***Anthoceros–Nostoc* Symbiosis: Immunoelectronmicroscopic Localization of Nitrogenase, Glutamine Synthetase, Phycoerythrin and Ribulose-1,5-bisphosphate Carboxylase/Oxygenase in the Cyanobiont and the Cultured (Free-living) Isolate *Nostoc* 7801**

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Localization of nitrogenase, glutamine synthetase (GS), phycoerythrin (PE) and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) was studied with immunocytochemical techniques in the cyanobiont and the free-living cultured isolate *Nostoc* 7801 of *Anthoceros punctatus*. In both cases nitrogenase was located in heterocysts only and was uniformly distributed within the cell. GS was located both in heterocysts and vegetative cells, with a uniform cellular distribution in each cell type. Whereas heterocysts of *Nostoc* 7801 had about twofold higher label than vegetative cells, labelling in heterocysts and vegetative cells of the cyanobiont was similar. While the GS content of the vegetative cells of the cyanobiont and *Nostoc* 7801 was comparable, the apparent GS content of the cyanobiont heterocysts was 60% less than that in *Nostoc* 7801 heterocysts. PE and RuBisCO were located in vegetative cells only. PE was located on thylakoid membranes and RuBisCO in the cytoplasm and carboxysomes. In each case the pattern of labelling in the cyanobiont and *Nostoc* 7801 was similar.

INTRODUCTION

N₂-fixing cyanobacteria form symbiotic associations with plants that range from algae to angiosperms (Gallon & Chaplin, 1987; Smith & Douglas, 1987). The cyanobacterial partner (cyanobiont) in all these symbioses is known to fix N₂ and make fixed nitrogen available to the eukaryotic partner (see Stewart *et al.*, 1983). In some symbioses (e.g. in lichens and *Azolla*) the cyanobiont is photosynthetically active. However, in bryophytes, cycads and *Gunnera*, the cyanobiont is photosynthetically inactive and dependent on the eukaryotic partner for its fixed carbon (Smith & Douglas, 1987; Stewart *et al.*, 1983). The transfer of metabolites between the cyanobiont and eukaryotic partner is linked to physiological and biochemical changes in the cyanobiont (Stewart *et al.*, 1983; Meeks *et al.*, 1985; Nierzwicki-Bauer & Haselkorn, 1986; Lindblad *et al.*, 1987).

Among the bryophyte–cyanobacterial symbioses, the hornwort *Anthoceros–Nostoc* symbiosis is best characterized. N₂-fixing *Nostoc* spp. develop in cavities on the undersurface of the *Anthoceros* gametophyte thallus. The cyanobiont in this association reportedly has an increased heterocyst frequency, is photosynthetically inactive and lacks phycobiliproteins (Rodgers & Stewart, 1977; Stewart & Rodgers, 1977). The increased heterocyst frequency does not correlate with an increase in N₂-fixation rates (Rodgers & Stewart, 1977; Stewart & Rodgers, 1977). Whether this is due to limited availability of fixed carbon or to the presence of nonfunctional heterocysts lacking nitrogenase is not known. The activity of glutamine synthetase (GS) in the

Abbreviations: GS, glutamine synthetase; GDH, glutamic dehydrogenase; PE, phycoerythrin; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; TEM, transmission electronmicroscopy.

cyanobiont is three- to fourfold lower than that in the free-living isolate; this reduction is apparently due to regulation at the level of enzyme inactivation rather than synthesis (Joseph & Meeks, 1987). This is in contrast to the situation in lichen and *Azolla* cyanobionts, where the reduction in GS activity has been attributed to low rates of enzyme synthesis (Stewart *et al.*, 1983; Rowell *et al.*, 1985; Nierzwicki-Bauer & Haselkorn, 1986). The relative distribution of GS between vegetative cells and heterocysts of the cyanobiont in *Anthoceros* is not known.

Using immunocytochemistry, the aims of this study were (1) to examine the content of GS protein and its relative distribution between heterocysts and vegetative cells, and (2) to check for the presence of nitrogenase, phycoerythrin (PE) and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) in vegetative cells and in single and multiple heterocysts in the cyanobiont from mature cyanobacterial colonies of the *Anthoceros punctatus* gametophyte. For comparison, the free-living cultured isolate *Nostoc* 7801 was also studied.

METHODS

Organisms. *Nostoc* sp. strain 7801 was obtained from Dr J. C. Meeks (Department of Bacteriology, University of California, Davis, USA). Axenic batch cultures were grown in BG-11₀ medium (Rippka *et al.*, 1979) at 25 °C and at a photon fluence rate of 70 µmol m⁻² s⁻¹. *Anthoceros punctatus* gametophyte thalli bearing cyanobacterial colonies were collected from North-Eastern Hill University campus (Shillong, India) at the end of September, at around 11 a.m. The thalli were cleaned using distilled water and mature colonies from middle portions of the thalli were picked using a needle and fine forceps.

Antibodies. Rabbit serum containing antibodies raised against Fe-protein of nitrogenase (dinitrogenase reductase), purified from *Rhodospirillum rubrum*, was a gift from Dr S. Nordlund (University of Stockholm, Stockholm, Sweden) and has been previously used for specific localization of nitrogenase in free-living and symbiotic cyanobacteria (Bergman *et al.*, 1986). Antibody against GS protein, purified from *Anabaena* PCC 7120, was a gift from Professor R. Haselkorn (University of Chicago, Chicago, USA); it has been shown to be monospecific, and has been used for GS localization in cyanobacteria (Orr & Haselkorn, 1982; Bergman *et al.*, 1985). Antibodies against PE, purified from the cyanobacterium *Phormidium persicinum*, were a gift from Dr D. Guard-Friar and its specificity has been shown by Guard-Friar *et al.* (1986). These antibodies were also seen to be specific from the labelling pattern observed, i.e. high labelling in thylakoids of vegetative cells and comparatively little labelling in heterocysts, which are known to have little or no phycobiliproteins (see Stewart, 1980).

Rabbit serum containing antibodies raised against RuBisCO purified from *Sinapis alba* was a gift from Dr R. Oelmüller (University of Freiburg, Freiburg, FRG). The antibodies have been shown to be specific against chloroplast RuBisCO (Oelmüller & Mohr, 1986). Their specificity with cyanobacterial RuBisCO was assumed, indirectly, from the labelling pattern observed, i.e. high labelling in vegetative cells, particularly carboxysomes (which are known to be RuBisCO-containing bodies in cyanobacteria), and very low labelling in heterocysts. This pattern of labelling is identical to that observed using RuBisCO antibodies raised against the enzyme purified from cyanobacteria (see Cossar *et al.*, 1985).

Immunolabelling and transmission electron microscopy (TEM). Preparation of samples for TEM and the protocols for immunolabelling were essentially as described by Bergman *et al.* (1985) except that uncoated gold grids were used, the goat anti-rabbit IgG was conjugated to 15 nm size colloidal gold particles and different primary antibodies were used as required (see figure legends). Ultrathin sections of glutaraldehyde-fixed and Epon-embedded samples were cut with an LKB Ultramicrotome and picked up on gold grids (300 µm mesh). These were then labelled for 1 h with primary antibody (rabbit anti-nitrogenase, GS, PE or RuBisCO) followed by 30 min incubation with secondary antibody (goat anti-rabbit IgG conjugated to 15 nm size colloidal gold particles). After cross-reaction, the sections on grids were stained with uranyl acetate and lead citrate. TEM observations were made using a Jeol 100B electron microscope operated at 60 kV. Five ultrathin sections from three mature *Nostoc* colonies from *Anthoceros punctatus* gametophyte were used in each labelling experiment. However, except for GS, where 15 electronmicrographs were taken, only five or six electronmicrographs were taken. We present one set of representative micrographs, for each labelling experiment, which we assume reflect the functional symbiosis.

RESULTS AND DISCUSSION

Biochemical characterization of the cyanobiont has been rather difficult because of the problem of obtaining enough clean material from the symbiotic association. Furthermore, in isolating the cyanobiont, whole thalli are generally used. This results in metabolically heterogeneous cyanobiont populations since cyanobionts are in different stages of developing symbiosis in different parts of the thallus, as indicated by varying heterocyst frequency and

enzymic activities (see Englund, 1977; Lindblad *et al.*, 1985; Rowell *et al.*, 1985). With the introduction of immunogold labelling these problems can be avoided and an antigen can be located precisely with little sample material. Here, we have used this method to study the *Anthoceros-Nostoc* association in mature cyanobacterial colonies from the mid-part of the *A. punctatus* thallus.

The secondary antibody (goat anti-rabbit IgG) was first tested for any nonspecific binding to the fixed cells. Virtually no label was detected in the absence of the primary antibody either in the cyanobiont or in *Nostoc* 7801 (Fig. 1*a, b*). This showed that the antibody was specific to rabbit IgG. The location of the specific antigens was then determined using primary antibodies to nitrogenase, GS, PE and RuBisCO.

Nitrogenase

In heterocystous cyanobacteria, nitrogenase is restricted to heterocysts under aerobic growth conditions (Stewart, 1980; Janaki & Wolk, 1982). However, nitrogenase may be expressed in vegetative cells of non-heterocystous cyanobacteria both under microaerobic (Stewart & Lex, 1970; Rippka & Waterbury, 1977) and aerobic (Stal & Krumbein, 1985) conditions. Whether nitrogenase can be induced in vegetative cells of heterocystous cyanobacteria is less certain (Flemming & Haselkorn, 1974; Murry *et al.*, 1984). Rippka & Stanier (1978) and Spence & Stewart (1987) isolated mutants of *Anabaena* lacking heterocysts in which N_2 -fixation still occurs. Bergman *et al.* (1986) have shown that in lichens and in root nodules of cycads, nitrogenase is restricted to heterocysts only. However, the possibility of N_2 -fixation in vegetative cells of the cyanobiont in *Anthoceros-Nostoc* symbiosis has been suggested (Rodgers & Stewart, 1977).

Immunolabelling experiments on the cyanobiont of *A. punctatus* showed that the nitrogenase was located in heterocysts only (Fig. 2*a*). The pattern and intensity of labelling was similar in the free-living *Nostoc* 7801 (Fig. 2*b*). The distribution of the antigen within the heterocysts was uniform. Labelling in vegetative cells was very low, suggesting that they contained little or no nitrogenase.

As in the *Nostoc* cyanobiont of lichens (Englund, 1977) and cycads (Lindblad *et al.*, 1985), the heterocyst frequency of the cyanobiont in bryophytes can vary with the age of the colony (Rodgers & Stewart, 1977). A maximum heterocyst frequency of 43%, as against 3–6% in the free-living *Nostoc*, has been observed in the cyanobiont of *Anthoceros* (Rodgers & Stewart, 1977). However, the increase in nitrogenase activity was much less than the increase in heterocyst frequency (Rodgers & Stewart, 1977), indicating the presence of nonfunctional heterocysts. Immunogold labelling showed that all the heterocysts of the cyanobiont, including the multiple ones, contained the nitrogenase protein (Fig. 2*a, b*). This suggests that the supply of fixed carbon to multiple heterocysts occurring in chains may be restricted. A similar case has been found in the cycad cyanobiont (Bergman *et al.*, 1986). Although the photosynthate is provided by the eukaryotic partner in *Anthoceros* (Rodgers & Stewart, 1977), it probably enters the heterocysts via the vegetative cells. Heterocysts with no direct contact with the vegetative cells may receive little or no fixed carbon. In the cycad cyanobiont, an increase in nitrogenase activity correlates with an increase in the frequency of single heterocysts throughout the root (tip to base), but with increasing number of multiple heterocysts this correlation is lost (Lindblad *et al.*, 1985).

Glutamine synthetase

GS (EC 6.3.1.2) is the primary ammonia-assimilating enzyme in heterocystous cyanobacteria (see Stewart, 1980). In lichens, *Azolla* and hornwort symbioses the GS activity in the cyanobiont is reduced, which may contribute to the liberation of N_2 -derived ammonia from the cyanobiont (see Stewart *et al.*, 1983; Meeks *et al.*, 1985).

In the *Anthoceros-Nostoc* association, GS activity in the cyanobiont has been reported to be reduced three- to fourfold without a similar decrease in the GS content (Joseph & Meeks, 1987). This led to the conclusion that GS activity in the cyanobiont is regulated by a post-translational mechanism. However, the relative distribution of the inactive GS protein between heterocysts and vegetative cells is not known.

Fig. 1. Background (unspecific) labelling due to secondary antibody (goat anti-rabbit IgG, gold conjugated) in the cyanobiont (a) and in the free-living cultured isolate *Nostoc* 7801 (b). In these experiments primary antibody was omitted during immunolabelling. V, Vegetative cells; H, heterocysts; C, cyanophycin. Bar marker, 1 micrometer.

Fig. 2. Localization of nitrogenase in the cyanobiont (a) and in the free-living cultured isolate *Nostoc* 7801 (b). Rabbit anti-*Rhodospirillum rubrum* nitrogenase (Fe-protein) was used as the primary antibody. Other details as in Fig. 1; A, *Anthoceros* tissue.

Fig. 3. Localization of GS in the cyanobiont (a) and in the free-living cultured isolate *Nostoc* 7801 (b). Rabbit anti-*Anabaena* 7120 GS was used as primary antibody. Other details as in Figs 1 and 2.

Table 1. GS content of heterocysts and vegetative cells of the cyanobiont and the free-living cultured isolate *Nostoc* 7801 from *Anthoceros punctatus*

The GS contents given below are comparative and were calculated by counting the number of gold particles per unit cell area using TEM photomicrograph prints (at $\times 15000$ magnification). The data are means $\pm$ SE of 70 counts from 15 photomicrographs taken from five ultrathin sections of three symbiotic colonies.

Sample	No. of gold particles cm^{-2}
<i>Nostoc</i> 7801	
Vegetative cells	8.21 $\pm$ 1.35
Heterocysts	17.30 $\pm$ 3.65
Cyanobiont	
Vegetative cells	6.96 $\pm$ 1.30
Heterocysts	7.15 $\pm$ 1.71

The GS protein was present in both heterocysts and vegetative cells (Fig. 3*a, b*) and its distribution was uniform within the cells. As in other free-living cyanobacteria (Bergman *et al.*, 1985), labelling in *Nostoc* 7801 heterocysts was nearly twofold higher than that in vegetative cells (Fig. 3*b*, Table 1). In contrast, the label in the cyanobiont was similar in heterocysts and vegetative cells (Fig. 3*a*, Table 1). Multiple heterocysts also contained similar amounts of the label. The GS content was similar in vegetative cells of the cyanobiont and *Nostoc* 7801 but the cyanobiont heterocysts had 60% less GS protein than the heterocysts of *Nostoc* 7801. Thus, reduction in GS activity of the cyanobiont is at least partly due to a reduction in the GS content of heterocysts. This contrasts with the situation in lichens and *Azolla*, where reduction in GS activity is due only to decreased synthesis of GS (see Stewart *et al.*, 1983; Nierzwicki-Bauer & Haselkorn, 1986). It also differs from the cycad cyanobiont, which has full GS activity and normal GS content, with a distribution pattern in heterocysts and vegetative cells similar to that in heterocysts and vegetative cells of the free-living isolate (Lindblad & Bergman, 1986). However, the selective decrease in GS content in heterocysts noted here resembles the situation in lichens (Hällbom *et al.*, 1986) and *Azolla* (B. Bergman, unpublished results), where the distribution of residual GS in the cyanobiont is similar in both heterocysts and vegetative cells. It is interesting to note here that when N_2 -grown cultures of *Anabaena cylindrica* are transferred to ammonium medium, the few heterocysts which remain show a GS content similar to that of vegetative cells (E. Renström, unpublished results).

Phycocerythrin

Phycobiliproteins are accessory photosynthetic pigments in cyanobacteria. They also serve as a nitrogen reserve and undergo degradation during nitrogen limitation (Cohen-Bazire & Bryant, 1982; Ho & Krogman, 1982). The cyanobionts in lichens, *Azolla* and cycads possess phycobiliproteins (see Stewart *et al.*, 1983; Lindblad, 1987). However, Stewart & Rodgers (1977) were unable to detect phycobiliproteins in the cyanobiont of *Anthoceros punctatus*, although structures similar to phycobilisomes were noted in their TEM photomicrographs.

Immunolabelling experiments showed that PE is present in the cyanobiont of *A. punctatus* (Fig. 4). The pattern of labelling was similar to that in free-living *Nostoc* 7801. The labelling of PE was associated with thylakoid membranes in vegetative cells. There was comparatively little labelling in heterocysts; this is consistent with the fact that heterocysts are generally deficient in phycobiliproteins (see Stewart, 1980). The intensity of labelling was similar in vegetative cells of the cyanobiont and *Nostoc* 7801. Cyanophycin granules (nitrogen reserves) were also noted in the cyanobiont (see Fig. 1), indicating that the cyanobiont is not nitrogen starved. Considering that the frequency of heterocysts (which have a very low phycobiliprotein content) is eight or nine times higher in the cyanobiont, the overall level of phycobiliproteins would be considerably lower than that of *Nostoc* 7801.

Fig. 4. Localization of PE in the cyanobiont (a) and in the free-living cultured isolate *Nostoc* 7801 (b). Rabbit anti-*Phormidium pericinum* PE was used as primary antibody. Other details as in Figs 1 and 2.

Fig. 5. Localization of RuBisCO in the cyanobiont (a) and in the free-living cultured isolate *Nostoc* 7801 (b). Rabbit anti-*Sinapis alba* RuBisCO was used as primary antibody. Other details as in Figs 1 and 2.

Ribulose-1,5-bisphosphate carboxylase/oxygenase

RuBisCO (EC 4.1.1.39) is the primary carboxylating enzyme in cyanobacteria, and cyanobacterial carboxysomes (polyhedral bodies) contain RuBisCO (see Codd & Marsden, 1984). The cyanobiont of *Anthoceros punctatus* has been shown to be deficient not only in photosynthetic O₂-evolution but also in CO₂-fixation (Rodgers & Stewart, 1977).

In both the cyanobiont and the free-living *Nostoc* 7801, RuBisCO was present in vegetative cells, with little or no labelling in heterocysts (Fig. 5a, b). This is consistent with the reported absence of RuBisCO in heterocysts (Codd *et al.*, 1980; Cossar *et al.*, 1985). The labelling within the vegetative cells was most prominent in carboxysomes, although label was also present in the cytoplasm. The pattern of labelling was similar in the cyanobiont and *Nostoc* 7801. Thus, the inability of the cyanobiont, in *A. punctatus*, to fix CO₂ is not due to lack of RuBisCO. In the case of the cycad cyanobiont, *in vitro* RuBisCO activity is similar to that of the free-living isolate; however, there is no *in vivo* CO₂-fixation by the cyanobiont (Lindblad *et al.*, 1987).

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