Characterization of a brown *Nostoc* species from Java that is resistant to high light intensity and UV

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A brown *Nostoc* species was isolated from a soil sample collected in Java, Indonesia. It has three physiological types of light-absorbing compounds: the photosynthetic pigments (chlorophyll *a*, carotenoids, phycocyanin, phycoerythrin and allophycocyanin), a brown pigment(s) and three UV-absorbing compounds. The organism undergoes complementary chromatic adaptation by varying its phycobilin content. It is resistant to photobleaching at high intensities of white light, and also to UV-C radiation, under which conditions a green-coloured *Nostoc* species (ATCC 27895) was killed. Synthesis of the brown pigment and UV-absorbing compounds was not observed at low oxygen levels. A brown pigment was released suddenly from cells during exponential growth, resulting in a deep brown-coloured medium. In addition, the absorption spectrum of the medium after pigment release had maxima in the UV region, at 256 nm, 314 nm and 400 nm. The appearance of the UV-absorbing compounds coincided with the brown pigment release and with the disappearance from the vegetative cells of peripheral granules. The *Nostoc* species reduced acetylene, a measure of nitrogen fixation, in both the light and the dark periods when grown on a 12/12 h light/dark cycle. On a 4/20 h light/dark cycle fixation occurred predominantly in the light period. When grown on this restricted light regime, or at very low light intensity, the cells did not produce the brown pigment or the UV-absorbing compounds.

Keywords: *Nostoc*, cyanobacteria, UV-absorbing compounds, photobleaching, photosynthetic pigments

INTRODUCTION

Cyanobacteria, in general, have adapted for growth in relatively dim light conditions, and will often actively seek suitable niches of favourable light intensity for optimum growth (Donkor & Häder, 1991). High solar radiation causes damage to their photosynthetic antennae and photobleaching of their cells (Carr & Whitton, 1982).

The phycobiliproteins of cyanobacteria serve as their primary light-gathering antennae, and greatly extend the range of wavelengths of light absorbed (Gantt, 1981). Engelmann (1902) reported that the pigmentation of certain cyanobacteria could be modified by altering the light quality and it was later shown by Boresch (1922) that the colour change of cyanobacterial cells is attributable to a change in the phycoerythrin/phycocyanin ratio. This chromatic adaptation enables the cells to make maximum use of the particular wavelengths of light available in their environment and is particularly useful in underwater

environments (Wyman & Fay, 1986a, b). Such chromatic adaptation can only occur in those cyanobacteria that synthesize phycoerythrin (Tandeau de Marsac, 1977). Phycobiliproteins can also serve as nitrogen reserves in some species (Wyman *et al.*, 1985; Heathcote *et al.*, 1992).

Depletion of the ozone layer has resulted in an increase in penetration of the shorter and most biologically injurious wavelengths (280–320 nm) of ultraviolet radiation (UV) (Smith *et al.*, 1992). Some cyanobacteria synthesize UV-absorbing compounds that appear to act as sunscreens and enable them to inhabit environments that are exposed to intense solar radiation (Garcia-Pichel & Castenholz, 1991; Garcia-Pichel *et al.*, 1992, 1993; Matsunaga *et al.*, 1993; Scherer *et al.*, 1988). Shibata (1969) isolated UV-absorbing substances that were later identified by Dunlap & Chalker (1986) as a series of mycosporine-like amino acids which in marine organisms absorb UV radiation between 310 and 360 nm (Dunlap *et al.*, 1989). Mycosporine-like amino acids in cyanobacteria have also been reported by Garcia-

Pichel & Castenholz (1993). They are water-soluble compounds that absorb in the UV-B region of the spectrum. Scherer *et al.* (1988) reported a UV-A/B-absorbing compound in *Nostoc commune* that was water-soluble and located outside the cells, in the polysaccharide matrix of the colony. An extracellular UV-A-absorbing pigment, scytonemin, was reported by Garcia-Pichel & Castenholz (1991). Matsunaga *et al.* (1993) reported a UV-A-absorbing bioprotein glucoside in a marine *Oscillatoria* species.

In this paper we describe the characterization of a brown *Nostoc* species that is resistant to high light intensity and UV.

METHODS

Cyanobacterial isolation and growth. The *Nostoc* sp. described here was from a soil crust sample collected in February 1992 from a village in Java, Indonesia. It was rendered unialgal using the light variation methodology of de Chazal *et al.* (1992), the sample being first enriched for cyanobacteria by growth in the nitrogen-free medium of Allen & Arnon (1955), diluted to 1/8 strength of all components except phosphate, and with a fourfold increase in NiSO_4 . *Nostoc* sp. ATCC 27895 was obtained from the American Type Culture Collection. For oxic growth the cells were sparged with 0.3% CO_2 in air at 25 °C (unless otherwise stated) and for anoxic growth they were sparged with 0.3% CO_2 in N_2 . The cells were illuminated with banks of fluorescent lights (Daday *et al.*, 1977). For photo-bleaching experiments at 'high light intensity', the cells were grown in tall, narrow (8 cm diameter) flasks placed on aluminium foil to provide a reflective surface and surrounded by vertical banks of fluorescent tubes; for other experiments at this light intensity the cells were grown in Erlenmeyer flasks. The light intensity was, in this lighting configuration, difficult to define precisely because the cells received light from 360°; the unidirectional intensity measured at one side of the flask was $200 \mu\text{E s}^{-1} \text{m}^{-2}$ photosynthetically active radiation. In the experiments at the 'high light intensity' the growth flasks were cooled by a fan in order to maintain the growth temperature of 25 °C; in other experiments the cells were deliberately temperature-stressed by switching off the fan, in which case the cultures reached a temperature of 39 °C. Cell growth was measured with a Klett–Summerson colorimeter (no. 66 filter; Mallette, 1969).

Cell extraction. Cells were concentrated by centrifugation (27000 g, 15 min, 4 °C), disrupted using a French pressure cell at 138 MPa and cell debris was subsequently removed by centrifugation (27000 g, 15 min, 4 °C). The brown pigment fraction was pelleted by further centrifugation in a Beckman TL-100 ultracentrifuge (81000 g, 1 h, 4 °C). The UV-absorbing compounds were extracted by centrifuging cells at 1000 g, aspirating the supernatant, then extracting the pellet of cells with water at 50 °C for 30 min and centrifuging (Scherer *et al.*, 1988).

Assays. The nitrogen-fixing capacity of the cells was determined by the acetylene reduction assay as described previously (Lambert & Smith, 1980). Phycoerythrin, phycocyanin and allophycocyanin were extracted and determined by the method of Tandeau de Marsac (1977). The scytonemin assay method was that of Garcia-Pichel & Castenholz (1991). The assay for proanthocyanidins (condensed tannins) was that of Sarker & Haworth (1976) and the cyanophycin assay was that of Daday *et al.* (1988).

Spectra. Spectra of growth media were measured on the supernatants obtained after centrifuging the cells, using a Varian Techtron model 635 spectrophotometer attached to a chart recorder.

Cell dry weights. These were determined by centrifuging a homogeneous mixture of cells (5–10 ml), washing once with water and then drying in preweighed tubes at 85 °C.

Exposure to UV. Cells in exponential growth were washed three times with water and added to 250 ml water (in which cells were viable for many days; the growth medium was not used because it partially absorbed UV) to a concentration of 20 Klett units in flat crystal dishes, 15 cm diameter, and stirred with a magnetic bar. They were placed 33 cm below each of the following lamps: a cool white fluorescent globe, a National Electric Company 40 W T10 black light tube with an emission range of 304–450 nm and peak emission at 366 nm (UV-A), and a Philips TL 40W/12 UV tube with an emission range of 260–360 nm and maximum emission in the UV-B region. The cells were covered with a Perspex sheet 2 mm thick that specifically blocked UV-C. For experiments with UV-C alone, the cells were exposed to a single germicidal lamp (253.7 nm emission). The viability of cells was determined qualitatively by monitoring their colour and trichome damage, and also by streaking them on to 1% (w/v) agar plates (prepared in the growth medium). In the latter case, cells were plated after 2.5 h exposure to the UV and then daily for 3 d; the plates were then placed in continuous light at 25 °C and checked for colony growth after a further week.

Light measurement. Light intensity was measured as photo-synthetically active radiation using a Li-COR meter, model LI-185 B.

Electron microscopy. Cells were fixed in 2.5% (v/v) glutaraldehyde, 4% (v/v) formaldehyde in 0.1 M sodium cacodylate buffer with 0.14 M sucrose (pH 7.2), fixed in 1% (w/v) osmium tetroxide in the same buffer, washed, dehydrated through an ethanol series and embedded in Araldite (Ciba-Geigy). Ultrathin sections were cut, stained for 15–20 min with uranyl acetate, washed, stained for 5–10 min with lead citrate, washed again and allowed to dry before viewing in an Hitachi H600 transmission electron microscope at 75 kV.

RESULTS

General characterization of the brown *Nostoc* species

Cyanobacterial soil crusts were inoculated into nitrogen-free medium and grown at a light intensity of $80 \mu\text{E s}^{-1} \text{m}^{-2}$. In such a mixed culture the *Nostoc* grew as isolated bundles within the resulting matted cyanobacterial mixture. Assignment as a *Nostoc* species was based on the microscopic criteria given by Rippka *et al.* (1979). The vegetative cells were pale brown and the heterocysts were green. The organism initially represented only a small proportion of the mixed culture. When such mixed cultures were subcultured several times by washing and inoculating into fresh medium at 'high light intensity' and maintained at 25 °C with a fan (see Methods), the brown *Nostoc* became the predominant species together with small numbers of a *Synechococcus* species that could be removed by repeated dilution streaking on 1% agar plates or by movement of the *Nostoc* on a scored agar plate (see de Chazal *et al.*, 1992, for details of methodology).

It was observed visually that the cells synthesized an intense brown pigment which was released suddenly at a

Fig. 1. Electron micrographs of brown *Nostoc* cells before (top) and after (bottom) pigment release. Associated with this release can be seen the disappearance of granules from the cells. The cells were grown at the 'high light intensity' as defined in Methods. Bars, 5 μm .

certain stage of exponential growth, resulting in a deep brown-coloured medium. The *Nostoc* showed an interesting adaptation with respect to its pigmentation: under normal growth conditions it was brown, but when grown at very low light intensity ($3.5 \mu\text{E s}^{-1} \text{m}^{-2}$) or on a 4/20 h light/dark cycle it became green. Microscopic examination of the brown cells revealed the presence of large intracellular granules (Fig. 1) that were not present in green cells. These granules did not fluoresce when examined in a confocal microscope with excitation wavelengths of 488, 568 or 647 nm.

In addition to the brown pigment, both brown and green cells contained phycocyanin, allophycocyanin and phycoerythrin, although the content of these biliprotein pigments was significantly less in green cells (0.021, 0.006 and 0.004 versus 0.066, 0.040 and 0.074 mg (mg cell dry weight) $^{-1}$, respectively). There was no measurable temporal change in the content of these pigments in cells grown either on a 12/12 h or a 4/20 h light/dark cycle

(results not shown), suggesting that the biliproteins do not function as a dynamic nitrogen store across the diurnal cycle (Carr, 1988). Nevertheless, when cells were incubated in the dark for a prolonged period, the content of phycocyanin, allophycocyanin and phycoerythrin decreased from 0.028 to 0.009, from 0.017 to 0.004 and from 0.034 to 0.008 mg (mg cell dry weight) $^{-1}$, respectively. The cells were still viable after three weeks of darkness. The organism did not produce significant cyanophycin (as measured biochemically) under any of the growth conditions described here (results not shown).

When grown on a 12/12 h light/dark cycle the brown *Nostoc* reduced acetylene, reflecting its capacity for nitrogen fixation, both in the light and in the dark (results not shown). Clearly, under these conditions the cells can maintain throughout most of the dark period a sufficient supply of those photosynthetically generated chemicals that provide reductant and ATP for the nitrogenase. The rate of acetylene reduction was related inversely to the intensity of the brown colour of the cells; dark brown cells needed a higher light intensity to maintain a given rate of acetylene reduction than did green cells. When green cells were incubated on a 4/20 h light/dark cycle, acetylene reduction occurred mainly in the light period (results not shown).

Properties of the brown pigment

An absorption spectrum of a total cell extract, which was brown, is shown in Fig. 2(a). When this cell extract was centrifuged at 81 000 g for 60 min, to pellet chlorophyll-containing membrane fractions (Tandeau de Marsac, 1977), the brown pigmentation was found in the pellet, leaving a pink supernatant, the spectrum of which is also shown in Fig. 2(a); the two peaks in the supernatant represent phycoerythrin and phycocyanin, and so clearly the brown colour is due to some other pigment. Interestingly, when the brown medium that had been obtained after centrifuging whole cells was similarly centrifuged, the brown coloration remained in the supernatant, indicating that the excreted pigment(s) was different, either chemically or in its attachment to cellular particles. Attempts to extract the brown pigment were unsuccessful; it was always found with the chlorophyll fraction. The difference spectrum calculated by subtracting the two spectra shown in Fig. 2(a) showed a shoulder at approximately 480 nm (Fig. 2b). When a normalized spectrum of pure chlorophyll *a* was in turn subtracted from this difference spectrum, a broad peak with an absorbance maximum at 480 nm was revealed (Fig. 2b). This is presumed to represent the brown pigment. Its nature is unknown. It is not tannin since no proanthocyanidins (condensed tannins) were detected. It is highly anionic, binding very strongly to DEAE-cellulose at pH 7.6.

Properties of the UV-absorbing compounds

The absorption spectrum of the medium after the spontaneous release of brown pigment from cells grown under oxic conditions, but not anoxic ones, revealed

Fig. 2. Absorption spectrum of a total cell extract, which was brown, obtained by disrupting the cells in a French pressure cell and removing cell debris by centrifugation (○, a). When this cell extract was further centrifuged at high speed (81000 g, 60 min) the brown pigmentation was found in the pellet, leaving a pink supernatant, the spectrum of which (●) is also shown in (a). The difference spectrum calculated by subtracting the two spectra from (a) is shown (□) in (b). When a normalized spectrum of pure chlorophyll *a* was subtracted from this difference spectrum a broad peak with an absorbance maximum at 480 nm was revealed (■, b). This is presumed to represent the brown pigmentation.

peaks in the UV region with maxima at 256 nm (UV-C), 314 nm (UV-B) and 400 nm (UV-A) (Fig. 3). The appearance of the UV-absorbing compounds in the medium coincided with the release of the brown pigment. When brown cells were extracted into water at 50 °C the spectrum of the extract revealed only the peak with an absorption maximum at 256 nm (result not shown). With green cells this peak was not found. No scytonemin was detected in brown cells. HPLC analysis (Dunlap & Chalker, 1986) of lyophilized cells revealed three distinct polar compounds (with maxima at 314 nm, 280 nm and 256 nm) which bore no relationship to mycosporin-like amino acids.

Physiological aspects of pigment and UV-absorbing compound formation and release

Production of the brown and UV-absorbing compounds appeared to be a response of the cells to light. When green cells were used to initiate growth they turned brown and, after a certain time of exponential growth, were clearly observed visually to suddenly release both the brown pigment and the UV-absorbing compounds into the growth medium. The time of release in the growth cycle varied with the light intensity. A typical result for the UV-

Fig. 3. Absorption spectra of growth media obtained after centrifuging cells that had been grown to 125 Klett units under either oxic (brown cells) or anoxic (orange-green cells) conditions at 'high light intensity' as defined in Methods.

Fig. 4. Sudden release of UV-absorbing compounds from exponential-phase cells grown under oxic conditions at 'high light intensity'. Absorbance was measured at 400 nm (●), 315 nm (□) and 255 nm (▲).

absorbing compounds is shown in Fig. 4; at 70 h the cells were growing with a doubling time of 12 h. The brown pigment release appeared to be related to the disappearance of granules from vegetative cells as observed by electron microscopy (Fig. 1). These granules subsequently re-formed within the cells and were released again; this was more readily observed when the cells were washed and resuspended in fresh medium. When brown cells were inoculated for growth on a 4/20 h light/dark cycle the supernatant remained clear and the cells turned green. If green cells were placed on such a cycle, they remained green and the supernatant remained colourless.

Oxygen appeared to be necessary for synthesis of both the brown and the UV-absorbing compounds since reduction

in oxygen levels prevented their formation; cells sparged with nitrogen/0.3% CO₂ during growth turned orange-green and did not release either brown pigment or UV-absorbing compounds (results not shown).

Response of cells to UV

When compared with a green *Nostoc* species (ATCC 27895) a striking resistance of the brown species to UV was observed. *Nostoc* sp. ATCC 27895 was rapidly photobleached and killed by UV-C, whereas brown cells of the Javan isolate survived after a 22 h exposure. UV-C did, however, turn the brown *Nostoc* cells green and caused granules to appear in the vegetative cells. Interestingly, exposure to UV-C also increased the resistance of the cells during subsequent exposure to UV-A together with UV-B; such cells were still viable after 56 h, whereas cells not previously exposed to UV-C did not produce granules, were photo-oxidized and almost all were killed within 50 h.

Resistance to photobleaching by white light

The brown *Nostoc* was able to grow at elevated temperature (39 °C) combined with high light intensity. When cells were incubated under oxic conditions on a 12/12 h light/dark cycle at 'high light intensity' (see Methods), and fanned to maintain them at ambient temperature 25 °C, they were resistant to photobleaching and turned dark brown over the course of 2 or 3 d, during which time granules appeared in the vegetative cells (cf. Fig. 1). Cells grown under anoxic conditions were also resistant to photobleaching but no granules appeared in the vegetative cells. A similar resistance was observed in cultures grown in continuous light, both at 25 °C and 39 °C.

In order to provide control observations we did similar experiments on the green *Nostoc* sp. (ATCC 27895). By contrast with the brown species it was susceptible to photobleaching. In continuous 'high light intensity', under both oxic and anoxic growth conditions, the cells survived at 25 °C, but at 39 °C they photobleached within 3 d as observed visually by the cells turning yellow (cf. Barabás & Laczkó, 1985) followed by the trichomes breaking up. On a 12/12 h light/dark cycle both oxic and anoxic cells survived at 25 °C but at 39 °C the oxic cells remained green and survived whereas the anoxic cells photobleached within 3 d.

DISCUSSION

The *Nostoc* species described here has a number of interesting properties that presumably enable it to survive and grow in the tropical environment, with its intense solar radiation, and successfully compete with other species (it became the dominant organism in a cyanobacterial mixture exposed to high light intensity). These properties include nitrogen fixation, resistance to photobleaching under both oxic and anoxic conditions at elevated temperature, and the presence of three types of

light-absorbing compounds: the photosynthetic light-harvesting pigments (including chlorophylls, carotenoids and phycobilins), a brown pigment, and UV-absorbing compounds. The brown *Nostoc* is clearly different in several respects from *Nostoc commune*, which also has UV-absorbing compounds. Unlike the brown *Nostoc*, *N. commune* has a life cycle (Fogg *et al.*, 1973) and has a different spectrum in the UV region (Scherer *et al.*, 1988). Although the brown pigmentation of cells was dominant under most growth conditions, a green form of the organism was observed under the following conditions: growth on a light/dark cycle with a long dark period (4/20 h); growth while sparging with 0.3% CO₂ in nitrogen; maintenance at very low light intensity and exposure to UV-C.

The ability of the cells to vary their phycoerythrin/phyocyanin ratio allows regulation of the balance of wavelengths of light absorbed by the organism, a phenomenon termed chromatic adaptation (Tandeau de Marsac, 1977). There was no evidence that the phycoerythrin has a major function as a nitrogen storage compound, as for example in the marine cyanobacterium *Synechococcus* (Wyman *et al.*, 1985), although this organism may be the exception rather than the rule. The brown *Nostoc* has a phycoerythrin/phyocyanin ratio of approximately one and both of these pigments decreased upon prolonged starvation in the dark. These pigments appeared to decline more slowly during nitrogen starvation than did phyocyanin in *Anacystis nidulans* (Allen & Smith, 1969), an organism in which phyocyanin appears to play a more dynamic role as a nitrogen storage compound. Phycoerythrin, phyocyanin and allophyocyanin all remained constant across the light/dark cycle. Interestingly, it has been reported that many phycoerythrin-containing cyanobacteria are markedly light-sensitive (Tandeau de Marsac, 1977), whereas the brown *Nostoc* investigated here thrives at high light intensity and is strikingly resistant to photo-oxidation.

An interesting property of the brown Javan *Nostoc* is the sudden release of brown pigment during growth. This pigment may be excreted as a waste-product generated from a form that functions as an antioxidant; it is not synthesized when cells are sparged with gas not containing oxygen. It is also possible that the brown pigment is synthesized constitutively and loss of brown pigmentation is a stress response of the cells. Extracellular products, including pigments, of cyanobacteria are well known (Carr & Whitton, 1982) but little is known about the physiology of their release. It may be that there is more than one brown pigment, and the form which is excreted is distinct from that which is tightly bound in the chlorophyll fraction and pelleted at high centrifugal force. It is interesting to speculate on whether the latter functions as an accessory light-harvesting pigment.

The brown *Nostoc* was resistant to photobleaching under conditions of high continuous light intensity and elevated temperature (39 °C), under which conditions a green *Nostoc* sp. (ATCC 27895) photobleached. The latter organism did not photobleach when incubated under oxic conditions on a 12/12 h light/dark cycle at the elevated

temperature of 39 °C; one possible explanation is that a period of respiration in the dark allows recovery. It should be noted in relation to experiments with the green *Nostoc* sp. (ATCC 27895), and other experiments that we have done on *Anabaena cylindrica* (results not shown), that photobleaching is a combined effect of high light intensity and elevated temperature; the photobleaching described for the latter organism by Laczkó & Barabás (1981) was partly due to a temperature effect in continuous light.

In addition to their resistance to high white light intensity, the brown cells were also resistant to UV, particularly UV-C. Exposure of the trichomes to UV-C increased the subsequent resistance of the *Nostoc* to UV-A plus -B. The physiological significance of this observation is not clear because UV-C is absorbed by the Earth's atmosphere. The nature of the UV-absorbing compounds remains to be determined.

The brown *Nostoc* fixed nitrogen in both the light and a substantial period in the dark when grown on a 12/12 h light/dark cycle, in contrast to *Nostoc* UCSB9 (Khamees *et al.*, 1987). It presumably does not therefore need nitrogen reserve compounds, such as the phycobilins or cyanophycin, to integrate growth across the light and dark periods on the normal diurnal cycle (Carr, 1988). Cyanobacteria appear to vary considerably in their capacities to support dark nitrogen fixation (Khamees *et al.*, 1987). When the brown *Nostoc* was grown on a 4/20 h light/dark cycle, fixation decreased much earlier in the dark period (results not shown).

In summary, the organism described here has properties that indicate it is well adapted to the tropical environment from which it was isolated. Further work is necessary to chemically characterize the pigments, and to determine their relationship to the intracellular granules and the effect of pigmentation on nitrogen fixation.

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