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# Immunogold Localization of Hydrogenase in the Cyanobacterial-Plant Symbioses *Peltigera canina*, *Anthoceros punctatus* and *Gunnera magellanica*

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## Abstract

Using immunoelectronmicroscopy, localization and levels of hydrogenase were studied in the cyanobionts of three cyanobacterial-plant symbioses: *Peltigera canina*, *Anthoceros punctatus* and *Gunnera magellanica*. Free-living (cultured) *Nostoc* isolates from all the three symbioses showed hydrogenase label in both vegetative cells and heterocysts; the former having consistently higher label than the latter. A similar pattern and level of hydrogenase labelling was found in the cyanobiont cells residing in *Anthoceros punctatus* tissue. In contrast, labelling in cyanobiont cells of *P. canina* and *G. magellanica* was much lower than that in the cells of their respective cultured cyanobacterial isolates. In the cultured cyanobacterial isolates as well as in the cyanobiont cells of all the symbioses studied, a higher intensity of hydrogenase labelling was observed along the plasma membranes between vegetative cells. The eukaryotic partners did not show any hydrogenase antigen in any of the symbioses studied. These data suggest lack of hydrogenase in the eukaryotic partners of all the three symbioses studied here and a reduction in the levels of hydrogenase protein in the cyanobionts of *P. canina* and *G. magellanica* but not of *A. punctatus*. The data are discussed in relation to the cyanobionts mode of carbon nutrition and oxygen levels in these cyanobacterial-plant symbioses.

Keywords: *Anthoceros punctatus*, cyanobacteria, *Gunnera magellanica*, hydrogenase, *Nostoc*, *Peltigera canina*, symbiosis

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## 1. Introduction

Hydrogenases are important in diazotrophs for the recycling of hydrogen evolved during nitrogen-fixation. It has been suggested that such a recycling improves the nitrogen-fixing efficiency because it regenerates ATP and reductant, consumes oxygen and thereby contributes to the oxygen protection of nitrogenase, and prevents buildup of hydrogen which is an inhibitor of nitrogenase and photosynthesis (see Adams et al., 1981; Lambert and Smith, 1981; Houchins, 1984; Antarikanonda et al., 1980). Hydrogen metabolism has been studied extensively in diazotrophic bacteria (Adams et al., 1981; Gogotov, 1986; Evans et al., 1987, 1988), cyanobacteria (Lambert and Smith, 1981; Houchins, 1984; Papen et al., 1986; Almon and Boger, 1988; Rao and Hall, 1988; Chen et al., 1989; Kentemich et al., 1989; Ewart and Smith, 1989a,b, 1990), and rhizobial and actinorhizal symbioses (Evans et al., 1987, 1988; Mellor and Werner, 1990; Huss-Danell, 1990). The aim has been to understand nitrogenase-hydrogenase relationships and to increase the efficiency of nitrogen-fixation in economically important plants like legumes and actinorhizal trees.

Cyanobacteria form diazotrophic symbioses with eukaryotic plants ranging from algae to angiosperms. In these symbioses, the cyanobacterial partner (cyanobiont) undergoes several structural and metabolic modifications resulting in increased rates of of nitrogen-fixation and transfer of fixed nitrogen from the cyanobiont to the eukaryotic partner (Stewart et al., 1983). Cyanobacterial symbioses have been studied extensively with regard to nitrogen and carbon metabolism (see Rai, 1988, 1990). A few studies have been done on hydrogen metabolism and localization of hydrogenase in the free-living (cultured) cyanobacterial isolates from cycads (Kumar et al., 1986; Daday and Smith, 1987; Lindblad and Sellstedt, 1990; Tredici et al., 1990) and *Azolla* (Chan-van-Ni and Gogotov, 1984). However, information about occurrence and localization of hydrogenase in cyanobacterial symbioses are virtually nonexistent, except for preliminary studies about hydrogen uptake and evolution in cycad root nodules (Perraju et al., 1986) and *Azolla* (Peters et al., 1977).

The aim of the present study was to investigate the occurrence and localization of hydrogenase in cyanobacterial symbioses, and to compare the levels of hydrogenase in free-living and symbiotic cyanobacteria. To cover the variety of conditions encountered by cyanobacteria in cyanobacterial-plant symbioses, three cyanobacterial symbioses and their free-living (cultured) cyanobionts were studied (*Peltigera canina* and *Nostoc* PC; *Anthoceros punctatus* and *Nostoc* ANTH; *Gunnera magellanica* and *Nostoc* GM). Our data indicate a significant reduction in the levels of hydrogenase protein in symbiosis which

correlates with reduced oxygen tention but not with the mode of carbon nutrition.

## 2. Materials and Methods

*Organisms*

*Nostoc* PC (the isolate from *P. canina*), *Nostoc* ANTH (the isolate from *A. punctatus*) and *Nostoc* GM (an isolate from *G. magellanica*) were grown axenically in BG-$11_0$ medium (Rippka et al., 1979) at 25°C and in continuous light (photon fluence rate: 50 $\mu$mol $m^{-2}s^{-1}$). Cells were harvested during mid log phase. *P. canina* and *A. punctatus* thalli were collected locally from the field. *G. magellanica* was grown outdoors, using non-fertilized sandy soil, in the Botanical Garden. In the cases of *A. punctatus* and *G. magellanica*, *Nostoc*-infected tissues were carefully separated using a scalpel and a stereomicroscope. Cells of the cultured isolates, small pieces of *P. canina* thallus, and the *Nostoc*-infected tissues of *A. punctatus* and *G. magellanica* were fixed, embedded, sectioned and immunolabelled as described below.

*Hydrogenase antibodies*

Hydrogenase antibodies, raised in rabbit against the hydrogenase holoenzyme purified from *Alcaligenes latus*, were a gift from Dr. D.J. Arp (University of California, USA; Doyle and Arp, 1987). These antibodies have been shown to be monospecific, recognizing a single polypeptide of Mr 55 kDa, and have been used for specific localization of hydrogenase in *Nostoc* PCC 73102 (Lindblad and Sellstedt, 1990).

*Immunogold labelling*

Fixation, embedding, sectioning and immunolabelling protocols were essentially the same as described previously (Bergman et al., 1985) with the following differences. Rabbit anti-*Alcaligenes latus* hydrogenase was used as primary antibody at a dilution of 1:1000 and the labelling with the primary antibody was done overnight at 4°C. The secondary antibody used was 1:20 dilution of goat anti-rabbit IgG conjugated to 10 nm size colloidal gold particles (obtained from Amersham International plc, Amersham, UK). In control experiments primary antibody was omitted.

*Transmission electron microscopy and estimation of hydrogenase label*

Transmission electron microscopy was performed using a Zeiss EM 10 Transmission Electron Microscope (TEM) operated at 60 kV. Relative levels of hydrogenase label was estimated by counting gold particles in various cell types using TEM photomicrograph prints. These values were converted to number of gold particles per $\mu m^2$ cell area taking into account the magnification of the prints and the cell area counted. A similar exercise was done to calculate background labelling by counting gold particles per unit area outside the cells/tissues.

## 3. Results

*Occurrence and localization of hydrogenase in free-living (cultured) cyanobacterial isolates*

Three *Nostoc* spp., isolated from *P. canina* (*Nostoc* PC), *A. punctatus* (*Nostoc* ANTH) and *G. magellanica* (*Nostoc*GM), and cultured in BG-$11_0$ medium, were used for immunolocalization of hydrogenase. As seen in Fig. 1, hydrogenase antigen was found to be present both in heterocysts and vegetative cells of *Nostoc* PC. Most of the heterocysts observed had a lower density of hydrogenase label as compared to vegetative cells. However, we did find some heterocysts with a labelling density similar to that in vegetative cells. Within the cell, the hydrogenase antigen was evenly distributed with no preferential association with any particular structure. However, a higher intensity of hydrogenase label was found along the plasma membranes, particularly between two vegetative cells and along the plasma membranes being formed during cell division. Similar patterns of hydrogenase labelling were found in *Nostoc* ANTH and *Nostoc*GM. Background (unspecific) labelling, as evidenced from the gold distribution outside the cells were less than 5% of the specific labelling observed inside the cells. In control experiments where the primary antibody was omitted, no gold particles were observed within or outside the cells (data not shown).

A quantitative analysis of the hydrogenase label distribution in heterocysts and vegetative cells showed that in all the cyanobacterial isolates, heterocysts had significantly lower levels of the hydrogenase antigen (per unit cell area) as compared to that in vegetative cells (Fig. 5). This decrease in the hydrogenase antigen level in the heterocysts, as compared to vegetative cells, was 47% in *Nostoc* PC, 70% in *Nostoc* ANTH, and 60% in *Nostoc* GM (Fig. 5).

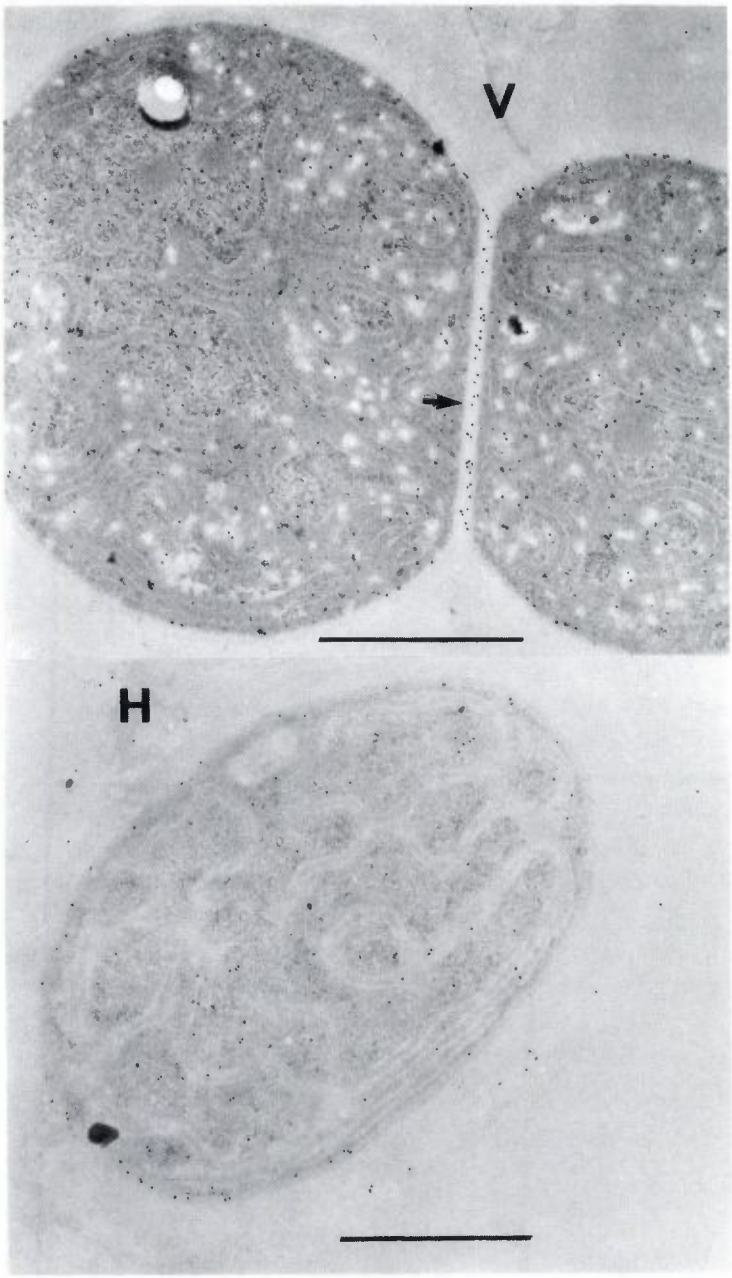


Figure 1. Immunogold localization of hydrogenase in *Nostoc* ANTH, the free-living cultured isolated from *A. punctatus*. Note the higher intensity of labelling along the cytoplasmic membranes at the cell junction (arrow). H, heterocyst; V, vegetative cell. Bar = 1 $\mu$m.

*Occurrence and localization of hydrogenase in* Peltigera canina

In the bipartite cyanolichen *P. canina*, hydrogenase antigen was detected both in heterocysts and vegetative cells of the cyanobiont however, labelling in the mycobiont was similar to the background (Fig. 2). The pattern of

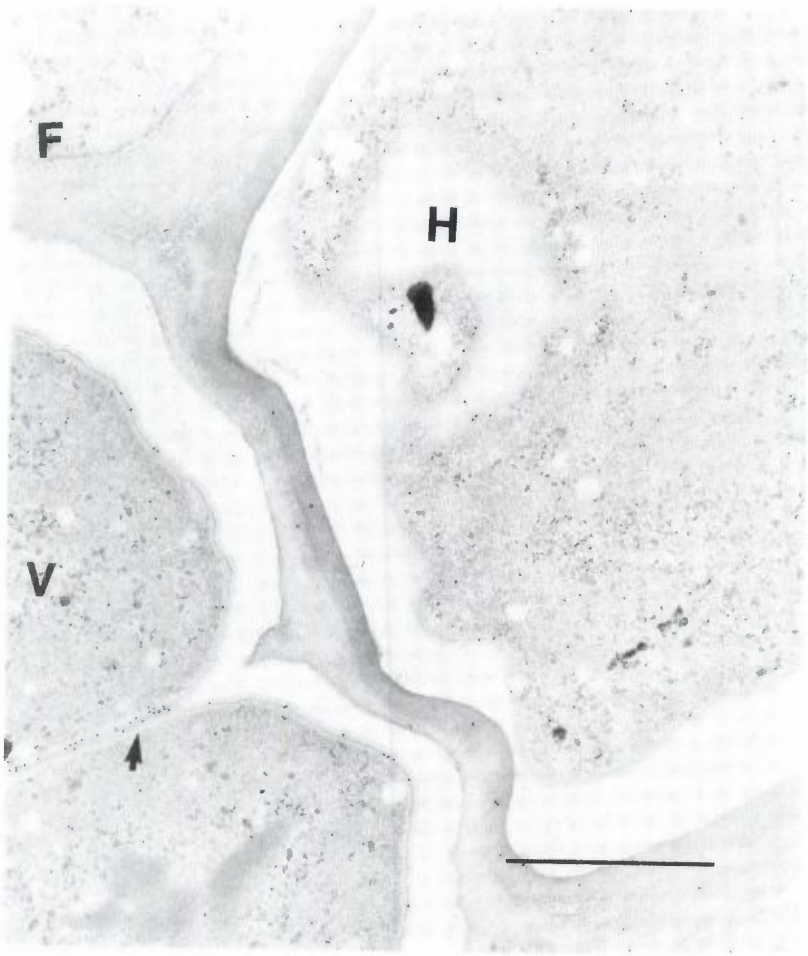


Figure 2. Immunogold localization of hydrogenase in *Nostoc* cells in *P. canina*. F, fungal hypha. Other symbols as in Fig. 1.

labelling in the cyanobiont cells was similar to that in the case of *Nostoc* PC including the high intensity of hydrogenase label along the plasma membranes between two vegetative cells. However, in contrast to the situation in *Nostoc* PC, heterocysts and vegetative cells of the cyanobiont had similar labelling intensities. Quantitative analysis of the hydrogenase antigen levels showed a

significant decrease in hydrogenase antigen level of the cyanobiont as compared to the free-living isolate *Nostoc* PC (Fig. 5). This decrease was much more pronounced in vegetative cells (65%) than in heterocysts (30%).

*Occurrence and localization of hydrogenase in* Anthoceros punctatus-Nostoc *symbiosis*

In the case of *A. punctatus*, hydrogenase antigen was found to be present in the cyanobiont cells but none was detected in the *Anthoceros* tissues (Fig. 3). Within the cyanobiont cells, the pattern of hydrogenase labelling was similar

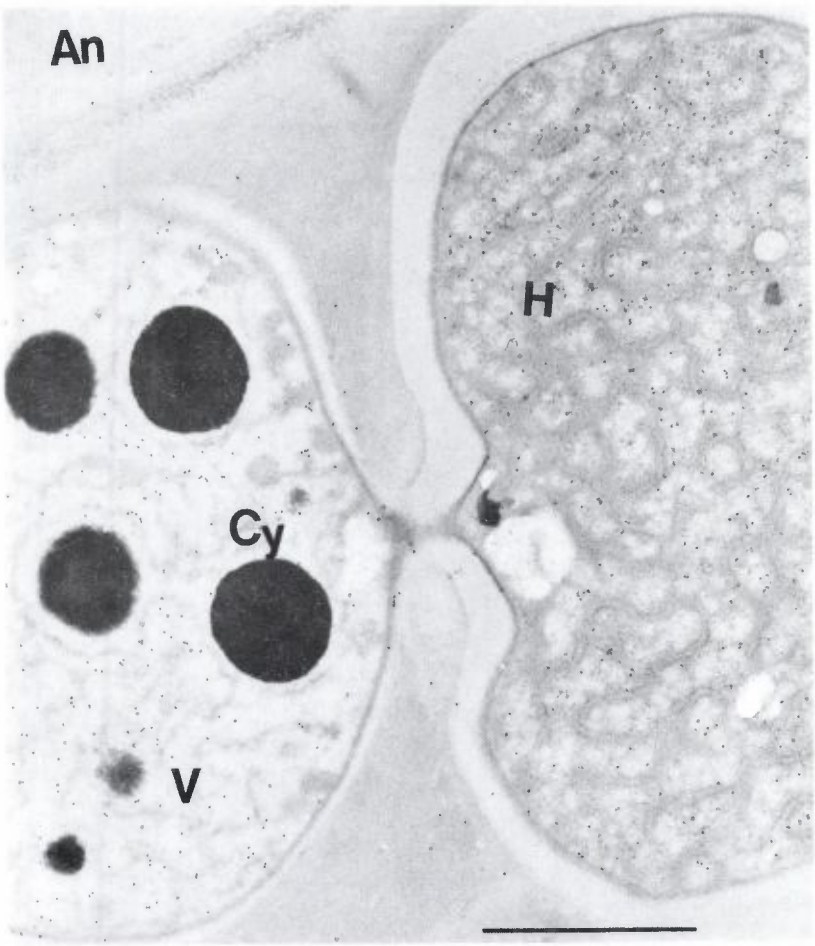


Figure 3. Immunogold localization of hydrogenase in *Nostoc* cells in *A. punctatus*tissue. An, *Anthoceros* tissue; Cy, cyanophycin granule. Other symbols as in Fig. 1.

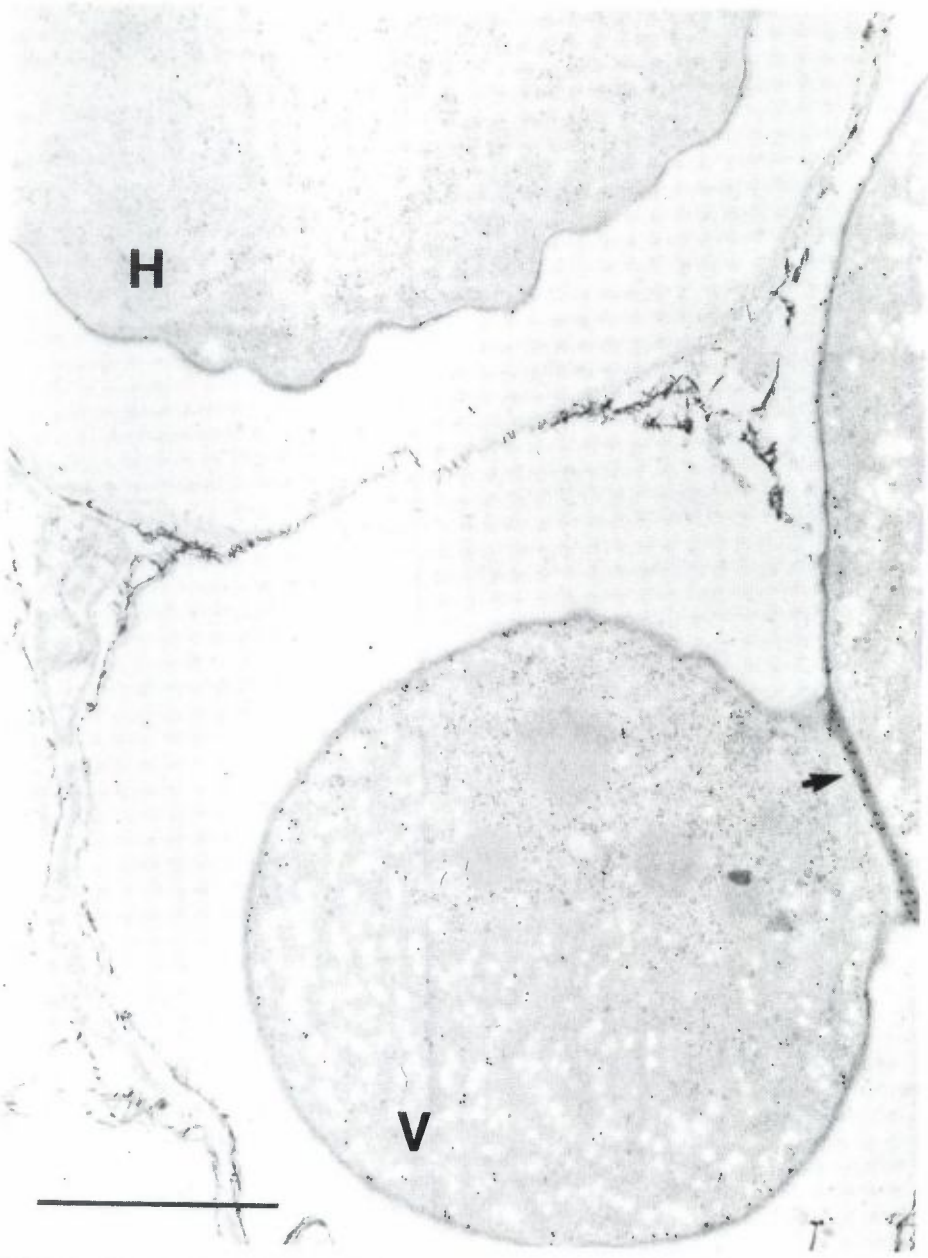


Figure 4. Immunogold localization of hydrogenase in *Nostoc* cells within a *G. magellanica* cell. Symbols as in Fig. 1.

to that in the cultured cyanobacterial isolates mentioned above. As in *Nostoc* ANTH, vegetative cells had a higher intensity of the hydrogenase label than the heterocysts. The hydrogenase antigen levels in the heterocysts of the cyanobiont in *A. punctatus* and *Nostoc* ANTH were comparable. However, the hydrogenase antigen levels in the vegetative cells were 20% lower than that in vegetative cells of *Nostoc* ANTH (Fig. 5). These results indicate that in *A. punctatus*, as in *P. canina*, hydrogenase was located in the cyanobiont cells and absent in the eukaryotic partner. However, in contrast to the situation in *P. canina*, there was little or no decrease in the hydrogenase levels of the cyanobiont in *A. punctatus*.

*Occurrence and localization of hydrogenase in* Gunnera magellanica-Nostoc *symbiosis*

In *Gunnera-Nostoc* symbiosis, the cyanobiont occurs intracellularly (Bonnett, 1990). Immunolabelling studies of *Nostoc* infected *G. magellanica* cells showed presence of hydrogenase antigen in the cyanobiont cells but no labelling was detectable in *Gunnera* cells (Fig. 4). The pattern of labelling was similar to that in the free-living (cultured) isolates, but the hydrogenase antigen levels were much lower in the cyanobiont cells (both in heterocysts and vegetative cells). A quantitative analysis of the hydrogenase label showed that hydrogenase level in heterocysts was 60% lower than that in vegetative cells. This is similar to the situation in the free-living (cultured) cyanobacterial isolate of *G. magellanica* (*Nostoc* GM; see Fig. 5). However, in symbiosis, there was a 75% decrease in the hydrogenase levels of both the heterocysts and the vegetative cells (Fig. 5). This decrease in the hydrogenase levels in the cyanobiont cells of *G. magellanica*bear resemblance to the situation in *P. canina* but differ from the situation in *A. punctatus*.

## 4. Discussion

In the present study we have discussed our data without making a distinction between uptake and reversible hydrogenases because of the following reasons. First, earlier studies on hydrogen uptake and evolution activities suggested occurrence of two hydrogenases in heterocystous cyanobacteria: a cytoplasmic reversible hydrogenase located both in heterocysts and vegetative cells and a membrane-bound uptake hydrogenase located in heterocysts (see Houchins, 1984). However, recent studies have shown hydrogen uptake by nitrogenase in heterocysts (Chen et al., 1986; Almon and Böger, 1988) and presence of both uptake and reversible hydrogenase activities in membrane bound as well

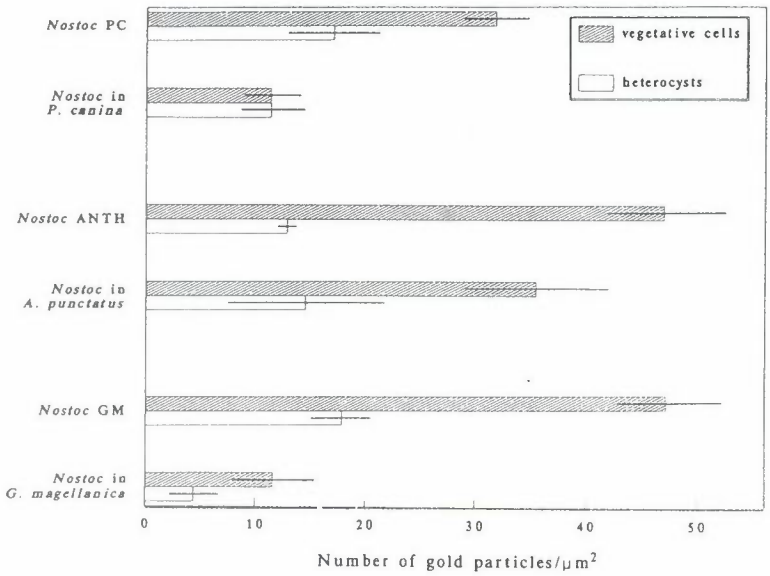


Figure 5. Cellular distribution and quantitative estimates of hydrogenase label in free-living (cultured) and symbiotic cyanobionts of *P. canina*, *Anthoceros punctatus*, and *G. magellanica*. In each case three grids containing several sections each were immunolabelled and 50–100 $\mu m^2$ cell area was used for counting gold particles (gold labelling associated with the plasma membranes was excluded in these counts). These values were plotted after subtracting the values for background labelling. The latter did not exceed 5% of the specific labelling. Bars indicate standard deviation.

as in soluble fractions (see Houchins, 1984; Rao and Hall, 1988; Ewart and Smith, 1989a,b; Kentemich et al., 1986; Papen et al., 1986). Indeed there have been suggestions of a single hydrogenase in cyanobacteria which may occur in different forms, working either in the direction of hydrogen oxidation or hydrogen evolution, depending on the physiological conditions (Adams et al., 1981; Lambert and Smith, 1981; Houchins, 1984). In view of this, the subcellular localization data (heterocysts vs vegetative cells or membrane-bound vs cytoplasmic) can not be interpreted in terms of uptake and reversible hydrogenases. Second, *A. latus* hydrogenase antibodies recognize a polypeptide of approximately 55 kDa in *Nostoc* 73102 cell extracts, which may correspond to the 50 kDa polypeptide reported to be present in both uptake and reversible hydrogenase proteins of *Anabaena cylindrica* (Lindblad and Sellstedt, 1990; Ewart and Smith, 1989a,b). Indeed the large subunits of uptake and reversible hydrogenases in cyanobacteria have molecular weights in the same range (see Houchins, 1984; Rao and Hall, 1988; Kentemich et al., 1989; Ewart and Smith,

1989a,b). Thus, in studies using antibodies raised against the large subunit, one can not exclude the possibility that the antibody recognizes both uptake and reversible hydrogenase antigens even if such antibodies recognize only one polypeptide.

Our results showing presence of hydrogenase both in heterocysts and vegetative cells, and the higher intensities of hydrogenase label along the plasma membranes (Figs. 1–4) are similar to the observations of Lindblad and Sellstedt (1990) in *Nostoc* 73102. However, in contrast to *Nostoc* 73102, where heterocysts and vegetative cells had similar levels of hydrogenase (Lindblad and Sellstedt, 1990), a higher levels of hydrogenase was found in vegetative cells than in heterocysts of all the three free-living (cultured) *Nostoc* cyanobionts studied here (Fig. 5). This difference in our findings may reflect the fact that different *Nostoc* strains were used in the two studies. Alternatively, it may be due to the difference in the immunolabelling protocols. For instance, the lower density of label in our studies may be better in highlighting the differences in subcellular levels of the hydrogenase antigen. We also found that while most heterocysts had lower levels of hydrogenase label than vegetative cells, a few heterocysts did have levels similar to that in vegetative cells. It is possible that hydrogenase levels are reduced during heterocyst differentiation and that young heterocysts still retain hydrogenase levels similar to that in vegetative cells. Such a decrease in hydrogenase levels of heterocysts may be the result of decreased synthesis of one or both hydrogenases if indeed heterocystous cyanobacteria have two hydrogenases.

Hydrogenase was absent in the eukaryotic partners of all the three cyanobacterial-plant symbioses (*P. canina*, *A. punctatus* and *G. magellanica*). This is consistent with the fact that among eukaryotic organisms only algae are known to have hydrogenase (see Adams et al. 1981). A comparison between the free-living and the symbiotic *Nostoc* in *A. punctatus* showed almost similar levels of hydrogenase as well as the labelling pattern (Figs. 3, 5). However, while the intensity of hydrogenase label along the plasma membrane remained unchanged, there was a significant decrease in the cytoplasmic levels of hydrogenase in the cyanobionts of *P. canina* and *G. magellanica*, particularly in the vegetative cells (Figs. 2, 4, 5). These results suggest that the synthesis of cytoplasmic hydrogenase, but not the plasma membrane associated hydrogenase, is significantly decreased in the cyanobionts of *P. canina* and *G. magellanica*. Such a decrease seems to correlate with a lowered oxygen tension. In *P. canina* and *G. magellanica* the cyanobionts reside in respiring nonphotosynthetic tissues but in *A. punctatus* the cyanobiont is surrounded by photosynthetically active tissue (see Stewart et al., 1983; Rai, 1988). This may also explain the

lower levels of hydrogenase in heterocysts which are known to have lower oxygen tension than that in vegetative cells (see Stewart, 1980). There has been an earlier suggestion that heterotrophic model of carbon nutrition may repress uptake hydrogenase in symbiotic cyanobacteria (Perraju et al., 1986) but this does not seem to be the case here. This is because the mode of carbon nutrition in cyanobionts of both *A. punctatus*and *G. magellanica* is heterotrophic but only in *G. magellanica* the cyanobiont showed decreased levels of hydrogenase. Furthermore, a decreease was also seen in the cyanobiont of *P. canina* which is autotrophic. Another possibility that Ni availability may explain this decrease was ruled out because while Ni is necessary for the hydrogenase activity, it is not necessary for hydrogenase synthesis (Ewart and Smith, 1989b).

Overall, our studies show that hydrogenase is present both in heterocysts and vegetative cells, the latter having higher levels of the enzyme; that in symbiosis the eukaryotic partners lack hydrogenase; and that while the levels of plasma membrane bound hydrogenase are similar in free-living and symbiotic cyanobionts, there is a decrease in the cytoplasmic levels of hydrogenase in cyanobionts occurring under microaerobic conditions. The fact that vegetative cells have nearly double the amount of hydrogenase than heterocysts, indicates an important role of the enzyme in cyanobacteria which is not directly related to nitrogen-fixation as suggested earlier.

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