Nitrogen fixation by *Oscillatoria* spp. under autotrophic and photoheterotrophic conditions

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Oscillatoria spp. UCSB8 and UCSB25 are both capable of aerobic N₂ fixation. The optimum temperature for C₂H₂ reduction was 22 °C for *Oscillatoria* sp. UCSB8 and 35 °C for *Oscillatoria* sp. UCSB25, whilst the optimum temperature for growth on N₂ was 25 °C and 30 °C, respectively. In *Oscillatoria* sp. UCSB25, but not in UCSB8, inhibition of N₂ fixation may limit diazotrophic growth at temperatures above 35 °C. When grown under alternating 12 h light and 12 h darkness, both isolates reduced C₂H₂ predominantly in the dark and both were capable of N₂ fixation and photoheterotrophic growth in the presence of 20 µM-DCMU to inhibit photosystem II activity. Under these conditions, the best exogenous carbon source for *Oscillatoria* sp. UCSB8 was glucose, whilst that for *Oscillatoria* sp. UCSB25 was fructose. In *Oscillatoria* sp. UCSB8, exogenous glucose was catabolized mainly through the oxidative pentose phosphate pathway. Although cultures grown photoheterotrophically showed higher specific activities of nitrogenase than photoautotrophic cultures, they grew more slowly. Furthermore, cultures grown photoheterotrophically under alternating light and darkness reduced C₂H₂ both in the light and in the dark, but the highest rates of C₂H₂ reduction were observed in the dark. This cyclic pattern of N₂ fixation was independent of photosystem II activity.

Introduction

Among cyanobacteria, aerobic N₂ fixation is relatively rare except in those genera that produce heterocysts (Haselkorn, 1978; Wolk, 1982; Neuer *et al.*, 1983). Non-heterocystous cyanobacteria that fix N₂ aerobically fall into two groups: unicellular organisms such as *Gloeotheca* (Wyatt & Silvey, 1969; Gallon *et al.*, 1988; Gallon & Chaplin, 1988; Huang & Chow, 1988; Gallon, 1989) and *Synechococcus* (Grobbelaar *et al.*, 1986, 1987; Huang & Chow, 1988; Mitsui *et al.*, 1986, 1987; Spiller & Shanmugan, 1987), and filamentous cyanobacteria such as *Microcoleus chthonoplastes* (Pearson *et al.*, 1979; Malin & Pearson, 1988) and *Oscillatoria* (Stal & Krumbein, 1981, 1985a, b; Khamees *et al.*, 1987). In virtually all diazotrophic non-heterocystous cyanobacteria, the O₂-sensitive nitrogenase complex seems to be located within the vegetative cell, together with the O₂-evolving photosynthetic system. A possible exception to this is *Trichodesmium* (*Oscillatoria*) *erythraea*, in which N₂ fixation may be confined to lightly pigmented cells located in the central region of bundles of individual

filaments (Carpenter & Price, 1976; Bryceson & Fay, 1981).

In all non-heterocystous cyanobacteria so far examined, except *Trichodesmium* (Bryceson & Fay, 1981), N₂ fixation occurred predominantly during the dark phase of an alternating cycle of light and darkness, thereby effecting a temporal separation between N₂ fixation and oxygenic photosynthesis (Khamees *et al.*, 1987). Under these conditions, ATP and reducing power for N₂ fixation must be generated by dark metabolism. In *Gloeotheca*, the ATP for N₂ fixation may be provided by respiration rather than photosynthesis even under continuous illumination (Maryan *et al.*, 1986). However, it is not clear whether this is the case in other non-heterocystous cyanobacteria. Furthermore, since *Gloeotheca* does not grow on exogenously supplied sugars (Rippka *et al.*, 1979) it has been impossible to examine the supply of energy and reductant to nitrogenase in the total, rather than temporal, absence of oxygenic photosynthesis. On the other hand, many *Oscillatoria* spp. can grow heterotrophically in the light when incubated with an inhibitor of photosynthesis and an appropriate sugar (Rippka *et al.*, 1979) and, if capable of aerobic N₂ fixation, should be more suitable organisms for such studies. In this paper, we describe some results on N₂

Abbreviation: DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea.

fixation by two facultatively heterotrophic *Oscillatoria* spp. grown under photoautotrophic and photoheterotrophic conditions.

Methods

Isolation and growth of organisms. *Oscillatoria* sp. UCSB8 (University College of Swansea, Biochemistry Culture Collection) was isolated from Landimore Marsh, North Gower, close to Swansea (Griffiths *et al.*, 1987). It was rendered unicyanobacterial by successive plating of gently homogenized cultures on the medium described by Khamees *et al.* (1987), solidified with 1% (w/v) Difco Bacto agar, followed by isolation of individual filaments. Homogenization was performed in a sterile Potter-Elvehjem glass homogenizer and served to disrupt cyanobacterial clumps into individual, undamaged trichomes. Unicyanobacterial cultures were treated with cycloheximide (0.1 mg ml⁻¹) to eliminate contaminating eukaryotes and were then freed from bacterial contamination on scored agar plates as described by Vaara *et al.* (1979). Cultures were routinely checked for contamination by microscopic examination of old cultures and by streaking on nutrient agar (Rippka *et al.*, 1979). Large-scale cultivation of strain *Oscillatoria* sp. UCSB8 was performed at 22 °C in 15 l of the same medium as used for isolation, which is free of combined nitrogen and has an overall salinity approximately 50% that of seawater (Khamees *et al.*, 1987). The light regime for growth was either continuous illumination or 12 h light/12 h dark, with illumination (25 µmol photons m⁻² s⁻¹) provided by Osram white fluorescent lights. All cultures were gassed continuously with air at 4.5 l min⁻¹. Alternatively, for growth experiments (see Table 4), cultures (100 ml) were incubated in 250 ml Erlenmeyer flasks on an orbital shaker at 120 oscillations min⁻¹. Microscopic examination of *Oscillatoria* sp. UCSB8 revealed filaments of diameter 12 µm, composed of disc-shaped cells, 1–2 µm long.

Oscillatoria sp. UCSB25 was isolated from mangrove roots adjacent to Tickle Belly Creek, near Townsville, Queensland, Australia. Isolation was performed as described above, except that the medium contained (mmol l⁻¹): NaCl, 320; MgCl₂, 18.7; KCl, 6.7; CaCl₂, 5.9; NaHCO₃, 1.8; Na₂SO₄, 0.9; KBr, 0.6; H₃BO₃, 0.37; Na₂CO₃, 0.18; MgSO₄, 0.15; K₂HPO₄, 0.088; SrCl₂, 0.067; NaF, 0.048; ferric citrate, 0.01; MnCl₂, 0.009; Na₂MoO₄, 0.0016; MgNa₂EDTA, 0.0012; ZnSO₄, 0.0008; CuSO₄, 0.0003; CoCl₂, 0.0002. The pH was 8.0 and the salinity was approximately 75% that of seawater. For experimental purposes the culture volumes, light regime and air supply were identical to those described for *Oscillatoria* sp. UCSB8 (see above), but the temperature was 30 °C. Filaments of *Oscillatoria* sp. UCSB25 are composed of disc-shaped cells, approximately 3 µm in width and 0.3 µm in length.

For growth on combined nitrogen, NaNO₃ (8 mM) was added to the media. For photoheterotrophic growth, the media were supplemented with 20 µM-DCMU. After autoclaving, filter-sterilized D-glucose, D-fructose or D-sucrose was added to a final concentration of 20 mM.

Immobilization of *Oscillatoria* spp. Immobilization of cultures of *Oscillatoria* in 0.5 cm diameter beads of calcium alginate was performed as described by Gallon *et al.* (1988). In order to avoid interference due to high salinity, cultures were suspended in nitrogen-free ASM-1 medium (Gallon *et al.*, 1978) at a cell density equivalent to 0.5 mg protein ml⁻¹ and gently homogenized prior to immobilization. Immobilized cultures were then transferred to a bioreactor (Musgrave *et al.*, 1982) containing 400 ml of appropriate growth medium and aerated at 0.1 l min⁻¹. Alternatively, homogenized cultures were immobilized at approximately 0.1 mg protein per cube in 0.125 cm³ cubes of polyvinyl foam, preincubated in appropriate growth medium (Gallon *et al.*, 1988).

Photosynthetic and respiratory O₂ exchange. O₂ exchange was measured polarographically at 22 °C (*Oscillatoria* sp. UCSB8) or 30 °C

(*Oscillatoria* sp. UCSB25), in the light (25 µmol photons m⁻² s⁻¹) for net O₂ production or in the dark for O₂ consumption (Gallon & Hamadi, 1984). In order to prevent cyanobacterial clumps from settling and interfering with efficient stirring during polarography, cultures were homogenized and immobilized in polyvinyl foam (see above) prior to transfer of five cubes to the O₂ electrode vessel.

Evolution of ¹⁴CO₂. Cultures of *Oscillatoria* sp. UCSB8 (100 ml) were transferred, 21–25 d after inoculation, to sterile Erlenmeyer flasks (250 ml) which were then fitted with seals equipped with gas inlets and outlets. Prior to use, photoheterotrophically grown cultures were washed three times with fresh medium containing 20 µM-DCMU but no carbon source, and finally resuspended in 100 ml of latter medium. By injection through the flask seal, 0.37 MBq of either D-[1-¹⁴C]glucose (2 TBq mol⁻¹; Amersham) or D-[6-¹⁴C]glucose (1.9 TBq mol⁻¹; New England Nuclear) was added to each culture. Each flask was then connected, in a fume cupboard, to a water-generated vacuum line such that the culture received a steady flow of air from the atmosphere at a rate of 50 ml min⁻¹. For autotrophic cultures incubated with [¹⁴C]glucose in the light in the absence of DCMU, the air was supplemented with 0.01 atm CO₂ in order to minimize refixation of ¹⁴CO₂. Before reaching the culture vessel, incoming air had been bubbled through a flask containing sterile distilled water, and after leaving the culture, the air passed through an ice trap and into a scintillation vial containing a mixture of 2 ml ethanolamine and 8 ml methanol. This mixture absorbed any ¹⁴CO₂ produced from the cyanobacterial culture. The air supply then passed through 0.2 M-KOH as a safety precaution before reaching the vacuum pump. During incubation (22 °C), the cultures were gently stirred either in the dark or in the light (25 µmol photons m⁻² s⁻¹). On each occasion, a culture was incubated with [1-¹⁴C]glucose in parallel with an identical culture incubated with [6-¹⁴C]glucose. No ¹⁴CO₂ was evolved from boiled cultures incubated with [¹⁴C]glucose.

After a suitable incubation period, air flow was temporarily interrupted. The scintillation vial was then removed and replaced by a fresh vial containing absorbant. The radioactivity in the removed vial was determined by liquid scintillation counting following addition of 10 ml Emulsifier Scintillant 299 (Packard Instrument International).

Enzyme assays. Nitrogenase (EC 1.18.6.1) activity was measured by the C₂H₂ reduction technique (Tözüm *et al.*, 1977). For some experiments (see Tables 2 and 3) aggregates of filaments were disrupted by gentle homogenization in a Potter-Elvehjem homogenizer to produce a uniform cell suspension prior to transfer to replicate assay flasks. To allow recovery of cultures from this treatment, assay flasks containing 50 ml culture (about 2.5 mg protein) were incubated for 48 h on an orbital shaker (120 oscillations min⁻¹) under identical conditions of temperature and illumination to those described for growth (see above). C₂H₂ was then added to a final concentration of 10% (v/v) and C₂H₄ was monitored at hourly intervals. Nitrogenase activity in immobilized cultures was measured using 20 beads of calcium alginate (equivalent to about 1 mg protein) suspended in 50 ml fresh growth medium.

For experiments involving regular sampling of cultures over a period of up to 144 h (see Figs. 1 and 2), an alternative approach was adopted. The entire cultures were subjected to gentle homogenization 48 h prior to the start of the experiments and the uniform cell suspensions returned to the culture vessels. Although reaggregation of filaments occurred during the recovery period, no large clumps were formed. In order to take into account any variation in the number of cells present in individual samples removed from these cultures for experimental purposes, the protein content of each sample was measured.

Unless stated otherwise, *Oscillatoria* sp. UCSB8 was incubated at 22 °C and *Oscillatoria* sp. UCSB25 at 30 °C. For kinetic determinations, C₂H₂ was added at concentrations ranging between 0.0025 and

0.1 atm. In both strains of *Oscillatoria*, the apparent K_m for C₂H₂ was between 0.008 and 0.01 atm.

The NADP⁺-dependent glucose-6-phosphate dehydrogenase (EC 1.1.1.49) of *Oscillatoria* UCSB8 was assayed spectrophotometrically at 340 nm, using cell-free extracts prepared as described by Tözüm & Gallon (1979). The reaction mixture (2 ml) contained 20 mM-HEPES/NaOH buffer, pH 7.5, 2 mM-MgCl₂, 0.1 mM-NADP⁺ and 0.5 ml extract (equivalent to 1 mg protein). The reaction was started by addition of 10 µmol glucose 6-phosphate, sodium salt. Incubation was at 25 °C. The absorption change was corrected for that observed in the absence of glucose 6-phosphate, and for that obtained with boiled extracts.

Photosynthetic pigments. Photosynthetic pigments were estimated as described by Bennett & Bogorad (1973).

Other measurements. Growth of cultures of *Oscillatoria* was estimated by measurement of their optical density at 436 nm (Fay *et al.*, 1976), after homogenization (see above) to disrupt aggregated filaments. To avoid interference caused by changes in the intracellular concentration of photosynthetic pigments during growth of cultures, measurements were also made at 900 nm. The doubling times calculated by measurements at 436 nm differed from those based on measurements at 900 nm by 7 ± 1% (SEM; 135 determinations).

Dry weights were determined by filtration of cultures through a 0.2 µm membrane (Millipore) followed by drying at 65 °C until constant weight was achieved.

Protein was measured as described by Bailey (1962). In the case of cultures immobilized in polyvinyl foam, protein was first extracted at 55 °C with 1 ml per cube of 0.19 M-Na₂CO₃ in 0.1 M-NaOH, as described by Price (1965). Blocks of foam containing no *Oscillatoria* served as reference samples.

Results and Discussion

As might be expected from the different temperature ranges encountered in their natural habitats, *Oscillatoria* sp. UCSB8, an isolate from a temperate region, reduced C₂H₂ maximally at 22 °C whereas the tropical strain, *Oscillatoria* sp. UCSB25, showed optimum C₂H₂ reduction at 35 °C (data not shown). Incubation at 15 °C decreased maximal activity by about 50% in *Oscillatoria* sp. UCSB8 whilst a much more pronounced inhibition (93%) was observed at this temperature in the tropical strain.

Optimum growth of *Oscillatoria* sp. UCSB8 on N₂ occurred at 25 °C whereas optimal growth rates for *Oscillatoria* sp. UCSB25 under these conditions were obtained at 30 °C (Table 1). The temperature optima for growth on N₂ of the two strains were therefore close to their optima for C₂H₂ reduction (see above). However, the two strains differed considerably in their response to temperature in the presence and absence of combined nitrogen (Table 1). The growth rates of *Oscillatoria* sp. UCSB8 were relatively similar under N₂-fixing conditions and in NO₃⁻-containing medium at 22–30 °C. Above 30 °C, growth in the presence of NO₃⁻ was completely arrested, whereas some residual growth was observed up to 40 °C with N₂ as the source of nitrogen. In

Table 1. Growth of *Oscillatoria* spp. UCSB8 and UCSB25 at various temperatures

Replicate cultures, inoculated with a homogeneous cell suspension, were incubated in medium free of combined nitrogen or in medium containing NaNO₃. At regular intervals during growth under continuous illumination, one of the replicate cultures was subjected to gentle homogenization to disrupt aggregates of filaments and its optical density measured at 436 and 900 nm. The doubling times shown are based on measurements at 436 nm and are the means of at least four separate experiments (±SD).

Growth temp. (°C)	Nitrogen source . . .	Doubling time (h)			
		UCSB8		UCSB25	
		N ₂	NO ₃ ⁻	N ₂	NO ₃ ⁻
15		283 ± 11	ND	1312 ± 46	ND
20		255 ± 9	ND	715 ± 28	ND
22		234 ± 8	202 ± 10	ND	ND
25		201 ± 6	197 ± 5	211 ± 7	232 ± 7
30		224 ± 7	200 ± 5	184 ± 6	197 ± 6
35		498 ± 25	∞	203 ± 8	165 ± 4
40		784 ± 16	∞	400 ± 16	160 ± 5

∞, No growth; ND, not determined.

contrast, growth of *Oscillatoria* sp. UCSB25 was similar on N₂ and NO₃⁻ at 25–30 °C, but in the range of 35–40 °C there was a severe reduction of growth under N₂-fixing conditions, whilst the presence of NO₃⁻ in the medium stimulated growth at these temperatures. These results suggest that elevated temperatures (30–40 °C) affect not only N₂ fixation but also general metabolism and cell function in *Oscillatoria* sp. UCSB8. In *Oscillatoria* sp. UCSB25, however, growth limitation under N₂-fixing conditions above 35 °C may result from a specific inhibition of N₂ fixation.

Both species of *Oscillatoria* formed large aggregates of filaments during growth in liquid culture. Disruption of these aggregates by gentle homogenization did not destroy individual filaments, as judged by microscopic examination, but N₂-fixing cultures treated in this way lost the ability to reduce C₂H₂. In *Oscillatoria* sp. UCSB8, this ability recovered during aerobic incubation more rapidly in standing cultures than in shaken cultures (Table 2). In each case, recovery coincided with reformation of aggregates. During microaerobic incubation under N₂/CO₂ (99:97:0.03, v/v) the lag period prior to recovery of C₂H₂ reduction was similar to that observed in aerobic cultures (Table 2), implying that aggregate formation did not provide O₂-depleted micro-zones for optimum aerobic nitrogenase activity as may occur in other cyanobacteria (Paerl & Carlton, 1988). Furthermore, immobilization of individual filaments of *Oscillatoria* sp. UCSB8 in beads of calcium alginate completely prevented any reaggregation, but the ability

Table 2. Recovery of C_2H_2 reduction by cultures of *Oscillatoria* sp. UCSB8 under different incubation conditions

Cells were collected from a batch culture, incubated under constant illumination, during the late exponential phase of growth (about 28 d after inoculation). Cultures designated as 'standing' or 'shaken' were gently homogenized to disrupt aggregates of filaments, after which replicate cultures were allowed to stand, or were shaken on an orbital shaker at 120 oscillations min^{-1} , under constant illumination. The gas phase was either air or N_2/CO_2 (99.97:0.03, v/v). Immobilized cultures were generated by entrapping homogenized filaments in beads of calcium alginate (see Methods). At hourly intervals thereafter C_2H_2 reduction was measured in one of the replicate cultures. The lag periods quoted are those that preceded recovery and during which C_2H_2 reduction remained at less than $0.2 \text{ nmol min}^{-1} (\text{mg protein}^{-1})$. The values are the means of at least four separate experiments ($\pm$ SD).

Incubation conditions	Gas phase	Lag period (h)	
		Air	N_2/CO_2
Standing cultures		14 ± 4	11 ± 3
Shaken cultures		34 ± 2	32 ± 4
Standing immobilized cultures		14 ± 3	ND

ND, Not determined.

to reduce C_2H_2 aerobically recovered as rapidly as in non-shaken unimmobilized cultures (Table 2). However, it may be argued that immobilization might affect parameters such as gas exchange or the level of illumination at filaments in the centre of each calcium alginate bead in a manner identical to that caused by aggregation. It therefore remains unclear whether aggregate formation is essential for aerobic N_2 fixation by *Oscillatoria* spp., as seems to be the case in *Trichodesmium erythraea* (Bryceson & Fay, 1981) but not in *Trichodesmium* sp. NIBB 1067 (Ohki & Fujita, 1988). Nevertheless, it is possible that, as in *Microcoleus chthonoplastes* (Malin & Pearson, 1988), aggregate formation in *Oscillatoria* spp. UCSB8 and UCSB25 is merely a typical property of filamentous cyanobacteria from benthic habitats whose beneficial effect is not linked to N_2 fixation. Aggregation in such strains may, for example, limit desiccation during periods when the organisms are not submerged.

C_2H_2 reduction by *Oscillatoria* sp. UCSB8, grown photoautotrophically and assayed in the light, was stimulated (Table 3) in the presence of DCMU, an inhibitor of non-cyclic photosynthetic electron transport and O_2 evolution. This is in agreement with the results of Stal & Krumbein (1985b) for *Oscillatoria* strain 23, but contrasts with the observations on *Gloeotheca* (Gallon, 1980) and *Synechococcus* (Grobbelaar *et al.*, 1987), in which DCMU did not significantly alter nitrogenase activity. The stimulation of C_2H_2 reduction by DCMU

Table 3. Effect of DCMU and glucose on C_2H_2 reduction by *Oscillatoria* sp. UCSB8

Cells were collected from a batch culture, incubated under constant illumination, during the late exponential phase of growth (about 28 d after inoculation). Aggregates of filaments were disrupted by gentle homogenization and 50 ml of the homogeneous cell suspension was then transferred to each assay flask. After 48 h under continuous illumination to allow recovery from this treatment, additions were made as indicated and after further incubation for the period shown, C_2H_2 was added to 10% (v/v) and the rate of C_2H_2 reduction was measured over the following 60 min. The rates of C_2H_2 reduction are the means of at least six observations; in parentheses these rates are expressed as percentages of the control culture without any addition.

Addition	C_2H_2 reduction ($\text{nmol min}^{-1} (\text{mg protein}^{-1})$) after incubation for:	
	0 h	3 h
None	2.56 (100)	3.78 (100)
20 μM -DCMU	7.73 (302)	15.38 (407)
20 μM -DCMU + 20 mM-glucose	7.99 (312)	19.50 (516)
20 mM-glucose	2.59 (101)	6.12 (162)

may be a consequence of the inhibition of photosystem II activity, thereby decreasing the concentration of dissolved O_2 in the vicinity of nitrogenase. If so, it would imply that, even in aggregates of *Oscillatoria* UCSB8, the protection of nitrogenase against O_2 is not optimal and that photosynthetically generated O_2 may inhibit N_2 fixation substantially. Addition of 20 mM-glucose enhanced C_2H_2 reduction in both the presence and absence of DCMU (Table 3), though only after a lag period, suggesting that this exogenously supplied carbohydrate might support N_2 fixation. That this is the case was demonstrated by growth experiments (Table 4). *Oscillatoria* sp. UCSB8 grew photoheterotrophically under N_2 -fixing conditions on glucose, fructose or sucrose, though not on ribose or glycerol. Most rapid photoheterotrophic growth was found with glucose as carbon source, but growth was always slower than under photoautotrophic conditions. *Oscillatoria* sp. UCSB25 also grew photoheterotrophically in the absence of combined nitrogen (Table 4), but fructose was the preferred carbon source. Furthermore, the presence of an appropriate carbon source stimulated growth in the light, and also enabled both species of *Oscillatoria* to grow diazotrophically in the dark, though only very slowly.

When grown photoautotrophically under alternating 12 h light and 12 h darkness, *Oscillatoria* spp. UCSB8 (Fig. 1a) and UCSB25 (data not shown) fixed N_2 predominantly during the period of darkness, with peak activities occurring 6–8 h after transfer to the dark. In this respect they resemble most other non-heterocystous cyanobacteria (see Introduction for references). Micro-

Table 4. Growth of *Oscillatoria* spp. UCSB8 and UCSB25 under photoautotrophic, photoheterotrophic and chemoheterotrophic conditions

Replicate cultures, inoculated with a homogeneous cell suspension, were incubated at 22 °C (UCSB8) or 30 °C (UCSB25) in continuous light, either with no additions or with 20 µM-DCMU (which completely inhibited photosynthetic O₂ evolution), or in the dark. Where indicated, an organic carbon source was also present, at a concentration of 20 mM. At regular intervals during growth, one of the replicate cultures was subjected to gentle homogenization to disrupt aggregates of filaments, and its optical density measured at 436 nm and 900 nm. The doubling times shown are based on measurements at 436 nm and are the means of at least four separate experiments (±SD).

Strain	Carbon source	Doubling time (h)		
		Light	Incubation conditions: Light + DCMU	Dark
UCSB8	None	234 ± 8	∞	∞
	Glucose	180 ± 8	330 ± 10	1363 ± 55
	Fructose	210 ± 14	418 ± 16	∞
	Sucrose	249 ± 10	485 ± 11	2400 ± 105
UCSB25	None	184 ± 11	∞	∞
	Glucose	146 ± 5	238 ± 12	∞
	Fructose	141 ± 5	178 ± 8	614 ± 13
	Sucrose	153 ± 6	258 ± 5	1032 ± 38

∞, No growth.

scopic examination of *Oscillatoria* spp. UCSB8 and UCSB25 failed to identify any obviously differentiated cells, and it is possible that all cyanobacteria that fix N₂ predominantly in the dark phase of an alternating cycle of light and darkness lack any spatial separation between N₂ fixation and photosynthesis.

Photoheterotrophic cultures of *Oscillatoria* UCSB8 grown in an alternating cycle of 12 h light and 12 h darkness also reduced C₂H₂ predominantly in the dark but, unlike photoautotrophic cultures, showed significant, though decreasing with time, nitrogenase activity during the light phase (Fig. 1b). Furthermore, the maximum specific activities of C₂H₂ reduction during the dark phase of photoheterotrophic cultures were about 10-fold greater than those of photoautotrophic cultures during the dark cycle (Fig. 1a), which may reflect the efficient use of exogenous glucose for N₂ fixation. The persistence of significant levels of nitrogenase activity during the light phase of photoheterotrophic cultures (equivalent to those observed in photoautotrophic cultures during the dark phase) can be explained by the effect of DCMU on photosystem II activity, thus providing protection of nitrogenase against photosynthetically generated O₂ (see above). However, the decrease of nitrogenase activities during the last few hours of the dark cycle and over the 12 h light period (Fig. 1b) is somewhat surprising, but it is clear that the cyclic pattern

Fig. 1. C₂H₂ reduction by cultures of *Oscillatoria* sp. UCSB8 growing under alternating 12 h light and 12 h darkness (a) photoautotrophically and (b) photoheterotrophically in the presence of 20 µM-DCMU and 20 mM-glucose. The data shown are from a single experiment but the observed fluctuations are typical (at least eight separate observations) of cultures grown under alternating light and darkness.

of nitrogenase activity imposed by this illumination regime is independent of photosystem II activity, which under these growth conditions is blocked by DCMU. In *Gloeotheca*, the pattern of nitrogenase activity observed in photoautotrophic cultures under alternating light and darkness is achieved by regulation of nitrogenase synthesis (Gallon *et al.*, 1988). This is dependent upon ATP, generated in the dark from breakdown of photosynthetically derived glucan, which may become limiting during the latter half of the dark phase. However, in photoheterotrophically grown cultures of *Oscillatoria* sp. UCSB8, exogenous glucose is available at all times during the light-dark cycle and therefore a limitation of carbon and energy is not to be expected during the dark phase and certainly cannot account for the decline of nitrogenase activity during the light period.

Under constant illumination, C₂H₂ reduction by *Oscillatoria* sp. UCSB8 showed peaks and troughs with successive troughs separated by about 120 h (Fig. 2a). These troughs coincided with periods of elevated net O₂ evolution in the light (Fig. 2b) and with periods of relatively low rates of O₂ consumption in the dark (Fig.

Fig. 2. C_2H_2 reduction (a), net O_2 evolution measured in the light (b), and O_2 consumption in the dark (c) in cultures of *Oscillatoria* sp. UCSB8 incubated under continuous illumination. The data shown are from a single experiment but the observed fluctuations are typical (four separate observations) of cultures grown under constant illumination.

2c). The relatively large fluctuations between some successive measurements resulted from the aggregation of the filaments during the course of the experiments, rendering homogeneous sampling for the assays difficult. Nevertheless, over and above these short-term fluctuations, there were distinct global changes in the rates of C_2H_2 reduction and O_2 exchange during photoautotrophic growth of *Oscillatoria* sp. UCSB8 under continuous illumination. The interval (about 120 h) that separated successive troughs in nitrogenase activity (Fig. 2a) is equivalent to about 50% of the doubling time of *Oscillatoria* sp. UCSB8 under these conditions (Table 4). This observation is in agreement with previous reports on the periodicity of nitrogenase activity under continuous illumination for *Gloeotheca* (generation time 72–80 h, successive peaks or troughs of nitrogenase activity 30–40 h: Mullineaux *et al.*, 1981) and for *Oscillatoria* sp. strain 23 (generation time 60 h, intervals between peaks of nitrogenase activity about 24 h: Stal & Krumbein, 1985a).

In photoheterotrophically grown cultures of *Oscillatoria* sp. UCSB8, the initial rate of $^{14}CO_2$ production was six- to seven-fold greater from $[1-^{14}C]$ glucose than from $[6-^{14}C]$ glucose (Table 5). This is consistent with the operation of the oxidative pentose phosphate pathway for glucose breakdown (Smith, 1982), and was observed in cultures grown photoheterotrophically or photoautotrophically, and incubated either in the light or in the dark (Table 5). It therefore appears that exogenous glucose is metabolized predominantly, if not exclusively, through the oxidative pentose phosphate pathway in both photoheterotrophically and photoautotrophically grown cultures of *Oscillatoria* sp. UCSB8. The activity of $NADP^+$ -dependent glucose-6-phosphate dehydrogenase, the first enzyme of the oxidative pentose phosphate pathway, was more than sevenfold greater in extracts of photoheterotrophic cultures of *Oscillatoria* sp. UCSB8 [$22 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$] than in extracts of photoautotrophic cultures [$3 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$]. Furthermore, O_2 consumption in the dark was greater [$10.2 \text{ } \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$] in photoheterotrophically grown cultures than in cultures grown photoautotrophically [$4.3 \text{ } \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$]. In *Oscillatoria* sp. UCSB8, therefore, exogenously supplied glucose, catabolized through the oxidative pentose phosphate pathway, can support N_2 fixation. This implies that there is no obligatory link between photosynthesis and N_2 fixation in this organism, and respiration rather than photosynthesis may support nitrogenase activity even under photoautotrophic conditions as has been reported for *Gloeotheca* (Maryan *et al.*, 1986). In agreement with this, photoautotrophically grown cultures of *Oscillatoria* sp. UCSB8, incubated under continuous illumination, showed peaks of nitrogenase activity coincident with low rates of O_2 evolution in the light and maximum rates of O_2 consumption in the dark (Fig. 2). Such a correlation has also been reported for *Synechococcus* spp. (Mitsui *et al.*, 1987; Grobbelaar *et al.*, 1987) but was not observed in *Gloeotheca* (Gallon & Chaplin, 1988; Gallon, 1989).

Both species of *Oscillatoria* appeared more pigmented under photoheterotrophic conditions than did photoautotrophically grown cultures. Expressed per unit dry weight, all of the photosynthetic pigments were more abundant in photoheterotrophically grown cultures of *Oscillatoria* sp. UCSB8 than in cultures grown photoautotrophically (Table 6). However, fluctuations in endogenous reserves of carbohydrates or in extracellular polysaccharides may distort data expressed per unit dry weight. On the basis of total protein, only phycoerythrin was more abundant in photoheterotrophic than in photoautotrophic cultures of *Oscillatoria* sp. UCSB8. In *Oscillatoria* UCSB25, phycocyanin per unit dry weight increased substantially in response to photoheterotro-

Table 5. Evolution of ¹⁴CO₂ from [1-¹⁴C]glucose and [6-¹⁴C]glucose by cultures of *Oscillatoria* sp. UCSB8

Cultures of *Oscillatoria* sp. UCSB8, grown either photoautotrophically or photoheterotrophically (with 20 µM-DCMU and 20 mM-glucose), were incubated with 0.37 MBq of D-[1-¹⁴C]glucose or D-[6-¹⁴C]glucose. Production of ¹⁴CO₂ was measured during the following 6 h. Cultures incubated in the light with [¹⁴C]glucose had previously been grown under constant illumination whilst cultures incubated in the dark had previously been grown under alternating light and darkness and were given [¹⁴C]glucose 3 h into the period of darkness. The rates of ¹⁴CO₂ evolution quoted represent initial rates and are the means of at least three separate experiments.

Growth and assay conditions	Evolution of ¹⁴ CO ₂ [Bq (mg dry wt) ⁻¹ h ⁻¹]		Ratio of CO ₂ evolution [1- ¹⁴ C]/[6- ¹⁴ C]
	Substrate:		
	[1- ¹⁴ C]Glucose	[6- ¹⁴ C]Glucose	
Photoautotrophic			
Light	3.3	0.5	6.6
Dark	5.8	1.0	5.8
Photoheterotrophic			
Light	7.9	1.1	7.2
Dark	10.0	1.6	6.3

Table 6. Pigment contents of photoautotrophic and photoheterotrophic cultures of *Oscillatoria* spp. UCSB8 and UCSB25

Batch cultures were grown for 28 d under continuous illumination at 22 °C (UCSB8) or 30 °C (UCSB25). Photoheterotrophic growth was performed in the presence of 20 µM-DCMU and 20 mM-glucose (UCSB8) or 20 mM-fructose (UCSB25). Cultures were subjected to gentle homogenization prior to removal of samples for the determinations of dry weight, pigments and total protein. The data presented are the means of at least three determinations on separate cultures at the same stage of growth. Abbreviations: Chl *a*, chlorophyll *a*; PC, phycocyanin; APC, allophycocyanin; PE phycoerythrin.

	UCSB8		UCSB25	
	Photoauto-trophic	Photohetero-trophic	Photoauto-trophic	Photohetero-trophic
Dry weight [mg (ml culture) ⁻¹]	1.30	1.32	1.10	1.22
Protein [µg (mg dry wt) ⁻¹]	158.4	312.0	161.0	244.2
Chl <i>a</i> [µg (mg dry wt) ⁻¹]	3.4	5.2	4.4	5.0
[µg (mg protein) ⁻¹]	21.5	16.6	27.3	20.5
PC [µg (mg dry wt) ⁻¹]	18.6	37.8	31.4	48.0
[µg (mg protein) ⁻¹]	117.4	121.2	195.0	196.6
APC [µg (mg dry wt) ⁻¹]	18.8	30.4	17.2	18.3
[µg (mg protein) ⁻¹]	118.7	97.4	106.8	74.9
PE [µg (mg dry wt) ⁻¹]	10.7	54.5	4.4	3.9
[µg (mg protein) ⁻¹]	67.6	174.7	27.3	16.0

phic growth, whereas the other photosynthetic pigments were relatively similar in concentration to those observed in photoautotrophic cultures. However, when expressed per unit of total protein, chlorophyll *a*, allophycocyanin and phycoerythrin decreased significantly in photo-

heterotrophic cultures of *Oscillatoria* UCSB25, whilst the content of phycocyanin was similar to that observed in photoautotrophic cultures. Therefore, the visual changes of pigmentation of photoheterotrophic cultures of *Oscillatoria* UCSB8 are most likely due to an increase of

phycoerythrin, whereas those observed in photoheterotrophic cultures of *Oscillatoria* UCSB25 are less readily evident, since neither chlorophyll *a* nor total phycobiliprotein content increased per unit of total protein. In addition to their role as accessory photosynthetic pigments, the phycobiliproteins may function as nitrogen reserves (Carr, 1988). Photoheterotrophic N_2 -fixing cultures of *Oscillatoria* UCSB8 may be more nitrogen-replete than photoautotrophic cultures and thus synthesize more phycoerythrin, since the specific activities of nitrogenase were greater under photoheterotrophic conditions both under constant illumination (Table 3) and under alternating light and darkness (Fig. 1). Nevertheless, such cultures grew less rapidly than photoautotrophic cultures (Table 4), and some factor other than the rate of N_2 fixation may limit growth under photoheterotrophic conditions.

In conclusion, it is proposed that in *Oscillatoria* sp. UCSB8, as in *Gloeotheca* (Maryan *et al.*, 1986) respiration may be more significant than photosynthesis itself in supporting N_2 fixation. Respiration may be supported either by endogenous glucan reserves or by exogenously supplied glucose, fructose or sucrose, all of which are most likely metabolized via the oxidative pentose phosphate pathway. Although *Oscillatoria* sp. UCSB25 differs in some respects from *Oscillatoria* sp. UCSB8, it shares the ability to fix N_2 photoheterotrophically and shows the same pattern of N_2 fixation during growth under alternating light and darkness. The ability of these organisms to use exogenous carbon should prove useful for future investigations of the mechanisms involved in the regulation of both nitrogen and carbon metabolism in non-heterocystous diazotrophic cyanobacteria.

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