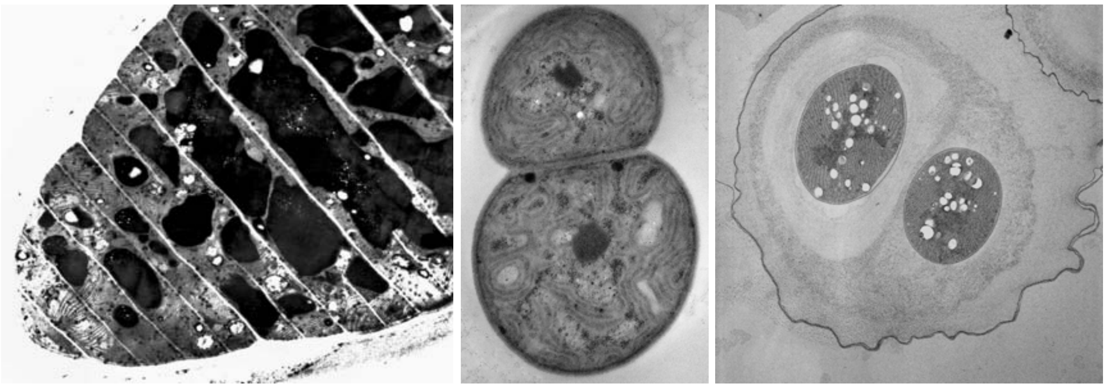
Rui Seabra Alves Martinho

Immunolocalization of the uptake hydrogenase in the marine *Lyngbya majuscula* CCAP 1446/4 and other cyanobacteria



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To Catarina

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ABSTRACT

In N_2-fixing cyanobacteria the reduction of N_2 to NH_3 is coupled with the production of molecular hydrogen (H_2), which is rapidly consumed by an uptake hydrogenase, an enzyme that is present in almost all the diazotrophic cyanobacteria. The cellular and sub-cellular localization of the cyanobacterial uptake hydrogenase remains unclear, and the previously available data focuses mainly on heterocystous cyanobacteria.

This work presents the first effort to localize the uptake hydrogenase in a non-heterocystous cyanobacterium, *Lyngbya majuscula* CCAP 1446/4. The data obtained revealed higher specific labelling associated with the thylakoid membranes of *L. majuscula*. As cyanobacteria exhibited a broad morphological diversity, and that may influence the cellular and sub-cellular localization of the uptake hydrogenase, a comparative analysis was performed using a unicellular - *Gloeothece* sp. ATCC 27152, and two heterocystous strains - *Nostoc* sp. PCC 7120 and *Nostoc punctiforme* PCC 73102. No labelling was found on the vegetative cells of *Nostoc* sp. PCC 7120, in contrast with the situation in *N. punctiforme*, in which labelling was detected in both vegetative cells and heterocysts.

In addition, sequences of *nifK*, structural gene encoding the β subunit of the nitrogenase (enzymatic complex responsible for N_2-fixation), were obtained for *Lyngbya* spp.. This work will allow additional studies, notably on the correlation between nitrogen fixation and hydrogen metabolism in non-heterocystous cyanobacteria, a line of research that is being followed by other members of the team.

Unexpectedly, the images of *L majuscula* grown in medium containing combined nitrogen using electron microscopy, revealed the presence of an extremely high number of large cyanophycin granules, opening a window of opportunity for future research.

The results presented within this work will contribute to a better understanding of the field of H_2 metabolism in cyanobacteria, particularly in the non-heterocystous strains effecting a temporal separation between the photosynthesis and the N_2 fixation/H_2 uptake processes.

RESUMO

Em cianobactérias fixadoras de azoto, a redução do azoto atmosférico a amónia é acompanhada pela produção de hidrogénio molecular. Este H_2 é rapidamente consumido por uma hidrogenase de assimilação, enzima que está presente, praticamente, em todas as cianobactérias diazotróficas. De momento, desconhece-se a localização precisa da hidrogenase de assimilação, a nível celular e sub-celular, em cianobactérias, embora haja alguns dados referentes especialmente a cianobactérias heterocísticas.

Com este trabalho pretendeu-se localizar a hidrogenase de assimilação numa cianobactéria não-heterocística, *Lyngbya majuscula* CCAP 1446/4, utilizando anticorpos específicos para a subunidade maior da enzima desta cianobactéria. Os resultados obtidos revelam a presença de um maior grau de marcação específica associada às membranas dos tilacóides, o que está de acordo com o facto da hidrogenase de assimilação ter sido previamente descrita como uma enzima ligada a membranas. Uma vez que as cianobactérias exibem uma ampla diversidade morfológica, e que esse facto pode influenciar a localização celular e sub-celular da hidrogenase de assimilação, foi realizada uma análise comparativa utilizando a cianobactéria unicelular *Gloeothece* sp. ATCC 27152, e as cianobactérias heterocísticas *Nostoc* sp. PCC 7120 e *Nostoc punctiforme* PCC 73102. Não foi encontrada qualquer marcação nas células vegetativas de *Nostoc* sp. PCC 7120, contrastando com o verificado para *N. punctiforme*, onde foi detectada marcação tanto nas células vegetativas como nos heterocistos. Estes resultados confirmam que a hidrogenase de assimilação está apenas presente nos heterocistos de *Nostoc* sp. PCC 7120, em contraste com *N. punctiforme*, no qual antigénios HupL foram detectados quer nas células vegetativas quer nos heterocistos. Contudo, com o trabalho efectuado não foi possível concluir se se trata ou não de uma forma activa da enzima. Os trabalhos referentes à cianobactéria unicelular estão, ainda, a decorrer.

Em paralelo com os trabalhos de imuno-localização, foram identificados e sequenciados os genes *nifK* (gene estrutural que codifica a subunidade β da nitrogenase - o complexo enzimático responsável pela fixação do azoto), em duas estirpes de *Lyngbya*. Estas sequências tornarão possível a realização de estudos suplementares, principalmente sobre a correlação entre a fixação de azoto e o metabolismo do hidrogénio em cianobactérias não-heterocísticas, uma linha de investigação que está a ser seguida por outros membros da equipa.

As imagens de microscopia electrónica de transmissão de *L. majuscula* cultivada em meio com nitrato, revelaram a presença de um número extremamente elevado de grandes grânulos de cianoficina, um polímero de reserva das cianobactérias que pode

ser utilizado na indústria para produzir bioplásticos, abrindo uma janela de oportunidade para investigações futuras.

Os resultados apresentados neste trabalho irão contribuir para uma melhor compreensão do metabolismo de hidrogénio em cianobactérias, particularmente em estirpes não-heterocísticas que levam a cabo uma separação temporal entre a fotossíntese e a fixação de azoto/assimilação de hidrogénio.

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INTRODUCTION

1 INTRODUCTION

Cyanobacteria are a wide and diverse group of prokaryotic photosynthetic microorganisms that mainly use water as the electron donor for the photosynthetic process, leading to oxygen production. This characteristic, and the presence of *a* and/or *b* chlorophyll, differentiate the cyanobacteria from other photosynthetic bacteria (Whitton and Potts, 2000). Moreover, the fact that respiration and photosynthesis occur in the same compartment, distinguishes them from the eukaryotic algae (Durán *et al.*, 2004).

Members of this group of microorganisms can be found in almost any ecological niche from fresh to salt water, terrestrial, and extreme environments (Whitton and Potts, 2000). The most primitive organisms attributed to this group were found in sedimentary rocks, dated from ca. 3500 million years ago (Schopf, 2000), and their ancestors most probably played a key role in the introduction of oxygen into the atmosphere (Mulkidjanian *et al.*, 2006). It is believed that, after a long period of endosymbiosis, the ancestors of actual cyanobacteria have evolved to become plastids, since there is a great resemblance specially between cyanobacteria and red algae chloroplasts, both at the structural, biochemical and genetic levels (Lopez-Juez and Pyke, 2005; Dyall *et al.*, 2004; Giovannoni *et al.*, 1988).

Besides the large variety of colours they exhibit, as a result of different pigment combinations, cyanobacteria also display a broad morphological diversity, with unicellular, filamentous and colonial forms. Some filamentous strains may form differentiated cells specialized in nitrogen fixation – heterocysts, and spore-like resting cells – akinetes (Whitton and Potts, 2000). The classification of cyanobacteria is mainly based on morphological and developmental differences and consists in five Subsections (Castenholz, 2001), which broadly correspond to the traditional five major groups. These groups are essentially based on their cell division patterns, on their ability to form filaments or not, and on the capacity for those filaments to differentiate heterocysts. The abovementioned groups are: the unicellular Chroococcales (Subsection I), that reproduce by binary fission or budding, and the Pleurocapsales (Subsection II), which divide by multiple fission; the filamentous Oscillatoriales (Subsection III), non-heterocystous dividing in only one plane, the Nostocales (Subsection IV), heterocystous which divide in only one plane and the Stigonematales (Subsection V) which divide in more than one plane (Castenholz, 2001).

Some cyanobacteria, both unicellular and filamentous, can establish symbiotic interactions with a large diversity of hosts (Adams, 2000b). In most cases the benefit to the host is metabolic provision, mostly in the form of combined nitrogen, or occasionally carbon. The benefit to the cyanobacteria is less evident, but probably the enclosed environment provided by the host may offer protection from environmental extremes and from predation (Adams, 2000b).

1.1 Cyanobacteria in the marine ecosystems

The marine environment provides a wide range of habitats, both at biogeochemical and trophic levels, in which the presence of cyanobacteria is almost ubiquitous and periodically dominant in the shape of superficial blooms, thus having a significant impact in microbial productivity, nutrient cycles and in the structure of communities from estuaries, coastal areas and oceans all over the planet (Paerl, 2000).

Cyanobacteria exert their influence in the marine ecosystems in two major ways: (i) they are important, and some times dominant, phototrophic CO_2 fixers and contribute significantly to the primary production and C cycling. The genera *Synechococcus*, *Synechocystis* and *Prochlorococcus* are examples of cosmopolite planktonic cyanobacteria that have been reported as responsible for a large portion (30 to 50%) of the planktonic biomass primary production in waters ranging from oligotrophic open ocean to more eutrophic ecosystems, like the coastal and estuarine areas (Ting *et al.*, 2002; Paerl, 2000); (ii) some cyanobacteria are capable of N_2 fixation, making it bioavailable and therefore contributing substantially – ~35% of the annual nitrogen loading to the Baltic Sea (Tuomainen *et al.*, 2003) – to the input of new nitrogen into the environment. Recent studies suggest that N_2 fixation by unicellular diazotrophic cyanobacteria may be responsible for a large flux of new nitrogen into the upper water column of the Pacific Ocean (Montoya *et al.*, 2004). This process is relevant in extensive oligotrophic ocean areas exhibiting nitrogen deficiency, where the input of ammonia by cyanobacteria extenuates the limitation imposed by the scarcity of nitrogen in growth, sustaining the primary and secondary production of other organisms in the pelagic foodweb (Dyhrman *et al.*, 2006; Mulholland and Capone, 2000; Paerl, 2000). Some cyanobacteria, as for example the filamentous non-heterocystous *Trichodesmium*, contribute substantially to both the primary production and the nitrogen fixation process in the vast oligotrophic regions of the oceans (Capone *et al.*, 1997; Carpenter and Romans, 1991).

The ability to fix nitrogen allows some marine planktonic cyanobacteria genera to proliferate, in the form of superficial blooms, in coastal, estuarine and oceanic areas with chronic deficiencies in nitrogen. Cyanobacterial bloom formation has long been recognized and is depicted throughout history. Blooms of *Trichodesmium* are presumably in the origin of the biblical description of the “blood-red waters” of the Red Sea (Paerl, 2000), as well as of its name (Capone *et al.*, 1997). Extensive blooms of *L. majuscula* have been recorded in Hawaii, Guam, Caribbean, Florida and Australia (Pittman and Pittman, 2005). These blooms can be a public health issue as *L. majuscula* has proven to be a prolific source of novel, structurally diverse secondary metabolites (Burja *et al.*, 2002), some of which are toxic to marine animals, including molluscs, fish and crustaceans, as well as to humans (Osborne *et al.*, 2001). Blooms of this cyanobacterium have been implicated as the causative agent in off-flavour in fish and intoxication due to ingestion of sea-turtle meat and macroalgae epiphytised by *L. majuscula* (Osborne *et al.*, 2001) and have resulted in detrimental impacts to affected areas including: (i) localised seagrass loss, (ii) poor crab and fish harvests (reported by fishermen), (iii) increase in bacterial biomass with bloom decomposition and (iv) significant localised input of bioavailable nitrogen through nitrogen fixation and release of organic and inorganic nitrogen through decay (Watkinson *et al.*, 2005). In addition, the local economy can suffer due to affected commercial and recreational fisheries, declining recreational use of the region due to health concerns, and the removal of large beach wracks of decaying *L. majuscula* by local government for health and aesthetic reasons (Watkinson *et al.*, 2005).

1.2 Nitrogen fixation

The biochemical machinery required for nitrogen fixation is provided by the nitrogenase enzymatic complex, consisting of two component metalloproteins: the dinitrogenase reductase (Fe-protein) and the dinitrogenase (MoFe-protein), that catalyse the ATP-dependent reduction of nitrogen (N_2) to ammonia (NH_3) (Rees *et al.*, 2005; Berman-Frank *et al.*, 2003; Halbleib and Ludden, 2000). The dinitrogenase reductase is a homodimer of about 64 kDa encoded by the *nifH* gene, and the dinitrogenase is a heterotetramer of approximately 250 kDa encoded by the *nifD* and *nifK* genes and contains molybdenum as part of the FeMo-cofactor, the site of substrate reduction (Figure 1) (Rees *et al.*, 2005; Berman-Frank *et al.*, 2003; Halbleib and Ludden, 2000). The structural genes of the nitrogenase complex represent one of the most highly conserved gene groups in bacteria (Böhme, 1998). Some cyanobacterial strains were found to be able to express alternative nitrogenases that are homologous to the described enzyme, yet have vanadium or iron substituting the molybdenum (Bergman *et al.*, 1997; Smith and Eady, 1992).

In all N_2-fixing non-heterocystous cyanobacteria studied to date the *nif* structural genes are contiguous and arranged in the order *nifHDK* (Bergman *et al.*, 1997). Such contiguous arrangement of *nif* structural genes resembles that in bacteria and in heterocysts of cyanobacteria but contrasts with the situation in vegetative cells of some heterocystous cyanobacteria, for e.g. *Nostoc* sp. PCC 7120, in which *nifK* is separated by 11 kb from *nifDH* (Bergman *et al.*, 1997). The presence of a DNA element interrupting the *nifHDK* genes has been reported at least in eight strains of *Anabaena* and *Nostoc* (Carrasco *et al.*, 2005). The excision of this element occurs during heterocyst differentiation and is required for the expression of these genes in heterocysts only. So far, there is no evidence for any similar genomic rearrangement in non-heterocystous cyanobacteria (Bergman *et al.*, 1997).

Since nitrogenase is very oxygen labile (Fay, 1992), all diazotrophs must protect the enzymatic complex from the deleterious effects of oxygen (O_2). The occurrence in cyanobacteria of both oxygenic photosynthesis and nitrogen fixation is, therefore, a remarkable achievement. Cyanobacteria have evolved different mechanisms and strategies, ranging from fixing nitrogen only under anoxic conditions to temporal or even spatial separation of nitrogen fixation and oxygen evolution, to protect their nitrogen-fixing machinery not only from atmospheric oxygen but also from the intracellularly generated oxygen (Berman-Frank *et al.*, 2003; Mulholland and Capone, 2000; Böhme, 1998; Fay, 1992). Temporal separation between photosynthetic oxygen evolution and nitrogen fixation seems to be the most common strategy adopted by non-heterocystous cyanobacteria (Misra and Tuli, 2000; Huang *et al.*, 1999; Reade *et al.*, 1999), whereas spatial separation of the two processes is achieved in many filamentous cyanobacteria by differentiation of vegetative cells into cells specialized in nitrogen fixation, i.e., the heterocysts (Golden and Yoon, 2003; Wolk, 2000; Adams, 2000a; Wolk, 1996). A remarkable exception is the marine filamentous non-heterocystous *Trichodesmium*, where a spatial separation occurs between the two processes without any obvious cellular differentiation. In this organism, only a small fraction of cells within a colony or along the filament are capable of nitrogen fixation, and in contrast to the irreversible changes occurring during heterocyst differentiation, those occurring in this cyanobacterium can be reversed (Mulholland and Capone, 2000; Fredriksson and Bergman, 1997; Bergman *et al.*, 1997).

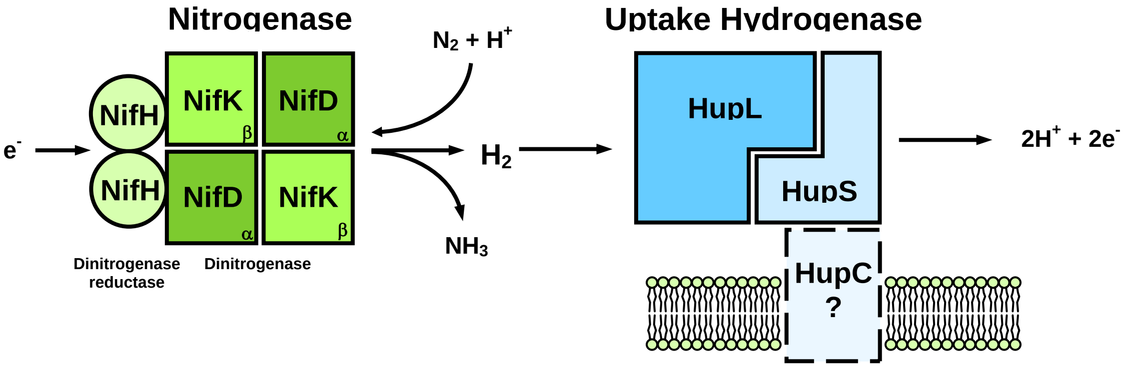

Figure 1. Schematic representation of the subunits and interaction between the nitrogenase and the uptake hydrogenase in N_2-fixing cyanobacteria. The existence of a third subunit anchoring the uptake hydrogenase to the membrane (HupC) is yet to be confirmed (adapted from Leitão, 2005).

1.3 H_2 uptake and the uptake hydrogenase

In cyanobacteria, as in any diazotrophic bacteria, the reduction of N_2 to NH_3 is coupled with the production of molecular hydrogen (H_2) (Hansel and Lindblad, 1998). The H_2 produced by the nitrogenase is rapidly consumed by an uptake hydrogenase, an enzyme that is present in almost all the nitrogen-fixing cyanobacteria (Tamagnini *et al.*, 2005; Tamagnini *et al.*, 2002), with one reported exception – *Synechococcus* sp. BG 043511 (Ludwig *et al.*, 2006). A correlation between the nitrogen fixation process and the uptake hydrogenase activity has been demonstrated for several cyanobacteria (Tamagnini *et al.*, 2005; Masukawa *et al.*, 2002; Happe *et al.*, 2000; Oxelfelt *et al.*, 1995; Weisshaar and Böger, 1985), indicating that the main physiological function of the uptake hydrogenase is to reutilize and regain the H_2/electrons produced by the nitrogenase. This recycling has been suggested to have at least three beneficial functions to the organism (i) it provides ATP via the oxyhydrogen reaction, minimizing the loss of energy; (ii) it removes the oxygen from nitrogenase, thereby protecting it from inactivation; and (iii) it supplies reducing equivalents (electrons) to various cell functions (Bothe, 1982; Bothe *et al.*, 1977).

The NiFe cyanobacterial uptake hydrogenase is at least a heterodimeric enzyme with a large subunit of about 60 kDa containing the active site (HupL), and a small subunit of approximately 35 kDa playing a role in electron transfer (HupS) (Figure 1) (Tamagnini *et al.*, 2002, 2005). The uptake hydrogenase is encoded by the *hup* – hydrogen uptake – genes and their physical arrangement is very similar in all cyanobacteria studied so far: *hupS* and *hupL* are contiguous, with the gene encoding the smaller subunit located upstream from the gene encoding the larger one (Leitão *et al.*, 2005; Oliveira *et al.*, 2004; Happe *et al.*, 2000; Lindberg *et al.*, 2000; Oxelfelt *et al.*, 1998; Carrasco *et al.*, 1995).

Similarly to what has been observed for the nitrogenase structural genes, a rearrangement occurring within the *hupL* of *Nostoc* sp. 7120 has been described by Carrasco *et al.* (1995, 2005). In the vegetative cells of this cyanobacterium, *hupL* is interrupted by a 9.5 kb element that is excised late during the heterocyst differentiation process by a site-specific recombination, therefore allowing its expression in heterocysts only. DNA sequences similar to the gene responsible for the rearrangement within *hupL* – *xisC* – have been identified in several other heterocystous strains (Tamagnini *et al.*, 2000). In contrast, no such rearrangement could be identified in *Anabaena variabilis* ATCC 29143 or *Nostoc punctiforme* PCC 73102 (Happe *et al.*, 2000; Oxelfelt *et al.*, 1998).

1.4 Ultrastructure of cyanobacteria

The cyanobacterial vegetative cells have a distinctive fine structure. Cyanobacteria are considered to be gram-negative bacteria, however the multilayered cell wall combines structural characteristics of gram-negative and some gram-positive bacteria (Hoiczyk and Hansel, 2000), consisting of an outer membrane, and an electron-opaque peptidoglycan layer which varies in thickness between 5 and 10 nm but can be considerably thicker, particularly in some species of *Oscillatoria*. In *Oscillatoria* sp. PCC 6407 the peptidoglycan is approximately 25 nm thick, whereas it reaches 200-250 nm in *Oscillatoria princes* (Stanier, 1988). Many cyanobacteria synthesize outermost investments, mainly of polysaccharidic nature, that differ in thickness and consistency and that can be referred to as sheaths, capsules and slimes (De Philippis and Vincenzini, 1998).

The most conspicuous cytoplasmic elements are the thylakoids (Figure 2). Each thylakoid is formed by two closely appressed membranes; they are the basic structures of oxygenic photosynthesis and contain the chlorophyll protein complexes, carotenoids, the photosynthetic reaction centres, and the electron transport system. In favourable sections, alternate rows of electron-opaque discoidal structures can be distinguished in the interthylakoidal space, namely, the phycobilisomes (PBs), multimolecular complexes of phycobiliproteins, the major light-harvesting pigments of cyanobacteria (Stanier, 1988). Within prokaryotes cyanobacteria are unique in that their highly differentiated internal system of thylakoid membranes that, as demonstrated for *Synechocystis* sp. PCC 6803 ,is discontinuous from the plasma membrane (Liberton *et al.*, 2006).

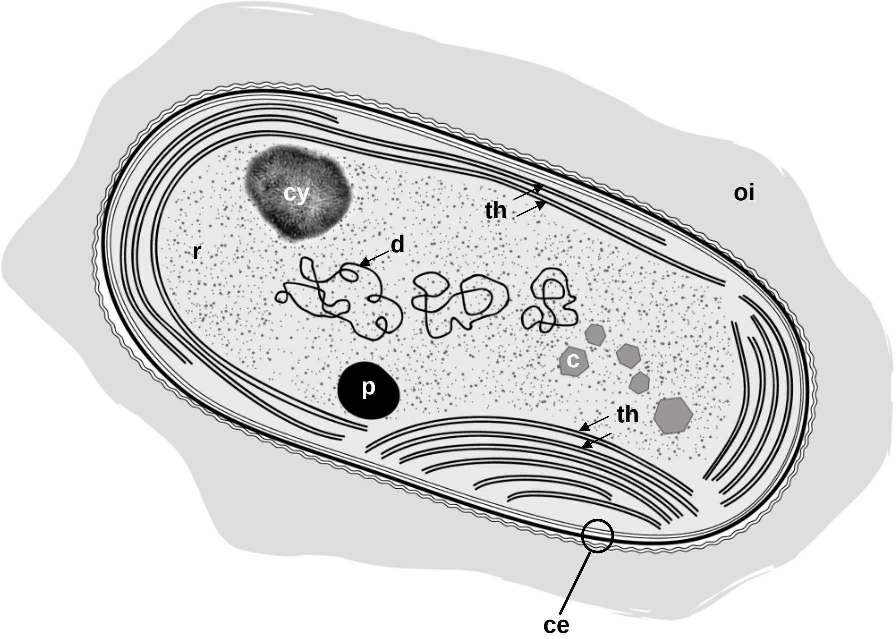

Figure 2. Schematic representation of a thin section of a cyanobacterial cell. ce – cell envelope of the gram-negative type, consisting in an outer membrane, a peptidoglycan layer and the cytoplasmic membrane; oi – outermost investment (sheath, capsule or slime); th – thylakoids; c – carboxysomes; d – DNA; r – ribosomes; cy – cyanophycin inclusion; p – polyphosphate granule.

Different thylakoid arrangements are observed among cyanobacteria. The simplest is characteristic of *Synechococcus* and of many filamentous strains. The cells contain 3 to 6 thylakoids parallel to one another and the outer surface of the cells, enclosing a central region, which contains the DNA, carboxysomes, polyphosphate granules, and many ribosomes (Stanier, 1988). In *Synechocystis* sp. PCC 6803 the thylakoid membrane pairs form layered sheets that follow the periphery of the cell and converge at various sites near the cytoplasmic membrane. At some of these sites, the margins of thylakoid membranes associate closely along the external surface of rod-like structures termed thylakoid centers, which sometimes traverse nearly the entire periphery of the cell. The thylakoid membranes surrounded the central cytoplasm that contained inclusions such as ribosomes and carboxysomes (van de Meene *et al.*, 2006).

In other cyanobacteria, unicellular and filamentous, particularly in heterocystous strains, the thylakoids are convoluted and occupy a very large portion of the cytoplasm. In unicellular strains of this type, the carboxysomes are often large and are usually located near the periphery of the cell, close to the cytoplasmic membrane; ribosomes and DNA fibrils are localized between the folds of the convoluted thylakoids. Arrangements of thylakoids between these two extreme types are frequent (Stanier, 1988). Only one cyanobacterium, *Gloeobacter violaceus*, has been identified that does not contain thylakoids. The only membrane present in this cyanobacterium is the cytoplasmic membrane, which plays a dual role: that of a typical cytoplasmic membrane and that of a photosynthetic membrane (Nakamura *et al.*, 2003).

The ultrastructure of a cyanobacterial cell is also characterized by the presence of a variety of cytoplasmic inclusions, for e.g. carboxysomes, glycogen granules, cyanophycin inclusions and polyphosphate granules. **Carboxysomes** are paracrystalline aggregates of the key enzyme of CO_2 fixation via the reductive pentose phosphate pathway, ribulose-bisphosphate carboxylase (Stanier, 1988; Shively, 1974; Shively *et al.*, 1973). They are easily distinguishable in thin sections, where they appear limited by a unilamellar protein shell or coat, and exhibit a granular substructure of medium electron density (Kaneko *et al.*, 2006; Stanier, 1988; Shively, 1974). Carboxysomes are absent from fully differentiated heterocysts that do not fix CO_2 (Stanier, 1988).

Glycogen is a general carbohydrate reserve material of cyanobacteria. Its accumulation is visible in thin sections as electron-transparent irregular dots, often located between the thylakoids and prominent in nitrogen-limited photosynthesizing cells. In exponentially growing cells, glycogen deposits are rarely visible (Schneegurt *et al.*, 2000; Stanier, 1988; Shively, 1974). In *Gloeobacter violaceus*, the glycogen deposits are scattered in the cytoplasm (Stanier, 1988).

Cyanophycin is a nitrogenous organic reserve whose distribution seems to be limited to cyanobacteria (Oppermann-Sanio and Steinbüchel, 2002). It is composed of long polymeric molecules of multi-L-arginyl-poly(L-aspartic acid) and is located only inside the producing cell (Oppermann-Sanio and Steinbüchel, 2002). Cells approaching the stationary phase of growth in light-limiting conditions accumulate this polymer as cyanophycin granules of irregular contour, often large enough to be resolved by light microscopy, while these granules are rarely present in exponentially growing cells (Oppermann-Sanio and Steinbüchel, 2002; Stanier, 1988). Their denomination as “structured granules” depicts their appearance in the electron microscope, where a radiating pattern of substructure can be resolved, as well as the absence of a surrounding membrane (Oppermann-Sanio and Steinbüchel, 2002). Cyanophycin granules are particularly numerous in akinetes, the resting cells of many heterocystous cyanobacteria (Stanier, 1988). In heterocysts the cyanophycin granules are primarily associated with the polar nodule and the neck, which connects the heterocyst to the adjoining vegetative cell (Sherman *et al.*, 2000).

Among other inclusions are **polyphosphate granules**, which are often volatilized under the electron beam in thin sections, leaving an empty space limited by a thin electron-opaque line. *Gloeobacter violaceus* contains particularly large polyphosphate granules that do not seem to be easily volatilized by the electron beam. **Lipid droplets** appear as electron-opaque, small, circular objects of variable size frequently seen among the thylakoids. **Gas vesicles** are found in some aquatic species of cyanobacteria and transiently during hormogonia differentiation of some filamentous heterocystous strains. Their fine structure in thin sections is characteristic: in longitudinal sections, they appear as small, empty cylinders with conical ends limited by a thin membrane; in cross section, they appear as circles. The gas vesicles of a particular species appear to have the same approximate dimensions (diameter and length), probably determined by the three-dimensional structure and packing of their major constituent protein (Stanier, 1988).

1.5 Cellular/sub-cellular localization of the uptake hydrogenase

The cellular/sub-cellular localization of the cyanobacterial uptake hydrogenase remains a controversial subject. Since the physiological and biochemical data point out to a membrane-bound enzyme (Rai *et al.*, 1992; Lindblad and Sellstedt, 1990; Houchins, 1984; Houchins and Burris, 1981a), and the hydropathy profiles of the HupL and the HupS proteins do not indicate any transmembrane domains (Tamagnini *et al.*, 2005), the existence of a polypeptide that anchors the HupSL heterodimer to the membrane seems likely. In fact, analysis of the available genomes revealed the presence of open reading frames whose products could potentially fulfil this anchoring role (Lindberg, 2003). However, to date no definitive proof was obtained, and the existence of both a soluble and a membrane-bound form of the enzyme cannot be excluded (see for e.g. Houchins and Burris, 1981a).

Immunolocalization studies, using antibodies produced against hydrogenases from other bacteria, showed that hydrogenase antigens are present in both the vegetative cells and heterocysts of *Nostoc punctiforme* PCC 73102 and several symbiotic *Nostoc* strains (Tamagnini *et al.*, 1995; Rai *et al.*, 1992; Lindblad and Sellstedt, 1990). However, these studies do not clarify if the enzyme is in its active form in both cell types. In *Nostoc* sp. PCC 7120 the uptake hydrogenase activity was essentially associated with the particulate fraction of the heterocysts (Houchins and Burris, 1981b), however one must bare in mind that in this strain the *hupL* gene suffers a rearrangement, as mentioned previously, allowing its expression in the heterocysts only. Moreover, the presence/levels of the cyanobacterial uptake hydrogenase are certainly dependent on the growth conditions. All the studies available on the immunolocalization of the uptake hydrogenase in cyanobacteria focus on filamentous heterocystous cyanobacteria, resulting in a lack of knowledge on the localization of this enzyme both in unicellular and filamentous non-heterocystous strains.

1.6 Aims of this work

This work's major aim was to identify the sub-cellular localization of the uptake hydrogenase in the filamentous non-heterocystous cyanobacterium *Lyngbya majuscula* CCAP 1446/4. The antiserum used was raised against the uptake hydrogenase of a cyanobacterium, thus having an increased specificity for the target protein. To that end the following experimental approaches were carried out: (a) transmission electron microscopy (TEM) studies in order to provide detailed insights on the organism's cell structure, and (b) TEM/immunolocalization of the enzyme. As different cyanobacteria exhibit different morphological and physiological characteristics that may influence the cellular and sub-cellular localization of the uptake hydrogenase, it was decided to perform comparison studies using: *Gloeothece* sp. ATCC 27152 (unicellular), *Nostoc* sp. PCC 7120 and *Nostoc punctiforme* PCC 73102 (filamentous heterocystous). The two heterocystous strains were chosen due to the presence (*Nostoc* sp. PCC 7120) or absence (*N. punctiforme*) of a DNA element interrupting the gene encoding the large subunit of the uptake hydrogenase – *hupL* – in the vegetative cells. This element is excised when a vegetative cell differentiates into a heterocyst allowing *hupL* expression in heterocysts only. Consequently, this event is suspected to result in differences in the cellular localization of the uptake hydrogenase.

The uptake hydrogenase is one of the enzymes directly involved in the hydrogen metabolism in cyanobacteria, together with the bidirectional hydrogenase and the nitrogenase, consuming the hydrogen produced by the enzymatic complex responsible for N_2 fixation. In a attempt to compile more knowledge on the H_2 metabolism, and since the genes encoding the uptake hydrogenase - *hupSL* - had already been identified and characterized for *L. majuscula*, it was pertinent to sequence and characterize the structural genes encoding the nitrogenase – *nif*.

2

MATERIAL AND METHODS

2 MATERIAL AND METHODS

2.1 Organisms and growth conditions

The cyanobacteria used in this study were the following: the unicellular *Gloeothece* sp. ATCC 27152/PCC 6909 (*Gloeothece* sp. ATCC 27152 throughout the text; Subsection I; American Type Culture Collection, Rockville, Md., USA); the filamentous non-heterocystous *Lyngbya aestuarii* CCY 9616 (Subsection III; Culture Collection of Yerseke, Yerseke, The Netherlands) and *Lyngbya majuscula* CCAP 1446/4 (Subsection III; Culture Collection of Algae and Protozoa, Norfolk, England, U.K.), and the filamentous heterocystous Anabaena/*Nostoc* sp. PCC 7120 (*Nostoc* sp. PCC 7120 throughout the text; Subsection IV; Pasteur Culture Collection, Paris, France), and *Nostoc punctiforme* PCC 73102 (Subsection IV; Pasteur Culture Collection, Paris, France).

Cells were grown in BG11 (medium with combined nitrogen, 1.5 g L^{-1} of $NaNO_3$) and $BG11_0$ (N_2-fixing conditions, BG11 without $NaNO_3$) (Stanier *et al.*, 1971), at 25º C, under a 12 h light (7-10 µmol photons m^{-2} s^{-1}) / 12 h dark regimen.

2.2 Optical Microscopy (OM)

Cyanobacteria cultures were observed with an Olympus BX50 microscope, and light micrographs were obtained with an Olympus DP50 digital camera.

2.3 Transmission Electron Microscopy (TEM)

2.3.1 Ultrastructure studies

Cells were collected and immediately fixed in 2% glutaraldehyde in 50 mM sodium cacodylate buffer (pH 7.2) for 2 h, washed three times in double strength buffer, post-fixed with 2% osmium tetroxide in 50 mM sodium cacodylate buffer (pH 7.2) for 2 h, washed three times in double strength buffer, and embedded in 3% agarose (except for *L. majuscula* that grows as cohesive mats, making this process unnecessary). The solidified agarose was cut into small blocks for the subsequent processing. The dehydration was performed by using an ethanol series (25-100%; v/v), and once propylene oxide. Samples were embedded in mixtures of propylene oxide and Epon, followed by Epon for at least 24 h before being placed in embedding moulds with Epon and allowed to polymerize at 55 º C. Thin sections were cut with a Leica Reichert Supernova ultramicrotome, and mounted in copper grids. The sections were contrasted with a saturated solution of uranyl acetate for 7 min and with lead citrate (Reynolds, 1963) for 7 min, before being visualized using an electron microscope Zeiss EM C10 operating at 80 kV.

2.3.2 Immunolocalization of the uptake hydrogenase

The samples from the non-heterocystous strains *Gloeothece* and *L. majuscula* were collected at 6 h into the dark phase (see Figure 3), taking into account the temporal separation between the photosynthesis (light) and the nitrogen fixation/hydrogen uptake (dark) and the levels of hydrogen uptake activity which exhibit a maximum towards the end of the dark phase (Leitão *et al.*, 2005) (discussed later). Samples from the heterocystous strains were collected during the light phase. In all cases the cells were grown under N_2-fixing conditions and re-culture into fresh medium 24 h before the harvesting.

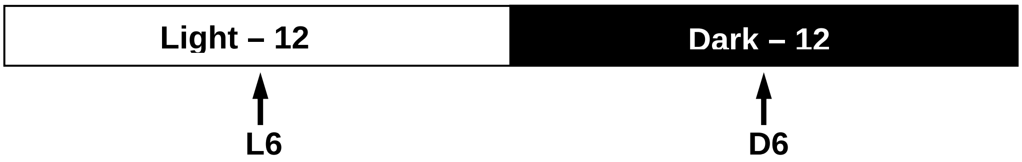

Figure 3. Time point selected for collection of the samples of non-heterocystous strains: D6 – 6h into the dark phase (high H_2 uptake activity). Samples from the heterocystous strains were collected at L6 – 6h into the light phase.

After being harvested, the cells were immediately fixed in 4% formaldehyde and 0.5% glutaraldehyde in 50 mM sodium cacodylate buffer (pH 7.2) for 1 h, then washed three times in double strength buffer, incubated with glycylglycine 1 mM in double strength buffer for 15 min, washed two times with double strength buffer and, if necessary, embedded in 3% agarose (see 3.1). The solidified agarose was cut into small blocks for the subsequent processing. The dehydration was performed by using an ethanol series (25-100%). Samples were embedded in mixtures of ethanol and LR-White resin, followed by LR-White resin for at least 24 h, and placed in gelatine capsules with LR-White resin before polymerization at 55º C. Thin sections were cut with a Leica Reichert Supernova ultramicrotome, and placed in formvar coated nickel grids.

Sections were washed in PBS-T [20 mM phosphate buffer, pH 7.4 supplemented with 0.9% NaCl (w/v) and 0.1% Tween-20 (v/v)] containing 1% BSA for 5 min and blocked in PBS-T containing 5% BSA for 20 min before being incubated overnight in the primary antiserum 1:150 diluted in PBS-T containing 1% BSA. The sections were then washed six times in PBS-T with 1% BSA for 5 min, incubated with the secondary antiserum diluted 1:20 in PBS-T with 1% BSA for 1 h, washed three times in PBS-T with 1% BSA for 5 min, and washed three times in filtered distilled water for 5 min. If necessary, the sections were contrasted as previously described for 2 minutes, before being visualized using an electron microscope Zeiss EM C10 operating at 80 kV.

The primary antiserum used in this study was previously obtained in our laboratory (Leitão *et al.*, 2005) and consists in polyclonal antibodies raised against *L. majuscula* HupL (large subunit of the uptake hydrogenase). To produce these antibodies, a recombinant protein comprising amino acids 1 to 521 and His-tagged was overexpressed in *Escherichia coli*, purified and injected in rats. Polyclonal rat-anti-HupL antiserum was collected, and the specificity of the antibodies was confirmed by testing them against both the recombinant purified protein and *L. majuscula* protein extracts in Western blotting. A single polypeptide of about 60 kDa was recognized in all samples (for details see Leitão *et al.*, 2005). The secondary antiserum used was a Goat-anti-Rat Immunoglobulin G, conjugated to 10 nm colloidal gold particles (Agar Scientific Ltd., UK). Controls were performed by substituting the primary antiserum with a similar amount of pre-immune serum.

2.4 Identification and sequencing of nitrogenase structural genes (*nifHDK*) in *Lyngbya majuscula* CCAP 1446/4 and *Lyngbya aestuarii* CCY 9616

2.4.1 Design of primers

To determine the sequence of the structural genes encoding the Mo-nitrogenase complex in *Lyngbya* spp., primers against conserved regions within the *nifHDK* genes from *Anabaena* sp. PCC 7120, *Fischerella* UTEX1931, and *Cyanothece* ATCC 51142 were designed and synthesized (Table 1, next page).

Table 1 – Oligonucleotide primers used for the amplification of fragments within *nifK* of *Lyngbya* spp.

Primers[a]	Position 5'-3' (bp)[b]	Sequence 5' → 3'
nifK0F[c]	*nifK* 265-285	`CAAGGTTCTCAAGGTTGCGTT`
nifK1F[c]	*nifK* 265-285	`CAAGGTTCTCAAGGTTGTGTG`
LMnifK2F	*nifK* 597-618	`GCTCAAGAGCATTCTGTCAGAT`
nifK3'F	*nifK* 651-672	`CGGCAAAATCAACTTCATCCCT`
nifK3'R	*nifK* 671-649	`GGGATGAAGTTGATTTTGCCGTT`
nifK4R	*nifK* 1403-1382	`TGTAAGTGGTGGCGATCAAAGA`

[a] F - forward) and R – reverse, designations refer to primer orientations in relation to the frame of the gene; LM- refer to *L. majuscula* specific primers
[b] the positions are referred to the positive strand of the coding sequences
[c] forward primer nifK01F is an equimolar mixture of nifK0F and nifK1F

2.4.2 Genomic DNA extraction

Subsequently, genomic DNA was extracted according to the methods described previously (Tamagnini *et al.*, 1997). To extract genomic DNA , the cells were resuspended in 50 mM Tris-HCl buffer containing 10 mM EDTA (pH 8.0), and disrupted by adding 0.6 g of 0.6-mm acid washed glass beads (Sigma, UK), 25 μL of 10% SDS, and 500 μL of a mixture of phenol-chloroform [1:1 (v/v)] and vortexing at high speed. The phases were separated by centrifugation at 14000 *g* for 15 min, and the upper aqueous phase was extracted twice with an equal volume of chloroform. The DNA was precipitated with 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol at -20° C for approximately 1 h before being washed, dried, and resuspended in water.

2.4.3 Polymerase Chain Reaction (PCR)

PCRs were carried out in the thermal cycler GeneAmp PCR system 2400 (Perkin-Elmer, Inc., Wellesley, MA) using 0.5 U of *Taq* polymerase (GE Healthcare, UK), 1x PCR buffer [10x PCR buffer is 500 mM KCl, 15 mM MgCl2, and 100 mM Tris HCl (pH 9.0), GE Healthcare, UK], 200 μM dNTPs, 1 μM of each primer (see Table 1), and 0.1-10 ng of genomic DNA. The PCR profile was: 40 cycles of 94° C for 1 min, 50° C for 1min, and 72° C for 1 min, followed by an extension at 72° C for 7 min.

2.4.4 Agarose gel electrophoresis

Agarose gel electrophoresis was performed by standard protocols using 1X TAE buffer (Sambrook *et al.*, 1989). Bands of DNA were visualized with the fluorescent dye ethidium bromide by direct examination of the gel under UV light.

2.4.5 DNA purification from agarose gel, sequencing and sequence analysis

DNA fragments were isolated from agarose gels using the GFX PCR – DNA and Gel Band Purification kit (GE Healthcare, UK), according to the manufacturer’s instructions. DNA fragments were sequenced at STAB Vida (Lisboa, Portugal), published sequences were retrieved from GenBank, and computer-assisted sequence comparisons were performed using ClustalW (Thompson *et al.*, 1994).

3

RESULTS AND DISCUSSION

3 RESULTS AND DISCUSSION

In N_2-fixing cyanobacteria, as in other diazotrophs, concomitantly with the conversion of atmospheric nitrogen into ammonia, molecular hydrogen is produced. This H_2 is recycled by an uptake hydrogenase. The sub-cellular location of this enzyme in cyanobacteria is still under discussion, and at the time this work started, the information regarding its localization in non-heterocystous cyanobacteria was virtually inexistent. Recently, the transcription regulation and the expression of the uptake hydrogenase was characterized for *Gloeothece* sp. ATCC 27152 – unicellular, and *Lyngbya majuscula* CCAP 1446/4 – filamentous non-heterocystous (Leitão *et al.*, 2005; Oliveira *et al.*, 2004). The major aim of this work was the sub-cellular localization of the uptake hydrogenase in *L. majuscula*, and for the comparison studies were chosen *Gloeothece* sp. ATCC 27152, and the heterocystous *Nostoc* sp. PCC 7120 and *Nostoc punctiforme* PCC 73102 (see Aims). To allow a proper interpretation of the immunolocalization data, the morphology and ultrastructure of the selected strains was studied under the same physiological conditions, and the methodology for TEM was optimized for each strain. Additionally, in order to achieve a broader knowledge of the enzymes involved in the H_2 metabolism in non-heterocystous cyanobacteria, the structural genes encoding the nitrogenase, enzymatic complex responsible for the nitrogen fixation, were sequenced and characterized for *Lyngbya* spp..

3.1 Morphology and ultrastructure of *Gloeothece* sp. ATCC 27152

Gloeothece sp. ATCC 27152 is a diazotrophic unicellular cyanobacterium that divides by transverse binary fission in a single plane. Even though it is characterized as being rod-shaped, during exponential growth the cells may be almost spherical (Rippka *et al.*, 2001). A prominent feature of the genus *Gloeothece* is a well defined sheath, that has been characterized by Tease *et al.* (1991). *Gloeothece* sp. ATCC 27152 has also been the subject of studies concerning the nitrogen fixation process (see for e.g. Reade *et al.*, 1999 or Kallas *et al.*, 1983) and its hydrogen metabolism (Oliveira *et al.*, 2004).

The analysis of the images of the structure of *Gloeothece* ATCC 27152 obtained within this study revealed the presence of well-defined sheath layers, separated by a region clearly visible on OM and electron-dense on TEM (Figures 4a and 4b). Within the cell, the thylakoids appeared dispersed throughout the cytoplasm in a conspicuous manner, and parallel to one another (Figure 4b). The remains of polyphosphate granules, which are often volatilized under the electron beam in thin sections, were also visible (Figures 4b and 4c). The images obtained from cells grown with combined nitrogen show the presence of noticeable cyanophycin inclusions (Figure 4c), a nitrogenous organic reserve almost exclusive to cyanobacteria (Neumann *et al.*, 2005), these inclusions are not frequent on images obtained from cells grown under N_2-fixing conditions.

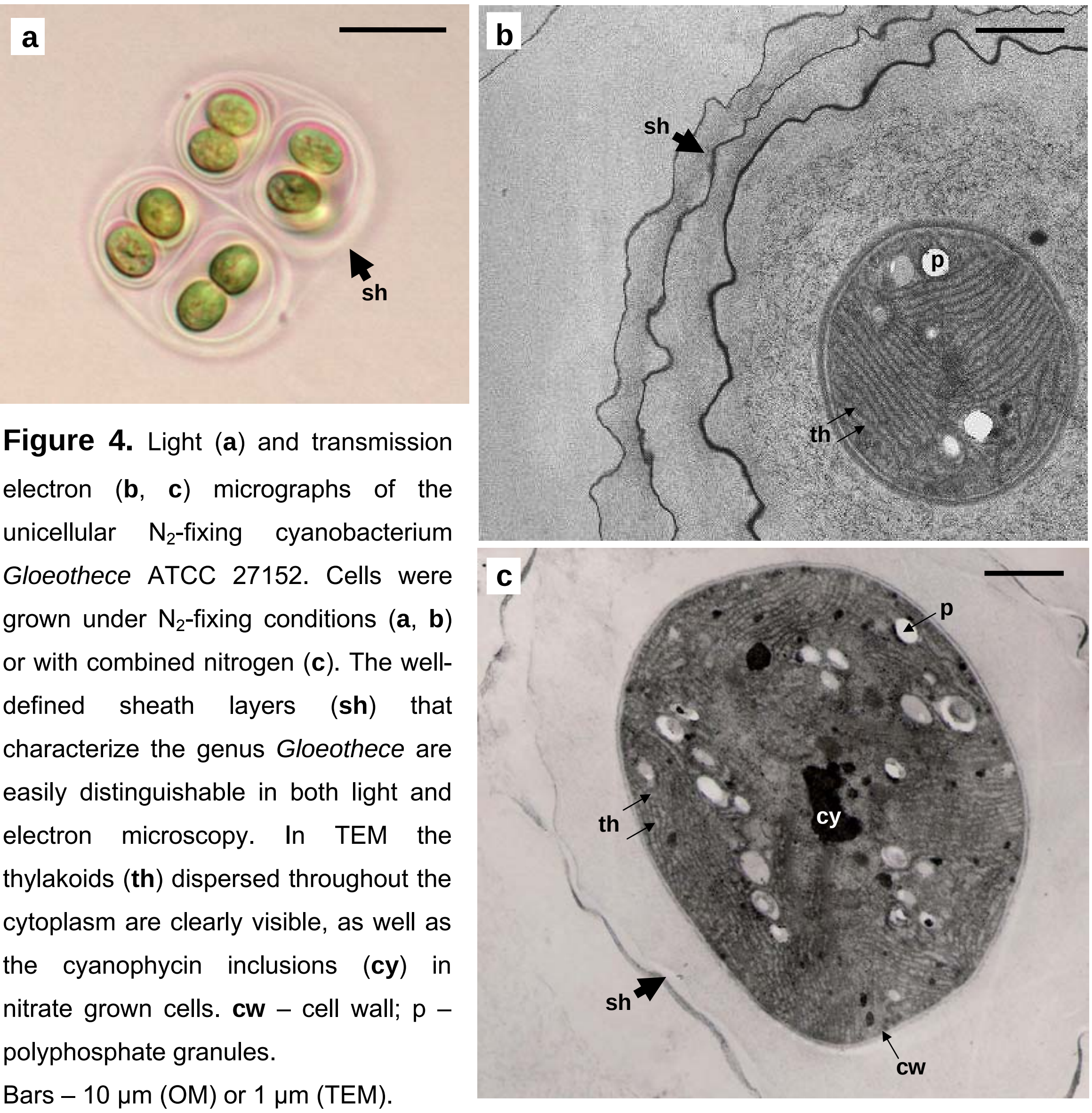

Figure 4. Light (**a**) and transmission electron (**b**, **c**) micrographs of the unicellular N_2-fixing cyanobacterium *Gloeothece* ATCC 27152. Cells were grown under N_2-fixing conditions (**a**, **b**) or with combined nitrogen (**c**). The well-defined sheath layers (**sh**) that characterize the genus *Gloeothece* are easily distinguishable in both light and electron microscopy. In TEM the thylakoids (**th**) dispersed throughout the cytoplasm are clearly visible, as well as the cyanophycin inclusions (**cy**) in nitrate grown cells. **cw** – cell wall; p – polyphosphate granules.
Bars – 10 µm (OM) or 1 µm (TEM).

3.2 Morphology and ultrastructure of *Lyngbya majuscula* CCAP 1446/4

Lyngbya majuscula CCAP 1446/4 is a filamentous non-heterocystous cyanobacterial strain, with disk shaped cells that undergo binary fission in only one plane, that differ from the closely related form-genus Oscillatoria (as defined by Castenholz *et al.*, 2001a) by producing a distinct, persistent, and firm sheath (Castenholz *et al.*, 2001b).

L. majuscula is found worldwide in tropical and subtropical estuarine and coastal habitats (Osborne *et al.*, 2001) and is capable of N_2 fixation (Roelfsema *et al.*, 2005; Omoregie *et al.*, 2004; Bergman *et al.*, 1997) and H_2 production (Kuwada and Ohta, 1987). It grows loosely attached to seagrass, macroalgae, rock, coral and anthropogenic structures forming benthic mats and detached floating masses that may rise to the surface by accumulation of gas bubbles when rapidly photosynthesizing (Roelfsema *et al.*, 2005; Arthur *et al.*, 2005; Stielow and Ballantine, 2003). These masses are generally found in the inter-tidal and sub-tidal habitat, although they have been observed as deep as 30 m (Arthur *et al.*, 2005).

The OM images of *Lyngbya majuscula* CCAP 1446/4 obtained within this work showed a distinct and firm sheath, extending beyond the end of the trichome that exhibits a conical terminal cell (Figure 5a). TEM images further revealed the laminated sheath, as well as the disk shaped nature of the cells. The lamellar system is well developed and constituting a significant part of the cells, with the thylakoids disposed in a radial pattern towards the periphery of the cells, and enclosing a core region where most inclusions are present. Dividing cells are often detected, and numerous incomplete septa can be observed (Figure 5b). Polyhedral carboxysomes could be seen in a number of cells and, as described for *Gloeothece* ATCC 27152, cyanophycin inclusions are mainly present in cells grown with combined nitrogen. However, in *Lyngbya majuscula* CCAP 1446/4 the number and size of these inclusions is remarkably high (Figures 5b and 5c). These findings are of great relevance since cyanophycin can be used to produce homo- and copolymers of poly-aspartate, a plastic-like biomaterial with many applications and, therefore, with biotechnological and economical significance (Neumann *et al.*, 2005; Conrad, 2005). In cells grown under N_2-fixing conditions during a short period of time, it was possible to observe that the cyanophycin inclusions had a “spongy-like” appearance, suggesting they were partially mobilized/metabolized (Figure 5d).

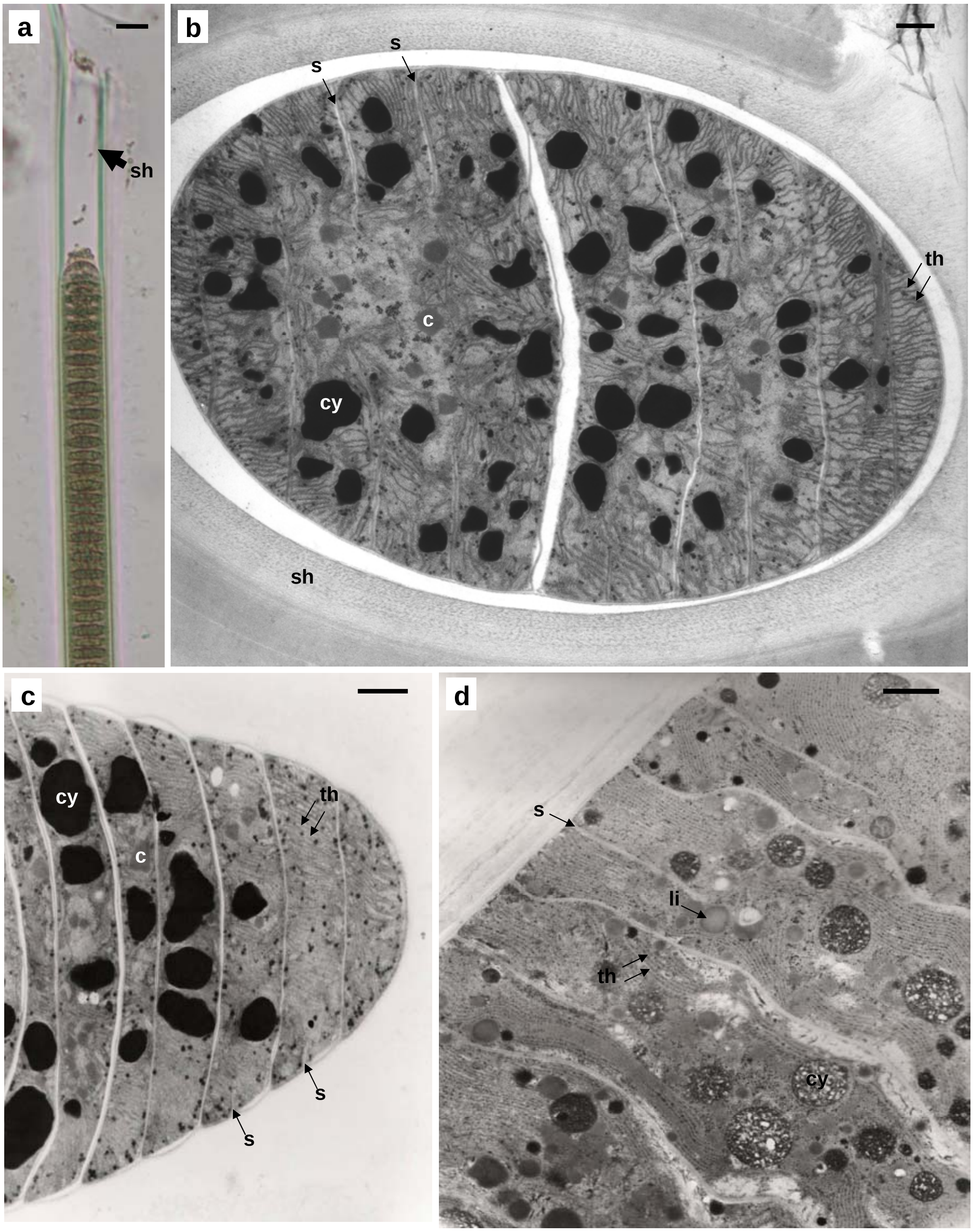

Figure 5. Light (**a**) and transmission electron (**b**, **c**, **d**) micrographs of the filamentous, N_2-fixing cyanobacterium *Lyngbya majuscula* CCAP 1446/4. Cells were grown with combined nitrogen (**a**, **b**, **c**) or under N_2-fixing conditions (**d**). *L. majuscula* filaments are composed of staked disk shaped cells, enclosed by a laminated sheath (**sh**). The conspicuous cyanophycin inclusions (**cy**), clearly visible in cells grown with nitrate (**b**, **c**), are mobilized/metabolized when the cells are grown without combined nitrogen (**d**). **th** – thylakoids; **s** – septum; **c** – carboxysome; **li** – lipid inclusion.

Bars – 10 µm (OM) or 1 µm (TEM).

3.3 Morphology and ultrastructure of *Nostoc* sp. PCC 7120 and *Nostoc punctiforme* PCC 73102

The *Nostoc* genus comprises filamentous heterocystous cyanobacteria that divide exclusively by binary fission in one plane. The trichomes never exhibit basal-apical polarity, and the cells appear uniformly sized (Herdman *et al.*, 2001). It is considered to be one of the most widespread phototrophic taxa known (Dodds *et al.*, 1995). Based on pigments content and DNA similarities, the strains were assigned to several clusters: *Nostoc punctiforme* PCC 73102 belongs to Cluster 1, being its reference strain, while *Nostoc* sp. PCC 7120 belongs to Cluster 3.1 (Herdman *et al.*, 2001). The most striking differences between these strains are: the shape of the cells in the trichomes, *Nostoc* sp. PCC 7120 displays barrel shaped cells, and *N. punctiforme* has the typical spherical/ovoid cells exhibited by the majority of the members of the group; *N. punctiforme* shows a high degree of trichome coiling during some stages of the developmental cycle (Herdman *et al.*, 2001); and their genome sizes are considerably different [6413 Kb for *Nostoc* sp. 7120 (Genomes Online Database: *Nostoc* sp. PCC 7120 Chromosome 1; Kaneko *et al.*, 2001) and 9760 Kb for *Nostoc punctiforme* PCC 73102 (Genomes Online Database: *Nostoc punctiforme* PCC 73102; Meeks *et al.*, 2001)].

As expected, the images obtained by OM and TEM revealed more morphological similarities than differences between the two *Nostoc* strains selected for this study. The trichomes from both strains are composed exclusively of vegetative cells in the presence of combined nitrogen, while in its absence heterocysts differentiate. The latter cell type vary in shape – in *Nostoc* sp. 7120 they appear spherical while in *Nostoc punctiforme* PCC 73102 the heterocysts have an ovoid shape – and both have the characteristic polar nodes and thick envelopes (Figures 6c and 7e). Numerous convoluted thylakoid membranes are visible in the vegetative cells of both strains (Figures 6b and 7b), as well as carboxysomes which were at times found in large numbers (Figures 6d and 7c). As mentioned previously, when grown with nitrate only vegetative cells could be found in samples from both strains, and most of these cells contained large cyanophycin inclusions (Figures 6d and 7f).

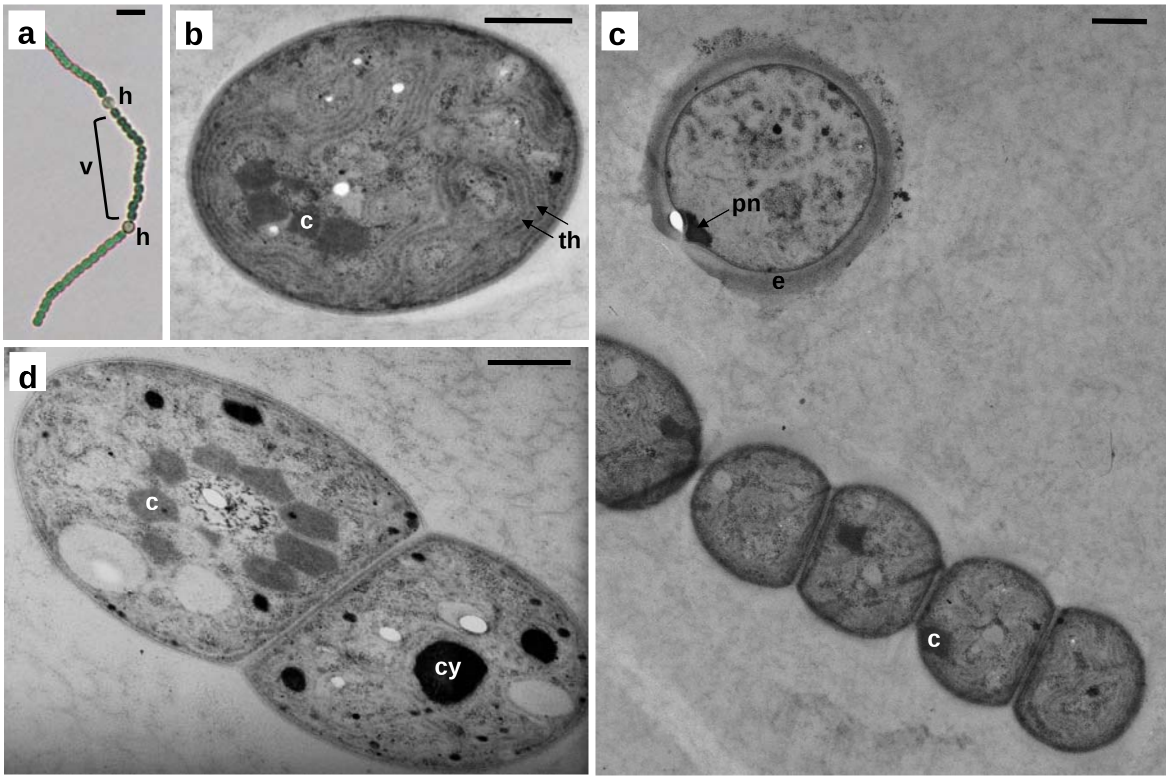

Figure 6. Light (**a**) and transmission electron (**b**, **c**, **d**) micrographs of the filamentous heterocystous cyanobacterium *Nostoc* sp. PCC 7120. The morphologic characteristics of this strain are very similar to the ones exhibited by *N. punctiforme* (see legend of Fig. 3) Cells were grown in N_2-fixing conditions (**a**, **b**, **c**) or with combined nitrogen (**d**). **v** – vegetative cells, **h** – heterocysts, **th** – thylakoids, **c** – carboxysome, **pn** – polar node, **e** – heterocyst's envelope, **cy** – cyanophycin. Bars – 10 µm (OM) or 1 µm (TEM).

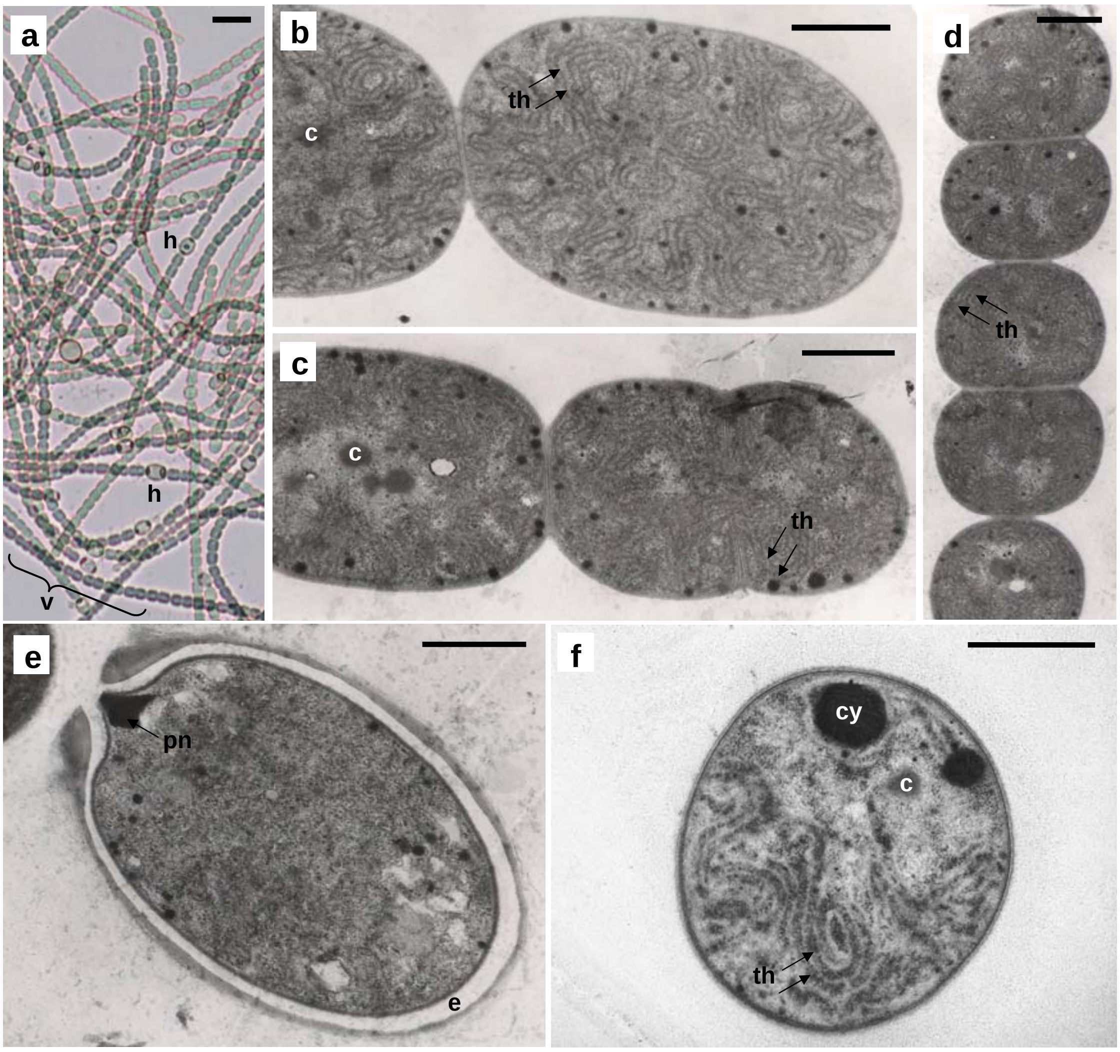

Figure 7. Light (**a**) and transmission electron (**b**, **c**, **d**, **e**, **f**) micrographs of the filamentous heterocystous cyanobacterium *Nostoc punctiforme* PCC 73102. Cells were grown in N_2-fixing conditions (**a**, **b**, **c**, **d**, **e**) or with combined nitrogen (**f**). Filaments of spherical or ovoid shaped vegetative cells (**v**) exhibit conspicuous convoluted thylakoids (**th**) and some carboxysomes (**c**). In filaments grown in N_2-fixing conditions some vegetative cells differentiate into heterocysts (**h**) which possess distinctive structural features such as a thick envelope (**e**) and polar node (**pn**). In cells grown with nitrate the most striking difference is the presence of cyanophycin inclusions (**cy**). Bars – 10 µm (OM) or 1 µm (TEM).

3.4 Immunolocalization of HupL in *Lyngbya majuscula* CCAP 1446/4

To investigate the sub-cellular localization of the uptake hydrogenase in *L. majuscula*, cell grown under N_2-fixing conditions were collected at 6 h into the dark phase. This specific time point was selected since it was previously shown that, under N_2-fixing conditions and a 12 h light/12 h dark regimen, the H_2 uptake activity of *Lyngbya majuscula* CCAP 1446/4 follows a daily pattern with a maximum towards the middle/end of the dark phase, preceded by an increase in the transcript levels during the transition between the light and the dark phase (for details see Leitão *et al.*, 2005). The same authors produced antibodies directed against the large subunit of the enzyme, HupL, and used them to monitor the protein levels throughout the 24 h period. Their results suggest that a protein turnover occurs, with degradation taking place during the light phase, and *de novo* synthesis occurring during the dark phase, in agreement with the pattern of H_2 uptake (Leitão *et al.*, 2005).

In this work, the above mentioned *L. majuscula* anti-HupL antibodies were used (kindly supplied by Elsa Leitão). The analysis of the immunolocalization data showed higher specific labelling associated with the thylakoid membranes, however a lower degree of labelling connected with the cytoplasm and vesicles could also be detected for *L. majuscula* (Figure 8).

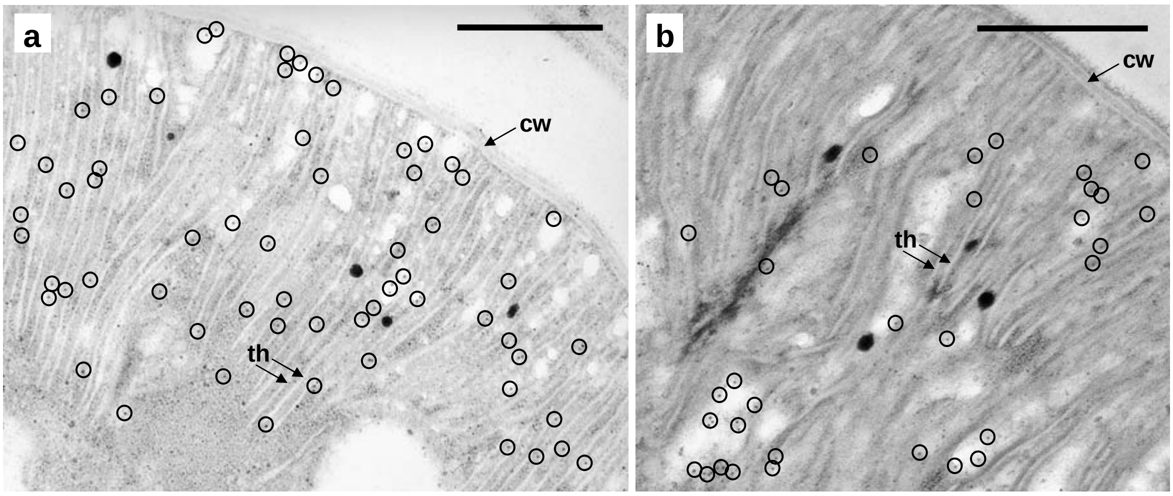

Figure 8. TEM/Immunogold localization of the uptake hydrogenase in *L. majuscula*. Cells were grown under N_2-fixing conditions and collected 6 h into the dark period (a, b). Specific labelling associated with the thylakoid membranes, but also in the cytoplasm and vesicles (encircled). **th** – thylakoids, **cw** – cell wall. Bars – 1 µm.

Previous studies refer the cyanobacterial uptake hydrogenase as a membrane-bound enzyme (Rai *et al.*, 1992; Lindblad and Sellstedt, 1990; Houchins, 1984; Houchins and Burris, 1981b). In agreement with the previously reported, the immunolocalization images of *L. majuscula* showed a higher specific labelling that appears to be associated to the membranes, either thylakoids or vesicles. The presence of labelling associated with vesicles in the cytoplasm is now less intriguing since Nevo *et al.* (2007) have found that these vesicles most probably serve in transport to and from the thylakoids, and often fused with the thylakoid membranes in cyanobacteria. As for the labelling associated with the cytoplasm, it could be explained by the existence of a soluble, and possibly inactive, form of the enzyme, a fact that has not yet been confirmed or excluded (Houchins and Burris, 1981b). In any case, caution needs to be taken when examining the abovementioned results since other factors as for e.g. distinct environmental conditions, like O_2 pressure, could lead to differences on the pattern of sub-cellular localization.

3.5 Immunolocalization of HupL in *Nostoc* sp. PCC 7120 and *Nostoc punctiforme* PCC 73102

For comparative analysis, the same immunolocalization technique was used with *Nostoc* sp. PCC 7120 and *N. punctiforme*, except that the cells were collected in the middle of the light phase, since the literature reported that the *in vivo* hydrogen uptake is stimulated by light (Oxelfelt *et al.*, 1995; Lindblad and Sellstedt, 1990). In *Nostoc* sp. PCC 7120 the labelling was only found in heterocysts, associated with the vesicular region, and absent from the vegetative cells (Figure 9), whereas in *Nostoc punctiforme* PCC 73102 labelling was observed on both cell types (Figure 10), mainly associated with the vesicular region of heterocysts and with the thylakoid membranes of the vegetative cells (Figure 9). The existence of a rearrangement occurring within the *hupL* of *Nostoc* sp. PCC 7120, which allows its expression in heterocysts only (Carrasco *et al.*, 1995), and the absence of such a rearrangement in *Nostoc punctiforme* PCC 73102 (Oxelfelt *et al.*, 1998) may be responsible for the differences observed. Once again, the labelling associated with the vegetative cells of *N. punctiforme* might not be connected to an active form of the enzyme. Moreover, it has been demonstrated that the *hup* genes are exclusively expressed under nitrogen fixing conditions in *N. punctiforme* (Lindberg *et al.*, 2000). Previous immunolocalization studies, using antibodies produced against hydrogenases from other bacteria, showed that the hydrogenase antigens are present in both the vegetative cells and heterocysts of *N. punctiforme*, and several symbiotic *Nostoc* strains (Tamagnini *et al.*, 1995; Rai *et al.*, 1992; Lindblad and Sellstedt, 1990), but the association with membranes was uncertain. However, at that time the authors did not correlate the harvesting of the cells with the H_2 uptake activity, which could provide crucial information. In contrast, the pattern observed for *Nostoc* sp. PCC 7120 is in agreement with all the genetic (rearrangement, see above) and physiological information available. Already three decades ago Houchins and Burris (1981b) reported the activity of the uptake hydrogenase to be confined to the heterocysts of *Nostoc* sp. PCC 7120.

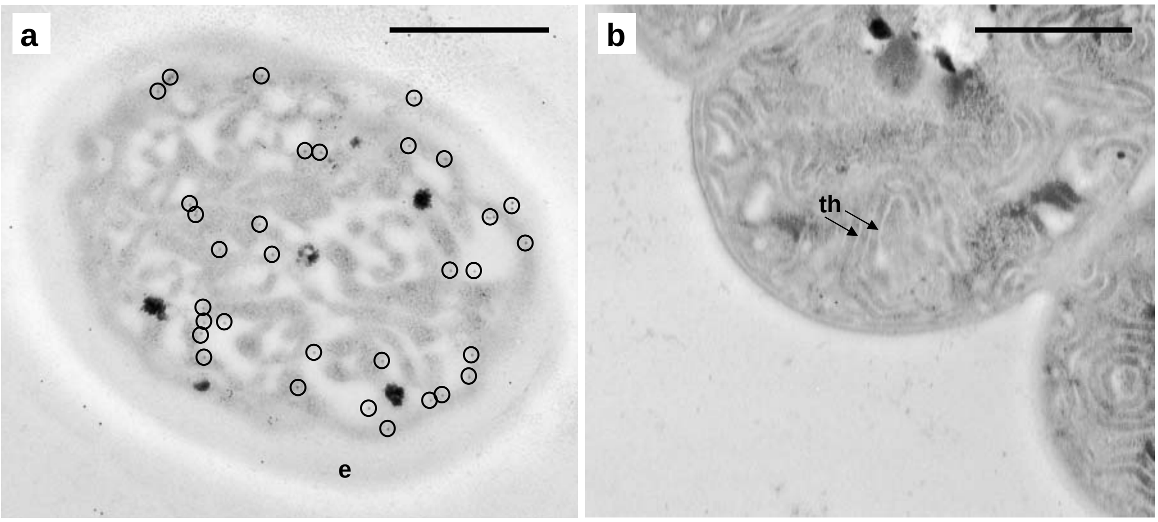

Figure 9. TEM/Immunogold localization of the uptake hydrogenase in *Nostoc* sp. PCC 7120 cells grown under N_2-fixing conditions. Specific labelling associated with the vesicular region of the heterocyst; vegetative cells show no labelling (encircled). Heterocyst (**a**), vegetative cells (**b**). **th** – thylakoids, **e** – heterocyst's envelope. Bars – 1 µm.

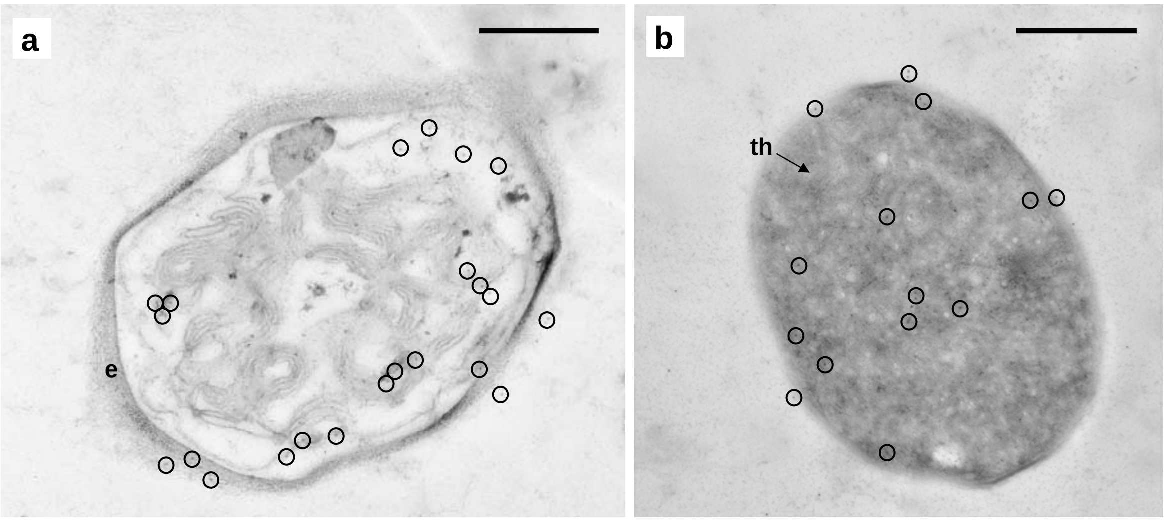

Figure 10. TEM/Immunogold localization of the uptake hydrogenase in *Nostoc punctiforme* PCC 73102 cells grown under N_2-fixing conditions. Specific labelling associated with the vesicular region of the heterocyst; vegetative cells also show labelling (encircled). Heterocyst (**a**), vegetative cells (**b**). **th** – thylakoids, **e** – heterocyst's envelope. Bars – 1 µm.

3.6 Identification and sequencing of nitrogenase structural genes (*nifHDK*) in *Lyngbya majuscula* CCAP 1446/4 and *Lyngbya aestuarii* CCY 9616

The nitrogenase enzymatic complex, which catalyses the ATP-dependent reduction of nitrogen to ammonia, is composed by two metalloproteins named the dinitrogenase reductase (Fe-protein) and the dinitrogenase (MoFe-protein). The dinitrogenase reductase is a homodimer encoded by the *nifH* gene and the dinitrogenase is a heterotetramer encoded by the *nifD* and *nifK* genes.

To determine the sequence of the structural genes encoding the Mo-nitrogenase complex in *Lyngbya* spp., oligonucleotide primers against conserved regions within the *nifHDK* genes from *Anabaena* sp. PCC 7120, *Fischerella* UTEX1931, and *Cyanothece* ATCC 51142 were designed and synthesized. Partial sequences of both *L. majuscula* and *L. aestuarii nifK* were obtained by sequencing PCR products amplified using the designed primers. Subsequently, these sequences were used to complete the *nifK* sequence and to identify other *nif* genes in *L. majuscula* (accession number: DQ78751) and *L. aestuarii* (accession number: DQ375443).

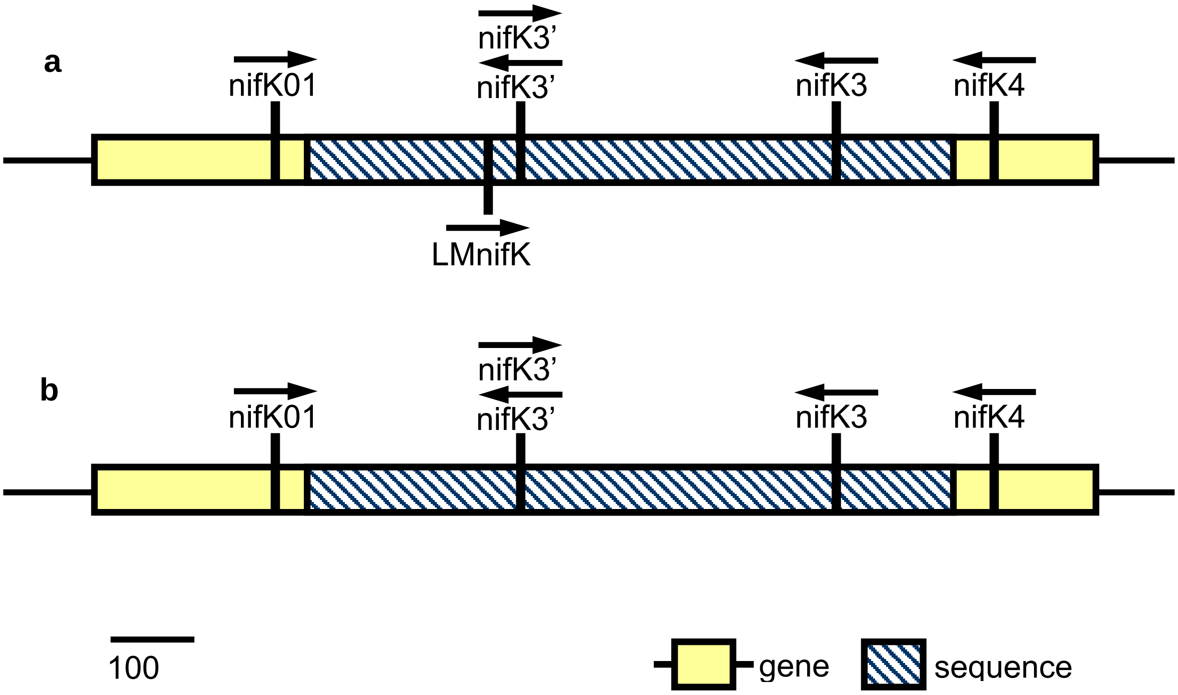

Figure 11. Schematic representation of the *nifK* genes from *Lyngbya majuscula* CCAP 1446/4 (**a**), and *Lyngbya aestuarii* CCY 9616 (**b**). These figures represent the *nifK* genes (yellow), the portion sequenced (shadow), and the primers used to obtain the sequences (vertical bars), as well as their positions and orientation within the *nifK* of both organisms. Bar – 100 bp.

4

CONCLUSIONS AND FUTURE PERSPECTIVES

4 CONCLUSIONS AND FUTURE PERSPECTIVES

HupL specific labelling is associated with the thylakoid membranes of *L. majuscula*

The analysis of the images of the immunolocalization of the uptake hydrogenase in *L. majuscula* revealed higher specific labelling associated with the thylakoid membranes. This is the first study on the immunolocalization of this enzyme in a non-heterocystous cyanobacterium. Future studies will include the purification of cell fractions, like thylakoids, cytoplasmic membrane, and cytoplasmic in order to perform enzyme activity assays and western blots. More time points within a 12 h light/ 12 h dark cycle will be considered for sampling. This will help to clarify the localization of the enzyme and whether or not it exists in a cytoplasmic (inactive) and/or a membrane-bound (active) form.

HupL specific labelling is absent from the vegetative cells of *Nostoc* sp. PCC 7120, and present in both cell types of *Nostoc punctiforme* PCC 73102

No labelling was found on vegetative cells of *Nostoc* sp. PCC 7120, in contrast with the situation in *N. punctiforme*, in which labelling was detected in both cell types. Further studies (similar to that suggested above for *L. majuscula*) should be performed with *N. punctiforme*.

Immunolocalization of the uptake hydrogenase in a unicellular cyanobacterium

The immunolocalization of the uptake hydrogenase in *Gloeothece* sp. ATCC 27152 is currently in progress, and it is expected that the results obtained together with those for *L. majuscula* will allow to reach conclusions about the sub-cellular localization of the uptake hydrogenase in non-heterocystous cyanobacteria effecting a temporal separation between photosynthesis and nitrogen fixation/H_2 uptake.

The sequencing of *nifK* will allow further studies on the H_2 metabolism

The sequences within *nifK* obtained during this work will allow additional studies, notably on the correlation between nitrogen fixation and hydrogen metabolism in *Lyngbya* spp., a line of research that is being followed by other members of the team.

L. majuscula grown with combined nitrogen accumulates high amounts of cyanophycin

The images of *L majuscula* grown in medium containing combined nitrogen, obtained through electron microscopy, revealed the presence of high numbers of large sized cyanophycin granules. Since cyanophycin can be used to produce plastic-like biomaterials, this finding may be interesting for future research and possibly biotechnological exploitation.

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Genomes Online Database: *Nostoc punctiforme* PCC 73102
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