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Photosynthate Partitioning and Fermentation in Hot Spring Microbial Mat Communities

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Patterns of $^{14}CO_2$ incorporation into molecular components of the thermophilic cyanobacterial mat communities inhabiting hot springs located in Yellowstone National Park and *Synechococcus* sp. strain C1 were investigated. Exponentially growing *Synechococcus* sp. strain C1 partitioned the majority of incorporated $^{14}CO_2$ into protein, low-molecular-weight metabolites, and lipid fractions (45, 22, and 18% of total incorporated carbon, respectively). In contrast, mat cores from various hot springs predominantly accumulated polyglucose during periods of illumination (between 77 and 85% of total incorporated $^{14}CO_2$). Although photosynthetically active, mat photoautotrophs do not appear to be rapidly growing, since we also detected only limited synthesis of macromolecules associated with growth (i.e., protein and rRNA). To test the hypothesis that polysaccharide reserves are fermented in situ under the dark anaerobic conditions cyanobacterial mats experience at night, mat cores were prelabeled with $^{14}CO_2$ under illuminated conditions and then transferred to dark anaerobic conditions. Radiolabel in the polysaccharide fraction decreased by 74.7% after 12 h, of which 58.5% was recovered as radiolabeled acetate, CO_2, and propionate. These results indicate tightly coupled carbon fixation and fermentative processes and the potential for significant transfer of carbon from primary producers to heterotrophic members of these cyanobacterial mat communities.

The laminated cyanobacterial mat communities inhabiting alkaline silicious hot springs in Yellowstone National Park have been intensively investigated in an effort to make fundamental observations about bacterial species diversity (16, 18, 45, 46, 55, 63) and microbial community ecology (6, 62, 65). Previous studies investigating the activity and growth of cells in these well-characterized communities suggested that hot spring cyanobacterial mats display high rates of metabolic activity and growth. Using oxygen and pH microelectrodes, Revsbech and Ward (49) measured oxygen production and pH variation corresponding to very high rates of gross primary productivity by oxygenic photosynthetic cyanobacteria (*Synechococcus* spp.). After darkening a mat and observing the decrease of *Synechococcus* cells over time, Brock (6) calculated washout rates for cells of this morphology. By assuming that washout rate equals replacement, or growth rate, cyanobacterial productivity was calculated to be 5.7×10^{11} to 1.6×10^{12} cells m^{-2} day^{-1}, corresponding to 0.098 to 0.263 doubling day^{-1}. In other experiments, rates of accretion of mat material above a silicon carbide layer sifted onto the mat surface were used to measure growth rates of 18 to 45 μm day^{-1} (13). Microautoradiographic studies of hot spring cyanobacterial mat samples demonstrated light-stimulated $^{14}CO_2$ uptake and glycolate excretion (up to 7% of total photosynthate) by cells conforming to the *Synechococcus* morphology (3). Clearly, cyanobacterial cells in the Octopus Spring mat are photosynthetically active and appear to be rapidly growing.

Analysis of molecular synthesis from carbon substrates can provide information about the physiological state of microbial cells. Synthesis of ribosomes and rRNA is closely associated with cellular growth regulation, since modulation of ribosome content is a primary mechanism by which growth rates are controlled (38). rRNA content is proportional to cellular growth rate in logarithmically growing cultures of *Escherichia coli* (21), and RNA content was found to increase exponentially with growth rate of the freshwater cyanobacterium *Anacystis nidulans* (39) and a marine *Synechococcus* strain (32). Synthesis of lipids can provide information about cellular activity (61), and protein synthesis is closely related to cellular growth rate (12). The relative incorporation of photosynthetically fixed carbon into protein, polysaccharide, lipid, and low-molecular-weight metabolites has been used to determine which environmental factors affect the physiological state of cyanobacterial cultures (28, 30, 31) and naturally occurring phytoplankton communities (e.g., see references 19, 22, 42, and 47). These studies show that nutrient availability, light conditions, and temperature can influence how photosynthate is partitioned into growth-related molecules (i.e., protein, low-molecular-weight metabolites, and membrane lipids) or storage polymers (polysaccharide and storage lipids) (19, 42).

A study of photosynthate partitioning in a hot spring cyanobacterial mat community revealed that mat cyanobacteria primarily synthesize polysaccharide under illuminated conditions (29). Other studies of cyanobacterial pure cultures have shown that carbon and energy are stored in the form of polyglucose during photoautotrophic growth (57). This polymer is accumulated when carbon assimilation exceeds nutritional requirements for the synthesis of cellular components, especially under conditions of nutrient limitation (1, 11, 36, 60). Fermentation of endogenous polyglucose reserves under dark anaerobic conditions has been shown in a variety of cyanobacterial species (58), including unicellular (41) and thermophilic (50) types. Although microbial mat communities undergo marked diel fluctuations in oxygen concentration (48, 49), and dark cyanobacterial fermentation of stored polyglucose has been considered an important mechanism of carbon flux through microbial mat communities (58), to our knowledge, no studies have investigated this phenomenon in situ.

In this study, we investigated the qualitative and quantitative

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incorporation of photosynthetically fixed carbon into molecular components of a logarithmically growing *Synechococcus* sp. isolate C1 culture and hot spring cyanobacterial mat communities. We showed that photosynthate is incorporated primarily into polyglucose during periods of illumination instead of other cellular components, including protein and rRNA. We also demonstrated the fermentation of these stored polyglucose reserves under the dark, anaerobic conditions cyanobacterial mats experience at night.

MATERIALS AND METHODS

Mat samples and cultures. Cyanobacterial mat samples were collected between June and November of 1994 and 1995 from similar-temperature regions of mildly alkaline silicious hot springs in Yellowstone National Park, Wyo. The locations of Octopus Spring (pH 8.7, 48 to 65°C) and Twin Butte Vista Spring (pH 9.1, 60 to 62°C) are described by Brock (6). Clearwater Springs site D (pH 7.8, 52 to 59°C) is located north of Norris Geyser Basin as described by Ruff-Roberts et al. (52). Cores of mat material were collected with a no. 4 cork borer, and the top 2 to 3 mm of photosynthetically active mat was separated from lower layers by using a razor blade. The top green layer was transferred to screw-cap glass vials containing 3 ml of water from the collection site before addition of radiolabel.

The Octopus Spring cyanobacterial mat isolate *Synechococcus* sp. strain C1 (18) was generously provided by M. J. Ferris. Cultures were grown to late log phase in 50 ml of medium D (8) in a 250-ml culture flask with shaking (150 rpm) at 50°C under constant illumination provided by three 50-W fluorescent bulbs. Irradiance measured with a Lutron (Coopersburg, Pa.) light meter was 1,150 to 1,200 lx. For comparison, ambient irradiance measured above the mat surface in Yellowstone National Park during field incubations was 40 to 55 klx, but light is rapidly attenuated in the top 1 to 2 mm of the mat (34). Klett absorbance readings were compared with a standard curve relating absorbance to cell number determined by direct microscopy to detect logarithmic growth (mean ± standard deviation [SD], 1.49 ± 0.53 doublings day^{-1}; $n = 5$). Cell densities in the culture flasks were 3.2×10^7 to 5.2×10^7 cells ml^{-1} before radiolabel administration. The pH of cultures was adjusted to 8.5 before addition of radiolabel.

Radiolabeling. Each cyanobacterial mat core received either 2 μCi of [^{14}C]sodium bicarbonate (54 mCi/mmol), 2 μCi of [^{32}P]sodium phosphate dibasic (1 Ci/mmol), or 1 μCi of [2-^{14}C]sodium acetate (3.1 mCi/mmol). Logarithmically growing C1 cultures received 2 μCi of [^{14}C]sodium bicarbonate. Radiolabel was added from a concentrated stock solution and dispersed by gentle agitation. All isotopes were procured from New England Nuclear (Boston, Mass.).

Triplicate samples were incubated in the presence of radiolabeled substrate for 3 h unless otherwise noted. Cyanobacterial mat cores were incubated at ambient hot spring temperatures and light intensities by placing vials in the effluent channel at the site of sample collection during time periods proximate to solar noon. Dark conditions were achieved by wrapping vials with aluminum foil and black tape. During nutrient addition experiments, nitrogen and phosphorus were added to 10% of the concentrations present in medium D (which contains 922 μM NO_3^- and 77 μM PO_4^{2-}) from a concentrated stock solution before addition of the mat core. During cell density reduction manipulations, individual mat cores were homogenized in 3 ml of springwater. Following incubation, field samples were frozen on dry ice to stop biological activity, and cultures were harvested immediately. Since frozen cells may leak organic carbon, measured amounts of incorporated radiolabel may underestimate actual radiolabel incorporation, especially into low-molecular-weight cellular components. Cells were concentrated by centrifugation (20 min, 15,000 × *g*) and washed once with sterile water to reduce unincorporated $^{14}CO_2$ and excreted photosynthate. We measured 98.5% ± 1.7% (mean ± SD; $n = 21$) removal of extracellular ^{14}C in the first cell wash. $^{14}CO_2$ uptake into cellular material during nutrient addition and homogenization experiments was compared with that of unmanipulated controls by using a two-sample *t* test.

During fermentation experiments, Clearwater Springs site D mat cores were preincubated in the presence of 2 μCi of $^{14}CO_2$ for 3 h in the light and then shifted to dark anaerobic conditions. Radiolabeled water was replaced with springwater rendered anoxic by bubbling with nitrogen. In some cases, 2-bromoethanesulfonic acid (BES) (to 50 mM) was added to inhibit methanogenesis (2) or formalin (to 4%) was added to terminate biotic activity. In a separate experiment, we determined that cells removed during the replacement procedure contained 4.7% ± 4.5% (mean ± SD; $n = 6$) of the radiolabel associated with cells remaining in the vial. After replacement, the vials were darkened as described above and the gas headspace of each sealed vial was flushed with nitrogen. Samples were then incubated for 0, 3, 6, 12, and 24 h at temperatures within 4°C of the ambient hot spring temperature.

Nucleic acid analysis. Cell lysates were prepared from radiolabeled Octopus Spring mat and C1 cultures in a FastPrep bead beater (Bio 101, Vista, Calif.), using sterile 0.1-mm-diameter zirconium beads and a high-salt lysis buffer containing 1 M NaCl, 5 mM $MgCl_2$, and 10 mM Tris base (pH 8.0) to minimize nucleic acid degradation (4). Nucleic acids were obtained from the lysate by standard phenol-chloroform extraction and ethanol precipitation procedures modified to minimize nuclease degradation of RNA (53). Nucleic acids were separated on a 3.5 to 10% linear polyacrylamide gradient denaturing gel or a 10% denaturing polyacrylamide gel overlaid with a 3.5% denaturing polyacrylamide gel (53). Equivalent amounts of radioactivity were loaded onto the gel without regard for nucleic acid concentration. Nucleic acids were stained with ethidium bromide and photographed by standard procedures (53). The gel was dried with a Drygel Sr. vacuum drying apparatus (Hoeffer Scientific Instruments, San Francisco, Calif.). Autoradiography of the dried gel was performed with a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, Calif.).

Polysaccharide identification. Aliquots of radiolabeled Octopus Spring mat nucleic acid extract or 1.5 mg of glycogen (Sigma Chemical Co., St. Louis, Mo.) were transferred to 4-ml, Teflon-lined, screw-cap glass test tubes. Inositol was added to each tube as an internal standard (20 μl of 10 mM inositol in glass-distilled water), and samples were lyophilized overnight. Polysaccharides were hydrolyzed, and the resulting monosaccharides were methylated in a single step by adding 1 M methanolic hydrochloric acid (500 μl) under a stream of nitrogen and allowing the reaction to proceed in the sealed tubes overnight at 80°C. Samples were dried under a stream of nitrogen at room temperature, and a trimethylsilyl (TMS) derivatizing reagent (Sigma Sil-A; Sigma Chemical Co.) was added (200 μl) to replace hydroxyl groups with ester-linked TMS moieties. The derivatization reaction was allowed to proceed for 20 min at 80°C. Samples were again dried under a stream of nitrogen and dissolved in 100 μl of hexane for coupled gas chromatography-mass spectrometry analysis.

TMS-derivatized monosaccharides were separated in a Varian (model 3700) gas chromatograph equipped with a 30-m DB-1 glass capillary column (0.25-mm inner diameter; J & W Scientific, Folsom, Calif.). A splitless injector operating 30 s after injection (injector temperature, 260°C) was used. A temperature program was begun at injection with an initial column temperature of 160°C for 3 min followed by a rise of 3°C min^{-1} to 260°C, which was then held for 15 min. The helium carrier gas flow rate was 30 cm s^{-1}. The gas chromatograph was directly coupled to a VG 70E-HF double-focusing magnetic mass spectrometer operating at a mass resolution of 1,500. The ion source temperature was 200°C, the electron energy was 70 eV, and the ions were accelerated with 6 keV of energy. Data were acquired on a VG 11-250 data system based upon a DEC 11-73 computer.

Radiolabeled Octopus Spring mat nucleic acid extract was incubated for 3 h at 37°C with 1 U of α-amylase and/or 1 U of amyloglucosidase (Boehringer Mannheim Corp., Indianapolis, Ind.) in an aqueous reaction buffer containing 5 mM NaCl and 2 mM phosphate buffer (pH 5.8). Samples were precipitated with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of absolute ethanol and then centrifuged for 20 min at 16,000 × *g*. The supernatant was removed, and the remaining pellet was dissolved in sterile water. Radioactivity in the supernatant and the dissolved pellet was determined as described below. Radiolabeled nucleic acid extracts from Octopus Spring mat and logarithmically growing C1 cultures were also incubated with 40 μg of proteinase K (Sigma Chemical Co.) ml^{-1} in water for 10 min at 45°C or with RNase A (1 μg; Sigma Chemical Co.) or RNase-free DNase I (10 U; Boehringer Mannheim Corp.) in a buffer containing 3 mM $MgCl_2$ for 1 h at 37°C. Protease and nuclease digestions were phenol-chloroform extracted and ethanol precipitated as described above. All samples were separated on a 10% denaturing polyacrylamide gel overlaid with a 3.5% polyacrylamide gel. The gels were dried and autoradiography was performed as described above.

Protein analysis. Radiolabeled cell lysates from Octopus Spring mat and C1 cultures were prepared as described for nucleic acid extractions but with sterile water instead of high-salt lysis buffer. Proteins were separated by denaturing polyacrylamide gel electrophoresis using the discontinuous system of Laemmli and Favre (35). The separating gel was 1.5 mm thick and contained a 7 to 20% linear polyacrylamide gradient. Equivalent amounts of radioactivity were loaded onto the gel without regard for protein concentration. The gel was dried and autoradiography was performed as described above. Silver staining was performed according to the procedure of Blum et al. (5).

Lipid analysis. Lipids were extracted from radiolabeled Octopus Spring mat and C1 cultures by using a chloroform-methanol-phosphate buffer extraction solvent as described by Guckert et al. (25). Total lipid was separated by two-dimensional thin-layer chromatography according to the procedure of Evans et al. (15) using the following solvent systems: chloroform-methanol-acetic acid-diethylamine-water (120:35:37.5:6:4.5, by volume) in the first dimension and chloroform-methanol-ammonium hydroxide (65:25:5, by volume) in the second dimension. Lipids were detected by charring with sulfuric acid (27). Equivalent amounts of radioactivity were loaded onto the plates without regard for lipid concentration. Autoradiography was performed as described above. Diagnostic stains for phospholipids (molybdenum blue), aminolipids (ninhydrin), and glycolipids (α-naphthol) were applied to the developed plates according to the directions supplied by the manufacturer (Alltech Association Inc., Deerfield, Ill.).

Photosynthate partitioning. The major end products of photosynthesis in mat samples and C1 cultures were determined by partitioning incorporated carbon into chloroform-soluble (lipid), methanol-water-soluble (low-molecular-weight metabolites), hot-trichloroacetic acid (TCA)-soluble (polysaccharide), and hot-TCA-insoluble (protein) fractions by the method of Morris et al. (44) as modified

TABLE 1. $^{14}CO_2$ uptake in various hot spring cyanobacterial mat samples and logarithmically growing *Synechococcus* sp. isolate C1 cells[a]

Sample	Light		Dark	
	$^{14}CO_2$ incorporation (10^{-6} dpm cell^{-1})	Label incorporated (% of total added)	$^{14}CO_2$ incorporation (10^{-6} dpm cell^{-1})	Label incorporated (% of total added)
Octopus Spring mat[b]	1,404 ± 409	26.8 ± 5.7	97 ± 7	2.0 ± 0.2
Twin Butte Vista Spring mat[b]	2,181 ± 310	39.1 ± 3.1	65 ± 53	1.3 ± 1.1
Clearwater Springs site D mat[b]	1,673 ± 88	70.5 ± 4.4	38 ± 6	1.9 ± 0.4
Synechococcus sp. isolate C1	1,250 ± 487	54.0 ± 9.2		

[a] Values are means ± 95% confidence intervals (Twin Butte Vista Spring and Clearwater Springs site D, $n = 3$; Octopus Spring, $n = 4$; *Synechococcus* sp. isolate C1, $n = 5$).

[b] Incorporation values assume 10^{10} cells ml^{-1} in mat material (13).

by Fitzsimons et al. (20) to maximize removal of polysaccharide from the TCA-insoluble pellet. Nucleic acids partition into the polysaccharide fraction in this fractionation scheme (43). An aliquot of radiolabeled mat nucleic acid extract was lyophilized overnight and subjected to the fractionation scheme described above. To validate cell fractionation procedures, the amount of anthrone-reactive polysaccharide in each of the four fractions was determined as described by Herbert et al. (26). Of the original anthrone-reactive material present in unfractionated cells, 80.8% was recovered in the polysaccharide fraction, 4.8% was recovered in both the protein and the lipid fractions, and 1.0% was recovered in the low-molecular-weight metabolite fraction. Radioactivity in each of these fractions was determined as described below. Total incorporated radiolabel was calculated by summing the radioactivity detected in each fraction. By comparison with whole-cell incorporation, recovery of radiolabel by this method was determined to be 94.6% ± 13.2% (mean ± SD; $n = 7$).

Headspace gas analysis. Headspace gases (carbon dioxide and methane) in the unopened vials after dark anaerobic incubations were measured with a gas chromatograph as previously described (64). Radioactivity in $^{14}CO_2$ and $^{14}CH_4$ was determined by coupling the gas chromatograph to a gas proportional counter (64).

VFA detection. The supernatants of samples incubated under dark anaerobic conditions were analyzed for volatile fatty acids (VFAs) after centrifugation (20 min, 15,000 × *g*) to remove cells. VFAs were separated by high-performance liquid chromatography using a DX300 ion chromatography system and detected with a conductivity detector (Dionex, Sunnyvale, Calif.). VFAs were separated isocratically by using an HPICE-AS10 ion-exchange column, an eluent consisting of 3.5 mM $K_2B_4O_7$, and a self-regenerating suppressor. A column regeneration step consisting of an increase in eluent strength to 100 mM $K_2B_4O_7$ was performed after each run. Calibration was achieved by comparison of retention times and peak heights with external standards. The detection limits for acetate, propionate, and butyrate were approximately 0.5 μg ml^{-1}, whereas the detection limits for *n*-butyrate and *i*-butyrate were approximately 1.0 μg ml^{-1}. The lowest reported acetate concentration was above 3 μg ml^{-1}. Column eluent fractions corresponding to the retention time of each VFA, as well as fractions prior to and after VFA elution, were collected manually. Radiolabeled carbon was determined as described below. VFA specific activity was calculated by dividing the total radioactivity detected in the collected fraction by the measured concentration of the VFA.

Radioassays. Water samples and molecular fractions were radioassayed for ^{32}P and ^{14}C content with a Tri-Carb liquid scintillation analyzer (model 1900 TR; Packard Instrument Co., Meriden, Conn.). Aliquots of aqueous samples were added to Optima Gold (Packard Instrument Co.) scintillation cocktail. Lipid samples were dried under a stream of nitrogen and dissolved in chloroform, and 10% was removed to a scintillation vial and dried prior to scintillant addition. The entire protein fraction was reacted with tissue solubilizer (Scintigest [Fisher Scientific, Pittsburgh, Pa.] or Soluene 350 [Packard Instrument Co.]) for 1 h prior to scintillant addition. Background counts were subtracted from sample counts before counts per minute were converted to disintegrations per minute, using an external-standards ratio method of quench correction. Counting continued until a constant coefficient of variation was achieved (0.5%) or until 10 min had elapsed.

RESULTS

Logarithmically growing C1 cultures and mat cores from three different hot springs actively incorporated $^{14}CO_2$ into cellular material under illuminated conditions (Table 1). When normalized to the number of cells present in the incubations, C1 cultures and intact mat cores incorporated approximately equivalent amounts of radioactivity. $^{14}CO_2$ incorporation did not deplete available radiolabel under either light or dark conditions during the 3-h incubation. Dark incorporation of $^{14}CO_2$ was 6.9, 3.0, and 2.3% of photoautotrophic carbon fixation in Octopus Spring, Twin Butte Vista Spring, and Clearwater Springs mats, respectively.

Nucleic acid synthesis. (i) Radiolabeling with $^{14}CO_2$. We detected nucleic acids in both logarithmically growing C1 cultures and cyanobacterial mat samples (Fig. 1A) which corresponded to known sizes of standard *E. coli* rRNA. Although we detected incorporation of $^{14}CO_2$ into 23S, 16S, and 5S rRNAs in logarithmically growing C1 cultures, we failed to detect incorporation of $^{14}CO_2$ into rRNAs in cyanobacterial mat samples (Fig. 1B). Instead, radiolabel was detected in material which only slightly entered a 3.5% polyacrylamide gel. This material accumulated in mat samples incubated in the light, but not in dark-incubated samples, and was also detected to a lesser extent in C1 cultures. We estimate the lower detection limit of radiolabeled rRNA by this method to be ca. 1.5×10^3 dpm, 60 times lower than the ca. 9.0×10^4 dpm associated with rRNAs in the C1 culture.

(ii) Radiolabeling with $^{32}PO_4^{2-}$. When $^{32}PO_4^{2-}$ was provided to the mat as a radiolabeled substrate, synthesis of rRNAs was detected (Fig. 2). Of the added $^{32}PO_4^{2-}$, 51.0% ± 2.9% was incorporated by the mat cells after 3.25 h of light incubation (mean ± 95% confidence interval; $n = 3$).

(iii) Effect of environmental manipulations on rRNA synthesis. Neither addition of nitrogen and phosphorus to the

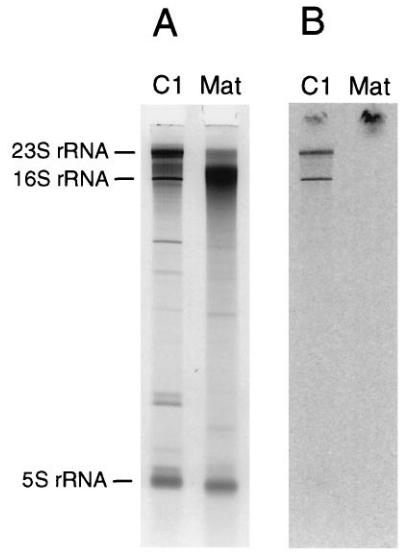

FIG. 1. Composition of nucleic acid extracts from a logarithmically growing *Synechococcus* culture (C1) and an Octopus Spring cyanobacterial mat community (Mat) after radiolabeling with $^{14}CO_2$ in the light. (A) Negative photographic image of ethidium bromide-stained nucleic acids separated on a 3.5 to 10% linear polyacrylamide gradient denaturing gel; (B) autoradiogram of the same gel.

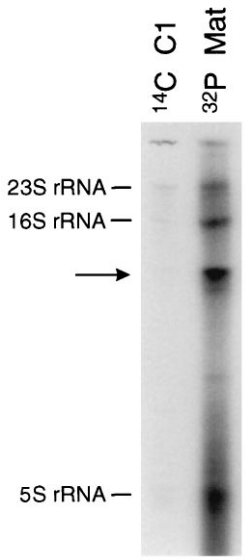

FIG. 2. Autoradiogram of polyacrylamide gel containing nucleic acid extract from an Octopus Spring cyanobacterial mat community after radiolabeling with $^{32}PO_4^{2-}$ in the light (^{32}P Mat) and ^{14}C-labeled *Synechococcus* sp. isolate C1 nucleic acid extract (^{14}C C1). The interface between the 3.5 and 10% polyacrylamide gel concentrations is indicated (arrow).

incubation water to alleviate possible nutrient imbalance nor homogenization and dilution of mat material to alleviate possible density-dependent regulation of cell physiology resulted in increased $^{14}CO_2$ uptake into cellular material compared with that of unmanipulated controls ($P = 0.5481$ and 0.8228, respectively). rRNA synthesis from $^{14}CO_2$ could not be demonstrated for treatments including nutrient addition, homogenization and dilution, extended light and dark incubations (5 h each), or shifting of incubations from 2.5-h light to 2.5-h dark conditions (data not shown).

(iv) Identification of radiolabeled material in the nucleic acid extract. Radiolabeled material in mat nucleic acid extract was resistant to protease (proteinase K) and nuclease (RNase A and DNase I) treatments (data not shown). To test the hypothesis that mat nucleic acid extract contained radiolabeled polysaccharide, the extract was hydrolyzed, TMS derivatized, and analyzed by combined gas chromatography-mass spectrometry. Gas chromatographic analysis showed that mat nucleic acid extract contained a compound which exhibited monomers with retention times identical to those of glucose derived from glycogen (Fig. 3A). The two peaks observed in the glycogen standard correspond to the α- and β-anomeric forms of TMS-derivatized D-glucose (10). The major peaks in the Octopus Spring mat nucleic acid extract detected by gas chromatography exhibit mass spectra similar to those of TMS-derivatized hexose sugars (Fig. 3B) by comparison with standard library spectra. Minor peaks were not identified.

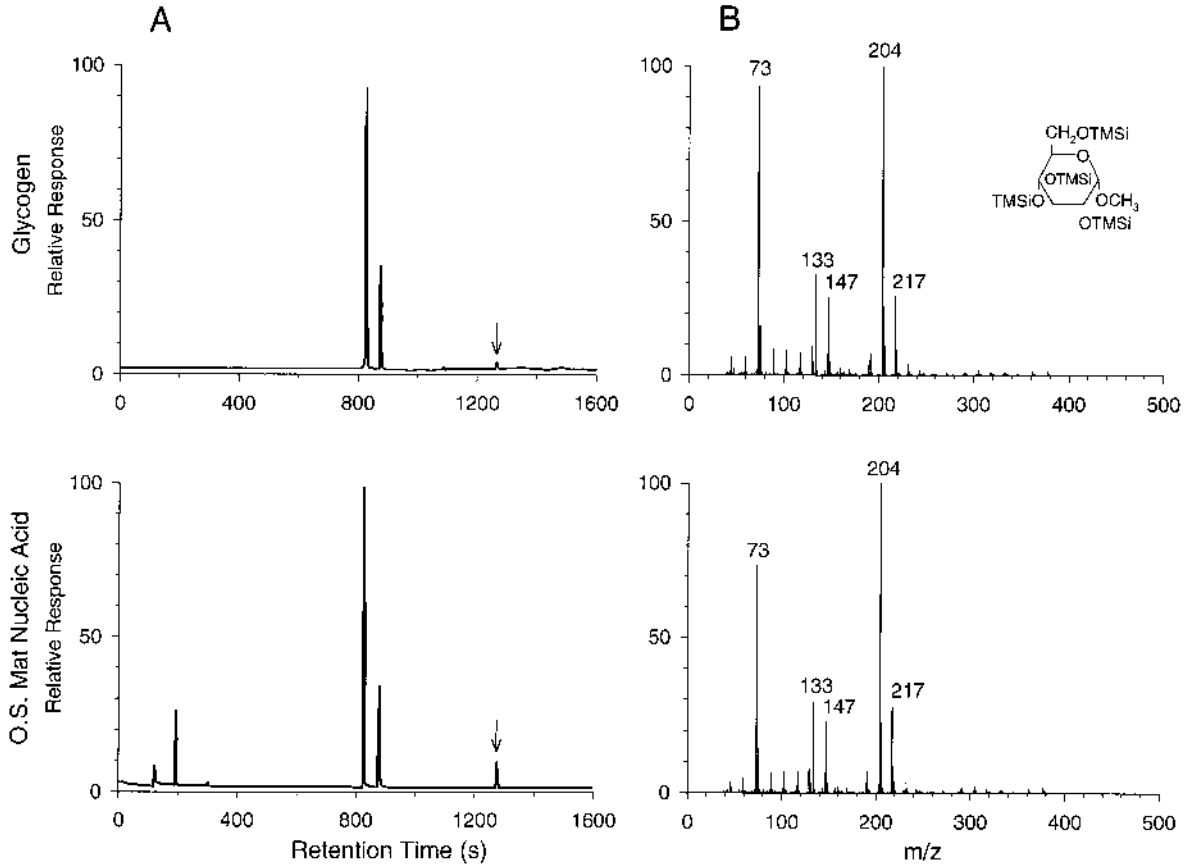

FIG. 3. (A) Hydrolyzed and TMS-derivatized glycogen (top panel) and Octopus Spring (O.S.) mat nucleic acid extract (bottom panel) samples analyzed by gas chromatography. (B) Mass spectra of peaks with retention times of 838 and 833 s in glycogen and mat samples, respectively. TMSi, TMS moiety. The inositol internal standard is indicated (arrows).

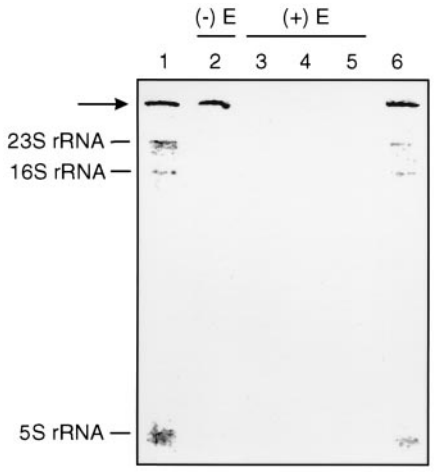

FIG. 4. Autoradiogram of polyacrylamide gel containing enzymatically treated Octopus Spring mat nucleic acid extract after radiolabeling with $^{14}CO_2$ in the light. (−) E, no enzymatic treatment; (+) E, samples treated with polyglucose-specific enzymes as follows: lane 3, α-amylase and amyloglucosidase; lane 4, α-amylase only; and lane 5, amyloglucosidase only. Lanes 1 and 6, radiolabeled *Synechococcus* sp. isolate C1 nucleic acid (no enzymatic treatment). The location of the well of the polyacrylamide gel is indicated (arrow).

Radiolabeled material in the mat nucleic acid extract was labile in the presence of α-amylase and amyloglucosidase, two polyglucose-specific enzymes (Fig. 4). White precipitate observed in untreated controls could not be observed in the enzyme-treated samples after ethanol precipitation, and radiolabel could not be detected in the pellet after centrifugation. Of the original radiolabel added to the enzymatic digest, 87.3% was recovered in the ethanol wash removed after centrifugation.

Protein synthesis. Although diverse proteins were detected in logarithmically growing C1 cultures and cyanobacterial mat material, there is little correspondence between the observed protein sizes (Fig. 5A). While C1 cells actively incorporated $^{14}CO_2$ into protein, we detected only limited protein synthesis in the cyanobacterial mat (Fig. 5B). Instead, radiolabel was detected primarily in the well of the polyacrylamide gel.

Lipid synthesis. Logarithmically growing C1 cultures and cyanobacterial mat material displayed similar lipids (Fig. 6A) and similar patterns of $^{14}CO_2$ incorporation into lipids (Fig. 6B). Aminolipids which were detected in the mat were not detected in C1 cultures (Fig. 6A). Nearly all the lipids which appear in Fig. 6A received radiolabel, including pigments (Fig. 6B).

$^{14}CO_2$ partitioning into cellular components. Various hot spring cyanobacterial mat samples and a logarithmically growing C1 culture were fractionated to quantify patterns of photosynthate partitioning into major molecular classes. Different hot spring cyanobacterial mat communities displayed remarkably similar patterns of photosynthate partitioning (Table 2). When incubated in the light, mat samples partitioned the majority of photosynthetically fixed carbon into the polysaccharide-containing fraction, whereas mat samples incubated in the dark synthesized primarily protein. In contrast, logarithmically growing C1 cultures partitioned photosynthate primarily into protein, and the remaining carbon was approximately equally divided between polysaccharide, lipid, and low-molecular-weight metabolites (Table 2). In an experiment to determine which cellular fraction contained the radiolabeled polyglucose detected in cyanobacterial mat nucleic acid extract, 98.1% ± 1.1% (mean ± SD; $n = 2$) was recovered in the polysaccharide fraction.

Polysaccharide fermentation. Cyanobacterial mat samples were preincubated with $^{14}CO_2$ in the light to accumulate radiolabeled polysaccharide and then shifted to dark anaerobic conditions for up to 24 h. Following incubation, radiolabel in cellular fractions and extracellular fermentation products was then determined. Upon transfer of photoautotrophically grown cells to dark anaerobic conditions, we observed a decrease in ^{14}C-labeled polysaccharide. Simultaneously, [^{14}C]acetate, [^{14}C]propionate, and $^{14}CO_2$ were produced (Fig. 7A). Overall, radiolabel in the polysaccharide fraction decreased 74.7% after 12 h of dark incubation, of which 58.5% was recovered in ^{14}C-labeled fermentation products. Acetate was the most abundant ^{14}C-labeled fermentation product. Radiolabel in the protein fraction remained constant or increased only slightly during dark anaerobic incubation. Addition of BES to inhibit methanogenic hydrogen and CO_2 consumption (2) resulted in a more rapid accumulation of radiolabeled acetate (compare panel B of Fig. 7 with panel A). Radiolabeled polysaccharide in BES-treated samples decreased 80.0% after 12 h, and recovery of radiolabeled fermentation products was higher in BES-treated samples (82.9%) than in unamended samples. Small amounts of $^{14}CH_4$ were detected in unamended samples after 24 h of dark incubation, but none was detected in equivalent BES-treated samples. We observed a linear decrease in the specific activity of acetate from 3 to 24 h of dark incubation (43.9 and 43.1% decreases in unamended and BES-treated samples after 24 h, respectively). The specific activity of propionate did not significantly change over time. Formalin-killed controls displayed no readily identifiable trend (Fig. 7C).

[^{14}C]acetate partitioning into cellular components. The fate of excreted acetate was investigated by fractionating mat ma-

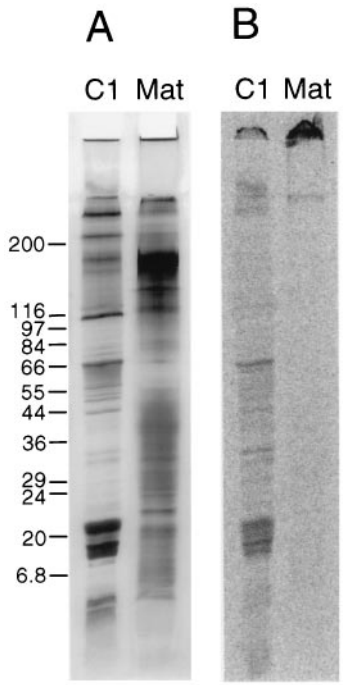

FIG. 5. Composition of proteins solubilized from whole-cell extracts of a logarithmically growing *Synechococcus* sp. isolate C1 culture and Octopus Spring cyanobacterial mat community after radiolabeling with $^{14}CO_2$ in the light. (A) Photographic image of silver-stained proteins separated by denaturing polyacrylamide gel electrophoresis; (B) autoradiogram of the same gel. Molecular mass markers (in kilodaltons) are shown on the left.

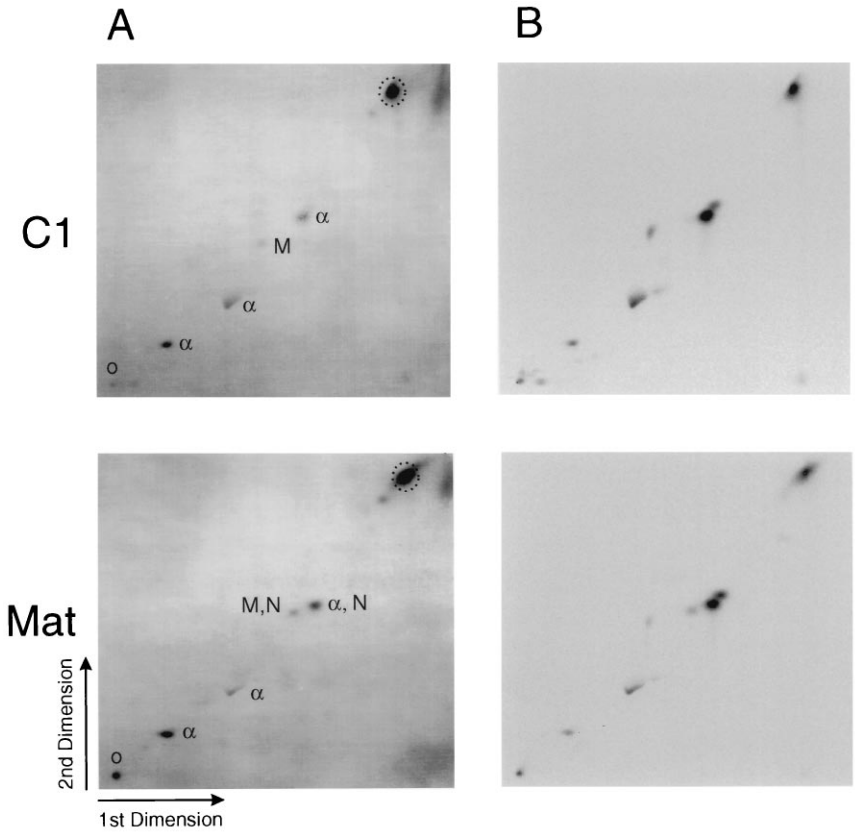

FIG. 6. Composition of lipids extracted from a logarithmically growing *Synechococcus* sp. isolate C1 culture and Octopus Spring cyanobacterial mat community after radiolabeling with $^{14}CO_2$ in the light. (A) Photographic image of sulfuric acid-charred two-dimensional thin-layer chromatography plates; (B) autoradiogram of the same plates. M, molybdenum blue reactive; N, ninhydrin reactive; α, α-naphthol reactive; O, origin. Encircled spots were pigmented green.

terial incubated under light and dark conditions in the presence of [^{14}C]acetate into molecular classes. Dark incorporation of [^{14}C]acetate was 29.9 and 60.5% of light incorporation in Octopus Spring and Clearwater Springs site D mats, respectively. Incorporated [^{14}C]acetate was partitioned primarily into polysaccharide and protein fractions in the light and into protein in the dark (Table 3). rRNA synthesis could not be detected when [^{14}C]acetate was added as a radiolabeled substrate. Instead, labeled material which was resistant to α-amylase and amyloglucosidase enzymatic digestion was detected in the well of a 3.5% polyacrylamide gel (data not shown).

DISCUSSION

Here, we present several lines of evidence which suggest that although the oxygenic photosynthetic cyanobacteria inhabiting hot spring mat communities are very active (49), they do not appear to be rapidly dividing. First, we failed to detect incorporation of $^{14}CO_2$ into rRNA, a sensitive indicator of the rate of cyanobacterial cell division (32, 39). Manipulation of nutrient levels, cell density, and light conditions failed to induce detectable rRNA synthesis from $^{14}CO_2$. Since rRNA synthesis from $^{14}CO_2$ was below detection limits, a high-specific-activity preparation of $^{32}PO_4^{2-}$ was provided as a radiolabeled substrate to increase the sensitivity of detecting synthesis of phosphorus-containing molecules, such as rRNA. Although the observed incorporation of $^{32}PO_4^{2-}$ indicates limited rRNA synthesis, we cannot necessarily associate this synthesis with cyanobacterial growth, since both photoautotrophic and heterotrophic organisms assimilate P_i. Second, we detected only limited synthesis of proteins and lipids in the mat. Instead, mat

TABLE 2. $^{14}CO_2$ partitioning among molecular fractions in logarithmically growing *Synechococcus* sp. isolate C1 cultures and hot spring cyanobacterial mat samples incubated under light and dark conditions

Fraction	% Label in fraction[a]						
	Light				Dark		
	OS	CWS	TBV	C1	OS	CWS	TBV
Polysaccharide	84.6 ± 3.6	77.0 ± 3.8	84.0 ± 2.8	14.9 ± 2.5	5.7 ± 2.8	5.2 ± 4.9	11.3 ± 3.2
Protein	9.3 ± 3.0	11.3 ± 2.7	9.9 ± 3.3	44.8 ± 2.7	88.4 ± 3.2	88.8 ± 9.3	78.8 ± 4.4
Lipid	1.9 ± 0.3	8.3 ± 1.6	2.1 ± 0.3	18.5 ± 3.2	3.1 ± 1.4	2.5 ± 1.8	6.2 ± 1.4
Low-molecular-weight metabolites	4.0 ± 0.5	3.5 ± 0.9	4.5 ± 0.3	21.8 ± 1.9	2.0 ± 0.3	3.4 ± 3.0	3.8 ± 2.5

[a] Percentage of total incorporated radiolabel detected in molecular fraction. Values are means ± 95% confidence intervals (n = 3 for Twin Butte Vista Spring [TBV] and Clearwater Springs site D [CWS]; n = 4 for Octopus Spring [OS] and C1).

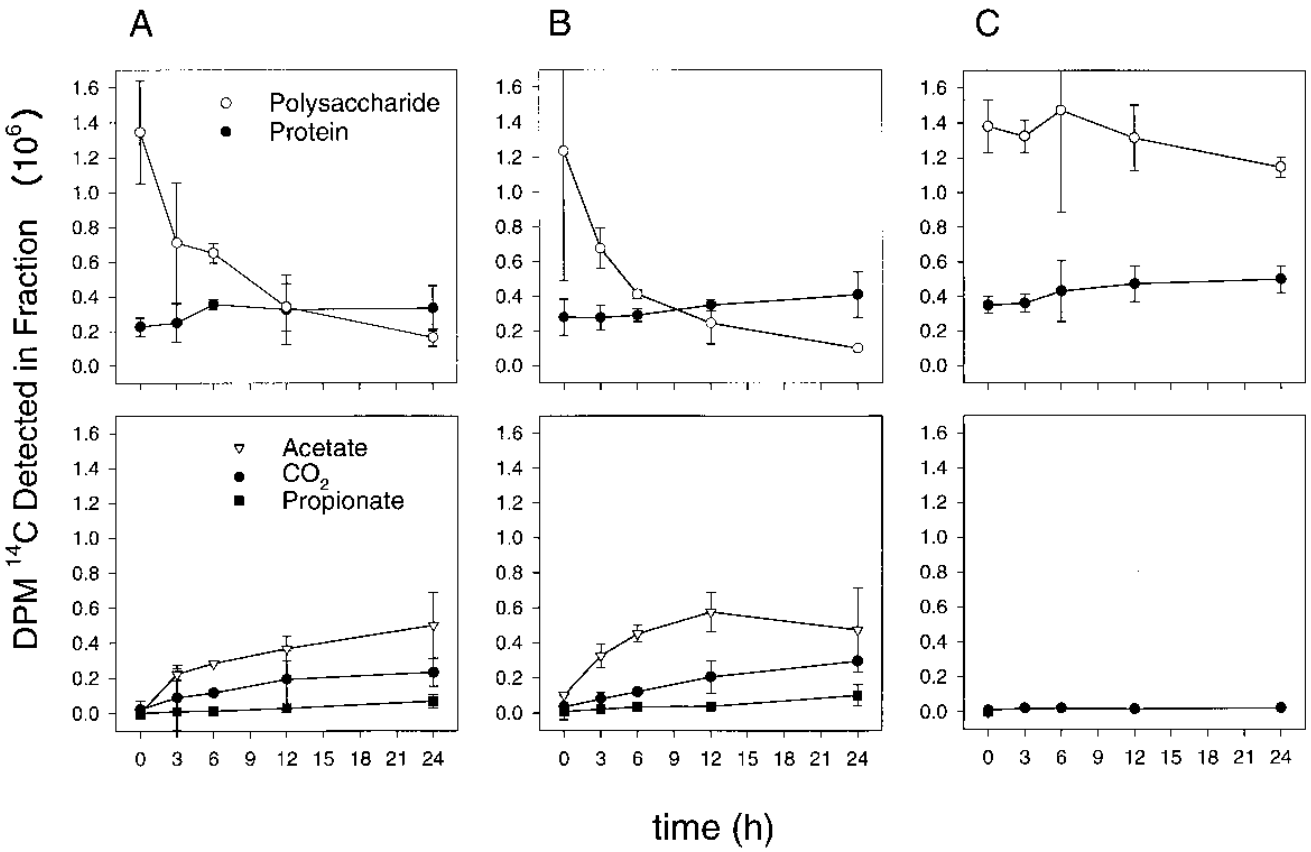

FIG. 7. (A) Changes in ^{14}C detected in polysaccharide and protein cellular fractions (top panel) and acetate, CO_2, and propionate fermentation products (bottom panel) in Clearwater Springs site D mat cores shifted from a 3-h light incubation in the presence of $^{14}CO_2$ to 0-, 3-, 6-, 12-, and 24-h dark anaerobic incubations. (B) Results with BES added to inhibit methanogenesis. (C) Formalin-killed control. Error bars indicate 95% (top panel) and 90% (bottom panel) confidence intervals about the mean ($n = 3$, except for 0-h BES-treated samples [$n = 2$] and 6-h unamended fermentation product values [$n = 1$], so only this value is reported).

samples accumulated primarily polyglucose reserves in the light (77.0 to 84.6% of total incorporated carbon). The extent of polyglucose synthesis measured in this study is similar to a previous determination (82%) (29). In contrast to mat samples, logarithmically growing C1 cultures readily incorporated $^{14}CO_2$ into rRNA, protein, lipid, and low-molecular-weight metabolites.

These results are consistent with the hypothesis that hot spring cyanobacterial mats are active but contradict earlier studies which suggested that mats are rapidly growing. Calculation of cyanobacterial productivity from measured washout rates following darkening (6) requires the assumption that washout rate equals replacement rate. Washout rates may more accurately measure the cyanobacterial death rate after elimination of light as the energy source for oxygenic photosynthesis. Rates of accretion above silicon carbide layers added to hot spring mat surfaces provide compelling evidence for rapid mat growth (13), but these experiments may merely reflect a disturbance artifact. The community above the silicon carbide layer might be in a postdisturbance successional phase which would exhibit a species composition and growth rate different from those of the original community below the layer (see below).

TABLE 3. [^{14}C]acetate partitioning among molecular fractions in Octopus Spring and Clearwater Springs site D cyanobacterial mat samples incubated under light and dark conditions

Fraction	% label in fraction[a]			
	Light		Dark	
	OS	CWS	OS	CWS
Polysaccharide	56.3 ± 2.3	37.2 ± 6.5	9.9 ± 3.5	7.6 ± 1.9
Protein	27.7 ± 3.5	52.2 ± 7.0	61.7 ± 23.1	76.2 ± 3.9
Lipid	12.3 ± 2.8	8.5 ± 2.3	25.7 ± 17.0	12.1 ± 4.4
Low-molecular-weight metabolites	3.7 ± 0.7	2.1 ± 0.4	2.6 ± 2.8	4.1 ± 1.1

[a] Percentage of total incorporated radiolabel detected in molecular fraction. Values are means ± 95% confidence intervals ($n = 3$ for Octopus Spring [OS] and $n = 4$ for Clearwater Springs site D [CWS]).

Studies of elemental composition, carbon partitioning, and molecular synthesis in natural phytoplankton populations have shown that phytoplankton are photosynthetically active and often exhibit growth at or near maximal rates (22, 23, 33, 42, 44). Phytoplankton populations are also subject to disturbance (i.e., removal of biomass [24]) by zooplankton grazing, which may force phytoplankton populations to grow and divide to replace lost biomass (e.g., see references 7, 40, and 56). In contrast, hot spring cyanobacterial mats inhabit high-temperature environments that are devoid of eukaryotic grazers and which allow microbial mats to accumulate without grazing disturbance (6). The low disturbance potential (59) which characterizes hot spring environments may be one reason we observe limited synthesis of growth-related molecules in mature

mat communities. In support of this hypothesis, we observed changes in cyanobacterial populations and patterns of carbon partitioning after disturbing the photosynthetically active layer of the Octopus Spring cyanobacterial mat. Five days after removing the green surface layer, we detected a pattern of photosynthate partitioning similar to that of logarithmically growing C1 cultures (i.e., protein and low-molecular-weight metabolite synthesis). After 21 days, the pattern of photosynthate partitioning had changed to more closely resemble that of undisturbed regions of the mat (i.e., primarily polysaccharide synthesis) (17).

The physiological state of hot spring cyanobacteria characterized by high rates of activity combined with relatively slow growth may occur in other microbial communities. For example, bacteroid-forming root nodule symbionts (i.e., *Rhizobium* spp.) are also found at high cell densities and actively fix elemental nitrogen but do not rapidly grow (67). The bioluminescent fish and squid light-organ symbiont *Vibrio fischeri* actively produces light but does not rapidly divide under the high-cell-density conditions which characterize these habitats (14, 51). Perhaps other microbial communities characterized by high activity and high cell densities (e.g., temperate cyanobacterial mats and biofilms) may also exhibit slow growth.

The decrease in photosynthetically fixed radiolabeled polyglucose and the corresponding increase in radiolabeled fermentation products suggest that *Synechococcus* spp. switch to a fermentative metabolism under dark anaerobic conditions. Interestingly, we detected no significant increase of radiolabel in the protein fraction during dark incubation. Other studies investigating photosynthate partitioning in phytoplankton and microbial mat communities have shown movement of radiolabel from storage products to protein at night (9, 22, 29, 37), but these experiments were performed with low-cell-density cell suspensions incubated under aerobic conditions. Our observation that protein synthesis does not readily occur even after an extended dark anaerobic incubation supports the conclusion that native mat *Synechococcus* populations are not rapidly growing.

The measured accumulation of fermentation products under dark anaerobic conditions closely parallels results obtained by Anderson et al. (2), who showed that acetate and propionate were the major fermentation products to accumulate overnight. The populations which perform fermentative metabolisms have, until now, not been identified, although numerous fermentative bacteria have been isolated from hot spring mats (e.g., see references 66, 68, and 69). By detecting the accumulation of radiolabeled fermentation products from radiolabeled polyglucose, we have identified cyanobacterial populations as contributors to the pool of fermentation products.

Cyanobacterial fermentation may not be the only anaerobic process contributing to acetate pools, since other acetate-producing processes such as acetogenesis and noncyanobacterial fermentation may be occurring simultaneously. Acetogenic bacteria (2) and the previously discussed fermentative bacteria have been cultivated from hot spring cyanobacterial mats, and acetogenic conversion of CO_2 and H_2 to acetate was suspected to occur in these habitats under dark anaerobic conditions (3). Here, we present evidence in support of the hypothesis that acetate production occurs by both cyanobacterial fermentation and other acetate-producing processes. If cyanobacterial fermentation was the sole source of anaerobic acetate production, acetate specific activity would remain constant over time. However, acetate specific activity decreased over time, indicating that unlabeled acetate accumulated concurrently with radiolabeled acetate. In addition, acetate accumulated more quickly in BES-treated samples than in unamended samples, possibly because of removal of competition between methanogens and acetogens for hydrogen, leading to increased acetate production. Finally, we detected greater recovery of fermentation products in BES-treated samples (82.9% recovery versus 58.5% in unamended samples), possibly because of $^{14}CO_2$ conversion to [^{14}C]acetate rather than to $^{14}CH_4$, a product which was not quantified. The lower recovery of fermentation products in unamended samples may result in an underestimation of the extent of cyanobacterial fermentation. Thus, cyanobacterial fermentation and other forms of acetate production must occur simultaneously. However, cyanobacteria must play an important role in acetate production, as acetate specific activity was diluted only ca. 43% during a 24-h dark incubation. Combined with the observation that acetoclastic methanogenesis does not occur in the Octopus Spring mat (54), these mat communities appear to be physiologically poised to produce and accumulate acetate under dark anaerobic conditions (2).

By combining the results from this study with those of previous physiological studies, it is possible to construct a plausible description of carbon flow through hot spring cyanobacterial mat communities. During the day, carbon is photoautotrophically incorporated by *Synechococcus* spp. and is mainly stored as polyglucose. Fixed carbon is also photoexcreted as glycolate, which is readily incorporated by *Chloroflexus*-like filaments (3). At night, *Synechococcus* spp. respond to anaerobic conditions by fermenting the majority of stored polyglucose, thereby producing acetate and propionate, most of which accumulates overnight (2). CO_2 produced from polyglucose may be further converted to acetate by acetogenic bacteria (2) or lost from the system as methane (54). CO_2 incorporated in the dark is synthesized primarily into protein, which could be either the product of dark cyanobacterial incorporation (57) or the product of carbon assimilation by acetogenic or methanogenic bacteria. The next day, *Chloroflexus*-like filamentous organisms photoheterotrophically incorporate accumulated fermentation products (2, 54) and synthesize primarily protein and polysaccharide but not rRNA. This scenario describes significant transfer of carbon from the primary producers (*Synechococcus* spp.) to the filamentous heterotrophic *Chloroflexus*-like primary consumers throughout the diel cycle. The extent of carbon transfer is nearly complete, leaving little carbon for *Synechococcus* cell replication. The basis for this symbiotic association is not understood.

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