshwater isolates *Gloeothece*

and *Synechococcus* strain RF-1. These strains were relatively slow growing, and reported doubling times were in the range from 60 to 70 h (16, 25).

Cyanothece strains BH63 and BH68 can grow photoheterotrophically in the presence of DCMU with glycerol as a carbon source. Although the majority of known cyanobacteria are obligate photoautotrophs, several strains of cyanobacteria are capable of photoheterotrophic growth in the presence of DCMU and glucose (33). *Synechococcus* strain PCC7002, a marine unicellular cyanobacterium, grows photoheterotrophically and utilizes glycerol as a carbon source. This strain could grow normally even in the presence of 30 mM glycerol in the medium (22). The majority of cyanobacteria tested use glucose, and very few strains which can grow in the presence of glycerol have been isolated. In certain cases, glycerol can kill cells growing photoautotrophically (22). In testing for photoheterotrophic growth, the strains first have to be adapted to low concentrations of glycerol, with the concentration of glycerol gradually increased. With this approach, it was possible to adapt *Cyanothece* strain BH68 to grow in a medium containing 5 mM glycerol.

The aerobic nitrogenase activity of *Cyanothece* strains BH63 and BH68 grown under a cycle of 12-h alternating light and dark periods occurred exclusively in the dark phase; no activity was seen in the light phase. This pattern of aerobic nitrogenase activity is similar to that of *Gloeothece* strain ATCC 27152 and *Synechococcus* strain BG043511 (9, 25, 26). Temporal separation of photosynthesis and nitrogen fixation was proposed to be the mechanism by which these unicellular cyanobacteria are able to maintain the oxygen-sensitive nitrogenase (5, 7). In the *Gloeothece* strain, aerobic respiration and not photosynthetic electron transport is responsible for supplying the reductant and energy for nitrogen fixation (24). We note that the nitrogenase activity assayed in the dark was lower than that assayed in the light. This suggests some additional role of light in enhancing aerobic nitrogenase activity. The nitrogen fixation reaction is very energy demanding and requires at least 16 ATP molecules per molecule of nitrogen fixed (2). One possibility is that the dark-assayed cultures are limited by ATP because of the lack of generation of ATP through cyclic photophosphorylation (1). An alternative explanation is limitation of carbohydrate or inefficient mobilization of these reserve materials to fully support nitrogenase activity.

Although the temporal separation of photosynthesis and nitrogen fixation is believed to be the main and preferred way to reduce molecular nitrogen in *Cyanothece* strains, this adaptation by itself cannot explain the diazotrophic growth of these bacteria in batch cultures under continuous illumination with constant bubbling of air through the medium (data not shown). Therefore, these *Cyanothece* strains must have a robust oxygen protection mechanism to support aerobic nitrogen fixation. It is evident from numerous studies that the in vitro nitrogenase activity is extremely sensitive to oxygen (5), and microorganisms have evolved various means to avoid nitrogenase inhibition by oxygen. The possible involvement of the inclusion granules in these processes is an important new line of research that we are currently pursuing.

These strains were isolated in order to develop a system with which to study the interrelationships between photosynthesis and N_2 fixation in an O_2-evolving, unicellular diazotroph. Such an organism should be capable of rapid growth in liquid culture and on agar plates and amenable to biochemical and molecular biological manipulation. We have shown in this report that these organisms grow well, even under photoheterotrophic conditions. Photoheterotrophy can be valuable for these studies because mutants defective in photosystem II can be generated and maintained on glycerol. This will allow further studies on the specific role of photosystem II-generated O_2 on the expression and regulation of aerobic nitrogenase activity. In other work, we have analyzed *Cyanothece* plasmids (38), and we have preliminary evidence for transformation in *Cyanothece* strain BH68 (39). The inclusion granules that form under N_2-fixing conditions are a novel structure that may be unique to *Cyanothece* spp. and important for aerobic N_2 fixation. We have determined that the granules are not composed of poly-β-hydroxybutyrate or cyanophycin (36a). However, we recently isolated these granules and found that they are composed of protein and carbohydrate (36). We have also determined that these intracellular inclusions are not static but are formed in the light (during photosynthesis) and used in the dark (36a). Therefore, these strains appear to represent a good model for detailed analysis of the strategies used by these unicellular diazotrophs to permit aerobic N_2 fixation.

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REFERENCES

1. **Almon, H., and H. Bohme.** 1982. Photophosphorylation in isolated heterocysts from the blue-green alga *Nostoc muscorum*. Biochim. Biophys. Acta **679**:279–286.
2. **Burris, R. H.** 1991. Nitrogenases. J. Biol. Chem. **266**:9339–9342.
3. **Carpenter, E. J., and C. C. Price.** 1976. Marine *Oscillatoria* (*Trichodesmium*): explanation for aerobic nitrogen fixation without heterocysts. Science **191**:1278–1280.
4. **Chou, H. M., and T. C. Huang.** 1991. Ultrastructure of the aerobic, nitrogen-fixing unicellular cyanobacterium *Synechococcus* sp. RF-1, p. 53–59. *In* O. Lhotsky (ed.), Archiv für hydrobiologie: algological studies 64. E. Schwiezerbartsche Verlagsbuchhandlung, Stuttgart, Germany.
5. **Fay, P.** 1992. Oxygen relations of nitrogen fixation in cyanobacteria. Microbiol. Rev. **56**:340–373.
6. **Gallon, J. R.** 1981. The oxygen sensitivity of nitrogenase: a problem for biochemists and microorganisms. Trends Biochem. Sci. **6**:19–23.
7. **Gallon, J. R., and A. E. Chaplin.** 1988. Nitrogen fixation, p. 148–173. *In* L. J. Rogers and J. R. Gallon (ed.), Biochemistry of the algae and cyanobacteria. Clarendon Press, Oxford.
8. **Gallon, J. R., and A. F. Hamadi.** 1984. Studies on the effects of oxygen on acetylene reduction (nitrogen fixation) in *Gloeothece* sp. ATCC27152. J. Gen. Microbiol. **130**:495–503.
9. **Gallon, J. R., S. M. Perry, T. M. A. Rajab, K. A. M. Flayeh, J. S. Yunes, and A. E. Chaplin.** 1988. Metabolic changes associated with the diurnal pattern of nitrogen fixation in *Gloeothece*. J. Gen. Microbiol. **134**:3079–3087.
10. **Gotto, J. W., F. R. Tabita, and C. Van Baalen.** 1979. Isolation and characterization of rapidly-growing, marine, nitrogen-fixing strains of blue-green algae. Arch. Microbiol. **121**:155–159.
11. **Grobbelaar, N., T. C. Huang, H. Y. Lin, and T. J. Chow.** 1986. Dinitrogen-fixing endogenous rhythm in *Synechococcus* RF-1. FEMS Microbiol. Lett. **37**:173–177.
12. **Johnson, J. L.** 1985. Determination of DNA base composition. Methods Microbiol. **18**:1–31.
13. **Haselkorn, R., and W. J. Buikema.** 1992. Nitrogen fixation in cyanobacteria, p. 166–190. *In* G. Stacey, H. J. Evans, and R. H. Burris (ed.), Biological nitrogen fixation. Chapman and Hall, New York.

14. **Howard, R. J., and K. L. O'Donnell.** 1987. Freeze substitution of fungi for cytological analysis. Exp. Mycol. **11:**250–269.
15. **Huang, T. C., and W. M. Chou.** 1991. Setting of the circadian N_2-fixing rhythm of the prokaryotic *Synechococcus* sp. RF-1 while its gene is repressed. Plant Physiol. **96:**324–326.
16. **Huang, T. C., and T. J. Chow.** 1986. New type of nitrogen-fixing unicellular cyanobacterium (blue-green alga). FEMS Microbiol. Lett. **36:**109–110.
17. **Huang, T. C., and T. J. Chow.** 1988. Comparative studies of some nitrogen-fixing unicellular cyanobacteria isolated from rice fields. J. Gen. Microbiol. **134:**3089–3097.
18. **Huang, T. C., and T. J. Chow.** 1990. Characterization of the rhythmic nitrogen-fixing activity of *Synechococcus* sp. RF-1 at the transcription level. Curr. Microbiol. **20:**23–26.
19. **Huang, T. C., J. Tu, T. J. Chow, and T. S. Chen.** 1990. Circadian rhythm of the prokaryote *Synechococcus* sp. RF-1. Plant Physiol. **92:**531–533.
20. **Kallas, T., R. Rippka, T. Coursin, M. C. Rebiere, N. T. DeMarsac, and G. Cohen-Bazire.** 1983. Aerobic nitrogen fixation by non-heterocystous cyanobacteria, p. 281–302. *In* G. C. Papageorgiou and L. Packer (ed.), Photosynthetic procaryotes. Elsevier, Amsterdam.
21. **Kratz, W., and J. Myers.** 1955. Nutrition and growth of several blue-green algae. Am. J. Bot. **42:**282–287.
22. **Lambert, D. H., and S. E. Stevens.** 1986. Photoheterotrophic growth of *Agmenellum quadruplicatum* PR-6. J. Bacteriol. **165:**654–656.
23. **Leon, C., S. Kumazawa, and A. Mitsui.** 1986. Cyclic appearance of aerobic nitrogenase activity during synchronous growth of unicellular cyanobacteria. Curr. Microbiol. **13:**149–153.
24. **Maryan, P. S., R. R. Eady, A. E. Chaplin, and J. R. Gallon.** 1986. Nitrogen fixation by *Gloeothece* sp. PCC 6909: respiration and not photosynthesis supports nitrogenase activity in the light. J. Gen. Microbiol. **132:**789–796.
25. **Millineaux, P. M., J. R. Gallon, and A. E. Chaplin.** 1981. Acetylene reduction (nitrogen fixation) by cyanobacteria grown under alternating light-dark cycles. FEMS Microbiol. Lett. **10:**245–247.
26. **Mitsui, A., S. Kumazawa, A. Takahashi, H. Ikemoto, S. Cao, and T. Arai.** 1986. Strategy by which nitrogen-fixing unicellular cyanobacteria grow photoautotrophically. Nature (London) **413:**514–530.
27. **Ni, C. V., D. Z. Khong, Z. D. Tien, and I. N. Gogotov.** 1988. Nitrogen fixation by the cyanobacterium, *Aphanothece palida*, isolated from a rice field soil. Microbiologiya **57:**458–463.
28. **Ohki, K., and Y. Fujita.** 1988. Aerobic nitrogenase activity measured as acetylene reduction in the marine non-heterocystous cyanobacterium *Trichodesmium* sp. grown under artificial conditions. Mar. Biol. **98:**111–114.
29. **Ortega, J., and L. J. Stal.** 1991. Diazotrophic growth of the unicellular cyanobacterium Gloeothece sp. PCC6909 in continuous culture. J. Gen. Microbiol. **137:**1789–1797.
30. **Provasoli, L., J. J. A. McLaughlin, and M. R. Droop.** 1957. The development of artificial media for marine algae. Arch. Mikrobiol. **25:**392–428.
31. **Reddy, K. J.** 1984. Studies on the hydrogen metabolism of marine cyanobacteria. Ph.D. thesis, University of Miami, Coral Gables, Fla.
32. **Reddy, K. J., and A. Mitsui.** 1984. Simultaneous production of hydrogen and oxygen as affected by light intensity in unicellular, aerobic nitrogen-fixing blue-green alga *Synechococcus* sp. Miami BG043511, p. 785–788. *In* C. Sybesma (ed.), Advances in photosynthesis research, vol. 2. Martinus Nijhoff/Dr W. Junk Publishers, The Hague, The Netherlands.
33. **Rippka, R.** 1972. Photoheterotrophy and chemoheterotrophy among unicellular blue-green algae. Arch. Mikrobiol. **87:**93–98.
34. **Rippka, R., and G. Cohen-Bazire.** 1983. The cyanobacteriales: a legitimate order based on the type strain *Cyanobacterium stanieri*? Ann. Microbiol. **134:**21–36.
35. **Rippka, R., J. Deruelles, J. B. Waterbury, M. Herdman, and R. Y. Stanier.** 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. **111:**1–61.
36. **Schneegurt, M. A., and L. A. Sherman.** Unpublished data.
36a. **Sherman, D. M., and L. A. Sherman.** Unpublished data.
37. **Singh, P. K.** 1973. Nitrogen fixation by the unicellular blue-green alga *Aphanothece*. Arch. Microbiol. **92:**59–62.
38. **Soper, B. W., and K. J. Reddy.** 1991. Characterization of plasmids in marine unicellular, aerobic nitrogen-fixing *Cyanothece* spp., p. 176. Abstr. VII Int. Symp. Photosynthetic Prokaryotes, Amherst, Mass.
39. **Soper, B. W., and K. J. Reddy.** Unpublished data.
40. **Spiller, H., and K. T. Shanmugam.** 1987. Physiological conditions for nitrogen fixation in a unicellular marine cyanobacterium, *Synechococcus* sp. strain SF1. J. Bacteriol. **169:**5379–5384.
41. **Stacey, G., C. Van Baalen, and F. R. Tabita.** 1977. Isolation and characterization of a marine *Anabaena* sp. capable of rapid growth on molecular nitrogen. Arch. Microbiol. **114:**197–201.
42. **Stal, L. J., and W. Krumbein.** 1981. Aerobic nitrogen fixation in pure cultures of a benthic marine *Oscillatoria* (cyanobacteria). FEMS Microbiol. Lett. **11:**295–298.
43. **Steinbrecht, R. A., and M. Mueller.** 1987. Freeze substitution and freeze-drying, p. 149–172. *In* R. A. Steinbrecht and K. Zierold (ed.), Cryotechniques in biological electron microscopy. Springer-Verlag, Berlin.
44. **Stevens, S. E., C. O. Patterson, and J. Myers.** 1973. The production of hydrogen peroxide by blue-green algae. J. Phycol. **9:**427–430.
45. **Van Baalen, C.** 1962. Studies on marine blue-green algae. Bot. Mar. **4:**129–139.
46. **Waterbury, J. B., and R. Rippka.** 1989. Order *Chroococcales* Wettstein 1924, emend, Rippka et al. 1979, p. 1728–1746. *In* J. T. Staley, M. P. Bryant, N. Pfenning, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol 3. Williams & Wilkins, Baltimore.
47. **Waterbury, J. B., S. W. Watson, R. R. Guillard, and L. E. Brand.** 1979. Widespread occurrence of unicellular, marine, plankotic cyanobacterium. Nature (London) **277:**293–294.
48. **Waterbury, J. B., S. W. Watson, F. W. Valois, and D. G. Franks.** 1986. Biological and ecological characterization of the marine unicellular cyanobacterium *Synechococcus*. Can. Bull. Fish. Aquat. Sci. **214:**71–120.
49. **Wolk, C. P.** 1982. Heterocysts, p. 359–386. *In* N. G. Carr and B. A. Whitton (ed.), The biology of cyanobacteria. University of California Press, Berkeley.
50. **Wyatt, J. T., and J. K. G. Silvey.** 1969. Nitrogen fixation by *Gloeocapsa*. Science **165:**908–909.