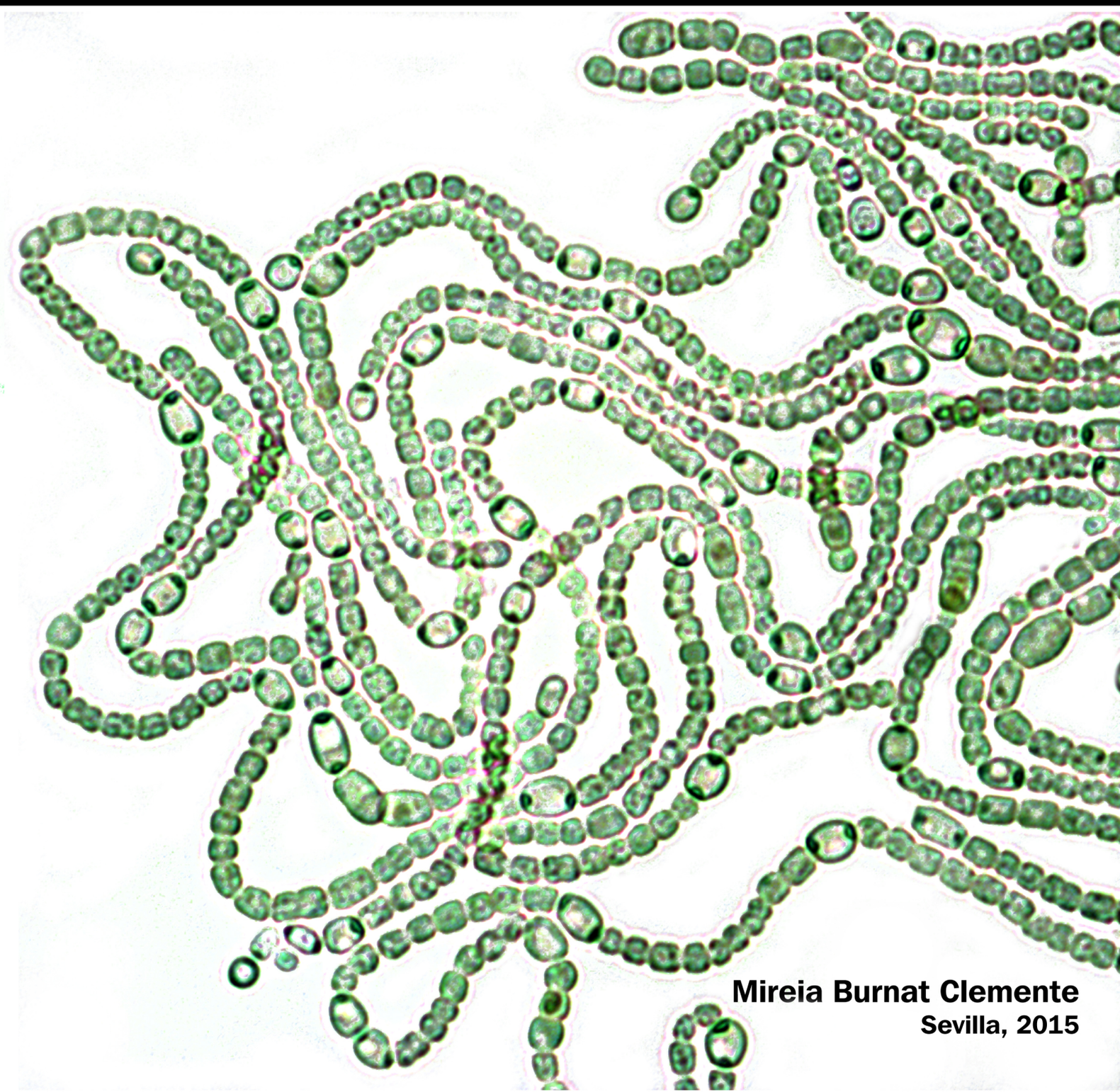
STRUCTURAL AND METABOLIC ASPECTS OF MULTICELLULARITY IN A HETEROCYST-FORMING CYANOBACTERIUM



Mireia Burnat Clemente

Sevilla, 2015

Cover: micrograph of filaments of the heterocyst-forming cyanobacterium *Anabaena* sp. PCC 7120 grown in nitrogen-free medium. *Back cover*: transmission electron micrograph of a heterocyst from *Anabaena* sp. PCC 7120. Micrograph from *Anabaena* was taken in TEM facilities at Göethe Universität Frankfurt am Main. Images have been modified with artistic filters using an image analysis program. Cover and back cover designed by Maria Sabater.

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Structural and metabolic aspects of multicellularity in a heterocyst-forming cyanobacterium

Trabajo presentado para optar al grado de
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A la meva família,
en especial
als meus pares

"Sometime after photosynthesis in the oxygen-poor atmosphere of the early Earth had been well established, however, a kind of blue-green bacteria solved the hydrogen crisis forever. These were the ancestors to modern cyanobacteria. (…) The blue-green alchemists, using light as energy, had extracted hydrogen from one of the planet's richest sources, water itself. This single metabolic change in tiny bacteria had major implications for the future history of all life on Earth."

"Microcosmos"
Lynn Margulis and Dorion Sagan

Agradecimientos

2 Agradecimientos

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Summary

Multicellularity appears to have arisen several times during the course of evolution and has evolved in different phylogenetic groups, including cyanobacteria, a highly diverse group of oxygenic photosynthetic prokaryotes that exhibit a wide range of developmental processes. Cyanobacteria represent one of the most diverse prokaryotic phyla, with morphotypes ranging from unicellular to multicellular filamentous forms. Some filamentous cyanobacteria can produce different types of cells, each one with specific functions. In some cases, cell differentiation allows the filament to carry out tasks that are functionally incompatible, such as oxygenic photosynthesis and the fixation of atmospheric nitrogen. The general plan in this thesis was to address the study of some structural and metabolic aspects of multicellularity in the model heterocyst-forming cyanobacterium *Anabaena* sp. PCC 7120.

Heterocyst-forming cyanobacteria grow as chains of cells (trichomes or filaments), and septal proteins, such as SepJ, are important for cell-cell contact and filament formation. From a structural point of view, cyanobacteria are diderm bacteria, bearing two cellular membranes: the cytoplasmatic membrane and an outer membrane, the latter residing outside of the peptidoglycan layer (or murein sacculus). Filamentous cyanobacteria present a continuous outer membrane along the filament, determining the presence of a continuous periplasmic space that contains the peptidoglycan layer. Although the cell envelope from cyanobacteria has been studied in some detail, the role of this structure in multicellularity has not been addressed until recently. Chapter 1 of this thesis focuses on the possible role of cell envelope components in filamentation, the process of producing and maintaining filaments, thus contributing to the growth of *Anabaena* forming long trichomes. In order to address this structural aspect of multicellularity in *Anabaena* sp. PCC 7120, a set of available peptidoglycan- and outer membrane-related gene mutants and strains with mutations in two genes encoding class B penicillin-binding proteins isolated in this work have been used to study filament length and the response of their filaments to mechanical fragmentation. The results obtained indicate that alteration of both the peptidoglycan layer and the outer membrane influence filamentation, although none of these elements is as important as the septal protein SepJ.

Because of the compartmentalization of photosynthetic CO_2 fixation and N_2 fixation processes in different cell types, an intercellular exchange of nutrients takes place in the cyanobacterial filament. Vegetative cells donate products of CO_2 fixation, such as sucrose, glutamate and alanine to the heterocysts. Heterocysts, in turn, provide N_2 fixation products to the vegetative cells, being glutamine a previously identified metabolite. However, the nitrogenous metabolites that are transferred from heterocysts to vegetative cells are still not fully known. The work presented in Chapter 2 aimed to study the role of cyanophycin, a biopolymer that serves as a dynamic nitrogen cellular reserve material, in the diazotrophic physiology, and of its derivative products as possible nitrogen vehicles in the diazotrophic filament of *Anabaena*. The results confirmed that ORF *all3922* of *Anabaena* sp. PCC 7120 is the gene encoding isoaspartyl dipeptidase, the enzyme involved in the second step of cyanophycin degradation. Under diazotrophic conditions, the enzyme accumulates in vegetative cells, implying that the β-aspartyl-arginine dipeptide produced by cyanophycinase

in heterocysts is transferred intercellularly to the vegetative cells, where it would be hydrolysed releasing aspartate and arginine. Thus, the β-aspartyl-arginine dipeptide has been identified as a nitrogen vehicle in the diazotrophic filament.

Arginine appears to be an important metabolite in the physiology of cyanobacteria, not only because it is found in cyanophycin, but also because it might function as a nitrogen vehicle for intercellular molecular exchange, at least in part as β-aspartyl-arginine. However, arginine catabolism is not well understood in these microorganisms and only few studies have been published regarding arginine catabolic enzymes. In Chapter 3 and Annex II of this thesis, the study of two genes, *alr2310*, encoding an ureohydrolase family protein, and *alr4995*, encoding a protein belonging to the guanidine-group modifying enzymes superfamily, has been addressed in order to investigate their possible roles in arginine catabolism. The results showed that Alr2310 is the *speB* gene of *Anabaena* sp. PCC 7120, encoding an agmatinase, which accumulates preferentially in vegetative cells during diazotrophic growth, and that its inactivation leads to a severe toxic effect that could result from interference of accumulated agmatine with heterocyst differentiation. On the other hand, Alr4995 is a novel enzyme that generates proline from arginine in a two-step reaction, with ornithine (or citrulline) as intermediate metabolite. The concluding remarks of this work is that the heterocyst-forming cyanobacteria present highly coordinated and unique features of compartmentalized metabolic pathways as a strategy of multicellular behavior.

Introduction

1.1. Multicellularity in the microbial world

The notion of bacteria as simple unicellular microbes has persisted over the years and many existing theories of bacterial growth, physiology and genetics have been formulated exclusively in terms of an isolated bacterium. That view started to change when some investigators observed that, in many ways, bacterial cells behave more as components of a multicellular consortium than as autonomous organisms. Observations from a microbial ecology point of view soon enlightened the behavior of bacteria as interactive elements of ecosystems. Studies from Beijerinck and Winogradsky elucidated the behavior of bacteria interacting with other species and playing specific roles in their habitats and ecosystems. Additionally, also under laboratory conditions, observations of some bacterial behaviors, such as colony development of myxobacteria or *Streptomyces*, or the swarming movement of *Proteus* colonies, were seen as coordinated and complex processes more related to multicellular organisms than to unicellular cells (Shapiro, 1988). Because these observations of social behavior were the exceptions in the models of prokaryotes studied in microbiology, bacteria were still seen, in general terms, as simple unicellular organisms. Another aspect that highlight that bacteria are not just single cells is the fact that some bacteria are indeed multicellular, and much effort has been put recently to understand the mechanism(s) and origins of multicellularity in bacteria.

Multicellularity appears to have arisen several times during the course of evolution and has evolved in different phylogenetic groups, including bacteria, fungi, algae, plants and metazoans (Bonner, 1998; Carroll, 2001). Nonetheless, despite their disparate forms and physiologies, the evolution and diversification of all these organisms has followed similar global trends (Carroll, 2001). Bonner (1998) argued that the first step in the evolution of multicellularity was a size increase due to an accident as, for example, a mutation that prevents the daughter cells to divide. Also, size could play a critical role in influencing the degree of differentiation, since the larger the organism, the more cell types it presents (Bonner, 2003). Bacteria are thought to have independently developed complex multicellular behaviors, which has resulted in several different routes that lead to multicellularity, with or without ensuring cellular differentiation, and these manifestations of multicellularity are evolutionary unrelated, as they emerged independently in distant groups. Bacterial responses that are induced by nutrient stress or predation stress and that give rise to heterogeneity, programmed cell death and division of labor might have necessitated cooperative activity between cells in the initial stages of multicellularity (Claessen *et al*., 2014).

Bacterial manifestations of multicellularity range from undifferentiated chains of cells (by an incomplete cell fission after cell division) or aggregation of individual cells (generally embedded by an extracellular matrix) to morphologically differentiated structures, and the behavior of cells within multicellular structures is coordinated by both common and unique molecular mechanisms (Claessen *et al*., 2014). Thus, multicellularity generally involves cell-cell adhesion, intercellular communication, and the differentiation of specialized cells.

Prokaryotes that morphologically differentiate structures that carry out distinct functions can be classified as patterned multicellular organisms (Claessen *et al*., 2014). These include myxobacteria, streptomycetes and cyanobacteria.

Myxobacteria are prokaryotes from soil that move as a coordinated assembly of cells during growth, but under nutrient starvation a developmental program is initiated that culminates in the formation of a well-defined structure, made of different cell types, known as the fruiting body. The formation of a fruiting body is thought to provide several benefits to cells in comparison to unicellular growth, among which maximizing the growth rate and increasing the efficiency in the use of growth substrates are the most evident ones. Intercellular signaling is essential for fruiting body formation, affecting the regulation of gene expression and cell motility (Zusman *et al*., 2007).

Streptomycetes are bacteria that grow as a complex hyphal network in the soil initiated by the germination of a single spore. Under stress conditions, the vegetative mycelium is submitted to programmed cell death and an aerial mycelium, which bears a differentiated spore-bearing structure, is formed, thus gaining new resources for growth (Flärdh and Buttner, 2009). Interestingly, the complex multicellular hyphae present channels that connect compartments, permitting the transport of nutrients and genetic material over long distances through the hyphae (Claessen *et al*., 2014), thus representing a communication pathway in this multicellular organism.

Finally, cyanobacteria, a highly diverse group of oxygenic photosynthetic prokaryotes, exhibit a wide range of developmental processes. Some filamentous forms of cyanobacteria can develop different types of cells, each one with a specific functional role. In some cases, cell differentiation allows the filament to carry out tasks that are functionally incompatible, such as oxygenic photosynthesis and the fixation of atmospheric nitrogen (Flores and Herrero, 2010; 2014).

1.2. The Cyanobacteria

Cyanobacteria are a phylogenetically coherent group of bacteria that perform oxygenic photosynthesis and are considered to be the precursors of plastids (Giovannoni *et al*., 1988). Cyanobacteria have become relevant both for their ecological role and their role in the evolution of the biosphere. As they were the first organisms to use oxygenic photosynthesis, cyanobacteria were key players in the early evolution of the Earth. Geochemical and fossil evidence indicate that they were responsible for the oxygenation of the primitive atmosphere in the Precambrian Era, around 2.4 to 2.2 billion years ago, during the so-called “Great Oxygenation Event”, and contributed significantly to primary productivity during the Proterozoic eon (Knoll, 2008). Some cyanobacteria can also fix atmospheric nitrogen, which makes them key players in the carbon and nitrogen biogeochemical cycles of the biosphere.

Cyanobacteria are one of the most diverse prokaryotic phyla, with morphotypes ranging from unicellular to multicellular filamentous forms, including those able to produce differentiated cells. These diverse growth strategies have enabled cyanobacteria to inhabit almost every terrestrial and aquatic habitat on Earth, including extreme environments. Moreover, some diazotrophic cyanobacteria (that is, cyanobacteria that can fix atmospheric N_2) establish symbioses with a wide variety of fungi, plants and other organisms (Meeks and Elhai, 2002).

Despite having been first assigned to and classified under the Botanical Code, Rippka *et al*. (1979) reappraised the classification of the group cyanobacteria and distinguished five sections, based on morphology, cell division and development (Figure 1). Section I (Chroococcales) is composed of unicellular organisms that reproduce either by binary fission or by budding, resulting in spherical, cylindrical or oval cells. Section II (Pleurocapsales) is composed of unicellular organisms able to reproduce by multiple fission, producing small cells, called baeocytes, that are enclosed in a fibrous wall layer and are subsequently released by breakage of such layer of the parental cell wall. Section III (Oscillatoriales) comprises filamentous non-heterocysts-forming cyanobacteria whose trichomes are uniseriated since cell division occurs regularly in a plane at a right angle to the long axis of the trichome. Section IV (Nostocales) is also composed of filamentous cyanobacteria, but distinguished from those of Section III by their capability to undergo cellular differentiation. Finally, Section V (Stigonematales) is composed of filamentous heterocyst-forming cyanobacteria in which cell division can occur in more than one plane, resulting in branched filaments. Besides heterocysts, some filamentous cyanobacteria can also form spores, which are known as akinetes, and hormogonia, which are short motile filaments that can serve a dispersal function in benthic environments (Rippka *et al*., 1979; Flores and Herrero, 2010). Therefore, the wide capability of cyanobacteria to undergo cellular differentiation processes should be highlighted.

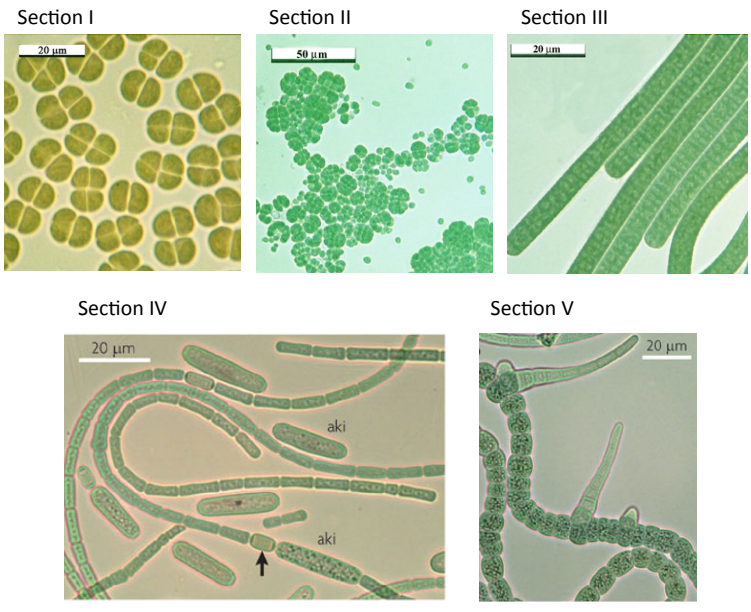

Figure 1: Diversity of cyanobacteria. Classification of cyanobacteria based on morphological features (Rippka *et al*., 1979). Some examples are shown: Section I: *Gloeocapsa* sp. PCC 9319; Section II: *Chroococcidiopsis* sp. PCC 7203; Section III: *Oscillatoria* sp. PCC 9325 (Images courtesy of José E. Frías, CSIC and Universidad de Sevilla); Section IV: *Anabaena cylindrica*; and Section V: *Fischerella muscicola* (Images from Flores and Herrero, 2010).

Molecular phylogenetic analysis, however, only partially supports Rippka's classification. Different studies support that filamentous heterocyst-forming cyanobacteria (Section IV and V) have a monophyletic origin, whereas there is some controversy about the phylogeny of cyanobacteria from other Sections (Tomitani *et al*., 2006; Shih *et al*., 2013). All cyanobacteria share a unicellular most recent common ancestor, although multicellularity evolved very early in the cyanobacterial lineage. By comparing the results from molecular phylogenetic analysis with the prokaryotic fossil record, it was possible to elucidate that multicellularity evolved in cyanobacteria earlier than 2.0 billion years ago, at the time of the "Great Oxygenation Event", coincident with a marked increase of diversification in cyanobacteria, that led to the current distribution of unicellular and multicellular forms (Figure 2) (Tomitani *et al*., 2006; Schirrmeister *et al*., 2011; 2013).

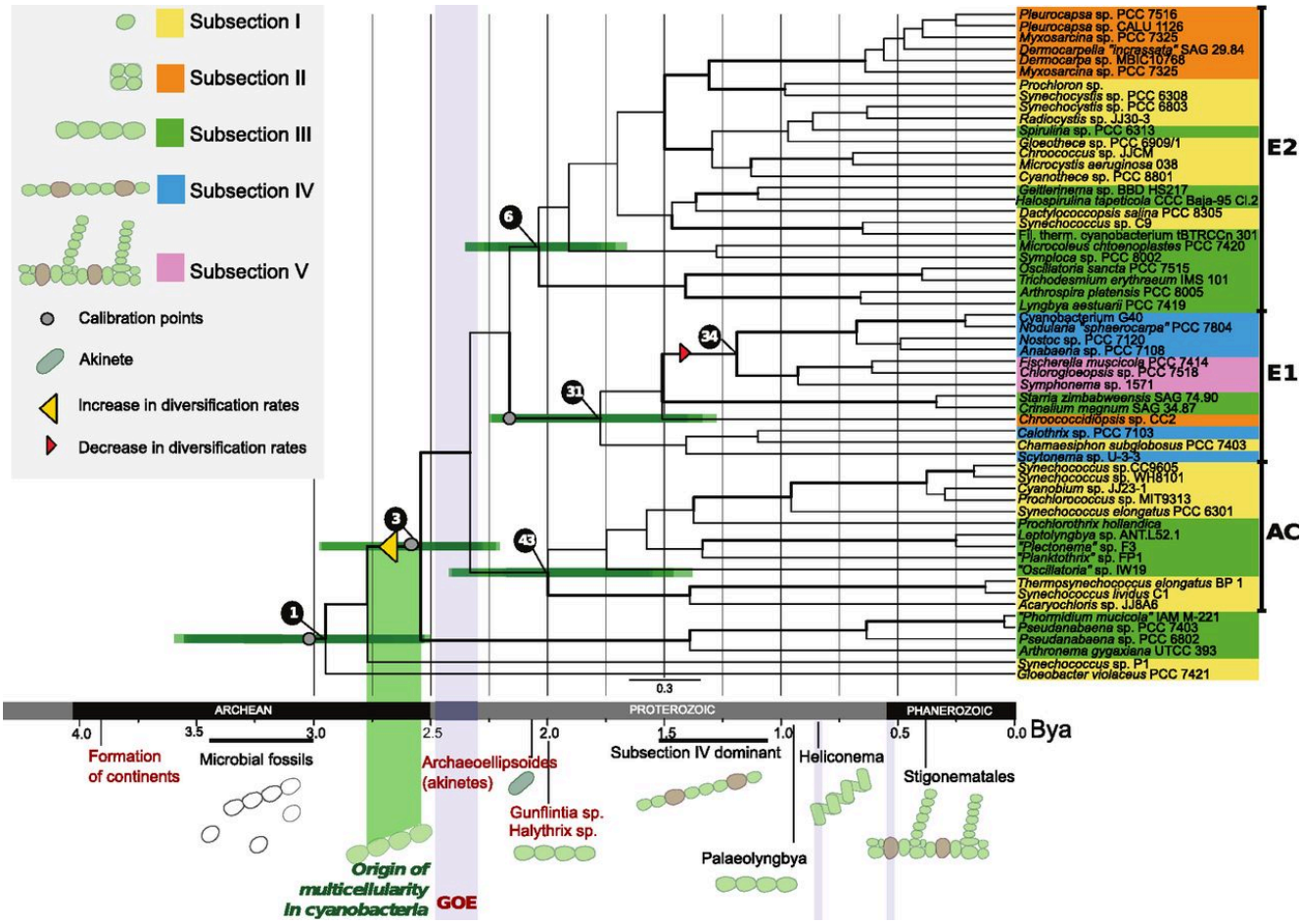

Figure 2: Origin and evolution of cyanobacteria. Time-calibrated phylogeny of cyanobacteria displaying divergence time estimates. Multicellularity (indicated by green shade) originated before or at the beginning of the Great Oxygenation Event (GOE). Figure from Schirrmeister *et al*. (2013).

Generally, all cyanobacteria perform oxygenic photosynthesis and fix atmospheric CO_2 through the Calvin cycle, which includes phosphoribulokinase and Rubisco (ribulose-1,5-biphosphate carboxylase/oxigenase) enzymes. Given the low atmospheric concentration of CO_2 and the low affinity of Rubisco for CO_2, cyanobacteria has developed the so-called CO_2 concentrating mechanism (CCM), which includes CO_2 and bicarbonate transporters, carbonic anhydrase and the carboxysome, a protein microcompartment in which Rubisco is confined, to enhance the activity of this enzyme (Kaplan and Reinhold, 1999; Cameron *et al*., 2014). Although cyanobacteria are generally photoautotrophs, some cyanobacteria can also grow as facultative photoheterotrophs or chemoheterotrophs, using sugars as a carbon source and as a carbon and energy source, respectively (Rippka, 1972). Assimilation of sugars in the form of glucose, fructose and sucrose has been reported in some cyanobacteria (Rippka *et al*., 1979), and sugar transporters have been identified, as in the case of the facultative heterotrophic plant-symbiont *Nostoc punctiforme*. Interestingly, in this organism sugar uptake is required for colonization of the plant tissue (Ekman *et al*., 2013; Picossi *et al*., 2013).

Regarding nitrogen metabolism, the nitrogen sources most commonly used by these organisms are ammonium, nitrate, urea, some amino acids and, in some species, dinitrogen. Among these, both ammonium and urea can diffuse through the cytoplasmatic membrane, but cyanobacteria also possess ammonium and urea transporters that mediate the uptake of these compounds when they are found at low concentrations in the medium. Ammonium transporters belong to the Amt protein family and have been identified in *Synechocystis* sp. PCC 6803 (Montesinos *et al*., 1998), *Synechoccocus elongatus* (Vázquez-Bermúdez *et al*., 2002a; Paz-Yepes *et al*., 2007) and *Anabaena* sp. PCC 7120 (Paz-Yepes *et al*., 2008). The urea Urt transporter of *Anabaena* sp. PCC 7120 is an "ATP-binding cassette" (ABC)-type transporter encoded by the *urtABCDE* operon and, once inside the cell, urea is hydrolyzed to CO_2 and ammonium by urease (Valladares *et al*., 2002). Ammonium is incorporated into organic compounds through the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway, which generates glutamate from 2-oxoglutarate (2-OG) and ammonium in a two-step reaction: first, glutamine is synthetized from ammonium and glutamate in an ATP-dependent reaction catalyzed by GS; second, the synthesis of two molecules of glutamate takes place from glutamine and 2-OG by GOGAT, which requires reductant provided by ferrodoxin in many cyanobacteria (Luque and Forchhammer, 2008). Thus, the GS/GOGAT pathway generates glutamate and glutamine, the main nitrogen-distributing compounds in the cell, and incorporates 2-OG, which derives from CO_2 fixation and serves as a carbon backbone. In addition, because cyanobacteria lack 2-oxoglutarate dehydrogenase in the Krebs cycle, the GS/GOGAT pathway links the carbon and nitrogen metabolisms, and 2-OG is the compound that senses the C-to-N balance in the cell (Stanier and Cohen-Bazire, 1977; Muro-Pastor *et al*., 2001).

Incorporation of nitrate (and nitrite) takes place through a nitrate/nitrite permease, which can be either an ABC-type uptake transporter or a Major Facilitator Superfamily (MFS) permease that concentrates the substrate inside the cell, where nitrate and nitrite

reductases convert them to ammonium, in turn incorporated into carbon skeletons through the GS/GOGAT pathway. Genes involved in nitrate/nitrite assimilation are commonly found in a gene cluster that behaves as an operon, including the nitrite reductase (*nirA*), ABC-type uptake transporter system (*nrtABCD*) or permease (*nrtP*) and nitrate reductase (*narB*) genes. In addition, closely linked to the *nirA* operon, a set of genes coding for proteins that influence its expression are found (Flores *et al*., 2005; Luque and Forchhammer, 2008).

As mentioned above, some cyanobacteria can use atmospheric N_2 as a source of nitrogen. The enzyme nitrogenase catalyzes the reduction of dinitrogen to two molecules of ammonia in an energy- and reductant-dependent manner. Nitrogenase is constituted by two components: dinitrogenase (comprised by NifD and NifK proteins) and dinitrogenase reductase (formed by NifH protein) (Rubio and Ludden, 2008). This enzymatic machinery is extremely sensitive to oxygen, and bacteria that perform N_2 fixation have developed diverse strategies to protect nitrogenase from O_2. In diazotrophic cyanobacteria, oxygenic photosynthesis and N_2 fixation are intrinsically incompatible processes occurring in the same microorganism. In order to solve this problem cyanobacteria separate spatially or temporally the processes of oxygenic photosynthesis and N_2 fixation. Thus, in unicellular and some filamentous cyanobacteria, N_2 fixation takes place during dark periods, when photosynthesis is not operative (Rippka and Waterbury, 1977; Bergman *et al*., 1997; Stal and Zehr, 2008), whereas some other filamentous cyanobacteria separate both processes through the differentiation of specialized cells, named heterocysts, where nitrogenase is confined (Flores and Herrero, 2010).

In cyanobacteria, the control of nitrogen metabolism is orchestrated by the NtcA protein, a transcriptional regulator influencing the expression of many genes whose products are involved in nitrogen assimilation and related functions. NtcA belongs to the cyclic AMP receptor protein family of regulators and is conserved in all cyanobacteria (Herrero *et al*., 2001; 2004). NtcA binds to specific DNA sites that bear the sequence signature $GTAN_8TAC$, which are found in the promoter regions of such genes (Luque *et al*., 1994). The DNA binding activity of NtcA is enhanced in the presence of 2-OG, and 2-OG is necessary for transcriptional activation by NtcA (Tanigawa *et al*., 2002; Vázquez-Bermúdez *et al*., 2002b; Valladares *et al*., 2008). Although first described as the factor that exerts nitrogen control in cyanobacteria (Frías *et al*., 1994), recently it has been found that NtcA controls the largest bacterial regulon characterized to date (Picossi *et al*., 2014), acting both as an activator and a repressor of transcription (Herrero *et al*., 2004). NtcA has also a crucial role in heterocyst differentiation.

This work has focused on the model organism *Anabaena* sp. PCC 7120, which is a heterocyst-forming cyanobacterium (Figure 3).

1.2.1. The heterocyst

As mentioned above, cyanobacteria from Sections IV and V of Rippka's classification (Rippka *et al*., 1979) perform N_2 fixation in differentiated cells termed heterocysts. The process of heterocyst differentiation takes place under nitrogen deprivation conditions. The structure and physiology of heterocysts provide the microoxic environment necessary for nitrogen fixation, so that the organism spatially separates oxygen-evolving photosynthesis (that takes place in vegetative cells) from nitrogen fixation. During the middle and later stages of differentiation, the differentiating cells undergo many metabolic and morphological changes (Golden and Yoon, 1998), and they lose the ability to divide or to revert to a vegetative cell, becoming committed to terminal differentiation. Heterocysts are typically distinguishable from vegetative cells by their larger and rounder shape, diminished pigmentation, thicker cell envelope, and usually prominent cyanophycin granules (see below) at poles adjacent to vegetative cells (Figure 3) (Kumar *et al*., 2010). The additional envelope layers surrounding heterocysts help to protect the enzyme nitrogenase from oxygen (Fay, 1992).

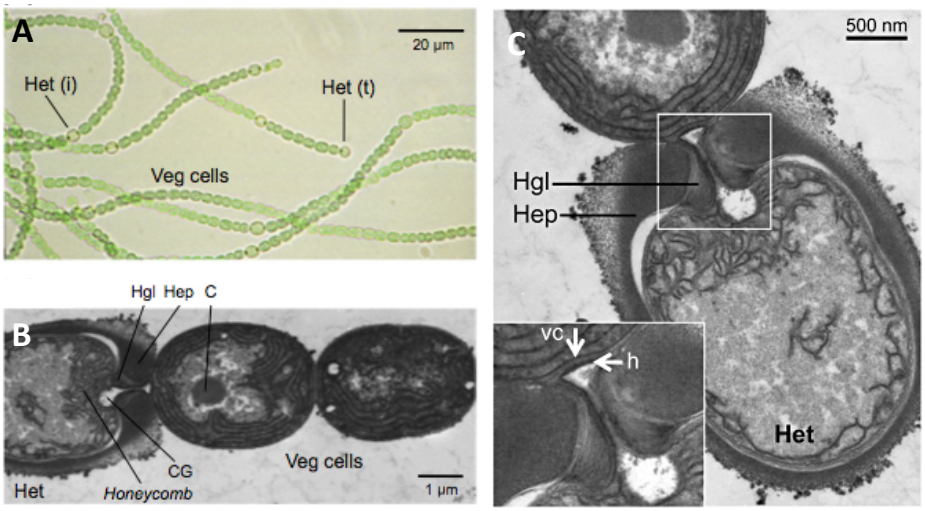

Figure 3: The filamentous heterocyst-forming cyanobacterium *Anabaena* sp. PCC 7120. Micrographs of *Anabaena* sp. PCC 7120 observed by light microscopy (A) or by transmission electron microscopy (B and C). (A, B) Filaments of cells. Veg cells, vegetative cells; Het, heterocyst, which may be placed intercalary (i) or terminally (t) in the filament; Hgl, heterocyst glycolipid layer; Hep, heterocyst envelope polysaccharide layer; C, carboxysome; honeycomb, heterocyst intracellular membrane system; CG, place where the cyanophycin granule (lost during sample preparation) was located (micrographs from Herrero *et al*., 2013). (C) Transmission electron micrograph of septa between a heterocyst and a vegetative cell; the inset shows a magnification of the area indicated by the square, in which white arrows indicate the cytoplasmatic membrane (CM) from the heterocyst (h) and the vegetative cell (vc) (micrographs from Merino-Puerto *et al*., 2011b).

1.2.1.1. Structure of the heterocyst

Morphological changes taking place during heterocyst differentiation include the deposition of two additional envelope layers: an inner "laminated" layer composed of heterocyst-specific glycolipids (HGL) and an outer polysaccharide (HEP) layer (Figure 3) (Cardemil and Wolk, 1979; 1981). The HGL layer is a permeability barrier for gases (Fay, 1992) and the HEP layer apparently protects the glycolipid layer from physical damage (Xu *et al*., 2008).

Mutants that lack either the HEP or HGL layer are unable to grow diazotrophically under oxic conditions (Wolk *et al*., 1988; Wolk, 1996; Fan *et al*., 2005; Huang *et al*., 2005). The heterocyst envelope might provide the right degree of gas permeability to allow the entry of sufficient N_2 for nitrogenase function but to maintain an adequate micro-oxic environment to avoid nitrogenase inactivation. However, it has also been suggested that the main gas diffusion path into the heterocyst is the cell envelope pores present at the vegetative cell–heterocyst junctions (Walsby, 2007). Regardless, the heterocyst envelope appears to have the role of limiting the entry of air, including poisoning oxygen, into the heterocyst.

Under laboratory conditions, most of the genes involved in heterocyst envelope formation, encoding both regulatory proteins and enzymes for the synthesis and export of envelope material, are induced after 6-12 h of nitrogen deprivation, which in *Anabaena* sp. PCC 7120 represents the medium phase of heterocyst differentiation, being deposition of the HEP layer one of the earliest morphological changes to take place. Some of the genes involved in HEP biosynthesis are clustered in the so-called "HEP island" (*alr2825-alr2841*) (Huang *et al*., 2005). The *hepA* gene (*alr2835*), formerly known as *hetA* (Holland and Wolk, 1990), is part of the HEP cluster and is involved in production of the HEP layer (Wolk *et al*., 1988; Wolk *et al*., 1993). HepA is an ABC-type transporter homologous to the *E. coli* exporter of the Lipid A of lipopolysaccharide (LPS). Other genes encoding enzymes including glycosyl transferases and LPS biosynthesis proteins are present in the HEP island, thus raising the question of whether HEP is a particular type of modified LPS in the heterocyst (Huang *et al*., 2005). Additional *hep* genes are located in other regions of the *Anabaena* chromosome and include the glycosyl transferases HepB (Alr3698) and Alr3699 (Maldener *et al*., 2003; Wang *et al*., 2007), a polysaccharide export protein (All4388) (Maldener *et al*., 2003; Lechno-Yossef *et al*., 2011), and HepP (All1711), a predicted membrane protein that belongs to the major facilitator superfamily (MFS) and could function as a glycoside exporter (López-Igual *et al*., 2012). In addition, specific regulatory mechanisms of HEP layer biosynthesis have been reported and are required for normal heterocyst maturation, including the two-component regulatory system HepK-DevR (Zhou and Wolk, 2003), HepN, HepS and HenR (Fan *et al*., 2006; Lechno-Yossef *et al*., 2006; Wang *et al*., 2007). However, the mechanistic bases of these regulatory systems in the context of heterocyst differentiation are still unknown. In summary, a number of genes encoding HEP biosynthesis and export proteins have been identified, but the process of production and deposition of the HEP layer remains to be elucidated in detail.

The HGL layer is assembled beneath the HEP layer and is composed of fatty alcohols glycosidically linked to sugar residues. Several genes involved in its synthesis and proper deposition have been identified and the function of their products assigned (Fan *et al*, 2005; Maldener *et al*., 2014). As in the case of the *hep* genes, a number of *hgl* genes are located in a cluster in the genome of *Anabaena* sp. PCC 7120 (*all5343-alr5357*), and their inactivation leads to formation of immature heterocysts lacking the HGL layer (Fan *et al*., 2005). The *hgl* cluster contains genes encoding putative fatty acid synthases and

polyketide synthases that might be involved in the biosynthesis of glycolipids, such as *hglB*, *hglC*, *hglD*, and *hglE* (Fan *et al*., 2005; Maldener *et al*., 2014). Indeed, the glycosyl transferase HglT, encoded by *all5341* in *Anabaena* sp, has been described to be necessary for glycosylation of the aglycone hexacosane-1,3,25-triol (Awai and Wolk, 2007). Genes involved in the correct deposition of HGL have been also identified and named *hgd* for Hgl deposition (Fan *et al*., 2005). These include the genes of the *devBCA* operon, which encodes an ABC-type exporter (Fiedler *et al*., 1998), and *hgdD*, which encodes an outer membrane TolC-like protein (Moslavac *et al*., 2007a). Differentiation and maturation of heterocysts is dependent on DevBCA and HgdD, which together form a secretion protein complex that traverses the cell wall (Staron *et al*., 2011). Other genes, such as *hglK* (encoding a pentapeptide repeat-containing protein that bears four transmembrane segments), are also required for the localization of glycolipids (Black *et al*., 1995). Finally, DevH, a trans-acting regulatory protein, has been shown to be required for the formation of the glycolipid layer, either by directly regulating gene expression or indirectly through other gene products (Ramirez *et al*., 2005).

The organization of the intracytoplasmatic membranes (thylakoids) is different in heterocysts and vegetative cells, probably reflecting the particular bioenergetics of the heterocyst. In the latter, the thylakoid membranes are arranged in the so-called *honeycomb* membranes, which are localized near the polar regions of the heterocysts, close to the heterocyst necks (Figure 3) (Lang and Fay, 1971). These *honeycomb* membranes harbor elements of the photosynthetic and respiratory apparatus (Wolk *et al*., 1994). Recently, the FraH protein has been shown to be involved in the formation of the *honeycomb* membranes (Merino-Puerto *et al*., 2011a).

Some intracellular structures present in vegetative cells, such as the carboxysomes and glycogen granules, disappear during heterocyst differentiation. However, the structured granules of cyanophycin (a polymer made of aspartate and arginine that functions as a nitrogen reserve material), which are present in different locations in the cytoplasm of vegetative cells, are localized close to the heterocyst poles, forming the so-called polar plugs, which enter each heterocyst neck (Figure 3) (Lang *et al*., 1972). The accumulation of cyanophycin at the polar regions, together with the narrowest septa between vegetative cells and heterocysts, produce a slower metabolite exchange between vegetative cells and heterocysts than that measured between vegetative cells (Mullineaux *et al*., 2008).

1.2.1.2. Heterocyst metabolism

Besides the structural adaptations, heterocysts also exhibit metabolic differences with regard to vegetative cells. Heterocysts exhibit a high rate of respiration, which is carried out by specific oxidases with dual functions: elimination of O_2 and generation of ATP (Wolk *et al*., 1994). In *Anabaena* sp. PCC 7120, three *cox* operons, coding for terminal respiratory oxidases, have been identified and characterized (Valladares *et al*., 2003). Among these, *cox1* is expressed in vegetative cells independently of the nitrogen source present in the

medium (Jones and Haselkorn, 2002; Valladares *et al*., 2003), whereas *cox2* and *cox3* are expressed in proheterocysts and mature heterocysts, and at least one of them must be present for diazotrophic growth to take place (Valladares *et al*., 2003). Indeed, *cox2* and *cox3* not only have a role in the protection of nitrogenase but also play a structural role in the formation of the *honeycomb* membranes, since a double *cox2 cox3* mutant lacks this structure (Valladares *et al*., 2007). Other O_2-removing enzymes, such as specific flavodiiron proteins expressed in the heterocyst have also been recently characterized (Ermakova *et al*., 2014).

The water-splitting activity of photosystem II (PSII) is lost during heterocyst differentiation, favoring the establishment of an intracellular micro-oxic environment (Wolk *et al*., 1994), and *de novo* synthesis of phycobiliproteins is repressed in heterocysts (Yamanaka and Glazer, 1983). Degradation of phycobiliproteins associated to PSII, which account for up to 50% of soluble protein in vegetative cells, may serve as a source of nitrogen during differentiation (Meeks and Elhai, 2002). In contrast, photosystem I (PSI) is operative in the heterocyst carrying out non-cyclic electron transport and photophosphorylation, generating ATP and reductant needed for N_2 fixation (Wolk *et al*., 1994).

Heterocysts lose the ability to fix CO_2 photosynthetically due to a lack of Rubisco enzyme. The fixed carbon needed as a source of energy and reductant for N_2 fixation, and as a hydrocarbon backbone for assimilation of the newly fixed N, is mainly received from the neighboring vegetative cells (Wolk, 1968) in the form of sucrose (Wolk *et al*., 1994; López-Igual *et al*., 2010), glutamate (Thomas *et al*., 1977; Martin-Figueroa *et al*., 2000) and alanine (Jüttner, 1983; Pernil *et al*., 2010).

1.2.1.3. Regulation of heterocyst differentiation

Nitrogen starvation is the environmental cue that triggers heterocyst differentiation. The differentiation process involves the execution of a specific program of gene expression that includes the induction of regulatory genes, some of which act early in the process, as well as genes encoding the proteins for the morphological and biochemical differentiation of the heterocyst, including nitrogenase (Xu *et al*., 2008; Herrero *et al*., 2013) (Figure 4). Global studies of gene expression during heterocyst differentiation have been performed, revealing the induction of 495 genes and the repression of 196 genes during the process (Ehira and Ohmori, 2006a; data re-evaluated in Xu *et al*., 2008). Many of these genes bear complex promoters producing multiple transcription start sites directed by different promoter determinants (Valladares *et al*., 2008; Mitschke *et al*., 2011; Herrero *et al*., 2013; Picossi *et al*., 2014). In addition, the presence of multiple non-coding RNA transcripts has led to suggest a possible role of these elements during heterocyst differentiation (Mitschke *et al*., 2011; Flaherty *et al*., 2011).

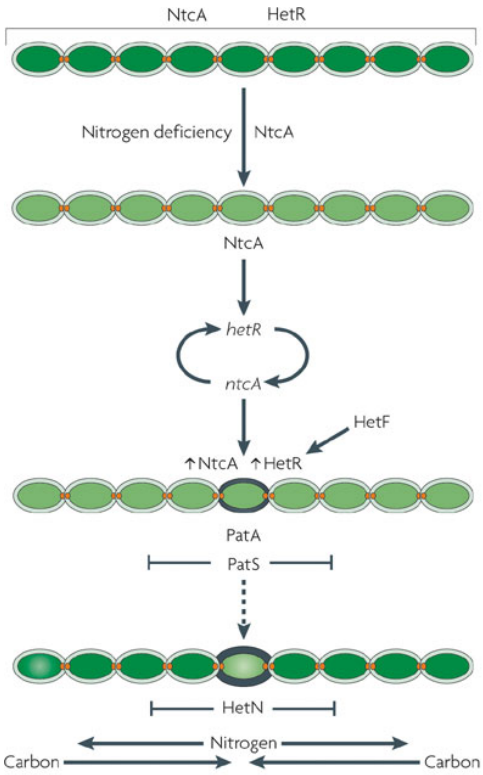

Figure 4: **The heterocyst differentiation process.** Under N deprivation, cyanobacteria undergo a specific program of gene expression that results in the formation of the mature heterocyst. Some of the regulatory proteins involved in heterocyst differentiation (see below) are indicated (from Flores and Herrero, 2010).

When combined nitrogen is not available, cyanobacteria experience an increase of the intracellular levels of 2-oxoglutarate (2-OG) that is an indicator of the C-to-N balance (Li *et al*., 2003). The accumulation of 2-OG is sensed by the global transcriptional regulator NtcA, which has a crucial role in the initial steps of heterocyst differentiation (Herrero *et al*., 2001; 2004). PipX, a protein found only in cyanobacteria, also influences the activity of NtcA, with a specific role in heterocyst maturation (Valladares *et al*., 2011). Another essential regulatory protein is HetR, which represents the master regulator of heterocyst differentiation and plays a key role in pattern formation (Buikema and Haselkorn, 1991a), being *hetR* one of the earliest genes induced in differentiating cells (Black *et al*., 1993; Buikema and Haselkorn, 2001). HetR also has DNA binding activity, which requires the formation of a HetR homodimer (Kim *et al*., 2011). During the initial steps of differentiation, *ntcA* and *hetR* are both positively autoregulated and induced in a mutually-dependent manner (Black *et al*., 1993; Frías *et al*., 1994; Muro-Pastor *et al*., 2002), leading to an increase of both proteins to the levels that are required for the establishment of the path to heterocyst differentiation (Black *et al*., 1993; Olmedo-Verd *et al*., 2008; Flores and Herrero, 2010). NrrA, a response regulator, has been identified as a regulatory link between NtcA and HetR (Ehira and Ohmori, 2006b), although its specific role in heterocyst differentiation has been questioned (Flores and Herrero, 2010).

One aspect of intense research is the establishment of the spatial pattern of heterocyst distribution along the filament. In *Anabaena* sp. PCC 7120, heterocysts are found at semiregular intervals along the filament, with 10-15 vegetative cells between two heterocysts. Differentiation often begins within a cluster of cells and proceeds to a certain

point, at which only one of the cells in the cluster is committed to continue differentiation (Yoon and Golden, 2001; Flores and Herrero, 2010). Several genes whose mutation, either by direct or indirect effects, results in an altered heterocyst pattern have been identified. Among these, *patS* is a key player in the establishment of the spatial pattern of heterocyst distribution acting as an inhibitor of differentiation, since its inactivation leads to a Multiple contiguous heterocysts (Mch) phenotype and its overexpression abolishes differentiation. The gene is early induced in the differentiating cells (Golden and Yoon, 1998; Yoon and Golden, 2001) and genes homologous to *patS* are widespread among filamentous cyanobacteria, both heterocyst- and non-heterocyst-forming strains (Zhang *et al*., 2009). The C-terminal part of PatS contains the ERGSGR sequence, and it has been suggested that a processed form of PatS containing at least the last five amino acids is exported from the differentiating cells to inhibit the differentiation of its neighbours, likely by acting on the transcription factor HetR (Yoon and Golden, 1998; Khudyakov and Golden, 2004; Risser and Callahan, 2009). Recently, a PatS concentration gradient in vegetative cells, decreasing from source differentiating cells, has been observed by the use of immunofluorescence, and the C-terminal octapeptide of PatS has been shown to be sufficient for regulation of heterocyst patterning along the filament (Corrales-Guerrero *et al*., 2013).

HetN is another negative regulator of heterocyst differentiation, having a role in the stabilization and maintenance of the heterocyst pattern over time (Callahan and Buikema, 2001). Interestingly, HetN bears an internal ERGSGR sequence and, as in the case of *patS*, mutation of this sequence leads to a Mch phenotype. Expression of *hetN* is induced when cells are committed to differentiation, and from that point, expression remains confined to differentiating and mature heterocysts. The HetN protein has been reported to be associated with cellular membranes (Li *et al*., 2002; Higa *et al*., 2012), and recently it has been shown specifically localized to the heterocyst periphery and cellular poles at the thylakoids, the N terminal part of HetN being required for localization (Corrales-Guerrero *et al*., 2014).

Besides PatS and HetN, which are the two predominant inhibitors of differentiation, several other gene products have been described to influence the pattern of heterocyst distribution along the filament, although their specific roles in establishing the heterocyst pattern need to be further investigated (see Herrero *et al*., 2013). In summary, the correct spatial pattern of heterocysts along the filament seems to rely on interference with the stream of gene transcription activation that sustains the differentiation of a given cell, although the molecular bases of the equilibrium between positive and negative factors that determine which cell in a neighborhood will differentiate are still unknown (Flores and Herrero, 2010). Why differentiation begins in a cluster of cells upon nitrogen starvation (Yoon and Golden, 2001) and how the sites of differentiation of new heterocysts are selected during diazotrophic growth (Wolk and Quine, 1975) are also unresolved aspects of the differentiation process, although it has been suggested that other factors, such as the stage in the cell cycle and the nitrogenous compounds exported from heterocysts, could also influence this process (Black and Wolk, 1994; Meeks and Elhai, 2002; Flores and Herrero, 2010).

1.3. The cyanobacterial cell envelope

Cyanobacteria are diderm bacteria (Sutcliffe, 2010), which means that they bear two cellular membranes: the cytoplasmatic membrane (CM) and an outer membrane (OM), the latter residing outside of the peptidoglycan (PG) layer (or murein sacculus). The CM and OM delimit an aqueous cellular compartment that Peter Mitchell (1961) first termed the periplasm (Figure 5). Although cyanobacteria are considered to bear a Gram-negative type of cell envelope, they present some features unusual for Gram-negative, and others more close to Gram-positive types of cell envelope. In filamentous cyanobacteria, whereas the CM surrounds cells individually, the OM is continuous along the filament, determining the presence of a continuous periplasmic space that contains the murein sacculus (Flores *et al*., 2006; Schneider *et al*., 2007; Wilk *et al*., 2011), and this seems to be the case both along the vegetative filament and the diazotrophic one constituted by vegetative cells and heterocysts. In some cyanobacteria, a surface layer (S-layer) is found outside of the OM. The S-layer is a paracrystalline array of proteinaceous material that forms a layer over the entire cell and could serve various functions, including protection, cell integrity, adhesion and, in some cyanobacteria, swimming motility (Liberton and Pakrasi, 2008).

1.3.1. The outer membrane

The outer membrane (OM), which is a distinguishing element of Gram-negative bacteria, is an asymmetrical bilayer consisting of phospholipids and lipopolysaccharide (LPS) in the inner and outer leaflet, respectively (Bos *et al*., 2007). Besides cell protection, an important function of the OM is to serve as a selective permeation barrier, and this is due not only to the intrinsic properties of the LPS, but also to the presence of porins, specific protein channels present in the OM (Nikaido, 2003) (Figure 5).

The bacterial LPS is composed of a hydrophobic membrane anchor, namely lipid A, an oligosaccharide core and the O-antigen, consisting in a repeating oligosaccharide chain (Bos *et al*., 2007; Raetz *et al*., 2007). The cyanobacterial LPS differs in some features from that of other well-known Gram-negative bacteria. One remarkable characteristic is that in some cyanobacteria, including *Anabaena* species, the LPS lacks heptose and 3-deoxy-D-manno-octulosonic acid (KDO), common components of enterobacterial LPS (Weckesser *et al*., 1974; Mikheyskaya *et al*., 1977; Hoiczyk and Hansel, 2000), and lacks or contains small amounts of phosphate (Schmidt *et al*., 1980; Hoiczyk and Hansel, 2000). A different fatty acid composition has also been found, and some components of cyanobacterial LPS are not usually present in other Gram-negative bacteria (Hoiczyk and Hansel, 2000; Snyder *et al*., 2009). The O-antigen is a repetitive polymer of homo- or hetero-oligosaccharide units of variable length and is the immunodominant portion of LPS. It often serves as a bacteriophage receptor and could have toxic activity on organisms of aquatic environments (Hoiczyk and Hansel, 2000). In *Anabaena* sp. PCC 7120 it was shown that the absence of the O-antigen resulting from mutation of genes related to its synthesis leads to resistance against bacteriophages (Xu *et al*., 1997). Although cyanobacterial O-antigen is reminiscent

of the *E.coli* O-antigen (Hoiczyk and Hansel, 2000), some differences in the chemical composition and extraction properties of O-antigen from *A. variabilis,* compared to the well-known enterobacterial one, have been described (Weckesser *et al*., 1974). Additionally, the structural sugar composition is very flexible and differs significantly not only between cyanobacterial genera but also between strains of the same genus or species (Hahn and Schleiff, 2014). Based on the study of a simple LPS from a marine *Synechococcus*, it has been suggested that cyanobacterial LPS is a more ancient form of LPS, from which a more complex LPS, such as that of enterobacteria, has evolved (Snyder *et al*., 2009).

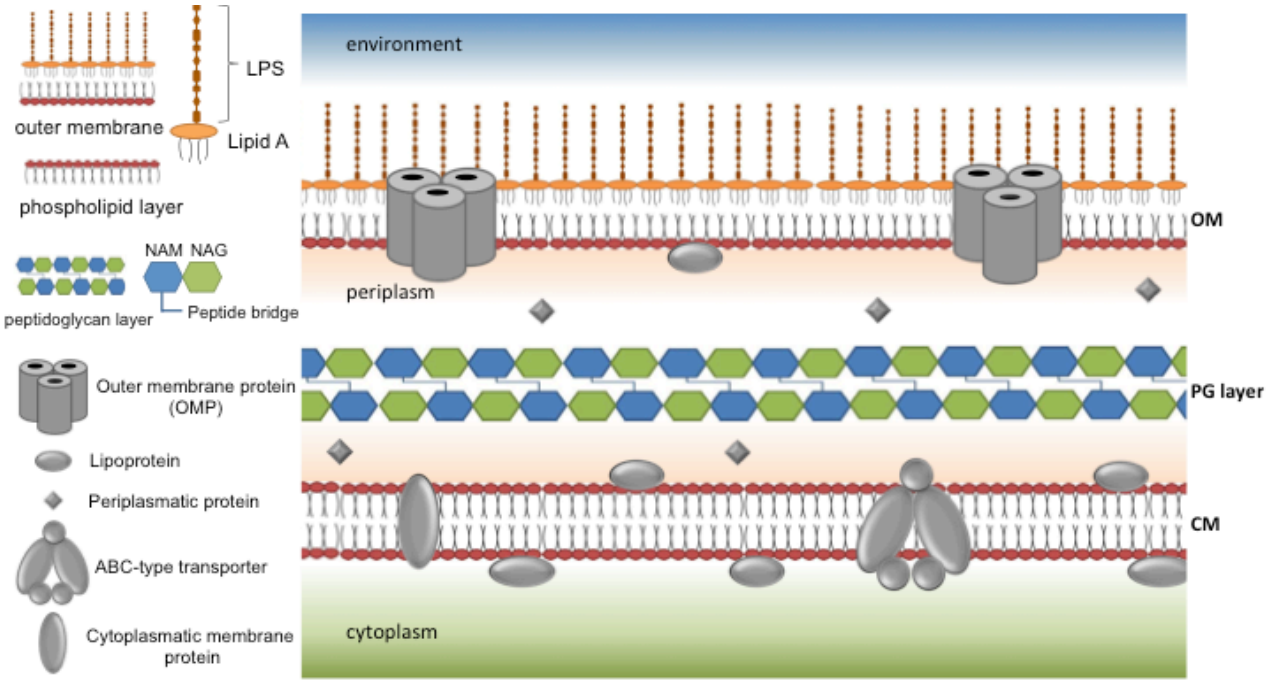

Figure 5: General structure of the cell envelope in Gram-negative bacteria. The cytoplasm is surrounded by a cell envelope, which is composed of the cytoplasmic membrane (CM), the peptidoglycan (PG) layer and the outer membrane (OM). The CM is a symmetric lipid bilayer composed of phospholipids (depicted in red and grey) and integral membrane proteins (depicted in grey). The periplasm is an aqueous compartment located between the CM and OM, and this is where the PG layer resides. The OM is an asymmetric lipid bilayer containing phospholipids in its inner leaflet and lipopolysaccharide (LPS) (depicted in orange) in its outer leaflet. The OM also contains integral proteins (OMP). Both membranes contain lipoproteins that, in the case of the OM are anchored to the periplasmic face (adapted from Ruiz *et al*., 2006).

LPS functions as a very effective barrier for hydrophobic molecules (Silhavy *et al*., 2010), which together with the fact that porins limit diffusion of hydrophilic molecules larger than 700 Da, makes the OM a selective permeability barrier (Nikaido, 2003). Porins are proteins with a β-barrel fold that permit the passage of small ions and molecules, such as amino acids and sugars (Nikaido, 2003). In *Anabaena* sp. PCC 7120, nine proteins have been assigned as porins (Nicolaisen *et al*., 2009a), although only four (Alr4550, All4499, Alr0834 and Alr3608) could be identified in an outer membrane proteomic analysis (Moslavac *et al*., 2005). Among these, All4499 and Alr4550, which show homology with the outer membrane porin OprB of *Pseudomonas aeruginosa*, were identified in membranes of vegetative cells and were the most abundant outer membrane proteins (OMP) in heterocysts (Moslavac *et al*., 2005; Moslavac *et al*., 2007b). Besides porins, other proteins or OM-associated proteins have been

identified, including proteins important for the biogenesis of the outer membrane (see below), TonB-dependent OM transporters, which are involved in iron and siderophore uptake (Nicolaisen *et al*., 2008; Mirus *et al*., 2009), and a TolC-like protein (HgdD) involved in the formation of mature heterocysts (Moslavac *et al*., 2007a), in the secretion of siderophores (Nicolaisen *et al*., 2010) and in the secretion of cytotoxic dyes and antibiotics (Hahn *et al*., 2012; 2014).

The OM also functions in protection of the cell, and carotenoids appear to play a role in this function (Liberton and Pakrasi, 2008). Carotenoids have been found to be true constituents of the OM in some cyanobacteria, such as *Synechocystis* sp. PCC 6714, suggesting that they could protect against high light intensities or have a structural function when associated with OM proteins (Jürgens and Weckesser, 1985).

The biogenesis of the OM involves two central systems based on the chemical nature of its components: outer membrane lipids (including the LPS) and OMP. Both proteins and LPS are synthetized in the cytoplasm and have to be translocated across the CM and inserted into the OM, which is carried out by the Lpt pathway and the Omp85 (also known as Bam or Imp/OstA) pathway, respectively (Silhavy *et al*., 2010) (Figure 6). Both systems are related, since ORF *alr2269* in *Anabaena* sp. PCC 7120, encoding an Omp85-like protein, is the first gene of an operon that also includes genes for Lipid A biosynthesis, *alr2270* to *alr2274*, (Nicolaisen *et al*., 2009b). Lipid A is synthetized in the inner leaflet of the CM and is transferred to the outer leaflet by MsbA, which in *Anabaena* may correspond to HepA, formerly known as HetA (Holland and Wolk, 1990). As mentioned earlier, HepA is also essential for the formation of the heterocyst-specific polysaccharide layer (Huang *et al*., 2005). LptB (*alr4068*) and LptF (*alr4069*) are suggested to function as the cytoplasmatic membrane-inserted unit facilitating the release of Lipid A from the CM. Then, LptA (*alr4067*) recognizes LPS in the periplasm and transfers it to the OM component LptD (*alr1278*), which is the central OM protein involved in Lipid A transport (Haarmann *et al*., 2010).

Regarding the biogenesis of OMPs, it has been shown that Omp85-like proteins are required for the incorporation of porins and other β-barrel proteins into the OM (Rigel and Silhavy, 2012). Omp85-like proteins are composed of a 16-stranded β-barrel and polypeptide transport-associated (POTRA) repeats, which serve as a receptor for OM proteins and as a docking site for Omp85 complex components (Schleiff *et al*., 2011). Three Omp85-like proteins have been identified in proteomic studies of the OM isolated from heterocysts or vegetative cells of *Anabaena* sp. PCC 7120, namely Alr0075, Alr2269 and Alr4893 (Moslavac *et al*., 2005), among which Alr2269 represents the best characterized Omp85-like protein in *Anabaena* (Ertel *et al*., 2005; Bredemeier *et al*., 2007). Alr2269 shows the highest similarity to Toc75 of the plant chloroplast protein import apparatus (Schleiff and Soll, 2005). Nicolaisen *et al.* (2009b) detected the other two Omp85-like proteins by immunodecoration, finding that these two have lower abundance than Alr2269. Indeed, Alr0075 is only expressed in vegetative cells, where it could have a specialized function. Thus, it seems that the three proteins, which are essential proteins since chromosomes mutated in their

corresponding genes could not be segregated (Nicolaisen *et al*., 2009b), may perform different functions. Omp85-like proteins in *Anabaena* sp. PCC 7120 play an important role in OM integrity, since mutations in their genes results in an increase of sensitivity to some toxic compounds and of uptake of some amino acid, suggesting an increased permeability of the outer membrane in these mutants. Indeed, the structure of the OM is extensively altered in the mutant of Alr2269 (Nicolaisen *et al*., 2009b; Tripp *et al*., 2012).

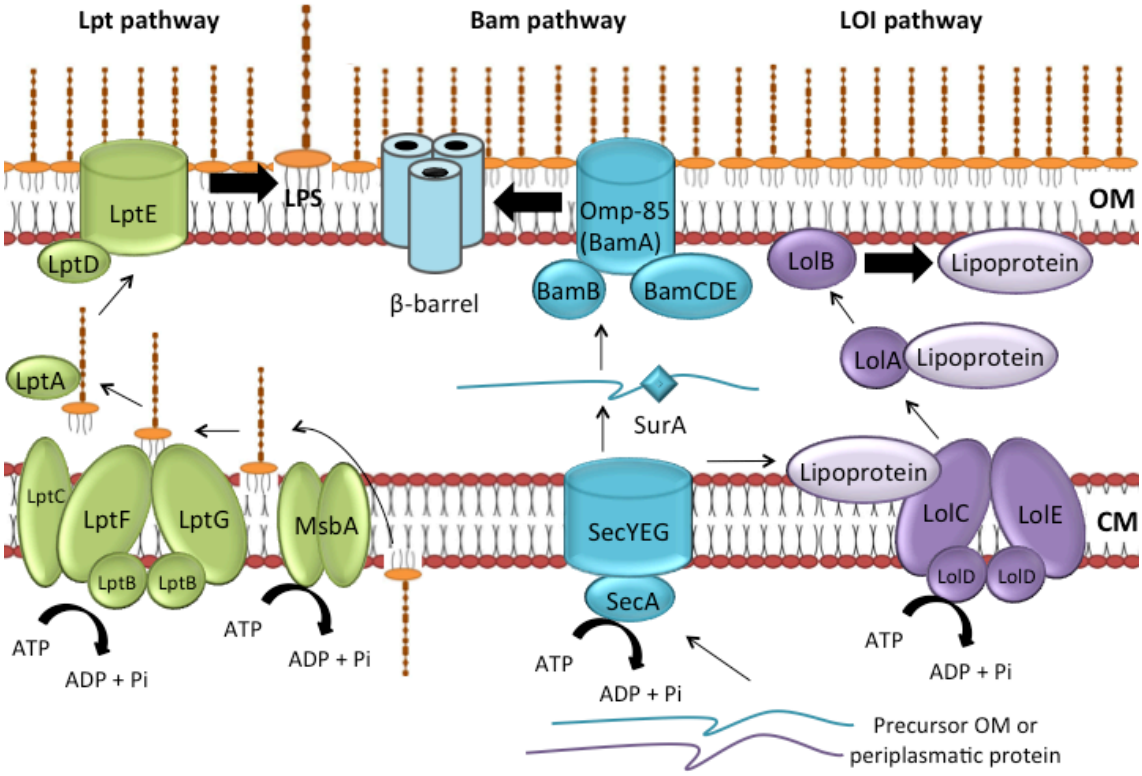

Figure 6: Biogenesis of the outer membrane in Gram-negative bacteria. The Lpt pathway, together with MsbA, transports LPS from the cytoplasm to the cell surface. Outer membrane proteins and lipoproteins are made in the cytoplasm in precursor forms and the Sec machinery translocates them to the periplasm. The β-barrel proteins are delivered by chaperones such as SurA to the Bam machinery for assembly in the OM. For lipoproteins, after being translocated and folded in the periplasm, the Lol machinery delivers them to the OM (adapted from Silhavy *et al*., 2010).

In Proteobacteria, once the OMPs have been translocated across the CM, the periplasmic chaperones SurA or Skp are responsible for protecting OMPs during their transit through the periplasm and for delivering them to Omp85 (Figure 6) (Bos *et al*., 2007; Silhavy *et al*., 2010). However, neither SurA nor Skp homologues have been found in cyanobacterial genomes (Bohnsack and Schleiff, 2010). Recent studies suggest that All0114 from *Anabaena* sp. PCC 7120, which is an homologue to Tic22 involved in protein translocation into plant chloroplasts, could function in a way similar to that of the periplasmatic chaperone SurA, since mutations in *all0114* result in an altered structure of the OM and also in sensitivity to some toxic compounds (Tripp *et al*., 2012), in a similar manner to the Omp85-like *alr2269* mutant.

1.3.2. The peptidoglycan layer

As mentioned earlier, the peptidoglycan layer is located in the periplasmic space, which is a highly viscous compartment occupied also by soluble proteins. The periplasm is an oxidizing environment that contains enzymes catalyzing the formation of disulphide bonds (Nakamoto and Bardwell, 2004). Periplasmic proteins participate in small molecule transport, the breakdown of polymers and protein-folding, and factors that build and maintain the cell envelope also occur in the periplasm. Interestingly, all of this protein folding, trafficking and degradation occur in the absence of an obvious energy source, since the periplasm is devoid of ATP (Ruiz *et al*., 2006).

The PG is a huge single molecule (the murein sacculus) surrounding the CM, and its function is to resist turgor in the cell wall of most bacteria, also determining the cell shape and playing an essential role in bacterial morphogenesis. Moreover, it is the anchoring site for other cell envelope components, such as proteins and teichoic acids in the case of Gram-positive bacteria (Vollmer *et al*., 2008a). The PG is made up of repeating units of a disaccharide containing N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), which are cross-linked by pentapeptide side chains (Figure 5) (Höltje, 1998; Vollmer *et al*., 2008a). Although the basic precursors of PG are conserved across species, it has been shown that the chemistry, structure and architecture of the final PG cell wall can be very different between species and even stages in the cell cycle of a bacterium (Turner *et al*., 2014).

In cyanobacteria, it has been described that the PG also differs from that of other Gram-negative bacteria, since it is thicker and also possess a higher degree of cross-linking between PG chains, features more related to Gram-positive than Gram-negative peptidoglycans (Hoiczyk and Hansel, 2000). Cyanobacteria appear to have more layers of PG than the model bacterium *E. coli* and dimensions of PG from different cyanobacterial species vary quite significantly. In *Anabaena* sp. PCC 7120, the murein sacculus seems to be composed of two or three layers that surround each cell of the filament (Wilk *et al*., 2011), and the PG layers of adjacent cells are fused in a number of intercellular septa, although in some other septa they are seen well separated, as observed by transmission electron microscopy and electron tomography (Flores *et al*., 2006; Wilk *et al*., 2011). Interestingly, in some heterocyst-forming cyanobacteria such as *Anabaena cylindrica* (Dunn and Wolk, 1970) and *Nostoc punctiforme* (Lehner *et al*., 2011), sacculi corresponding to several cells have been isolated, indicating that the PG layers from adjacent cells are fused in a considerable number of intercellular septa of the filament (Figure 7).

Growth of the murein sacculus is a dynamic process requiring synthases to make new PG and to attach it to the existing sacculus, and hydrolases that cleave covalent bonds in the old sacculus allowing the insertion of the new building blocks. Thus, the activity of these enzymes has to be highly regulated in order to maintain the cell shape (Typas *et al*., 2012). PG biogenesis is well documented in model microorganisms, such as *E. coli* (Sauvage *et al*., 2008; van Heijenoort, 2011; Typas *et al*., 2012), whereas in cyanobacteria the pathway of

PG synthesis has not yet been described in detail. However, it has been reported that some enterobacterial PG biogenesis and metabolism enzymes, which includes penicillin-binding proteins (PBPs) and peptidoglycan hydrolases, are conserved in cyanobacteria (Leganés *et al.*, 2005; Hahn and Schleiff, 2014).

PBPs catalyze the polymerization of the glycan chains (by transglycosylation) and the cross-linking between them (by transpeptidation), and some PBPs can also function as endopeptidases (Sauvage *et al*., 2008). PBPs with transpeptidase and glycosyltranferase activities are known as class A PBPs, and are involved in PG polymerization; monofunctional transpeptidases are known as class B PBPs and are involved in the modification of the PG layer during cell elongation and division. Class C PBPs are involved in the regulation of PG synthesis and its modification, and can have carboxypeptidase or endopeptidase activity (Sauvage *et al*., 2008; Typas *et al.*, 2012). In *Anabaena* sp. PCC 7120, genes encoding PBPs have been identified (Leganés *et al*., 2005), and it has been shown that genes *alr5101*, *all2981* and *alr4579*, encoding class A PBPs, are specifically important for heterocyst differentiation (Lázaro *et al*., 2001; Leganés *et al*., 2005), suggesting that these PBPs are involved in the modification of the PG during the differentiation process. In *Synechocystis* sp. PCC 6803, mutations in genes encoding different classes of PBPs result in alteration of cell size or, in some cases, in a lethal phenotype, suggesting that they play essential roles in cell survival (Marbouty *et al*., 2009). Interestingly, it was found that one of these PBPs belonging to class B, namely FtsI, is essential and might be involved in the synthesis of PG at the septum that is required for completing separation of daughter cells after cell division. Thus, it appears that FtsI from *Synechocystis* plays a role similar to that of the homologue FtsI in *E. coli,* since the latter is essential for cell division, forms part of the divisome and interacts with other PBPs and proteins associated to the Z-ring (Typas *et al*., 2012).

Degradation of PG is carried out by autolysins, which are proteins located in the periplasm and can lyse the cell using their hydrolase activity. Autolysins can have muramidase, glucosaminidase, amidase and/or endopeptidase activity (Typas *et al*., 2012). Not much is known about PG degradation in cyanobacteria, although cyanobacterial genomes contain genes homologous to those of autolysins from *E. coli* (Hahn and Schleiff, 2014). Thus, it is assumed that the PG degradation pathway would be similar to that described for the well-known enterobacterial system, although this should be further investigated due the complexity of the cyanobacterial PG.

Filamentous cyanobacteria present a distinct scenario compared to unicellular bacteria regarding cell division and septation. Instead of concluding cell division with daughter cell separation by PG amidases and invagination of the OM mediated by a Pal-Tol complex during septation (Vollmer *et al*., 2008b; Typas *et al*., 2012), cell division in filamentous cyanobacteria does not involve PG hydrolysis and OM invagination, resulting in the aforementioned continuous OM (Flores *et al*., 2006; Mariscal, 2014). In *E. coli*, amidases AmiA, AmiB and AmiC (N-acetylmuramyl-L-alanine amidases, which are enzymes that hydrolyze the septal PG) are important for septation and cell separation, since the triple

mutant *amiABC* results in a filamentous strain (Heidrich *et al*., 2002). In *Anabaena* sp. PCC 7120, amidase HcwA (Zhu *et al*., 2001) or AmiC2 (Berendt *et al*., 2012) was found to be required for heterocyst maturation, suggesting that an amidase participates in degradation or remodeling of the PG layer for heterocyst cell wall biosynthesis, perhaps increasing PG permeability and, thus, facilitating the penetration of polysaccharides (Zhu *et al*., 2001). In contrast to amidases from *E. coli*, inactivation of AmiC2 in *Anabaena* has no effect on filament morphology (Berendt *et al*., 2012). Interestingly, recent studies have reported that the PG at the septum between cells in filamentous cyanobacteria is perforated. An array of nanopores has been observed in isolated murein sacculus from *Nostoc punctiforme,* which presumably represents a framework for cell-cell communication (Lehner *et al.*, 2013; see below). These authors investigated the role of AmiC2 and suggested that this cell wall amidase is the responsible to drill the holes in the cross-walls (Lehner *et al.*, 2013) (Figure 7).

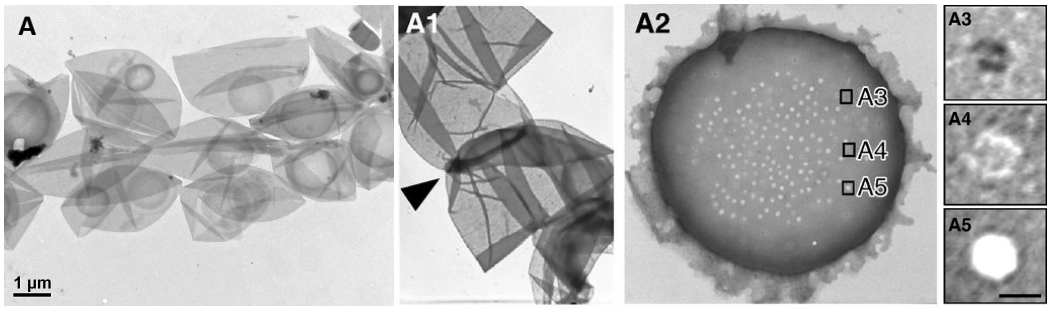

Figure 7: Transmission electron microscopy of purified peptidoglycan sacculi. (A) The isolation of PG sacculi was performed by repeated incubations in boiling 6% SDS, and isolated PG was stained with 1% uranyl acetate, as described in Lehner *et al*. (2013). Purified sacculi from several cells of *Anabaena* sp. PCC 7120. Note that some sacculi are broken. (A1-A5) Isolated PG sacculi from *Nostoc punctiforme.* Arrowhead in A1 points to a septum. Septal PG containing an array of nanopores (A2). Magnification of single pores (A3-A5) shows the progression of pore formation. Micrograph from *Anabaena* (A) was taken in TEM facilities at Göethe Universität Frankfurt am Main. Pictures from *N. Punctiforme* (A1-A5) are from Lehner *et al*. (2013).

Another protein involved in septal cell wall synthesis has been identified in *Anabaena* sp. PCC 7120. ConR, the product of ORF *all0187*, is predicted to be part of a family of proteins that contain the LytR-CpsA-Psr (LCP) domain associated with septum formation and cell wall maintenance. Inactivation of *conR* caused a septum-formation defect in vegetative cells and the presence of an abnormal septum between vegetative cells and heterocysts, which appear abnormally long and often with partially open poles (Fan *et al*., 2006; Mella-Herrera *et al*., 2011). Consequently, more oxygen can enter into heterocysts, which would explain the lower nitrogenase activity and impaired diazotrophic growth of the mutant. These observations point out that ConR might regulate proteins involved in the synthesis and maintenance of the cell wall, particularly those involved in heterocyst septum remodelling during heterocyst differentiation (Mella-Herrera *et al*., 2011). In Gram-positive bacteria, LCP proteins have been shown to mediate the formation of capsules or the attachment of glycopolymers, such as teichoic acids, to the PG layer (Bender *et al*., 2003; Chan *et al*., 2014; Wu *et al*., 2014).

1.3.3. The cytoplasmatic membrane

The cytoplasmatic membrane (CM) is a phospholipid bilayer that, besides delimiting the cell, is also important because it contains different transport systems essential for viability. Regarding membrane lipid composition, the cyanobacterial membrane resembles that of higher plant chloroplasts rather than of other bacterial membranes. The differences in lipid composition could be explained by the availability of phosphate in cyanobacterial habitats (Hahn and Schleiff, 2014). Carotenoids have been also found in the CM of cyanobacteria, and it is though that they function ensuring CM stability and functionality (Hahn and Schleiff, 2014).

Regarding the CM proteome of *Anabaena* sp. PCC 7120, a large variety of proteins are involved in the uptake, export or exchange of solutes across the CM. Among them, transporters involved in the uptake of nitrate, bicarbonate, urea, phosphate, ammonium and amino acids have been described (reviewed in Hahn and Schleiff, 2014). Enzymes involved in photosynthetic and respiratory processes, metabolic enzymes, proteins involved in signal transduction, as well as proteins anchored to the membrane for structural purposes are also found in the CM. In addition, in filamentous cyanobacteria, different proteins localized at the septum and anchored to the CM, constituting septal junction complexes (see below), have been shown to have an essential role in filament biology, and thus represent an important structural aspect of the cyanobacterial filament.

1.4. Intercellular molecular exchange in *Anabaena*

Because of the compartmentalization of the photosynthetic CO_2 fixation and N_2 fixation processes in different cell types, growth of the heterocyst-containing cyanobacterial filament as an organismic unit relies on intercellular exchange of nutrients (Wolk *et al*., 1994) and regulatory factors, such as the C-terminal fragment of PatS (Yoon and Golden, 1998; Corrales-Guerrero *et al*., 2013). Filament integrity is therefore essential for the diazotrophic growth of heterocyst-forming cyanobacteria. As mentioned above, vegetative cells donate products of CO_2 fixation to the heterocysts (Wolk, 1968), and heterocysts, in turn, provide N_2 fixation products to the vegetative cells (Wolk *et al.*, 1974). The identification of the exchanged products, as well as the mechanism of transference, have been aspects of intense study regarding the physiology of heterocyst-forming cyanobacteria (Wolk *et al*., 1994; Flores *et al*., 2006; Flores and Herrero, 2010).

The identification of the exchanged metabolites has been largely addressed by the study of the localization of expression of enzymes that metabolize such substrates, that is, the absence or presence of those enzymes in a particular cell type (Thomas *et al*., 1977; Martín-Figueroa *et al*., 2000; López-Igual *et al*., 2010; Pernil *et al*., 2010), but also by labelling studies. Regarding reduced carbon compounds, based on experiments performed with

radiolabelled $^{14}CO_2$, Jüttner (1983) suggested that alanine, glutamate and some sugars, such as glucose-6-phosphate, could function as carbon vehicles from vegetative cells to heterocysts. As heterocysts lack GOGAT but have high activity of GS (Thomas *et al*., 1977; Martín-Figueroa *et al*., 2000), glutamate has to be transferred from vegetative cells to heterocysts, which may serve as a hydrocarbon backbone for the incorporation by GS of ammonium produced by nitrogenase, generating glutamine. In *Anabaena* sp. PCC 7120, it has been shown that the catabolic enzyme alanine dehydrogenase (Ald) is expressed in the heterocyst, and that an *ald* mutant is impaired both in diazotrophic growth and nitrogenase activity (Pernil *et al.*, 2010). These results support the hypothesis that also alanine is transferred from vegetative cells to heterocysts, where this amino acid would be metabolized producing reductant to be used in these differentiated cells. Additionally, Ald also participates in the synthesis of aspartate, a constituent of cyanophycin.

Finally, sucrose, a universal vehicle of reduced carbon in plants and a key sugar for carbon metabolism in cyanobacteria, has been proposed to be an important compound transferred from vegetative cells to heterocysts, since diazotrophic cultures from some heterocyst-forming cyanobacteria have a high activity of sucrose metabolism enzymes (Schilling and Ehrnsperger, 1985; Wolk *et al*., 1994). In *Anabaena* sp. PCC 7120, two sucrose-phosphate synthases (encoded by *spsA* and *spsB*), and a sucrose–phosphate phosphatase (*sspA*) are involved in sucrose biosynthesis (Porchia and Salerno, 1996; Cumino *et al*., 2002), whereas three proteins, namely sucrose synthase (*susA*) and two alkaline/neutral invertases (encoded by *invA* and *invB*) have a role in sucrose degradation (Schilling and Ehrnsperger, 1985; Curatti *et al*., 2002). SusA is regulated under diazotrophic conditions and has a role in vegetative cells (Curatti *et al*., 2002). InvA is required for a correct distribution of carbon compounds along the filament and for the maintenance of the C-to-N balance, whereas InvB is expressed only in heterocysts and is required for diazotrophic growth (López-Igual *et al*., 2010; Vargas *et al*., 2011). As inactivation of *invB* results in a stronger impairment of diazotrophic growth than inactivation of *ald*, it seems that sucrose is a more important carbon vehicle into heterocysts than alanine (López-Igual *et al*., 2010; Vargas *et al*., 2011).

Concerning the vehicles of fixed N, glutamine has been identified as an important candidate for amino acid transferred from heterocysts to vegetative cells, as demonstrated by $^{13}N_2$ radiolabeling experiments (Wolk *et al*., 1674; 1976; Thomas *et al*., 1977). As mentioned above, GOGAT activity in vegetative cells converts the glutamine imported from heterocysts into glutamate (Thomas *et al*., 1977; Martín-Figueroa *et al*., 2000). Therefore, there is a glutamine-glutamate exchange, conforming a compartmentalized GS/GOGAT cycle in the diazotrophic filament. Besides glutamine, other amino acids have been proposed to function as nitrogen vehicles, such as arginine and aspartate, being the former a nitrogen-rich compound and a constituent of the nitrogen reserve polymer cyanophycin (see below).

Regarding the mechanism of transference for molecular exchange along the filament, two possible pathways have been suggested: the continuous periplasm and a direct intercellular pathway via the septal junction complexes. A scheme of intercellular molecular exchange through the filament is shown in Figure 8.

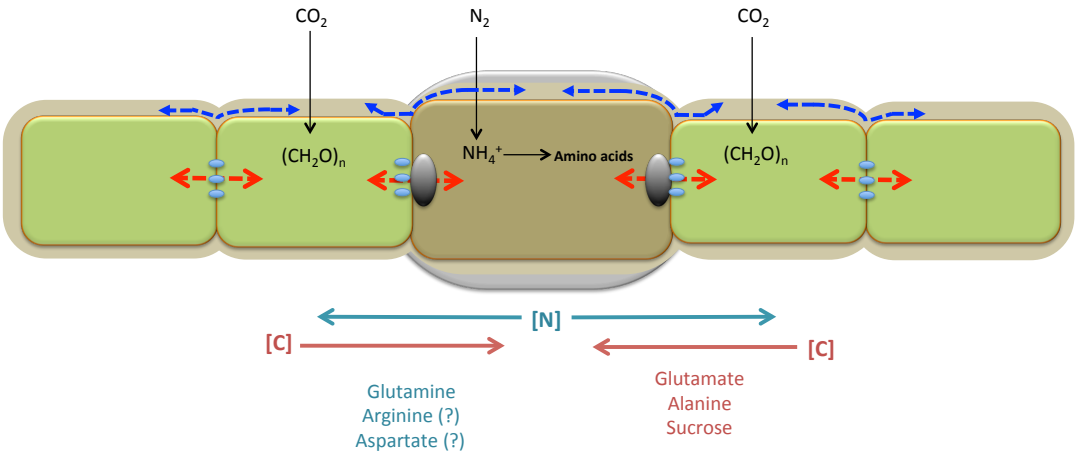

Figure 8: Intracellular molecular exchange under diazotrophic conditions. Vegetative cells provide heterocysts with reduced C fixed photosynthetically, which serves as source of energy and reductant, in the form of amino acids such as glutamate and alanine or sugars (sucrose). Heterocysts transfer combined nitrogen to the vegetative cells in the form of glutamine and likely other amino acids. The continuous periplasmic pathway (indicated as blue dashed lines) and the intracellular transference via septal junction complexes (red dashed lines) are indicated (adapted from Flores *et al*., 2006).

1.4.1. The continuous periplasm as a functional unit

It has been suggested that the continuous periplasm, permitting the movement of molecules, could function as a communication conduit along the filament (Flores *et al*., 2006; Mariscal *et al*., 2007). This is in accordance with the fact that soluble periplasmic GFP produced in the heterocysts in *Anabaena* sp. PCC 7120 can be found at a distance from the producing cells, suggesting that the GFP moves through the periplasm, whereas if the GFP is produced as a membrane-anchored protein, no diffusion is observed (Mariscal *et al*., 2007). However, barriers for the diffusion of the 27-kDa GFP seem to exist, which could correspond to the PG in the intercellular septa that may have a size exclusion limit of 25-50 kDa (Flores and Herrero, 2010). Interestingly, the OM is a specific permeability barrier to metabolites such as sucrose and glutamate, which are subjected to intercellular exchange during diazotrophic growth, and the continuous OM would help to keep these metabolites within the filament (Nicolaisen *et al*., 2009b). Furthermore, it has been reported that transport system NI for neutral amino acids and glutamine is expressed solely in vegetative cells and is required for optimal diazotrophic growth. This may be indicative of a role of NI in the uptake of amino acids released from heterocysts to the continuous periplasm (Picossi *et al*., 2005; Flores *et al*., 2006; Pernil *et al*., 2008).

1.4.2. Septal junctions

Septa between cells in filamentous cyanobacteria are structured sections containing proteinaceous complexes that link the CM of the neighboring cells. The first observations of intercellular septal structures between heterocysts and vegetative cells were done by electron microscopy by Lang and Fay (1971), who reported thin structures perpendicular to the CM of the adjacent cells. These authors introduced the term "microplasmodesmata" to emphasize that, like plasmodesmata of plants, these structures might represent sites of intimate contact and could have a role at connection of adjacent cells. Using freeze-fracture electron microscopy of *Anabaena cylindrica*, Giddings and Staehelin (1978) showed the presence of distinct deep pits in the external leaflet of the CM and corresponding protrusions in the cytoplasmic leaflet that could correspond to microplasmodesmata, and established that heterocyst-forming cyanobacteria produced more microplasmodesmata (200-300 microplasmodesmata per septum) than species unable to form such differentiated cells (30-40 microplasmodesmata per septum). These structures could be analogous to gap junctions from animals (Flores *et al*., 2006), which are intercellular channels made of proteins that join the CM of adjacent cells and permit the passage of small molecules and ions between cells (Koval, 2006). As there are not ORFs coding for proteins homologous to gap junction proteins in the *Anabaena* genome (Kaneko *et al*., 2001), these structures joining the cells in heterocyst-forming cyanobacteria have been named "septal junctions".

Wilk *et al*. (2011), using electron tomography after chemical fixation, emphasized the proteinaceous nature of septal junctions (Figure 9). Additionally, they showed that septal junctions between vegetative cells occur in a structured manner in the center of the septum and have a length of 27 nm and a diameter of 5.5 nm. These "channels" have also been described in the septa between vegetative cells and heterocysts (Omairi-Nasser *et al*., 2014). As mentioned earlier, perforations of the PG layer have been also reported (Wilk *et al*., 2011; Lehner *et al*., 2013). Their dimensions are similar to those of the protein structures, consistent with the idea that the nanopores are holes that serve as a framework for the septal junction complexes to traverse the PG layer.

The identification of the proteins that form the septal junctions, together with their role in the physiology of the diazotrophic filament, has called the attention in this field in the last years. Several genes from *Anabaena* sp. PCC 7120, whose mutation results in filament fragmentation are known (Buikema and Haselkorn, 1991b; Ernst *et al.,* 1992; Bauer *et al*., 1995; Flores *et al*., 2007; Nayar *et al*., 2007; Merino-Puerto *et al*., 2010), and some of them encode cell-cell joining proteins. Up-to-date, proteins SepJ, FraC and FraD have been identified to be putatively involved in the septal junction structures, having a role in maintaining the integrity of the filament. The use of fluorescent tracers, such as calcein or 5-carboxyfluorescein (5-CFDA), together with FRAP (Fluorescence Recovery After Photobleaching) analysis has been crucial for the identification of the role of these proteins in intercellular communication.

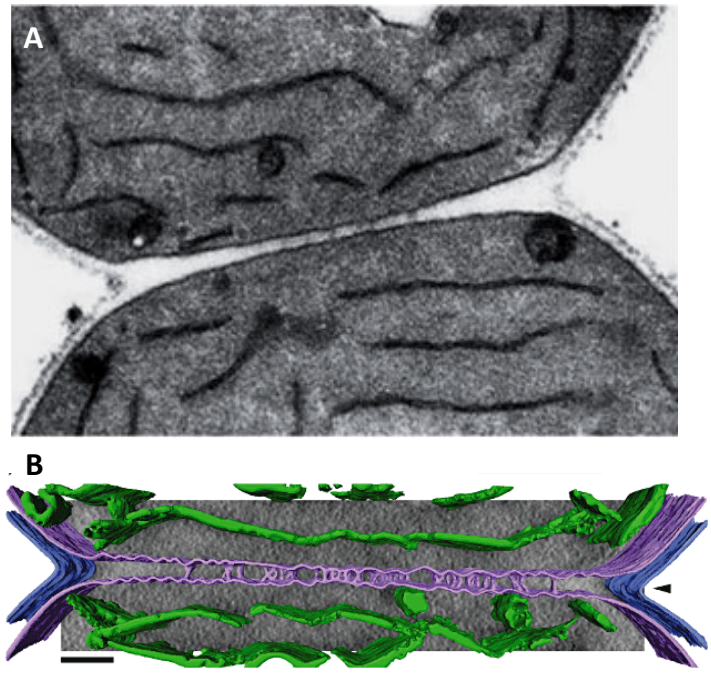

Figure 9: Intercellular septa in the filament of *Anabaena* sp. PCC 7120. (A) A transmission electron micrograph showing an intercellular septum, in which thin structures perpendicular to the cytoplasmic membranes of the two adjacent cells can be observed. Courtesy of Iris Maldener, Universität Tübingen, Germany (Flores and Herrero, 2010). (B) Tomographic volumes of cellular structures in the cell–cell contact region based on potassium permanganate treatment. Septosomes (another name for septal junctions) and CM (purple), thylakoids (green) and OM (blue) are represented as single volumes for simplicity. Scale bar is 200 nm (Wilk *et al*., 2011).

1.4.2.1. The SepJ protein

SepJ (also known as FraG; Nayar *et al*., 2007) is a 751 amino acid protein encoded by ORF *alr2338*, which is downstream of *hetR* in the genome of *Anabaena* sp. PCC 7120 (Flores *et al*., 2007). *Anabaena* strains lacking the SepJ protein make short filaments when growing in the presence of combined nitrogen (Flores *et al*., 2007; Nayar *et al*., 2007; Mariscal *et al*., 2011), although filament fragmentation is most evident under N deprivation, resulting in filaments of only two or three cells. *sepJ* mutants are impaired in N_2 fixation (Fox^- phenotype) and nitrogenase activity, both under oxic and anoxic conditions (Fix^- phenotype) (Flores *et al*., 2007). *sepJ* mutants have been also analyzed by means of electron tomography, and it has been described that its septal junctions are comparable to those observed in the wild-type strain, but the distance between the CM of the adjacent cells is considerably shorter in the mutant, which is indicative of altered septa (Wilk *et al*., 2011).

Fluorescent tracers, including calcein and 5-CFDA, have been used to investigate the possible role of SepJ in intercellular molecular exchange. It has been shown that mutants lacking the SepJ protein are severely impaired in calcein transfer, both after growth in the presence of nitrate and after incubation in the absence of combined nitrogen (Mullineaux *et al*., 2008). Intercellular transfer of 5-CFDA is also affected in cells of some *sepJ* mutants grown with nitrate, but to a lesser extent than calcein transfer (Mariscal *et al*., 2011).

SepJ contains three main domains: an N-terminal coiled-coil domain, a central linker domain, and a C-terminal permease domain. The coiled-coil domain could be involved in protein-protein interactions (Lupas and Gruber, 2005) and has been shown to be required for filament integrity and diazotrophic growth (Mariscal *et al*., 2011). The linker domain is rich in proline and serine residues and, although not required for filament integrity, influences the effective size of the channel, its deletion impairing the intercellular transfer of calcein (Mariscal *et al*., 2011). The permease domain is similar to CM proteins belonging to the Drug and Metabolite Transporter (DMT) family, and has been reported to be involved in intercellular molecular exchange functions required for diazotrophy (Flores *et al*., 2007; Mariscal *et al*., 2011). A SepJ protein containing these three domains is generally found in filamentous heterocyst-forming cyanobacteria, whereas filamentous non-heterocyst-forming cyanobacteria contain proteins similar to SepJ but with only the coiled-coil and permease domains. Furhermore, some unicellular cyanobacteria contain proteins belonging to the DMT family, showing some similarity to the permease domain of SepJ (Mariscal *et al*., 2011; Nürnberg *et al*., 2014). SepJ is conspicuously located at the cell poles in the intercellular septa between vegetative cells and between vegetative cells and heterocysts, as demonstrated by Green Fluorescent Protein (GFP) fusions (Flores *et al*., 2007; Mariscal and Flores, 2010). This focused septal localization has been shown to require the presence of the coiled-coil domain but not of the linker domain (Mariscal *et al*., 2011). This observation, together with the predicted topology of SepJ, suggests that the C terminal part of the protein is located in the cytoplasm, whereas the linker and coiled-coil domains would reside in the periplasm, where they could have a role in joining adjacent cells by means of intercellular interactions of the coiled-coil domains (Mariscal, 2014). These results suggested that SepJ contributes to make a channel that connects adjacent cells and allows the intercellular transfer of small molecules.

1.4.2.2. The Fra proteins

Besides SepJ, other proteins including FraC, FraD and FraE influence filament integrity in *Anabaena* sp. PCC 7120 (Bauer *et al*., 1995; Merino-Puerto *et al*., 2010), and it is likely that they are also components of septal junction complexes. Inactivation of each of the corresponding *fra* genes results in a strain impaired in diazotrophic growth that shows extensive filament fragmentation upon incubation in the absence of combined nitrogen. FraC, FraD and FraE are integral membrane proteins and are encoded by the *fraCDE* operon, which is expressed at relatively low and constant levels both in the presence and absence of combined nitrogen (Merino-Puerto *et al*., 2010). FraD contains a coiled-coil periplasmic domain, whereas FraE is homologous to type-2 ABC transporters. By electron microscopy, it has been observed that *fra* mutants present an altered heterocyst neck and a wider septum between vegetative cells and heterocysts than the wild type. Specifically, the *fraE* mutant lacks the heterocyst neck and the heterocyst polysaccharide layer is missing (Merino-Puerto *et al*., 2011b). In order to study the FraC and FraD subcellular localization, GFP fusions have been used, showing that both FraC and FraD are localized at the intercellular septa, although

not as focused as SepJ. In addition, FraC and FraD are needed for a correct localization of SepJ (Merino-Puerto *et al*., 2010; 2011b).

Calcein and 5-CFDA transfer are significantly decreased in *fraC* and *fraD* mutants, as well as in a *fraC fraD* double mutant (Merino-Puerto *et al*., 2010; 2011b). It has been suggested that the impairment of calcein transfer in the *fra* mutants could be an indirect effect, since *fraC* and *fraD* mutants show an alteration of the SepJ localization and SepJ is required for calcein transfer. Therefore, FraC and FraD could together be involved in a separate pathway for the transfer of 5-CFDA, independently of SepJ (Merino-Puerto *et al*., 2011b). In summary, SepJ, FraC and FraD proteins appear to contribute to septal junction complexes involved not only in filament integrity, but also in intercellular molecular exchange in the diazotrophic filament of filamentous cyanobacteria.

1.5. Cyanophycin

Cyanophycin is a biopolymer that is thought to represent a dynamic reservoir of nitrogen. Cyanophycin (CGP, cyanophycin granule polypeptide) was discovered in 1887 by the Italian botanist Borzi, who introduced the term "cyanoficina" for the refractile inclusions seen in the cytoplasm of cells of cyanobacteria during microscopic observations (Borzi, 1887). Since then, cyanophycin has been a subject of intense research for cyanobacteriologists.

Characterization and description of these inclusions have been extensively reported since the 1950s. Fogg (1951) observed that the material could be stained by the Sakaguchi reaction (which is specific for free and protein-bound arginine) (Messineo, 1966) and was located at the heterocyst poles in the cyanobacterium *Anabaena cylindrica*. Simon and co-workers carried out a detailed characterization of CGP at a structural, physiological and biochemical level. This polymer is a non-ribosomically synthesized peptide composed of aspartate and arginine (multi-L-arginyl-poly-L-aspartic acid). It is accumulated in the cells in the form of membrane-less cytoplasmatic inclusions and occurs in most, but not all, cyanobacteria, both unicellular and multicellular forms. The molecular mass of CGP ranges between 25 and 100 kDa, and these non-uniform chains are spontaneously self-associated into granules that are water insoluble and contain a high degree of β-pleated sheet structure (Simon, 1971; Lang *et al*., 1972; Simon and Weathers, 1976; Simon *et al*., 1980; Allen and Weathers, 1980; Allen, 1984). Other authors established the conditions in which cyanophycin is accumulated inside the cells, such as in the stationary growth phase, under unbalanced growth (Allen, 1984; Simon, 1987) or during transitions between utilization of different nitrogen sources (Mackerras *et al*., 1990). Studies carried out by Lang and co-workers (Lang *et al*., 1972) and Sherman and co-workers (Sherman *et al*., 1998; Sherman *et al*., 2000) have focused on the dynamics of accumulation as well as the cellular localization of this reserve material in unicellular and filamentous cyanobacteria. In Figure 10, an illustration of conditions of cyanophycin accumulation in diazotrophic unicellular cyanobacteria is presented.

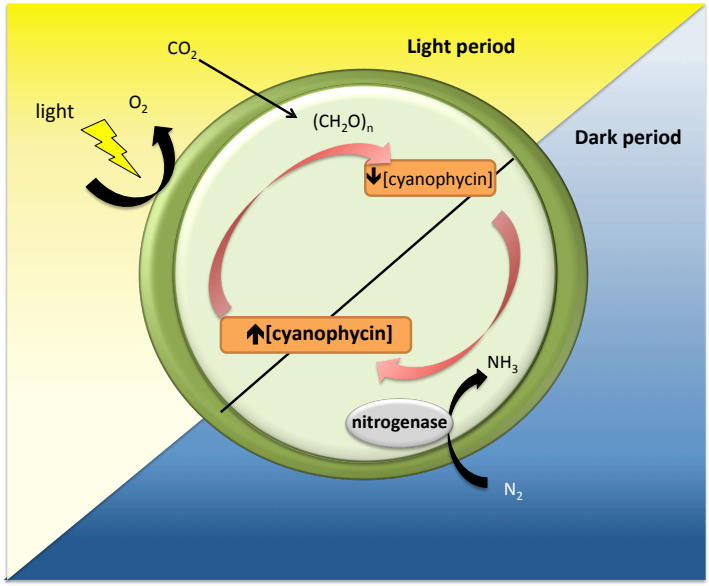

Figure 10: Cyanophycin accumulation in unicellular diazotrophic cyanobacteria. Unicellular cyanobacteria fix carbon and nitrogen in the same cell, but these processes are temporally separated: during the dark period, when atmospheric nitrogen is fixed, cyanophycin is synthetized and accumulated in the cytoplasm. During light periods, carbon is fixed through photosynthesis while the stored cyanophycin is consumed.

Work regarding the identification of the CGP synthetizing enzyme and its coding gene was mainly contributed by Lockau and co-workers (Ziegler *et al*., 1998; Berg *et al*., 2000). Cyanophycin synthetase is the single enzyme involved in cyanophycin synthesis and requires L-aspartic acid, L-arginine, ATP, magnesium ions and a cyanophycin primer to catalyze the biosynthetic reaction. The enzyme is a homodimer of 96 kDa of the product of the *cphA* gene, which was first identified in *Synechocystis* sp. PCC 6803 (Ziegler *et al*., 1998).

Cyanophycin is highly resistant to degradation by all conventional proteases, and the only enzyme known to be capable to hydrolyze this polymer is cyanophycinase. Degradation of CGP was first described in *Anabaena cylindrica* by Simon *et al*. (1980). Later, the studies of Gupta and Carr (1981a) and Allen (1984) reported CGP degradation in crude extracts prepared from cyanobacterial cells. Purification of cyanophycinase from *Synechocystis* sp. PCC 6803 and identification of the corresponding coding gene (*cphB*) were carried out by Richter *et al*. (1999). The enzyme is a serine-type protease highly specific for the polypeptide cyanophycin (Richter *et al*., 1999; Law *et al*., 2009). The crystal structure of cyanophycinase from *Synechocystis* sp. PCC 6803 was accomplished at 1.5 Å resolution, showing that the enzyme is a dimer with the individual protomers resembling aspartyl dipeptidase enzymes. Some studies (Gupta and Carr, 1981a; Richter *et al*., 1999) could identify a dipeptide, β-aspartyl-arginine, as the main product of the reaction catalyzed by cyanophycinase. Thus, it was suggested that a second enzyme participated in the degradation of cyanophycin in order to make available the free amino acids, aspartate and arginine. Much effort has been put to identify the second enzyme involved in cyanophycin degradation, but it was not until 2002 that the group of Lockau reported that the putative plant-type asparaginases from *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120 expressed in *E. coli* showed not only asparaginase but also isoaspartyl dipeptidase activity, suggesting that these enzymes could be involved in the final step of cyanophycin degradation in cyanobacteria (Hejazi *et al*., 2002). A scheme illustrating cyanophycin metabolism is presented in Figure 11.

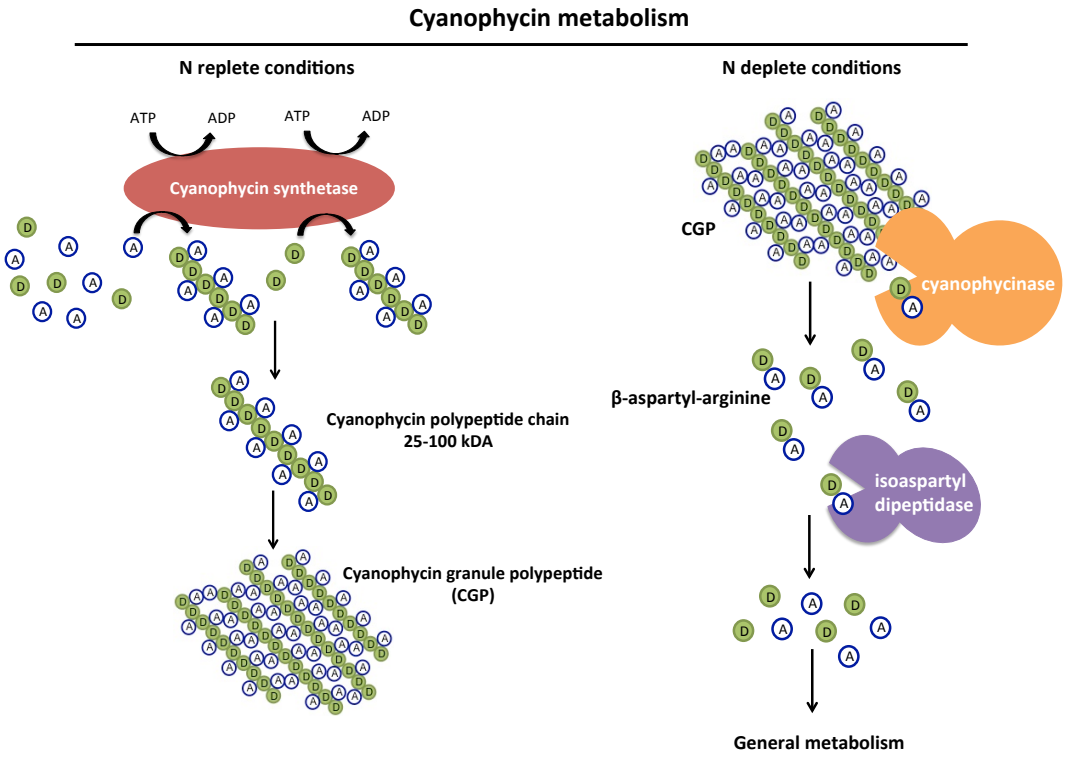

Figure 11: Cyanophycin metabolism. Under conditions of abundant nitrogen, cyanophycin is synthesized by cyanophycin synthetase, which has two catalytic domains, one adding aspartate (D) and the other arginine (A) residues to a cyanophycin primer. The cyanophycin polypeptide chains of variable length self-associate to form the cyanophycin granule. Under nitrogen-limiting conditions, cyanophycin is degraded by two enzymes: first, cyanophycinase hydrolyses cyanophycin and releases the dipeptide β-aspartyl-arginine. Second, an aspartyl dipeptidase cleaves the dipeptide to release aspartate and arginine (adapted from Law *et al*., 2009).

Although initially thought to be present only in cyanobacteria, CGP has been also detected in the heterotrophic bacterium *Acinetobacter calcoaceticus* (Krehenbrink *et al*., 2002), and an extracellular cyanophycinase (CphE) from *Pseudomonas anguillispetica* strain B1 was found to hydrolyze cyanophycin with high specificity (Obst *et al*., 2002). In addition, with the current vast availability of bacterial genome sequences, it has been possible to identify putative cyanophycin metabolism genes in bacteria belonging to different phylogenetic taxa not closely related to cyanobacteria (Füser and Steinbüchel, 2007).

Nowadays, cyanophycin is seen as a valuable biopolymer with potential biotechnological applications for industrial purposes. Thus, research on cyanophycin has focused on obtaining strains that accumulate high amounts of this polymer, both by recombinant and engineered versions of cyanophycin metabolism genes from cyanobacteria expressed in different organisms, including yeast and plants (Frey *et al*., 2002; Neumann *et al*., 2005; Hai *et al*., 2006; Steinle *et al*., 2008; 2010; Neubauer *et al*., 2012).

Detailed aspects of the structure and physiology, as well as the enzymology, regulation and gene expression of cyanophycin are presented as a bibliographic research in the review article presented in Annex I of Chapter 2 of this thesis, entitled *Cyanophycin, a cellular nitrogen reserve material*, which has been recently published in the book "The Cell Biology of Cyanobacteria" (E. Flores and A. Herrero, Eds., 2014).

1.6. Arginine metabolism

L-arginine is a metabolically versatile amino acid that not only serves as a building block for protein synthesis and as a precursor of polyamines, but also as a source of nitrogen, carbon and energy in different bacteria. Over the past decades, a variety of arginine catabolic pathways have been described in bacteria (Abdelal, 1979; Cunin *et al*., 1986), and research regarding the identification of genes and enzymes involved in biosynthetic and catabolic routes has continued to date (Xu *et al*., 2007).

1.6.1. Arginine biosynthesis

Arginine is synthetized from glutamate, and three different routes have been described for arginine biosynthesis in bacteria. In the so-called "linear pathway", the biosynthesis of arginine proceeds from glutamate in eight enzymatic steps, where the five first steps generate ornithine involving *N*-acetylated intermediates: *N*-acetylglutamate, *N*-acetylglutamate-5-phosphate, *N*-acetylglutamate-5-semialdehyde and N^2-acetylornithine (Figure 12). The synthesis of these *N*-acetylated intermediates is catalyzed by ArgA, ArgB (also known as NAGK), ArgC and ArgD, respectively. The subsequent step, mediated by the hydrolytic enzyme acetylornithinase, ArgE, removes the acetyl group of N^2-acetylornithine to yield ornithine. Enterobacteriaceae and Bacillaceae use this biosynthetic pathway, in which *N*-acetylglutamate synthetase (ArgA), the first enzyme of the pathway, is the target for feedback inhibition by arginine. In the "cyclic pathway", occurring in pseudomonads, photosynthetic bacteria, cyanobacteria and *Thermus aquaticus*, the acetyl group of *N*-acetylornithine is recycled by ArgJ, an acetyltransferase, to yield *N*-acetylglutamate, and feedback inhibition by the end-product arginine appears to be on *N*-acetylglutamate kinase (NAGK or ArgB) (Hoare and Hoare, 1966; Lu, 2006) (Figure 12). Therefore, the difference between the linear and the cyclic pathways of arginine biosynthesis resides in the strategy used to remove the acetyl group from N^2-acetylornithine. Both pathways converge in ornithine, and three additional steps are required to generate arginine. First, a carbamoyl-phosphate group is added to ornithine generating citrulline in a reaction catalyzed by ornithine carbamoyltransferase (ArgF). Then, citrulline is used by argininosuccinate synthase (ArgG), involving the addition of one molecule of aspartate and using ATP to generate the intermediate argininosuccinate, which is used by argininosuccinase (ArgH) to finally produce arginine with the release of fumarate (Cunin *et al*., 1986; Lu, 2006; Xu *et al*., 2007).

Recently, a novel family of transcarbamylases has been described in *Xanthomonas campestris*, thus defining a third pathway for arginine biosynthesis. In this microorganism, *N*-acetylornithine is converted into *N*-acetylcitrulline by an enzyme encoded by *argF'*, and ArgE deacetylates *N*-acetylcitrulline to form citrulline. Homologues of *argF'* have been found in other Eubacteria (Shi *et al*., 2005; Lu, 2006).

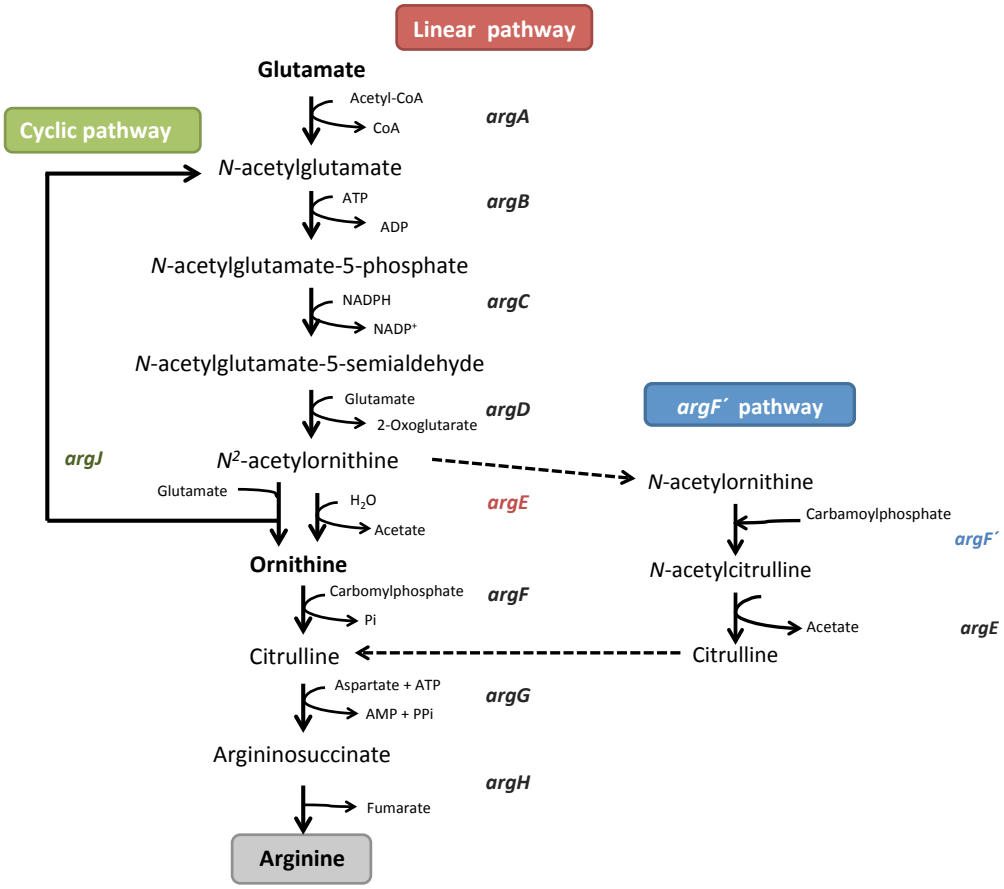

Figure 12: Arginine biosynthesis. ArgA, *N*-acetylglutamate synthetase; ArgB, *N*-acetylglutamate-5-phosphotransferase; ArgC, *N*-acetylglutamate-5-semialdehyde dehydrogenase**;** ArgD, N^2-acetylornithine-5-aminotransferase; ArgE, acetylornithinase; ArgJ, ornithine acetyltransferase; ArgF, ornithine carbamoyltransferase; ArgG, argininosuccinate synthetase; ArgH, argininosuccinase. The key enzymes of each pathway (see text) are indicated in colour.

In *Anabaena* sp. PCC 7120 some genes coding for enzymes of the arginine biosynthesis pathway have been annotated, namely, *N*-acetylglutamate kinase (*argB*; *alr1245*), *N*-acetylglutamate semialdehyde dehydrogenase (*argC*; *all2498*), *N*-acetylornithine aminotransferase (*argD*; *alr1080*), ornithine carbamoyltransferase (*argF*; *alr4907*) and argininosuccinate synthetase (*argG*; *alr4798*) (Floriano *et al*., 1994; Kaneko *et al*., 2001). Expression of *argC* and *argD* has been studied in detail in this cyanobacterium (Floriano *et al*., 1992), and it has been shown that they are expressed monoscistronically and constitutively in vegetative cells (Floriano *et al.,* 1994).

Besides feedback inhibition by arginine, the biosynthetic pathway is also under the control of the C-to-N sensitive signal transduction protein PII, the *glnB* gene product. In *Synechococcus* sp. PCC 7942 and other oxygenic photosynthetic organisms including *Arabidopsis*, PII regulates the activity of NAGK, the second enzyme involved in arginine biosynthesis. It has been shown that, under conditions of nitrogen excess, non-phophorylated PII stimulates the activity of NAGK, thus favoring arginine synthesis (Burillo *et al*., 2004; Heinrich *et al*., 2004; Ferrario-Méry *et al*., 2006). The crystal structure of the

PII-NAGK complex and the sophisticated activation mechanism has been studied in detail (Llácer *et al*., 2007; 2008). In addition, PII mutants of *Synechocystis* sp. PCC 6803 (Maheswaran *et al*., 2006) and *Anabaena* sp. PCC 7120 (Paz-Yepes *et al*., 2009) show no accumulation of cyanophycin, thus connecting the regulation of arginine synthesis and the accumulation of this nitrogen-rich polymer. Interestingly, in *Nostoc ellipsosporum* a mutant lacking an arginine-biosynthetic gene was unable to accumulate cyanophycin in heterocysts and akinetes. The product of this gene, which shows 58% similarity to ArgC from *Bacillus subtilis* but only 26% similarity to the product of *argC* from *Anabaena* sp. PCC 7120, was denoted *argL*. The authors suggested that *N. ellipsosporum* has two different genes involved in arginine biosynthesis, *argC* and *argL*, and that whereas the product of the former would be involved primarily in arginine biosynthesis for protein synthesis, the product of *argL* might be involved specifically in the formation of cyanophycin (Leganés *et al*., 1998).

1.6.2. Arginine catabolism

Arginine serves as a source of nitrogen, carbon and energy through a variety of catabolic pathways in Archaea and Eubacteria. During the past decades, six major catabolic routes have been described in Eubacteria (Figure 13), and it has been demonstrated that arginine catabolism in a given bacterium could occur either via only one pathway, such as in many heterotrophic bacteria, or through multiple catabolic pathways, as it occurs in some *Pseudomonas* species.

1.6.2.1. Arginase pathway

Arginase hydrolyzes arginine releasing urea and ornithine. The resulting ornithine is further catabolized to glutamate by ornithine transaminase (OAT) and Δ^1pyrroline-5-carboxylate dehydrogenase. Urea is further degraded by urease to ammonium and CO_2. The fate of ornithine and urea may differ from one microorganism to another. The last reaction of this pathway, the oxidation of pyrroline-5-carboxylate to glutamate, is shared with the proline catabolic pathway (Cunin *et al*., 1986). The arginase pathway has been found in yeast, *Neurospora crassa*, *Aspergillus nidulans*, in many *Bacillus* and *Streptomyces* species, *Proteus vulgaris*, *Thermus aquaticus*, mycobacteria and cyanobacteria (see below) (Abdelal, 1979; Cunin *et al*., 1986), although relatively few arginases from prokaryotes have been purified and characterized.

Besides being used as a carbon and nitrogen source, arginine could have other roles in different biological processes. In *Agrobacterium tumefaciens*, arginine utilization through the arginase pathway is closely linked to the Ti plasmid containing genes for the uptake and catabolism of opines formed in the tumor tissues of plants (Dessaux *et al*., 1986; Lu, 2006). Another example occurs in *Helicobacter pylori* in which arginase, encoded by *rocF*, is involved and plays important roles in pathogenesis (Gobert *et al*., 2001).

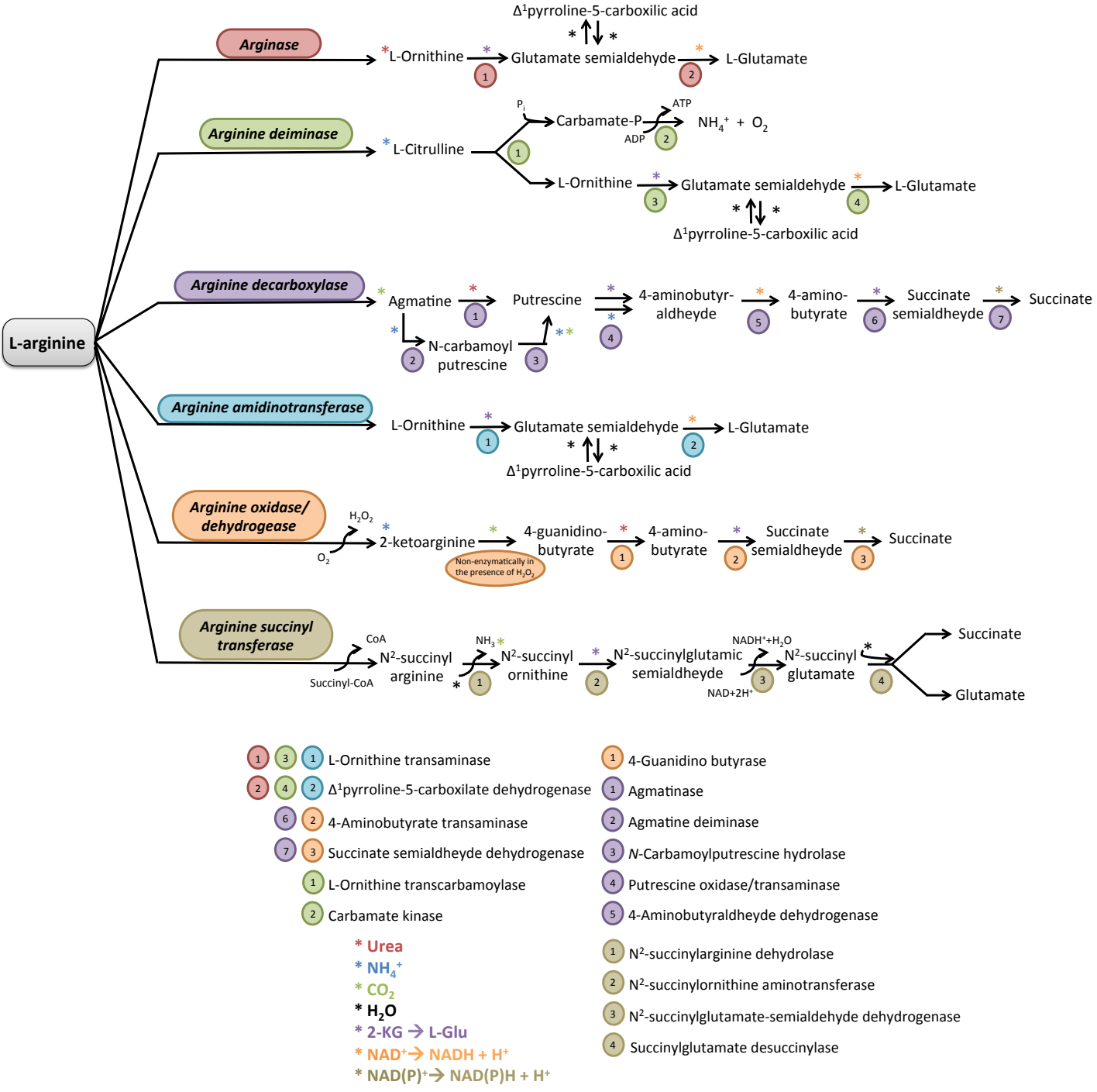

Figure 13: **Schematic representation of arginine catabolic pathways found in bacteria.** Enzymes, intermediate metabolites and final products are indicated. Asterisks represent intermediate products released in the reaction. Circles (see color code) represent enzymes involved in the corresponding pathway. Note that some enzymes catalyze reactions that are shared by different pathways (adapted from Schriek *et al.*, 2007).

1.6.2.2. Arginine deiminase pathway

Arginine deiminase (ADI), which produces citrulline and ammonium from arginine, ornithine transcarbamylase (OTCase) and carbamate kinase are the enzymes involved in this pathway, in which arginine is converted into ornithine, ammonia and CO_2 with the final production of 1 mol of ATP per mol of arginine consumed. The pathway is widely distributed in Eubacteria and Archea, and it is characterized by an abundant ornithine excretion, suggesting that only the guanidino group of ornithine is used (Cunin *et al*., 1986). The major physiological function of this pathway seems to be to provide ATP under anaerobic conditions (Lu, 2006).However, in some bacteria ornithine is further metabolized to glutamate as in other pathways.

1.6.2.3. Arginine decarboxylase pathway

In the arginine decarboxylase pathway (ADC), arginine is decarboxylated by arginine decarboxylase (*speA*) to generate agmatine, and agmatine is converted into putrescine and urea by an agmatinase (*speB*). Putrescine can also be formed from agmatine via carbamoylputrescine by agmatine deiminase (*aguA*) and *N*-carbamoylputrescine hydrolase (*aguB*), as it occurs in *Pseudomonas aeruginosa* (Nakada *et al*., 2001), *Aeromonas* sp. and lactic bacteria (Cunin *et al*., 1986). Further metabolism of putrescine depends on the organism considered. In lactic bacteria, putrescine is accumulated in the medium, whereas in other organisms putrescine can be utilized as a carbon and nitrogen source or for polyamine biosynthesis.

The conversion of putrescine into succinate in the second part of the arginine decarboxylase pathway that takes place in some bacteria involves four enzymatic steps. The first step is either the oxidation of one nitrogen atom of putrescine to give ammonia and 4-aminobutyraldehyde or the transamination between putrescine and 2-ketoglutarate to render glutamate and 4-aminobutyraldehyde, reactions catalyzed by putrescine oxidase and putrescine transaminase, respectively. The next step is the oxidation of 4-aminobutyraldehyde to 4-aminobutyrate (GABA) by 4-aminobutyraldehyde dehydrogenase. The subsequent steps involve the enzymatic activities of 4-aminobutyrate transaminase and succinate semialdehyde dehydrogenase to finally yield succinate (Cunin *et al*., 1986). Thus, the arginine decarboxylase pathway would provide carbon and nitrogen sources, as it has been described in *Escherichia coli* and *Pseudomonas* sp. (Kurihara *et al*., 2005; Chou *et al*., 2008).

Polyamines are polycationic compounds that have been implicated in a wide variety of biological reactions, including synthesis of DNA, RNA and proteins, both in eukaryotic and prokaryotic cells, and are essential for normal growth (Tabor and Tabor, 1985). The most common polyamines are putrescine, spermidine and spermine. It has been suggested that part of the enzymatic polyamine biosynthetic pathway of *Arabidopsis* and other plants was

acquired from the cyanobacterial ancestor of chloroplasts (Illingworth *et al*., 2003; Fuell *et al*., 2010).

1.6.2.4. Arginine amidinotransferase pathway

Besides by arginases, ornithine may also be formed by arginine amidinotransferases, which catalyze the reversible transfer of an amidino group from a donor compound to the amino moiety of an acceptor. Thus, the first step of this route is the transfer of the amidino group of arginine to another substrate, such as glycine, releasing ornithine. Ornithine transaminase catalyzes then the conversion of ornithine to glutamate semialdehyde, and Δ^1pyrroline-5-carboxylate dehydrogenase produces glutamate as the final product, as it is also the case for the arginase pathway.

Arginine amidinotransferases have been shown to function as arginine:glycine amidinotransferases in creatine biosynthesis in vertebrates, as arginine:glycine amidinotransferases in the biosynthesis of toxins, such as cylindrospermopsin, in various cyanobacteria, as arginine:inosamine phosphate amidinotransferases in streptomycin biosynthesis in *Streptomyces* sp. and as arginine:lysine amidinotransferases in phaseolotoxin biosynthesis in *Pseudomonas syringae* pv. *phaseolica* (Schriek *et al*., 2007).

1.6.2.5. Arginine oxidase/dehydrogenase pathway

In this route, arginine is converted to 2-ketoarginine by an arginine oxidase/dehydrogenase. The product of this first reaction could be decarboxylated either via 2-ketoarginine decarboxylase and 4-guanidinobutyraldehyde dehydrogenase, or non-enzymatically in the presence of H_2O_2, producing 4-guanidinobutyate. This product is then converted into 4-aminobutyrate (GABA) by a 4-guanidinobutyrase, which is further metabolized by two reactions that are shared with the arginine decarboxylase pathway to produce succinate (Cunin *et al*., 1986).

1.6.2.6. Arginine succynil transferase pathway

The first reaction of this pathway is catalyzed by an arginine succinyltransferase; N^2-succinylarginine dehydrolase then converts the resulting N^2-succinylarginine into N^2-succinylornithine, ammonia and CO_2. The following reaction is catalyzed by N^2-succinylornithine aminotransferase, which will transfer the nitrogen atom of N^2-succinylornithine to 2-ketoglutarate, yielding glutamate and N^2-succinylglutamate semialdehyde. The latter is oxidized by a dehydrogenase into N^2-succinylglutamate, which is cleaved into succinate and glutamate (Cunin *et al*., 1986). This pathway is suggested to be mainly limited to those heterotrophic eubacteria that have the ability to use L-arginine as a source of both nitrogen and carbon (Cunin *et al*., 1986; Lu, 2006).

1.6.3. Arginine catabolism in cyanobacteria

As it has been mentioned previously, arginine appears to be an important metabolite in the physiology of cyanobacteria, because it is found in cyanophycin, and also because it might function as a nitrogen source and nitrogen vehicle for intracellular molecular exchange in filamentous heterocysts-forming cyanobacteria. In cyanobacteria, arginine catabolism is not extensively understood and only few studies have been published regarding arginine catabolic enzymes. Most of the catabolic pathways described in Eubacteria have also been detected in cyanobacteria by bioinformatics analysis (Schriek *et al*., 2007). However, identification of genes and detection of arginine catabolism-related enzymes *in vivo* is more difficult, and only three routes have been detected by physiological studies: the arginase pathway, the arginine deiminase pathway and the arginine decarboxylase pathway (Quintero *et al*., 2000; Schriek *et al*., 2007; Wegener *et al*., 2010). The only pathway not yet related to cyanobacteria is the arginine succinyl transferase, as no evidence for the presence of genes involved in this pathway was found in the genome sequences from 24 cyanobacterial strains (Schriek *et al*., 2007).

In an early report, it was described that *Aphanocapsa* 6308 catabolized arginine through either the arginase pathway, which would provide nitrogen for the cells, or the arginine deiminase (cited as arginine dihydrolase) pathway, that provides carbon, nitrogen and energy for the cells (Weathers *et al*., 1978). In that study, no product from the arginine decarboxylase pathway was detected. One of the best-studied cyanobacteria regarding arginine catabolism, both by physiological and proteomics analysis, is *Synechocystis* sp. PCC 6803. By *in vivo* studies with ^{14}C-labeled substrates and mutational analysis of various amino acid metabolism-related genes, Quintero *et al*. (2000) proposed a model for arginine catabolism, suggesting the operation of an arginase-like pathway combined with a sort of urea cycle as the main route for the utilization of arginine in this cyanobacterium. The authors also detected the arginine decarboxylase pathway, but it was apparently less important than the arginase-like route under the experimental conditions assayed. Nevertheless, despite detecting arginase activity in cells of strain PCC 6803 and two putative genes, *sll0228* and *sll1077,* showing homology to ureohydrolase proteins, the arginase-encoding gene in this cyanobacterium was not identified (Quintero *et al*., 2000). The model proposed in that study provided a suitable explanation of how nitrogen is made available to the cells during arginine assimilation and uptake from the medium, as well as during metabolism of arginine from cyanophycin granule mobilization. In addition, in a large-scale proteomic study, in which *Synechocystis* sp. PCC 6803 was subjected to conditions of environmental perturbations, enzymes of the arginine decarboxylation pathway (arginine decarboxylase and agmatinase) were found up-regulated compared to control conditions, suggesting a role of this pathway during perturbations (Wegener *et al*., 2010). Furthermore, cyanophycinase and proteins involved in the transport and utilization of urea and arginine, such as those of the Bgt transporter, were also up-regulated. Thus, it seems that under stress conditions, the arginine decarboxylase pathway converting putrescine to succinate could provide carbon and nitrogen, as it occurs in *E. coli* and *Pseudomonas* sp. (Kurihara *et*

al., 2005; Chou *et al*., 2008). This is in contrast with the results obtained in a study based on the bioinformatic analysis of 24 cyanobacterial genomes, including that of *Synechocystis* sp. PCC 6803, in which genes coding for putrescine oxidase or putrescine transaminase, needed in the arginine decarboxylase pathway that ends in succinate, were not found in any of the investigated genomes (Schriek *et al*., 2007). These authors suggested that the arginine decarboxylase pathway in cyanobacteria could be mainly involved in the synthesis of polyamines and release of ammonium from arginine (Schriek *et al*., 2007).

In heterocyst-forming cyanobacteria such as those of the genus *Anabaena*, arginase and arginine deiminase activities were detected in cell-free extracts prepared from *Anabaena cylindrica* and *Anabaena* sp. PCC 7120 (Gupta and Carr, 1981b). Specifically, both catabolic activities were found to be higher in cell-free extracts from vegetative cells than from heterocysts, whereas the arginine biosynthesis enzymes investigated were similarly active in heterocyst and vegetative cell extracts. The authors considered that this compartmentalization of arginine metabolism in the diazotrophic filaments might be related to the specific activities of cyanophycinase and cyanophycin synthetase, which are enhanced in heterocysts as compared to vegetative cells (Gupta and Carr, 1981a). Arginase activity has been also found in *Anabaena cycadeae*, where it was described to be repressible by NH_4^+ (Singh and Bisen, 1994).

Besides these three arginine catabolizing routes detected by physiological studies, an amino acid oxidase, namely AoxA, has been found in some cyanobacteria. The enzyme was first purified and characterized from *Anacystis nidulans* (Pistorius *et al*., 1979; Pistorius and Voss, 1980), and it was reported to have high specificity for basic amino acids, especially arginine, releasing large amounts of ammonium into the medium, suggesting that the activity of this enzyme accounts for the ability of *Anacystis nidulans* to use arginine as a nitrogen source for growth (Flores *et al*., 1982). By genome sequence comparisons, *aoxA* and an additional gene with similarity to *aoxA*, namely *aoxB*, were detected in other cyanobacteria, including some marine *Synechococcus* strains, *Synechococcus elongatus* PCC 6301 and PCC 7942, *Synechocystis* sp. PCC 6803, *Gloeobacter violaceus* PCC 7421, *Nostoc punctiforme* PCC 73102 and *Nostoc* (*Anabaena*) sp. PCC 7120 (Gau *et al*., 2007).

Recently, a novel arginine:glycine amidinotransferase from two different cyanobacteria, *Cylindrospermopsis raciborskii* (Muenchhoff *et al*., 2010) and *Aphanizomenon ovalisporum* (Shalev-Alon *et al*., 2002; Barón-Sola *et al*., 2013) have been identified and characterized. The enzyme, named CyrA in *Cylindrospermopsis raciborskii* and AoaA in *Aphanizomenon ovalisporum*, is involved in the first step of the biosynthesis of cylindrospermopsin, a potent hepatoxin. This arginine:glycine amidinotransferase represents a novel group of amidinotransferases that use only arginine and glycine as substrates with a complex kinetic mechanism that differs from that of the eukaryotic arginine:glycine amidinotransferases (Muenchhoff *et al*., 2010). Thus, it has been suggested that arginine:glycine amidinotransferase and other prokaryotic amidinotransferases participate in secondary

metabolite biosynthesis rather than in the primary catabolism of arginine as a nitrogen, carbon and energy source (Shalev-Alon *et al*., 2002; Muenchhoff *et al*., 2010).

It appears that the arginase pathway is one of the main catabolic routes to utilize arginine in cyanobacteria, producing glutamate, which is an important carbon vehicle for intercellular exchange and involved in the GS/GOGAT cycle, as mentioned above. Much effort has been put in the identification of the gene encoding a putative arginase, but it still remains unknown. This could be due, in part, to the fact that automatic annotation procedures relying on sequence comparisons may lead to wrong assignments of enzyme activities (Sekowska *et al*., 2000). In fact, this is the case of arginases and agmatinases, enzymes involved in the arginase and arginine decarboxylase pathways, respectively. Both arginases and agmatinases belong to the ureohydrolase protein family. In a detailed study of this family based on functional homology, Sekowska *et al*. (2000) showed that the ureohydrolase family is indeed split into two major groups, agmatinases and arginases, further suggesting that agmatinases predated arginases, which implies that the arginase pathway evolved later than the arginine decarboxylase pathway. An examination of the phylogenic trees revealed that some enzymes annotated as agmatinases are grouped with arginases, and *vice versa*. An example of this is the case of *Synechocystis* sp. strain PCC 6803, in which the ureohydrolase protein in the database is annotated as an arginase, but the identified function turned out to be that of an agmatinase (Sekowska *et al*., 2000; Quintero *et al*., 2000; Schriek *et al*., 2007). This puzzling picture has emerged also from other studies attempting to identify genes encoding arginine-related enzymes by genome sequence comparison, as is the case of arginine deiminases and arginine amidinotransferases (Schriek *et al*., 2007), both enzymes belonging to the superfamily of guanidine-group modifying enzymes.

In the *Anabaena* sp. PCC 7120 genome, genes encoding arginine decarboxylase and enzymes corresponding to the second and subsequent steps of the arginase pathway can be identified (Kaneko *et al*., 2001). On the other hand, although the presence of an arginase-encoding gene is not evident, ORF *alr2310* is annotated as "similar to agmatinase" (Kaneko *et al*., 2001). Owing to the missannotation of ureohydrolase family proteins previously discussed (Sekowska *et al*., 2000), and given that by comparative studies of cyanobacterial genomes *alr2310* has been annotated as an arginase and as an agmatinase (Schriek *et al*., 2007), the actual function of the product of this gene is unclear. Similarly, ORF *alr4995* from *Anabaena* sp. PCC 7120 has been annotated as an arginine deiminase as well as an arginine amidinotransferase (Schriek *et al*., 2007), leaving unresolved the question of which catabolic pathway could be operative in this filamentous heterocyst-forming cyanobacterium.

1.7. Objectives of this study

The general plan in this thesis was to address the study of some structural and metabolic aspects of multicellularity in the model heterocyst-forming cyanobacterium *Anabaena* sp. PCC 7120.

Although the cell envelope from cyanobacteria has been studied in some detail, the role of this structure in multicellularity has not been addressed until recently. Taking into account that filamentous cyanobacteria present a continuous outer membrane, the aim of this study was to elucidate a possible role of cell envelope components, the peptidoglycan layer(s) and the outer membrane in filamentation, the process of producing and maintaining filaments, thus contributing to the growth of *Anabaena* forming long trichomes.

On the other hand, despite the large amount of published work on diazotrophic metabolism, the nitrogenous metabolites that are transferred from heterocysts to the vegetative cells are still not fully known. This work aimed to study the role of cyanophycin in the diazotrophic physiology, and of its derivative products as possible nitrogen vehicles in the diazotrophic filament of *Anabaena*.

Research objectives:

In the model heterocyst-forming strain *Anabaena* sp. PCC 7120, we addressed:

1. The study of the role of cell envelope components in filamentation.

2. The study of ORF *all3922*, annotated as an L-asparaginase, and its possible role in the degradation of cyanophycin and in the diazotrophic physiology.

3. The study of ORF *alr2310*, annotated as similar to agmatinase, and of ORF *alr4995*, encoding a possible guanidine-group modifying enzyme, and their role in arginine catabolism.

Chapter 1:

Cell envelope components influencing filament length in the heterocyst-forming cyanobacterium *Anabaena* sp. PCC 7120

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Cell Envelope Components Influencing Filament Length in the Heterocyst-Forming Cyanobacterium *Anabaena* sp. Strain PCC 7120

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Heterocyst-forming cyanobacteria grow as chains of cells (known as trichomes or filaments) that can be hundreds of cells long. The filament consists of individual cells surrounded by a cytoplasmic membrane and peptidoglycan layers. The cells, however, share a continuous outer membrane, and septal proteins, such as SepJ, are important for cell-cell contact and filament formation. Here, we addressed a possible role of cell envelope components in filamentation, the process of producing and maintaining filaments, in the model cyanobacterium *Anabaena* sp. strain PCC 7120. We studied filament length and the response of the filaments to mechanical fragmentation in a number of strains with mutations in genes encoding cell envelope components. Previously published peptidoglycan- and outer membrane-related gene mutants and strains with mutations in two genes (*all5045* and *alr0718*) encoding class B penicillin-binding proteins isolated in this work were used. Our results show that filament length is affected in most cell envelope mutants, but the filaments of *alr5045* and *alr2270* gene mutants were particularly fragmented. All5045 is a DD-transpeptidase involved in peptidoglycan elongation during cell growth, and Alr2270 is an enzyme involved in the biosynthesis of lipid A, a key component of lipopolysaccharide. These results indicate that both components of the cell envelope, the murein sacculus and the outer membrane, influence filamentation. As deduced from the filament fragmentation phenotypes of their mutants, however, none of these elements is as important for filamentation as the septal protein SepJ.

Multicellularity appears to have arisen several times during the course of evolution and has evolved in different phylogenetic groups, including bacteria, fungi, algae, plants, and metazoans (1, 2). Multicellularity generally involves cell-cell adhesion, intercellular communication, and the differentiation of specialized cells. Bacterial manifestations of multicellularity range from undifferentiated chains of cells to morphologically differentiated structures, and the behavior of cells within multicellular structures is coordinated by both shared and unique molecular mechanisms (3). Because they present differentiated cells that carry out distinct functions, some prokaryotes, including myxobacteria, streptomycetes, and cyanobacteria, are classified as patterned multicellular organisms (3). Cyanobacteria are characterized by performing oxygenic photosynthesis, but they show very diverse morphologies that include both unicellular and multicellular forms (4). Among multicellular forms, some cyanobacteria, such as those of the genera *Anabaena* and *Nostoc*, make filaments (also known as trichomes) that can be hundreds of cells long. When deprived of nitrogen, the cells of these genera are characterized by specialized cells that have differing nutritional tasks, oxygenic photosynthesis and nitrogen fixation, which contribute to the growth of the filament as an organismic unit (5, 6).

Because an exchange of carbon and nitrogen compounds occurs between CO_2-fixing vegetative cells and N_2-fixing heterocysts (5, 6), filament integrity is essential for the diazotrophic growth of heterocyst-forming cyanobacteria. In the model heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120 (here referred to as *Anabaena*), several genes whose mutation results in filament fragmentation are known (7–13), and some of these genes encode cell-cell joining proteins. The *fraCDE* operon and the *sepJ* gene encode integral membrane proteins that are required for *Anabaena* to make long filaments, mainly when deprived of fixed nitrogen, although inactivation of *sepJ* produces a stronger filament fragmentation phenotype than inactivation of any of the *fra* genes (9–12). Available evidence suggests that SepJ on one hand and FraC and FraD on the other are part of septal junction complexes that are likely involved not only in filament integrity but also in intercellular molecular exchange (14, 15).

Cyanobacteria are diderm bacteria (16) that bear a cytoplasmic membrane (CM) and an outer membrane (OM), the latter lying outside the peptidoglycan (PG) layer or murein sacculus (17, 18). Whereas the CM is a phospholipid bilayer, the OM is an asymmetrical bilayer consisting of phospholipids and lipopolysaccharide (LPS) in the inner and outer leaflet, respectively (19). The permeability of the OM is due to the presence of porins, proteins with a β-barrel fold that permit the passage of small ions and molecules (20). In a proteomic analysis of *Anabaena*, some OM porins could be identified (21). Omp85 proteins are required for the incorporation of porins and other β-barrel proteins into the OM (22). The *Anabaena* genome encodes three Omp85-like proteins, Alr0075, Alr2269, and Alr4893, among which Alr2269 likely represents the principal Omp85-like protein in this cyanobacterium (23–26). LPS is composed of a hydrophobic membrane anchor (lipid A), an oligosaccharide core, and an O antigen consisting of a repeating oligosaccharide (19, 27). LPS is a barrier for hydrophobic molecules (28), which, coupled with a porin diffusion limit for hydrophilic molecules of about 700 Da, makes the OM an effective selective permeability barrier (20). Although showing some char-

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TABLE 1 Cell envelope mutant strains used in this work

Strain	Genotype	Protein product/predicted function	Reference
AFS-I-*alr2269*[a]	*alr2269*::pCSV3	Chloroplast outer envelope membrane protein homolog (Omp-85-like protein); involved in outer membrane protein biogenesis	26
AFS-I-*alr0075*[a]	*alr0075*::pCSV3	Omp-85-like protein; not expressed in heterocysts	26
AFS-I-*alr4893*[a]	*alr4893*::pCSV3	Omp-85-like protein	26
AFS-I-*alr2270*[a]	*alr2270*::pCSV3	LpxC; UDP-3-*O*-acyl *N*-acetylglucosamine deacetylase; involved in lipid A biosynthesis	26
DR1822	Tn*5*::Ω within *all4829*	RfbP; UDP-galactophosphotransferase; involved in O antigen biosynthesis	29
DR1963	Tn*5*::Ω within *all4830*	RfbZ; mannosyl transferase; involved in O antigen biosynthesis	29
DR1967[a]	*all4828*::Ω	RfbD; GDP-D-mannose dehydratase; involved in O antigen biosynthesis	29
AFS-I-*all1861*[a]	*all1861*::pCSV3	Peptidoglycan-binding protein	Rudolf et al., unpublished
CSR27[a]	*alr2458*::pCSV3	Alanine racemase; likely involved in peptidoglycan biosynthesis	
DR1992	*alr0093*::Tn*5*-1058	HcwA (AmiC2); *N*-acetylmuramoyl–L-alanine amidase; involved in peptidoglycan metabolism	38
CSMI23	Δ*alr5045*::C.K3	PBP	This study
CSMI24[a]	Δ*alr0718*	PBP; FtsI	This study
CSVM34	Δ*alr2338*	Septal protein SepJ	55
CSR10	*alr4167*::pCSV3	BgtA; ATPase subunit of a basic amino acid ABC transporter	56

[a] PCR analysis with genomic DNA and primers listed in Table S1 in the supplemental material showed that the strain is heterozygous, containing both wild-type and mutant chromosomes (see Fig. S2 to S4 in the supplemental material).

acteristic differences, the cyanobacterial OM conserves the main features of the OM of Gram-negative bacteria (18). In *Anabaena*, genes encoding lipid A biosynthetic proteins (*alr2270* to *alr2274*) are clustered in an operon with Omp85-encoding *alr2269* (26), and a cluster of genes encoding O antigen-related enzymes has also been characterized (29). The cyanobacterial PG differs from the best-investigated peptidoglycans in that it is thicker and possesses a higher degree of cross-linking between PG chains than those in other Gram-negative bacteria (17, 18). Nonetheless, the essential enzymatic machinery of PG biogenesis and metabolism, including glycosyltransferases, DD-transpeptidases, and hydrolases (30), is conserved in cyanobacteria (18, 31). Bifunctional enzymes with transpeptidase and glycosyltransferase activities are known as class A penicillin-binding proteins (PBPs), and monofunctional transpeptidases are known as class B PBPs (30).

In filamentous cyanobacteria, the OM is continuous along the filament, determining the presence of a continuous periplasmic space that contains the murein sacculus (32–35). *Anabaena* bears a murein sacculus composed of two or three PG layers, which surround each cell in the filament, so that PG is also present in the intercellular septa (35). As observed by transmission electron microscopy (TEM) and electron tomography, the PG layers of adjacent cells are fused in a number of intercellular septa, but in some other septa, they are seen well separated (33, 35). The murein sacculus has been isolated from some heterocyst-forming cyanobacteria, and sacculi corresponding to several cell units have been observed, implying that the PG layers from adjacent cells are indeed fused (chemically interlinked) in a substantial number of the intercellular septa of the filament (36, 37).

The aim of this study was to elucidate a possible role of cell envelope components, PG and OM, in filamentation, the process of producing and maintaining filaments, in heterocyst-forming cyanobacteria. For this study, available *Anabaena* strains with mutations in genes encoding proteins involved in the biosynthesis of the cell envelope were investigated (Table 1). With respect to the OM, strains with mutations in genes encoding Omp85-like proteins and a lipid A biosynthesis protein (26), as well as proteins involved in the biosynthesis of the O antigen of LPS (29), have been studied. For the murein sacculus, strains with mutations in genes encoding an amidase known as HcwA (38) or AmiC2 (39), the All1861 protein that contains two PG-binding domains, and an alanine racemase (M. Rudolf, N. Tetik, N. Flinner, G. Ngo, M. Stevanovic, M. Burnat, R. Pernil, E. Flores and E. Schleiff, unpublished data) have been studied. In addition, two genes, *alr0718* and *alr5045*, that encode class B PBPs have been inactivated and characterized. Below, we describe the last of these and then present our analysis of cell envelope properties and filament integrity. We have found that both components of the cell envelope, PG and OM, contribute to make *Anabaena* filaments long.

MATERIALS AND METHODS

Strains and growth conditions. *Anabaena* sp. (also known as *Nostoc* sp.) strain PCC 7120 was grown axenically in BG11 medium (containing $NaNO_3$), $BG11_0$ medium (free of combined nitrogen), or $BG11_0$ NH_4^+ medium {$BG11_0$ containing 4 mM NH_4Cl and 8 mM TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid]-NaOH buffer, pH 7.5}. In every case, ferric citrate replaced the ferric ammonium citrate used in the original recipe (4). For plates, the medium was solidified with 1% separately autoclaved Difco agar. Cultures were grown at 30°C in the light (25 $\mu E\ m^{-2}\ s^{-1}$ [1 E is the energy in 1 mol of photons]), with shaking (80 to 90 rpm) for liquid cultures. For the mutants described below, antibiotics were used at the following concentrations: streptomycin sulfate (Sm) and spectinomycin dihydrochloride pentahydrate (Sp), 5 $\mu g\ ml^{-1}$ each for both liquid and solid media, and neomycin sulfate (Nm), 40 $\mu g\ ml^{-1}$ for solid media and 25 $\mu g\ ml^{-1}$ for liquid media. DNA was isolated from *Anabaena* by the method of Cai and Wolk (40). All *Anabaena* strains used in this work are listed in Table 1. *Escherichia coli* DH5α was used for plasmid constructions. It and strains HB101 and ED8654, used for conjugation with *Anabaena*, were grown in Luria-Bertani medium supplemented when appropriate with antibiotics at standard concentrations.

Plasmid construction and genetic procedures. To inactivate *alr5045*, two DNA fragments, one encompassing 566 bp from sequences upstream of the central region of the gene and the other one including 601 bp from sequences downstream of the central region of the gene, were amplified by standard PCR using DNA from *Anabaena* as the template and primer pairs alr5045–1/alr5045-2 and alr5045-3/alr5045-4, respectively. (All oli-

godeoxynucleotide primers and plasmids used in this work are listed in Table S1 in the supplemental material.) The two DNA fragments were cloned in pSpark (Canvax, Biotech SL), producing plasmid pCSMI50, and, after corroboration by sequencing and digestion, ligated in direct orientation separated by gene cassette C.K3 encoding neomycin phosphotransferase, producing plasmid pCSMI51. The insert of the resulting plasmid, excised with SacI, was transferred to SacI-digested pCSRO (41), producing pCSMI52.

To inactivate *alr0718*, two DNA fragments, one encompassing 710 bp from sequences upstream of the central region of the gene and the other one including 554 bp from sequences downstream of the central region of the gene, were amplified by standard PCR using *Anabaena* DNA as the template and primer pairs alr0718-7/alr0718-8 and alr0718-9/alr0718-10, respectively. The two DNA fragments were cloned in pSpark (Canvax, Biotech SL), producing plasmid pCSMI48, and after corroboration by sequencing, the insert of the resulting plasmid, excised with SacI, was transferred to SacI-digested pCSRO (41), producing pCSMI49.

Conjugation of *Anabaena* with *E. coli* HB101 carrying the cargo plasmid (pCSMI52 or pCSMI49) with the helper and methylation plasmid pRL623 was effected by the conjugative plasmid pRL443, carried in *E. coli* ED8654, and performed as described previously (42), with selection for resistance to Sm and Sp for pCSMI49 and to Nm for pCSMI52. Exconjugants were isolated, and double recombinants were identified as clones resistant to sucrose (and Nm for pCSMI52) (40) and sensitive to the antibiotic for which the resistance determinant was present in the vector portion of the transferred plasmid (Sm/Sp). The genetic structures of selected clones were studied by PCR with DNA isolated from those clones and primers alr5045-5/alr5045-6 and alr5045-5/C.K3-5′ for *alr5045* and alr0718-11/alr0718-12 and alr0718-11/alr0718-1 for *alr0718*. A clone homozygous for the chromosomes with mutations in *alr5045* was chosen for further analysis and named strain CSMI23 (see Fig. S1 in the supplemental material). Although no clone containing only chromosomes with *alr0718* mutated was obtained, one with a low number of wild-type chromosomes was selected for further analysis and named CSMI24 (see Fig. S2 in the supplemental material).

Mutant chromosome segregation. Genomic DNA from strains AFS-I-*alr2269*, AFS-I-*alr0075*, AFS-I-*alr4893*, and AFS-I-*alr2270* (26) and DR1822, DR1963, and DR1967 (29), and from DR1992 (38), was subjected to PCR analysis to test the segregation of mutant chromosomes. The primers used are shown in Table S1 and the PCR results in Fig. S3 and S4 in the supplemental material.

Growth tests. The protein concentrations and chlorophyll *a* (Chl) contents of the cultures were determined by a modified Lowry procedure (43) and by the method of Mackinney (44), respectively. The growth rate constant ($\mu = \ln 2/t_d$, where t_d is the doubling time) was calculated from the increase of the protein content, determined in 0.2-ml samples, of shaken liquid cultures (45). The cultures were inoculated with an amount of cells containing about 5 μg of protein ml^{-1} and grew logarithmically until they reached about 40 μg of protein ml^{-1}. For tests of growth on solid media, cultures grown in BG11 medium (supplemented with antibiotics when appropriate) were harvested and washed three times with 50 ml of $BG11_0$ medium, and dilutions were prepared in $BG11_0$ medium. Ten-microliter samples of the resulting suspensions were spotted on agar plates with different nitrogen sources and incubated at 30°C in the light (25 μE m^{-2} s^{-1}).

Sensitivity to harmful compounds was tested by spotting 2 μl of a cell suspension containing 2.5 μg Chl ml^{-1} on agar plates with BG11 medium supplemented with SDS, lysozyme, or proteinase K at 5 to 30 μg ml^{-1} or with erythromycin at 0.005 to 0.1 μg ml^{-1}. Because some of the mutants still contained wild-type copies of the mutated genes, for these mutants, the plates were supplemented with 5 μg ml^{-1} each of Sp and Sm to maintain the selection pressure for the mutated chromosomes. In these plates, strain CSR10 (Sm^r Sp^r) was used as a control instead of PCC 7120. The plates were incubated at 30°C in the light (25 μE m^{-2} s^{-1}) and observed over a period of 2 weeks.

Nitrogenase activity. Filaments grown in BG11 medium were harvested, washed, and resuspended in $BG11_0$ medium. After 24 h of incubation under growth conditions, the filaments were used in acetylene reduction assays performed under oxic or anoxic conditions, as described previously (45). For the anoxic assays, the cell suspensions, placed in sealed flasks, were supplemented with 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), bubbled with argon for 3 min, and incubated for 90 min under assay conditions before starting the reaction by addition of acetylene.

Substrate uptake assays. Cells grown in BG11 medium were harvested by centrifugation at 4,000 rpm at room temperature, washed twice with 25 mM Tricine [*N*-tris(hydroxymethyl)-methylglycine]-NaOH buffer (pH 8.1), and resuspended in the same buffer. Transport assays were carried out at 30°C in the light (white light from fluorescent lamps; about 175 μE m^{-2} s^{-1}). Amino acid uptake was determined as described previously (45) in 10-min transport assays that were started by mixing a suspension of cells (1.1 ml) containing 10 μg of Chl with a solution (0.1 ml) of L-[U-^{14}C]glutamic acid (253 mCi $mmol^{-1}$) or L-[U-^{14}C]aspartic acid (207 mCi $mmol^{-1}$) (both radiolabeled amino acids were purchased from Amersham, GE Healthcare, United Kingdom). The final concentration of amino acids in the assays was 10 μM. The amount of amino acid taken up was determined in a 1-ml sample of the cell suspension. The sample was filtered (0.45-μm-pore-size Millipore HA filters were used), and the cells on the filters were washed with 5 to 10 ml of 5 mM Tricine-NaOH buffer (pH 8.1). The filters carrying the cells were then immersed in 5 ml of scintillation cocktail, and their radioactivity was measured. The retention of radioactivity by boiled cells was used as a blank.

Microscopy. Cells grown in shaken liquid BG11 medium or incubated in shaken liquid $BG11_0$ medium were observed and photographed with a Zeiss Axioscop microscope equipped with a Zeiss ICc1 digital camera. To determine the percentage of heterocysts, about 3,000 cells of each strain from each tested growth condition were counted. For TEM, 50-ml cultures from the wild type and strain CSMI23 grown in BG11 medium were harvested and fixed in 2.5% glutaraldehyde-$BG11_0$ medium for 1 h at room temperature and washed two times with 10 ml of wash buffer (0.05 M Na-cacodylate, 0.4 M sucrose, pH 7.2) for 15 min at room temperature. Samples were postfixed in 1% OsO_4 at 4°C for 1 h and washed three times with wash buffer. They were then dehydrated in a graded series (30, 50, 70, 80, 90, and 100%) of ethanol and embedded in araldite resin. Ultrathin sections (40 to 50 nm) were mounted on carbon-coated copper grids and stained with uranyl acetate. Samples were visualized in a transmission electron microscope (Philips CM12). Septa and longitudinal axes of the cells were measured in TEM micrographs using ImageJ software (http://imagej.nih.gov/ij).

In vivo labeling with fluorescent vancomycin (Van-FL) (Bodipy FL Conjugate; Invitrogen) was done as described by Lehner et al. (46). Samples (50 μl) from cultures grown in BG11 medium were used. Filaments were incubated for 1 h in the dark with Van-FL (1 mg ml^{-1}) and washed twice with BG11 medium. The samples were visualized with a Leica DM6000B fluorescence microscope and an Orca-ER camera (Hamamatsu). Fluorescence was monitored using a fluorescein isothiocyanate (FITC) L5 filter (excitation, band-pass [BP] 480/40 filter; emission, BP 527/30 filter) and analyzed with ImageJ. The fluorescence per surface area was determined for the indicated numbers of septal regions, and the background fluorescence per surface area in the micrograph was subtracted.

Mechanical fragmentation of filaments. To determine the filament size distribution, samples from BG11-grown cultures were taken with great care to prevent disruption of the filaments and visualized by standard light microscopy. For mechanical fragmentation of filaments, 10-ml samples from the same cultures were passed three times through a 25-gauge (G) (length, 5/8 in.) syringe, and the filaments in the resulting suspension were visualized by standard light microscopy. To determine the filament size (the number of cells per filament), 170 to 1,700 filaments were counted from each strain. Comparison of the filament lengths from

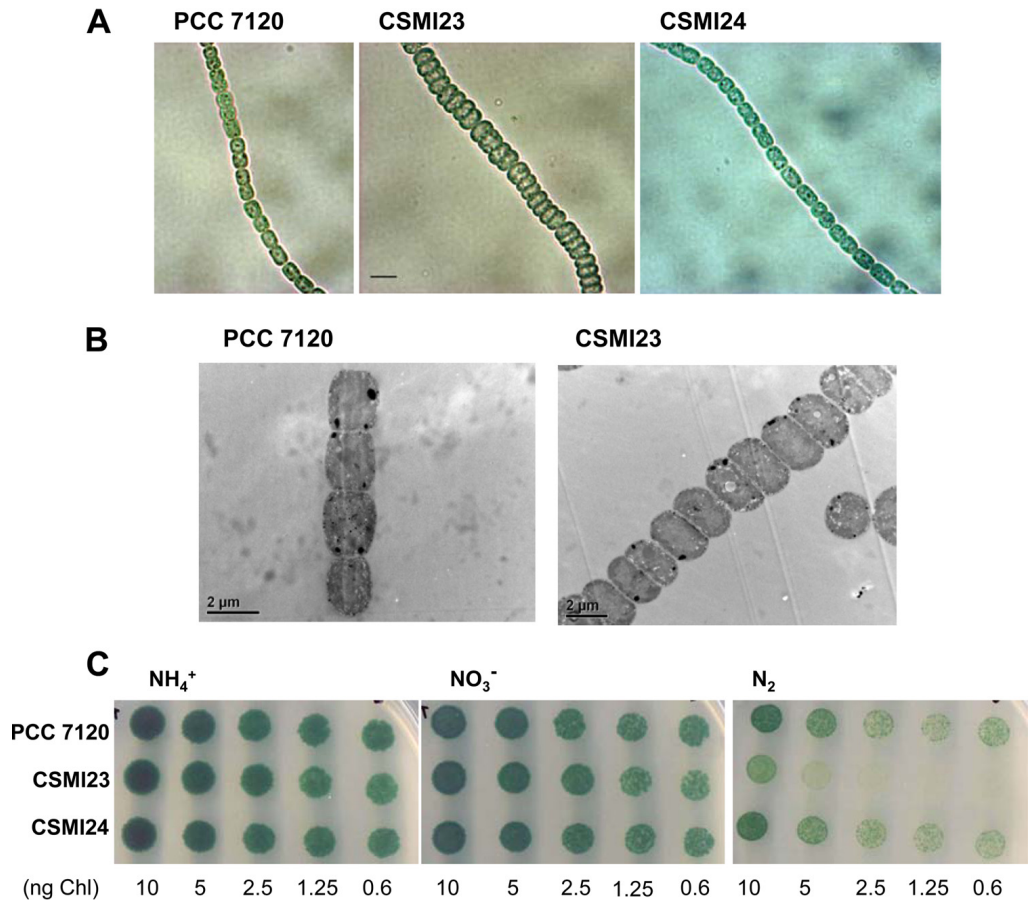

FIG 1 Characterization of *Anabaena alr5045* and *alr0718* mutants. (A) Filaments of *Anabaena* sp. strains PCC 7120, CSMI23, and CSMI24 from cultures incubated in BG11 medium visualized by light microscopy (scale bar, 5 μm; the same magnification was used for the three micrographs). (B) Filaments of *Anabaena* sp. strains PCC 7120 and CSMI23 from cultures incubated in BG11 medium visualized by transmission electron microscopy. (C) Growth tests of strains PCC 7120, CSMI23, and CSMI24 in solid medium using ammonium ($BG11_0$ NH_4^+), nitrate (BG11), or N_2 ($BG11_0$) as the nitrogen source. Each spot was inoculated with an amount of cells containing the indicated amount of chlorophyll *a* (Chl), and the plates were incubated under culture conditions for 9 days and photographed.

mechanically fragmented filaments and the distributions of filament sizes in different strains were assessed by Student's *t* test and the χ^2 test, respectively.

RESULTS

Isolation and characterization of *alr5045* and *alr0718* mutants. In order to extend the set of cell envelope mutants used in this study, two genes, *alr5045* and *alr0718*, both predicted to encode class B PBPs (31, 47), were inactivated. Based on their protein sequences, Alr0718 and Alr5045 are 609- and 610-amino-acid-residue proteins, respectively, both bearing two functional domains: a dimerization domain close to their N termini and a β-lactamase-type transpeptidase fold in their C-terminal halves (47). Open reading frame (ORF) *alr5045* was inactivated by deletion of a 1,329-bp internal fragment of the gene with insertion of a neomycin resistance-conferring gene cassette (C.K3). After transferring the gene construct by conjugation into *Anabaena*, a selected clone that was homozygous for chromosomes bearing the Δ*alr5045*::C.K3 mutation was named strain CSMI23 (see Fig. S1 in the supplemental material). To create an *Anabaena alr0718* mutant, a 1,392-bp fragment internal to the gene was deleted without leaving any gene marker behind to avoid polar effects on neighboring genes (see Fig. S2 in the supplemental material). Several clones bearing this deletion were obtained, but genotypic characterization performed by PCR using genomic DNA from those clones indicated that none was homozygous for the mutant chromosome. Thus, *alr0718* is likely an essential gene in *Anabaena*. Although not fully segregated, a clone with a low number of wild-type chromosomes, strain CSMI24 (see Fig. S2 in the supplemental material), was used in this study.

As observed by light microscopy, strain CSMI23 showed an evident alteration in cell morphology both in medium containing (BG11) and medium lacking ($BG11_0$) combined nitrogen (Fig. 1A shows filaments from BG11 medium). In contrast, no evident alteration in cell morphology was observed in mutant CSMI24. In order to determine the sizes of the cells from these mutants, the cell axis parallel to the filament axis (which is called the longitudinal axis) and, as an indication of cell width, intercellular septa were measured for a number of cells in filaments visualized by light microscopy. Strain CSMI23 cells showed a shorter longitudinal axis and wider septum than the cells from the wild type, whereas strain CSMI24 cells presented dimensions that differed little from those of the wild-type cells (Table 2). Nonetheless, Student's *t* tests indicated that the dimensions of the cells were significantly different ($P \leq 10^{-6}$) in each of the mutants and the wild type. In order to corroborate the altered cell dimensions of strain CSMI23, filaments of the mutant were also visualized by TEM,

TABLE 2 Cell dimensions, growth rates, and nitrogenase activities of CSMI23 and CSMI24 mutants

Strain	Cell dimensions[a] (μm)		Growth rate constant (μ) (day^{-1})[b]		Nitrogenase activity (μmol mg Chl^{-1} min^{-1})[c]	
	Longitudinal axis	Septum	NO_3^-	N_2	Oxic	Anoxic
PCC 7120	3.74 ± 0.88 (415)	2.36 ± 0.36 (428)	0.83 ± 0.13 (4)	0.76 ± 0.20 (6)	8.35 ± 1.58 (3)	15.26 ± 3.05 (5)
CSMI23	2.68 ± 0.68 (302)	3.58 ± 0.69 (288)	0.82 ± 0.12 (4)	0.71 ± 0.31 (6)	2.34 ± 0.60 (3)	3.66 ± 0.83 (3)
CSMI24	3.39 ± 0.74 (231)	2.13 ± 0.32 (235)	0.88 ± 0.16 (4)	0.77 ± 0.24 (6)	6.71 ± 1.89 (3)	ND

[a] The cell dimensions were determined in filaments grown in BG11 (nitrate-containing) medium and viewed by light microscopy. Septum dimensions were used as a measure of cell width. Shown are means and standard deviations of the data from the number of cells indicated in parentheses.
[b] Growth rate constants were determined from the increase in protein content of the cultures with the indicated nitrogen source, measured as described in Materials and methods. Shown are means and standard deviations of the data from the number of independent cultures indicated in parentheses.
[c] Nitrogenase activity was determined as the ethylene produced in acetylene reduction assays performed under oxic and anoxic conditions in filaments incubated for 24 h in the absence of combined nitrogen. Shown are means and standard deviations of the data from the number of independent cultures indicated in parentheses. Student's *t* test analysis indicated that nitrogenase activity was significantly different in strain CSMI23 and the wild type under both oxic ($P = 0.007$) and anoxic ($P = 0.001$) conditions. ND, not done.

which confirmed the morphological alteration of the cells (Fig. 1B). Strain CSMI23 cells showed a shorter longitudinal axis (1.51 ± 0.33 μm [mean ± standard deviation]; $n = 136$) and wider septum (1.94 ± 0.51 μm; $n = 114$) than the cells from the wild type (2.57 ± 0.48 μm, $n = 33$, and 1.16 ± 0.26 μm, $n = 28$, respectively). Student's *t* test showed that both the lengths of longitudinal axes ($P = 10^{-31}$) and widths of the septa ($P = 10^{-11}$) differed significantly in the mutant relative to the wild type. The cell dimensions determined by light or electron microscopy were somewhat different, but the cells could have been affected by the fixation procedure used for electron microscopy.

Strains CSMI23 and CSMI24 grew well on solid media supplemented with a source of combined N, either nitrate or ammonium, but on solid medium free of combined nitrogen, strain CSMI23 exhibited weak growth compared to the wild type (Fig. 1C). Determination of the growth rate constants (μ) in liquid cultures showed, however, that the growth rate of CSMI23 under diazotrophic conditions was only 7% lower than that of the wild type, whereas no difference was found between the growth rate constant of strain CSMI24 and that of the wild type (Table 2). The growth rates of strains CSMI23 and CSMI24 in nitrate-containing medium were similar to that of the wild type (Table 2). The nitrogenase activities of strains PCC 7120, CSMI23, and CSMI24 were determined in filaments incubated for 24 h without combined nitrogen (Table 2). The nitrogenase activities assayed under oxic conditions were about 28% and 80% of the wild-type activity for strains CSMI23 and CSMI24, respectively. Under anoxic conditions, the activity of CSMI23 was still about 24% that of the wild type. At 24 and 48 h after removal of combined nitrogen, heterocysts were 2.7 and 7.4% of total cells, respectively, in strain CSMI23 and 7.6 and 9.2%, respectively, in the wild type. Thus, heterocyst differentiation appears delayed in strain CSMI23. Whereas these results confirm a deficit in heterocyst differentiation and physiology in strain CSMI23, we cannot rule out the possibility that lack of alteration in the growth phenotype of strain CSMI24 is due to lack of segregation of the mutant chromosomes.

Vancomycin recognizes the free D-Ala-D-Ala dipeptide of lipid II or uncrosslinked murein, and a fluorescently labeled derivative of vancomycin, Van-FL, binds to regions of the cell that are actively synthesizing PG (46, 48, 49). Here, we used Van-FL to look into the morphology of CSMI23 and CSMI24. In the wild type, Van-FL labeling was mainly observed at intercellular septa, and different filaments showed intercellular septa labeled to different levels, perhaps indicative of differences in the growth stage, although most septa showed fluorescence of 90 to 190 units (frequencies above 0.02) (Fig. 2). In strain CSMI23, strong label was also observed in the intercellular septa, which showed a tendency to accumulate more label than wild-type septa (Fig. 2). In contrast, relatively weak labeling was observed in the intercellular septa of strain CSMI24 (Fig. 2).

OM permeability. Because the OM is generally a permeability barrier for some harmful substances (20) and, in the case of *Anabaena*, also for some metabolites, including acidic amino acids (26), we tested OM permeability in the different cell envelope mutants by studying sensitivity to erythromycin, SDS, lysozyme, and proteinase K and uptake of aspartate and glutamate. *Anabaena* is polyploid (50), and although some of the investigated mutants were not homozygous for the mutant chromosomes, they could be studied as heterozygous strains. Strains AFS-I-*alr2269*, AFS-I-*alr0075*, AFS-I-*alr4893*, and AFS-I-*alr2270* have been previously investigated and showed the presence of an OM with increased permeability in the strains bearing a low number of wild-type *alr2269* or *alr2270* genes (26). The sensitivity of strains AFS-I-*all1861* and CSR27 to some harmful compounds will be described in detail elsewhere (Rudolf et al., unpublished). Examples of tests of the sensitivities of the other mutants investigated in this work to the harmful compounds are shown in Fig. S5 in the supplemental material. Relevant information is summarized in Table 3. Strains DR1822, DR1963, and DR1967 (strains with mutations in genes related to the biosynthesis of the O antigen of LPS) were sensitive to SDS and, in the cases of DR1822 and DR1963, also to lysozyme, proteinase K, and erythromycin. Strains AFS-I-*all1861*, CSR27, DR1992, and CSMI23 (with genes related to PG biogenesis mutated) were sensitive to SDS and, in the cases of CSR27 and CSMI23, also to lysozyme and proteinase K. These results show that the OM has increased permeability in LPS mutants (AFS-I-*alr2270*, DR1822, DR1963, and DR1967) and also that some PG mutants (AFS-I-*all1861*, CSR27, DR1992, and CSMI23) are sensitive mainly to SDS.

The abilities of aspartate and glutamate to be taken up were tested in the cell envelope mutants and compared with those of the wild type and of strains AFS-I-*alr2269*, AFS-I-*alr2270*, AFS-I-*alr4893*, and AFS-I-*alr0075*, which have been previously reported (26). Substantial increases in glutamate and aspartate uptake in strains AFS-I-*alr2269* and AFS-I-*alr2270* has been interpreted to result from increased permeability of the OM in these mutants (26). OM mutants DR1963 and DR1967 showed increased transport of glutamate (1.9-fold and 1.7-fold, respectively) and aspar-

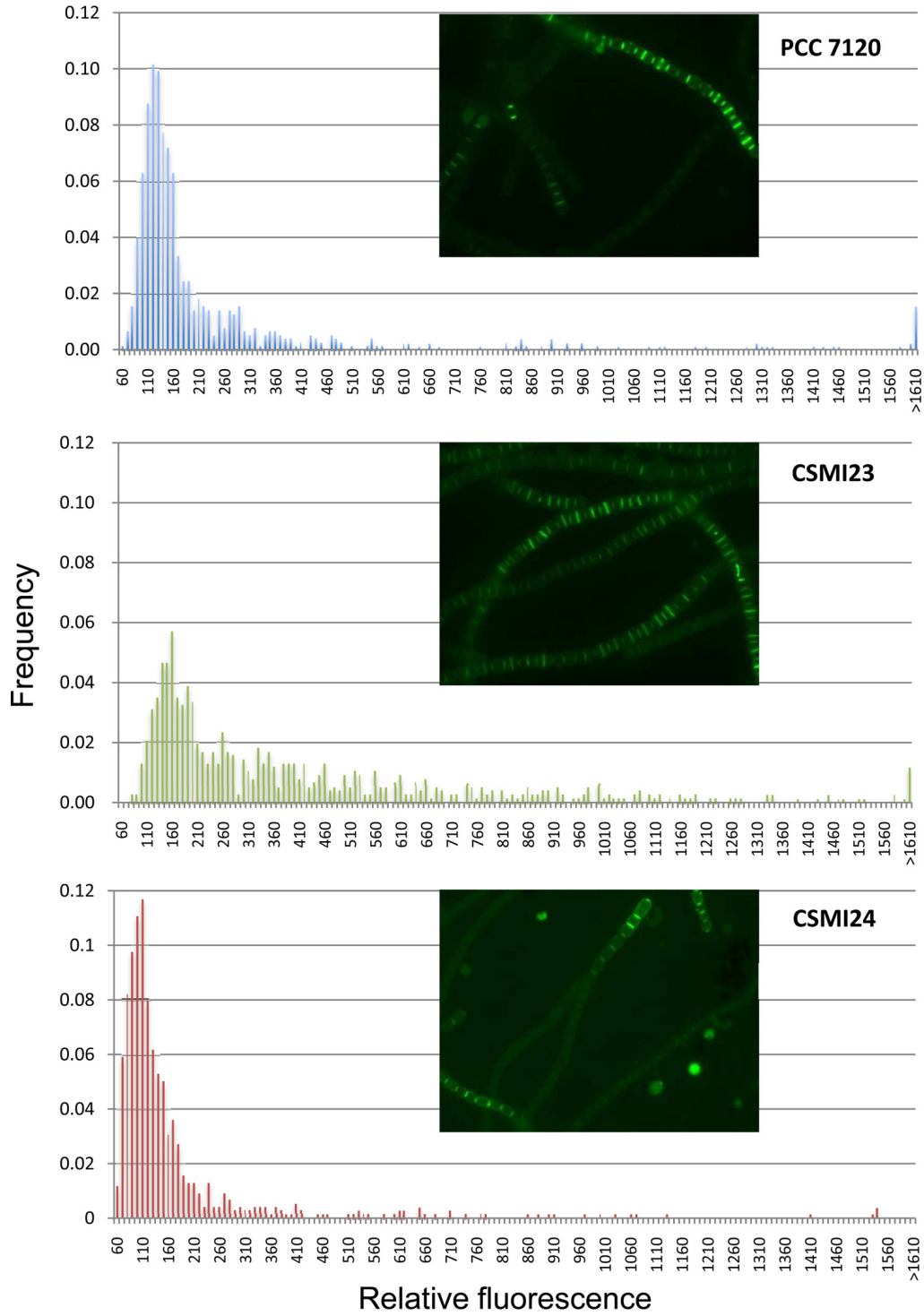

FIG 2 Staining with Van-FL of *Anabaena* wild type and *alr5045* and *alr0718* mutants and quantification of fluorescence from the intercellular septa. The insets show representative examples of filaments of strains PCC 7120, CSMI23, and CSMI24 from cultures incubated in BG11 medium, stained with Van-FL, and visualized by fluorescence microscopy. (The brightness and contrast were increased to improve visibility.) The histograms show the distribution of labeling quantified in 777 (strains PCC 7120 and CSMI24) or 769 (strain CSMI23) intercellular septa.

tate (2.2-fold and 1.6-fold, respectively) compared to the wild type (Table 3). The PG biosynthesis mutant CSR27 showed increased glutamate (2.1-fold) and aspartate (2.1-fold) uptake compared to the wild type. All other cell envelope mutants, however, showed transport activities for the two amino acids that could not be considered significantly different from those of the wild type (with the possible exception of strain CSMI24 and, only for aspartate uptake, strain DR1822). Thus, the increased permeability resulting from inactivation of some components of the cell envelope was also observed as increased uptake of aspartate and glutamate in strains DR1963, DR1967, and CSR27.

Filament lengths. Once the permeability of the OM in the cell envelope mutants was assessed through studies of sensitivity to harmful compounds and transport of acidic amino acids, we de-

TABLE 3 Sensitivity to harmful compounds and uptake of acidic amino acids in *Anabaena* cell envelope mutants

Strain	Segregation of mutant chromosomes[a]	Sensitivity to harmful compounds[b]	Uptake (nmol mg Chl^{-1})[c]			
			Glu	*P*	Asp	*P*
PCC 7120	Wild type		18.23 ± 7.75 (11)		43.48 ± 11.32 (11)	
AFS-I-*alr2269*[d]	No	Em, SDS, Lsz, PK	152.7 ± 13.1 (5)		161.9 ± 15.1 (5)	
AFS-I-*alr0075*[d]	No	NS	13.8 ± 0.7 (12)		40.7 ± 1.7 (10)	
AFS-I-*alr4893*[d]	No	NS	13.6 ± 0.5 (11)		39.9 ± 2.0 (12)	
AFS-I-*alr2270*[d]	No	Em, SDS, Lsz, PK	238.5 ± 11.3 (4)		250.7 ± 8.0 (4)	
DR1822	Yes	Em, SDS, Lsz, PK	30.32 ± 22.63 (6)	0.170	63.10 ± 20.85 (6)	0.039
DR1963	Yes	Em, SDS, Lsz, PK	34.18 ± 9.20 (6)	0.004	93.73 ± 31.64 (6)	0.001
DR1967	No	SDS	31.06 ± 9.45 (6)	0.015	70.95 ± 11.22 (5)	0.001
AFS-I-*all1861*	No[e]	SDS[e]	23.30 ± 6.23 (2)	0.445	57.58 ± 3.40 (2)	0.142
CSR27	No[e]	SDS, Lsz, PK[e]	38.24 ± 0.74 (2)	0.008	90.09 ± 9.65 (2)	0.001
DR1992	Yes	SDS	16.75 ± 8.15 (4)	0.774	39.48 ± 15.91 (4)	0.634
CSMI23	Yes	SDS, Lsz, PK	25.76 ± 4.72 (4)	0.119	38.75 ± 13.79 (4)	0.551
CSMI24	No	NS	28.72 ± 7.72 (4)	0.055	55.46 ± 7.63 (4)	0.097
CSVM34	Yes[f]	ND	ND		ND	

[a] The presence of wild-type and mutant chromosomes was tested by PCR, as shown in Fig. S1 to S4 in the supplemental material.
[b] Sensitivity to erythromycin (Em), SDS, lysozyme (Lsz), or proteinase K (PK) was tested on solid BG11 medium as described in Materials and Methods and shown in Fig. S5 in the supplemental material (see also the work of Nicolaisen et al. [26]). The substance(s) to which the indicated strain was more sensitive than the control strain (PCC 7120 or CSR10 [see Fig. S5 in the supplemental material and reference 26]) are indicated. NS, the indicated strain was not more sensitive than the control strain to any of the tested substances; ND, not determined.
[c] Uptake assays were performed as described in Materials and Methods, with 10 μM glutamate (Glu) or aspartate (Asp) as the substrate in 10-min assays. Shown are the means and standard deviations from the number of experiments indicated in parentheses, which were performed with samples from independent cultures. The significance (*P*) of the differences between the mutants and the wild-type figures was assessed by Student's *t* test.
[d] Data from Nicolaisen et al. (26).
[e] Data from Rudolf et al., unpublished.
[f] Mariscal et al. (55).

termined the lengths of the filaments of the mutants. Samples from cultures of the wild type and cell envelope mutants grown for 4 days in liquid BG11 medium were visualized by light microscopy, and filament sizes were determined as the number of cells per filament. The distributions of filament sizes in the mutants and the wild type were compared (Fig. 3). Strain CSVM34, a *sepJ* gene mutant whose filaments fragment extensively, was used for comparison. As shown in Fig. 3, each of the cell envelope mutants presented a different distribution of filament sizes. Strain AFS-I-*alr0075* was the only mutant in which the distribution of filament sizes did not differ significantly from that in the wild type. Other than strain CSVM34, strains AFS-I-*alr2270*, CSMI23, DR1992, and DR1967 had the highest proportions of short filaments. In contrast, strains AFS-I-*all1861* and DR1822 had a smaller proportion of short filaments than the wild type but did not show an increased percentage of long filaments. Thus, except for strain AFS-I-*all1861*, the PG mutants produced shorter filaments than the wild type, although mutant AFS-I-*alr2270*, altered in lipid A biosynthesis, was the strain showing the shortest filaments, with more than 60% of filaments with a length not greater than 20 cells. Therefore, alteration by mutation of almost any component of the cell envelope results in an altered distribution of filament sizes. Because mutants AFS-I-*alr2269*, AFS-I-*alr0075*, AFS-I-*alr4893*, AFS-I-*alr2270*, DR1967, AFS-I-*all1861*, CSR27, and CSMI24 were heterozygous, i.e., contained both wild-type and mutant chromosomes, a decrease in the numbers of the corresponding wild-type genes appears sufficient to alter filament length.

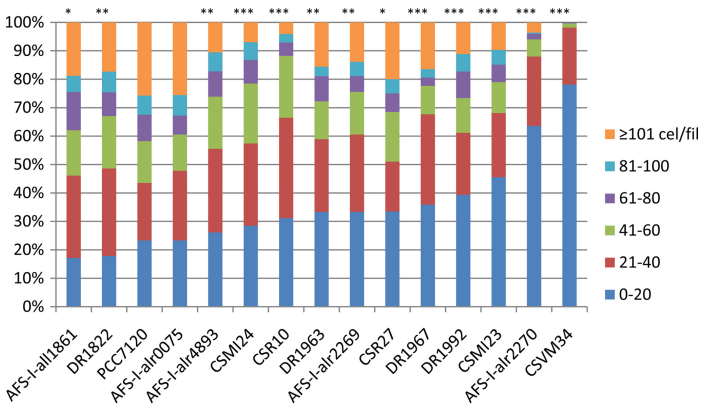

FIG 3 Distribution of filament lengths in *Anabaena* cell envelope mutants. Samples of shaken cultures of the wild type and mutants grown in BG11 medium (with the corresponding antibiotics for the mutants) were taken with great care to avoid disruption of the filaments and counted. A total of 170 filaments (from three or four independent cultures) from each strain were ascribed to the indicated size intervals (size is expressed as cells per filament [cel/fil], with the color code on the right; percentages of filaments are indicated on the left). The distribution of the filament sizes in each mutant was compared to that of the wild type using the χ^2 test; *P* values are indicated with asterisks to denote the level of significance (*, $P \leq 0.05$; **, $P \leq 0.001$; ***, $P \leq 0.0001$). Strain CSVM34 (*sepJ*) was used for comparison. The wild-type and mutant strains are arranged based on increasing percentages of the 0- to 20-cell/filament size interval.

We then asked whether the cell envelope mutants were more sensitive to mechanical fragmentation than the wild type. Samples from the same cultures used for the analysis of the distribution of the filament sizes were subjected to mechanical fragmentation by passing them through a syringe (see Materials and Methods) and visualized by light microscopy. As a result of this treatment, the filaments from most mutants had a mean size significantly different from that of the wild-type filaments ($P < 0.05$) (Table 4), including two mutants (strains DR1967 and CSR27) showing somewhat larger filaments. The filaments from strains AFS-I-*alr2270* and CSMI23 were particularly fragmented by the treat-

TABLE 4 Lengths of mechanically fragmented filaments from *Anabaena* wild type and cell envelope mutants

Strain	Segregation of mutant chromosomes[a]	Size of mechanically fragmented filaments[b]	
		No. of cells per filament	*P*
PCC 7120	Wild type	22.03 ± 17.18 (1,700)	
AFS-I-*alr2269*	No	20.35 ± 19.91 (210)	0.175
AFS-I-*alr0075*	No	21.70 ± 16.11 (209)	0.794
AFS-I-*alr4893*	No	22.32 ± 16.30 (210)	0.815
AFS-I-*alr2270*	No	12.71 ± 11.31 (343)	10^{-16}
DR1822	Yes	25.27 ± 19.80 (209)	0.012
DR1963	Yes	21.05 ± 17.56 (210)	0.438
DR1967	No	25.26 ± 21.17 (207)	0.013
AFS-I-*all1861*	No[c]	19.41 ± 15.53 (290)	0.015
CSR27	No[c]	26.64 ± 21.45 (217)	0.0003
DR1992	Yes	20.00 ± 16.14 (1,091)	0.002
CSMI23	Yes	14.14 ± 10.12 (1,703)	10^{-56}
CSMI24	No	20.47 ± 16.57 (1,642)	0.008
CSVM34	Yes[d]	6.27 ± 3.36 (310)	10^{-54}

[a] The presence of wild-type and mutant chromosomes was tested by PCR, as described in Fig. S1 to S4 in the supplemental material.
[b] To determine filament lengths, samples (10 ml) were subjected to mechanical fragmentation by passing them three times through a 25-G (length, 5/8 in.) syringe and visualized by light microscopy, as described in Materials and Methods. Shown are the means and standard deviations of the number of filaments indicated in parentheses, which were taken from 3 or 4 independent cultures. The significance (*P*) of the differences between the wild type and each mutant was assessed by Student's *t* test.
[c] Rudolf et al., unpublished.
[d] Mariscal et al. (55).

ment (Table 4). However, none of these mutants fragmented as much as the *sepJ* mutant. Thus, the strains that showed more short filaments in the distribution of filament sizes (AFS-I-*alr2270* and CSMI23) were also more sensitive to mechanical fragmentation, indicating that the cell envelope contributes to the maintenance of the integrity of the filament. Microscopic inspection of fragmented filaments predominantly showed filaments ending in round cells and only a few filament ends evidently corresponding to a broken cell, but we have not addressed the mechanism through which filaments break down.

DISCUSSION

Class B PBPs have only transpeptidase activity, with a role in the processes of elongation and division of rod-shaped bacterial cells (51, 52). *Anabaena* Alr5045 is a predicted class B PBP that is homologous to *E. coli* PBP2 (31), which is involved in cell elongation (30). The inactivation of *alr5045* results in a strain (CSMI23) whose vegetative cells show an altered morphology. Specifically, the cells of strain CSMI23 are shorter and wider than the wild-type cells. This morphology could result from a deficit in cell elongation, which would be consistent with a role of Alr5045 similar to that of *E. coli* PBP2 in cell elongation. Strain CSMI23 shows a delay in heterocyst differentiation and is impaired in aerobic diazotrophic growth specifically on solid media. In *Anabaena*, *alr5101* encodes a class A PBP (18) whose inactivation results in a deficit in aerobic N_2 fixation and in altered morphology of both vegetative cells and heterocysts (53). Two other class A PBPs, All2981 and Alr4579, are also specifically required for aerobic N_2 fixation, and their inactivation results in an altered polysaccharide layer of the heterocyst envelope (31). In addition, an *N*-acetylmuroamoyl-L-alanine amidase is required specifically for heterocyst maturation (38, 39). Therefore, PG metabolism-related enzymes appear to be important during the differentiation of heterocysts: they may be needed for the proper assembly of the glycolipid and polysaccharide layers of the heterocyst envelope or for remodeling of the vegetative-cell–heterocyst intercellular septa.

In contrast to strain CSMI23, the *alr0718* mutant CSMI24 could not be segregated. Alr0718 likely represents the *Anabaena* ortholog of *E. coli* FtsI/PBP3 (47), a protein specifically involved in septal-PG synthesis during cell division (30). It seems that whereas other PBPs can fulfill, at least partly, the role of Alr5045 in cell elongation, no protein can replace Alr0718. The *ftsI* ortholog of the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 is also an essential gene (54). Labeling of strains CSMI23 and CSMI24 with Van-FL increased in the septa of the former and decreased in the septa of the latter compared to the wild type, consistent with a role of Alr5045 and Alr0718 in PG synthesis during cell elongation and septation, respectively. Perhaps cells of strain CSMI23 respond to diminished cell elongation by increasing synthesis of septal PG.

To investigate a possible role of the cell envelope in filamentation, we examined PG and OM mutants of *Anabaena*. Regarding PG-related genes, in addition to *alr5045* (encoding a PBP2 protein), *alr0718* (*ftsI*), and *alr0093* (*hcwA* or *amiC2*) mutants, strains CSMI23, CSMI24, and DR1992, respectively, we investigated *alr2458* (encoding an alanine racemase) and *all1861* (encoding a PG-binding protein) mutants, strains CSR27 and AFS-I-*all1861*, respectively. Strains CSMI23 and DR1992 formed filaments significantly shorter than those of the wild type when grown in standard BG11 medium, whereas filaments of strain CSR27 were not very different in length compared to those of the wild type and those of AFS-I-*all1861* were somewhat longer than those of the wild type. Although all strains were somewhat affected, only the filaments of strain CSMI23 were severely fragmented by mechanical shearing. Short filaments in BG11 medium and, in the case of strain CSMI23, the response to mechanical fragmentation indicate a role of PG in maintaining long filaments in *Anabaena*. The role of peptidoglycan in filamentation may be related to its chemical nature as a covalently linked macromolecule that can span several cell units in the filament. On the other hand, the sensitivity of most PG-related mutants to SDS may imply a malformation of their OMs resulting from impaired anchoring to an altered PG.

Regarding OM-related genes, mutants of the Omp85-like protein Alr2269 and of the lipid A biosynthesis protein Alr2270 have previously been shown to produce an OM with increased permeability (26), resulting in very high activities of transport of acidic amino acids (Table 3). We have shown here that mutations that affect the biosynthesis of the O antigen of the LPS may also increase the permeability of the OM, because at least strains DR1963 and DR1967 show significantly increased levels of uptake of acidic amino acids. In addition, these strains and DR1822 are sensitive to SDS and, in the cases of DR1822 and DR1963, to the other harmful substances tested. Strains DR1963 and DR1967 form significantly more abundant short filaments than does the wild type, whereas among the OM mutants investigated, strain AFS-I-*alr2270* is notable for producing a large fraction of short filaments under standard growth conditions and being very sensitive to mechanical fragmentation. Available data suggest that this mutant bears a much altered OM, correlating with its production of short, weak filaments. Although the components of the OM are kept together

mainly, if not exclusively, by noncovalent chemical interactions, our results imply that the OM also contributes to filamentation. The molecular basis for the role of the OM in filamentation, however, remains to be determined. We note, for instance, that strain DR1822, which completely lacks the O antigen of LPS, unexpectedly makes longer filaments than strain DR1967, which produces substantial amounts of LPS (29).

In summary, we have found in *Anabaena* that a PG metabolism mutant, strain CSMI23, and a lipid A biosynthesis mutant, AFS-I-*alr2270*, make very short, weak filaments, although other cell envelope mutants are also affected in the length of the filaments that they produce. None of the investigated cell envelope components, however, is as important as the septal protein SepJ for filamentation. Our results are consistent with the idea that specific elements, such as SepJ, are key for filamentation but also that filaments become and remain lengthy, in part, due to characteristics of their cell envelopes.

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Supplemental material

Cell envelope components influencing filament length in the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120

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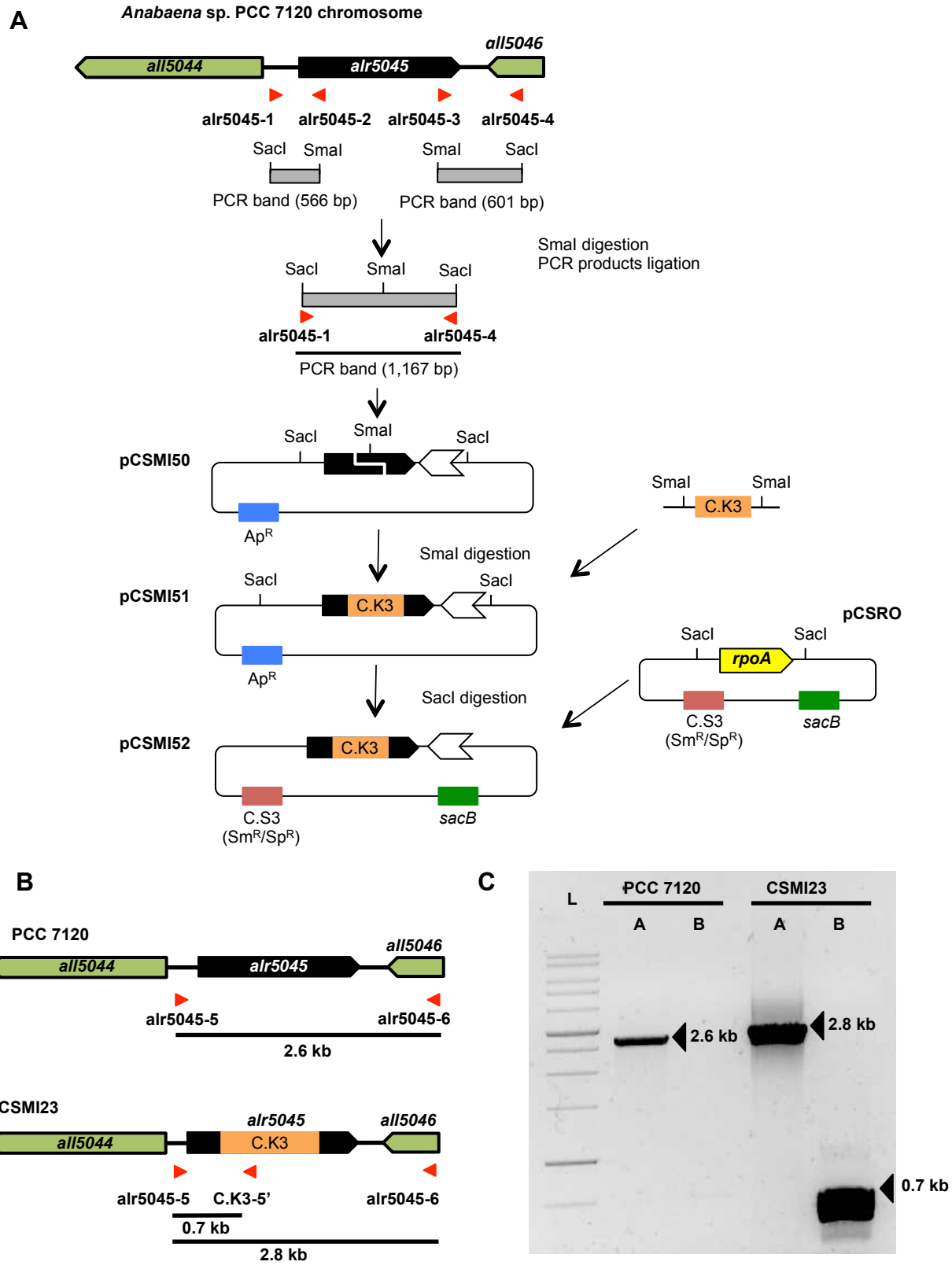

Figure S1. Construction and verification of mutant CSMI23. (A) Construction of pCSMI52. Amplification by PCR of regions upstream and downstream from the central part of *alr5045,* digestion with SmaI and cloning of the PCR products in pSPARK produced pCSMI50. Insertion of the C.K3 cassette on the SmaI site of pCSMI50 produced pCSMI51. The SacI insert of pCSMI51 was transferred to SacI-digested pCSRO, producing pCSMI52, which was transferred to *Anabaena*. (B) Scheme of the *alr5045* genomic region in *Anabaena* sp. PCC 7120 and CSMI23. Block arrows denote genes and direction of transcription. Red arrowheads represent primers. (C) Verification of strain CSMI23 by PCR. L, 1-kb DNA ladder (Biotools). Lane A, PCR with primers alr5045-5 and alr5045-6; lane B, PCR with primers alr5045-5 and C.K3-5'.

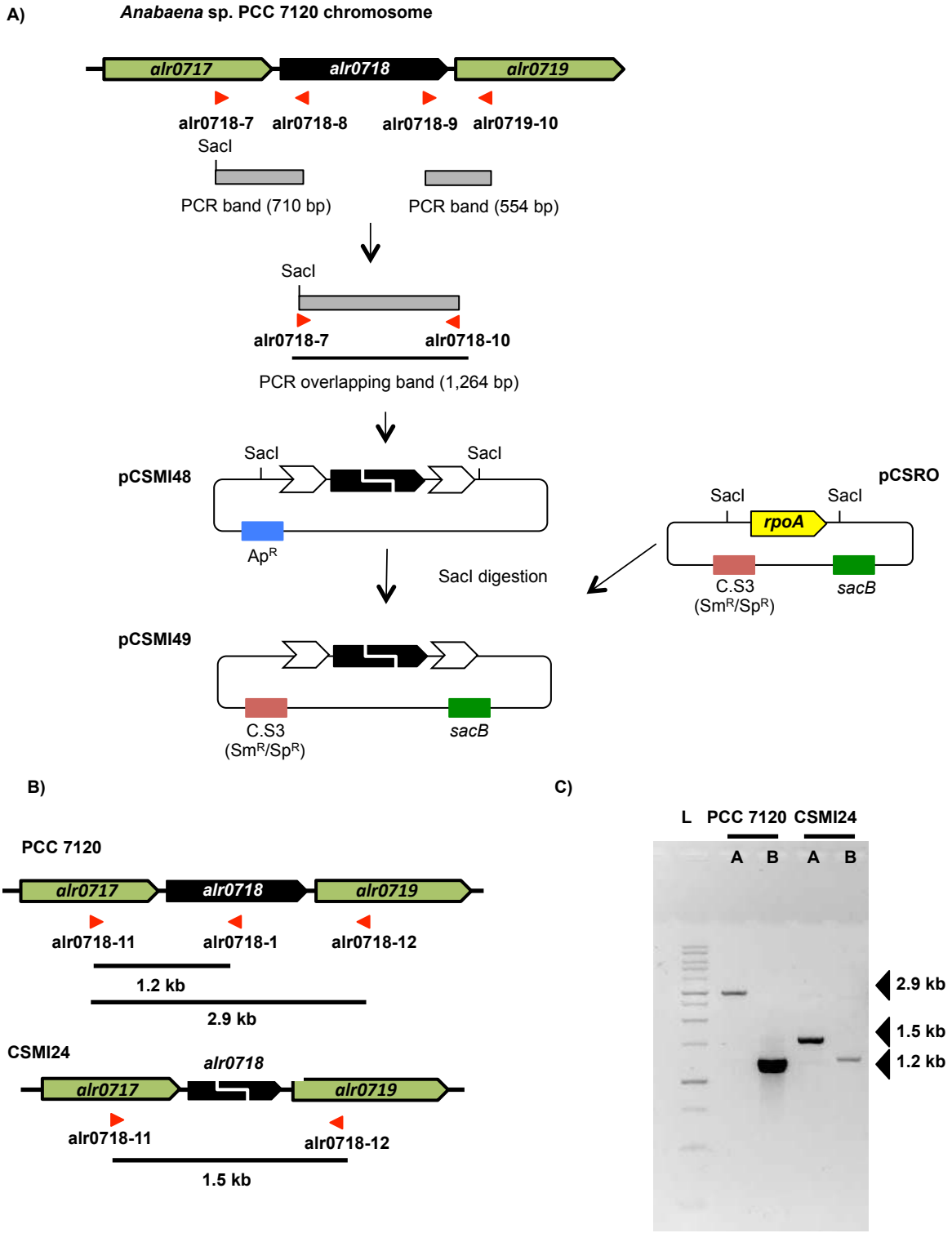

Figure S2. Construction and verification of mutant CSMI24. (A) Construction of pCSMI49. Amplification by PCR of regions upstream and downstream from the central part of *alr0718,* cloning of the PCR products in pSPARK produced pCSMI48. The SacI insert of pCSMI48 was transferred to SacI-digested pCSRO, producing pCSMI49, which was transferred to *Anabaena*. (B) Scheme of the *alr0718* genomic region in *Anabaena* sp. PCC 7120 and CSMI24. Block arrows denote genes and direction of transcription. Red arrowheads represent primers. (C) Verification of strain CSMI24 by PCR. L, 1-kb DNA ladder (Biotools). Lane A, PCR with primers alr0718-11 and alr0718-12; lane B, PCR with primers alr0718-11 and alr0718-1.

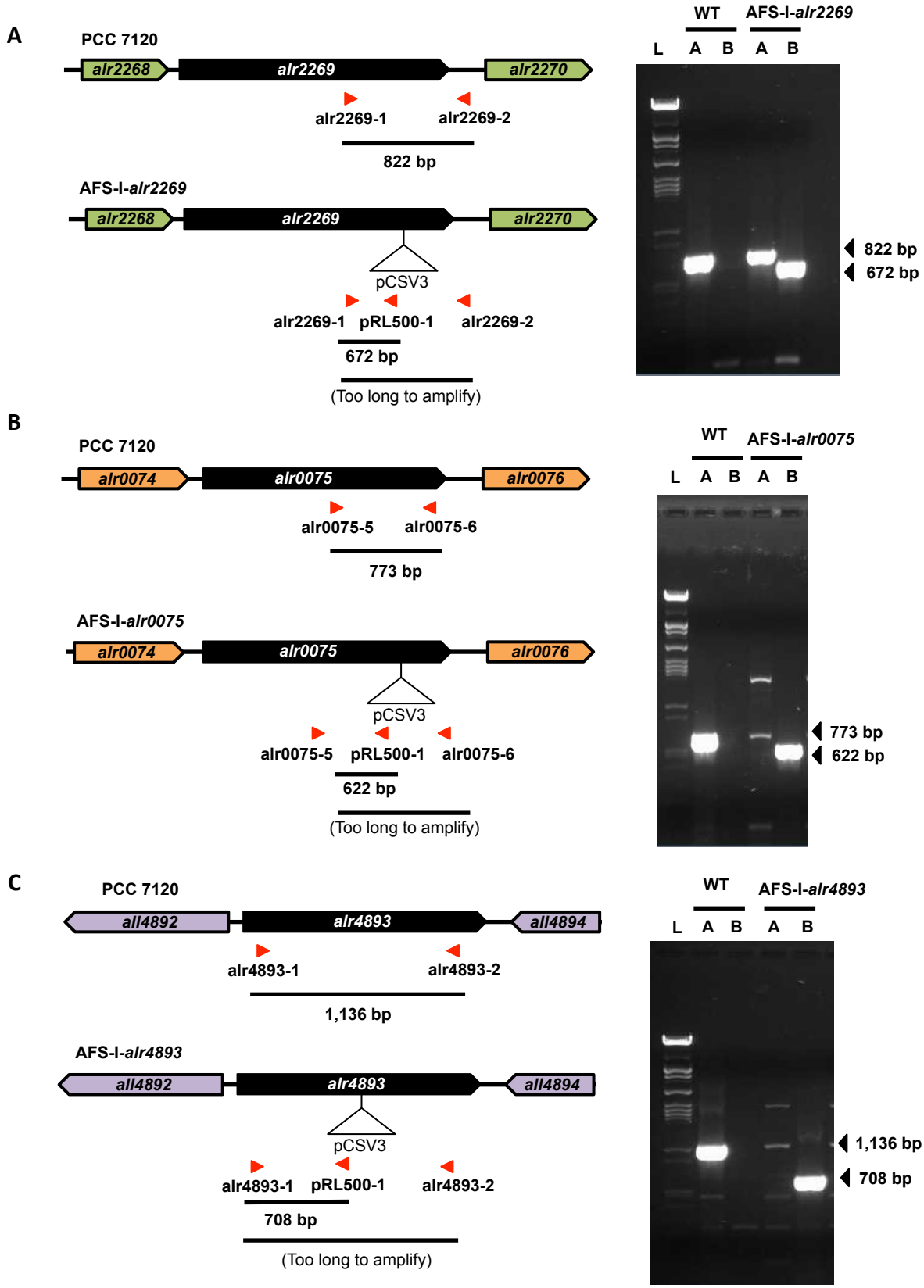

Figure S3. Scheme of genomic regions and verification by PCR of Omp85-like protein mutant strains AFS-I-*alr2269* (A), AFS-I-*alr0075* (B) and AFS-I-*alr4893* (C). The approximate positions of the primers used (red arrowheads) and position of the pCSV3 insertion inactivating the ORF are indicated. Block arrows denote genes and direction of transcription. Triangle represents pCSV3 plasmid inserted. L, lambda DNA digested with ClaI. Lane A, PCR with primers alr2269-1and alr2269-2 (A), alr0075-5 and alr0075-6 (B) and alr4893-1 and alr4893-2 (C); lane B, PCR with primers alr2269-1 and pRL500-1 (A), alr0075-5 and pRL500-1 (B) and alr4893-1 and pRL500-1. Genes not to scale.

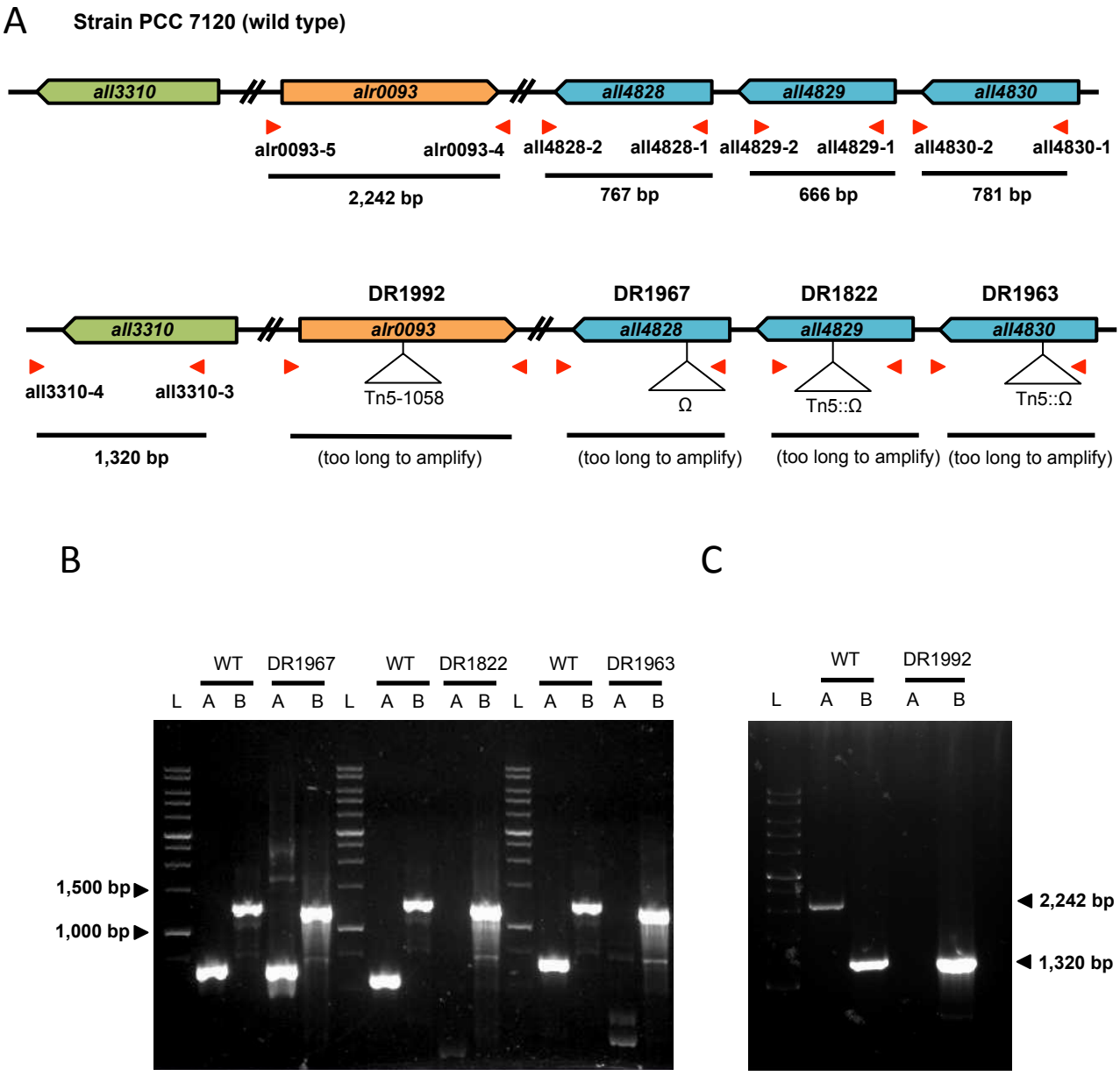

Figure S4. Scheme of genomic regions (A) and verification by PCR of O antigen biosynthesis mutants DR1822, DR1963 and DR1967 (B) and *hcwA* (*amiC2*) mutant DR1992 (C). The name of the strain bearing a mutated gene is indicated above the corresponding gene. The approximate positions of the primers used (red arrowheads) and of the insertion sites of Tn*5* or the omega (Ω) cassette inactivating the different ORFs are indicated. Block arrows denote genes and direction of transcription. Triangle represents Tn*5* or Ω cassette inserted. Because the exact position where the transposon is inserted is not known, the PCR analyses were carried out with primer pairs from the regions close to the edges of the genes (lanes A). As a control, PCR from an unrelated gene located in a different chromosome location was performed with primers all3310-3 and all3310-4 (lanes B). L, 1-kb DNA ladder (Biotools). Genes not to scale. In panel B the size of two standards is shown to the left; in panel C, the sizes of amplified bands are indicated to the right. Mutated chromosomes were completely segregated in strains DR1822, DR1963 and DR1992. Strain DR1967 bears a substantial level of wild-type chromosomes.

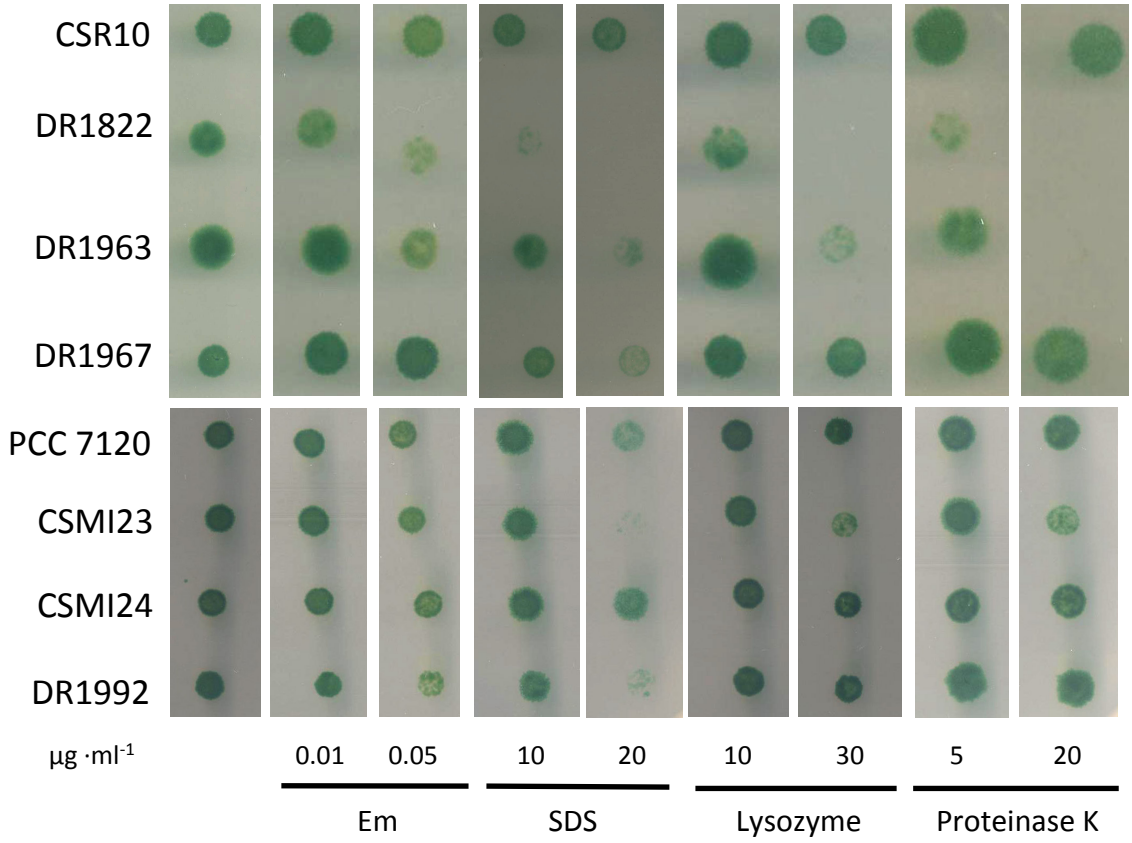

Fig S5. Sensitivity to harmful compounds. Strains DR1822, DR1963, and DR1967 were incubated on solid BG11 medium supplemented with Sm and Sp (5 µg ml^{-1} each), and strains CSMI23, CSMI24 and DR1992 and were incubated on solid BG11 medium. Erythromycin (Em), SDS, lysozyme or proteinase K were added at the indicated concentrations. Strain CSR10, which is not affected in the outer membrane but can grow in the presence of Sm and Sp, was used as a control for the strains grown in the presence of these antibiotics, whereas strain PCC 7120 was used as a control for the strains tested in the absence of Sm and Sp.

TABLE S1. Plasmids and oligodeoxynucleotide primers used in this work

Plasmid	Relevant characteristic(s)[a]	Source or reference
pSpark	Cloning vector; Ap^R	Canvax, Biotech SL
pRL443	Conjugative plasmid; Ap^R	1
pRL623	Helper plasmid; carries *mob* and DNA methylases; Cm^R	2
pCSRO	*sacB*-containing negative selection vector; $Sm^RSp^R\ Suc^S$	3
pCSMI48	*alr0718* lacking a 1,392-bp internal fragment cloned in pSpark; Ap^R	This study
pCSMI49	SacI fragment from pCSMI48 cloned into SacI-digested pCSRO; $Sm^RSp^R\ Suc^S$	This study
pCSMI50	*alr5045* lacking a 1,330-bp internal fragment cloned in pSpark; Ap^R	This study
pCSMI51	Δ*alr5045*::C.K3 cloned in pSpark; Ap^RNm^R	This study
pCSMI52	SacI fragment from pCSMI51 cloned into SacI-digested pCSRO; $Sm^RSp^RNm^R\ Suc^S$	This study
Primer	**Sequence (5' → 3')** [b]	
alr5045-1	CGAGCTCTCAGACCACAGCGTTGTC	
alr5045-2	CCCCCGGGACGAGGATAGCGTGTA	
alr5045-3	CCCCCGGGACTGCTGAAGCTTG	
alr5045-4	CGAGCTCAAAGCGGCAACGGC	
alr5045-5	CATTGCTGGAGTGACCTATGA	
alr5045-6	CCTATGCGTCTACAAGATGAGC	
alr0718-1	GGTGCAGATGATCCAACGCCTAA	
alr0718-7	CCAGAGCTCGTGGAGTCAAGGTTACAGCCG	
alr0718-8	ataacggggtgattcaacCAAGCTTACCATTTG	
alr0718-9	tggtaagcttgGTTGAATCACCCCGTTATGTA	
alr0718-10	AGCCCCATTAATTGCCCTGGAAAC	
alr0718-11	CAAATTCACAGCACCTGCCT	
alr0718-12	CTTGTGCAAGTTCCCACTTCTG	
alr2269-1	AGCCCAACCCCGTCTTA	
alr2269-2	CTTGCCCGTTTGTATCATTAG	
alr0075-5	GCGCCGCTTTCCTAGTTG	
alr0075-6	CACTGGCGAGTTTTCCTGAAC	
alr4893-1	ATCGGCAAACCCATCAG	
alr4893-2	CCACGGACGGAGTTAGAA	
alr0093-4	AGATTTGACGCAGTACCGTTAGCC	
alr0093-5	GCTGAAGTTTGAACGGTGATGGT	
all4828-1	TCGCCGGACTTCCACATTCAATAC	
all4828-2	CTGGACGCAGATAACGCTCATCAA	
all4829-1	ACGCTTACGGCAAGATGCTA	
all4829-2	CCATAAGTCCAACCAGAGAGTCCT	
all4830-1	GGGATGGGTGACTCGATACTTGTT	
all4830-2	GTGCTTCTGGGGTTAAATTGCC	
all3310-3	TATTTCAGCACCTACGGCCCTACA	
all3310-4	AAGTCAAGTCGCTTCGCTCCAA	
pRL500-1	ATAGGCGTATCACGAGGC	
C.K3-5'	CCTTAAAACATGCAGGAATTGACG	
SacB-1	CTTGAGGTACAGCGAAGTG	
SacB-2	TCTGCAAAAGGCCTGGAGG	

[a] Resistance to the indicated antibiotic (denoted by ^{R}): Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Nm, neomycin; Sm, streptomycin; and Sp, spectinomycin. Suc^S, sensitivity to 5% sucrose.
[b] Introduced restriction enzyme cutting sites are underlined.

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Chapter 2: Compartmentalized cyanophycin metabolism in the diazotrophic filaments of a heterocyst-forming cyanobacterium

Compartmentalized cyanophycin metabolism in the diazotrophic filaments of a heterocyst-forming cyanobacterium

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Heterocyst-forming cyanobacteria are multicellular organisms in which growth requires the activity of two metabolically interdependent cell types, the vegetative cells that perform oxygenic photosynthesis and the dinitrogen-fixing heterocysts. Vegetative cells provide the heterocysts with reduced carbon, and heterocysts provide the vegetative cells with fixed nitrogen. Heterocysts conspicuously accumulate polar granules made of cyanophycin [multi-L-arginyl-poly (L-aspartic acid)], which is synthesized by cyanophycin synthetase and degraded by the concerted action of cyanophycinase (that releases β-aspartyl-arginine) and isoaspartyl dipeptidase (that produces aspartate and arginine). Cyanophycin synthetase and cyanophycinase are present at high levels in the heterocysts. Here we created a deletion mutant of gene *all3922* encoding isoaspartyl dipeptidase in the model heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120. The mutant accumulated cyanophycin and β-aspartyl-arginine, and was impaired specifically in diazotrophic growth. Analysis of an *Anabaena* strain bearing an All3922-GFP (green fluorescent protein) fusion and determination of the enzyme activity in specific cell types showed that isoaspartyl dipeptidase is present at significantly lower levels in heterocysts than in vegetative cells. Consistently, isolated heterocysts released substantial amounts of β-aspartyl-arginine. These observations imply that β-aspartyl-arginine produced from cyanophycin in the heterocysts is transferred intercellularly to be hydrolyzed, producing aspartate and arginine in the vegetative cells. Our results showing compartmentalized metabolism of cyanophycin identify the nitrogen-rich molecule β-aspartyl-arginine as a nitrogen vehicle in the unique multicellular system represented by the heterocyst-forming cyanobacteria.

intercellular communication | nitrogen fixation

Heterocyst-forming cyanobacteria represent a unique group of multicellular bacteria in which growth requires the activity of two interdependent cell types: the vegetative cells that perform oxygenic photosynthesis and the N_2-fixing heterocysts (1, 2). Vegetative cells fix CO_2 photosynthetically and transfer reduced carbon to heterocysts (3), in which nitrogenase fixes N_2 producing ammonia that is incorporated into amino acids via the glutamine synthetase–glutamate synthase (GS/GOGAT) pathway (4). Heterocysts transfer fixed N to the vegetative cells in the filament (5). In a cyanobacterium such as the model organism *Anabaena* sp. strain PCC 7120 (hereafter *Anabaena*), the filament can be hundreds of cells long. Under nitrogen fixing conditions, which are established when no combined nitrogen is available in the medium (1, 2), about one in 10–20 cells are heterocysts, which are semiregularly distributed along the filament ensuring an even exchange of nutrients between the two cell types. A full understanding of this multicellular system needs the identity of the intercellularly exchanged compounds to be known.

Because the heterocysts bear high levels of glutamine synthetase but lack glutamate synthase (6, 7), an exchange of glutamine (transferred from heterocysts to vegetative cells) for glutamate (transferred from vegetative cells to heterocysts) has been proposed for the GS/GOGAT cycle to work in the diazotrophic cyanobacterial filament (4, 6). Indeed, when incubated under proper conditions, isolated heterocysts can produce glutamine that is released to the surrounding medium (6). In addition to glutamate, two compounds that can be transferred from vegetative cells to heterocysts are alanine and sucrose (8). Alanine can be an immediate source of reducing power in the heterocyst, where it is metabolized by catabolic alanine dehydrogenase, the product of *ald* (9). Sucrose, a universal vehicle of reduced carbon in plants, appears to have a similar role within the diazotrophic cyanobacterial filament, because diazotrophic growth requires that sucrose is produced in vegetative cells and hydrolyzed by a specific invertase, InvB, in the heterocysts (10–13). Sucrose is a more important carbon vehicle to heterocysts than alanine, because inactivation of *invB* (12, 13) has a stronger negative effect on diazotrophic growth than inactivation of *ald* (9). In summary, there is evidence for transfer of sucrose, glutamate, and alanine from vegetative cells to heterocysts and of glutamine in the reverse direction.

Heterocysts bear inclusions in the form of refractile granules that are located at the cells poles (close to the heterocyst "necks") and are made of cyanophycin [multi-L-arginyl-poly (L-aspartic acid)], a nitrogen-rich polymer (14, 15). Although production of cyanophycin is not required for diazotrophic growth (16, 17), its conspicuous presence in the heterocysts suggests a possible role of this cell inclusion in diazotrophy (18, 19), for instance as a nitrogen buffer to balance nitrogen accumulation and transfer (20). Cyanophycin is synthesized by cyanophycin synthetase that adds both aspartate to the aspartate backbone of the polymer and arginine to the β-carboxyl group of aspartate residues in the backbone (21, 22). Cyanophycin is degraded by

Significance

The heterocyst-forming cyanobacteria represent a true multicellular system found in the bacterial world that adds unique features to biodiversity. They grow as chains of cells containing two metabolically interdependent cellular types, the vegetative cells that fix carbon dioxide performing oxygenic photosynthesis and the dinitrogen-fixing heterocysts. Heterocysts accumulate a cell inclusion, cyanophycin [multi-L-arginyl-poly (L-aspartic acid)], as a nitrogen reservoir. We have found that the second enzyme of cyanophycin catabolism, isoaspartyl dipeptidase, is present at significantly higher levels in vegetative cells than in heterocysts, identifying the nitrogen-rich dipeptide β-aspartyl-arginine as a nitrogen vehicle to the vegetative cells and showing that compartmentalization of a catabolic route is used to improve the physiology of nitrogen fixation.

Author contributions: M.B., A.H., and E.F. designed research; M.B. performed research; M.B., A.H., and E.F. analyzed data; and E.F. wrote the paper.

The authors declare no conflict of interest.

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cyanophycinase that releases a dipeptide, β-aspartyl-arginine (23), which is hydrolyzed to aspartate and arginine by an isoaspartyl dipeptidase homologous to plant-type asparaginases (24). Cyanophycin synthetase is the product of *cphA* (21) and cyanophycinase of *cphB* (23). *Anabaena* bears two gene clusters, *cph1* and *cph2*, each containing both *cphA* and *cphB* genes, of which *cph1* contributes most to cyanophycin metabolism (17). All these genes are expressed in vegetative cells and heterocysts, but differential expression leads to levels of both cyanophycin synthetase and cyanophycinase that are much higher (about 30- and 90-fold, respectively) in heterocysts than in vegetative cells (25). The fate of the β-aspartyl-arginine released in the cyanophycinase reaction has, however, not been investigated until now.

As deduced from heterologous expression in *Escherichia coli*, ORF *all3922* of the *Anabaena* chromosome encodes an isoaspartyl dipeptidase (24). In this work, we have generated *Anabaena* mutants of *all3922*, including inactivation and reporter-expressing strains, and have found that *all3922* is required for optimal diazotrophic growth and that isoaspartyl dipeptidase is present mainly in the vegetative cells of the diazotrophic filament. Our results imply that a substantial fraction of the β-aspartyl-arginine dipeptide produced in the heterocysts is hydrolyzed in the vegetative cells and, thus, that this peptide is an intercellularly transferred nitrogen vehicle in the diazotrophic filament.

Results

Isolation and Characterization of an *all3922* Mutant. To create an *Anabaena* mutant of *all3922*, a 763-bp fragment internal to the gene was deleted (Fig. 1*A*) without leaving any gene marker behind to avoid polar effects on neighboring genes (Fig. S1). Several clones bearing this deletion were obtained that were homozygous for the mutant chromosomes (Fig. S1). Strain CSMI6, which was selected for further analysis, exhibited weak growth under diazotrophic conditions on solid medium, although it grew well in the presence of combined nitrogen, either nitrate or ammonium (shown in Fig. 1*B* for nitrate-supplemented medium). Complementation of CSMI6 with a plasmid bearing wild-type *all3922* (see *SI Materials and Methods*) allowed diazotrophic growth corroborating that growth impairment resulted from the *all3922* mutation (see strain CSMI6-C in Fig. 1*B*). Determination of growth rate constants in liquid culture showed that the growth rate of CSMI6 in the presence of combined nitrogen (nitrate or ammonium) was comparable to that of the wild type, but it was about 46% slower in the absence of combined nitrogen (Table 1). Nitrogenase activity, measured by the acetylene reduction assay, was only 15% lower in the mutant than in the wild type (Table 1).

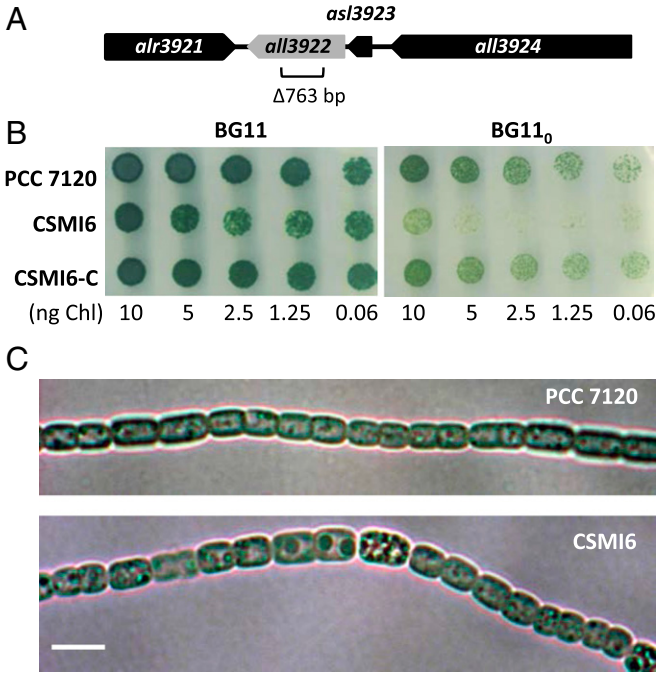

Fig. 1. Characterization of an *Anabaena all3922* mutant. (*A*) Schematic of the *all3922* genomic region in *Anabaena* with indication of the DNA fragment removed to create strain CSMI6. (*B*) Growth tests in solid medium using nitrate (BG11) or N_2 (BG11$_0$) as the nitrogen source. Each spot was inoculated with an amount of cells containing the indicated amount of Chl, and the plates were incubated under culture conditions for 11 d and photographed. (*C*) Filaments of *Anabaena* sp. strains PCC 7120 and CSMI6 from cultures incubated for 4 d in BG11 medium and visualized by light microscopy. (Scale bar, 5 μm.)

Microscopic observation of strain CSMI6 showed the presence of abundant granulation in the cytoplasm of the cells (Fig. 1*C*). To test the possibility that the granulation corresponded to cyanophycin, cyanophycin granule polypeptide (CGP) isolation was carried out and the isolated material was measured with the Sakaguchi reaction for arginine. CSMI6 cells grown for 8 d in the presence of nitrate had about ninefold the amount of CGP present in the control wild-type cells [1365.24 ± 483.91 and 147.06 ± 6.62 μg arginine (mg Chl)$^{-1}$, respectively; mean and SD (n = 3)]. An experiment of accumulation and degradation of cyanophycin was then performed. Cells grown in three successive batch cultures with ammonium were incubated for 24 h in medium with ammonium, with nitrate, or lacking combined nitrogen and then used for determination of CGP. In the presence of ammonium, cells of the mutant had about 2.8 times the CGP detected in the wild-type cells (Table 1). However, whereas the wild type contained similar levels of CGP under the three conditions, CSMI6 cells contained less amounts of CGP after incubation with nitrate or, especially, in the absence of combined nitrogen than in the presence of ammonium (Table 1). These results showed that the CGP present in the ammonium-grown CSMI6 cells was degraded to some extent upon incubation for 24 h in media with nitrate or without combined nitrogen. Because strain CSMI6 was expected to be impaired in hydrolysis of the β-aspartyl-arginine produced in cyanophycin degradation, we asked whether the dipeptide could be detected in the filaments of this strain.

Detection of β-Aspartyl-arginine. Because β-aspartyl-arginine has an amino group that can be derivatized with phenylisothiocyanate, we subjected cell-free extracts from filaments incubated under different conditions to standard HPLC analysis of amino acids. A compound not found (or observed at very low levels; see below) in wild-type extracts was observed in the region of the chromatogram between glutamate and serine in extracts from strain CSMI6 (Fig. 2). Cochromatography with authentic β-aspartyl-arginine identified that compound as the β-aspartyl-arginine dipeptide (Fig. S2). The amount of β-aspartyl-arginine detected in CSMI6 extracts ranged from 5 to 25 μmol (mg Chl)$^{-1}$ under different conditions, and it was generally higher than the levels of the two most abundant amino acids detected, glutamate [2–18 μmol (mg Chl)$^{-1}$] and alanine [1.25–9 μmol (mg Chl)$^{-1}$].

The dipeptide could also be detected after extraction of the cells with 0.1 M HCl or by boiling (Table S1). The levels of β-aspartyl-arginine found in filaments that had been incubated without combined nitrogen for 48 h were about 4.2 μmol (mg Chl)$^{-1}$ in strain CSMI6 and 0.035 μmol (mg Chl)$^{-1}$ in the wild-type strain. This indicates that the dipeptide is indeed produced in the wild type, but its cellular levels are kept very low by the action of the All3922 dipeptidase. To check whether some of the dipeptide accumulated in the filaments of strain CSMI6 leaked out to the extracellular medium, the supernatant from a culture incubated for 48 h in the absence of combined nitrogen was analyzed and

Table 1. Growth rate constants, nitrogenase activity, and cyanophycin granule polypeptide (CGP) levels in *Anabaena* sp. strains PCC 7120 (wild type) and CSMI6 (*all3922*)

Strain	Growth rate, μ, day^{-1}			Nitrogenase activity, μmol (mg Chl)$^{-1}$ h^{-1}	CGP, μg arginine (mg Chl)$^{-1}$		
	NH_4^+	NO_3^-	N_2		NH_4^+	NO_3^-	N_2
PCC 7120	0.55 ± 0.08 (4)	0.79 ± 0.12 (8)	0.67 ± 0.22 (9)	16.52 ± 4.27 (6)	166.52 ± 34.64 (3)	160.06 ± 24.02 (3)	166.66 ± 53.88 (3)
CSMI6	0.58 ± 0.14 (3)	0.77 ± 0.17 (4)	0.36 ± 0.12 (3)	13.97 ± 3.16 (5)	465.05 ± 97.85 (3)	366.94 ± 22.19 (3)	248.01 ± 40.74 (3)
CSMI6 vs. PCC 7120	$P = 0.80$	$P = 0.83$	$P = 0.053$*	$P = 0.34$	$P = 0.015$*	$P = 0.001$*	$P = 0.164$

The growth rate constant (μ) was determined in photoautotrophic shaken cultures with the indicated nitrogen sources. To determine nitrogenase activity, filaments grown in $BG11_0NH_4^+$ medium and incubated in nitrogen-free $BG11_0$ medium for 48 h were used in assays of reduction of acetylene to ethylene under oxic conditions. Cyanophycin was determined by the Sakaguchi reaction for arginine on CGP granules isolated from ammonium-grown filaments incubated for 24 h in media with the indicated nitrogen sources. Figures are the mean and SD of data from the number of independent experiments indicated in parentheses. The significance of the differences between the mutant and the wild-type figures was assessed by the Student's *t* test (*P* indicated in each case); asterisks denote likely significant differences. In strain CSMI6, the amount of CGP was significantly different ($P = 0.044$) in the cultures incubated in the absence of combined nitrogen compared with the cultures kept in the presence of ammonium.

found to contain 0.108 μmol β-aspartyl-arginine (mg Chl)$^{-1}$, indicating that only a limited leakage takes place.

Cell-Specific Expression of All3922. To investigate the expression and cell localization of All3922, an *all3922-sf-gfp* fusion gene [*sf-gfp* encodes a superfolder green fluorescent protein (26)] was constructed and transferred to *Anabaena* (Fig. S3). *Anabaena* clones bearing this construct as the only *all3922* gene were readily isolated (Fig. S3). Strain CSMI27, which was selected for further analysis, exhibited growth properties similar to those of the wild type (Fig. S3), indicating that the All3922-sf-GFP fusion protein retained All3922 function. The mature isoaspartyl dipeptidase is a tetramer of two subunits (α and β) produced, both isolated from diazotrophic filaments by cleavage of the primary gene product (24). In the mature protein, the sf-GFP is bound to the β subunit, and no substantial release of the sf-GFP from the fusion polypeptide was observed (Fig. S4).

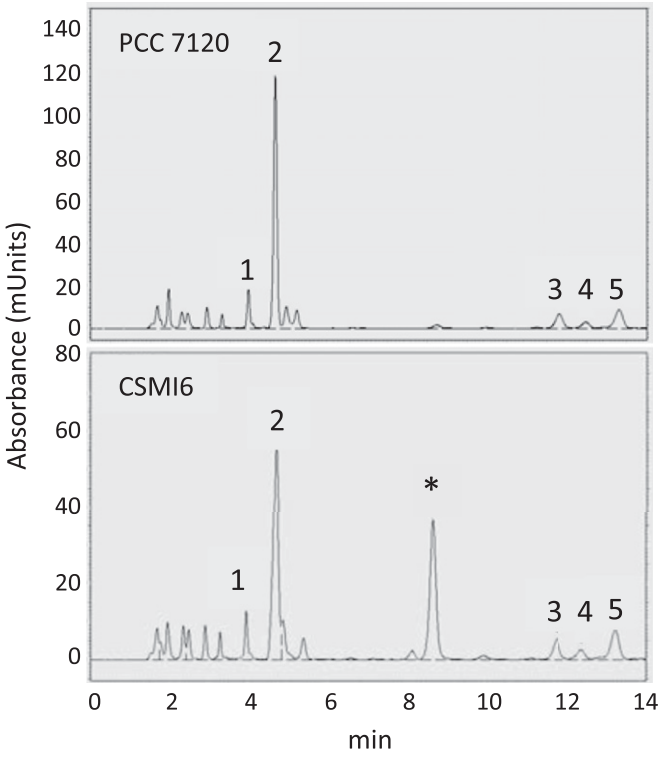

Fig. 2. Accumulation of β-aspartyl-arginine in *Anabaena* sp. strain CSMI6. Chromatographs of cell-free extracts from strains PCC 7120 (*Upper*) and CSMI6 (*Lower*) grown on ammonium and incubated for 24 h in $BG11_0$ medium (lacking combined nitrogen) are shown. Peaks corresponding to aspartate (1), glutamate (2), serine (3), asparagine (4), and glycine (5) are indicated. The peak marked with an asterisk (*) corresponds to β-aspartyl-arginine (see Fig. S2) and represents 25.38 μmol (mg Chl)$^{-1}$ in the cell-free extract.

When strain CSMI27 was grown with nitrate, GFP fluorescence was observed in all of the cells of the filament, but when grown diazotrophically, GFP fluorescence was observed in vegetative cells but not (or much less) in heterocysts (Fig. 3). Quantification of GFP fluorescence along the filaments confirmed the visual impression of low sf-GFP fluorescence in the heterocysts (Fig. 3). Quantification of the GFP fluorescence in a large number of cells indicated that, in filaments incubated for 24 h without combined nitrogen, heterocysts had on average 19.7% of the fluorescence detected in vegetative cells (630 vegetative cells and 108 heterocysts counted; Student's *t* test $P = 6 \times 10^{-114}$). In filaments incubated for 48 h without combined nitrogen, heterocysts had on average 11.2% of the fluorescence detected in vegetative cells (662 vegetative cells and 65 heterocysts counted; $P = 5 \times 10^{-99}$). These results suggest that All3922 is lost from heterocysts as they age.

Isoaspartyl dipeptidase can use a number of substrates other than β-aspartyl-arginine (24). To determine its activity in cell-free extracts, we used β-aspartyl-lysine as a convenient, commercially available substrate (Fig. S5). Levels of isoaspartyl dipeptidase activity were similar in extracts of *Anabaena* filaments grown in the presence and absence of combined nitrogen, but the activity was undetectable in extracts from mutant CSMI6 (Table 2). Heterocysts can be isolated from diazotrophic filaments with a protocol based on treatment with lysozyme to disrupt vegetative cells by mild mechanical treatments (27, 28). We then assessed isoaspartyl dipeptidase in extracts of vegetative cells and heterocysts, both isolated from diazotrophic filaments. Isoaspartyl dipeptidase levels in extracts of vegetative cells were similar to (just somewhat higher than) those of whole filaments, but they were about fivefold lower in heterocyst extracts than in vegetative cell extracts (Table 2). To assess that heterocyst extracts had been isolated properly, we determined glutamine synthetase that is known to be present at high levels in the heterocysts (6, 29). In contrast to isoaspartyl dipeptidase, glutamine synthetase levels were threefold higher in heterocyst extracts than in vegetative cell extracts (Table 2). Our results indicate that isoaspartyl dipeptidase accumulates at appreciable levels in both nitrate-grown and diazotrophic filaments, but in the latter, it is distributed differentially between the two cell types, being preferentially accumulated in vegetative cells.

Production of β-Aspartyl-arginine by Isolated Heterocysts. We reasoned that if the product of the cyanophycinase reaction, β-aspartyl-arginine, is metabolized to a limited extent in the heterocysts, being rather exported by them, isolated heterocysts might release the dipeptide into the incubation medium. We then studied the production of β-aspartyl-arginine in suspensions of heterocysts that were largely devoid of vegetative cells (Fig. 4*A*). After incubation in a buffer for different times up to 4 h, supernatants from these

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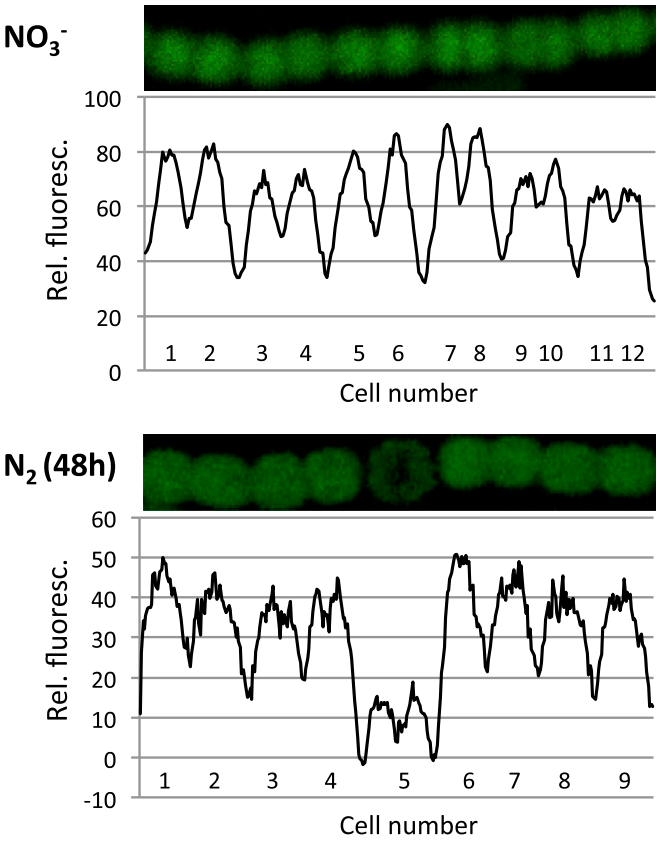

Fig. 3. All3922 is expressed mainly in vegetative cells. Filaments of strain CSMI27 (*all3922-sf-gfp*) grown with nitrate (*Upper*) or incubated for 48 h in medium lacking combined nitrogen (*Lower*) visualized by confocal microscopy and quantification of sf-GFP fluorescence from each cell along the filaments. Average background fluorescence from wild-type cells (lacking the sf-GFP) was subtracted. Cell number 5 in the *Lower* panel is a heterocyst. Rel. fluoresc., relative fluorescence.

heterocyst suspensions were subjected to HPLC analysis. As shown in Fig. 4*B*, β-aspartyl-arginine was released at a rate of 447 nmol ± 107 (mg Chl)$^{-1}$ h^{-1} (mean and SD, $n = 4$), whereas three other amino acids, arginine, aspartate and glutamate, were released at appreciable but lower rates [about 100 nmol (mg Chl)$^{-1}$ h^{-1} for the three of them]. β-Aspartyl-arginine could result from cyanophycin degradation catalyzed by cyanophycinase, and arginine and aspartate could be produced, at least in part, by hydrolysis of the dipeptide catalyzed by the low level of isoaspartyl dipeptidase present in the heterocysts. On the other hand, glutamate could be released because reactants (such as ammonia) needed for biosynthesis of glutamine (6) were not provided in these experiments.

Heterocysts isolated from the dipeptidase mutant, strain CSMI6, produced β-aspartyl-arginine at rates [393 ± 55 nmol (mg Chl)$^{-1}$ h^{-1}; mean and SD, two experiments] similar to those observed with heterocysts from the wild type. Heterocysts isolated from a mutant of gene *cphA1*, which encodes the main cyanophycin synthetase in *Anabaena* (17), produced the dipeptide only at low rates [12.25 ± 2.30 nmol (mg Chl)$^{-1}$ h^{-1}; mean and SD, two experiments]. These results indicate that production of the dipeptide by isolated heterocysts is essentially dependent on cyanophycin.

Amino Acid-Dependent Growth. Our results imply that β-aspartyl-arginine is a nitrogen vehicle to feed the vegetative cells in the diazotrophic filament. Therefore, arginine, aspartate, and the previously known nitrogen vehicle glutamine may together provide nitrogen to sustain the growth of the vegetative cells. We then asked whether these three amino acids, for which cytoplasmic membrane transporters are present in *Anabaena* (28, 30), could support growth of nitrogen fixation mutants of this cyanobacterium in the absence of a readily assimilated nitrogen source such as nitrate or ammonium. The growth of *Anabaena* sp. strains 216, a mutant of the heterocyst differentiation transcription factor HetR (31), and FQ163, a mutant of the Major Facilitator Superfamily protein HepP needed to form the heterocyst envelope polysaccharide layer (32), was compared with that of the wild type in BG11$_0$ medium supplemented with 1 mM (Fig. 5) or 0.5 mM (Fig. S6) each of the three amino acids. Whereas no growth of the mutants was observed in the absence of combined nitrogen, as expected, robust growth, similar to that obtained with nitrate as the nitrogen source, was observed with the three amino acids together. With the single amino acids, the strongest growth was obtained with glutamine (see Fig. S6 for tests with the single amino acids or amino acid pairs). These results are consistent with the idea that arginine, aspartate, and glutamine can together represent the source of nitrogen for vegetative cells in the diazotrophic *Anabaena* filament.

Discussion

The polar accumulation of cyanophycin granules is a distinctive feature of cyanobacterial heterocysts. Consistent with a role of cyanophycin as a dynamic reservoir of nitrogen (20), activities of cyanophycin synthetase and cyanophycinase are detected at high levels in heterocysts (25). In contrast, as shown in this work with an All3922-GFP fusion and by determination of the enzyme activity,

Table 2. Isoaspartyl dipeptidase and glutamine synthetase activities in cell-free extracts of *Anabaena* sp. strains PCC 7120 (WT) and CSMI6

Sample	Isoaspartyl dipeptidase, nmol (mg Chl)$^{-1}$ min^{-1}	Glutamine synthetase, μmol (mg Chl)$^{-1}$ min^{-1}
WT, Fil, BG11	23.94	18.96
WT, Fil, BG11$_0$	22.53	21.54
WT, Vgt	26.81	22.45
WT, Het ($n = 3$)	4.82 ± 0.46	61.37 ± 2.75
CSMI6, Fil, BG11	nd	23.18
CSMI6, Fil, BG11$_0$	nd	22.36
CSMI6, Het	nd	61.33

Isoaspartyl dipeptidase was assayed using β-aspartyl-lysine as a substrate in cell-free extracts of whole filaments grown in bubbled BG11 or BG11$_0$ medium or of vegetative cells or heterocysts isolated from filaments grown in bubbled BG11$_0$ medium; figures refer to dipeptide hydrolyzed in the reaction. Glutamine synthetase was determined in the same cell-free extracts by the transferase assay; figures are γ-glutamyl-hydroxamate produced in the reaction. Data for wild-type heterocysts are the mean and SD of the values obtained with three independent heterocyst preparations. Note that the Het values for isoaspartyl dipeptidase are likely an overestimation, because the presence of some contamination of vegetative cell extracts in the heterocyst extracts is unavoidable. Fil, extracts from whole filaments; Het, extracts from heterocysts; nd, not detected; Vgt, extracts from vegetative cells.

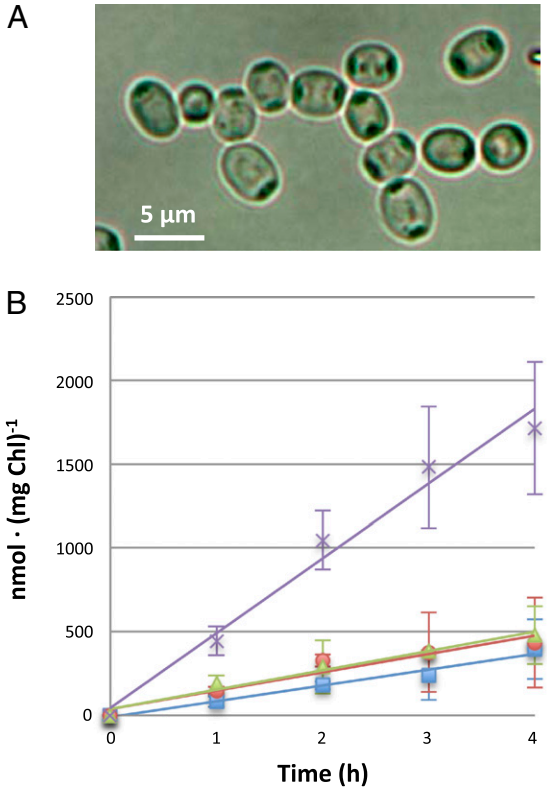

Fig. 4. Release of β-aspartyl-arginine and amino acids by heterocysts isolated from wild-type *Anabaena*. (*A*) Light micrograph of heterocysts isolated from bubbled cultures as described in *SI Materials and Methods*. Preparations contained 90.5 ± 2.5% heterocysts that were undamaged as judged by visual inspection, 8.0 ± 2.5% heterocysts that might be damaged, and 1.5 ± 0.5% vegetative cells (mean and SD; $n = 4$). (*B*) Heterocyst preparations described in *A* were incubated in 10 mM TES-NaOH buffer (pH 7.5), and suspension supernatants from the indicated time points were analyzed by HPLC. Data, presented as nmol of amino acid (or dipeptide) released normalized by the concentration of Chl in the heterocyst suspension, are the mean and SD from four (three in the case of arginine) independent experiments. Amino acids that were consistently detected in the supernatants (glutamate, red circles; aspartate, blue squares; arginine, green triangles) and β-aspartyl-arginine (magenta crosses) are shown.

isoaspartyl dipeptidase accumulates preferentially in vegetative cells. This observation implies a compartmentalized degradation of cyanophycin, with the first step (catalyzed by cyanophycinase) taking place in the heterocysts and the second step (catalyzed by the dipeptidase) taking place mainly in the vegetative cells. Because β-aspartyl-arginine is not accumulated at high concentrations in wild-type *Anabaena* filaments, this compartmentalized metabolism of cyanophycin implies in turn that during diazotrophic growth, the dipeptide is transferred from heterocysts to be hydrolyzed in the vegetative cells. Consistently, isolated heterocysts release substantial amounts of β-aspartyl-arginine when incubated in a buffer, although the mechanism of release is currently unknown. In the dipeptidase mutant, strain CSMI6, a substantial accumulation of cyanophycin granules was observed in the cells of the filament. This suggests that high levels of β-aspartyl-arginine impair cyanophycin degradation, perhaps through a feedback inhibition of cyanophycinase.

As observed with *cphA* (cyanophycin synthetase) mutants from different species of *Anabaena* sp., under diazotrophic conditions, cyanophycin synthesis is required only for optimal growth (16, 17). Blocking cyanophycin degradation in *Anabaena* by inactivation of *cphB* (cyanophycinase) genes has, however, a clear impact on the rate of diazotrophic growth, which is reduced to about 62–64% of the wild-type rate (17). Inactivation of the *Anabaena* isoaspartyl dipeptidase (All3922) reduces the diazotrophic growth rate to about 54% of the wild-type value. Thus, blocking cyanophycin degradation by inactivation of either cyanophycinase or the dipeptidase has similar effects on diazotrophic growth. These observations imply that in *Anabaena*, nitrogen does not need to take the route of cyanophycin, but that once synthesized, cyanophycin has to be degraded to permit normal growth. Lack of degradation makes cyanophycin into a nitrogen sink (17). As previously discussed (18), when cyanophycin synthesis is not possible, arginine might be transferred directly from heterocysts to vegetative cells. Interestingly, we have observed that the isolated heterocysts can also release arginine and aspartate. These amino acids were found among the first products of nitrogen fixation in heterocyst-forming cyanobacteria (4).

Our results implying intercellular transfer of β-aspartyl-arginine, together with the possible transfer of arginine (alone or with aspartate), are to be considered together with previous results that identified glutamine as a nitrogen vehicle in the diazotrophic filaments of heterocyst-forming cyanobacteria (5, 6). Glutamine, arginine, and aspartate can together feed the vegetative cells for nitrogen, as evidenced by the robust growth of two heterocyst differentiation mutants of *Anabaena* when supplied with a mixture of the three amino acids as the sole nitrogen source (Fig. 5 and Fig. S6). Growth of different species of *Anabaena* sp. using glutamine or arginine as nitrogen source has also been reported previously (33–35). Although the exact equation of intercellular nutrient exchange in the diazotrophic *Anabaena* filament is unknown, movement of sucrose, glutamate, and alanine from vegetative cells to heterocysts and of glutamine and β-aspartyl-arginine from heterocysts to vegetative cells could result in a net transfer of reduced carbon to heterocysts and of fixed nitrogen to vegetative cells. A possible gradient of arginine or of an arginine-containing compound in the diazotrophic filament of *Anabaena* has been noted (19).

The compartmentalized metabolism of cyanophycin shown in this work represents an optimized way of using this nitrogen reservoir in heterocyst-forming cyanobacteria. Cyanophycin synthesis after nitrogen fixation has been suggested to serve an important function by removing from solution the products of nitrogen fixation, which could have a negative feedback effect on nitrogenase (18–20). Interestingly, this strategy appears to be generally used in nitrogen-fixing cyanobacteria independently of whether they are unicellular (36) or filamentous (37). However, a limited hydrolysis of β-aspartyl-arginine in the heterocysts adds the benefit of avoiding the release of the constituent amino acids back in the cytoplasm of the nitrogen-fixing cell. Thus, cyanophycin metabolism appears to have evolved to increase the efficiency of nitrogen fixation taking advantage of the multicellular nature of heterocyst-forming cyanobacteria.

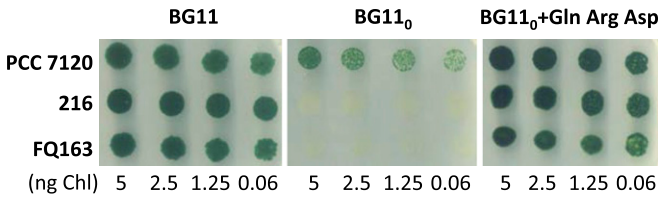

Fig. 5. Growth tests of *Anabaena* sp. strains PCC 7120, 216 (*hetR*) and FQ163 (*hepP*) in BG11$_0$ solid medium supplemented with 10 mM TES-NaOH buffer (pH 7.5) and nitrate (BG11) or 1 mM each glutamine (Gln), arginine (Arg), and aspartic acid (Asp). Spots were inoculated with an amount of cells containing the indicated amount of Chl, and the plates were incubated under culture conditions for 10 d and photographed.

Materials and Methods

Anabaena sp. strain PCC 7120 was grown photoautotrophically at 30 °C with the nitrogen source indicated in each experiment, in shaken cultures with air levels of CO_2 or in bubbled cultures supplemented with bicarbonate/CO_2. Anabaena mutants used in this work are summarized in Table S2. Strain CSMI6 bears an *all3922* gene with an internal fragment deleted and was constructed in a way such that it bears no antibiotic resistance marker. Strain CSMI6-C (Sp^R/Sm^R) is mutant CSMI6 complemented with wild-type *all3922* present in plasmid pCSAM200 (plasmids used in this work are summarized in Table S2), which includes the pDU1 replicon from a *Nostoc* sp. that can replicate in *Anabaena*. Strain CSMI27 (Sp^R/Sm^R) bears an *all3922-sf-gfp* fusion gene integrated as part of a nonreplicative plasmid in the *all3922* locus. The protein encoded by the fusion gene contains the superfolder GFP fused through a four-glycine linker to the C terminus of All3922. Construction of these strains is detailed in *SI Materials and Methods*, and oligodeoxynucleotide primers used for strain construction and verification are listed in Table S3.

Growth tests in liquid and solid media, quantification of protein and chlorophyll *a* (Chl), isoaspartyl dipeptidase, glutamine synthetase and nitrogenase activity measurements, and microscopic inspection of cultures and heterocyst preparations were performed as detailed in *SI Materials and Methods*. In strain CSMI27, sf-GFP fluorescence was visualized by confocal microscopy (excitation at 488 nm; emission collected from 500 to 520 nm) and quantified using the wild-type strain PCC 7120 as a control.

CGP preparations were obtained by disruption of filaments in a French pressure cell at 20,000 p.s.i., collecting the granules by centrifugation and dissolving them in 0.1 N HCl as detailed in *SI Materials and Methods*. The amount of cyanophycin was then estimated by determining arginine by the Sakaguchi reaction. Heterocysts were isolated from filaments grown in bubbled cultures in media lacking combined nitrogen. Filaments treated with 1 mg lysozyme mL^{-1} were disrupted by passage two or three times through a French pressure cell at 3,000 psi, and the heterocysts were collected by low-speed centrifugation. Determination of amino acids (including β-aspartyl-arginine) in cell-free extracts, in material extracted from whole filaments with 0.1 HCl or by boiling, or in supernatants from heterocyst suspensions was performed by high-pressure liquid chromatography as described in *SI Materials and Methods*.

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Supporting Information

Burnat et al. 10.1073/pnas.1318564111

SI Materials and Methods

Bacterial Strains and Growth Conditions. *Anabaena* sp. (also known as *Nostoc* sp.) strain PCC 7120 was grown in BG11 (containing $NaNO_3$), $BG11_0$ (free of combined nitrogen), or $BG11_0$ + ammonium ($BG11_0$ containing 4 mM NH_4Cl and 8 mM TES-NaOH buffer, pH 7.5) medium at 30 °C in the light (25 μE m^{-2} s^{-1}), in shaken (80–90 rpm) liquid cultures or in medium solidified with 1% Difco agar. Alternatively, cultures (referred to as bubbled cultures) were supplemented with 10 mM of $NaHCO_3$ and bubbled with a mixture of CO_2 and air (1% vol/vol) in the light (50–75 μE m^{-2} s^{-1}). *Anabaena* sp. strain 216 [*hetR* mutant (1)], strain FQ163 [*hepP* mutant (2)], and strain CSS7 [*cphA1* mutant (3)] were grown in BG11 medium, supplemented when appropriate with antibiotics. For the mutants described below, antibiotics were used at the following concentrations: 5 μg mL^{-1} streptomycin sulfate (Sm) and 5 μg mL^{-1} spectinomycin dihydrochloride pentahydrate (Sp). DNA was isolated from *Anabaena* sp. as described (4). *Escherichia coli* DH5α, used for plasmid constructions, and other strains of *E. coli* (see *Strain Construction* below) were grown in LB medium, supplemented when appropriate with antibiotics at standard concentrations (5). *Anabaena* sp. strains are listed in Table S2.

Strain Construction. ORF *all3922* of the *Anabaena* chromosome (6) was inactivated by deleting a 763-bp internal fragment. DNA fragments upstream (674-bp) and downstream (410-bp) from the central region of the gene were amplified by standard PCR using *Anabaena* DNA as template and oligonucleotide primers all3922-1/all3922-2 and all3922-3/all3922-4 (all oligodeoxynucleotide primers are listed in Table S3). The external primers al3922-1 and all3922-4 included SacI-sites in their 5′ ends. The amplified DNA fragments were cloned in pMBL-T (Dominion MBL) producing plasmid pCSMI28, which was verified by sequencing and transferred as a SacI-ended fragment to SacI-digested pCSRO (7) producing pCSMI29. Conjugation of *Anabaena* with *E. coli* HB101 carrying pCSMI29 with helper and methylation plasmid pRL623 (all plasmids are listed in Table S2) was effected by the conjugative plasmid pRL443, carried in *E. coli* ED8654, and performed as described (8) with selection for resistance to Sm and Sp. Exconjugants were spread on $BG11_0NH_4^+$ medium supplemented with 5% sucrose (4), and individual Suc^R colonies were checked by PCR looking for clones that had replaced the wild-type locus by a locus bearing the deletion. The genetic structure of selected clones was studied by PCR with DNA from those clones and primers all3922-4/all3922-5 and all3922-5/all3922-6 (Fig. S1). A clone homozygous for the mutant chromosomes was named strain CSMI6 (Δ*all3922*).

The plasmid carrying fusion gene *all3922-sf-gfp* (where *sf-gfp* denotes the gene encoding a superfolder GFP from Theranostech Inc.) was prepared as follows. A 597-bp fragment from the 3′-terminal part of *all3922* was amplified by PCR using primers all3922-11 and all3922-13 [which lacks the stop codon of the gene and contains a sequence encoding a four-glycine (four-Gly) linker and an NheI site close to its 5′ end] and *Anabaena* DNA as template, and the resulting fragment was cloned in vector pMBL-T producing plasmid pCSMI57. This plasmid was corroborated by sequencing and transferred as a NotI/NheI-ended fragment to NotI/NheI-digested pCSAL34 (which contains, cloned in vector pMBL-T, the *sf-gfp* gene with an Ala-encoding codon instead of the Met start codon) producing plasmid pCSMI60 that carries the fusion of the *sf-gfp* gene to the 3′ end of *all3922*. The resulting fusion was finally transferred as a KpnI-ended fragment to KpnI-digested pCSV3 (9) producing pCSMI61. This plasmid was transferred to *Anabaena* by triparental mating with *E. coli* strains HB101 [pCSMI61, pRL623] and ED8654 [pRL443] as described (8). Insertion into *all3922* and segregation of chromosomes carrying the fusion was confirmed by PCR analysis using template DNA from exconjugant clones and primers all3922-5 and pRL500-1 for testing insertion of *sf-gfp* and all3922-5 and all3922-8 for testing segregation of the mutated chromosomes.

Complementation was performed using a replicative vector. The *all3922* gene was amplified by PCR using *Anabaena* DNA as template and primers all3922-7, which includes an EcoRI site close to its 5′ end, and all3922-8, and ligated into pSPARK (Canvax; Biotech SL) producing pCSMI55, which was corroborated by sequencing. The insert of pCSMI55 was excised with EcoRI and transferred to EcoRI-digested pCSAM200 (10) producing pCSMI56. The resulting plasmid was transferred to strain CSMI6 (Δ*all3922*) with selection for resistance to Sm and Sp.

Growth Tests and Nitrogenase Activity. Protein concentration and chlorophyll *a* (Chl) content of cultures or cell-free extracts were determined by a modified Lowry procedure (11) and by the method of Mackinney (12), respectively. The growth rate constant ($\mu=\ln 2/t_d$, where t_d is the doubling time) was calculated from the increase of protein content, determined in 0.2-mL samples, of shaken liquid cultures (13). Cultures were inoculated with an amount of cells containing about 2 μg of protein mL^{-1} and grew logarithmically until reaching about 40 μg of protein mL^{-1}.

For growth tests in solid media, cultures grown in medium $BG11_0$ + ammonium supplemented with antibiotics when appropriate were harvested and washed three times with $BG11_0$ medium. Samples of 10 μL of serial dilutions of the resulting suspensions were spotted on agar plates with different nitrogen sources and incubated at 30 °C in the light (25 μE m^{-2} s^{-1}). When indicated, solid media was supplemented with filter-sterilized L-glutamine, L-arginine, and L-aspartic acid (purchased from Sigma-Aldrich) at the concentrations indicated in the corresponding experiments.

Nitrogenase activity was determined by the acetylene reduction assay under oxic conditions as described previously (13). Cells grown in $BG11_0$ + ammonium medium were incubated in $BG11_0$ medium (without combined nitrogen) for 48 h before carrying out the nitrogenase assays.

Cyanophycin Granule Polypeptide Extraction and Determination. For cyanophycin measurements, filaments grown in 50 mL of $BG11_0$ + ammonium medium were used in two different experimental approaches. In the first one, cells grown in $BG11_0$ + ammonium medium were harvested, washed three times with $BG11_0$ medium, and used to inoculate 50-mL cultures of BG11 medium at 0.5 μg Chl mL^{-1}, which were incubated for 8 d under culture conditions. In the second approach, cells were grown in $BG11_0$ + ammonium medium during 3 wk (readding weekly new media and ammonium to prevent depletion of the nitrogen source), harvested, washed three times with $BG11_0$ medium, and used to inoculate 50-mL cultures of $BG11_0$ + ammonium, BG11, or $BG11_0$ medium, which were then incubated for 24 h under culture conditions.

Filaments from the different cultures were collected at room temperature, washed twice with, and resuspended in, milliQ-purified and autoclaved H_2O, and disrupted by passage through a French pressure cell (twice at 20,000 psi). After measuring

the obtained volume of cell extract, Chl was determined in 100-μL samples. The remainder of each extract was centrifuged at 27,216 × *g* (15,000 rpm in a SS34 rotor; 15 min at 4 °C), the resulting supernatants (which constitute the cell-free extracts) were discarded or stored at −20 °C as indicated, and the pellets were washed twice with 11 mL of sterile milliQ-purified H_2O and resuspended in 1 mL of 0.1 M HCl for solubilization of Cyanophycin Granule Polypeptide (CGP). After 2–4 h of incubation at room temperature and centrifugation under the same conditions, the resulting supernatants were removed and stored at 4 °C. The pellets were resuspended in 1 mL of 0.1 M HCl to complete solubilization of CGP, incubated overnight at room temperature, and again subjected to centrifugation. The obtained supernatants were combined with those obtained after the first centrifugation and stored at 4 °C. These preparations were used for arginine determination carried out by the Sakaguchi method as modified by Messineo (14).

Western Blot Analysis. Filaments from 100 mL of cultures of *Anabaena* sp. strains PCC 7120 and CSMI27 grown in bubbled BG11 and $BG11_0$ medium were harvested, washed with buffer 1 (50 mM Tris-HCl buffer, pH 7.5, 10% glycerine, 100 mM NaCl), and resuspended in 4 mL of the same buffer. To isolate heterocysts, 800 mL of cultures grown for 48 h in bubbled $BG11_0$ medium were harvested and washed with buffer 2 (50 mM imidazole and 0.5 mM EDTA, pH 8.0), resuspended in 10 mL of the same buffer, incubated with 1 $mg{\cdot}mL^{-1}$ of lysozyme for 15 min at room temperature, and centrifuged at 1,935 × *g* (4 °C, 10 min). The pellets were resuspended in 5 mL of buffer 2 and passed through a French pressure cell at 3,000 psi three times. Samples were enriched in heterocysts after successive steps of low-speed centrifugation (200 × *g*, 10 min, 4 °C) and washing with buffer 1, and the isolated heterocysts were resuspended in 4 mL of the same buffer. The filament and heterocyst suspensions were both supplemented with a protease inhibitor mixture tablet (cOmplete Tablets, Mini EDTA-free; Roche) and passed through a French pressure cell at 20,000 psi two times. Cell debris was removed by centrifugation at 16,100 × *g* (4 °C, 10 min). Proteins in the supernatants were resolved by SDS/PAGE and transferred to Hybond-P PVDF membranes (GE Healthcare). Western blot was performed incubating with an anti-GFP antibody (A6455 from Invitrogen) diluted 1:2,000 in Tween 20, Tris-buffered saline with 5% nonfat milk powder. Antigen–antibody complexes were visualized with a peroxidase-conjugated anti-rabbit-IgG antiserum (Sigma) and developed with the WesternBright Western blotting detection kit (Advansta). A cell-free extract from *Anabaena* carrying pAM1954 (15) was used as a free GFP-containing control.

Enzymatic Assays in Cell-Free Extracts. Cell-free extracts from whole filaments were obtained as described earlier for the Western analysis but omitting the protease mixture. For obtaining cell-free extracts from isolated vegetative cells and heterocysts, filaments from 1,600 mL of cyanobacterial cultures grown for 4 d in bubbled $BG11_0$ medium were used. Heterocysts were isolated as described above, with the modification that after the French pressure cell step, the suspension was centrifuged at 27,216 × *g* (4 °C, 10 min). The resulting supernatant constituted the cell-free extract from vegetative cells. The pellet was resuspended and washed with buffer 1 by low-speed centrifugation (200 × *g*, 10 min, 4 °C) four or five times, until the suspension was enriched in heterocysts to about 95–98% as examined by microscopy. Cell-free extracts of heterocysts were obtained by passing the heterocyst preparation two times through a French pressure cell at 20,000 psi and centrifugation at 27,216 × *g* (4 °C, 10 min). The pellet was discarded, and the supernatant corresponded to the heterocyst cell-free extract.

For determining the enzymatic activity of All3922, cell-free extracts from filaments grown in bubbled BG11 or $BG11_0$ medium, vegetative cells, or heterocysts were incubated at 30 °C with or without (as a control) 10 mM β-aspartyl-lysine (purchased from Bachem Distribution Services GmbH) in buffer 1 (total reaction volume, 1 to 1.35 ml). Samples of 0.27 mL of the reaction mixtures were taken at different times, the enzymatic reaction was stopped by addition of 0.1 N HCl (final concentration), and the mixture was incubated at 0 °C for 1 h and centrifuged at 16,100 × *g* at 4 °C, 5 min. Production of aspartate and lysine from β-aspartyl-lysine was determined in the supernatants by HPLC.

Glutamine synthetase transferase activity was determined by the formation of γ-glutamylhydroxamate as described previously (16).

Preparation of Samples for Determination of Metabolites. Heterocysts were isolated from filaments collected from 800-mL cultures incubated for 48 h in bubbled $BG11_0$ medium. Cultures were harvested by filtration, and filaments were washed with buffer 2, resuspended in 5–10 mL of the same buffer, incubated with 1 $mg{\cdot}mL^{-1}$ of lysozyme for 10–15 min at room temperature, centrifuged at 1,935 × *g* (4 °C, 10 min), and resuspended in 5 mL of the same buffer. Filaments were then broken by passage through a French pressure cell at 3,000 psi two or three times. Samples were enriched in heterocysts after successive steps of low-speed centrifugation (200 × *g*, 10 min, 4 °C) and washing with buffer 2. Purity of heterocyst preparations was examined by microscopy.

Heterocysts were resuspended in 7 mL of 10 mM TES-NaOH buffer (pH 7.5) and incubated at 30 °C in the light (25 $\mu E\ m^{-2}\ s^{-1}$) with shaking (80–90 rpm). Samples of 1 mL were taken at 0, 1, 2, 3, and 4 h and centrifuged at 16,100 × *g* at 4 °C, 10 min. Supernatants were transferred to new centrifuge tubes, treated with 0.1 N HCl for 1–1.5 h at 0 °C for protein precipitation, and centrifuged at 16,100 × *g* at 4 °C, 15 min. The resulting supernatants were used for determination of amino acids by HPLC as described below.

For determination of amino acids in samples of filaments, 50-mL cultures inoculated at 1 μg Chl mL^{-1} in $BG11_0$ medium were incubated for 48 h, harvested, and washed three times with 50 mL of $BG11_0$. Samples of 0.9 mL of the pellets from the last centrifugation were treated with 0.1 N HCl for 1–1.5 h at 0 °C for metabolite extraction and protein precipitation and centrifuged at 16,100 × *g* at 4 °C, 10 min. The resulting supernatants were used for determination of amino acids as described below. Alternatively, washed filaments from the cultures were resuspended in water, incubated at 100 °C for 5 min, and centrifuged to remove cell debris. The supernatants were supplemented with HCl (0.1 N, final concentration) for analysis by HPLC.

For determination of amino acids in cell-free extracts from the CGP isolation (described in *Cyanophycin Granule Polypeptide Extraction and Determination* above), frozen samples were thawed, treated with 0.1 N HCl for 1–1.5 h at 0 °C for protein precipitation, and centrifuged at 16,100 × *g* at 4 °C, 10 min. The resulting supernatants were used for amino acid determination.

Determination of Amino Acids. Amino acids were determined by HPLC in the samples prepared as described above. The method used for the analysis of amino acids involves a derivatization of amino acids with phenylisothiocyanate (PITC) (17), which binds to primary or secondary amines producing a derivative, phenylthiocarbamyl, that is detected by measuring the absorbance at 254 nm. Sixty microliters of sample were mixed with 60 μL of the derivatizing solution (ethanol:H_2O:triethanolamine:PITC, 7:1:1:1), incubated at room temperature for 30 min, and dried under flowing N_2. The pellet was resuspended in 60 μL of 4 mM sodium phosphate (pH 7.4) and 2% (vol/vol) acetonitrile and injected in a HPLC Elite LaChrom (Hitachi) system. The separation (at 1.5 $mL{\cdot}min^{-1}$, 46 °C) was performed in a reverse phase Sugesphere

C_{18} (4.6 mm × 25 cm) column. A step-wise gradient of two solvents was used. Solvent A contained 70 mM sodium phosphate (pH 6.55) and 2% acetonitrile (vol/vol); solvent B contained 50% (vol/vol) acetonitrile. An amino acid standard solution, AA-S-18 (from Sigma), supplemented with Gln and Asn, was used for calibrating the apparatus. The standard amino acids detected with this method were: Asp, Glu, Gln, Asn, Ser, Gly, His, Thr, Ala, Arg, Pro, Tyr, Val, Met, Cys, Ile, Leu, Phe, and Lys. β-Aspartyl-arginine was also detected as shown with a standard of this dipeptide (18).

Microscopy. Cells grown in liquid cultures or heterocyst preparations were observed and photographed with a Zeiss Axioscop microscope equipped with a Zeiss ICc1 digital camera. GFP fluorescence was analyzed by confocal microscopy. Samples from cultures of *Anabaena* sp. strain CSMI27 or the wild-type PCC 7120 as a control grown in BG11 or $BG11_0$ medium were visualized using a Leica HCX PLAN-APO 63× 1.4 NA oil immersion objective attached to a Leica TCS SP2 confocal laser-scanning microscope. GFP was excited using 488-nm irradiation from an argon ion laser, and fluorescence emission was monitored by collection across a window of 500–520 nm. Under the conditions used, optical section thickness was about 0.4 μm. GFP fluorescence intensity was analyzed using ImageJ 1.43m software.

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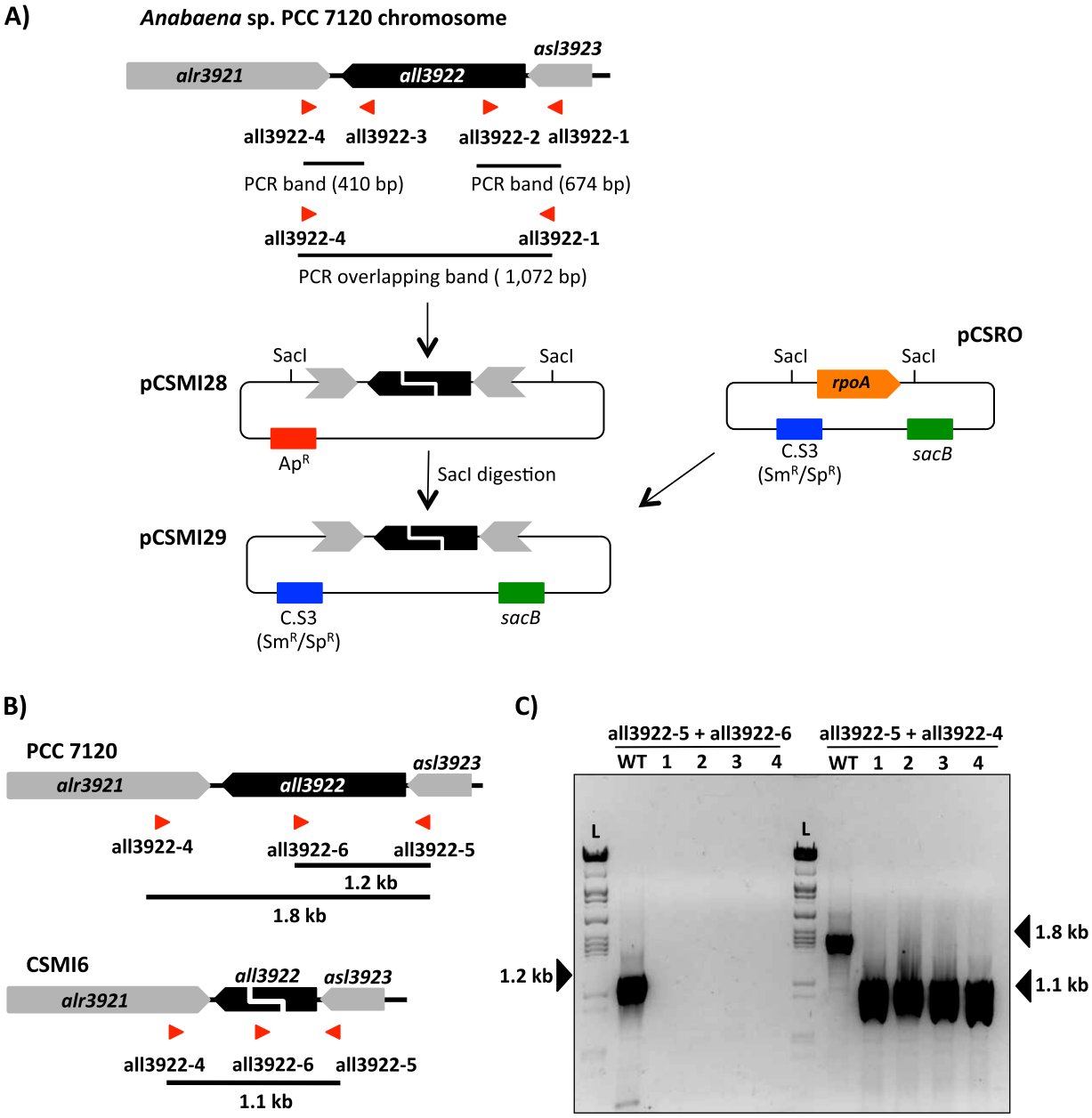

Fig. S1. Construction and verification of mutant CSMI6. (*A*) Construction of pCSMI29. Amplification by PCR of regions upstream and downstream from the central part of *all3922* and cloning of the PCR products in pMBL-T produced pCSMI28. The SacI insert of pCSMI28 was transferred to SacI-digested pCSRO, producing pCSMI29, which was transferred to *Anabaena*. (*B*) Scheme of the *all3922* genomic region in *Anabaena* sp. PCC 7120 and CSMI6. Block arrows denote genes and direction of transcription. Red arrowheads represent primers (all listed in Table S3). (*C*) Verification of strain CSMI6 by PCR. Primer pairs are indicated on top. Templates: WT, wild type DNA; 1–4, DNA from four clones in which the wild-type locus was replaced by a gene bearing the deletion. One of these clones, which could be complemented by wild-type *all3922*, was chosen for further analysis and named CSMI6. L, lambda DNA digested with ClaI.

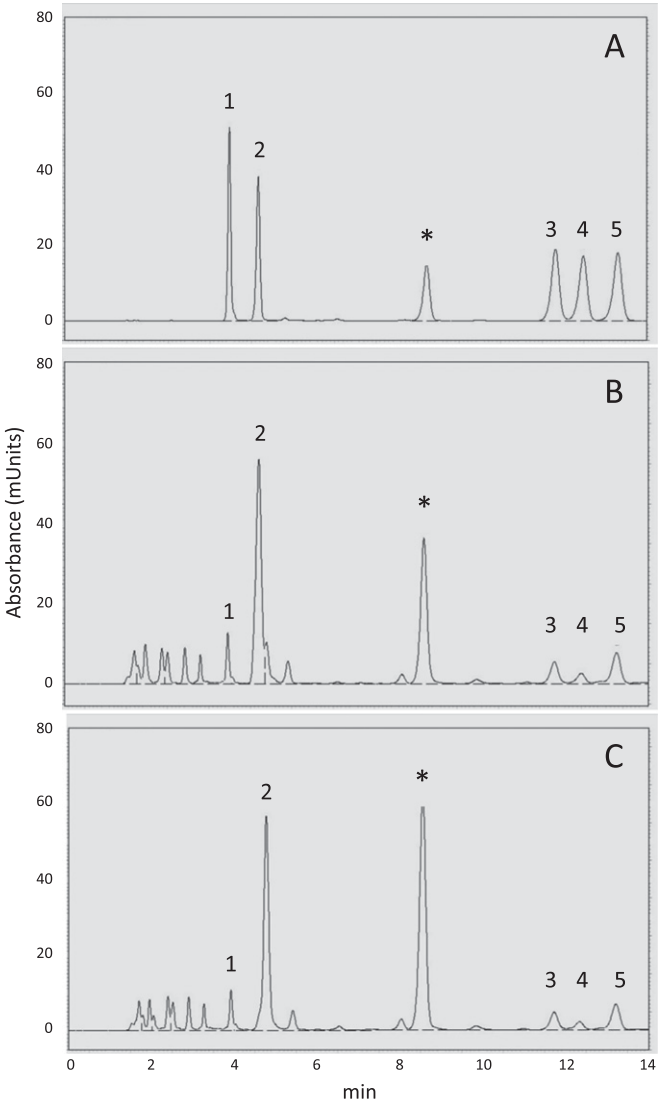

Fig. S2. Identification of β-aspartyl-arginine in *Anabaena* sp. strain CSMI6. (*A*) Chromatogram of standard amino acids including β-aspartyl-arginine. (*B*) Chromatogram of a cell-free extract from mutant CSMI6 incubated for 24 h in $BG11_0$ medium (lacking combined nitrogen). (*C*) Chromatogram of the same extract shown in *B* supplemented with 7.5 nmol of β-aspartyl-arginine. Peaks corresponding to aspartate (1), glutamate (2), serine (3), asparagine (4), and glycine (5) are indicated. The peak marked with an asterisk (*) corresponds to β-aspartyl-arginine. Note the increase in size of the β-aspartyl-arginine peak (*) in *C* compared with *B* due to the addition of the dipeptide.

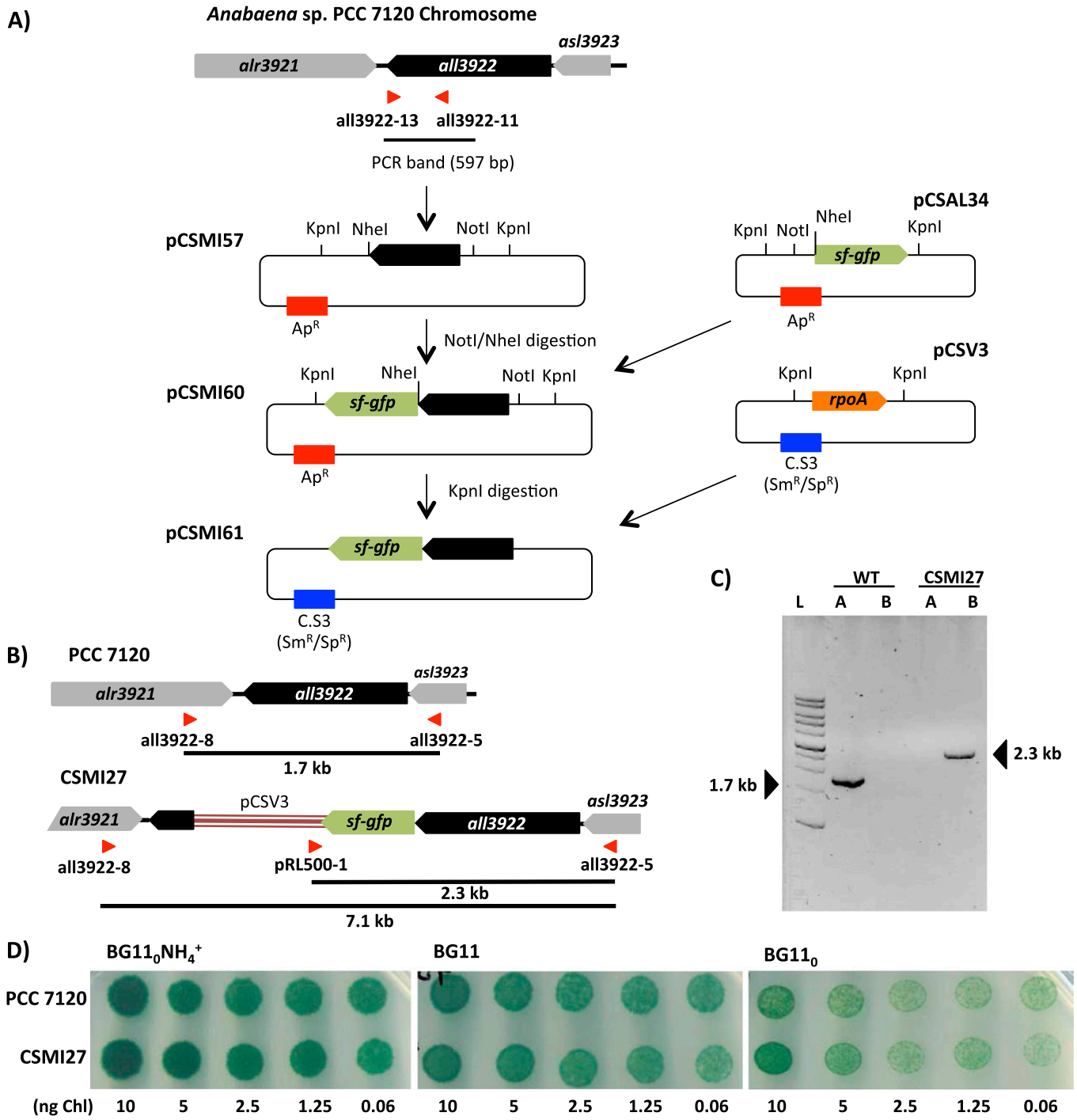

Fig. S3. Construction, verification, and growth test of strain CSMI27. (*A*) Construction of pCSMI61. The 3′ part of *all3922* was amplified by PCR and cloned in pMBL-T, producing pCSMI57. The NotI-NheI insert was transferred to NotI/NheI-digested pCSAL34, which carries *sf-gfp*, generating pCSMI60. The KpnI insert from pCSMI60 was transferred to KpnI-digested pCSV3, producing pCSMI61, which was transferred to *Anabaena*. (*B*) Scheme of the *all3922* genomic region in *Anabaena* sp. PCC 7120 and CSMI27. Block arrows denote genes and direction of transcription. Red arrowheads represent primers (Table S3). Red tripled line represents the pCSV3 vector. (*C*) Verification of strain CSMI27 by PCR. Lanes A, PCR performed with primers all3922-5 and all3922-8; lanes B, with primers all3922-5 and pRL500-1. Template DNA from the wild type (WT) or CSMI27 was used. L, 1-kb DNA ladder. (*D*) Growth tests in solid medium using ammonium ($BG11_0NH_4^+$), nitrate (BG11), or N_2 ($BG11_0$) as the nitrogen source. Spots were inoculated with cells containing the indicated ng of Chl, and the plates were incubated under culture conditions for 7 d and photographed. Note that strain CSMI27 grows similarly to strain PCC 7120 with the different nitrogen sources, indicating that the sf-GFP fusion is not impairing All3922 function.

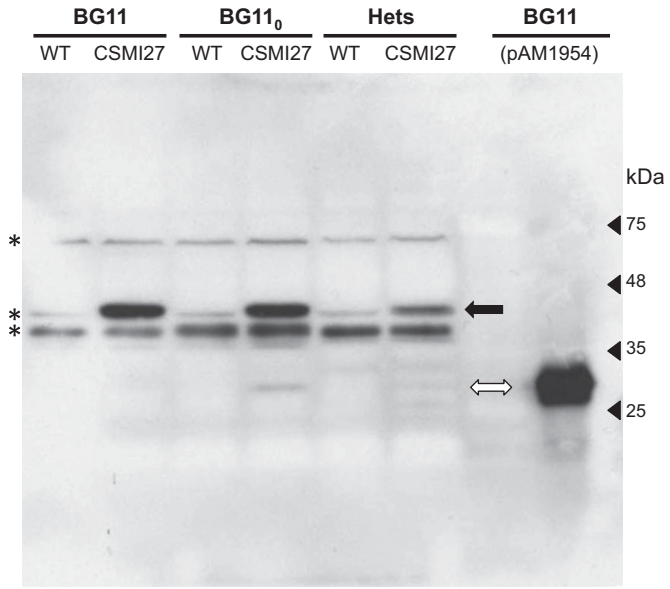

Fig. S4. Western blot analysis of the GFP in strain CSMI27 and control strains. The *Anabaena* strains used were the wild type (WT), mutant CSMI27 (*all3922-sf-gfp*), and a strain carrying pAM1954, which is a replicative plasmid bearing the *gfp* gene expressed from the *rbc* gene promoter. Cell-free extracts were prepared from whole filaments grown in bubbled cultures with BG11 or BG11$_0$ medium or from heterocysts (Hets) isolated from the latter. *Anabaena* sp. [pAM1954] was grown in shaken cultures containing BG11 medium. Proteins (40 μg, or 2.5 μg in the case of *Anabaena* [pAM1954]) were subjected to SDS/PAGE electrophoresis and transferred to a membrane. Protein detection was performed with anti-GFP antibodies. Native GFP is 26.9 kDa, and the All3922-sf-GFP fusion, which includes the terminal 138 amino acids of All3922 (β subunit of the mature protein) and a tetraglycine linker peptide, is 41.3 kDa. As observed with the wild-type extracts, the antibodies marked three unspecific bands (asterisks). Strain CSMI27 produced mainly a band corresponding to the fusion protein (black arrow) and only a faint band corresponding to free GFP, which was identified with the extract of *Anabaena* [pAM1954] that only produced, as expected, free GFP (open arrow). Note that the levels of the fusion protein were lower in heterocyst extracts than in extracts from whole filaments.

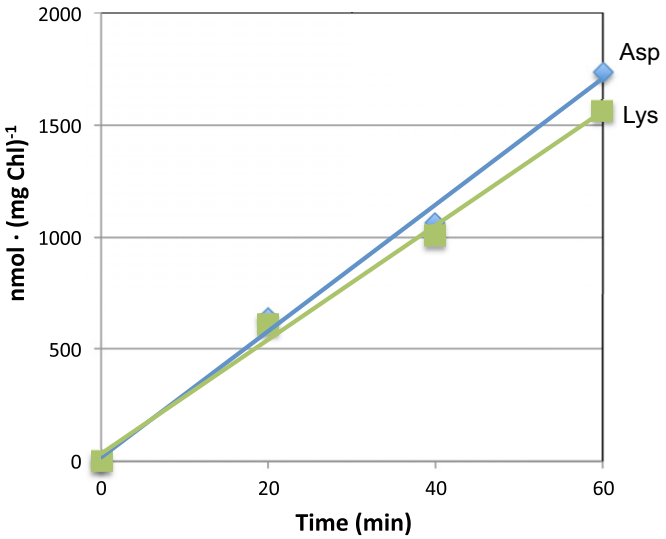

Fig. S5. Isoaspartyl dipeptidase assay. The enzyme was assayed in cell-free extracts of vegetative cells from wild-type filaments grown in bubbled BG11$_0$ medium. β-Aspartyl-lysine (10 mM) was used as a substrate, and the production of aspartate and lysine was determined by HPLC. The amount of amino acids present in control extracts incubated without added β-aspartyl-lysine was subtracted. Note that the amounts of aspartate (Asp) and lysine (Lys) produced from β-aspartyl-lysine showed a stoichiometry close to 1:1, as expected from hydrolysis of the dipeptide; therefore, isoaspartyl dipeptidase activity can be presented as the mean of the aspartate and lysine produced in the reaction (see Table 2). Hydrolysis of β-aspartyl-lysine in extracts of strain CSMI6 was undetectable, indicating that All3922 is the only enzyme catalyzing a significant hydrolysis of β-aspartyl-lysine in *Anabaena* and substantiating the use of this dipeptide as a substrate to assay isoaspartyl dipeptidase.

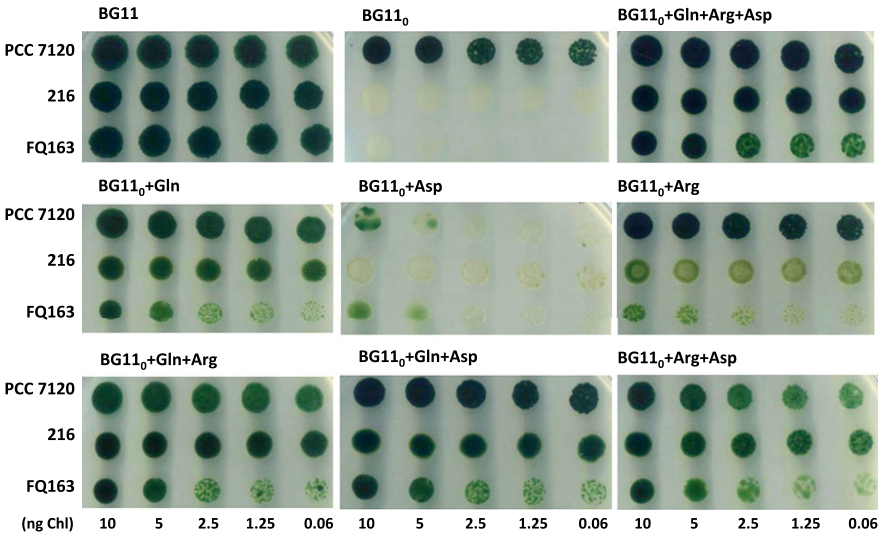

Fig. S6. Growth test of *Anabaena* sp. strains PCC 7120 (wild type), 216 (*hetR*), and FQ163 (*hepP*) in BG11$_0$ + 10 mM TES-NaOH buffer (pH7.5) solid media supplemented with nitrate (BG11) or with glutamine (Gln), arginine (Arg), and aspartic acid (Asp) as indicated. The final concentration of each amino acid was 0.5 mM. Each spot was inoculated with an amount of cells containing the indicated amount of Chl, and the plates were incubated under culture conditions for 13 d and photographed. Note that Asp alone was inhibitory, Arg alone permitted some growth of the mutants, and these two amino acids together supported appreciable growth. Gln alone or in combination with Arg or Asp was an excellent nitrogen source. However, the most robust growth, similar to that observed with nitrate, was obtained with Gln, Arg, and Asp together.

Table S1. Intracellular amino acids in filaments of *Anabaena* sp. strains PCC 7120 and CSMI6

	PCC 7120		CSMI6		
Amino acids	Mean ± σ	(n)	Mean ± σ	(n)	Boiled
Aspartic acid	335.70 ± 60.30	(2)	162.27 ± 23.97	(2)	794.31
Glutamic acid	4112.67 ± 442.97	(3)	2629.83 ± 401.26	(2)	3342.22
Serine	930.85 ± 35.80	(2)	375.69 ± 68.77	(2)	555.49
Asparagine	46.74 ± 14.50	(2)	16.22 ± 11.33	(2)	65.36
Glycine	307.90 ± 45.75	(2)	321.54 ± 63.57	(3)	475.72
Glutamine	159.70 ± 25.96	(2)	145.46 ± 76.85	(2)	295.99
Histidine	27.78 ± 2.38	(2)	ND		ND
Threonine	73.15 ± 8.94	(2)	154.81	(1)	ND
Alanine	560.75 ± 12.54	(2)	325.50 ± 9.28	(2)	611.75
Arginine	308.27 ± 57.25	(3)	136.31 ± 96.38	(3)	159.13
Proline	230.48 ± 17.35	(2)	136.95 ± 24.51	(2)	167.87
Tyrosine	212.16 ± 24.09	(2)	344.42 ± 109.77	(2)	218.68
Valine	225.13	(1)	298.62	(1)	258.46
Methionine	61.22 ± 39.55	(2)	103.86 ± 94.98	(2)	140.25
Cystine	ND		ND		ND
Isoleucine	140.96	(1)	121.05	(1)	138.80
Leucine	107.53	(1)	120.12	(1)	155.01
Phenylalanine	119.49	(1)	76.09	(1)	122.56
Tryptophan	ND		173.42	(1)	310.96
Lysine	ND		ND		ND
β-Asp-Arg	34.64 ± 10.92	(2)	4163.74 ± 1339.66	(3)	3077.57

Ammonium-grown filaments were inoculated at 1 μg Chl mL^{-1} and incubated for 48 h in BG11$_0$ medium (lacking combined nitrogen). Figures are the mean and SD of data from the number of independent determinations indicated (n), in which amino acid extraction was carried out with 0.1 N HCl. In one case, amino acids were also extracted from CSMI6 filaments by boiling, and the results are included for comparison. Amino acids were determined by HPLC as described in *SI Materials and Methods*. Figures are nmol (mg Chl)$^{-1}$. β-Asp-Arg, β-aspartyl-arginine; ND, not detected.

Table S2. Cyanobacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristic(s)	Source or reference
Anabaena strains		
PCC 7120	Wild type	1
216	*hetR* S179N	2
FQ163	*hepP(all1711)*::Tn5-1063; Nm^R	3
CSS7	*cphA1(all3879)*::C.S3; Sm^RSp^R	4
CSMI6	Δ*all3922*	This study
CSMI6-C	Δ*all3922* bearing wild-type *all3922* in pCSAM200; Sm^RSp^R	This study
CSMI27	*all3922*-C-*sf-GFP*; Sm^RSp^R	This study
Plasmids		
pMBL-T	Cloning vector; Ap^R	Dominion MBL
pSPARK	Cloning vector; Ap^R	Canvax, Biotech SL
pCSRO	*sacB*-containing negative selection vector with: Sm^RSp^R Suc^S	5
pCSV3	Positive selection vector; Sm^RSp^R	6
pRL443	Conjugative plasmid; Ap^R	7
pRL623	Helper plasmid; carries *mob* and DNA methylases; Cm^R	8
pCSAM200	Replicative vector used for complementation; Sm^RSp^R	9
pCSAL34	Plasmid carrying *sf-gfp;* Ap^R	A. López-Lozano and A. Herrero
pCSMI28	*all3922* lacking a 763-bp internal fragment cloned in pMBL-T; Ap^R	This study
pCSMI29	SacI fragment from pCSMI28 cloned into SacI-digested pCSRO; Sm^RSp^R Suc^S	This study
pCSMI55	*all3922* cloned in pSPARK; Ap^R	This study
pCSMI56	EcoRI fragment from pCSMI55 cloned into EcoRI-digested pCSAM200; Sm^RSp^R	This study
pCSMI57	597-bp fragment from the 3′ part of *all3922* cloned in pMBL-T; Ap^R	This study
pCSMI60	NotI/NheI fragment from pCSMI57 cloned into NotI/NheI-digested pCSAL34; Ap^R	This study
pCSMI61	KpnI fragment from pCSMI61 cloned into KpnI-digested pCSV3; Sm^RSp^R	This study

Abbreviations: ^{R} denotes resistance to the indicated antibiotic: Ap, ampicillin; Cm, chloramphenicol; Nm, neomycin; Sm, streptomycin; and Sp, spectinomycin. Suc^S, sensitivity to 5% sucrose.

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9. Ionescu D, Voss B, Oren A, Hess WR, Muro-Pastor AM (2010) Heterocyst-specific transcription of NsiR1, a non-coding RNA encoded in a tandem array of direct repeats in cyanobacteria. *J Mol Biol* 398(2):177–188.

Table S3. Oligonucleotide primers used in this work

Primer name	Sequence (5′ to 3′)
all3922-1	GAGCTCGCGCAAACTTGA
all3922-2	CCAGGCGCGCACTGCTTC
all3922-3	GTGCGCGCCTGGGGTAAAACT
all3922-4	GAGCTCGCACTAAAACAGGTTCT
all3922-5	AGTCAGGTCATGCCTTCCATC
all3922-6	CTCAAAGCCTTTGCCACCTG
all3922-7	TTTGAATTCAGCAGACGGTAACAG
all3922-8	TCCTTAGTAATTGCCCGGACTC
all3922-11	GCTGAATTAGCCAGGGAAATG
all3922-13	ATTGCTAGC*ACCTCCACCGCC*ACTGATACTACCCAC
pRL500-1	ATAGGCGTATCACGAGGC
SacB-1	CTTGAGGTACAGCGAAGTG
SacB-2	TCTGCAAAAGGCCTGGAGG
pRL277-1	TCAAGGATCTGGATTTCGAT
Universal	GTAAAACGACGGCCAGT
M13REV	CAGGAAACAGCTATGAC

Introduced restriction enzyme cutting sites are underlined. The nucleotide sequence that, inverted, encodes the four-Gly linker is in italics.

Annex I:

Cyanophycin, a cellular nitrogen reserve material

Cyanophycin, a Cellular Nitrogen Reserve Material

9

Antonia Herrero and Mireia Burnat

Abstract

Cyanophycin is a biopolymer that serves as a nitrogen cellular reserve and occurs in most, albeit not all cyanobacteria. In this chapter, available information on the enzymes of cyanophycin metabolism and on the expression of the corresponding genes is reviewed. Regulation of cyanophycin production in response to the C to N balance of the cells and, in the case of heterocyst-forming cyanobacteria, the cellular specificity of cyanophycin metabolism are also addressed. Genes encoding cyanophycin metabolism proteins have also been found in bacteria other than cyanobacteria, and the polymer has been considered of potential biotechnological use. Some studies aiming at the production of cyanophycin in different bacteria and eukaryotes for its large-scale accumulation are also summarized.

Introduction

Cyanophycin is thought to represent a dynamic reservoir of nitrogen that is accumulated in the cells in the form of membraneless cytoplasmic granules (Fig. 9.1). As an example, the unicellular cyanobacterium *Cyanothece* sp. ATCC 51142, which temporarily separates photosynthetic O_2 evolution and N_2 fixation along daily variations, accumulates cyanophycin in the dark periods when it fixes N_2 and degrades cyanophycin in the light periods, when photosynthesis is performed (Sherman *et al.*, 1998). Daily dynamics of cyanophycin accumulation coincident with the periods of N_2 fixation has also been reported for the filamentous cyanobacterium *Trichodesmium* spp., a globally relevant marine diazotroph (Finzi-Hart *et al.*, 2009). Cyanophycin accumulates conspicuously under unbalanced growth conditions, e.g. in the stationary growth phase, under unbalanced growth including high light or CO_2 supply, or under stress conditions (phosphate or sulfur starvation) that do not involve nitrogen (N) starvation (Allen, 1984; Simon, 1987). Cyanophycin is also synthesized under conditions of N sufficiency during transitions between different N

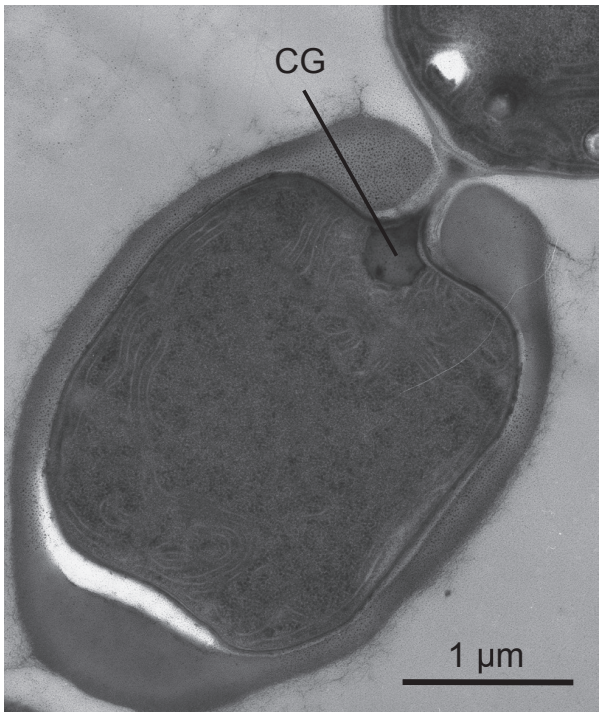

Figure 9.1 Transmission electron micrograph of a heterocyst of *Anabaena* sp. PCC 7120 showing a polar cyanophycin granule (CG). Sample prepared by high-pressure cryo-fixation as described by Merino-Puerto *et al.* (2011). Micrograph courtesy of Victoria Merino-Puerto, Heinz Schwarz and Iris Maldener.

supplies (Mackerras *et al.*, 1990) and, as shown by the use of $^{15}NO_3^-$ or $^{15}NH_4^+$ as a N source, it can be synthesized from external N sources as well as from N made available by degradation of cellular proteins (Allen *et al.*, 2005). Both the rate and the extent of nitrogen incorporation into cyanophycin depend on the source of N (ammonium or nitrate) available and whether the cells had been starved for N (Kolodny *et al.*, 2006).

In the filamentous heterocyst-forming cyanobacteria, during diazotrophic growth conditions, cyanophycin accumulates conspicuously at the heterocyst poles besides in vegetative cells (Lang *et al.*, 1972; Sherman *et al.*, 2000). In this case, how the N contained in the heterocyst granules is mobilized, and the mechanism of a putative transfer of this N to the neighbouring vegetative cells represent issues of long-standing interest in the biology of these organisms.

Although formerly thought to be a cyanobacterial characteristic, with the massive arrival of bacterial genomic sequences, genes encoding cyanophycin metabolism enzymes have been detected to occur in a wide range of prokaryotic organisms with different metabolic options including phototrophy, anaerobic and aerobic respiration, fermentation and chemolitoautotrophy (Krehenbrink *et al.*, 2002).

The cyanophycin granule polypeptide

The cyanophycin granule is composed of a multi-L-arginyl-L-aspartate (arginine residues linked to a polyaspartate backbone) polypeptide (Simon and Weathers, 1976). In this polymer, the amino acids aspartate and arginine are present in an almost 1:1 ratio because nearly all the aspartate residues in the backbone are linked to arginine residues. Rather than having a uniform molecular mass, the polymer is in the range between 25 and 100 kDa (Simon, 1971, 1976). The unitary multi-Arg-Asp chains that constitute cyanophycin are water insoluble and spontaneously associate into granules that include a substantial fraction of β-pleated sheet structure (Simon *et al.*, 1980).

Although in most cases cyanophycin is composed exclusively of Asp and Arg, some other constituent amino acids have been reported occasionally. Thus, besides Arg and Asp, Lys has been found, in place of Arg, in cyanophycin isolated from *E. coli* expressing cyanophycin synthetase (see below) from *Synechocystis* sp. PCC 6803 or from *Anabaena variabilis* ATCC 29413 (Ziegler *et al.*, 1998; Berg *et al.*, 2000). Additionally, in an *in vitro* reaction catalysed by *Synechocystis* cyanophycin synthetase, Lys, citrulline and ornithine have been shown to be incorporated into cyanophycin-like polymers, although at lower rates than Arg (see Berg *et al.*, 2000). Regarding glutamic acid, it has been described to occur *in vivo*, in place of Arg, in *Synechocystis* sp. PCC 6308 incubated under N-limiting conditions (Merritt *et al.*, 1994). Glu has also been found in cyanophycin synthesized *in vitro* with cell extracts from this cyanobacterium (Aboulmagd *et al.*, 2000).

Enzymes and genetics of cyanophycin metabolism

Cyanophycin synthetase

Cyanophycin is non-ribosomically synthesized by the action of cyanophycin synthetase [multi-L-arginyl-poly(L-aspartic acid) synthetase], which catalyses the elongation of a cyanophycin primer with L-Asp and, afterwards, the addition of L-Arg involving the conversion of ATP to ADP (Simon, 1976) (1 mol of ATP per mol of amino acid incorporated [Aboulmagd *et al.*, 2001]). Thus, cyanophycin synthetase catalyses the formation of two types of amide bonds: α-peptide bonds between the aspartyl residues that form the backbone of the polymer, and isopeptide bonds between the β-carboxyl group of aspartyl residues in the backbone and the α-amino group of arginine in the branches (Fig. 9.2). The enzyme was first purified from *Anabaena variabilis* (Ziegler *et al.*, 1998), and it is a homodimer of the 96 kDa product of the *cphA* gene that was first identified in the genomic sequence of *Synechocystis* sp. PCC 6803 (Ziegler *et al.*, 1998). Interestingly, cyanophycin synthetase shows similarity to peptide synthetases involved in the biosynthesis of murein. Indeed, the N- and C-terminal parts of cyanophycin synthetase show similarity to different superfamilies of ATP-dependent ligases. This observation led to the prediction of two ATP-binding and catalytic

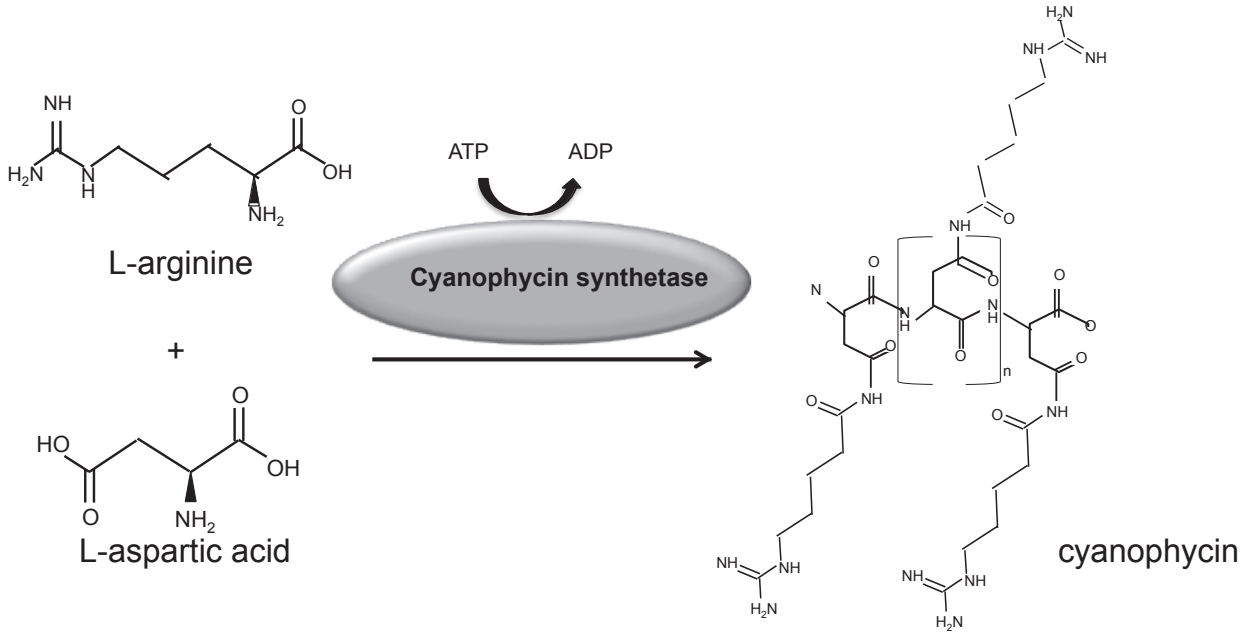

Figure 9.2 Synthesis of cyanophycin. A single enzyme, cyanophycin synthetase, is involved in cyanophycin synthesis. The enzyme requires L-arginine, L-aspartic acid, ATP, magnesium ions and, *in vitro*, a cyanophycin primer (not depicted). Cyanophycin synthetase alternately adds aspartate to the _-carboxylate and arginine to the β-carboxylate group of the terminal aspartate of the growing chain.

sites in cyanophycin synthetase, each involved in the addition of one of the amino acid substrates during the elongation reaction, bearing the C-terminal part the active site for the incorporation of aspartate and the N-terminal part the one for arginine incorporation (Berg *et al.*, 2000).

Extracts of *E. coli* expressing the *cphA* gene from *Synechocystis* show cyanophycin synthetase activity. Moreover, these cells synthesize a cyanophycin-like material, indicating that CphA catalyses *de novo* cyanophycin synthesis, in contrast to the *in vitro* activity that requires a cyanophycin primer. Interestingly, the enzyme from *Thermosynechococcus elongatus* strain BP-1 has been reported to catalyse primer-independent synthesis of cyanophycin (Arai and Kino, 2008). *In vitro*, the elongation reaction takes place at the C-terminal part of the cyanophycin primer, and the incorporation of amino acids to the cyanophycin polymer occurs step-by-step and not in the form of Asp-Arg dipeptides (Berg *et al.*, 2000). Finally, the fact that Glu can be incorporated into cyanophycin *in vivo* in *Synechocystis* sp. PCC 6308, but not *in vitro* with purified cyanophycin synthetase from this strain has led to suggest that the *in vivo* Glu incorporation could respond to an as yet unknown enzyme component (Aboulmagd *et al.*, 2001).

Cyanophycinase

Cyanophycin degradation is catalysed by a specific hydrolytic enzyme, cyanophycinase, the 29.4 kDa product of the *cphB* gene, which was first identified in the genome of *Synechocystis* sp. PCC 6803 as a gene directing the expression in *E. coli* of a product catalysing as a C-terminal exopeptidase the hydrolysis of cyanophycin to a peptide consisting of β-Asp-Arg (Richter *et al.*, 1999) (Fig. 9.3). The crystal structure of cyanophycinase from *Synechocystis* has been solved at 1.5 Å resolution, showing that the enzyme is a dimer and confirming that it is a serine-type protease. The enzyme would recognize the dipeptide up from the cleavable bond in the polypeptide chain, and details of the sterics of substrate recognition are available (Law *et al.*, 2009). CphB shows similarity, at both the protein sequence and structural levels, to an aspartyl dipeptidase from *E. coli* and the PepE dipeptidase from *Salmonella typhimurium* (Richter *et al.*, 1999; Law *et al.*, 2009). Based on sequence comparisons between the *Synechocystis* CphB and *Salmonella* PepE, residues Ser^{132},

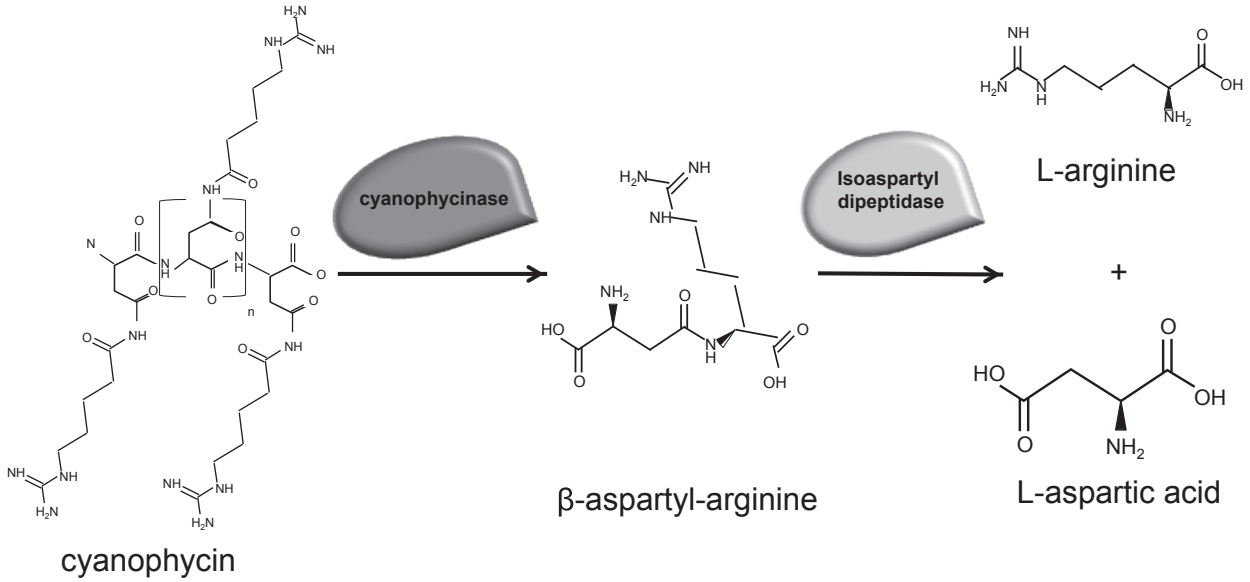

Figure 9.3 Degradation of cyanophycin. Two enzymes are involved in cyanophycin degradation: cyanophycinase, an exopeptidase that hydrolyses cyanophycin releasing β-aspartyl-arginine peptides, and an isoaspartyl dipeptidase that hydrolyses the peptide bond of β-aspartyl-arginine.

Glu[150] and His[174] (numeration of *Synechocystis*) have been proposed to constitute the catalytic triad of ChpB (Richter *et al.*, 1999).

cphA and chpB gene expression

In *Synechocystis* sp. PCC 6803, it has been suggested that the *cphA* (cyanophycin synthetase) and *cphB* (cyanophycinase) genes are expressed independently (Richter *et al.*, 1999). In *Anabaena* sp. PCC 7120, two gene clusters containing *cphB* and *cphA* genes have been identified (Picossi *et al.*, 2004) (Fig. 9.4). The *cph1* cluster is expressed at higher levels and constitutes an operon, although *cphA1* could additionally be expressed from an independent promoter. In the *cph2* cluster, the *cphB2* and *cphA2* genes are divergently oriented and thus expressed in separate transcripts. Expression of the *cph2* cluster is detected at levels considerably lower than the *cph1* cluster and, indeed, a mutational analysis indicated that the latter cluster contributes more than the former to cyanophycin accumulation in the *Anabaena* filaments (Picossi *et al.*, 2004).

Aspartyl-arginine dipeptidase

The β-L-aspartyl-L-arginine (β-Asp-Arg) dipeptide resulting from cleavage of cyanophycin by cyanophycinase is further hydrolysed to its amino

Figure 9.4 Clusters of cyanophycin metabolism related genes in *Anabaena* sp. PCC 7120. Cyanophycin synthetase (*cphA*), cyanophycinase (*cphB*) and the isoaspartyl dipeptidase (*all3922*) genes are highlighted in black.

acid constituents, Asp and Arg, by isoaspartyl dipeptidase (Fig. 9.3). β-Asp-Arg hydrolysing activity was found in extracts of *Synechocystis* sp. PCC 6803, which was attributed to the product of gene *sll0422* annotated as 'plant-type asparaginase' (Richter *et al.*, 1999). Confirming this identification, isoaspartyl-dipeptidase activity was shown in the asparaginases from *Synechocystis* sp. PCC 6803 (Sll0422) and *Anabaena* sp. PCC 7120 (All3922) expressed in *E. coli*, as well as in plant-type asparaginases in general (Hejazi *et al.*, 2002). Besides

deamidating asparagine, the enzymes hydrolyse a range of isoaspartyl dipeptides, including those that could result from proteolytic degradation of modified proteins containing isoaspartyl residues and, with high catalytic efficiency, also β-Asp-Arg from the degradation of cyanophycin.

The mature isoaspartyl dipeptidases of *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120 have molecular weights of around 70 kDa and consist of dimers of two subunits (α-subunit derived from the N-terminal part of the precursor; β-subunit derived from the C-terminal part) that arise from autoproteolytic cleavage at the Gly–Thr bond (G^{179}-T^{180}, numeration of the *Anabaena* polypeptide) within the conserved sequence GT(I/V)G of a single primary gene product (Hehazi *et al.*, 2002). Recently, a mutant of *Anabaena* devoid of All3922 has been generated, and this mutant accumulates the β-Asp-Arg peptide confirming the involvement of the enzyme in cyanophycin metabolism (M. Burnat *et al.*, unpublished).

The complete sequence of the genomes of about 150 cyanobacteria is currently available (Shih *et al.*, 2013). Genes encoding the three cyanophycin metabolism enzymes, cyanophycin synthetase, cyanophycinase and isoaspartyl dipeptidase, are widely distributed among cyanobacteria. Although these genes are not present in every cyanobacterium, they are found in phylogenetically very distant strains, from morphologically complex cyanobacteria belonging to *Rivulariaceae* to the phylogenetically deep-branching cyanobacterium *Gloeobacter violaceaus* (see http://genome.microbedb.jp/cyanobase/;http://img.jgi.doe.gov/cgi-bin/w/main.cgi).

C-to-N balance regulation

As mentioned above, cyanophycin accumulation has been shown in various cyanobacteria, both unicellular non-nitrogen fixing and filamentous or unicellular N_2 fixers, to respond to the N regime. In both *Synechocystis* sp. PCC 6803 (Maheswaran *et al.*, 2006) and *Anabaena* sp. PCC 7120 (Paz-Yepes *et al.*, 2009), it has been shown that the C-to-N sensory regulator P_{II} (the *glnB* gene product) influences cyanophycin accumulation. In *Synechocystis*, cyanophycin is absent in a P_{II}-null mutant, and in cells grown under nitrate-limiting conditions cyanophycin rapidly accumulates upon the supply of ammonium in the wild-type but not in the P_{II} mutant. *N*-acetyl-L-glutamate kinase, which catalyses the committed step of the cyclic pathway for arginine biosynthesis, is a direct target of P_{II}. This enzyme is strongly activated by complex formation with the unphosphorylated form of P_{II}, which indicates a low C–to-N ratio (Heinrich *et al.*, 2004). Thus, P_{II} affects cyanophycin accumulation through an effect on arginine synthesis under N sufficiency (Maheswaran *et al.*, 2006). Under diazotrophic conditions, a P_{II} mutant (Paz-Yepes *et al.*, 2009) or a *prpS* (encoding a putative P_{II} phosphatase) mutant (Laurent *et al.*, 2004) of *Anabaena* does not exhibit the cyanophycin polar granules characteristic of the wild-type heterocysts. However, whether in these mutants, which are impaired in diazotrophic growth, the lack of heterocyst cyanophycin responds to an effect of the mutation on arginine accumulation, on the impaired nitrogenase activity, or on other uncharacterized effects remains to be studied.

Cell specificity of cyanophycin metabolism in heterocyst-forming cyanobacteria

In both *Anabaena cylindrica* and *Anabaena* sp. PCC 7120 incubated under diazotrophic conditions, higher activities of cyanophycin synthetase and cyanophycinase have been found in cell extracts of heterocysts than of vegetative cells (Gupta and Carr, 1981). In strain PCC 7120, the cell specificity of the expression of the gene cluster responsible for the bulk of cyanophycin metabolism, *cph1*, has been studied. Globally, both *cphA1* (cyanophycin synthetase) and *cphB1* (cyanophycinase) appear to be expressed in both vegetative cells and heterocysts under diazotrophic growth conditions (Picossi *et al.*, 2004). The genes are cotranscribed in both cell types, but at increased levels in heterocysts. Additionally, *cphA1* is expressed monocistronically also preferably in the latter cells. At the molecular level, cotranscription takes place from three promoters located upstream of *cphB1* that show different cell specificities: $P_{cphB1-1}$, operating in heterocysts and

dependent on the global transcriptional regulator NtcA; $P_{cphB1-2}$, a low-activity consensus-type promoter with no cell specificity; and $P_{cphB1-3}$, an NtcA-dependent promoter preferentially used in vegetative cells. On the other hand, monocistronic expression of *cphA1* would take place from at least two promoters located in the *cphB1-cphA1* intergenic region: $P_{cphA1-3}$, representing a consensus-type promoter active in vegetative cells; and $P_{cphA1-2}$, directing NtcA-dependent expression in heterocysts. This complex promoter arrangement ensures certain level of expression of *cphB1* and *cphA1* in both cell types, vegetative cells and heterocysts, and also leads to a stronger expression of *cphA1* than of *cphB1* in heterocysts, responding for the conspicuous accumulation of cyanophycin observed in these differentiated cells (Picossi *et al.*, 2004).

With regard to isoaspartyl dipeptidase, in strain PCC 7120 the *all3922* gene product is present in vegetative cells but absent from heterocysts (M. Burnat *et al.*, unpublished). Thus, under diazotrophic conditions, the metabolism of cyanophycin appears to be compartmentalized in different cell types. Whereas synthesis from fixed N and accumulation into granules would take place preferentially in the heterocysts, degradation would take place stepwise in different cells: in heterocysts cyanophycin would be hydrolysed producing dipeptides that would be transferred to the vegetative cells, where they would be further broken down releasing the constituent amino acids to be used in metabolism

In *Anabaena* sp. PCC 7120 a mutational study has shown that strains lacking cyanophycin synthetase (*cphA1* and/or *cphA2*) are little affected in diazotrophic growth (Picossi *et al.*, 2004) (this is also the case for an *A. variabilis* ATCC 29413 cyanophycin synthetase mutant; Ziegler *et al.*, 2001). However, diazotrophic growth is significantly decreased in cyanophycinase (*cphB1* and *cphB1 cphB2*) mutants (Picossi *et al.*, 2004) or in isoaspartyl dipeptidase mutants (M. Burnat *et al.*, unpublished), which show conspicuous cyanophycin granules in both vegetative cells and heterocysts. Thus, although the path of the fixed nitrogen through cyanophycin seems to be dispensable, the capacity of mobilization of the nitrogen stored in this reservoir once it has been synthesized appears to significantly influence N nutrition.

Cyanophycin and cyanophycin-metabolism enzymes in other bacteria

Genes putatively encoding cyanophycin synthetase and cyanophycinase have been identified in the sequenced genomes of quite diverse bacteria. For instance, by 2002 genes homologous to the *Synechocystis* sp. PCC 6803 *cphA* were reported to occur in the genomic sequence of seven bacteria, out of 65 searched for, of the genera *Acinetobacter, Bordetella, Clostridium, Desulfobacterium* and *Nitrosomonas,* with identities in the range of 37–40% at the amino acid level with regard to the *Synechocystis* sequence (Krehenbrink *et al.*, 2002). Moreover, the aerobic bacterium *Acinetobacter* sp. strain DSM 587 accumulates cyanophycin under phosphate-limited conditions, and the *cphA* gene from *Acinetobacter* (Krehenbrink *et al.*, 2002) or from the anaerobe *Desulfitobacterium hafniense* (Ziegler *et al.*, 2002) cloned in *E. coli* leads to expression of cyanophycin synthetase activity and accumulation of cyanophycin-like polymers. Regarding enzymes of cyanophycin degradation, already by 2002 eleven eubacteria were selected as able to use cyanophycin as a carbon source for growth through the use of extracellular cyanophycinases. The purified extracellular enzyme from *Pseudomonas anguilliseptica* strain B1, CphE, exhibits high specificity for cyanophycin and, as its cyanobacterial counterparts, hydrolyses cyanophycin to β-Asp-Arg dipeptides, despite exhibiting only 27–28% identity to intracellular cyanophycinases from cyanobacteria (Obst *et al.*, 2002).

More recently, the presence of cyanophycin-metabolism genes has been searched for in 570 microbial genomes, identifying putative cyanophycin synthetases in 44 prokaryotes, of which 31 also possess cyanophycinase genes. In addition, 24 of the strains encoding cyanophycinases also bear putative isoaspartyl dipeptidases. Based on the organization of these genes in the genome, a classification of ten groups of ChpA and eight groups of ChpB enzymes has been proposed. Thus, the ability to synthesize and degrade cyanophycin

appears to be widely distributed among prokaryotes, although it seems to be a eubacterial trait found neither in Eukarya nor in Archaea (Füser and Steinbüchel, 2007).

Applied aspects

Cyanophycin as well as its derived dipeptides have been considered of potential industrial use. In the chemical industry, cyanophycin has been considered as a potential source of biopolymers, e.g. the polyaspartate polymers that can be generated by dearginylation of cyanophycin represent a biodegradable alternative for polyacrilates, and the cyanophycin constituent amino acids can be used as feedstock for the production of various other chemicals, e.g. nylons. Also, the dipeptides have potential uses in medicine and pharmacy as natural food additives and therapeutic source of amino acids (Mooibroek *et al.*, 2007).

To increase the extent of cyanophycin accumulation and of source biomass production beyond the levels attained with the wild-type strains of cyanobacteria, *E. coli* strains bearing the *cphA* gene from *Synechocystis* sp. PCC 6803 (Frey *et al.*, 2002) or engineered versions of *cphA* from *Nostoc ellipsosporum* (Hai *et al.*, 2006) have been used to produce cyanophycin, which accumulates to up 24% and 34.5% (wt/wt dry matter), respectively, although requiring complex growth media. These systems could yield isolated cyanophycin at the kilogram scale. The industrially relevant host *Saccharomyces cerevisiae* has also been used for expression of the *cphA* gene from *Synechocystis* sp. PCC 6308, although the accumulation of cyanophycin was lower in this system (15.3% wt/wt), even when production of cyanophycin containing amino acids alternative to arginine was improved by the use of *Saccharomyces* strains defective in arginine synthesis (Steinle *et al.*, 2009). Interestingly, in the latter case, in addition to Asp–Arg only and Lys-containing polymers, variants including citrulline or ornithine were obtained. Also the yeast *Pichia pastoris* has been used as a host of mutant derivatives of the *Synechocystis* sp. PCC 6308 *cphA* gene, yielding up to 23.3% (wt/wt) cyanophycin (Steinle *et al.*, 2010). In addition to differences in the chemical composition, the polymer produced in some of these recombinant strains contains soluble and insoluble forms of the polypeptide, which has a smaller, albeit more homogenous, molecular mass (approximately, 25–30 kDa) than the natural one. Finally, procedures have been developed for the large-scale isolation of cyanophycin from biomass (Sallam *et al.*, 2009; Steinle and Steinbüchel, 2010) and for the further production of β-dipeptides by treatment with massively produced extracellular cyanophycinase from *Pseudomonas alcaligenes* (Sallam *et al.*, 2009, 2011).

Production of cyanophycin has also been attempted in plants, in transgenic tobacco and potato, where the cyanophycin synthetase gene from *Thermosynechococcus elongatus* BP-1 was constitutively expressed and a cyanophycin-like polymer was produced up to 1.1% dry weight (Neumann *et al.*, 2005). Improvement of large-scale production and isolation of cyanophycin has been achieved by localizing the production in potato amyloplasts and chloroplasts, which also reduced the negative phenotypic effects of cyanophicin production in the plant (Neubauer *et al.*, 2012). Thus, the industrial exploitation of cyanophycin as a biotechnological resource appears promising.

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Erratum

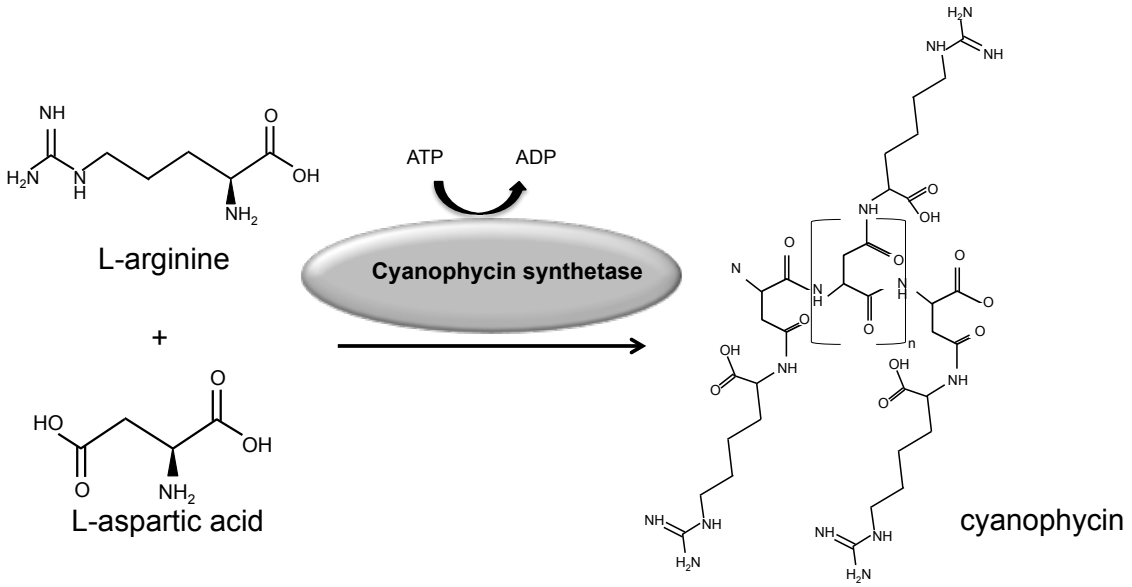

Figure 9.2. Synthesis of cyanophycin. A single enzyme, cyanophycin synthetase, is involved in cyanophycin synthesis. The enzyme requires L-arginine, L-aspartic acid, ATP, magnesium ions and, *in vitro*, a cyanophycin primer (not depicted). Cyanophycin synthetase alternately adds aspartate to the a-carboxylate and arginine to the b-carboxylate group of the terminal aspartate of the growing chain. The cyanophycin molecule has been corrected.

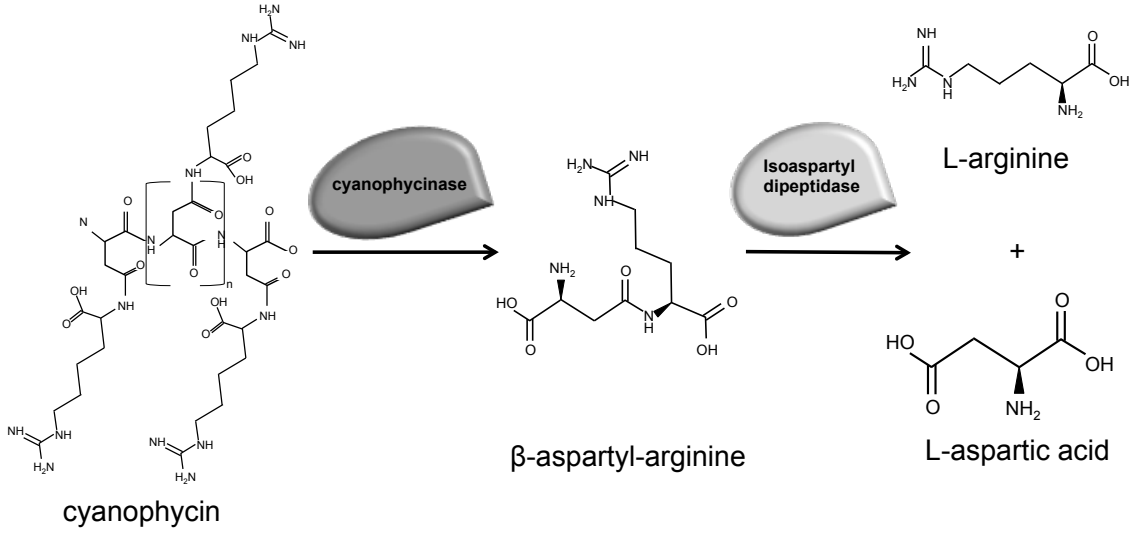

Figure 9.3. Degradation of cyanophycin. Two enzymes are involved in cyanophycin degradation: cyanophycinase, an exopeptidase that hydrolyses cyanophycin releasing b-aspartyl-arginine peptides, and an isoaspartyl dipeptidase that hydrolyses the peptide bond of b-aspartyl-arginine. The cyanophycin molecule has been corrected.

Chapter 3:

Inactivation of agmatinase expressed in vegetative cells alters arginine catabolism and prevents diazotrophic growth in the heterocyst-forming cyanobacterium *Anabaena*

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ORIGINAL RESEARCH

Inactivation of agmatinase expressed in vegetative cells alters arginine catabolism and prevents diazotrophic growth in the heterocyst-forming cyanobacterium *Anabaena*

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Keywords
Agmatinase, *Anabaena*, arginine catabolism, nitrogenase.

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Abstract

Arginine decarboxylase produces agmatine, and arginase and agmatinase are ureohydrolases that catalyze the production of ornithine and putrescine from arginine and agmatine, respectively, releasing urea. In the genome of the filamentous, heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120, ORF *alr2310* putatively encodes an ureohydrolase. Cells of *Anabaena* supplemented with [^{14}C]arginine took up and catabolized this amino acid generating a set of labeled amino acids that included ornithine, proline, and glutamate. In an *alr2310* deletion mutant, an agmatine spot appeared and labeled glutamate increased with respect to the wild type, suggesting that Alr2310 is an agmatinase rather than an arginase. As determined in cell-free extracts, agmatinase activity could be detected in the wild type but not in the mutant. Thus, *alr2310* is the *Anabaena speB* gene encoding agmatinase. The Δ*alr2310* mutant accumulated large amounts of cyanophycin granule polypeptide, lacked nitrogenase activity, and did not grow diazotrophically. Growth tests in solid media showed that agmatine is inhibitory for *Anabaena*, especially under diazotrophic conditions, suggesting that growth of the mutant is inhibited by non-metabolized agmatine. Measurements of incorporation of radioactivity from [^{14}C]leucine into macromolecules showed, however, a limited inhibition of protein synthesis in the Δ*alr2310* mutant. Analysis of an *Anabaena* strain producing an Alr2310-GFP (green fluorescent protein) fusion showed expression in vegetative cells but much less in heterocysts, implying compartmentalization of the arginine decarboxylation pathway in the diazotrophic filaments of this heterocyst-forming cyanobacterium.

Introduction

Cyanobacteria are oxygenic photosynthetic prokaryotes that constitute a phylogenetically coherent group of organisms (Giovannoni et al. 1988). However, they show very diverse morphologies, presenting both unicellular and multicellular forms (Rippka et al. 1979). Cyanobacteria such as those of the genera *Anabaena* and *Nostoc* grow as filaments of cells (trichomes) that, when incubated in the absence of a source of combined nitrogen, present two cell types: vegetative cells that fix CO_2 performing oxygenic photosynthesis and heterocysts that carry out N_2 fixation (Flores and Herrero 2010). Heterocysts differentiate from vegetative cells in a process that involves execution of a specific program of gene expression and intercellular transfer of regulators (Herrero et al. 2013). In the N_2-fixing filament, heterocysts are spaced along the filament and provide the vegetative cells with fixed nitrogen; for this, heterocysts need to receive in turn photosynthate from the vegetative cells (Wolk et al. 1994). An intercellular exchange of glutamine for glutamate (Thomas et al. 1977; Martín-Figueroa et al. 2000) and transfer

of β-aspartyl-arginine (Burnat et al. 2014) can move fixed nitrogen from heterocysts to vegetative cells. Conversely, alanine (Jüttner 1983; Pernil et al. 2010) and, mainly, sucrose (Jüttner 1983; Schilling and Ehrnsperger 1985; Curatti et al. 2002; López-Igual et al. 2010; Vargas et al. 2011) can transfer reduced carbon from vegetative cells to heterocysts.

Heterocysts conspicuously accumulate cyanophycin (multi-L-arginyl-poly [L-aspartic acid]), a nitrogen-rich reserve polymer (Lang et al. 1972; Sherman et al. 2000), which is seen as refractile granules that are located at the heterocyst poles (close to and inside the heterocyst "necks"). Although production of cyanophycin is not required for diazotrophic growth (Ziegler et al. 2001; Picossi et al. 2004), its conspicuous presence in the heterocysts suggests a possible role in diazotrophy, likely as dynamic nitrogen storage (Carr 1983; Haselkorn 2007). Cyanophycin is synthesized by cyanophycin synthetase and degraded by cyanophycinase, which releases β-aspartyl-arginine (Richter et al. 1999). In the diazotrophic filaments, both cyanophycin synthetase and cyanophycinase are present at high levels in the heterocysts (Gupta and Carr 1981a; Picossi et al. 2004). The β-aspartyl-arginine dipeptide is hydrolyzed to aspartate and arginine by an isoaspartyl dipeptidase (Hejazi et al. 2002), which in the model heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120 (hereafter *Anabaena*) has been found to be expressed preferentially in vegetative cells (Burnat et al. 2014). This implies that the dipeptide released by cyanophycinase in the heterocyst is transferred to vegetative cells serving as a nitrogen vehicle in the diazotrophic filament. The isoaspartyl dipeptidase products, arginine and aspartate, must be further catabolized to make their nitrogen atoms available for cellular metabolism.

Arginine serves as a source of nitrogen, carbon, and energy in different bacteria, and five possible catabolic pathways have been detected in cyanobacteria by bioinformatic analysis (Schriek et al. 2007). Of these, three (shown schematically in Fig. 1) have been detected by physiological studies: the arginase pathway, the arginine deiminase pathway, and the arginine decarboxylase pathway (Quintero et al. 2000; Schriek et al. 2007; Weathers et al. 1978). In the arginase pathway, arginine is converted to ornithine releasing urea, and ornithine is further metabolized to glutamate (Cunin et al. 1986). Arginine deiminase produces citrulline and ammonium, with citrulline being further catabolized producing ornithine and carbamoyl phosphate (Cunin et al. 1986). In the arginine decarboxylase pathway, arginine is decarboxylated to agmatine (1-[4-aminobutyl]guanidine), which is then metabolized to putrescine (1,4-diaminobutane) releasing urea and further to 4-aminobutyrate and succinate (Cunin et al. 1986). In *Aphanocapsa* 6308 (currently known as *Synechocystis* sp. strain PCC 6308), it was reported that, besides the arginase pathway, which would provide only nitrogen for the cells, the arginine deiminase (also known as arginine dihydrolase) pathway provides carbon, nitrogen, and energy (Weathers et al. 1978). In *Synechocystis* sp. strain PCC 6803, based on in vivo studies with ^{14}C-labeled substrates and mutational analysis, a model for arginine catabolism was proposed involving an arginase-like pathway combined with a urea cycle that would at the same time degrade aspartate (Quintero et al. 2000). The activity of arginine decarboxylase was also detected,

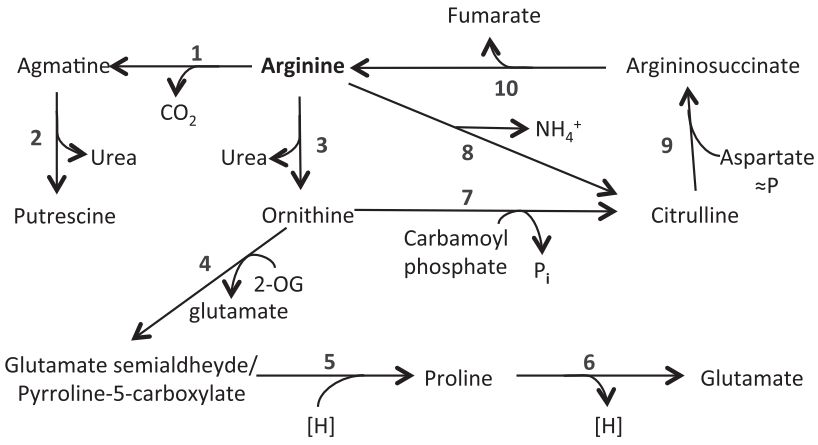

Figure 1. Schematic representation of the arginine decarboxylase, arginase and arginine deiminase pathways. Enzymes and the possible corresponding ORFs in the genome of *Anabaena* sp. strain PCC 7120 (Kaneko et al. 2001) are as follows: 1, arginine decarboxylase (*all3401*); 2, agmatinase (*alr2310*); 3, arginase; 4, ornithine transaminase (*alr1080*); 5, Δ^1pyrroline-5-carboxylate reductase (*alr0488*); 6, proline oxidase (*alr0540*); 7, ornithine carbamoyltransferase (*alr4907*); 8, arginine deiminase; 9, argininosuccinate synthetase (*alr4798*); 10, argininosuccinate lyase (*alr3887*). Note that no gene is annotated as encoding arginase or arginine deiminase. 2-OG, 2-oxoglutarate; [H], reducing power; ≈P, energy-requiring reaction (energy provided by the hydrolysis of ATP).

but it was apparently less important than the arginase-like route under the experimental conditions investigated (Quintero et al. 2000). On the other hand, in a large-scale proteomic study of *Synechocystis* sp. strain PCC 6803, the enzymes of the arginine decarboxylation pathway (arginine decarboxylase and agmatinase) were observed to be up-regulated under certain environmental perturbations (Wegener et al. 2010). The arginine decarboxylase pathway, including conversion of putrescine to succinate, might serve as a source of carbon and nitrogen, as it has been described in *Escherichia coli* and *Pseudomonas* sp. (Kurihara et al. 2005; Chou et al. 2008). However, bioinformatic analysis of the genomes of 24 cyanobacteria (including *Synechocystis* sp. strain PCC 6803) failed to identify genes encoding putrescine oxidase or putrescine transaminase (needed to produce 4-aminobutyraldehyde in the arginine decarboxylase pathway that ends in succinate), suggesting that the arginine decarboxylase pathway in cyanobacteria could be mainly involved in the synthesis of polyamines and the production of ammonium from arginine (Schriek et al. 2007). Additionally, an amino acid oxidase with specificity for basic amino acids (especially arginine) that can release ammonium for growth is present in some cyanobacteria (Flores et al. 1982; Gau et al. 2007). On the other hand, arginine:glycine amidinotransferases involved in secondary metabolism have been described in some cyanobacteria (Muenchhoff et al. 2010; Barón-Sola et al. 2013).

In the *Anabaena* genome, genes encoding arginine decarboxylase and enzymes corresponding to the second and subsequent steps of the arginase pathway can be identified (Kaneko et al. 2001; see Fig. 1). On the other hand, although the presence of an arginase-encoding gene is not evident, open reading frame (ORF) *alr2310* is annotated as "similar to agmatinase" (Kaneko et al. 2001). Arginases and agmatinases belong to the ureohydrolase protein family, which has led to wrong genomic annotations in the predicted functions of some ureohydrolase family proteins from different organisms, as has been the case for *Synechocystis* sp. strain PCC 6803 (Sekowska et al. 2000; see also Quintero et al. 2000). Therefore, it may be asked whether the product of ORF *alr2310* is an agmatinase or an arginase. In this work, we created and characterized *alr2310* mutants of *Anabaena* including deletion and reporter-expressing strains. We found that *alr2310* encodes an agmatinase that is mainly present in vegetative cells and is required for diazotrophic growth.

Materials and Methods

Strains and growth conditions

Anabaena sp. (also known as *Nostoc* sp.) strain PCC 7120 was grown axenically in BG11 medium (containing $NaNO_3$), $BG11_0$ medium (free of combined nitrogen) or $BG11_0$ NH_4^+ medium ($BG11_0$ containing 4 mmol/L NH_4Cl and 8 mmol/L 2-[Tris(hydroxymethyl)-methylamino]-ethanesulfonic acid (TES)-NaOH buffer, pH 7.5). In every case, ferric citrate replaced the ferric ammonium citrate used in the original receipt (Rippka et al. 1979). For plates, medium was solidified with 1% separately autoclaved Difco agar. Cultures were grown at 30°C in the light (25 μE m^{-2} sec^{-1}), with shaking (80–90 rpm) for liquid cultures. Alternatively, cultures (referred to as bubbled cultures) were supplemented with 10 mmol/L of $NaHCO_3$ and bubbled with a mixture of CO_2 and air (1% v/v) in the light (50–75 μE m^{-2} sec^{-1}). For mutants described below, antibiotics were used at the following concentrations: erythromycin (Em), 5 μg mL^{-1} for liquid cultures and 10 μg mL^{-1} for solid media; streptomycin sulfate (Sm) and spectinomycin dihydrochloride pentahydrate (Sp), 5 μg mL^{-1} each for both liquid and solid media; and neomycin sulfate (Nm), 40 μg mL^{-1} for solid media. DNA was isolated from *Anabaena* sp. by the method of Cai and Wolk (1990). *Anabaena* sp. strain FQ163, the *hepP* mutant (López-Igual et al. 2012), was grown in BG11 medium, supplemented when appropriate with antibiotics. All *Anabaena* strains used in this work are listed in Table S1.

Escherichia coli DH5α was used for plasmid constructions. It and strains HB101 and ED8654, used for conjugations with *Anabaena* sp., were grown in Luria–Bertani medium supplemented when appropriate with antibiotics at standard concentrations.

Plasmid construction and genetic procedures

Open reading frame *alr2310* of the *Anabaena* chromosome (Kaneko et al. 2001) was inactivated by deleting an internal fragment of 927 bp. DNA fragments upstream (667 bp) and downstream (501 bp) from the central region of the gene were amplified by standard PCR using as template DNA from *Anabaena* and primers alr2310-3/alr2310-6 and alr2310-4/alr2310-5 (all oligodeoxynucleotide primers are listed in Table S2). The external primers alr2310-3 and alr2310-4 included SacI-sites in their 5′ ends and primers alr2310-5 and alr2310-6 included EcoRV-sites in their 5′ ends. The upstream and downstream DNA fragments from this gene were cloned in pMBL-T (Dominion MBL, Córdoba, Spain), sequenced, and transferred as a SacI-ended fragment to SacI-digested pCSBN1 (which is a plasmid derived from pCSV3 and pRL278 containing a Nm^R gene cassette and the *sacB* gene for positive selection), producing pCSBN5 (all plasmids are listed in Table S1).

Conjugation of *Anabaena* with *E. coli* HB101 carrying the cargo plasmid (pCSBN5) with helper and methylation plasmid pRL623 was effected by the conjugative plasmid

pRL443, carried in *E. coli* ED8654, and performed as described (Elhai et al. 1997) with selection for resistance to Nm. Filaments of eight Nm^R clones were spread on $BG11_0$ NH_4^+ medium supplemented with 5% sucrose (Cai and Wolk 1990), and individual Suc^R colonies were checked by PCR looking for clones that had substituted the wild-type locus by a deleted locus. The genetic structure of selected clones was studied by PCR with DNA from those clones and primers alr2310-3/alr2310-7 and alr2310-3/alr2310-8. A clone homozygous for the mutant chromosomes was named strain CSMI11 (Δ*alr2310*).

The plasmid carrying fusion gene *alr2310-gfp* was prepared as follows. A 681-bp fragment from the 3′-terminal part of *alr2310* was amplified by PCR using primers alr2310-11 and alr2310-13 (which lacks the stop codon of the gene and contains a sequence encoding a 4-Gly linker and a NheI site in its 5′ end) and *Anabaena* DNA as template, and the resulting fragment was cloned in vector pMBL-T producing plasmid pCSMI42. This fragment was corroborated by sequencing and transferred as a SacI/NheI-ended fragment to SacI/NheI-digested pCSAL33 (which contains, cloned in vector pMBL-T, the *gfp-mut2* gene with an Ala-encoding codon instead of the Met start codon) producing plasmid pCSMI44 that carries the fusion of the *gfp* gene to the 3′ end of *alr2310*. The resulting fusion was finally transferred as a KpnI-ended fragment to KpnI-digested pCSV3, which provides resistance to Sm and Sp (Valladares et al. 2011), producing pCSMI46. This plasmid, which bears the *alr2310-gfp* fusion gene, was transferred to *Anabaena* by triparental mating as described above, with selection for Sm^R/Sp^R. Insertion into *alr2310* and segregation of chromosomes carrying the fusion was confirmed by PCR analysis using template DNA from exconjugant clones and primers alr2310-13 and pRL500-1 for testing insertion of *gfp-mut2* and alr2310-13 and alr2310-16 for testing segregation of the mutated chromosomes. A homozygous clone bearing the *alr2310-gfp* construct was named strain CSMI21.

Complementation of the *alr2310* deletion mutant was performed with a replicating plasmid. Using DNA from *Anabaena* as template and primers alr2310-15/alr2310-16, which include SmaI sites close to their 5′ ends, ORF *alr2310* was amplified by PCR and ligated into pSpark (Canvax Biotech S.L., Córdoba, Spain) producing plasmid pCSMI53, whose insert was corroborated by sequencing. This insert was excised from pCSMI53 by digestion with SmaI and transferred to SmaI-digested pRL3845, replacing *all1711* by *alr2310*, producing pCSMI54. (pRL3845 is a Cm^R Em^R-plasmid that contains a P_{glnA}-*all1711* construct and can replicate in *Anabaena*; López-Igual et al. 2012.) As is the case for *all1711* in pRL3845, *alr2310* in pCSMI54 is expressed from the *Anabaena glnA* promoter. This plasmid was conjugated into strain CSMI11 (Δ*alr2310*) as described above, with selection for Em^R, and tested for complementation of the mutant phenotype.

Agmatinase assay in cell-free extracts

Filaments from 800 mL of cultures of *Anabaena* wild type and strain CSMI11 grown for 5 days in bubbled BG11 medium were harvested and washed with 10 mmol/L N-tris(hydroxymethyl)-methylglycine (Tricine)-NaOH buffer (pH 8.5) and resuspended with 100 mmol/L Tricine-NaOH buffer (pH 8.5) supplemented with 1 mmol/L dithiothreitol, 1 mmol/L $MnCl_2$ and a protease inhibitor mixture tablet (cOmplete Tablets, Mini Ethylenediaminetetraacetic acid (EDTA)-free, Roche, Basel, Switzerland). Filament suspensions were passed twice through a French pressure cell at 20,000 psi. Cell debris was removed by centrifugation at 27,216*g*, 10 min at 4°C. The resulting supernatant constituted the cell-free extract from vegetative cells.

For determining agmatinase activity, cell-free extracts were supplemented with 5 mmol/L acetohydroxamic acid (a urease inhibitor) and incubated for 30 min at 30°C before adding 1 mmol/L agmatine sulfate (final concentration). The reaction was carried out at 30°C for 180 min. Reactions run without added agmatine were used as controls. Samples of 0.1 mL of the reaction mixture were taken at different times, supplemented with perchloric acid (final concentration, 5%), incubated at 0°C for 10 min and centrifuged at 16,100*g*, 5 min, at 4°C. The urea produced in the reaction was determined colorimetrically in 0.1 mL of the resulting supernatant by the method of Boyde and Rahmatullah (1980).

Growth tests and nitrogenase activity

Protein concentration and chlorophyll *a* (Chl) content of the cultures or cell-free extracts were determined by a modified Lowry procedure (Markwell et al. 1978) and by the method of Mackinney (1941), respectively. The growth rate constant ($\mu = \ln 2/t_d$, where t_d is the doubling time) was calculated from the increase of protein content, determined in 0.2 mL samples, of shaken liquid cultures (Montesinos et al. 1995). Cultures were inoculated with an amount of cells containing about 5 μg of protein mL^{-1} and grew logarithmically until reaching about 40 μg of protein mL^{-1}.

For growth tests in solid media, cultures grown in $BG11_0$ NH_4^+ medium (supplemented with antibiotics when appropriate) were harvested and washed three times with 50 mL of $BG11_0$ medium, and dilutions were prepared in $BG11_0$ medium. Ten microliter samples of the resulting suspensions were spotted on agar plates with

different nitrogen sources and incubated at 30°C in the light (25 μE m^{-2} sec^{-1}). When indicated, solid media were supplemented with filter-sterilized L-putrescine, agmatine sulfate or L-arginine purchased from Sigma-Aldrich (St. Louis, MO, USA).

Nitrogenase activity was determined by the acetylene reduction assay as described previously (Montesinos et al. 1995). Cells grown in 50 mL of $BG11_0$ NH_4^+ medium were incubated 24 h without combined nitrogen ($BG11_0$ medium) under growth conditions and used in the acetylene reduction assays performed under oxic or anoxic conditions. For the latter, the cell suspensions were placed in sealed flasks and supplemented with 10 μmol/L 3-(3,4-dichlorophenyl)-1,1-imethylurea (DCMU), bubbled with argon for 3 min, and incubated for 90 min under assay conditions before starting the reaction by addition of acetylene.

Cyanophycin measurements

To determine cyanophycin, filaments grown in $BG11_0$ NH_4^+ medium were washed with $BG11_0$ medium, inoculated at 0.5 μg Chl mL^{-1} in 50-mL cultures with $BG11_0$ NH_4^+ or BG11 media, and incubated for 8 days under growth conditions. Cyanophycin granule polypeptide (CGP) was isolated from filaments collected from these cultures. The filaments were harvested by centrifugation at 1,935*g* at room temperature, washed twice with, and resuspended in, sterile double destilled-purified H_2O, and disrupted with a French pressure cell (two passages at 20,000 psi). After measuring the obtained volume of cell extract, Chl was determined in a 100-μL sample. The remnant of each extract was centrifuged for 15 min, at 4°C and 27,216*g* and the pellets were washed twice with 11 mL of sterile milliQ-purified H_2O and resuspended in 1 mL of 0.1 mol/L HCl for solubilization of CGP. After 2–4 h of incubation at room temperature and centrifugation under the same conditions, the resulting supernatants were removed and stored at 4°C. The pellets were resuspended in 1 mL of 0.1 mol/L HCl and incubated overnight at room temperature to complete solubilization of CGP, and the solution was subjected to centrifugation as above. The supernatants were combined with those obtained after the first centrifugation and stored at 4°C. These preparations were used for arginine guanidine group determination carried out by the Sakaguchi reaction as modified by Messineo (1966).

Arginine catabolism

Cells grown in $BG11_0$ NH_4^+ medium were harvested by centrifugation at 1,935*g* at room temperature, washed twice with 25 mmol/L Tricine–NaOH buffer (pH 8.1), and resuspended in the same buffer. The uptake assays were carried out at 30°C in the light (white light from fluorescent lamps, about 175 μE m^{-2} sec^{-1}) and were started by mixing a suspension of cells (2.1 mL) containing 5–10 μg of Chl mL^{-1} with a solution (0.1 mL) of L-[U-^{14}C]arginine (274 mCi $mmol^{-1}$, from PerkinElmer, Waltham, MA, USA). The final concentration of arginine in the experiment was 1 μmol/L. The rate of arginine uptake in the 10- and 30-min assays was estimated by taking a 0.5-mL sample of the cell suspension. The sample was filtered (0.45 μm-pore-size Millipore HA filters were used) and the cells on the filters were washed with 5–10 mL of 5 mmol/L Tricine–NaOH buffer (pH 8.1). The filters carrying the cells were then immersed in 5 mL of scintillation cocktail, and their radioactivity was measured. Retention of radioactivity by boiled cells was used as a blank.

To determine metabolites produced from the labeled arginine at the end of the 10- and 30-min incubations, samples of 0.5 mL of the cell suspension were immediately (<15 sec) mixed, without filtering the cells, with 1.5 mL of water at 100°C and further incubated for 5 min in a bath of boiling water. The resulting suspensions were centrifuged, and samples (1–1.5 mL) from the supernatants were lyophilized and dissolved in 50 μL of water. Samples of the resulting solutions were applied to 0.1-mm-thick cellulose thin-layer chromatography (TLC) plates (20 × 20 cm; Merck, Darmstadt, Germany). Two-dimensional separation of amino acids was effected by using the following solvents: the first dimension solvent consisted of *n*-butanol–acetone–ammonium hydroxide–water (20:20:10:4, vol/vol/vol/vol), and the second dimension solvent consisted of isopropanol-formic acid-water (20:1:5, vol/vol/vol). The TLC plates were analyzed by electronic autoradiography using a two-dimensional scanner for β particles (Cyclone Plus Phosphor Imager, PerkinElmer, Waltham, MA, USA), which allows a quantitative analysis of the radioactive spots. Identification of the metabolite originating a radioactive spot was made by co-chromatography by supplementing the samples with stable amino acids as markers and visualizing the amino acids after chromatography with a solution of ninhydrin in acetone in the presence of cadmium acetate (Atfield and Morris 1961).

Determination of in vivo protein synthesis

To determine the radioactivity incorporated into macromolecules, a sample of the cell suspension in growth medium was incubated with 10 μmol/L L-leucine supplemented with L-[U-^{14}C]leucine (316 mCi $mmol^{-1}$, from American Radiolabeled Chemicals, Inc., St. Louis, MO, USA). Samples of 0.5 mL were collected at different times

and filtered to determine uptake into whole cells as above. Additionally, samples of 0.5 mL were added to ice-cold trichloroacetic acid (TCA; final concentration, 10%), incubated in ice-water for 60 min, and filtered as above. The filters were washed with 5 mL of ice-cold 10% TCA and immersed in a scintillation cocktail, and their radioactivity was measured.

Western blot analysis

Filaments from 100 mL of cultures of *Anabaena* sp. strains PCC 7120 and CSMI21 grown in bubbled BG11 and $BG11_0$ media were harvested, washed with buffer 1 (50 mmol/L Tricine-HCl buffer, pH 7.5, 10% glycerine, 100 mmol/L NaCl), and resuspended in 4 mL of the same buffer. To isolate heterocysts, 800 mL of cultures grown for 48 h in bubbled $BG11_0$ medium were harvested and washed with buffer 2 (50 mmol/L imidazol and 0.5 mmol/L EDTA, pH 8.0), resuspended in 10 mL of the same buffer, incubated with 1 mg mL^{-1} of lysozyme for 15 min at room temperature and centrifuged at 1935*g* (4°C, 10 min). The pellets were resuspended in 5 mL of buffer 2 and passed three times through a French pressure cell at 3,000 psi. Samples were enriched in heterocysts after successive steps of low-speed centrifugation (200*g*, 10 min, 4°C) and washing with buffer 1, and the isolated heterocysts were resuspended in 4 mL of buffer 1. The filament and heterocyst suspensions were both supplemented with a protease inhibitor cocktail tablet (cOmplete Tablets, Mini EDTA-free, Roche) and passed two times through a French pressure cell at 20,000 psi. Cell debris was removed by centrifugation at 16,100*g* (4°C, 10 min). Proteins in the supernatants were resolved by SDS-PAGE and transferred to Hybond-P PVDF membranes (GE Healthcare, Buckinghamshire, England). Western analysis was performed incubating with an anti-GFP antibody (A6455 from Invitrogen, Carlsbad, CA, USA) diluted 1:2000 in Tween 20, Tris-buffered saline (TTBS) with 5% non-fat milk powder. Antigen–antibody complexes were visualized with a peroxidase-conjugated anti-rabbit-IgG antiserum (Sigma-Aldrich, St. Louis, MO, USA) and developed with the WesternBright™ Western blotting detection kit (Advansta, Menlo Park, CA, USA). A cell-free extract from *Anabaena* carrying pAM1954 (Yoon and Golden 1998) was used as a free GFP-containing control.

Microscopy

Cells grown during 5–7 days in shaken liquid $BG11_0$ NH_4^+ medium or heterocyst preparations were observed and photographed with a Zeiss (Oberkochen, Germany) Axioscop microscope equipped with a Zeiss ICc1 digital camera. GFP fluorescence was analyzed by confocal microscopy. Samples from cultures of *Anabaena* sp. strain CSMI21 or the wild-type PCC 7120 as a control grown in bubbled cultures with BG11 or $BG11_0$ medium were visualized using a Leica HCX PLAN-APO 63X 1.4 NA oil immersion objective attached to a Leica TCS SP2 confocal laser-scanning microscope. GFP was excited using 488-nm irradiation from an argon ion laser. Fluorescence emission was monitored by collection across windows of 500–520 nm (GFP imaging) and 630–700 nm (cyanobacterial autofluorescence). Under the conditions used, optical section thickness was about 0.4 μm. GFP fluorescence intensity was analyzed using ImageJ 1.43 m software (http://imagej.nih.gov/ij/).

Results

Construction and phenotype of an *alr2310* mutant

The *Anabaena* ORF *alr2310* is located between *alr2309* encoding a single-stranded nucleic acid binding protein and *alr2311* encoding an RNA-binding protein (Fig. 2). Therefore, there is no obvious functional relation between the product of *alr2310* and those of its surrounding genes, and there is no evidence for co-transcription of these genes (Flaherty et al. 2011). Nonetheless, to investigate the role of the *alr2310* avoiding any possible polar effects of the mutation, a 927-bp in-frame internal fragment of the gene was removed without leaving any genetic marker in it (Fig. 2A; see Fig. S1 and Materials and Methods for details). The Δ*alr2310* mutant, strain CSMI11, was homozygous for mutant chromosomes (Fig. S1). Tests in solid media showed that strain CSMI11 is impaired in growth in the presence of combined nitrogen (ammonium or nitrate) and is unable to grow under diazotrophic conditions (Fig. 2B). In shaken liquid cultures, the growth rate constant of strain CSMI11 in ammonium- or nitrate-containing medium was, respectively, similar to or somewhat lower than that of the wild type, whereas the growth rate constant in combined *N*-free media was about 9% that of the wild type (Table 1), confirming that strain CSMI11 is hampered in diazotrophic growth. Complementation of CSMI11 with a plasmid bearing wild-type *alr2310* allowed diazotrophic growth, corroborating that growth impairment resulted from the *alr2310* mutation (see strain CSMI11-C in Fig. 2B). After incubation without a source of combined nitrogen, strain CSMI11 produced heterocysts that appeared immature (Fig. S2) and showed no nitrogenase activity, measured under both oxic and anoxic conditions (Table 1). The production of aberrant heterocysts that lack nitrogenase activity can explain the impairment of strain CSMI11 in diazotrophic growth.

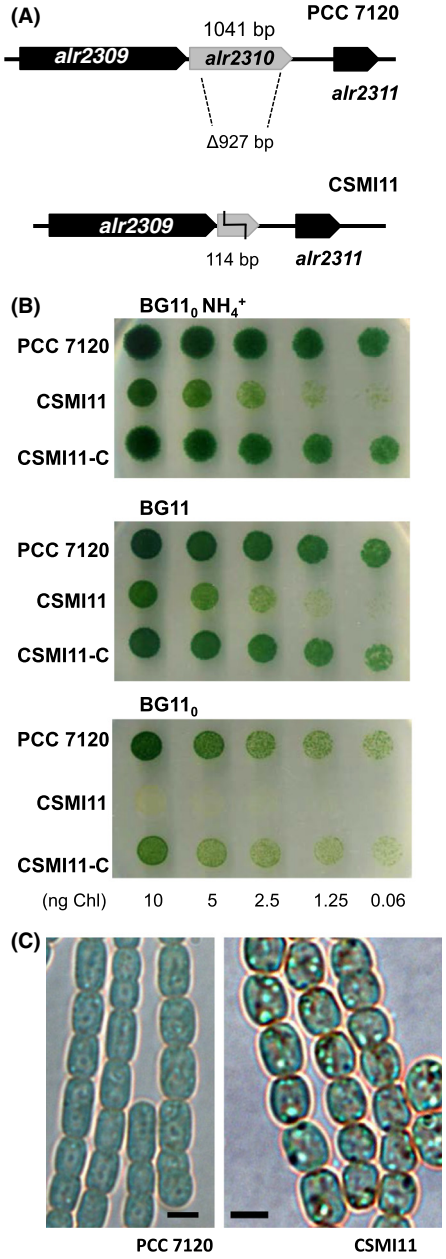

Figure 2. Characterization of an *Anabaena alr2310* mutant. (A) Schematic of the *alr2310* genomic region in *Anabaena* with indication of the DNA fragment removed to create strain CSMI11. (B) Growth tests in solid medium using ammonium (BG11$_0$ NH$_4^+$), nitrate (BG11) or N_2 (BG11$_0$) as the nitrogen source. Each spot was inoculated with an amount of cells containing the indicated amount of Chl, and the plates were incubated under culture conditions for 7 days and photographed. Strain CSMI11-C is strain CSMI11 complemented with *alr2310*. (C) Filaments of *Anabaena* wild type (PCC 7120) and strain CSMI11 from cultures incubated for 5 days in BG11 medium and visualized by light microscopy. Scale bars, 2 μm.

Table 1. Growth rate constants, nitrogenase activity, and cyanophycin granule polypeptide (CGP) levels in *Anabaena* sp. strains PCC 7120 (wild type) and CSMI11 (Δ*alr2310*).

	Growth rate, μ (day^{-1})			Nitrogenase activity (μmol [mg Chl]$^{-1}$ h^{-1})		CGP (μg arginine [mg Chl]$^{-1}$)	
Strain	NH_4^+	NO_3^-	N_2	Oxic	Anoxic	NH_4^+	NO_3^-
PCC 7120	0.55 ± 0.08 (4)	0.79 ± 0.12 (8)	0.67 ± 0.22 (9)	7.13 ± 2.58 (3)	15.26 ± 3.05 (5)	115.12 ± 85.80 (3)	147.06 ± 6.61 (3)
CSMI11	0.56 ± 0.11 (4)	0.62 ± 0.12 (4)	0.06 ± 0.04 (4)	0.00 ± 0.00 (4)	0.003 ± 0.003 (3)	516.71 ± 123.89 (3)	1211.11 ± 429.58 (3)
t test	*P* = 0.90	*P* = 0.06	*P* = 0.0003*	*P* = 0.005*	*P* = 0.0003*	*P* = 0.02*	*P* = 0.02*

The growth rate constant (μ) was determined in photoautotrophic shaken cultures with the indicated nitrogen source. To determine nitrogenase activity, filaments grown in BG11$_0$ NH_4^+ medium and incubated in nitrogen-free BG11$_0$ medium for 24 h were used in assays of reduction of acetylene to ethylene under oxic and anoxic conditions. Cyanophycin was determined by the Sakaguchi reaction for arginine on CGP granules isolated from ammonium- or nitrate-grown filaments. Figures are the mean and SD of data from the number of independent experiments indicated in parenthesis. The significance of the differences between the mutant and the wild-type figures was assessed by the Student's *t* test (*P* indicated in each case); asterisks denote likely significant differences.

Microscopic examination of strain CSMI11 showed the presence of abundant granulation in the cytoplasm of the cells growing in the presence of nitrate or ammonium (shown in Fig. 2C for nitrate-grown filaments). To test whether that granulation corresponded to cyanophycin, CGP isolation was carried out and the isolated material was measured with the Sakaguchi reaction for arginine. CSMI11 cells grown for 8 days in the presence of nitrate or ammonium had, respectively, about 8.3- and 4.5-fold higher amounts of CGP than the control wild type cells (Table 1).

Arginine catabolism

Wild-type *Anabaena* and strain CSMI11 were used in uptake assays with [^{14}C]arginine, and the fate of arginine was studied by TLC analysis of the radiolabeled compounds produced in filaments that had been incubated in BG11 or BG11$_0$ medium. Consistent with results previously published for *Anabaena* (Montesinos et al. 1995), arginine was taken up at appreciable levels in filaments from either BG11 or BG11$_0$ medium, and uptake was significant in both mutant CSMI11 and the wild type (Table 2). Arginine was metabolized in both strains. After 30 min of incubation the amount of radioactivity that remained associated to arginine accounted for only 21.6 and 19.2% in BG11-grown cells of the wild type and the mutant, respectively, and for 50.3 and 17.4% in cells of the corresponding strain that had been incubated in BG11$_0$ medium (Table 2). Because metabolism was observed to proceed to a somewhat larger extent in mutant CSMI11 than in the wild type, catabolism of arginine is not inhibited by the inactivation of *alr2310*.

In the suspensions of wild-type filaments from BG11 medium (Fig. 3, Table 2), after 10 min of incubation, radioactivity from [^{14}C]arginine was mainly distributed among proline, glutamate, glutamine/citrulline (which overlap in the TLC system of solvents used in this study), ornithine, aspartate, and unidentified spot #6. After 30 min, an additional unidentified spot, #7, became visible, and a notable accumulation of radioactivity in glutamate and proline was observed. In strain CSMI11, the general pattern of labeled compounds was similar to that observed in the wild type but, remarkably, a spot that could be identified as agmatine was detected (Fig. 3) that accounted for a significant 10.4% of the cell-associated radioactivity after 30 min of incubation. Additionally, substantially more radioactivity accumulated in glutamate, and less in proline, in strain CSMI11 than in the wild type.

In the suspensions of filaments that had been incubated in BG11$_0$ medium, the patterns of [^{14}C]arginine-derived products were similar to those found in the filaments from BG11 medium, although the accumulation of radioactivity in aspartate and, especially, in glutamate was lower in the filaments of both strains from BG11$_0$ medium (Fig. S3; Table 2). However, in the filaments from BG11$_0$ medium, the accumulation of radioactivity in glutamate was also higher in the mutant than in the wild type (Table 2). Additionally, a specific accumulation of

Table 2. L-[^{14}C]arginine uptake and metabolic products in *Anabaena* sp. strains PCC 7120 (wild type) and CSMI11 (Δ*alr2310*).

	BG11				BG11$_0$			
	PCC 7120		CSMI11		PCC 7120		CSMI11	
	10 min	30 min	10 min	30 min	10 min	30 min	10 min	30 min
Arginine taken up (nmol·[mg Chl]$^{-1}$)	73.3	115.9	57.3	72.4	93.7	162.3	91.3	124.5
Labeled compounds (%)								
Origin	7.5	18.6	1.8	8.4	10.0	12.4	10.2	14.2
Arginine	54.8	21.6	52.6	19.2	66.7	50.3	51.1	17.4
Agmatine	0.4	0.4	2.9	10.4	0.2	0.3	7.1	15.4
Aspartate	1.5	1.5	2.1	1.8	0.5	0.8	1.1	1.2
Glutamate	7.0	33.0	24.6	52.0	1.6	10.7	8.6	37.1
Glutamine/Citrulline	6.4	3.2	3.7	1.2	2.5	2.0	1.8	1.1
Ornithine	2.2	1.0	1.6	1.2	2.5	1.6	2.4	1.5
Proline	15.4	17.4	8.6	2.7	9.5	15.8	9.3	6.4
Spot #6	4.6	2.5	2.1	1.5	6.6	5.1	8.4	3.3
Spot #7	–	0.8	–	1.7	–	1.3	–	2.4

Filaments of the indicated strains grown in BG11$_0$ NH_4^+ medium and incubated for 24 h in BG11 or BG11$_0$ medium were used at 5–10 μg Chl mL^{-1} in assays of uptake of 1 μmol/L L-[^{14}C]arginine as described in Materials and Methods. After 10 and 30 min of incubation, uptake of arginine was determined and metabolites in the suspensions were analyzed by TLC. Cell-associated metabolites are presented as the percentage of the sum of radioactivity in ^{14}C-labeled chromatographic spots after subtraction of extracellular arginine. Compounds that do not move with the used solvents accumulate in the origin of the chromatography.

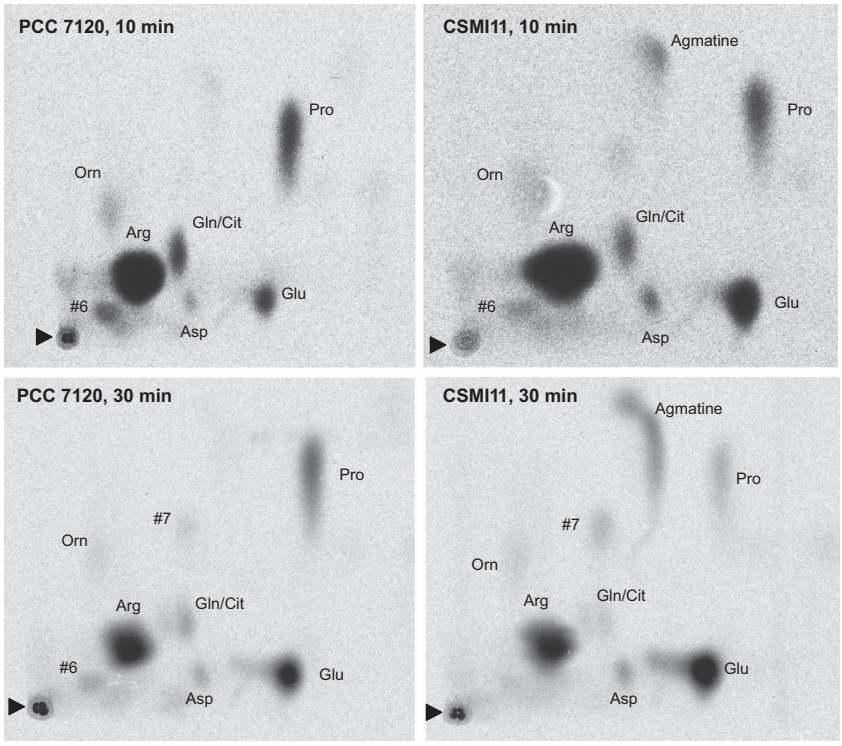

Figure 3. Production of ^{14}C-labeled metabolites from L-[^{14}C]arginine in filaments of *Anabaena* wild type (PCC 7120) and strain CSMI11 grown in $BG11_0$ NH_4^+ medium and incubated in BG11 (nitrate-containing) medium for 24 h. Suspensions of filaments containing 5–10 μg of Chl mL^{-1} were incubated for 10 and 30 min with 1 μmol/L L-[^{14}C-(U)]arginine. Metabolites in the cell suspensions were extracted and analyzed by TLC and autoradiography as described in Materials and Methods. The amino acids identified were as follows: arginine (Arg), citrulline (Cit), proline (Pro), glutamate (Glu), glutamine (Gln), ornithine (Orn), aspartate (Asp), and agmatine. Two unidentified spots, indicated as #6 and #7, were also detected. Note that the glutamine and citrulline spots overlap. The black triangles point to the origin of the chromatography.

agmatine, accounting for 15.4% of the cell-associated radioactivity, was also detected in filaments of the mutant from $BG11_0$ medium (Fig. S3).

The results showing that agmatine accumulates specifically in mutant CSMI11 suggest that *alr2310* is an agmatinase. We therefore determined agmatinase in cell-free extracts from BG11-grown filaments as production of urea from agmatine. Whereas an activity of about 10 nmol urea produced (mg Chl)$^{-1}$ h^{-1} was detected in extracts from the wild type, the activity was undetectable in extracts from strain CSMI11.

Growth tests with arginine and arginine catabolism-related compounds

Alterations in growth of the Δ*alr2310* mutant, strain CSMI11 (lacking the activity of reaction 2 in Fig. 1), could be explained by a lack of putrescine, which might be required as a polyamine or for production of other polyamines, or by the accumulation of agmatine, which might be detrimental to the cells. The effects of putrescine and agmatine on *Anabaena* were assessed by growth tests in solid medium, which were performed at a fixed pH of 7.5. Growth tests with different nitrogen sources and L-putrescine at 100 or 300 μmol/L were performed, but no (or a very poor) recovery of the growth of strain CSMI11 in $BG11_0$ medium was observed (Fig. S4). Although we cannot rule out that putrescine is not taken up by *Anabaena*, this possibility is unlikely because putrescine uptake has been reported in several cyanobacteria (Guarino and Cohen 1979; Raksajit et al. 2006) and the *Anabaena* genome bears genes encoding a putative polyamine ABC-type transporter (Kaneko et al. 2001).

To test whether the impairment of strain CSMI11 in diazotrophic growth could result from an accumulation of agmatine, growth tests with different nitrogen sources and agmatine were performed. Whereas good growth was observed in the presence of 10 or 100 μmol/L agmatine (not shown), 1 mmol/L agmatine was inhibitory for both the wild type and strain CSMI11, although in the presence of nitrate or ammonium the mutant was more sensitive than the wild type (Fig. 4). The higher sensitivity of the mutant can be related to its inability to metabolize agmatine. In addition, these results show that the degree of agmatine toxicity depends on the nitrogen source.

To assess the possibility that agmatine is competing with arginine in cellular metabolism, we investigated whether the growth defect of strain CSMI11 in media lacking combined nitrogen could be rescued by arginine. Strain FQ163, a *hepP* gene mutant that is unable to grow fixing N_2 under oxic conditions (Fox$^-$ phenotype; López-Igual et al. 2012), was used as a control. Supplementation of the medium with 0.1–30 mmol/L arginine rescued the growth of both strains, CSMI11 and FQ163, but growth was stronger in the case of FQ163 than in the case of CSMI11 (Fig. 5). Arginine can be utilized as a nitrogen source by *Anabaena*

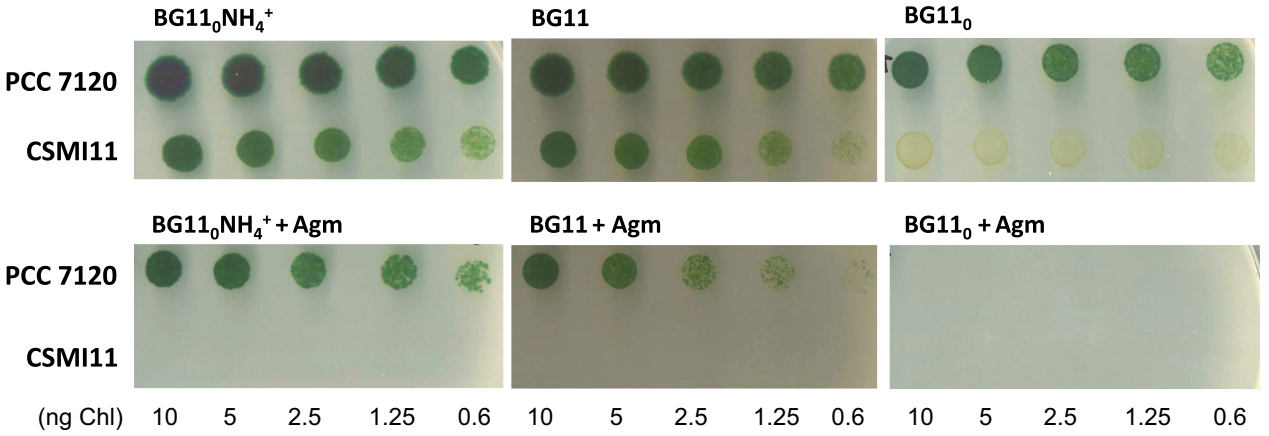

Figure 4. Growth test of *Anabaena* wild type (PCC 7120) and strain CSMI11 in BG11$_0$ NH_4^+-, BG11-, and BG11$_0$-solid media supplemented with 10 mM TES-NaOH buffer (pH 7.5) and, when indicated, 1 mmol/L agmatine sulfate (Agm). Each spot was inoculated with an amount of cells containing the indicated amount of Chl, and the plates were incubated for 8 days under culture conditions and photographed.

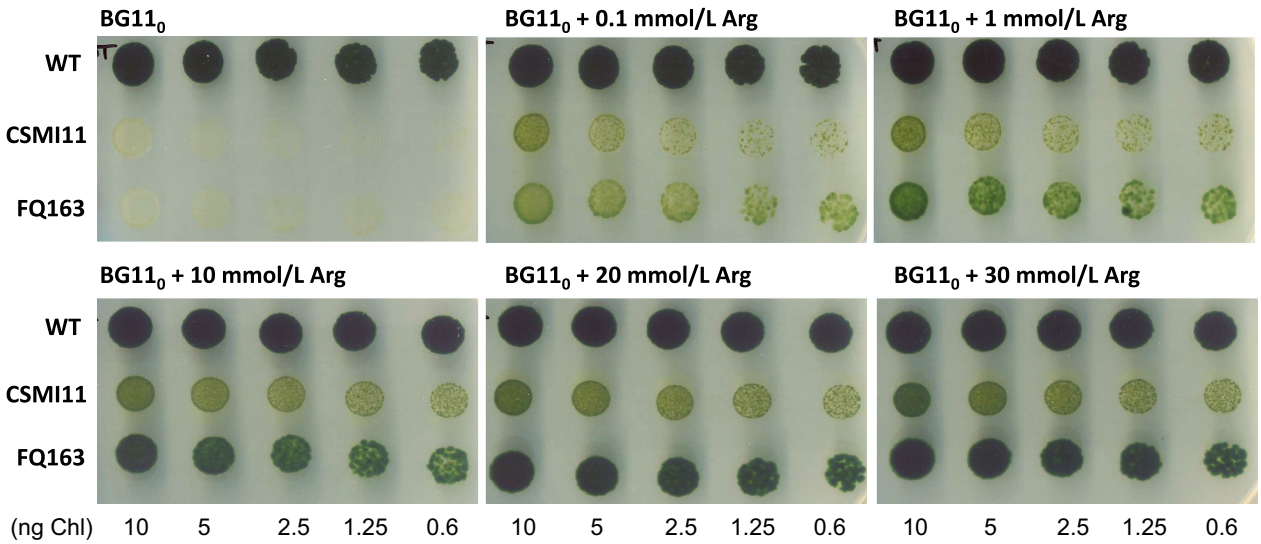

Figure 5. Growth tests of *Anabaena* sp. strains PCC 7120 (WT), CSMI11 (Δ*alr2310*) and FQ163 (*hepP*) in BG11$_0$ solid medium supplemented with 10 mmol/L TES-NaOH buffer (pH 7.5) and, when indicated, arginine (Arg) at the specified concentration. Spots were inoculated with an amount of cells containing the indicated amount of Chl, and the plates were incubated for 14 days under culture conditions and photographed. Strain FQ163 was used as a Fox$^-$ control.

(Herrero and Flores 1990; Burnat et al. 2014), and it clearly serves as a nitrogen source for the Fox$^-$ strain FQ163. In strain CSMI11, however, arginine compensates only to a limited extent the effect of inactivation of *alr2310*. It is possible that agmatine produced from arginine contributes to hamper growth.

Protein synthesis in strain CSMI11

To determine if strain CSMI11 could be affected in protein synthesis, radioactivity incorporated from [^{14}C] leucine into macromolecules was assessed. Leucine is an amino acid of low pool size that is primarily incorporated into protein, which makes it an appropriate amino acid to study protein synthesis in vivo (Padan et al. 1971). Cultures of wild-type *Anabaena* and strain CSMI11 were grown in BG11$_0$ NH_4^+ medium, incubated for 24 h in BG11$_0$ or BG11 medium and analyzed. As shown in Table 3, leucine uptake was about 1.8-fold higher in strain CSMI11 than in the wild type when incubated in BG11 medium, whereas the rates of uptake were similar for both strains in BG11$_0$ medium. Incorporation of

Table 3. [^{14}C]Leucine uptake and incorporation into TCA-precipitable material in *Anabaena* sp. strains PCC 7120 (wild type) and CSMI11 (Δ*alr2310*).

Growth conditions	Strain	[^{14}C]Leucine incorporation (nmol (mg Chl)$^{-1}$ min^{-1})		
		Uptake	TCA-precipitable material	TCA-precipitable material/uptake
BG11	PCC 7120	14.00 ± 8.59	11.91 ± 7.59	0.851
	CSMI11	25.03 ± 17.53	18.52 ± 13.43	0.740
$BG11_0$	PCC 7120	41.50 ± 28.49	25.48 ± 14.07	0.614
	CSMI11	39.97 ± 32.40	18.44 ± 14.12	0.461

To determine leucine uptake (incorporation into whole cells) and the radioactivity incorporated into macromolecules, suspensions of filaments from cultures with BG11 or $BG11_0$ medium were incubated in the same media with 10 μmol/L L-[^{14}C]leucine for 1 h, as described in Materials and Methods. At several time points, samples were filtered to determine leucine uptake rates or mixed with ice-cold TCA and then filtered to determine rates of incorporation into macromolecules. Figures are the mean ± SD of four independent experiments. For the fraction of radioactivity taken up that was precipitable with TCA, Student's *t* tests indicated that the differences between the mutant and the wild type were likely significant in both BG11 ($P = 0.006$) and $BG11_0$ ($P = 0.008$) medium.

radioactivity from [^{14}C]leucine into TCA-precipitable material was 1.6-fold and 0.72-fold in strain CSMI11 as compared to the wild type when incubated in BG11 or $BG11_0$ medium, respectively. Because the amount of [^{14}C]leucine that is incorporated into protein can be expected to depend on the amount of [^{14}C]leucine taken up, we compared the fraction of [^{14}C]leucine taken up that was incorporated into TCA-precipitable material in the mutant and the wild type in each of the two growth conditions. Relative incorporation into TCA-precipitable material was about 13% lower in the mutant than in the wild type in BG11 medium, and about 25% lower in $BG11_0$ medium. Therefore, the hampered diazotrophic growth of strain CSMI11 is hardly only a consequence of a low level of protein synthesis.

Cell-specific expression of Alr2310

To investigate the expression and cell localization of Alr2310, an *alr2310-gfp* fusion gene was constructed and transferred to *Anabaena* (Fig. S5). *Anabaena* clones bearing this construct as the only *alr2310* gene were readily isolated. Strain CSMI21, which was selected for further analysis, exhibited growth properties similar to those of the wild type (Fig. S5), indicating that the Alr2310-GFP fusion protein retained Alr2310 function.

When strain CSMI21 was grown with nitrate as the nitrogen source, GFP fluorescence was observed in all the cells of the filament, but when incubated in the absence of combined nitrogen, GFP fluorescence was observed in vegetative cells at higher levels than in heterocysts (Fig. 6A). Quantification of the GFP fluorescence in vegetative cells and heterocysts indicated that in filaments incubated for 24 or 48 h without combined nitrogen, heterocysts had on average 46.7% and 12.6%, respectively, of the fluorescence detected in vegetative cells (Fig. 6B).

Western blot analysis with anti-GFP antibodies identified a band corresponding to the Alr2310-GFP fusion protein in cell-free extracts from filaments of strain CSMI21 grown in bubbled BG11 medium or incubated in bubbled $BG11_0$ medium for 48 h (Fig. 7). Only a very low amount of free GFP released from the fusion protein was observed. Noteworthy, the fusion protein was not detected in cell-free extracts from heterocysts of strain CSMI21. The results of the western blot analysis are consistent with those of the GFP fluorescence analysis showing that the Alr2310-GFP fusion protein is present at significantly lower levels in heterocysts than in vegetative cells.

Discussion

ORF *alr2310* from the genome of the heterocyst-forming cyanobacterium *Anabaena* putatively encodes an ureohydrolase family protein. Based on bioinformatic analysis, Alr2310 has been proposed to be the arginase of *Anabaena* (Schriek et al. 2007). However, as demonstrated by the accumulation of agmatine from [^{14}C]arginine in the Δ*alr2310* mutant, strain CSMI11, and from lack of agmatinase activity in cell-free extracts from this mutant, *alr2310* encodes an agmatinase and therefore is the *speB* gene of *Anabaena*. Two possible agmatinases, Sll0228 and Sll1077, are encoded in the genome of *Synechocystis* sp. strain PCC 6803, and Alr2310 is most similar to Sll0228 (Schriek et al. 2007) whose inactivation also leads to lack of any agmatinase activity in *Synechocystis* cell-free extracts (Quintero et al. 2000). Accumulation of agmatine in strain CSMI11 in turn implies the operation of arginine decarboxylase, the product of ORF *all3401*, in *Anabaena* (Fig. 1). We have not consistently detected in the wild type a TLC spot that could be identified as putrescine. We do not know, however, whether in our

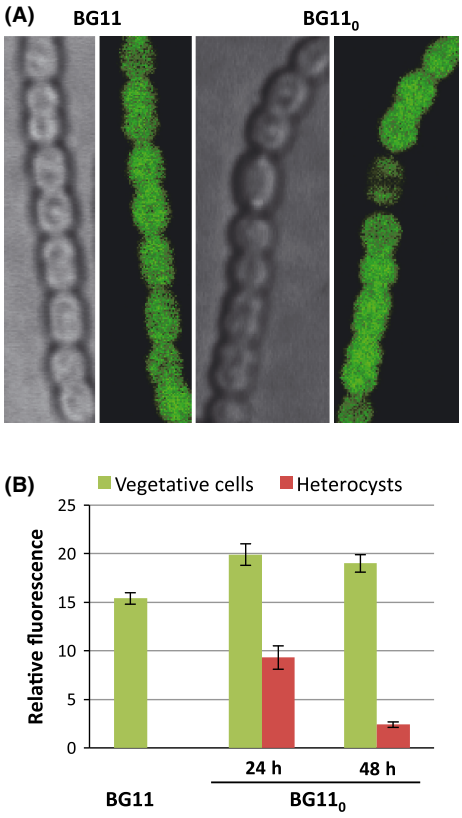

Figure 6. Cellular localization of Alr2310-GFP in the *Anabaena* filaments. (A) Filaments of *Anabaena* sp. strain CSMI21 grown in bubbled BG11 medium or incubated in bubbled $BG11_0$ medium (without combined nitrogen) for 48 h were visualized by confocal microscopy as described in Materials and Methods. Bright field and GFP fluorescence images are shown Brightness and contrast were increased to improve visibility. (B) Quantification of GFP fluorescence in cells of strain CSMI21. Average background fluorescence from wild-type cells (which lack GFP) was subtracted. Figures are the mean and standard deviation of the mean of the fluorescence recorded in cells grown in bubbled BG11 medium (487 cells counted) or incubated in bubbled $BG11_0$ medium for 24 h (309 vegetative cells and 58 heterocysts counted; Student's *t* test $P = 2.7 \times 10^{-18}$) or 48 h (409 vegetative cells and 67 heterocysts counted; $P = 1.3 \times 10^{-56}$).

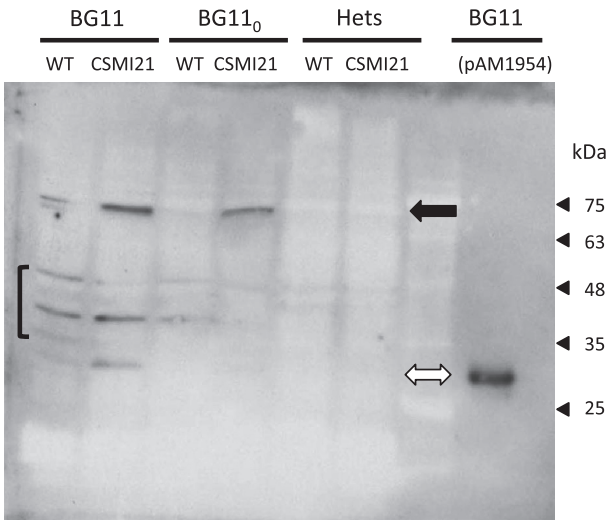

Figure 7. Western blot analysis of the GFP in strain CSMI21 and control strains. The *Anabaena* strains used were PCC 7120 (WT), mutant CSMI21 (*alr2310-gfp*) and a strain carrying pAM1954, which is a replicative plasmid bearing the *gfp* gene expressed from the *rbc* gene promoter. Cell-free extracts were prepared from whole filaments grown in bubbled cultures with BG11 medium or incubated in $BG11_0$ medium for 48 h, or from heterocysts (Hets) isolated from the latter. *Anabaena* sp. [pAM1954] was grown in shaken cultures containing BG11 medium. Proteins were subjected to SDS-PAGE electrophoresis and transferred to membranes. Protein detection was performed with anti-GFP antibodies. Native GFP is 26.9 kDa and the Alr2310-GFP fusion, which includes Alr2310, a tetra-glycine linker peptide, and the GFP is 65.7 kDa. Protein loaded was 40 μg per well (0.6 μg in the case of *Anabaena* [pAM1954]). As observed with the wild-type extracts, the antibodies marked 3 or 4 unspecific bands (brackets). Strain CSMI21 produced mainly a band corresponding to the fusion protein (black arrow) and only a faint band corresponding to free GFP, which was identified with the extract of *Anabaena* [pAM1954] that only produced, as expected, free GFP (open arrow). Note that some material from lane BG11/CSMI21 may have contaminated lane BG11/WT.

experiments the radioactivity from putrescine could pass to other metabolites or whether our methodology is inadequate to extract this polyamine.

Cells of the CSMI11 mutant grown with combined nitrogen (nitrate or ammonium) show an extensive granulation in the cytoplasm (Fig. 1C) that correlates with the accumulation of CGP (Table 1). It has been reported that the end products of cyanophycin mobilization, arginine and aspartate, inhibit cyanophycinase (Gupta and Carr 1981a). Agmatine is known to inhibit moderately cyanophycin synthetase (Aboulmagd et al. 2001), but we do not know whether it could also inhibit cyanophycinase as to account for the observed accumulation of CGP in the CSMI11 mutant. Alternatively, if, as is the case for amino acids such as lysine, glutamate, citrulline and ornithine (Merrit et al. 1994; Ziegler et al. 1998; Berg et al. 2000; Aboulmagd et al. 2001), agmatine were incorporated into cyanophycin at significant levels, accumulation of CGP could result from an inefficient degradation of agmatine-containing cyanophycin.

Strain CSMI11 is impaired in diazotrophic growth and produces only immature heterocysts, and CSMI11 filaments incubated for 24 h without combined nitrogen do not show measurable nitrogenase activity (Table 1). Inactivation of *alr2310* likely results in accumulation of agmatine (as observed with [^{14}C] arginine) and a lack of putrescine, but growth inhibition appears to be related specifically to accumulation of agmatine. Inhibi-

tion of the growth of some bacteria by agmatine has previously been noted, and competitive inhibition of amino acid transport or interference with translation were considered as possible mechanisms of inhibition (Griswold et al. 2006). Agmatine may compete with arginine for charging tRNAs resulting in a general inhibition of protein synthesis. We have found that incorporation of [^{14}C]leucine into macromolecules is somewhat inhibited in strain CSMI11 as compared to the wild type, especially in cells that had been incubated in the absence of combined nitrogen, but the observed effect seems insufficient to prevent growth. Because diazotrophic growth of *Anabaena* requires heterocyst differentiation and, as mentioned earlier, strain CSMI11 produces only immature heterocysts, interference of agmatine with heterocyst differentiation may contribute to prevent growth of this mutant in the absence of combined nitrogen.

Because the pattern of [^{14}C]arginine metabolic products in *Anabaena* is very similar to that reported for *Synechocystis* sp. strain PCC 6803 (Quintero et al. 2000), it is possible that the arginine catabolism pathway proposed for this cyanobacterium, which combines the arginase catabolic route and a urea cycle generating proline, glutamate and glutamine as final products (Quintero et al. 2000), is operative also in *Anabaena*. An interesting aspect of this pathway is that it incorporates aspartate at the level of the urea cycle (Fig. 1), thus providing a rationale for the combined utilization of the amino acids released in cyanophycin mobilization, arginine and aspartate. Appreciable levels of arginase activity have been detected in cell-free extracts of *Anabaena* (Gupta and Carr 1981b), but the gene encoding an enzyme with arginase activity remains to be identified in this cyanobacterium. The possibility that Alr2310 has also arginase activity is not supported by the results of inactivation of *alr2310*, which does not prevent production of proline and glutamate from [^{14}C]arginine. Instead, production of glutamate from [^{14}C]arginine is significantly increased in mutant CSMI11 as compared to the wild type (Table 2). This result suggests that if the arginine decarboxylase pathway is not operative, arginine degradation through the arginase-like pathway is increased. A small amount of [^{14}C]aspartate produced from [^{14}C]arginine could also be observed in our experiments, but we do not know the possible pathway involved in this conversion.

As described in the Introduction, we have recently found that in the diazotrophic filament of *Anabaena,* the second step of cyanophycin degradation, hydrolysis of β-aspartyl-arginine, takes place mainly in vegetative cells (Burnat et al. 2014). This dipeptide appears to be an important vehicle for the transfer of nitrogen from heterocysts to vegetative cells, consistent with the possible existence of a gradient of arginine or an arginine-containing compound in the diazotrophic filament (Ke and Haselkorn 2013). These results imply an active catabolism of arginine (and aspartate) in the vegetative cells of the diazotrophic filament. Consistently, arginase and a low level of arginine deiminase activity detected in *Anabaena* are present at significantly higher levels in vegetative cells than in heterocysts (Gupta and Carr 1981b). Our results showing the presence of the Alr2310-GFP fusion protein at significantly higher levels in vegetative cells than in heterocysts indicate that also agmatinase, and hence the arginine decarboxylase pathway, is mainly operative in the vegetative cells of the diazotrophic filament. These observations highlight the importance of arginine catabolism to make available in the vegetative cells the nitrogen transiently stored as cyanophycin and transferred from the heterocysts.

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Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Construction and verification of mutant CSMI11.

Figure S2. Microscopic observation of filaments of mutant strain CSMI11.

Figure S3. Catabolism of L-[^{14}C]arginine in filaments of *Anabaena* wild type and strain CSMI11 incubated in medium lacking combined nitrogen.

Figure S4. Growth tests of *Anabaena* wild type and strain CSMI11 in solid media supplemented with putrescine.

Figure S5. Construction, verification and growth test of strain CSMI21.

Table S1. Cyanobacterial strains and plasmids used in this work.

Table S2. Oligodeoxynucleotide primers used in this work.

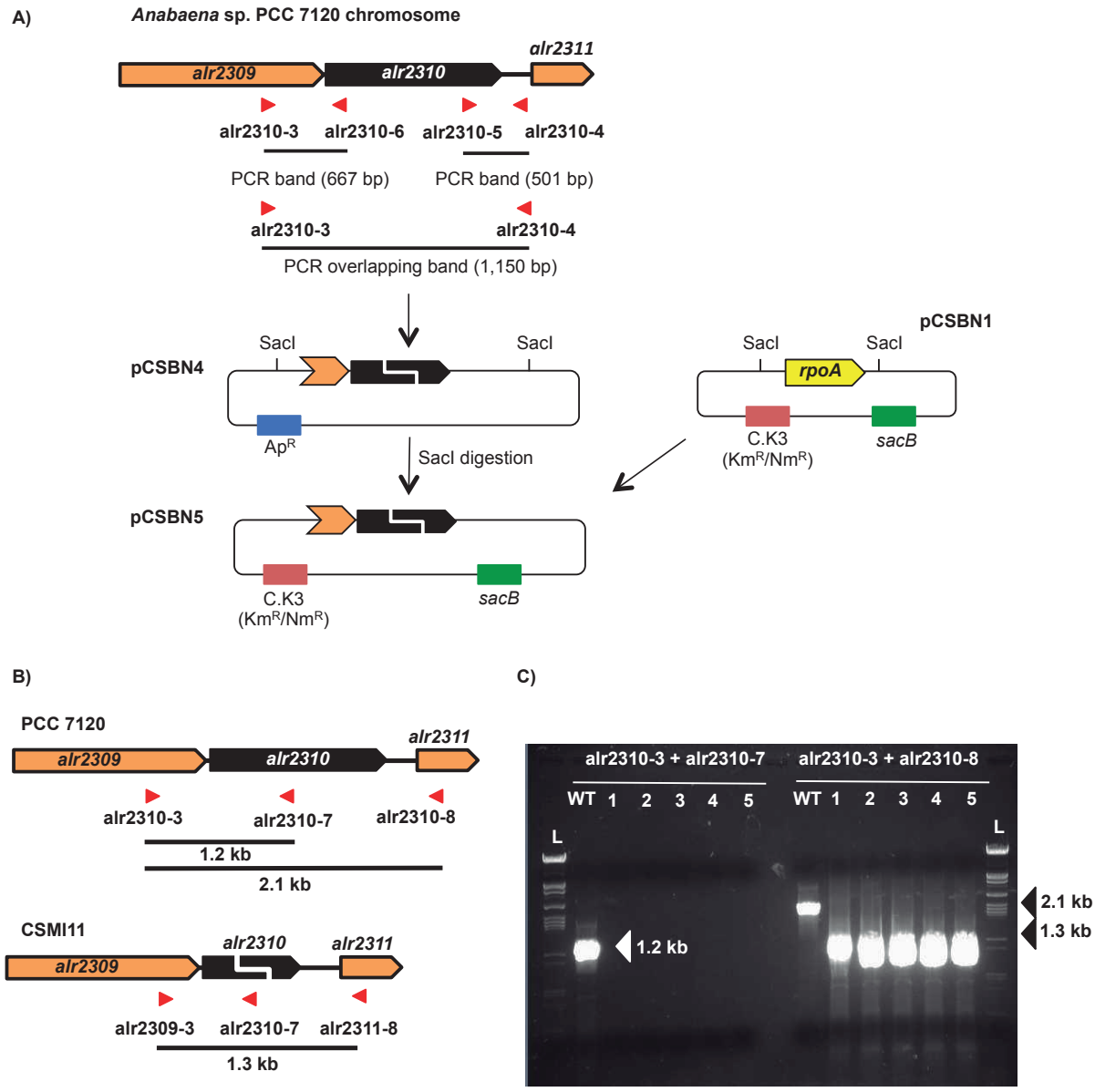

Figure S1. Construction and verification of mutant CSMI11. (A) Construction of pCSBN5. Amplification by PCR of regions upstream and downstream from the central part of *alr2310* and cloning of the PCR products in pMBL-T produced pCSBN4. The SacI insert of pCSBN4 was transferred to SacI-digested pCSBN1, producing pCSBN5, which was transferred to *Anabaena*. (B) Scheme of the *alr2310* genomic region in *Anabaena* sp. PCC 7120 and CSMI11. Block arrows denote genes and direction of transcription. Red arrowheads represent primers (all listed in Table S2). (C) Verification of strain CSMI11 by PCR. L, lambda DNA digested with ClaI ladder. Primer pairs are indicated on top. Templates: WT, wild type DNA; 1 to 5, DNA from five clones in which the wild-type locus was replaced by a deleted locus. One of these clones, which could be complemented by wild-type *alr2310*, was chosen for further analysis and named CSMI11.

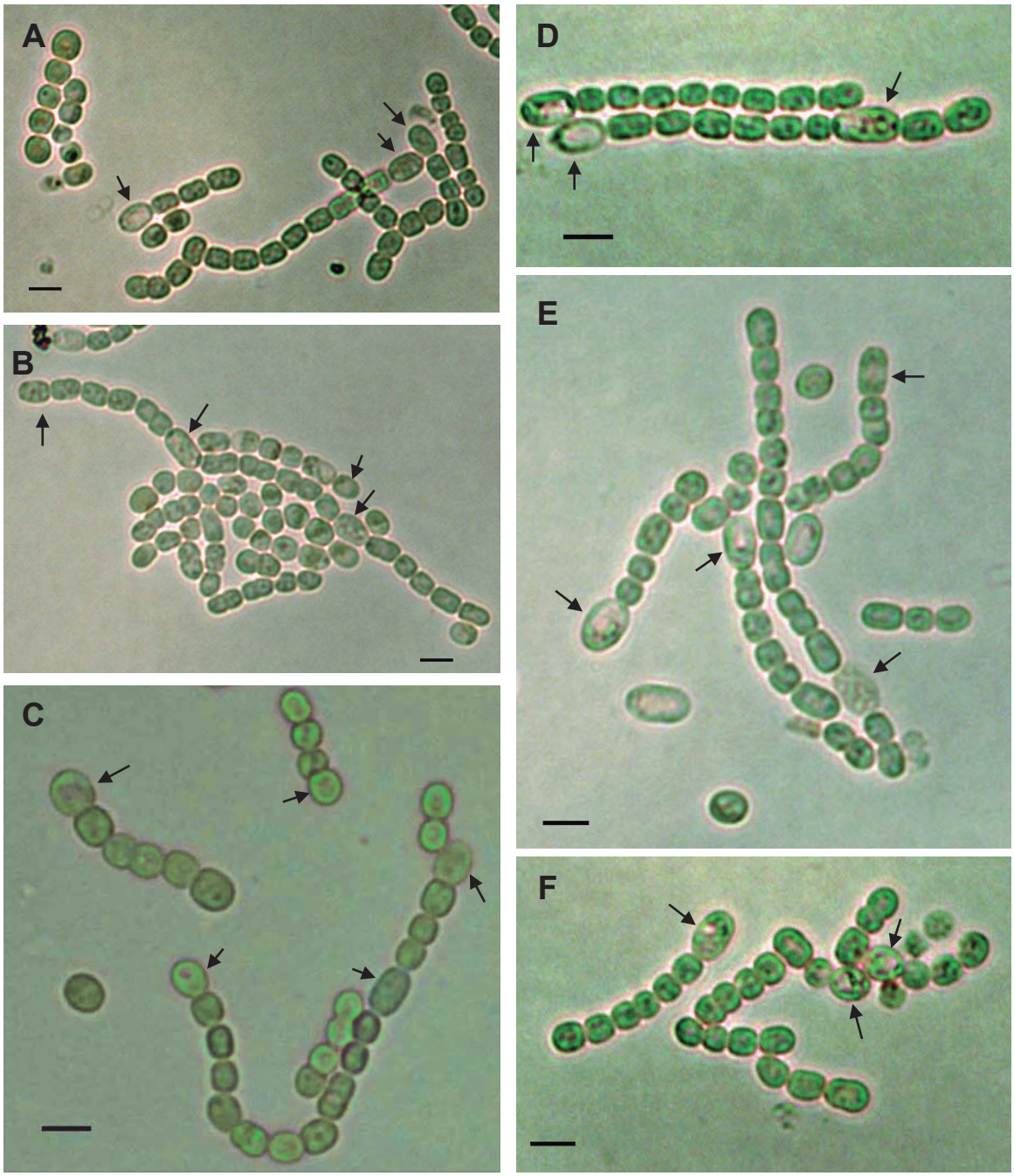

Figure S2. Examples of filaments of strain CSMI11 grown in $BG11_0$ NH_4^+ medium and incubated in $BG11_0$ medium for 24 h (A, B, C) or 48 h (D, E, F). Scale bars are 5 μm (A, B, D, E, F) or 2 μm (C). Some heterocysts are indicated by black arrows.

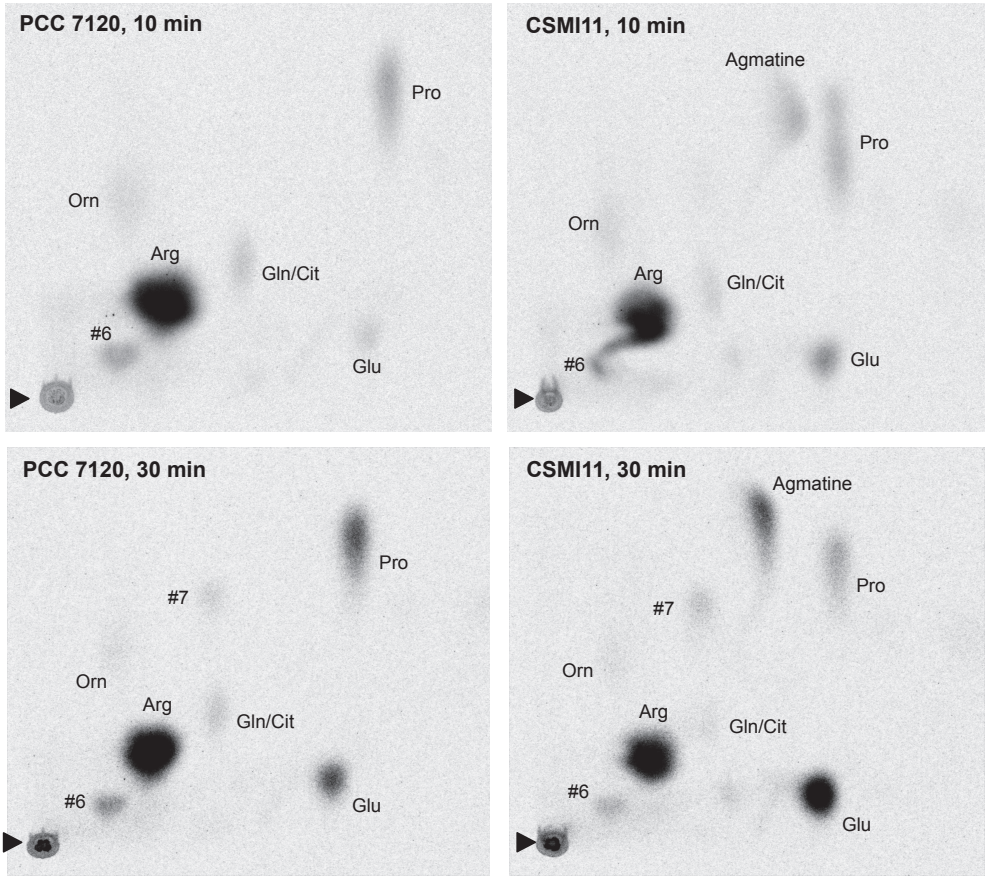

Figure S3. Production of ^{14}C-labeled metabolites from L-[^{14}C]arginine in filaments of *Anabaena* wild type (PCC 7120) and strain CSMI11 grown in $BG11_0$ NH_4^+ medium and incubated in $BG11_0$ medium (lacking combined nitrogen) for 24 h. Suspensions of filaments containing 5 to 10 µg of Chl·ml^{-1} were incubated for 10 and 30 min with 1 µM L-[^{14}C-(U)]arginine. Metabolites in the cell suspensions were extracted and analyzed by TLC and autoradiography as described in Materials and Methods. Detected spots as in Fig. 3.

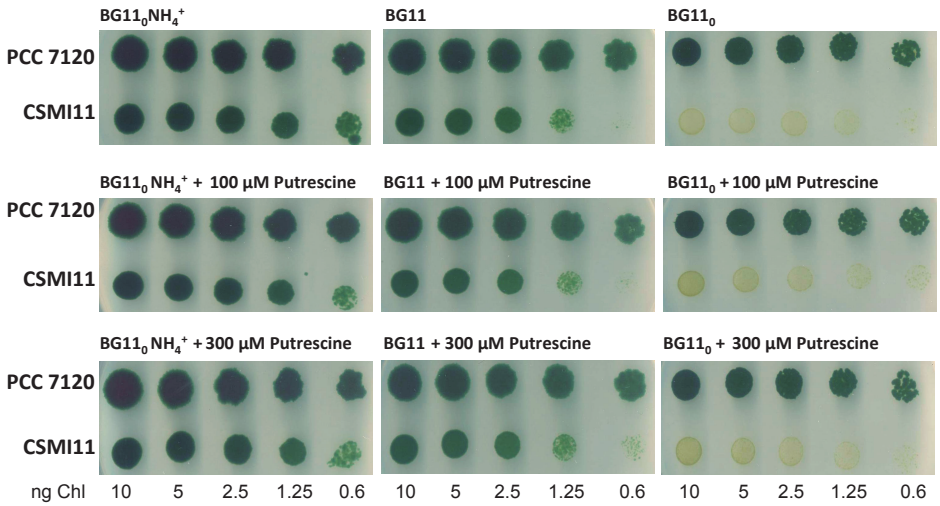

Figure S4. Growth test of *Anabaena* wild type (PCC 7120) and strain CSMI11 in BG11$_0$ NH$_4^+$-, BG11-, and BG11$_0$-solid media supplemented with 10 mM TES-NaOH buffer (pH 7.5) and, when indicated, putrescine at the specified concentration. Each spot was inoculated with an amount of cells containing the indicated amount of Chl, and the plates were incubated for 14 days under growth conditions and photographed.

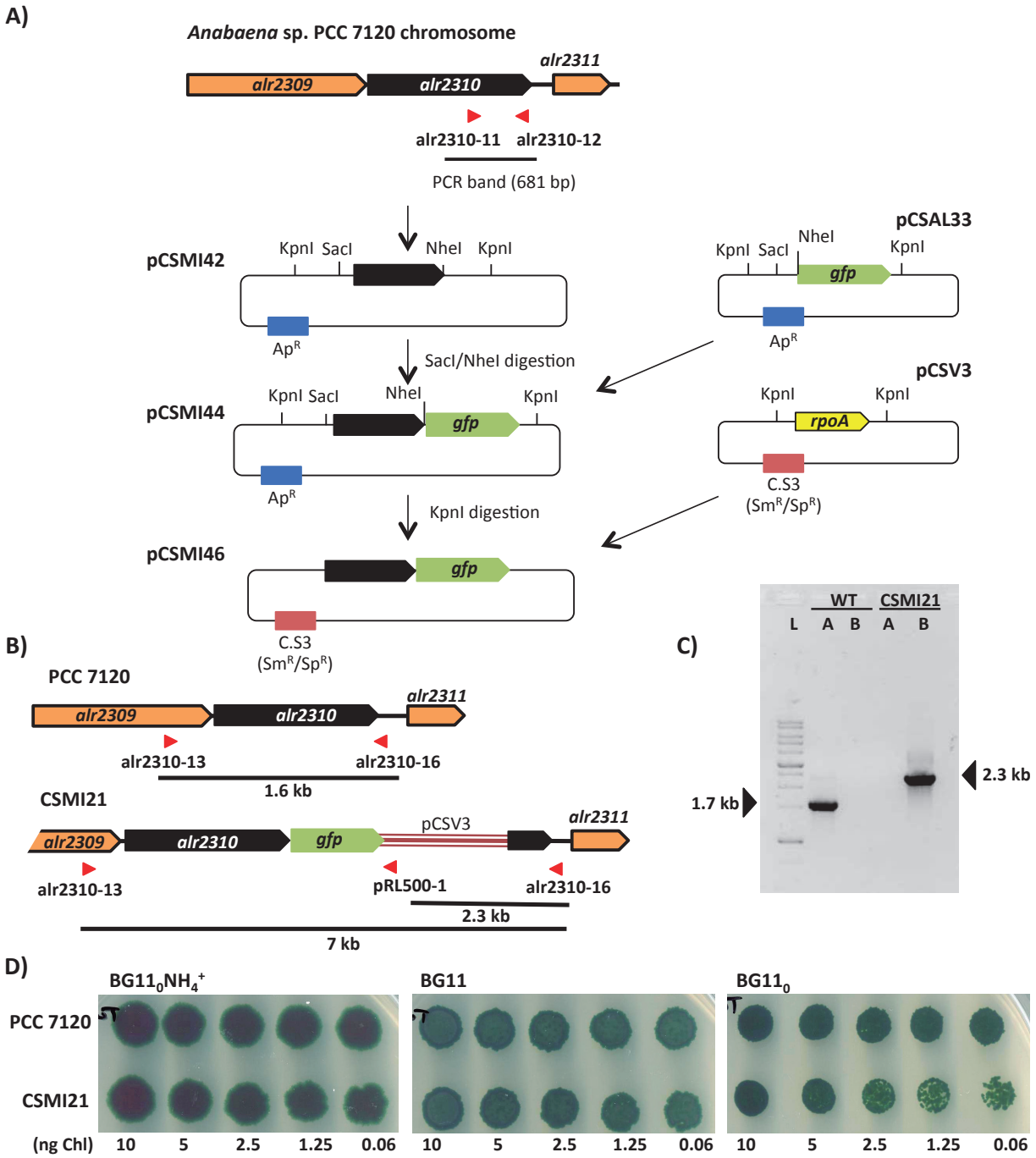

Figure S5. Construction, verification and growth test of strain CSMI21. (A) Construction of pCSMI46. The 3' part of *alr2310* was amplified by PCR and cloned in pMBL-T, producing pCSMI42. The SacI-NheI insert was transferred to SacI/NheI-digested pCSAL33, which carries *gfp*, generating pCSMI44. The KpnI insert from pCSMI44 was transferred to KpnI-digested pCSV3, producing pCSMI46, which was transferred to *Anabaena*. (B) Scheme of the *alr2310* genomic region in *Anabaena* sp. PCC 7120 and CSMI21. Block arrows denote genes and direction of transcription. Red arrowheads represent primers (Table S2). Red tripled line represents the pCSV3 vector. (C) Verification of strain CSMI21 by PCR. L, 1-kb DNA ladder (Biotools). Lanes A, PCR performed with primers alr2310-13 and alr2310-16; lanes B, with primers alr2310-13 and pRL500-1. Template DNA from the wild type (WT) or CSMI21 was used. (D) Growth tests in solid medium using ammonium ($BG11_0NH_4^+$), nitrate (BG11) or N_2 ($BG11_0$) as the nitrogen source. Spots were inoculated with cells containing the indicated ng of Chl, and the plates were incubated under culture conditions for 12 days and photographed. Note that strain CSMI21 grows well with the different nitrogen sources, indicating that the GFP fusion is not impairing Alr2310 function.

Table S1. Cyanobacterial strains and plasmids used in this work.

Strain or plasmid	Relevant characteristic(s)	Source or reference
***Anabaena* strains**		
PCC 7120	Wild type	1
FQ163	*hepP(all1711)*::Tn5-1063; Nm^R	2
CSMI11	Δ*alr2310*	This study
CSMI11-C	Δ*alr2310* bearing wild type *alr2310* in replicative plasmid pCSMI54; Em^RCm^R	This study
CSMI21	*alr2310*-C-*gfp*; Sm^RSp^R	This study
Plasmids		
pMBL-T	Cloning vector; Ap^R	Dominion MBL
pSPARK	Cloning vector; Ap^R	Canvax, Biotech SL
pRL278	*sacB*-containing negative selection vector with: Km^R Suc^S	3
pRL443	Conjugative plasmid; Ap^R	4
pRL623	Helper plasmid; carries *mob* and DNA methylases; Cm^R	5
pCSRO	*sacB*-containing negative selection vector with: Sm^RSp^R Suc^S	6
pCSV3	Positive selection vector; Sm^RSp^R	7
pAM1954	Plasmid carrying the fusion P_{rbcL}::*gfp*	8
pRL3845	Replicative vector used for complementation; Em^RCm^R	2
pCSBN1	Positive selection vector derived from pRL278 and pCSV3; Km^R Suc^S	This study
pCSBN4	*alr2310* lacking a 910-bp internal fragment cloned in pMBL-T; Ap^R	This study
pCSBN5	SacI fragment from pCSBN4 cloned into SacI-digested pCSBN1; Km^R Suc^S	This study
pCSMI42	681-bp fragment from the 3' part of *alr2310* cloned in pMBL-T; Ap^R	This study
pCSMI44	SacI/NheI fragment from pCSMI42 cloned into SacI/NheI-digested pCSAL33; Ap^R	This study
pCSMI46	KpnI fragment from pCSMI44 cloned into KpnI-digested pCSV3; Sm^RSp^R	This study
pCSMI53	*alr2310* cloned in pSPARK; Ap^R	This study
pCSMI54	SmaI fragment from pCSMI53 cloned into SmaI-digested pRL3845; Em^RCm^R	This study
pCSAL33	Plasmid carrying *gfp;* Ap^R	A. López-Lozano and A. Herrero

^{R} denotes resistance to the indicated antibiotic: Ap, ampicillin; Cm, chloramphenicol; Em, erythromycin; Km, kanamycin; Nm, neomycin; Sm, streptomycin; and Sp, spectinomycin. Suc^S, sensitivity to 5% sucrose.

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8. Yoon, H.S., Golden, J.W. (1998) Heterocyst pattern formation controlled by a diffusible peptide. *Science* **282**:953-938

Table S2. Oligodeoxynucleotide primers used in this work.

Primer name	Sequence (5' to 3')
alr2310-3	GAGCTCCCAGGCCCCAACGGTGAAG
alr2310-4	GAGCTCCAAAATCATGGCTGTGTAGAGA
alr2310-5	CCTAGCGATATCGAATGGGATGGTAATGTA
alr2310-6	CCATTCGATATCGCTAGGGTTATAGTCTTG
alr2310-7	ATGCGTCCATGGGATTCCTCAA
alr2310-8	GCACAACCCGTTTTACAGAACCA
alr2310-11	CAAGCTTCCCAACAGGTAAATCAATGGCTG
alr2310-12	GCTAGC*ACCTCCACCGCC*TTGTTTGTGAGATAAATC
alr2310-13	CACTGCACGTTTACCCTTATGGTGTGAGTAGG
alr2310-15	TGCCCGGGTTTGTCGAGTAAATCCA
alr2310-16	TTCCCGGGTGTCTAGGCAATGACTGA
SacB-1	CTTGAGGTACAGCGAAGTG
SacB-2	TCTGCAAAAGGCCTGGAGG
Universal	GTAAAACGACGGCCAGT
M13REV	CAGGAAACAGCTATGAC
pRL500-1	ATAGGCGTATCACGAGGC

Introduced restriction enzyme cutting sites are underlined. The nucleotide sequence that, inverted, encodes the four-Glycine linker is in italics.

Annex II:

Studies on ORF *alr4995* from *Anabaena* sp. PCC 7120

Studies on ORF *alr4995* from *Anabaena* sp. PCC 7120

Introduction

As has been described in the previous chapter, the arginine decarboxylase pathway is operative in *Anabaena* and its inactivation leads to an increase in the levels of the end products of a possible arginase-like pathway, proline and glutamate, suggesting the existence of an alternative route for arginine catabolism. Gupta and Carr (1981b) detected the presence of arginase and arginine deiminase activities in cellular extracts from *Anabaena*, but genes encoding these proteins are not evident in the genome of this cyanobacterium.

In a genomic screening searching for genes involved in arginine catabolism, Schriek *et al*. (2007) identified ORF *alr4995* from *Anabaena* that could encode an arginine amidinotransferase or an arginine deiminase. Arginine deiminases produce L-citrulline from L-arginine, releasing ammonium, with a subsequent enzymatic reaction step producing ornithine, whereas arginine amidinotransferases catalyze the transfer of the amidino group of arginine to the amino moiety of an acceptor, such as glycine, producing ornithine (Muenchhoff *et al*., 2010). Both arginine amidinotransferases and arginine deiminases belong to the "guanidino-group modifying enzymes superfamily" (Shirai *et al*., 2006). Exploring the architecture domains of Alr4995, Shirai *et al*. (2006) found that this protein presents unusual features that differ from proteins belonging to the same protein superfamily. Based on its protein sequence, Alr4995 shows homology to amidinotransferase family proteins in its N terminal part (Figure 14).

Figure 14: Predicted primary structure of Alr4995 showing regions of homology: amidinotransferase/arginine deiminase (AT/ADI) domain; lysine-oxoglutarate reductase/saccharopine dehydrogenase (LOR/SDH) domain; and deoxyhypusine synthase (DHS) domain.

The amidinotransferase (AT) family contains glycine and inosamine amidinotransferases, enzymes that are involved in creatine and streptomycin biosynthesis respectively, and also includes arginine deiminases (ADI). The AT/ADI domain adopts a unique three-dimensional structure known as the α/β propeller, which is a catalytic domain that can accommodate a diverse set of reactions (Humm *et al*., 1997; Shirai *et al*., 2001). The second domain of Alr4995 has similarity to a region of lysine-oxoglutarate reductase/saccharopine dehydrogenase (LOR/SDH), which is a bifunctional enzyme. Specifically, Alr4995 shows homology to the C-terminal part of the LOR domain. This conserved region is commonly found immediately N-terminal to a saccharopine dehydrogenase conserved region in eukaryotic proteins (saccharopine is an intermediate in the metabolism of lysine). Finally, in its C terminal part, Alr4995 shows homology to human deoxyhypusine synthases (DHS

domain), a type of aminobutyl transferase (hypusine is an amino acid related to lysine). Until now, no other protein has been described to contain these three domains in the same protein sequence. A BLAST search of the Alr4995 protein in the available databases shows that this protein is widely distributed and only found in cyanobacteria, with the only exception of a *Bacillus subtilis* strain.

In this work, the role of *alr4995* in arginine catabolism was investigated by using an *in vivo* approach. We have analyzed the fate of exogenously supplied [U-^{14}C]arginine and [1-^{14}C]ornithine in the *Anabaena* wild type and a Δ*alr4995* mutant strain.

Materials and methods

Strains and growth conditions. *Anabaena* sp. strain PCC 7120 was grown axenically in BG11 medium (containing $NaNO_3$), $BG11_0$ medium (free of combined nitrogen) or $BG11_0NH_4^+$ medium ($BG11_0$ containing 4 mM NH_4Cl and 8 mM TES-NaOH buffer, pH 7.5). In every case, ferric citrate replaced the ferric ammonium citrate used in the original recipe (Rippka *et al.*, 1979). For plates, medium was solidified with 1% separately autoclaved Difco agar. Cultures were grown at 30ºC in the light (25 μE m^{-2} s^{-1}), with shaking (80-90 rpm) for liquid cultures. DNA was isolated from *Anabaena* sp. by the method of Cai and Wolk (1990). The *Anabaena* strains used in this study are listed in Table 1.

Escherichia coli DH5α was used for plasmid constructions. It and strains HB101 and ED8654, used for conjugations with *Anabaena* sp., were grown in Luria–Bertani medium supplemented when appropriate with antibiotics at standard concentrations.

Plasmid constructions and genetic procedures. Open reading frame (ORF) *alr4995* of the *Anabaena* chromosome (Kaneko *et al.*, 2001) was inactivated by deleting an internal fragment of 1,755 bp. DNA fragments upstream (490 bp) and downstream (500 bp) from the central region of the gene were amplified by standard PCR using DNA from *Anabaena* as the template and primers alr4995-3/alr4995-4 and alr4995-5/alr4995-6 (all deoxyoligonucleotide primers are listed in Table 1). The external primers, alr4995-3 and alr4995-6, included a PstI-site in their 5′ ends and primers alr4995-4 and alr4995-5 were complementary in their 5′ ends. The upstream and downstream DNA fragments from this gene were cloned in pSpark (Canvax Biotech S.L., Spain), sequenced, and transferred as a PstI-ended fragment to PstI-digested pCSRO (Merino-Puerto *et al*., 2013), producing pCSDH8 (all plasmids are listed in Table 1).

Conjugation of *Anabaena* with *E. coli* HB101 carrying the cargo plasmid (pCSDH8) with helper and methylation plasmid pRL623 was effected by the conjugative plasmid pRL443, carried in *E. coli* ED8654, and performed as described (Elhai *et al.*, 1997) with selection for resistance to Sm/Sp. Exconjugants were spread on $BG11_0NH_4^+$ medium supplemented with 5% sucrose (Cai and Wolk, 1990), and individual Suc^R colonies were checked by PCR looking for clones that had replaced the wild-type locus by a locus bearing the deletion. The genetic structure of selected clones was studied by PCR with DNA from

those clones and primers alr4995-6/alr4995-7 and alr4995-7/alr4995-8. A clone homozygous for the mutant chromosomes was named strain CSDH8 (Δ*alr4995*).

Growth tests and microscopy. Protein concentration and chlorophyll *a* (Chl) content of the cultures were determined by a modified Lowry procedure (Markwell *et al.*, 1978) and by the method of Mackinney (1941), respectively. The growth rate constant ($\mu=\ln 2/t_d$, where t_d is the doubling time) was calculated from the increase of protein content, determined in 0.2-ml samples, of shaken liquid cultures (Montesinos *et al*., 1995). Cultures were inoculated with an amount of cells containing about 5 µg of protein ml^{-1} and grew logarithmically until reaching about 40 µg of protein ml^{-1}.

For growth tests in solid media, cultures grown in BG11 medium were harvested and washed three times with 50 ml of $BG11_0$ medium, and dilutions were prepared in $BG11_0$ medium. 10-µl samples of the resulting suspensions were spotted on agar plates with different nitrogen sources and incubated at 30°C in the light (25 µE m^{-2} s^{-1}).

Cells grown during 5 days in shaken liquid cultures were observed and photographed with a Zeiss Axioscop microscope equipped with a Zeiss ICc1 digital camera.

Table 1. Cyanobacterial strains, plasmids and oligodeoxynucleotide primers used in this work.

***Anabaena* strains**	**Relevant characteric(s)[a]**	**Source or reference**
PCC 7120	Wild type	Rippka et al., 1979
CSDH8	Δ*alr4995*	This study
Plasmids		
pSpark	Cloning vector; Ap^R	Canvax, Biotech, S.L.
pCSRO	*sacB*-containing negative selection vector; $Sm^R Sp^R Suc^S$	Merino-Puerto *et al*., 2013
pRL443	Conjugative plasmid; Ap^R	Elhai and Wolk, 1988
pRL623	Helper plamid; carries *mob* and DNA methylases; Cm^R	Elhai *et al*., 1997
pCSV3	Positive selection vector; $Sm^R Sp^R$	Valladares *et al*., 2011
pCSDH7	Alr4995 lacking 1,755-bp internal fragment cloned in pSpark; Ap^R	This study
pCSDH8	PstI fragment from pCSDH7 cloned into PstI-digested pCSRO; $Sm^R Sp^R Suc^S$	This study
Primer name	**Sequence (5′ to 3′)[b]**	
alr4995-3	CTCTGCAGGGCTAAAATAGGGCGAGATTG	
alr4995-4	ACCGCGATCGACAACGTTATCACC	
alr4995-5	AACGTTGTCGATCGCGGTTCTATCGAA	
alr4995-6	TAAAGCCGCACCATTCCAACCA	
alr4995-7	TGCATCAAAACGCCCGGAAGA	
alr4995-8	GTTCACCAGCTTTGAGGTCACGTA	

[a]Abbreviations: ^{R}denotes resistance to the indicated antibiotic: Ap, ampicillin; Cm, chloramphenicol; Sm, streptomycin; and Sp, spectinomycin. Suc^S, sensitivity to 5% sucrose.

[b]Introduced restriction enzyme cutting sites are underlined.

Arginine and ornithine catabolism. Cells grown in BG11 medium were harvested by centrifugation at 4,000 rpm at room temperature, washed twice with 25 mM N-tris(hydroxymethyl)-methylglycine (Tricine)–NaOH buffer (pH 8.1), and resuspended in the same buffer. The uptake assays were carried out at 30°C in the light (white light from fluorescent lamps, about 175 μE m^{-2} s^{-1}) and were started by mixing a suspension of cells (2.1 ml) containing 5 to 10 μg of Chl· ml^{-1} with a solution (0.1 ml) of L-[U-^{14}C]arginine (274 mCi·$mmol^{-1}$) or L-[1-^{14}C]ornithine (57.1 mCi·$mmol^{-1}$), both purchased from Perkin Elmer (Massachusetts, USA). The final concentration of both arginine and ornithine in the experiments was 1 μM. The amount of metabolite taken up in the 10- and 30-min assays was estimated in 0.5-ml samples of the cell suspensions. The samples were filtered (0.45 μm-pore-size Millipore HA filters were used) and the cells on the filters were washed with 5 to 10 ml of 5 mM Tricine–NaOH buffer (pH 8.1). The filters carrying the cells were then immersed in 5 ml of scintillation cocktail, and their radioactivity was measured. Retention of radioactivity by boiled cells was used as a blank.

To determine metabolites produced from the provided labeled amino acids at the end of the 10- and 30-min incubations, samples of 0.5 ml of the cell suspension were immediately (<15 s) mixed, without filtering the cells, with 1.5 ml of water at 100°C and further incubated for 5 min in a bath of boiling water. The resulting suspensions were centrifuged, and samples (1 to 1.5 ml) from the supernatants were lyophilized and dissolved in 50 μl of water. Samples of the resulting solutions were applied to 0.1-mm-thick cellulose thin-layer chromatography (TLC) plates (20 by 20 cm; Merck, Darmstadt, Germany). Two-dimensional separation of amino acids was effected by using the following solvent systems. In the first system of solvents, the first dimension solvent consisted of *n*-butanol–acetone–ammonium hydroxide–water (20:20:10:4, vol/vol/vol/vol), and the second dimension solvent consisted of isopropanol-formic acid-water (20:1:5, vol/vol/vol). In the second system of solvents, the first-dimension solvent consisted of phenol-water (100:28, vol/vol) and the second-dimension solvent consisted of *n*-butanol-acetic acid-water (12:3:5, vol/vol/vol). The TLC plates were analyzed by electronic autoradiography using a two-dimensional scanner for β particles (PhosphorImager), which allows a quantitative analysis of the radioactive spots. Identification of the metabolite originating a radioactive spot was made by co-chromatography, supplementing the samples with stable amino acids as markers and visualizing the amino acids after chromatography with a solution of ninhydrin in acetone in the presence of cadmium acetate (Atfield and Morris, 1961).

Results

Construction and phenotype of an *alr4995* mutant

The *Anabaena* ORF *alr4995* is located between *asl4994* and *alr4996*, both of them encoding proteins of unknown function. To investigate the role of the *alr4995* gene, a 1,755-bp in-frame internal fragment of the gene was deleted without leaving any gene marker behind to avoid polar effects on neighboring genes. The Δ*alr4995* mutant, strain CSDH8, was homozygous for mutant chromosomes (Figure 15).

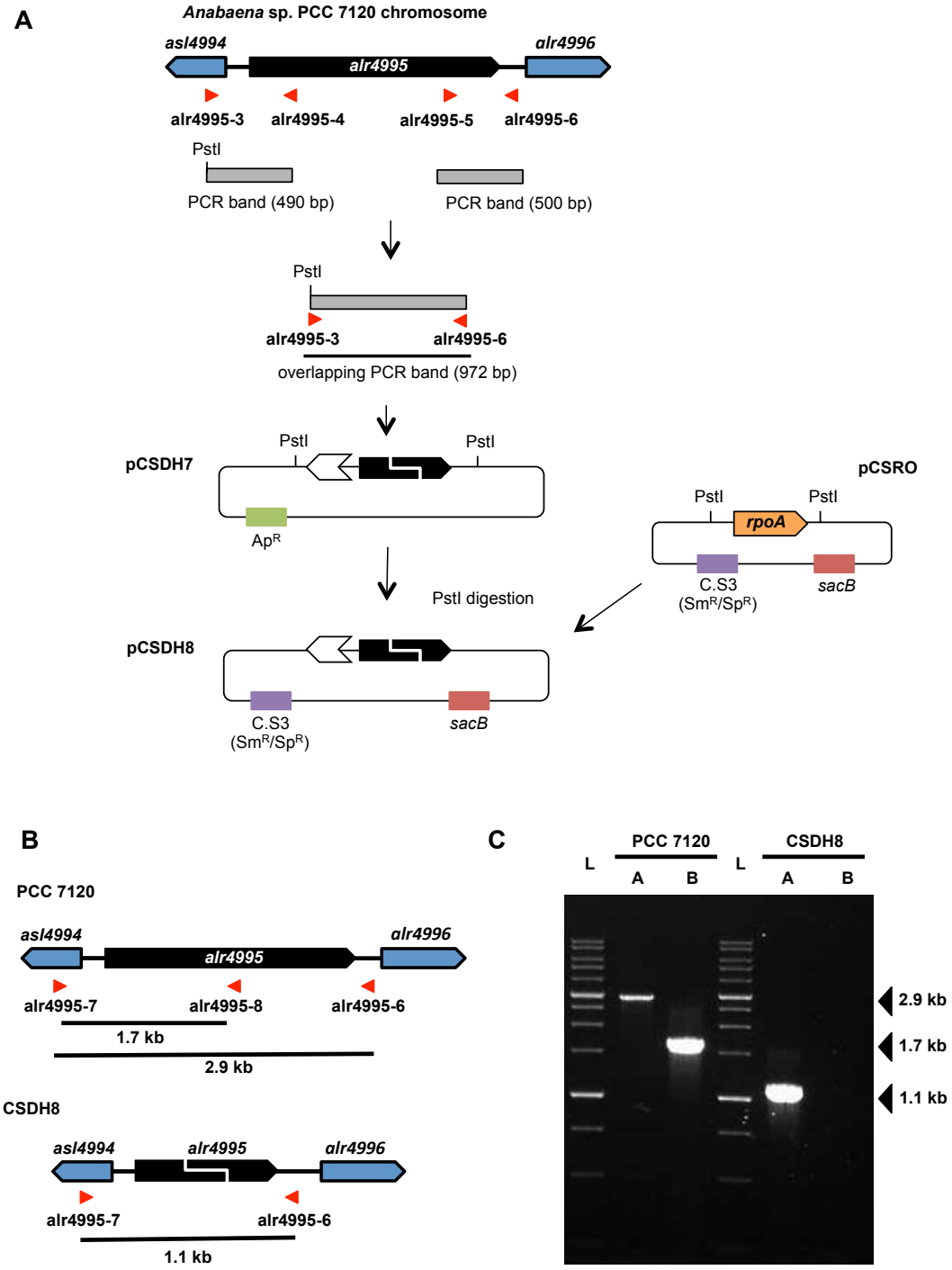

Figure 15: Construction and verification of mutant CSDH8. (A) Construction of pCSDH8. Amplification by PCR of regions upstream and downstream from the central part of *alr4995* and cloning of the PCR products in pSPARK produced pCSDH7. The PstI insert of pCSDH7 was transferred to PstI-digested pCSRO, producing pCSDH8, which was transferred to *Anabaena*. (B) Scheme of the *alr4995* genomic region in *Anabaena* sp. PCC 7120 and CSDH8. Block arrows denote genes and direction of transcription. Red arrowheads represent primers. (C) Verification of strain CSDH8 by PCR. L, 1-kb DNA ladder (Biotools). Lane A, PCR with primers alr4995-6 and alr4995-7; lane B, PCR with primers alr4995-7 and alr4995-8.

Tests in solid media showed that apparent growth of strain CSDH8 was similar to that of the wild-type strain independently of the nitrogen source supplied in the medium, although in N-free medium, the mutant strain may exhibit a slight growth defect (Figure 16).

However, determination of growth rate constants in liquid cultures indicated that there were no significant differences between strain CSDH8 and the wild type when utilizing ammonium, nitrate or N_2 (Table 2).

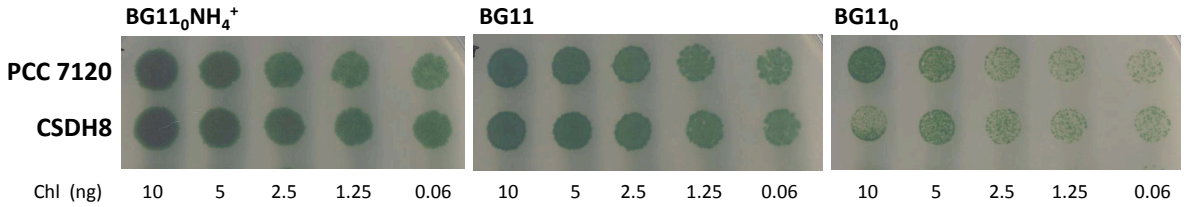

Figure 16: Growth tests in solid medium. *Anabaena* sp. strains PCC 7120 and CSDH8 were incubated with ammonium ($BG11_0NH_4^+$), nitrate (BG11) or N_2 ($BG11_0$) as the nitrogen source. Each spot was inoculated with an amount of cells containing the indicated amount of Chl, and the plates were incubated under culture conditions for 7 days and photographed.

Table 2: Growth rate constants in *Anabaena* sp. strains PCC 7120 (wild type) and CSDH8 (Δ*alr4995*).

	Growth rate, μ (day^{-1})		
	BG11$_0$NH$_4^+$	**BG11**	**BG11$_0$**
PCC 7120	0.53 ± 0.16	0.86 ± 0.19	0.87 ± 0.56
CSDH8	0.56 ± 0.08	0.91 ± 0.42	0.83 ± 0.51

Figures are the mean ± standard deviation from three independent assays.

Microscopic observation of strain CSDH8 showed the presence of granulation in the cytoplasm of the cells, which was more evident in filaments grown in the presence of nitrate or combined nitrogen-free media than in the presence of ammonium, compared to the wild-type cells (Figure 17). Under diazotrophic conditions, there were no evident differences in the morphology of the heterocysts between the CSDH8 and the wild type; both strains presented mature heterocysts with their characteristic cyanophycin granules at the heterocyst poles.

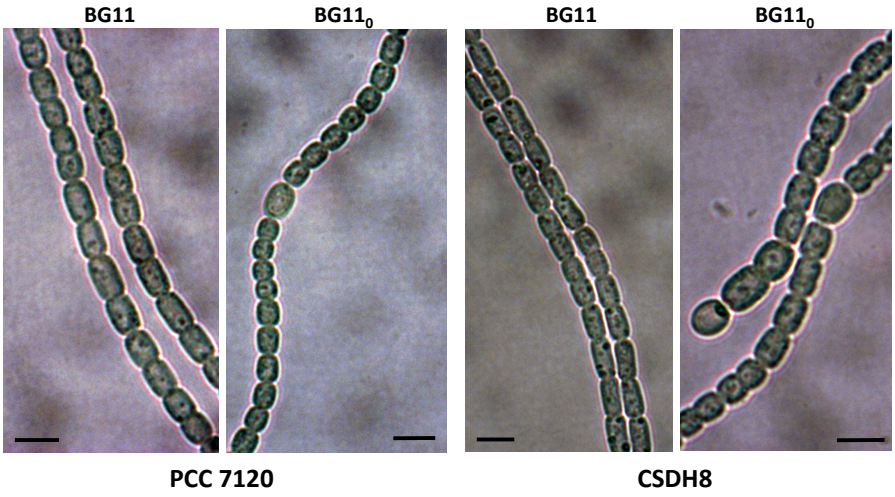

Figure 17: Filaments of *Anabaena* wild type (PCC 7120) and strain CSDH8 from cultures incubated for 5 days in BG11 or $BG11_0$ medium and visualized by light microscopy. Scale bars, 5 μm.

Arginine catabolism

Wild type *Anabaena* and strain CSDH8 were used in uptake assays with [^{14}C]arginine, and the fate of arginine was studied by TLC analysis of the radiolabeled compounds produced in filaments that had been incubated in BG11 or $BG11_0$ medium. Arginine was taken up at appreciable levels in filaments of both strains from either BG11 or $BG11_0$ medium (Table 3). After 30 min of incubation the amount of radioactivity that remained associated to arginine accounted for only 30.4 % and 47.2 % in cells of strain PCC 7120 from BG11 and $BG11_0$ medium, respectively, indicating that arginine was metabolized. However, in strain CSDH8 the amount of radioactivity associated to arginine remained at higher levels than in the wild type, 65.2% and 93.4 %, in cells from BG11 and $BG11_0$ medium, respectively, indicating that arginine catabolism is impaired in this mutant (Table 3).

The distribution of radioactivity among metabolites present in the soluble fractions of the cells was analyzed by TLC and electronic autoradiography as described in Materials and methods. In the suspensions of wild-type filaments from BG11 medium (Figure 18, Table 3), after 10 min of incubation, radioactivity from [^{14}C]arginine was mainly distributed among proline (8.0 %), glutamate (11.2 %) and glutamine/citrulline (5.1 %), which overlap in the TLC system of solvents used in this study. Other metabolites, such as ornithine and aspartate, could be also identified, but their accumulation was much lower than that of the previously mentioned metabolites. After 30 min, a notable accumulation of radioactivity in glutamate (32.5 %), ornithine (7.5 %) and proline (7.3 %) was observed, and glutamine/citrulline remained at similar levels (5.0 %) than after 10 min of incubation.

Remarkably, in strain CSDH8, radioactivity from [^{14}C]arginine was almost totally accumulated in arginine after 10-min of incubation, and no ornithine, proline or glutamate was detected (Figure 19). After 30-min of incubation, radioactivity was mainly distributed among ornithine (19.8 %), glutamine/citrulline (2.6%), aspartate (1.5 %) and a few unidentified spots that accounted for less than 2.2 % of radioactivity (spots #8, #9 and #10). Neither proline nor glutamate were produced in strain CSDH8 (Figure 19; Table 3). It has to be mentioned that glutamine is synthetized from glutamate, and then, the accumulation of radioactivity found in the spot glutamine/citrulline likely corresponds solely to citrulline. A spot in the same position as ornithine accounting for 6.3 % of radioactivity was detected with the 30-min samples from boiled filaments, used as a control (Figure S1).

Table 3: L-[^{14}C]arginine uptake and metabolic products in *Anabaena* sp. PCC 7120 (wild type) and CSDH8 (Δ*alr4995*).

	BG11				**BG11$_0$**			
	PCC 7120		**CSDH8**		**PCC 7120**		**CSDH8**	
	10′	**30′**	**10′**	**30′**	**10′**	**30′**	**10′**	**30′**
Arginine taken up (nmol· [mg Chl]$^{-1}$)	45.0	88.1	43.1	77.8	51.0	102.3	47.4	95.8
Labeled compounds (%)								
Origin	3.5	9.6	3.4	5.2	7.3	14.0	1.9	1.2
Arginine	56.2	30.4	96.5	65.2	64.4	47.2	94.1	93.4
Ornithine	0.3	7.5		19.8	2.1	7.7		4.1
Proline	8.0	7.3			4.9	5.9		
Glutamate	11.2	32.5			2.1	10.0		
Glutamine/Citrulline	5.1	5.0		2.6	4.3	4.2		
Aspartate	1.0	2.3		1.5	1.5	2.4		
#8	1.9	2.4		2.2	4.6	3.5		
#9	5.1	2.9		1.5	4.0	2.3		
#10	1.3			2.0	1.8	2.7		1.3
#11 (Agmatine)	1.0				1.5			
#12	5.4				1.5			
#13							4.0	

Filaments of the wild type and mutant strains grown in BG11 medium and incubated for 24h in BG11 or BG11$_0$ medium were used at 5 to 10 μg Chl·ml^{-1} in assays of uptake of 1 μM L-[^{14}C]arginine as described in Materials and methods. After 10 and 30 min of incubation, uptake of arginine was determined and metabolites in the suspensions were analyzed by TLC. Cell-associated metabolites are presented as the percentage of the sum of radioactivity in ^{14}C-labeled chromatographic spots after subtraction of extracellular arginine. Compounds that do not move with the used solvents accumulate in the origin of the chromatography.

The patterns of [^{14}C]arginine-derived products in the suspensions of filaments that had been incubated in BG11$_0$ medium were similar to those found in the filaments from BG11 medium. In general terms, the filaments of both strains from BG11$_0$ medium contained lower levels of cell-associated radioactive products of arginine catabolism than BG11-grown filaments (Figures 17 and 18; Table 3). Importantly, no accumulation of arginine-derived products was found in strain CSDH8 at 10 min (Figure 19). The results showing the absence of arginine-derived products in strain CSDH8 indicate that inactivation of *alr4995* prevents the production of proline and glutamate from [^{14}C]arginine. It has also to be noted, however, that there was a slow production of ornithine both in wild-type and CSDH8 strains observed at 30 min of incubation. We do not know whether this production has a biological origin, since it was also observed in the boiled filaments used as controls (Figure S1).

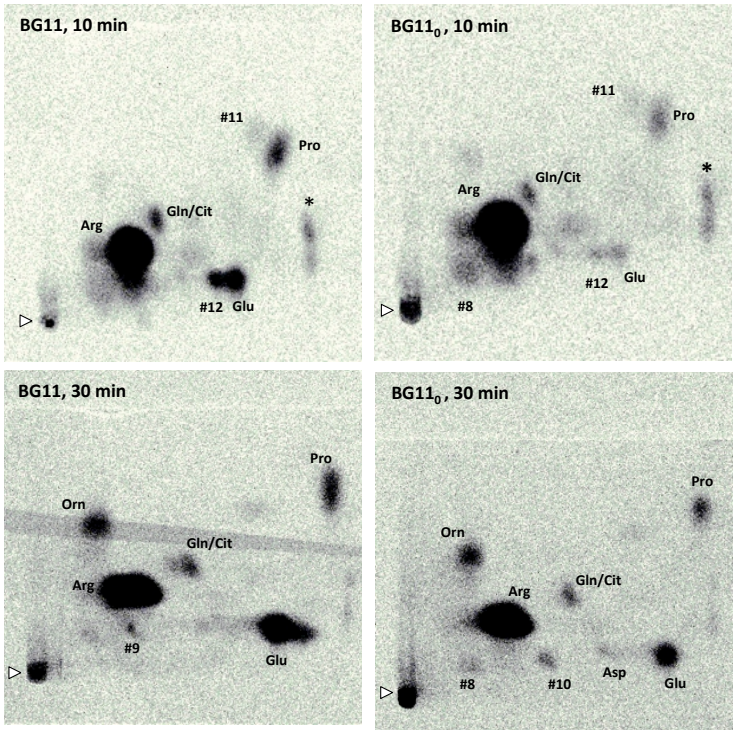

Figure 18: Production of ^{14}C-labeled metabolites from L-[^{14}C]arginine in filaments of *Anabaena* wild type (PCC 7120) grown in BG11 medium and incubated in BG11 and BG11$_0$ medium for 24 h. Suspensions of filaments containing 5 to 10 µg of Chl·ml^{-1} were incubated for 10 and 30 min with 1 µM L-[^{14}C-(U)]arginine. Metabolites in the cell suspensions were extracted and analyzed by TLC and autoradiography as described in Materials and methods. The amino acids identified were: Arg, Cit, Pro, Glu, Gln, Orn, Asp and agmatine (#11). Unidentified spots, indicated as #8, #9, #10 and #12 were also detected, although spot #12 could be part of the glutamate spot. Note that the glutamine and citrulline spots overlap. White triangles point to the origin of the chromatography. Asterisks (*) mark a spot found in the commercial arginine solution.

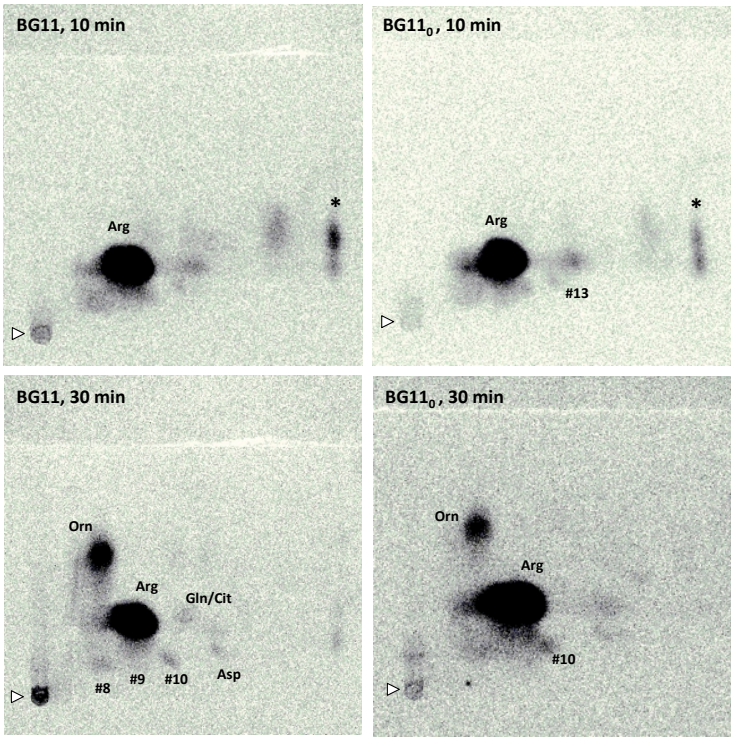

Figure 19: Production of ^{14}C-labeled metabolites from L-[^{14}C]arginine in filaments of strain CSDH8 (Δ*alr4995*) grown in BG11 medium and incubated in BG11 and BG11$_0$ medium for 24 h. Suspensions of filaments containing 5 to 10 µg of Chl·ml^{-1} were incubated for 10 and 30 min with 1 µM L-[^{14}C-(U)]arginine. Metabolites in the cell suspensions were extracted and analyzed by TLC and autoradiography as described in Materials and methods. The amino acids identified and unidentified spots (#8, #9 and #10) are indicated. Note that the glutamine and citrulline spots overlap. White triangles point to the origin of the chromatography. Asterisks (*) mark a spot present in the commercial arginine solution.

Ornithine catabolism

Once the [^{14}C]arginine-derived products had been analyzed in the studied strains, we asked whether the same products observed in arginine catabolism could be observed from labeled ornithine, a possible product of arginine through an arginase-like pathway. For this, wild type *Anabaena* and the mutant strain were used in uptake assays with [1-^{14}C]ornithine, and the fate of ornithine was studied by TLC of the radiolabeled compounds produced in filaments that had been incubated in BG11 or $BG11_0$ medium. Ornithine was taken up at rates similar to [^{14}C]arginine (Table 4). After 30 min of incubation, ornithine had been metabolized substantially in both strains, since the amount of radioactivity that remained associated to ornithine accounted for 31.4 % in the wild type and 37.4 % in CSDH8 mutant in cells from BG11 medium, and 31.8 % and 23.9 %, respectively, in cells that had been incubated in $BG11_0$ (Table 4).

Table 4: L-[1-^{14}C]ornithine uptake and metabolic products in *Anabaena* sp. PCC 7120 (wild type) and CSDH8 (Δ*alr4995*).

	BG11				**$BG11_0$**			
	PCC 7120		**CSDH8**		**PCC 7120**		**CSDH8**	
	10′	**30′**	**10′**	**30′**	**10′**	**30′**	**10′**	**30′**
Ornithine taken up (nmol· [mg Chl]$^{-1}$)	59.9	110.8	56.8	94.6	59.9	92.5	55.3	99.4
Labeled compounds (%)								
Origin	4.4	13.7	3.3	2.9	4.9	7.5	2.7	2.1
Ornithine	55.8	31.4	53.5	37.4	58.7	31.8	43.9	23.9
Proline	7.1	7.6			11.4	12.3		
Glutamate	19.9	37.4			8.8	24.9		
Glutamine/Citrulline	4.1	5.8	19.0	27.3	10.7	9.9	27.2	54.5
Arginine	8.7	4.1	10.7	32.3	5.4	13.4	3.9	19.5
#9			7.5				8.4	
#10			6.0				8.2	
#11							5.8	

Filaments of the wild-type and mutant strains grown in BG11 medium and incubated for 24h in BG11 or $BG11_0$ medium were used at 5 to 10 μg Chl·ml^{-1} in assays of uptake of 1 μM L-[1-^{14}C]ornithine as described in Materials and methods. After 10 and 30 min of incubation, uptake of ornithine was determined and metabolites in the suspensions were analyzed by TLC. Cell-associated metabolites are presented as the percentage of the sum of radioactivity in ^{14}C-labeled spots. Percentage of ornithine corresponds to intra- and extracellular-[1-^{14}C]ornithine in the assay. Compounds that do not move with the used solvents accumulate in the origin of the chromatography.

The pattern of [1-^{14}C]ornithine-derived products in the suspensions of filaments that had been incubated both in BG11 and $BG11_0$ medium indicated a clear metabolism, as illustrated in Figures 20 and 21. In the suspensions of wild-type filaments from BG11 medium (Figure 20, Table 4), after 10 min of incubation, radioactivity from [1-^{14}C]ornithine was distributed among proline (7.1 %), glutamate (19.9 %), glutamine/citrulline (4.1 %) and arginine (8.7 %). After 30 min of incubation, proline remained at a similar level (7.6 %), and arginine decreased its levels to 4.1 %. In contrast, glutamate and glutamine/citrulline increased to 37.4 % and 5.8 %, respectively. In mutant strain CSDH8, radioactivity from [1-^{14}C]ornithine

was basically distributed among the glutamine/citrulline spot (19.0 % and 27.3 %, at 10 and 30 min of incubation, respectively) and arginine (10.7 % and 32.3 %). No production of proline or glutamate was detected in this strain, either at 10 or 30 min of incubation (Figure 21). It has to be noted the higher accumulation of arginine and the glutamine/citrulline spot in strain CSDH8 compared to the wild-type strain. Two unidentified spots, #9 and #10, that account for 7.5 % and 6.0 % of radioactivity, respectively, were also detected after 10 min of incubation. However, a spot similar to unidentified #9 was detected in the TLC from the boiled filaments used as a control (see Figure S2).

The pattern of [1-^{14}C]ornithine-derived products in the suspensions of filaments that had been incubated in $BG11_0$ medium were the same as those found in the filaments from BG11 medium. Proline (11.4 %), glutamine/citrulline (10.7 %), glutamate (8.8 %) and arginine (5.4 %) were the metabolites found derived from [1-^{14}C]ornithine in filaments from the wild-type strain at 10 min of incubation. After 30 min of incubation, the mentioned amino acids were also found, but with increased levels of radioactivity accumulation, especially in the glutamate spot (Table 4). In strain CSDH8, no production of glutamate or proline was found, and the radioactivity was mainly distributed among glutamine/citrulline (27.2 % and 54.5 % at 10 and 30 min, respectively) and arginine (3.9 % and 19.5 %). Two unidentified spots, namely #10 and #11, which accounted for 8.2 % and 5.8 % of the radioactivity , respectively, was found at 10 min of incubation. None of the unidentified spots corresponded to γ-aminobutyric acid (GABA), a metabolite related to arginine catabolism, tested by co-chromatography. In conclusion, as in the case of the [^{14}C]arginine catabolism assay, the inactivation of *alr4995* abolishes the production of glutamate and proline from [1-^{14}C]ornithine.

As mentioned above, it should be noted that, in all assays with [1-^{14}C]ornithine, the glutamine/citrulline spot should correspond to citrulline, which is synthetized from ornithine in the arginine biosynthesis pathway. Because glutamine is produced from glutamate in the glutamine synthase (GS) reaction, and glutamate is not produced from ornithine in the mutant CSDH8, radiolabeled glutamine is not expected to be found in the mutant. Using a second TLC solvent systems (see Material and methods), which permits to clearly separate the glutamine and citrulline spot, it could be confirmed that the spot detected in samples from CSDH8 strain corresponded to citrulline (data not shown).

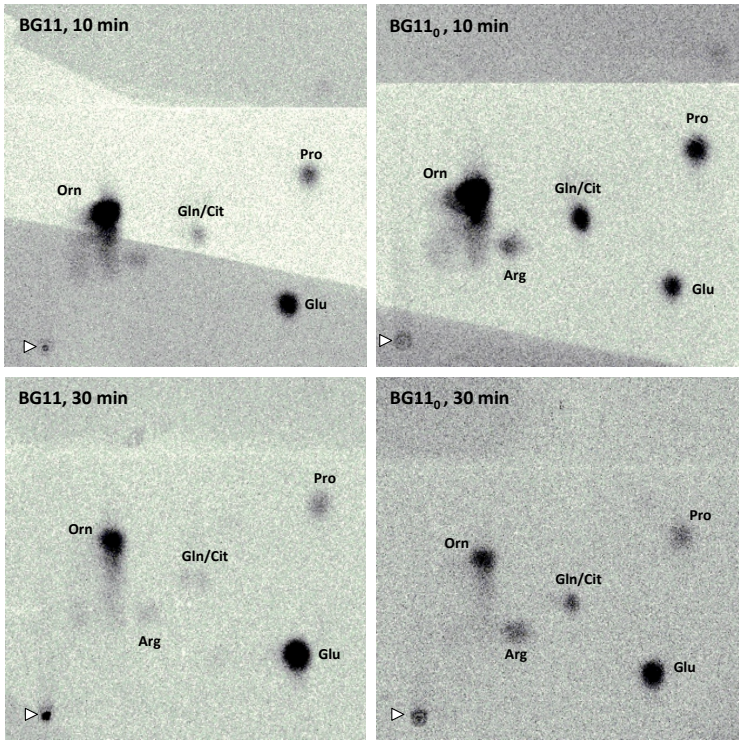

Figure 20: Production of ^{14}C-labeled metabolites from L-[1-^{14}C]ornithine in filaments of *Anabaena* wild type (PCC 7120) grown in BG11 medium and incubated in BG11 and BG11$_0$ medium for 24 h. Suspensions of filaments containing 5 to 10 µg of Chl·ml^{-1} were incubated for 10 and 30 min with 1 µM L-[1-^{14}C]ornithine. Metabolites in the cell suspensions were extracted and analyzed by TLC and autoradiography as described in Materials and methods. The amino acids identified were: Arg, Cit, Pro, Glu, Gln and Orn. Note that the glutamine and citrulline spots overlap. White triangles point to the origin of the chromatography.

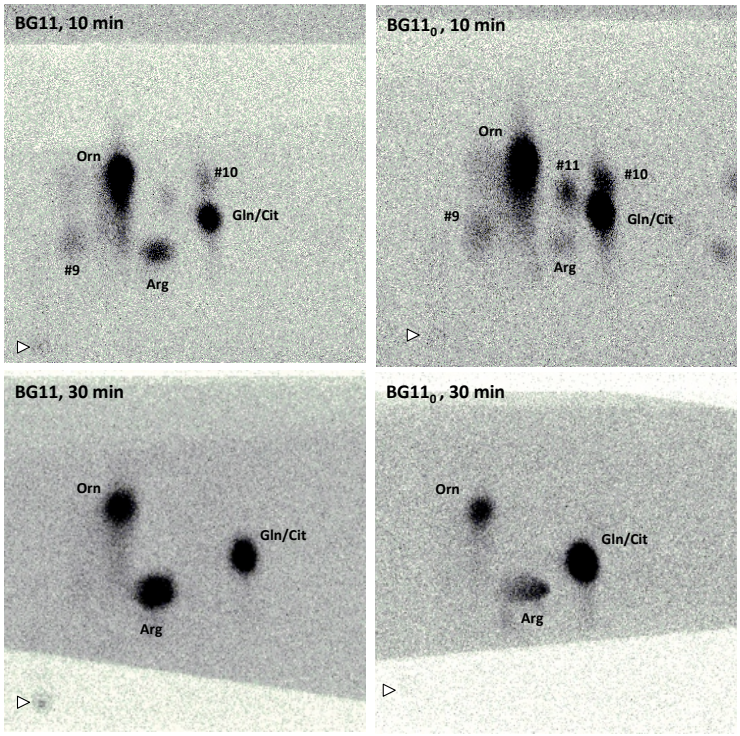

Figure 21: Production of ^{14}C-labeled metabolites from L-[1-^{14}C]ornithine in filaments of strain CSDH8 (Δ*alr4995*) grown in BG11 medium and incubated in BG11 and BG11$_0$ medium for 24 h. Suspensions of filaments containing 5 to 10 µg of Chl·ml^{-1} were incubated for 10 and 30 min with 1 µM L-[1-^{14}C]ornithine. Metabolites in the cell suspensions were extracted and analyzed by TLC and autoradiography as described in Materials and methods. The amino acids identified were: Arg, Cit, Gln and Orn. Unidentified spots, indicated as #9, #10 and #11 were also detected. Note that the glutamine and citrulline spots overlap. White triangles point to the origin of the chromatography.

Discussion

Based on bioinformatic analysis (Schriek *et al*. 2007), *alr4995* from the genome of the heterocyst-forming cyanobacterium *Anabaena* was noted as a possible L-arginine amidinotransferase as well as a possible L-arginine deiminase, both enzymes belonging to the guanidine-group modifying enzyme superfamily. Similarly, in *Synechocystis* sp. PCC 6803, ORF *sll1336* is assigned both an arginine deiminase and arginine amidinotransferase function (Schriek *et al*. 2007). Although it was not possible to distinguish the specific function of this gene by the methods used in that study, the authors favored the idea that the gene was more likely to encode an arginine deiminase than an arginine amidinotransferase, since the latter had been shown to function in antibiotic or toxin biosynthesis in different prokaryotes (Fritsche *et al*., 1998; Hernández-Guzman and Alvarez-Morales, 2001), including some cyanobacteria (Barón-Sola *et al*., 2013; Muenchhoff *et al*., 2010), rather than in arginine catabolism.

Two observations based on the results obtained in this work point to the notion that Alr4995 is the enzyme responsible for the arginine catabolic pathway producing proline and glutamate in *Anabaena*. First, the addition of [^{14}C]arginine to wild-type filaments results in production of proline, glutamate and glutamine/citrulline as the main [^{14}C]arginine-derived products, whereas mutant strain CSDH8 (Δ*alr4995*) shows an impaired catabolism. Second, whereas the addition of [1-^{14}C]ornithine to wild-type filaments produces proline, glutamate, glutamine/citrulline and also arginine, in the mutant strain no production of proline or glutamate is observed. Thus, the results obtained here suggest that Alr4995 catalyzes not only the conversion of arginine to proline, but also the conversion of ornithine to proline. Therefore, ornithine may function as an intermediate metabolite in the reaction catalyzed by Alr4995, which may have two catalytic domains as suggested in Shirai *et al*. (2006). The origin of ornithine in arginine catabolism could then be due to the bifunctional nature of the enzyme. In turn, when [1-^{14}C]ornithine is added, proline, glutamate, glutamine/citrulline and arginine are the products in the wild type, whereas in the mutant only citrulline and arginine are produced and their accumulation is increased compared to the wild type. These metabolites could result from anabolic reactions in the mutant, as has been also described in *Synechocystis* sp. PCC 6803 (Quintero *et al*., 2000). Citrulline could be synthetized from ornithine by ornithine carbamoyltransferase, the product of the gene *argF*, and citrulline is converted successively to argininosuccinate and arginine by argininosuccinate synthase and argininosuccinate lyase, respectively (Cunin *et al.,* 1986). Thus, the accumulation of arginine and, probably, citrulline in mutant strain CSDH8 could take place through the arginine biosynthetic pathway. Argininosuccinate is an intermediate metabolite that accumulates at low levels and this could be the reason for not clearly detecting it in the TLC assays (Quintero *et al*., 2000). Nonetheless, unidentified spot #9 in Figures 17 and 18 could correspond to argininosuccinate (Quintero, 2000; Quintero *et al*., 2000).

An alternative for the first step of the reaction catabolized by Alr4995 would be production of citrulline instead of ornithine. In this case, the [1-^{14}C]ornithine observed in arginine catabolism could result from the action of ornithine transcarbamoylase on citrulline. In a similar way, production of proline from [1-^{14}C]ornithine could proceed via citrulline synthetized by ornithine transcarbamoylase (Figure 22). Further studies with and *argF* mutant will be needed to clarify the nature of the intermediate in the reaction.

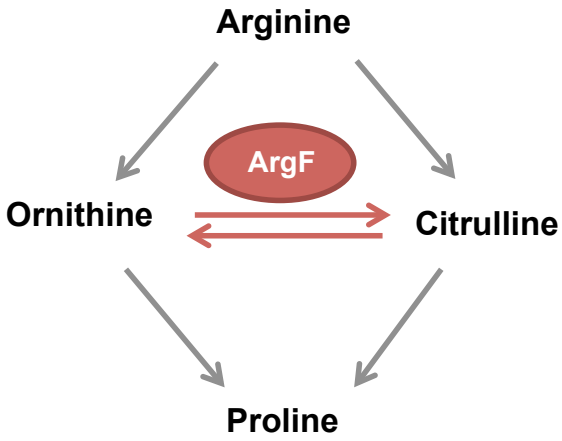

Figure 22: Production of proline from arginine by Alr4995 may proceed via ornithine or citrulline.

The second part of an arginase-like pathway, the generation of glutamate from ornithine, has been studied in *Synechocystis* sp. PCC 6803, and enzymes Δ^1pyrroline-5-carboxylate reductase and proline oxidase, encoded by *proC* and *putA*, respectively, have been identified and characterized in this cyanobacterium (Quintero *et al*., 2000). Inactivation of *putA* results in an accumulation of proline without production of glutamate (Quintero *et al*., 2000). Studies of the *putA* gene in *Anabaena* showed that its inactivation results in a phenotype similar to that of *Synechocystis* (Picossi, 2003). In addition, inactivation of proline oxidase in *Anabaena* affects diazotrophic growth, and expression of *putA* is localized in vegetative cells (Picossi, 2003). Inactivation of *proC* in *Synechocystis* results in a reduction of the accumulation of glutamate and glutamine but, unexpectedly, only in a partial reduction in proline accumulation (Quintero *et al*., 2000). Then, if arginine catabolism in *Synechocystis* is similar to that in *Anabaena*, the production of proline observed in *proC* mutants could come from the arginine catabolized by the homolog of Alr4995 in *Synechocystis* (Sll1336), which would generate proline from arginine. In summary, the results obtained in this study could not confirm or distinguish if *alr4995* encodes an arginine deiminase, an arginine amidinotransferase or an arginase, but they suggest that Alr4995 is a novel enzyme that generates proline from arginine in a two-step reaction: the first reaction involving the conversion of arginine to ornithine or citrulline, and the second reaction involving the production of proline from ornithine or citrulline. In addition, the novel enzyme described in this study also implies a new arginine catabolism route consisting of Alr4995 protein and the *putA* gene product, proline oxidase. Further investigation of *alr4995* and generation of mutants with deletions of its distinct protein domains will be needed in order to characterize in detail the biochemical reaction carried out by Alr4995.

Supplementary material

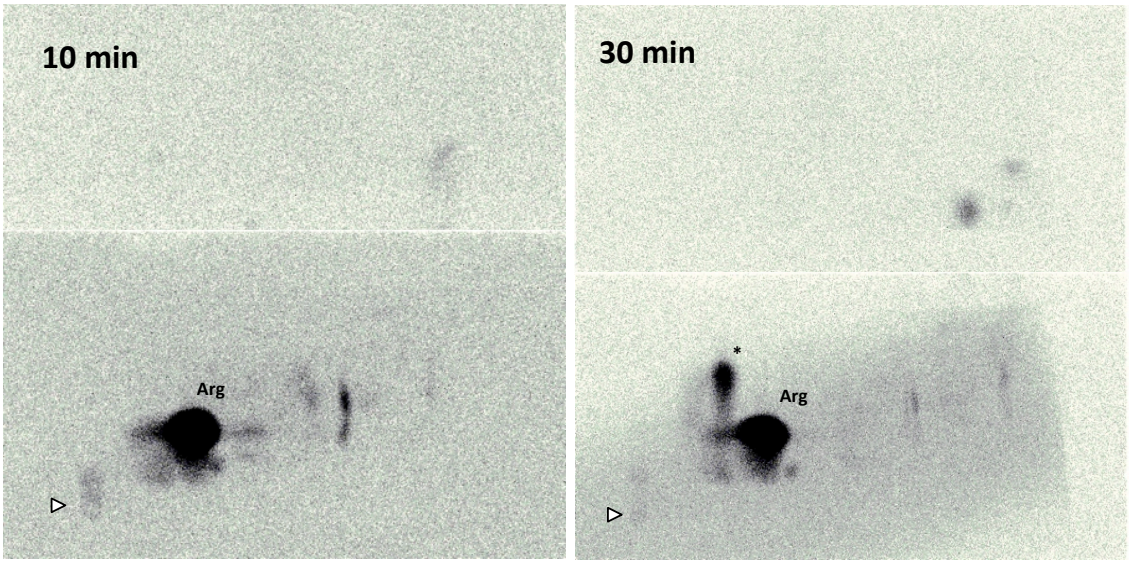

Figure S1: Production of ^{14}C-labeled metabolites from L-[^{14}C]arginine in boiled filaments (used as a control) in the [^{14}C]arginine uptake and catabolism assay. Filaments were treated at 100ºC during 5-10 min before starting the assay and were incubated for 10 and 30 min with 1 µM L-[^{14}C-(U)]arginine. Metabolites in the cell suspensions were extracted and analyzed by TLC and autoradiography as described in Materials and methods. As expected, a spot detected with boiled filaments was the labeled substrate [^{14}C]arginine. Unexpectedly, a spot that coincides with ornithine was detected at 30 min (asterisk in the right panel). White triangles point to the origin of the chromatography.

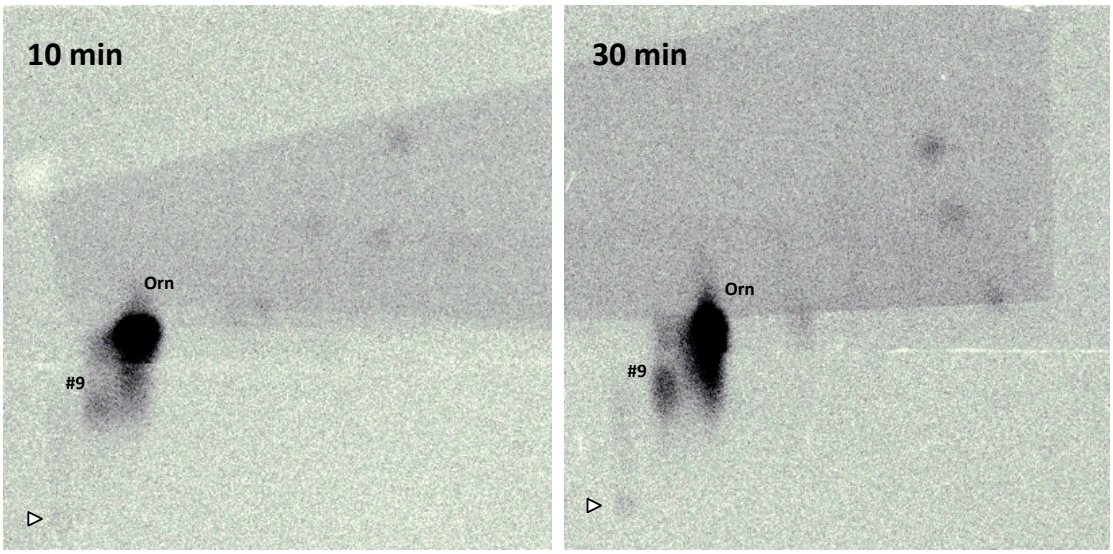

Figure S2: Production of ^{14}C-labeled metabolites from L-[1-^{14}C]ornithine in boiled filaments (used as a control) in the [1-^{14}C]ornithine uptake and catabolism assay. Filaments were treated at 100ºC during 5-10 min before starting the assay and were incubated for 10 and 30 min with 1 µM L-[1-^{14}C-(U)]ornithine. Metabolites in the cell suspensions were extracted and analyzed by TLC and autoradiography as described in Materials and methods. As expected, a spot detected with boiled filaments was the labeled substrate [1-^{14}C]ornithine. Unexpectedly, a spot that coincides with spot #9 (in Figure 21) was also detected. White triangles point to the origin of the chromatography.

General discussion

Recently published information has shown that proteins found in the septa of *Anabaena* sp. PCC 7120, such as SepJ, FraC and FraD, play an essential role in cell-cell adhesion, being indispensable for the integrity of long filaments (Flores *et al*., 2007; Merino-Puerto *et al*., 2010; Mariscal *et al*., 2011). However, cyanobacteria present a continuous outer membrane (OM) along the filament, whose role in filamentation, the process of producing and maintaining filaments, has not been asked before. This work has approached this question through the analysis of *Anabaena* strains with mutations in genes encoding proteins involved in the biosynthesis of the cell envelope.

We examined a collection of previously available peptidoglycan (PG) and OM mutants of *Anabaena* sp. PCC 7120 and mutants of two genes encoding class B PBPs, *alr5045* and *alr0718*, isolated in this work. The PG mutants included mutants of *alr0093* (encoding the amidase *hcwA*/*amiC2*), *alr2458* (encoding an alanine racemase), *all1861* (encoding a PG-binding protein) and the two genes encoding class B PBPs, *alr5045* and *alr0718*. Regarding OM mutants, these included mutants of three genes, *alr2269*, *alr0075* and *alr4893* encoding Omp85-like proteins, *alr2270* encoding *lpxC* involved in lipid A biosynthesis, and three genes involved in antigen 0 biosynthesis, *all4828*, *all4829* and *all4830*. The PG metabolism-related proteins Alr5045 and N-acetylmuroamoyl-L-alanine amidase (HcwA/AmiC2), and the lipid A biosynthesis protein Alr2270 have been found to have an important role in filamentation, since their inactivation leads the cyanobacterium to form short filaments, and among them, although all investigated strains were somewhat affected, inactivation of Alr5045 and Alr2270 resulted in filaments very sensitive to mechanical fragmentation. Thus, cell envelope components both of the PG and OM contribute to filamentation in *Anabaena* sp. PCC 7120. The function of PG in filamentation may be related to the fact that it is a macromolecule that encompasses several cells along the filament, but how the OM contributes to filamentation is unknown. It is possible, however, that the continuous of molecular non-covalent interactions in the OM provides a strength sufficient to increase filament stability.

Regarding properties of the OM, our results show that inactivation of either OM proteins or LPS (both O antigen and lipid A) biosynthesis proteins affects its permeability function. Thus, inactivation of these components results, to a higher or lesser extent, in an increase of the activities of transport of acidic amino acids and in an increased sensitivity to harmful compounds, indicating that the mutants bear an altered OM. Some PG mutants also show sensitivity to toxic compounds, concretely to SDS, which in turn may imply a malformation of their OM that could result from an impairment in its anchoring to an altered PG.

Although some cell envelope mutants are affected in the length and/or strength of the filaments that they produce, cell envelope components are not as important as the septal proteins, such as SepJ or FraCD, for filamentation, since inactivation of the genes encoding these septal proteins leads to a more severe phenotype of fragmentation. Thus, septal proteins, and notably SepJ, are indispensable for filamentation, but cell envelope components, such as the PG and Lipid A have a role in helping to maintain long filaments. The maintenance of filament integrity is an essential structural aspect of multicellularity, especially during

diazotrophic conditions, in which intercellular molecular exchange is needed for the growth of the filament as an organismic unit. Moreover, besides intercellular exchange via septal joining complexes, compounds could also be transferred via the continuous periplasm of filamentous cyanobacteria. In this case, the maintenance of long filaments and the delimitation of the periplasm as a continuous conduit would structurally require a continuous OM.

Anabaena Alr5045 is homologous to *E. coli* PBP2 (Leganés *et al*., 2005), which is involved in cell elongation (Typas *et al*., 2012), whereas Alr0718 likely represents the *Anabaena* ortholog of *E. coli* FtsI/PBP3 (Kaneko et al., 2001), a protein specifically involved in septal PG synthesis during cell division (Typas *et al*., 2012). The *alr0718* mutant (strain CSMI24) could not be segregated, meaning that *alr0718* is an essential gene, as is the case of the *ftsI* ortholog of the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 (Marbouty *et al*., 2009). The observations of an altered morphology of vegetative cells in the *alr5045* mutant (strain CSMI23), which are shorter and wider than those of the wild type, likely as a result of a deficiency in cell elongation, and the labeling of PG with fluorescent vancomycin in both strains, which is increased in the septa of the *alr5045* mutant (strain CSMI23) and decreased in the septa of the *alr0718* mutant (strain CSMI24) compared to the wild-type, support a role in PG synthesis during cell elongation and septation, respectively. It is known that in a given organism PBPs are often redundant and functionally overlapping. As a result, a mutation in a single PBP-encoding gene often does not produce a phenotype different from that of the wild-type strain. Indeed, some mutants lacking multiple PBPs could be still viable. It is possible that the role of Alr5045 in cell elongation can be fulfilled at least in part by other PBPs, whereas no other protein can substitute for Alr0718. Inactivation of some other PBPs results in altered cellular morphology, both of vegetative cells and heterocysts (Lázaro *et al*., 2001; Lehner *et al*., 2011). Strain CSMI23 also shows a delay in heterocyst maturation, which could be related with the low nitrogenase activity detected in this mutant. Other PBPs in *Anabaena* sp. PCC 7120 have also been shown to be required for diazotrophic growth, since their inactivation leads to an impaired aerobic N_2 fixation (Lázaro *et al*., 2001; Zhu *et al*., 2001; Leganés *et al*., 2005; Berendt *et al*., 2012), highlighting the role of PG metabolism-related enzymes during heterocyst differentiation, probably for PG septal remodeling for the correct deposition of the Hep and Hgl envelope layers.

Cyanophycin is a distinctive feature of cyanobacteria, and heterocysts are characterized by a polar accumulation of this polymer. It has been shown that, as a structural element, this accumulation of the cyanophycin granule polypeptide (CGP) at the heterocyst poles, possibly together with the narrow septa between vegetative cells and heterocysts, reduces the metabolite exchange rate between vegetative cells and heterocysts with regard to that measured between vegetative cells (Mullineaux *et al*., 2008). Besides being a structural element of heterocysts, cyanophycin plays an important metabolic function as a nitrogen reservoir during diazotrophic growth. Cyanophycin is synthetized by a single enzyme, cyanophycin synthetase and, under N starvation conditions, CGP is degraded by the consecutive activities of cyanophycinase, which hydrolyzes the CGP releasing the dipeptide β-aspartyl-arginine, and isoaspartyl dipeptidase, that hydrolyzes this dipeptide releasing its

constituent amino acids, arginine and aspartate. This work aimed at increasing our understanding of the role of cyanophycin in the diazotrophic physiology of *Anabaena*, including the possible role of its derivative products as nitrogen vehicles in the diazotrophic filament.

Previous studies detected high activities of cyanophycin synthetase and cyanophycinase in heterocysts (Gupta and Carr, 1981a), which is consistent with the role of cyanophycin as a dynamic reservoir of nitrogen in these differentiated cells (Carr, 1983). The results obtained here have shown that the second enzyme involved in cyanophycin degradation, isoaspartyl dipeptidase, which in *Anabaena* sp. PCC 7120 is encoded by *all3922*, accumulates in vegetative cells during diazotrophic conditions, as demonstrated by a GFP fusion and determination of its enzymatic activity. This result clarifies important aspects of the physiology and metabolism of diazotrophic cyanobacteria. Because cyanophycinase is highly active in heterocysts (Gupta and Carr, 1981a) and isoaspartyl dipeptidase accumulates in vegetative cells, cyanophycin degradation in filamentous cyanobacteria is compartmentalized, with the first step occurring in the heterocysts and the second step taking place in the vegetative cells (Figure 23). This compartmentalization of cyanophycin degradation identifies β-aspartyl-arginine (the product of hydrolysis of cyanophycin by cyanophycinase) as a nitrogen vehicle from heterocysts to vegetative cells, as corroborated by the substantial amounts of the dipeptide found in the supernatants of isolated heterocyst suspensions. Over the last decades, besides glutamine (Wolk *et al*., 1974; Thomas *et al*., 1977), arginine has been suggested to feed vegetative cells, although its role as a nitrogen vehicle had never been confirmed. Only recently, Ke and Haselkorn (2013) proposed that arginine was the principal nitrogen carrier since they noted a gradient of arginine (or of an arginine-containing compound) in diazotrophic filaments of *Anabaena* sp. PCC 7120. The method used in that study was based on the treatment of filaments with the Sakaguchi reagent and measuring the resulting quenching of fluorescence from cells. Taking into account that the Sakaguchi reagent reacts with arginine, both free and combined, the β-aspartyl-arginine could also have been detected by this method. Our results confirm that arginine can be transferred from heterocysts to vegetative cells, but the most part of it is transferred together with aspartate in the form of the dipeptide. Hence, it appears that, in the diazotrophic filament, the nitrogen carriers responsible to feed the vegetative cells are glutamine, arginine and aspartate, these last two in the form of the dipeptide. This is also consistent with the results of growth tests performed with two Fox^- mutants. When a mixture of arginine, aspartate and glutamine is supplied as a nitrogen source, both mutants show a growth that is more robust than if only arginine, glutamine or arginine and glutamine is supplied, which is consistent with the results identifying β-aspartyl-arginine as a nitrogen vehicle. Thus, intercellular nutrient exchange in diazotrophic filaments of heterocyst-forming cyanobacteria involves the transfer of reduced carbon in the form of glutamate, alanine and sucrose from vegetative cells to heterocysts (see Introduction), and the transfer of fixed nitrogen in the form of glutamine and β-aspartyl-arginine from heterocysts to vegetative cells. Although a new nitrogen vehicle has been identified in this work, further research will be needed to understand the mechanism of release of the β-aspartyl-arginine from heterocysts and its transfer to vegetative cells.

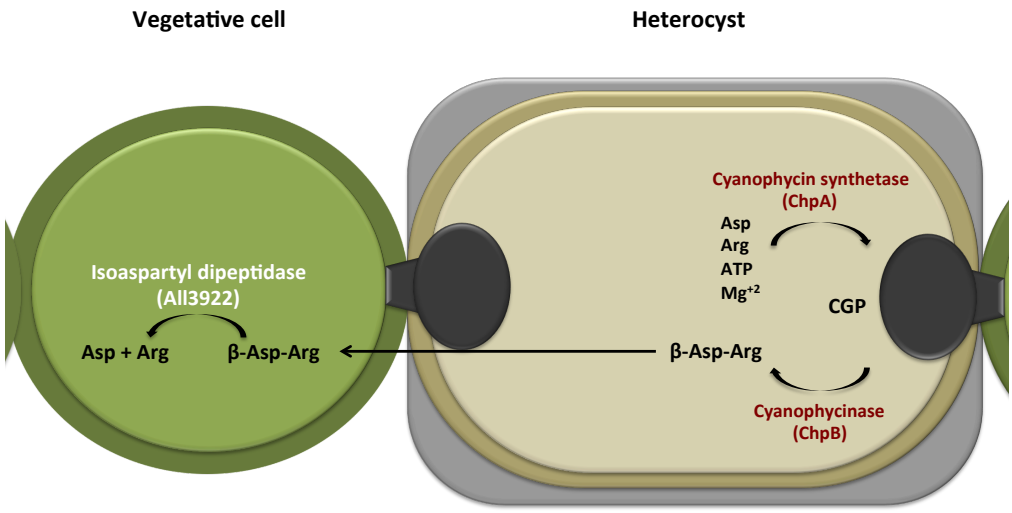

Figure 23: Compartmentalization of cyanophycin metabolism in *Anabaena* sp. PCC 7120. Under N limitation, cyanophycin is synthetized in heterocysts by cyanophycin synthetase (CphA) and accumulated in the form of CGP. In heterocysts, cyanophycinase (CphB) hydrolyses CGP producing β-aspartyl-arginine. The β-aspartyl-arginine dipeptide represents a nitrogen carrier that is transferred from heterocysts to vegetative cells, where it is hydrolyzed by isoaspartyl dipeptidase, releasing aspartate and arginine.

Inactivation of *all3922* encoding isoaspartyl dipeptidase (in strain CSMI6) results in an accumulation of cyanophycin and in a reduced growth rate under diazotrophic conditions. It is known that the constituent amino acids of cyanophycin, arginine and aspartate, inhibit cyanophycinase (Gupta and Carr, 1981a). Therefore, it is possible that high levels of β-aspartyl-arginine also inhibit cyanophycinase and impair cyanophycin degradation leading to extensive accumulation of CGP. Furthermore, the reduced growth of strain CSMI6 under diazotrophic conditions is similar to that of the cyanophycinase (*cphB*) mutant of *Anabaena* sp. PCC 7120, whereas the cyanophycin synthetase (*cphA*) mutant only shows a slightly decrease in its growth rate constant (Picossi *et al*., 2004). Hence, the results obtained here are in agreement with the notion that, although cyanophycin synthesis is not necessary for growth, once synthetized, this polymer has to be degraded for optimal growth under diazotrophic conditions (Ziegler *et al*., 2001; Picossi *et al*., 2004). This also implies that, if cyanophycin synthesis is not feasible, arginine may be transferred from heterocysts to vegetative cells. In agreement with this, we have observed that isolated heterocysts can also release arginine and aspartate.

Synthesis of cyanophycin can be considered an optimized way to confine products of nitrogen fixation, which have been found to include arginine and aspartate (Wolk *et al*., 1976), and thus to avoid possible negative feedback effects on nitrogenase (Carr, 1983; Haselkorn, 2007; Ke and Haslekorn, 2013). This strategy seems to be also present in filamentous non-heterocyst-forming and unicellular nitrogen-fixing cyanobacteria, which temporally separate photosynthesis and N_2 fixation, since they accumulate cyanophycin as they carry out N_2 fixation during the dark periods, thus removing soluble products of nitrogen fixation from the cytoplasm (Li *et al*., 2001; Finzi-Hart *et al*., 2009). The compartmentalized metabolism of

cyanophycin shown in this work represents a further optimized way of using this nitrogen reservoir in heterocyst-forming cyanobacteria since besides avoiding inhibition of nitrogenase, it serves intercellular transfer functions as discussed above. The compartmentalized cyanophycin metabolism can therefore be seen as an evolved strategy that increases the efficiency of the N_2 fixation process in multicellular heterocyst-forming cyanobacteria.

The metabolism of arginine in cyanobacteria is of particular interest since the urea cycle contributes significantly to CO_2 fixation in these organisms and because they characteristically contain, as above discussed, cyanophycin. Given that the end product of the arginase pathway, glutamate, is an important amino acid for the physiology of cyanobacteria, this pathway might be an important arginine catabolic route in these organisms. Although some studies point to the existence of arginase activity (Gupta and Carr, 1981b; Schriek *et al*., 2007), the gene encoding an arginase has remained unidentified in *Anabaena* sp. PCC 7120.

ORF *alr2310* from the *Anabaena* genome putatively encodes an ureohydrolase family protein that, based on bioinformatic analysis, has been proposed to be the arginase of *Anabaena* (Schriek *et al*. 2007). However, the results presented here on the inactivation of *alr2310* (mutant CSMI11) clearly show that *alr2310* encodes an agmatinase, as demonstrated by the accumulation of agmatine and the absence of this enzymatic activity in the mutant cells. Indeed, the hypothesis of Alr2310 having both enzymatic activities, arginase and agmatinase, is not sustained, since inactivation of *alr2310* not only does not impair the production of proline and glutamate from radiolabeled arginine but, instead, increases production of glutamate as compared to the wild type. The presence of agmatinase implies the function of arginine decarboxylase, the product of ORF *all3401*, in *Anabaena* sp. PCC 7120 (see Figure 13).

Strain CSMI11 (the *alr2310* mutant) is severely impaired in diazotrophic growth, produces immature heterocysts and shows negligible nitrogenase activity. It has been noted that agmatine can inhibit bacterial growth through competitive inhibition of amino acid transport or interference with charging tRNAs resulting in a inhibition of protein synthesis (Griswold *et al*. 2006). Although strain CSMI11 shows a slight inhibition of general protein synthesis, as demonstrated by the incorporation of radiolabeled leucine into macromolecules, this seems not to be enough to explain the growth impairment of strain CSMI11. Instead, impairment of diazotrophic growth in the mutant strain could be due, at least partly, to the fact that CSMI11 produces immature heterocysts, which could result from interference of agmatine with heterocyst differentiation. Cells of strain CSMI11 accumulated CGP when growing with a source of combined nitrogen. CGP is visible by the extensive granulation observed in the cytoplasm, meaning that cyanophycin degradation is blocked. As previously discussed, arginine and aspartate inhibit cyanophycinase (Gupta and Carr, 1981a). As agmatine is highly similar to arginine, one hypothesis could be that accumulation of CGP in CSMI11 results from an inhibitory effect of agmatine on cyanophycinase. Another hypothesis could be that an incorporation of agmatine into cyanophycin at significant levels, as it has been found for amino acids such as lysine, glutamate, citrulline and ornithine (Merritt *et al*. 1994; Ziegler *et al*.

1998; Berg *et al*. 2000; Aboulmagd *et al*. 2001), resulted in agmatine-containing cyanophycin that cyanophycinase is incapable to hydrolyze. Although the results of this study cannot elucidate the general mechanism of agmatine toxicity in *Anabaena* sp. PCC 7120, its important contribution is the identification of *alr2310* as the *speB* gene encoding agmatinase in this organism.

Once the enzymatic function of ORF *alr2310* was established, we focused on another possible arginine degrading enzyme, which belongs to a protein family that also could lead to missannotated functions. This is the case of arginine deiminases and arginine amidinotransferases, both belonging to the guanidine-group modifying enzymes superfamily, which, according to a bioinformatic analysis, could be encoded by ORF *alr4995* in the genome of *Anabaena* sp. PCC 7120 (Schriek *et al*., 2007). The study presented here, inactivating *alr4995* (strain CSDH8) and performing catabolic assays using radiolabeled arginine, shows that Alr4995 is involved in the production of proline and glutamate from arginine in *Anabaena* sp. PCC 7120. Unexpectedly, strain CSDH8 is also impaired in the production of proline and glutamate when radiolabeled ornithine is added, implying that Alr4995 also carries out a second reaction converting ornithine to proline. Thus, ornithine may function as an intermediate metabolite in the reaction catalyzed by Alr4995. However, as discussed in Annex II, the intermediate metabolite of the reaction carried out by Alr4995 could not be confirmed here to be ornithine, since citrulline could also function as an intermediate, with ornithine transcarbamoylase, the product of *argF* (encoded by *alr4907*), producing citrulline from ornithine. As previously mentioned, Gupta and Carr (1981b) detected arginase and a low level of arginine deiminase activity in cell free extracts from several *Anabaena* strains. The method used to measure these enzymatic activities was to quantify the urea and citrulline released in these reactions, respectively. Attempting to conciliate their results with ours, it is possible that these compounds are also released during the two-step reaction carried out by Alr4995.

Strain CSDH8 grows properly when using nitrate, ammonium or N_2 as the nitrogen source, and heterocysts do not show any apparent defect or delay in maturation. Thus, it seems that *alr4995* is not an essential gene for the growth of *Anabaena sp*. PCC 7120. However, vegetative cells of the CSDH8 mutant grown with combined nitrogen (nitrate or ammonium) show an evident granulation in the cytoplasm (Figure 17, Annex II). Although this aspect has to be further investigated, the observed granulation might correspond to CGP, as it has been shown for the agmatinase (strain CSMI11) and the isoaspartyl dipeptidase (strain CSMI6) mutants (see Chapters 2 and 3). It is possible that altering any element involved in arginine catabolism and perturbing the cellular levels of this amino leads to an abnormal accumulation of the polymer. An interrelation between net cyanophycin accumulation and the activity of arginine degrading enzymes has also been observed in *Synechocystis* sp. PCC 6803, where this relation is partly regulated by the activity status of photosystem II (Stephan *et al*., 2000).

Examining the domain architecture of the guanidine-group modifying enzyme superfamily, which includes metabolic enzymes from pathogenic bacteria and key regulatory proteins from higher eukaryotes, Shirai *et al*. (2006) found that Alr4995 and its homologues (among them,

Sll1336 from *Synechocystis* sp. PCC 6803, Tll0507 from *Thermosynechococcus elongatus* BP-1 and Glr1758 from *Gloeobacter violaceus* PCC 7421) are modular proteins that contain three domains. The module close to the N-terminus presents an α/β propeller catalytic domain (the amidinotransferase domain), whereas the second module of the protein contains two subdomains, the LOR/SDH bifunctional enzyme conserved domain in the middle, and a conserved uncharacterized region close to the C-terminus (Shirai *et al*., 2006). Analysis of the C-terminal region with a sequence-structure homology recognition software (Apweiler *et al.*, 2004) predicts that this region adopts a structure similar to that of human deoxyhypusine synthase (DHS), a protein involved in post-translational processing of the eukaryotic initiation factor 5A (eIF-5A). DHS adopts a structure similar to that of the protein structural motif that binds nucleotides, especially the cofactor nicotinamide adenine dinucleotide (NAD). Besides, in the C-terminal domain of Alr4995 and its homologues, many of the NAD binding sites are conserved, so that this domain may possess a function similar to DHS. Regarding the LOR/SDH domain, by sequence comparison analysis Alr4995 presents homology specifically to the LOR domain, and the presence of this domain suggests a reaction of condensation of 2-oxoglutarate, in a reductant-dependent manner, with another substrate that may be ornithine or citrulline. The presence of the guanidine-group modifying (α/β propeller catalytic) domain, on one hand, and of the LOR/SDH and DHS domains, on the other, makes Alr4995 and its homologues likely to be bifunctional enzymes (Shirai *et al*., 2006). This prediction is in agreement with the results obtained in this study: if Alr4995 had two catalytic domains, then it would be feasible that the enzyme catalyzes the two-step reaction to produce proline using arginine as a substrate. The amidinotransferase module might use arginine producing ornithine or citrulline, which would be used with appropriate cofactors and reductants in the reaction carried out by the second module to produce proline. As mentioned above, our data does not allow to distinguish whether *alr4995* encodes an arginine deiminase producing citrulline or an arginine amidinotransferase producing ornithine. Nevertheless, we would like to note that an enzyme responsible for the production of proline from arginine in *Anabaena* sp. PCC 7120 is described for the first time in this study. Further research will be needed to determine the biological aspects of Alr4995 function in *Anabaena* and to characterize in detail the unusual features of this novel enzyme.

In summary, arginine catabolism in *Anabaena* sp. PCC 7120 may involve an arginase-like route generating proline and glutamate as final products. The enzymes involved in the arginase-like pathway would be the Alr4995 and the proline oxidase, encoded by *alr0540* (*putA*), which converts proline to glutamate (Picossi, 2003) (Figure 24). Interestingly, if some ornithine or citrulline is released from Alr4995, this route of arginine catabolism could operate together with the urea cycle, incorporating aspartate and, then, using the two amino acids released through cyanophycin mobilization, as has been reported for *Synechocystis* sp. PCC 6803 (Quintero *et al*., 2000).

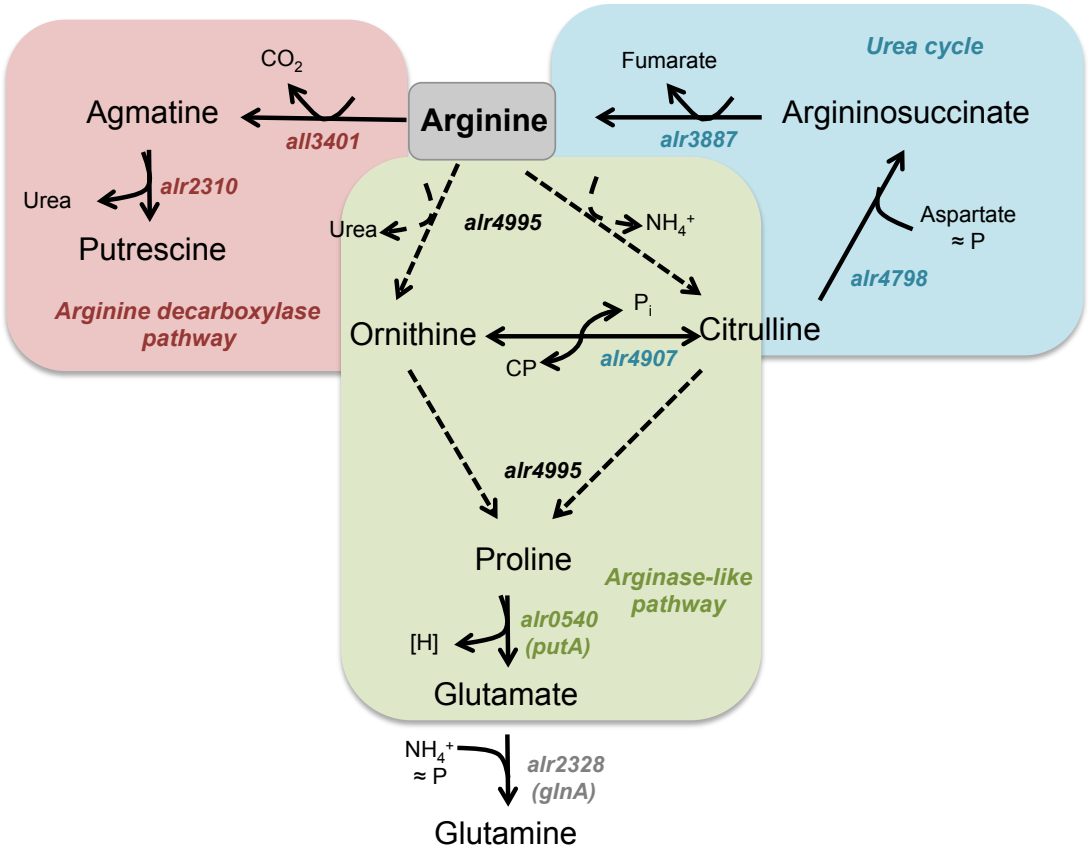

Figure 24: Schematic representation of the proposed arginine catabolism pathways and the possible corresponding ORFs in *Anabaena* sp. PCC 7120 (Kaneko *et al.*, 2001). The arginine decarboxylase pathway involves the activity of arginine decarboxylase (All3401) and agmatinase (Alr2310) (characterized in this study). The "arginase-like" route would involve Alr4995 (described in this study) in a two-step reaction, with ornithine or citrulline as an intermediate (indicated as dashed lines), and the proline oxidase enzyme (Alr0540; PutA). The urea cycle involves the ornithine carbamoyltransferase (Alr4907), the argininosuccinate synthetase (Alr4798) and the argininosuccinate lyase (Alr3887). [H], reducing power; ≈P, energy-requiring reaction (energy provided by the hydrolysis of ATP); CP, carbamoyl phosphate.

Some investigated enzymes involved in arginine catabolism have been shown to be expressed in a compartmentalized manner in the diazotrophic filaments of *Anabaena* sp. PCC 7120, being present at higher levels in vegetative cells than in heterocysts. These enzymes include arginase, arginine deiminase (Gupta and Carr, 1981b), proline oxidase (Picossi, 2003) and, as discussed above, agmatinase (see chapter 3). In addition, in cell-free extracts prepared from *Anabaena* sp. PCC 7120, four arginine biosynthesis enzyme activities were detected at higher levels in heterocyst extracts than in vegetative cell extracts (Gupta and Carr, 1981b). As mentioned above, the dipeptide resulting from CGP degradation can be an important nitrogen vehicle transferred from heterocysts to vegetative cells. This implies an active catabolism of arginine (and aspartate, perhaps through the urea cycle) in the vegetative cells, in accordance with the cellular localization of arginine-related enzymes, highlighting the importance of arginine catabolism in the vegetative cells to make available the nitrogen transiently stored as cyanophycin and transferred from the heterocysts. Thus, the correlation between arginine metabolism and cyanophycin metabolism in a compartmentalized manner, as proposed previously by Gupta and Carr (1981a), appears to be reinforced taking into account the results

presented in this work regarding the cellular localization of agmatinase and isoaspartyl dipeptidase. Two aspects worth of further research are the study of the cellular localization of arginine decarboxylase, encoded by ORF *all3401* in the genome of *Anabaena* sp. PCC 7120, and the newly identified Alr4995. These enzymes act in the first step of the arginine decarboxylase and arginase-like catabolic pathways, respectively, and could contribute to compartmentalization of arginine and cyanophycin metabolism in this filamentous heterocyst-forming cyanobacterium.

To conclude, the results obtained in this study regarding cyanophycin and arginine metabolism reinforce the view of the highly coordinated and unique features of compartmentalized metabolic pathways that patterned multicellular bacteria have developed as a strategy of multicellular behavior. These compartmentalized metabolisms involve intercellular communication between cells, which, besides the transfer of compounds through the septal joining complexes, could be also carried out via the continuous periplasm, where the OM plays an important role confining this space along the filament. Therefore, both structural and metabolic aspects of multicellularity are interrelated, determining fundamental aspects of the biology of filamentous heterocyst-forming cyanobacteria.

Conclusions

1) The alteration of the two components of the cell envelope, peptidoglycan and outer membrane, affects the length and/or strength of the filaments in *Anabaena* sp. PCC 7120. In particular, the peptidoglycan metabolism-related proteins Alr5045 and N-acetylmuroamoyl-L-alanine amidase (HcwA/AmiC2), and the lipid A biosynthesis protein Alr2270 are important for making long filaments. However, the cell envelope is not as important as septal proteins such as SepJ for filamentation.

2) Alr0718 represents the *Anabaena* ortholog of *E. coli* FtsI/PBP3, an essential protein specifically involved in septal peptidoglycan synthesis during cell division, whereas Alr5045 is an ortholog of *E. coli* PBP2, which is involved in peptidoglycan synthesis during cell elongation. Inactivation of Alr5045 results in a Fox^- phenotype, implying a role of Alr5045 during heterocyst differentiation.

3) The *all3922* gene of *Anabaena* sp. PCC 7120 encodes the isoaspartyl dipeptidase involved in the second step of cyanophycin degradation. Under diazotrophic conditions, the enzyme accumulates in vegetative cells, implying that the β-aspartyl-arginine dipeptide produced by cyanophycinase in heterocysts is transferred intercellularly to the vegetative cells as a nitrogen vehicle in the diazotrophic filament.

4) The putative ureohydrolase-encoding ORF *alr2310* of *Anabaena* sp. PCC 7120 has been identified as the *speB* gene, which determines an agmatinase. During diazotrophic growth, Alr2310 accumulates preferentially in vegetative cells and its inactivation leads to a severe toxic effect, which could result from to interference of accumulated agmatine with heterocyst differentiation.

5) *Anabaena* Alr4995 is a novel enzyme that generates proline from arginine in a two-step reaction, the first reaction involving the conversion of arginine to ornithine or citrulline, and the second reaction involving the production of proline from ornithine or citrulline. The operation of Alr4995 implies a new arginase-like route in arginine catabolism, which would include Alr4995 and proline oxidase, the product of *putA* (*alr0540*).

Resumen de la tesis

La multicelularidad ha surgido numerosas veces a lo largo de la evolución, habiendo aparecido en diferentes grupos filogenéticos, incluidas las cianobacterias, un grupo de procariotas que realizan la fotosíntesis oxigénica y exhiben un amplio rango de procesos de desarrollo. Las cianobacterias representan uno de los grupos más diversos del mundo bacteriano, incluyendo organismos con distintas morfologías, desde estirpes unicelulares hasta otras que forman filamentos multicelulares. Algunas cianobacterias filamentosas pueden presentar células especializadas en distintas funciones fisiológicas. En algunos casos, esta diferenciación celular permite que el filamento cianobacteriano lleve a cabo simultáneamente procesos que son funcionalmente incompatibles, como la fotosíntesis oxigénica y la fijación del nitrógeno atmosférico. El objetivo general de esta tesis ha sido el estudio de algunos aspectos estructurales y metabólicos de la multicelularidad en la cianobacteria formadora de heterocistos modelo *Anabaena* sp. PCC 7120.

Las cianobacterias filamentosas formadoras de heterocistos crecen formando cadenas de células (filamentos o tricomas), y presentan proteínas septales, como SepJ, que son elementos importantes para la adhesión celular y la filamentación (el proceso de formación y mantenimiento de los filamentos). Desde un punto de vista estructural, las cianobacterias son organismos didérmicos, presentando dos membranas celulares: la membrana citoplasmática y la membrana externa, esta última rodeando externamente a la capa de peptidoglicano (o saco de mureina). En el caso de las cianobacterias filamentosas, la membrana externa es continua a lo largo del filamento, determinando así un espacio periplásmico continuo que contiene la capa de peptidoglicano. A pesar de que la envuelta celular de las cianobacterias se ha estudiado en detalle recientemente, el papel de esta estructura en la multicelularidad no se había planteado previamente. El Capítulo 1 de esta tesis aborda el posible papel de los componentes de la envuelta celular en la filamentación. Para ello, se ha estudiado la longitud de los filamentos y la respuesta a la fragmentación mecánica de una colección de mutantes de genes relacionados con la formación del peptidoglicano y de la membrana externa, incluyendo dos estirpes con mutaciones en genes que determinan *penicillin-binding proteins* (proteínas de unión a la penicilina) de clase B generados en este trabajo. Los resultados obtenidos indican que la alteración tanto de los componentes de la capa de peptidoglicano como de la membrana externa afectan a la filamentación, contribuyendo así al crecimiento de *Anabaena* formando largos tricomas, aunque ninguno de estos elementos de la envuelta celular es tan relevante para la filamentación como la proteína septal SepJ.

Los procesos de la fotosíntesis y la fijación del N_2 están compartimentados en el filamento diazotrófico, lo que conlleva un intercambio de nutrientes entre las células vegetativas y los heterocistos. Las células vegetativas exportan a los heterocistos productos derivados de la fijación del CO_2, como la sacarosa, el glutamato y la alanina, y a su vez los heterocistos aportan a las células vegetativas productos de la fijación del N_2 atmosférico. Previamente se había identificado la glutamina como un vehículo de nitrógeno, pero se consideraba que podía haber otros metabolitos nitrogenados adicionales que se transfiriesen de los heterocistos a las células vegetativas. El Capítulo 2 de esta tesis aborda el papel de la

cianoficina, un polímero de aspartato y arginina que funciona como reservorio dinámico de nitrógeno, en la fisiología cianobacteriana, y de sus productos derivados como posibles vehículos nitrogenados en el filamento diazotrófico. Los resultados confirman que en *Anabaena* la ORF *all3922* es el gen que determina la isoaspartil dipeptidasa, enzima implicada en el segundo paso de la degradación de cianoficina. En condiciones diazotróficas, la enzima se expresa en las células vegetativas, lo cual implica que el dipéptido β-aspartil-arginina producido por la cianoficinasa en los heterocistos se transfiere intercelularmente a las células vegetativas, donde se hidroliza liberando aspartato y arginina. De este modo, se ha identificado el dipéptido β-aspartil-arginina como vehículo nitrogenado en el filamento diazotrófico.

La arginina es un metabolito importante en la fisiología de las cianobacterias, no sólo porque es un constituyente de la cianoficina sino también porque puede servir como vehículo intercelular de nitrógeno, al menos como parte del dipéptido β-aspartil-arginina. Sin embargo, el catabolismo de la arginina en las cianobacterias no se conoce en detalle, y sólo se han publicado unos pocos estudios acerca de las enzimas relacionadas con el mismo. En el Capítulo 3 y el Anexo II de esta tesis se han caracterizado dos genes, *alr2310*, que determina una proteína de la familia de las ureohidrolasas, y *alr4995*, que determina una proteína perteneciente a la familia de enzimas modificadoras del grupo guanidino, investigándose sus posibles funciones en el catabolismo de la arginina. Los resultados muestran que *alr2310* es el gen *speB* de *Anabaena*, que determina una agmatinasa cuya inactivación causa un efecto tóxico severo que puede ser consecuencia de una interferencia de la agmatina acumulada en el mutante con la diferenciación de los heterocistos. Se encontró asimismo que la agmatinasa se acumula preferentemente en las células vegetativas durante el crecimiento diazotrófico. Por otra parte, Alr4995 es una nueva enzima que genera prolina a partir de arginina en dos pasos, siendo la ornitina (o la citrulina) un metabolito intermediario de dicha reacción.

Los resultados de este trabajo indican que la envuelta celular es esencial para la filamentación en las cianobacterias formadoras de heterocistos, y que éstas presentan características únicas y altamente coordinadas de compartimentación celular de vías metabólicas como estrategia de organización multicelular.

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