Cell Differentiation as Survival Strategy in the Filamentous Cyanobacterium *Anabaena variabilis* ATCC 29413

Dissertation

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I dedicate this thesis to my parents and hubby!

Ritu

Contents

Abbreviations

×g	gravitational acceleration
AF	auto-fluorescence
ATCC	American type culture collection
ATP	adenosine triphosphate
BF	bright-field illumination
BLAST	Basic Local Alignment Search Tool
BODIPY	borondipyrromethene difluoride
Bp	base pairs
Chl a	chlorophyll a
cDNA	complementary deoxyribonucleic acid
CM	cellular membrane
Cm	chloramphenicol
CO_2	carbon dioxide
CP	cyanophycin
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
E. coli	Escherichia coli
Em	erythromycin
Fox	nitrogen fixation in the presence of oxygen
FRAP	fluorescence recovery after photobleaching
GFP	green fluorescence protein
GOE	Great Oxygenation Event
HEP	heterocyst envelope polysaccharide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGL	heterocyst-specific glycolipid
HGs	heterocyst glycolipids
HPLC-MS	high-performance liquid chromatography-mass spectrometry
Km	kanamycin
LB	lysogeny broth
mRNA	messenger RNA
Nm	neomycin
OD	optical density
OM	outer membrane
ORF	open reading frame
PBS	phosphate-buffered saline
PCC	Pasteur culture collection
PCR	polymerase chain reaction
PG	peptidoglycan

PSI	photosystem I
PSII	photosystem II
RNA	ribonucleic acid
RNase	ribonuclease
Rpm	revolutions per minute
RT	room temperature
RT	reverse transcriptase
RT-PCR	reverse transcription polymerase chain reaction
SD	standard deviation
SEM	scanning electron microscopy
sfGFP	superfolder GFP
Sm	streptomycin
Sp	spectinomycin
sp.	species
TEM	transmission electron microscopy
TLC	thin-layer chromatography
TM	thylakoid membrane
TMD	transmembrane domain
TTC	triphenyl tetrazolium chloride
UV	ultraviolet
WT	wild type

1. Zusammenfassung

Das planktonische Cyanobakterium *Anabaena variabilis* ATCC 29413 (syn. *Trichormus variabilis*) wächst im Süßwasser und bildet lange Filamente aus Hunderten von Zellen. Dieses photosynthetische Bakterium ist in der Lage, stickstofffixierende Heterozysten, bewegliche Hormogonien und ruhende Akineten aus vegetativen Zellen als Reaktion auf verschiedene Stressbedingungen zu differenzieren. Unter Bedingungen der Stickstofflimitierung bilden sich Heterozysten in einem fast regelmäßigen Muster und versorgen die Filamente durch Fixierung von N_2 mit organischem Stickstoff. Akineten sind transiente sporenähnliche Zellen, die es diesen Bakterien ermöglichen, rauen Umweltbedingungen zu widerstehen. Wenn geeignete Wachstumsbedingungen verfügbar sind, können die Akineten auskeimen und neue vegetative Filamente produzieren, wodurch sich diesen Cyanobakterien eine Überlebensmöglichkeit in sich verändernden Lebensräumen bietet.

Heterozysten und Akineten sind durch das Vorhandensein einer dicken mehrschichtigen Hülle gekennzeichnet, einschließlich der äußersten Polysaccharidschicht und der inneren Glykolipidschicht. Bisher war die Rolle einer Glykolipidschicht, die bei Heterozysten den Sauerstoffeintrag für die Aufrechterhaltung eines sauerstoffarmen Milieus für die Stickstofffixierung verhindert, bei sporenähnlichen Akineten unbekannt. Daher wurde in dieser Arbeit ein Gen in *A. variabilis* identifiziert, *Ava_2595*, das zu dem bekannten *hglB*-Gen homolog ist, welches eine mutmaßliche Polyketidsynthase kodiert und an der Synthese der Glykolipidschicht von Heterozysten in *Anabaena* sp. PCC 7120 beteiligt ist. Die Charakterisierung der erzeugten *hglB*-Mutante von *A. variabilis* ergab, dass bei Fehlen von HglB aberrante Heterozysten und Akineten-ähnliche Zellen gebildet werden. Ihnen fehlte die spezifische Glykolipidschicht, was die Notwendigkeit von HglB bei der Bildung dieser Glykolipide beider Zelltypen zeigte. Folglich war die Mutante nicht in der Lage, N_2 unter aeroben Bedingungen zu fixieren und diazotroph zu wachsen. Darüber hinaus haben wir die Rolle der Glykolipide beim Schutz der Akineten vor rauen Bedingungen wie Einfrieren, Austrocknung, oxidativem Stress und enzymatische Lyse gezeigt. Eine stark reduzierte Toleranz gegenüber Stressbedingungen zeigten die Akineten, denen die Glykolipide fehlten, aber unter Standardbedingungen konnten sie normal auskeimen. Diese Studie hat die zweifach Rolle der Glykolipidschicht bei der Erfüllung

verschiedener Funktionen in den evolutionär verwandten spezialisierten Zellen von Cyanobakterien nachgewiesen und die Existenz eines gemeinsamen Biosynthesewegs für die Glykolipidsynthese in Heterozysten und Akineten unter Beteiligung des gleichen Gens *hglB* aufgezeigt.

Akineten reichern während ihrer Differenzierung große Mengen zytoplasmatischer Reserveprodukte an, hauptsächlich Glykogen und das Stickstoffspeicherpolymer Cyanophycin. In dieser Arbeit wurde die physiologische Funktion von Cyanophycin bei der Akineten-Differenzierung und -Keimung untersucht und die signifikanten morphologischen und physiologischen Veränderungen zusammengefasst, die während dieser zellulären Prozesse auftreten. Wir haben gezeigt, dass die Cyanophycin-Produktion für die Bildung und Keimung der Akineten in *A. variabilis* ATCC 29413 nicht erforderlich ist.

Die morphologischen Veränderungen der Akinetenhülle, während Bildung und Keimung von Akineten wurde durch Rasterelektronenmikroskopie genauer untersucht. Es zeigte sich, dass bei reifen Akineten die Oberfläche aufgefaltet ist. Während der Keimung kam es zunächst zur Volumenzunahme bei der die Hülle glatter wurde, bis sie schließlich riss, um das kurze sich im Innern der Akinete gebildete Filament freizugeben. Auch konnte in dieser Arbeit die Akinetenhüllenarchitektur verschiedener Schichten, der Exopolysaccharid- und Glykolipidschicht, visualisiert werden und ihre Bedeutung bei osmotischen Stress die strukturelle Integrität aufrechtzuerhalten, demonstriert werden. Schließlich zeigten wir, dass die interzelluläre Kommunikation während der Akinetenbildung im Vergleich zu den vegetativen Zellen abnahm. Im Gegensatz dazu wurde die Zellkommunikation in frisch keimenden Filamenten wiederhergestellt.

2. Summary

The planktonic freshwater filamentous cyanobacterium *Anabaena variabilis* ATCC 29413 (syn. *Trichormus variabilis*) grows as filaments of hundreds of cells and is capable of differentiating nitrogen fixing heterocysts, motile hormogonia and dormant akinetes from vegetative cells in response to different stress conditions. Under conditions of nitrogen limitation, heterocysts form in a semi-regular pattern and provide the filaments with organic nitrogen by fixing N_2. Akinetes are transient spore-like cells enabling these bacteria to withstand harsh environmental conditions. When suitable growth conditions are available, the akinetes can germinate and produce new vegetative filaments, thereby providing cyanobacteria with a means of survival in changing habitats.

Heterocysts and akinetes are characterized by the presence of a thick multilayered envelope, including an outermost polysaccharide and an inner glycolipid layer. Until now, the role of a glycolipid layer, which reduces the entry of oxygen into the heterocysts for the maintenance of a microoxic environment and nitrogen fixation, was unknown in spore-like akinetes. Therefore, in this work, the function of the gene *Ava_2595* in *A. variabilis*, which is homolog to the known *hglB* gene, that encodes a putative polyketide synthase involved in heterocyst glycolipid synthesis in *Anabaena* sp. PCC 7120, a species which does not form akinetes, was elucidated. The *hglB* mutant was created and its phenotype was characterized and further investigated for the functionality of heterocysts and akinetes. This work revealed that the *hglB* mutant strain formed aberrant heterocysts and akinete-like cells, in which the specific glycolipid layers were absent demonstrating the requirement of HglB in glycolipid layer formation in both heterocyst and akinete envelope. Consequently, the mutant was unable to fix N_2 under aerobic condition and to grow diazotrophically. This study also confirmed that both cell types use a glycolipid of identical chemical composition in their special envelopes. Furthermore, we unraveled the role of the glycolipids in protecting the akinetes against harsh conditions, like freezing, desiccation, oxidative stress and lytic enzymes. Severely reduced tolerance to stress conditions was exhibited by the akinetes lacking the glycolipids but under standard conditions, they could germinate normally. Our study established the dual role of the glycolipid layer in fulfilling different functions in the

evolutionary-related specialized cells of cyanobacteria and indicated the existence of a common biosynthetic pathway for glycolipid synthesis in heterocysts and akinetes involving the same gene *hglB*.

Akinetes accumulate large quantities of cytoplasmic reserve products, mainly glycogen and the nitrogen storage polymer cyanophycin during their differentiation. In this work, the physiological function of cyanophycin in akinete differentiation and germination was investigated which showed that the cyanophycin production is not required for these cellular processes. This study also summarized the significant morphological and physiological changes that occur during formation and germination of the akinetes in *A. variabilis* ATCC 29413.

Further analysis of changes occurring during akinete formation and germination using scanning electron microscopy (SEM) found that the mature akinetes have a wrinkled envelope and during germination, the surface of the envelope smoothens upon increase in the cell size, and finally, the akinete envelope ruptures to release the short emerging filament. Also, in this work, the akinete envelope architecture of different layers, the exopolysaccharide and glycolipid layer, could be visualized and showed that this multilayered envelope helps to withstand the osmotic stress and to maintain the structural integrity. Finally, we demonstrated that the intercellular communication decreased during akinete formation as compared to the vegetative cells. In contrast, the cell communication was restored in freshly germinating filaments.

3. List of publications

Publication 1

Garg, R., Maldener, I. (2021). The dual role of the glycolipid envelope in different cell types of the multicellular cyanobacterium *Anabaena variabilis* ATCC 29413. *Front. Microbiol*. 12.

Publication 2

Garg, R., Maldener, I. (2021). The formation of spore-like akinetes: A survival strategy of filamentous cyanobacteria. *Microb. Physiol.* 31, 296–305.

Publication 3

Garg, R., Luckner, M., Berger, J., Hipp, K., Wanner, G., Forchhammer, K., Maldener, I. (2022). Changes in envelope structure and cell–cell communication during akinete differentiation and germination in filamentous cyanobacterium *Trichormus variabilis* ATCC 29413. *Life* 12, 429.

Publication 4

Garg, R., Perez, R., Maldener, I. (2022). Analysis of heterocyst and akinete specific glycolipids in cyanobacteria using thin-layer chromatography. *Bio-protoc.* 12(06): e4355.

4. Declaration of personal contribution

Publication 1: ***"****The dual role of the glycolipid envelope in different cell types of the multicellular cyanobacterium Anabaena variabilis ATCC 29413."*

I designed and performed all the experiments except mutant creation. I got technical support by Claudia Menzel and Thomas Härtner in some experiments. I analyzed and interpreted the data, prepared all the figures, wrote the manuscript under the supervision of Dr. Iris Maldener and took part in the revision process.

Publication 2: ***"****The formation of spore-like akinetes: A survival strategy of filamentous cyanobacteria."*

I designed and conducted all the experiments in this publication. I prepared all the figures, wrote the manuscript under the supervision of Dr. Iris Maldener and took part in the revision process.

Publication 3: ***"****Changes in envelope structure and cell–cell communication during akinete differentiation and germination in filamentous cyanobacterium Trichormus variabilis ATCC 29413."*

I performed all the experiments, except SEM and FIB/SEM analysis for which I prepared the samples. I analyzed and interpreted the data, prepared all the figures, wrote the manuscript under the supervision of Dr. Iris Maldener and took part in the revision process of the manuscript.

Publication 4: ***"****Analysis of heterocyst and akinete specific glycolipids in cyanobacteria using thin-layer chromatography."*

I prepared all the figures, wrote the manuscript under the supervision of Dr. Iris Maldener and took part in the revision process.

5. Introduction

5.1 Cyanobacteria- an overview

Cyanobacteria are Gram-negative, photoautotrophic bacteria classified in the phylum Cyanophyta and are the only known prokaryotes capable of performing oxygenic photosynthesis. They are among the most ancient organisms on Earth; appeared approximately 2.5 billion years ago (Schopf, 1994). Cyanobacteria are characterized by their ability to produce oxygen and carbohydrate products from carbon dioxide and water using light energy. Upon release of O_2, the primordial anoxic atmosphere was transformed to an oxidizing environment. This process is known as the Great Oxygenation Event, which pushed evolution of cells performing aerobic respiration (Fay, 1992; Kasting and Siefert, 2002; Schirrmeister et al., 2013). Despite their common metabolism, cyanobacteria represent a diverse group of prokaryotes and are widely distributed in various habitats on our planet (Whitton, 2012). Their morphological characteristics range from single cells to branched filamentous forms (Rippka et al., 1979). They can be found in various aquatic and terrestrial environments, including extreme environments like deserts, hot springs and polar regions (Elster and Kvíderová, 2014). A wide range of eukaryotic partners, including marine organisms and plants form symbiotic associations with cyanobacteria (Adams and Duggan, 2008; Stewart et al., 1983). Besides fixing CO_2 into organic matter and O_2 production, cyanobacteria also contribute to global nitrogen fixation (Garcia-Pichel et al., 2003; Whitton and Potts, 2012).

Cyanobacteria have been classified and divided into five different sections based on their morphological diversity and development (Rippka et al., 1979). Section I and II represent unicellular organisms that reproduce by budding/binary division or multiple fission. Sections III to V are filamentous species, characterized by the ability to form trichomes. Species of sections III and IV divide in one plane; section III includes filamentous species with no cell differentiation, and section IV consists of filamentous cyanobacteria capable of differentiating specialized cells. Section V includes filamentous cyanobacteria that can divide in multiple planes resulting in branched filaments and have the capability to differentiate specialized cells (Rippka et al., 1979). These specialized cells are termed heterocyst, hormogonia and akinete [reviewed in (Maldener et al., 2014)]

5.1.1 The life cycle of filamentous cyanobacteria of section IV

Cyanobacteria of the orders Nostocales such as *Anabaena variabilis* and *Nostoc punctiforme*, belong to taxonomic section IV. They are multicellular bacteria that grow in filaments, which are composed of hundreds of vegetative cells. These vegetative cells grow photosynthetically and have the potential to differentiate specialized cells such as heterocysts, akinetes and hormogonia under certain conditions (Figure 1) (Flores and Herrero, 2010; Maldener et al., 2014; Meeks and Elhai, 2002). For instance, under nitrogen limited condition, about 10% of semi-randomly spaced cells can differentiate into heterocysts, which fix atmospheric nitrogen and support the filaments with nitrogen. Another example is the small motile hormogonia – which enable dispersal of the cyanobacteria and represent the infectious agents during symbiosis (Meeks et al., 2002). Finally, many filamentous species can form spore-like resting cells, called akinetes in response to diverse environmental changes (Kaplan-Levy et al., 2010; Sukenik et al., 2018). While heterocysts represent a terminal differentiated cell, akinetes and hormogonia formation are transient processes in the life cycle of filamentous cyanobacteria (Adams and Duggan, 1999; Flores and Herrero, 2010; Maldener et al., 2014; Meeks et al., 2002; Meeks and Elhai, 2002). Under suitable growth conditions, akinetes can germinate again to produce new filaments and start their life cycle over (Maldener et al., 2014). The hormogonia can also differentiate back into the vegetative cells (Meeks and Elhai, 2002).

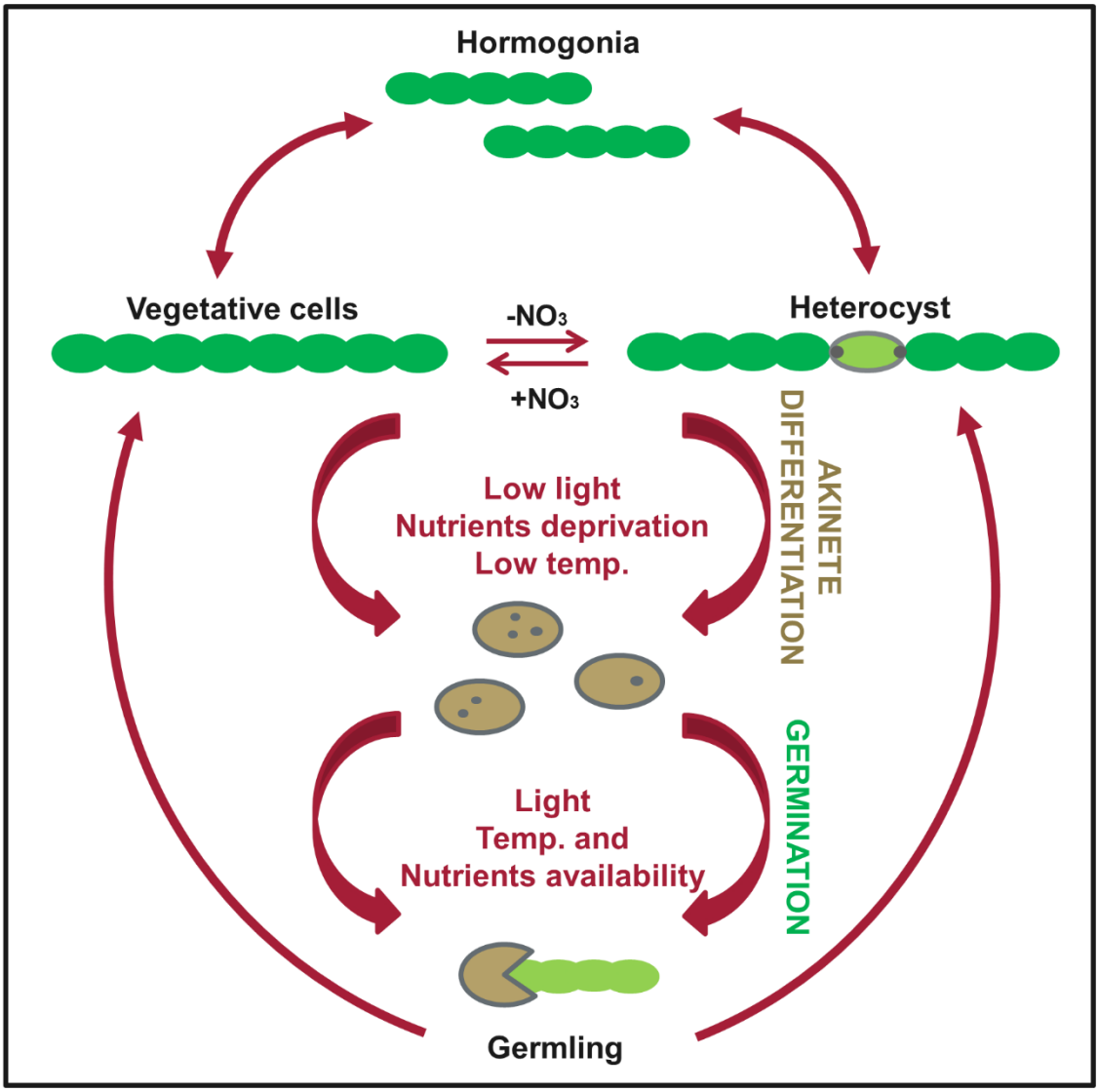

Figure 1. Schematic representation summarizing the life cycle of filamentous cyanobacteria of the order Nostocales. When nutrients are sufficient, vegetative cells can successively grow and divide forming long filaments. In response to deprivation of combined nitrogen sources, some vegetative cells differentiate into heterocysts, which can fix atmospheric nitrogen. Akinetes are formed under energy-limiting conditions, such as nutrient starvation, low light, and low temperature. Akinetes are spore-like resting cells, which can survive under adverse growth conditions. Finally, if the conditions are sufficiently favorable, the wall of akinetes ruptures, allowing germination and re-growth of the filaments with or without heterocysts, depending on the availability of combined nitrogen source. Some filamentous cyanobacteria also produce hormogonia, a short motile filament that can function as dispersal units for the establishment of symbiotic associations with plants. Environmental conditions, such as nitrogen deprivation, and a plant hormogonium-inducing factor stimulate the differentiation of hormogonia. When hormogonia resume their growth, they produce filaments with or without heterocysts, depending on the availability of combined nitrogen source. Adapted from (Garg and Maldener, 2021).

5.2 Multicellularity and cell-cell communication

The multicellularity in filamentous cyanobacteria, especially in species with cell differentiation and division of labor along the filaments, requires communication between the cells in the filament. Heterocyst-containing filaments depend on the intercellular exchange of metabolites and signaling molecules for nutrient supply and coordinating and maintaining heterocyst pattern formation, respectively (Flores et al., 2006; Flores and Herrero, 2010). In filamentous cyanobacteria, cell-cell communication occurs by direct diffusion of molecules from cytoplasm to cytoplasm via cell-cell joining structures termed septal junctions (Flores et al., 2016; Kieninger and Maldener, 2021; Mariscal, 2014; Mariscal et al., 2007; Mullineaux et al., 2008; Nieves-Morión et al., 2017; Nürnberg et al., 2015) (Figure 2). These septal junctions are gated proteinaceous complexes, which resemble eukaryotic gap junctions (Weiss et al., 2019). In the septal walls of isolated peptidoglycan sacculi, an array of nanopores (circular perforations) have been discovered which are drilled by AmiC amidases (Bornikoel et al., 2017; Kieninger et al., 2019; Lehner et al., 2013; Nürnberg et al., 2015). These nanopores are the framework for formation of septal junction complexes involving the septal proteins FraC and FraD, between the adjacent cells (Kieninger et al., 2019; Weiss et al., 2019).

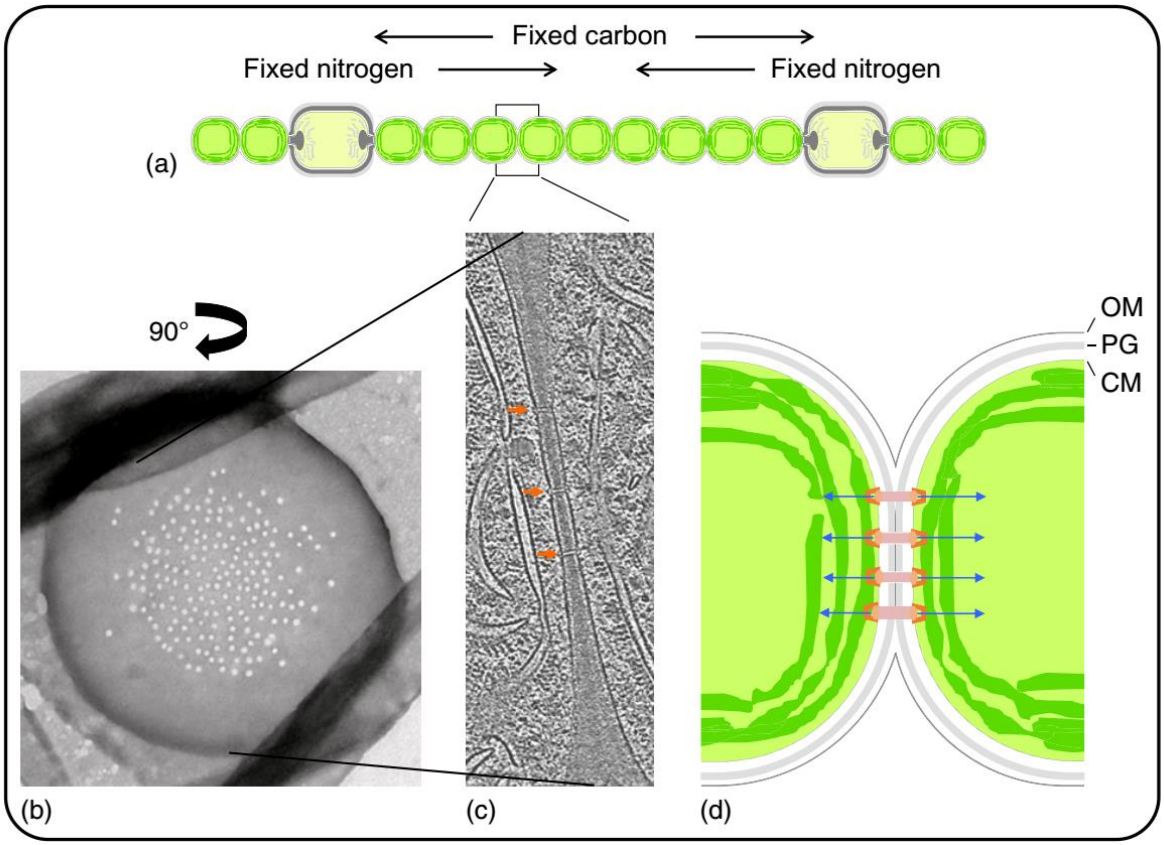

Figure 2. Molecule exchange in filamentous cyanobacteria occurs by diffusion via septal junctions. (a) Scheme showing molecule exchange between vegetative cells and heterocysts in a filament. Fixed nitrogen is transferred as glutamine and β-aspartyl-arginine while fixed carbon is transferred in the form of glutamate and sucrose. (b) Electron micrograph of isolated septal peptidoglycan with central nanopore array consisting of several nanopores. (c) Cryo-electron tomogram of a septum between two vegetative cells with the arrows pointing towards septal junctions. (d) Schematic model of putative septal junctions (shown in orange) between vegetative cells. Adopted from (Muro-Pastor and Maldener, 2019).

5.3 Cell differentiation in filamentous cyanobacteria

5.3.1 Nitrogen-fixing cells: Heterocysts

Filamentous cyanobacteria, such as *Anabaena or Nostoc,* under favorable conditions grow in the form of filaments, which are composed of hundreds of vegetative cells morphologically indistinguishable from one another. When inorganically bound nitrogen source like nitrate or ammonia is removed or exhausted, about 5-10% of semi-randomly spaced cells can differentiate into nitrogen-fixing heterocysts, which provide the filaments with nitrogen (Figure 3) (Fay, 1992; Muro-Pastor and Maldener, 2019). Heterocysts differ morphologically from

vegetative cells by their larger size and rounder shape, special cell envelope, loss of blue pigmentation, and prominent cyanophycin-accumulating polar bodies at poles adjacent to vegetative cells (Kumar et al., 2010).

Nitrogen fixation and oxygenic photosynthesis are intrinsically incompatible processes as the enzyme nitrogenase, responsible for reduction of N_2 to ammonia, is highly sensitive to oxygen. Therefore, the major purpose of heterocysts is to provide a micro-oxic environment necessary for the protection and proper functioning of nitrogenase complex (Bergman et al., 1997; Fay, 1992; Kumar et al., 2010; Maldener et al., 2014; Muro-Pastor and Maldener, 2019; Wolk, 1996). The microoxic environment in the heterocysts is maintained by increased respiration, by inactivation of oxygen-evolving photosynthesis, and the deposition of a multilayered envelope outside the cell wall (Magnuson, 2019; Murry et al., 1984; Murry and Wolk, 1989; Valladares et al., 2007, 2003; Walsby, 1985). The heterocyst envelope consists of an inner laminated heterocyst glycolipid layer (HGL), which acts as a barrier to oxygen and prevents its diffusion into the heterocyst; and an outermost polysaccharide layer (HEP), which protects the HGL layer from physical damage (Adams, 2000; Maldener et al., 2014; Muro-Pastor and Maldener, 2019; Wolk et al., 1994). Heterocysts supply reduced nitrogen to vegetative cells and get provided with reduced carbon in return (Wolk et al., 1994; Yoon and Golden, 1998).

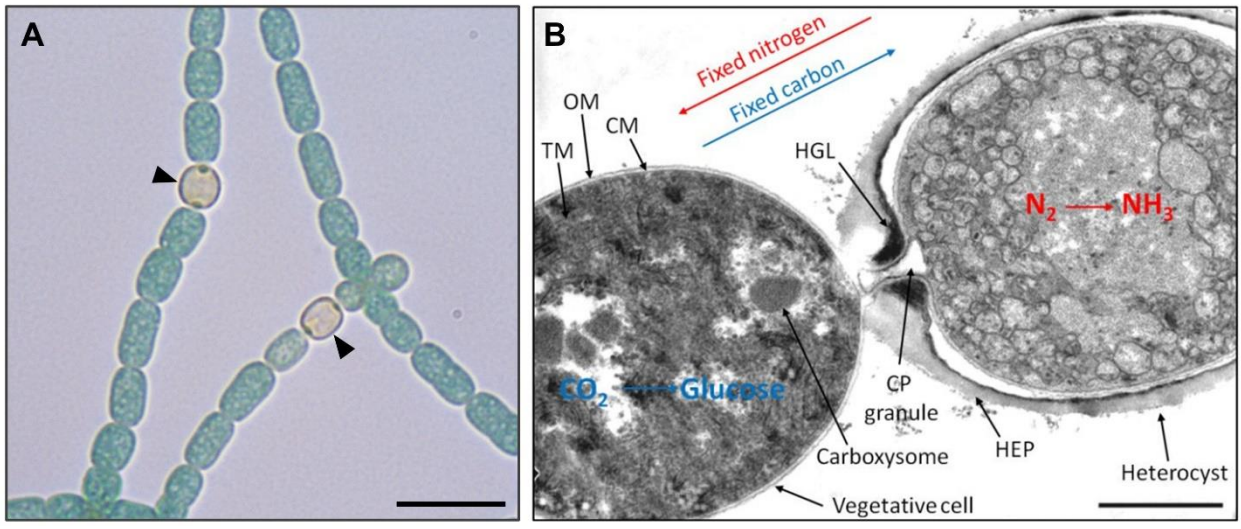

Figure 3. Heterocyst differentiation in *A. variabilis*. (A) Light micrograph of vegetative filaments containing heterocysts, indicated by black arrowhead. Bar, 10 µm. (B) Transmission electron micrograph of a terminal heterocyst and a vegetative cell. CM, cell membrane; OM, outer membrane; TM, thylakoid membrane; CP, cyanophycin; HGL, heterocyst-specific glycolipid layer; HEP, polysaccharide layer; adopted from (Perez, 2016).

5.3.2 Spore-like cells: Akinetes

Some cyanobacteria of the order Nostocales and Stigonematales form spore-like dormant cells, called akinetes (in Greek “akinetos” means motionless), that allow survival during adverse environmental conditions (Figure 4) (see also chapter 5.4). They differentiate from the vegetative cells in response to environmental changes in a species-specific way, including changes in light quality and intensity, temperature, and nutrient limitations. Akinetes are resistant to dryness and cold, while being sensitive to high temperatures unlike spores, which can endure heat. For this reason, they are just called “spore-like” cells. Akinetes are easily distinguished from the vegetative cells by their larger size, thicker cell envelope and transient accumulation of storage compounds visible in cellular granulation (Adams and Duggan, 1999; Kaplan-Levy et al., 2010).

Akinete formation is a transient process, and they can germinate into vegetative cells when environmental conditions are suitable for growth (Adams and Duggan, 1999; Maldener et al., 2014). The major stimuli identified for akinete germination are light, temperature, and nutrient conditions favorable for growth (Perez et al., 2016; Sukenik et al., 2018; Van Dok and Hart, 1997; Yamamoto, 1976). Akinetes provide a longer period of survival to cyanobacterial species living in harsh and fluctuating conditions due to their resistance to environmental extremes such as cold and desiccation, and therefore serve a perennation role (Kaplan-Levy et al., 2010; Maldener et al., 2014). Akinete forming species follow a periodic life cycle involving two phases: pelagic and benthic phase (Hense and Beckmann, 2006). Vegetative growth and akinete differentiation take place during the pelagic phase, and akinete maturation and dormancy occur in the benthic phase.

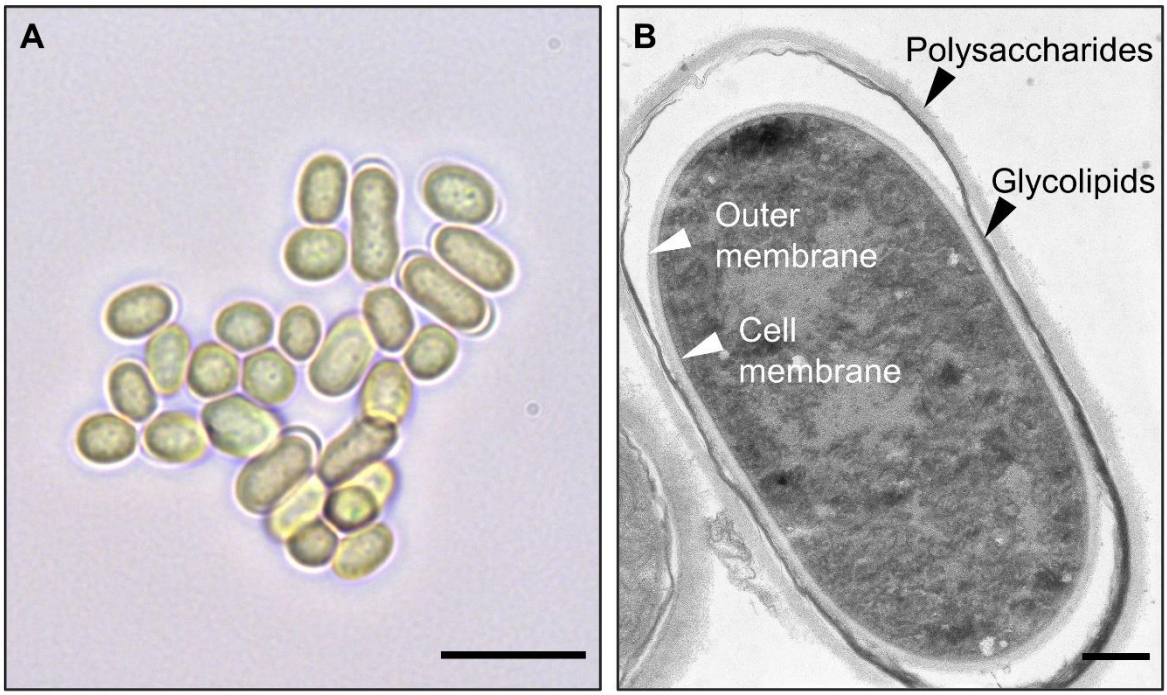

Figure 4. Akinete differentiation in *A. variabilis*. (A) Light micrograph of akinetes induced under low light condition. Scale bar, 10 µm. (B) Transmission electron micrograph of single akinete. Scale bar, 0.5 µm.

5.3.3 Motile filaments: Hormogonia

Hormogonia are differentiated short motile filaments that can be distinguished from vegetative filaments primarily by their gliding motility, visibly different shape and smaller cell size (Meeks and Elhai, 2002). Multiple cell divisions that are not accompanied by the cell growth result in smaller cells (Figure 5) (Herdman and Rippka, 1988). Hormogonia formation is a transient process where hormogonia remain in the gliding state for about 36-72 h, after which they cease to move and return to the vegetative state (Campbell and Meeks, 1989). Hormogonia play a major role in dispersal and symbiotic association in several filamentous cyanobacteria (Meeks, 1998). Hormogonia of many strains are able to move at short distance in response to photo- and chemotaxis stimulus (Meeks and Elhai, 2002). Several factors are reported to induce hormogonia differentiation, such as varying concentration of nutrients, changes in light intensity and quality (Marsac, 1994). They are also induced by the hormogonium-inducing factor (HIF) produced by a symbiotic plant partner (Meeks and Elhai, 2002). A gliding mechanism drives the motility of hormogonia, where association with the substrate is required and type IV pili along with directional

secretion of extracellular polysaccharides may also be involved (Duggan et al., 2007; Hoiczyk and Baumeister, 1998; Khayatan et al., 2015; Risser et al., 2014).

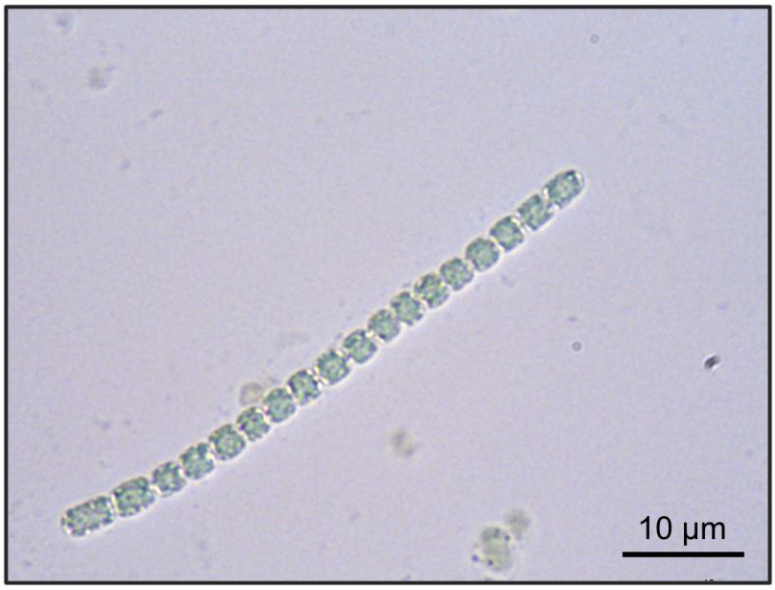

Figure 5. Light micrograph showing hormogonium of *A. variabilis*. Scale Bar, 10 µm.

5.4 Akinetes

5.4.1 Structure and composition

Akinetes are usually larger (sometimes up to 10-fold) than vegetative cells and heterocysts. They are quite distinguishable from vegetative cells by their cellular composition and ultra-structure as they undergo many morphological and physiological changes (Adams and Duggan 1999; Sukenik et al. 2018). During akinete differentiation, the cells transiently accumulate reserve materials such as glycogen, cyanophycin globules (non-ribosomal produced polymer composed of arginine and aspartate) and nucleic acids (Kaplan-Levy et al., 2010; Perez et al., 2016; Sarma et al., 2004; Simon, 1987; Sukenik et al., 2012; Sutherland et al., 1985). In *Nostoc* PCC 7524 and *Anabaena torulosa*, glycogen accumulation was reported during akinete formation (Sarma and Khattar, 1986; Sutherland et al., 1979). For many Nostocales species, the accumulation of cyanophycin granules was observed in akinetes (Perez et al., 2016; Sarma et al., 2004; Sarma and Khattar, 1986). In *A. torulosa* and *Aphanizomenon ovalisporum*, akinetes accumulate cyanophycin during their development (Sarma and Khattar, 1986; Sukenik et al., 2015). In *Nostoc ellipsosporum*, previous

study showed that cyanophycin granule formation is necessary for the function of heterocysts and akinetes (Leganés et al., 1998). During akinete germination, the degradation of cyanophycin was observed in *Cylindrospermum sp.*, *Aphanizomenon flos-aquae* and *Anabaena cylindrica* (Fay, 1969; Miller and Lang, 1968; Wildman et al., 1975). Mutation of the arginine biosynthesis gene (*argL*) in *N. ellipsosporum* (Leganés et al., 1998) and incubation of *A. cylindrica* with the arginine analog, canavanine (Nichols and Adams, 1982), resulted in the production of akinetes lacking cyanophycin that were unable to germinate. This suggested that cyanophycin accumulation is not required for akinetes formation but is essential for their germination. Further, glycogen and cyanophycin also accumulated during the stationary growth phase and under stress conditions in vegetative cells (Herdman, 1987; Lawry and Simon, 1982), which indicates that the reserve material's accumulation is not confined to akinete development. Upon akinete maturation, degradation of the accumulated reserve storage granules was observed in *A. variabilis* and *N. punctiforme* (Perez et al., 2016).

Furthermore, massive accumulation of nucleic acids in developed akinetes of *A. ovalisporum* and *A. cylindrica* was reported, which may be corroborated by abundant presence of inorganic polyphosphate bodies in vegetative cells which were absent in mature akinetes (Simon, 1977; Sukenik et al., 2012, 2007). Likewise, polyphosphate bodies were absent in mature akinetes and were rarely observed in immature akinetes of both *N. punctiforme* and *A. variabilis* (Perez et al., 2016). The thylakoid system and metabolic activities of akinetes are also drastically reduced but never completely lost (Miller and Lang, 1968; Perez et al., 2016).

Akinetes possess a thickened cell wall and a multilayered extracellular envelope. The specialized envelope composed of the outermost polysaccharide layer, similar to the homogeneous exopolysaccharide layer of the heterocyst envelope, the mucilaginous layer and the glycolipids layer, which is identical in composition to that of the heterocyst envelope (Cardemil and Wolk, 1981; Nichols and Adams, 1982; Perez et al., 2018; Soriente et al., 1993; Wolk et al., 1994).

5.4.2 Factors affecting akinete differentiation

Various environmental factors have been identified, which trigger akinete differentiation in different cyanobacterial species and strains. These include changes in light quality and quantity,

temperature fluctuations, inorganic nutrients limitations such as phosphate or carbon-to nitrogen (C:N) ratio (Maldener et al., 2014). Light intensities play an important role in akinete differentiation in many cyanobacterial species (Adams and Duggan, 1999). For example, high light intensities trigger the formation of akinetes in *Cylindrospermopsis raciborskii* (Moore et al., 2005). However, in *Nostoc* sp. PCC 7524 cultivated in the presence of excess inorganic nutrients, akinetes differentiated as light availability was reduced due to the culture self-shading (Sutherland et al., 1979). Light quality also determines akinete formation. In *Gloeotrichia*, akinete differentiation was induced by green rather than white light (Wyman and Fay, 1986). As green light is the dominant spectral component during bloom development, akinete differentiation in natural populations during surface blooms formation could be explained (Rother and Fay, 1977). In *A. circinalis*, red or green irradiance was proved more effective for akinete formation than blue light and even short exposures to blue light substantially reduced the number of akinetes, suggesting that blue light inhibits akinetes formation in cyanobacteria (Sukenik et al., 2018; Thompson et al., 2009). In *A. ovalisporum*, deprivation of potassium ions (K^+) in combination with other environmental conditions such as high light and optimal temperature (18-24°C) triggered the formation of akinetes (Sukenik et al., 2013, 2007). Furthermore, in *C. raciborskii*, the akinetes differentiation was triggered by an initial temperature shock, temperature fluctuations and by high light intensity (Moore et al., 2005). Similarly, in various *Anabaena* species, temperature plays an important role for inducing akinete differentiation (Li et al., 1997). Phosphate deficiency has been implicated as a trigger for akinete development (Herdman, 1988, 1987; Nichols and Adams, 1982) and the differentiation of akinetes was reported during phosphorus deficiency in *A. variabilis* and *N. punctiforme* (Perez et al., 2016). In *N. punctiforme*, akinete differentiation was induced within two weeks of phosphate starvation (Meeks et al., 2002; Perez et al., 2016). Phosphate limitation was also reported to be the major trigger for akinete development in *A. circinalis*, whereas no effect was observed by limiting N, inorganic C, iron, trace elements, or light (Van Dok and Hart, 1996). Similarly, phosphorus was needed for the full development of akinetes in *C. raciborskii* (Moore et al., 2005, 2003) and in *A. circinalis* (Fay et al., 1984). In addition to phosphate, other nutrients' status, and abiotic factors also play an important role in the akinete formation, such as deficiencies in Mg, Ca, Fe, and S led to a decrease in the number of akinetes in *Gloeotrichia ghosei* (Sinclair and Whitton, 1977). The importance of a critical C:N ratio was also demonstrated for akinete differentiation in *Anabaena doliolum* (Rao et al., 1987) and *A. torulosa* (Sarma and Khattar, 1993).

When a *N. punctiforme zwf* mutant was incubated in dark in the presence of fructose, it formed functional akinetes exhibiting a potential link to carbon balance (Argueta and Summers, 2005). Overall, the diverse environmental factors that trigger akinete formation are mostly consistent with cellular energy limitation and decreased cell division as primary signals (Adams and Duggan, 1999). These stimuli get translated into a secondary internal signal in specific vegetative cells which are at a certain phase of the cell cycle within a trichome and initiate the differentiation process. However, the question of how an external signal promotes akinete differentiation and activates the signal transduction pathways is still unclear.

5.4.3 Genes required for akinete differentiation

In a global gene expression analysis of the *N. punctiforme zwf* mutant strain, which shows synchronized differentiation into akinetes, 497 genes (~ 7% of the genome) were differentially expressed in 3-day akinetes. Most of the down-regulated genes encoded the core metabolic proteins, consistent with entry into a non-growth state, whereas up-regulation of 255 genes encoding unknown proteins was observed. Furthermore, some overlap with the expression profile of heterocyst developmental genes was found (Campbell et al., 2007).

Several putative transcriptional regulators of heterocyst differentiation and homologs of heterocyst specific genes are also involved in akinete differentiation in a species-specific way. For example, the gene *hepA* encoding an ABC transporter, is required for the deposition of polysaccharides layer in heterocysts envelope (Wolk et al., 1994) as well as normal envelope formation in akinetes of *A. variabilis* (Leganés, 1994). The overexpression of gene *devR*, encoding a response regulator of a two-component system involved in polysaccharide synthesis (Zhou and Wolk, 2003), results in an increased akinete differentiation in *N. punctiforme* (Campbell et al., 1996). Furthermore, the transcriptional regulatory gene *hetR*, which is a master regulator in heterocyst formation, is downregulated in akinetes of *N. punctiforme*, and a *hetR* mutant formed cold-resistant akinete-like cells (Wong and Meeks, 2002). However, *hetR* mutation resulted in a failure to differentiate both heterocyst and akinete in *N. ellipsosporum* (Leganés et al., 1994). Mutation of gene *argL*, which encodes for an N-acetylglutamate semialdehyde dehydrogenase (an enzyme involved in L-arginine biosynthesis in *N. ellipsosporum*), resulted in smaller than wild-type akinetes that lacked

cyanophycin granules and were unable to germinate (Leganés et al., 1998). So far, only one akinete marker gene, *avaK*, was identified which is shown to be differentially expressed in akinetes of *A. variabilis*, *N. punctiforme* and *A. ovalisporum* (Argueta and Summers, 2005; Campbell et al., 2007; Kaplan-Levy et al., 2010; Zhou and Wolk, 2002), but its cellular function is unknown yet. However, its homologous gene product AcaK43 was recently shown to be found in abundance in heterocysts of *A. cylindrica* (Qiu et al., 2020). A sigma/anti-sigma factor pair Npun_F4153 SigG/Npun_F4154 SapG was shown to be involved in stress resistance mechanisms of akinetes in *N. punctiforme*. The transcription of *sigG* increases in akinetes and heterocysts, and its regulon includes genes that are mainly related to cell envelope formation (Bell et al., 2017).

5.4.4 Role of akinetes in stress resistance and survival

Akinetes have remained an important aspect of cyanobacterial life cycle as they can sustain in different harsh environmental conditions. They are cold- and desiccation-tolerant, thereby allowing perennation and long-term survival of the species (Hori et al., 2003; Kaplan-Levy et al., 2010). While vegetative cells die in a temperate climatic zone during winter, akinetes survive and serve as a key factor of the life cycle in Nostocales (Kaplan-Levy et al., 2010; Sukenik et al., 2018). Their ability to survive desiccation and cold is generally far greater than that of vegetative cells in several cyanobacterial species.

Akinetes do not resemble the endospores structurally and are not as heat resistant as endospores. While initially reported to be heat sensitive (Adams and Duggan, 1999), akinetes were shown to resist higher temperatures as a result of the accumulation of betaine, glucosylglycerol, and glycine in *Nostoc* sp. HK-01 (Kimura et al., 2017). Similarly, akinetes of *A. cylindrica* were able to germinate after drying at 60ºC or under sunlight (Hori et al., 2003). Extended survival was reported for akinetes of *A. cylindrica* to survive 5-7 years of desiccation and retain the ability to germinate after storage in the darkness for 5 years, whereas vegetative cells survived no longer than 2 weeks under similar conditions (Yamamoto, 1975). Akinetes in *Cyanospira* sp. can also germinate with high efficiency after surviving 7 years in a desiccated state (Sili et al., 1994). In *Nostoc* sp. PCC 7524, akinetes are able to survive in the dark condition for several months at 4°C (Sutherland et al., 1979). Akinetes of *Aphanizomenon* and *Anabaena*, isolated from sediments as old as 64 years, were also found to be viable and germinate successfully (Livingstone and Jaworski, 1980).

Furthermore, immature pre-akinetes in some cyanobacterial strains are more tolerant to abiotic stress conditions such as freezing (Trumhová et al., 2019), osmotic stress (Kaplan et al., 2013; Pichrtová et al., 2014a), and desiccation (Pichrtová et al., 2014b). Thus, akinetes do not only provide a temporary resting function, but also ensure the long-term survival of a species by enabling it to cope with various harsh conditions.

5.4.5 Akinete germination

Germination of akinetes is triggered by various environmental signals suitable for growth such as optimal light, moderate temperature and, nutrient availability. In addition, sediment mixing and resuspension play an important role in triggering akinete germination as they relocate the akinetes into the water column and photic zone from the bottom sediment (Karlsson-Elfgren and Brunberg, 2004; Perez et al., 2016; Reynolds, 1972).

Light was identified as a significant factor for triggering akinete germination. In *A. cylindrica*, light intensity was shown to be important for germination of akinetes and, in dark or in the presence of the photosynthesis inhibitor 3-(3,4 dichlorophenyl)-1,1-dimethylurea (DCMU), germination was impaired (Braune, 1979; Yamamoto, 1976). This suggested that the photosynthetic apparatus is directly involved in akinete germination. In addition, the presence of light is also reported to be required for akinete germination in *A. variabilis* (Perez et al., 2018), *A. circinalis* (Van Dok and Hart, 1997), *Nodularia spumigena* (Huber, 1985), and *A. flos-aquae* (Karlsson-Elfgren et al., 2004; Karlsson-Elfgren and Brunberg, 2004). In *N. spumigena*, akinetes were not able to germinate in the dark but very low light intensities (0.5 µmol photons m^{-2} s^{-1}) were reported to be enough to initiate germination (Huber, 1985). Dilution of akinete-containing culture with fresh medium also induces germination, apparently due to increased light intensity and availability of nutrients (Adams and Duggan, 1999; Herdman, 1988).

Germination in *A. cylindrica* requires light and oxygen, suggesting that the energy and carbon for this process come from photosynthesis and respiration of reserve material (Yamamoto, 1976). As mentioned above, in *A. circinalis*, akinete germination requires light, but phosphate is also needed (Van Dok and Hart, 1997). Thus, photosynthesis provides the energy for akinete germination in this species, and the rate of germination is regulated by respiration in a temperature-dependent

manner (Fay, 1988; Kezhi et al., 1985). In *A. variabilis,* in response to medium light and optimal temperature, germination begins inside the akinete envelope with the energy for cell division initially supplied by the respiration of glycogen and subsequently by photosynthesis (Perez et al., 2018).

The akinetes of *Anabaenopsis arnoldii*, *N. spumigena*, and *Anabaena vaginicola* when incubated in extreme temperatures, a reduced germination rate was observed (Pandey and Talpasayi, 1981; Rai and Pandey, 1981; Reddy, 1983). In *Gloeotrichia echinulata*, akinete germination was influenced by high temperature and their exposure to an appropriate level of light and oxygen, and significantly enhanced by mixing of bottom sediment imposed by bioturbation and physical processes (Baker and Bellifemine, 2000; Fay, 1988; Karlsson-Elfgren et al., 2004; Rengefors et al., 2004; Ståhl-Delbanco and Hansson, 2002).

In several cyanobacterial strains including *A. variabilis* and *N. punctiforme,* the germination of akinetes is a highly asynchronous process (Braune, 1980; Miller and Lang, 1968; Perez et al., 2018; Wildman et al., 1975), while in some species such as *Nostoc* PCC 7524, synchronous germination was observed (Sutherland et al., 1985). Under appropriate growth conditions, akinete start to germinate visible by morphological changes, which include reorganization of cellular material followed by elongation and initial cell division, which occurs inside the akinete's envelope. In *A. variabilis*, the developing small filament penetrates the akinete envelope mostly at one pole (Perez et al., 2018). Similar behavior was also observed for many other cyanobacterial species (Baker and Bellifemine, 2000; Braune, 1980; Herdman, 1988; Hori et al., 2003; Kaplan-Levy et al., 2010; Moore et al., 2004; Sutherland et al., 1985). The open envelope may remain associated with the developing filament for some time or in some strains, the entire akinete wall may dissolve during germination (Adams and Duggan, 1999; Meeks et al., 2002; Moore et al., 2004; Perez et al., 2018; Sili et al., 1994; Skill and Smith, 1987). It is unclear whether the envelope rupture occurs solely due to the increased internal pressure resulting from the growing filament or whether it is assisted by some enzymatic activity. Finally, morphological and biochemical alterations in the germling lead to a fully developed short filament, which emerges from the akinete envelope (Moore et al., 2004).

The germination process usually starts with the reconstitution of the thylakoid membranes and carboxysomes to allow recovery and functioning of the photosynthetic machinery to fix CO_2.

However, recently it was shown that photosynthetic O_2 production and fully developed inner membrane system appeared at the late state of germination of a filament, just before emerging from the akinete envelope in *A. variabilis* and *N. punctiforme* (Perez et al., 2018). Germination of akinetes in *Cyanospira*, was accompanied by *de novo* protein synthesis that took place prior to the first cell division (Sili et al., 1994). In addition, fast cell divisions occurred during germination in several strains, indicating that all enzymes needed for division are already present and do not need to be synthesized. An additional stage during the germination process in some species is the formation of gas vacuoles in the new filament to allow floating of germlings and trichomes to the upper parts of the water column (Karlsson-Elfgren and Brunberg, 2004).

5.5 Research objectives

The cyanobacterium *Anabaena variabilis* ATCC 29413 is a true multicellular organism, capable of differentiating heterocysts specialized for nitrogen fixation, or akinetes, the dormant cells that allow survival under diverse environmental stress conditions. In contrast to a profound knowledge on the differentiation of ecologically important heterocysts, little is known about the akinete differentiation process and their adaptation strategy. In the last years, techniques in molecular genetics and advanced microscopy have been developed and the genome sequence is available for *A. variabilis* ATCC 29413, which offered the opportunity to explore the cellular and molecular biology of akinete differentiation and germination in more detail.

The glycolipid layer of heterocysts is responsible for the inhibition of oxygen diffusion into the cell to protect the nitrogenase from oxygen. Presumably, akinetes contain an envelope layer with the same composition (Perez et al., 2018). The function of this layer in akinetes was completely unknown and was addressed in this study. For this, a gene known to be involved in heterocyst glycolipid synthesis was identified, a mutant was created, and the phenotype related to heterocyst and akinete formation and function was studied.

Previous studies showed the accumulation of reserve granules such as cyanophycin during akinete differentiation but the function of this reserve material in akinete formation and germination was

not known. Therefore, a mutant, which cannot produce cyanophycin, was created and its survival performance and ability to differentiate akinetes was investigated in this study.

The morphological, and structural changes that take place during akinete differentiation and germination are not completely understood and were further addressed in this work using various microscopic techniques. Several environmental signals are known to trigger vegetative cells in the filaments to differentiate into akinetes and induce akinete germination when conditions are favorable. So, it can be assumed that regulatory molecules are exchanged between the cells using the septal junctions and allows the cell-cell communication during akinete differentiation and germination. However, nothing was known so far and was investigated in this study using fluorescence recovery after photobleaching (FRAP) analysis.

Results of the following publications are summarized and discussed in the "Results" and "Discussion" section:

Publication 1

Garg, R., Maldener, I. (2021). The dual role of the glycolipid envelope in different cell types of the multicellular cyanobacterium *Anabaena variabilis* ATCC 29413. *Front. Microbiol*. 12.

Publication 2

Garg, R., Maldener, I. (2021). The formation of spore-like akinetes: A survival strategy of filamentous cyanobacteria. *Microb. Physiol.* 31, 296–305.

Publication 3

Garg, R., Luckner, M., Berger, J., Hipp, K., Wanner, G., Forchhammer, K., Maldener, I. (2022). Changes in envelope structure and cell–cell communication during akinete differentiation and germination in filamentous cyanobacterium *Trichormus variabilis* ATCC 29413. *Life* 12, 429.

6. Results

6.1 HglB is involved in the glycolipid layer formation of heterocyst and akinete envelope and is important for the functionality of both cell types

The cluster of genes *alr5354* (*hglD*), *alr5355* (*hglC*) and *alr5357* (*hglB*) in *Anabaena* sp. PCC 7120 have been shown to be required for the synthesis of heterocyst envelope glycolipids (HGLs) (Fan et al., 2005). Also, a transposon mutant of *hglB* was not able to synthesize HGLs during heterocyst differentiation in *Anabaena* sp. PCC 7120, a species which does not form akinetes (Maldener et al., 2003). Using BLAST analysis, we identified the gene *Ava_2595* in the genome of *Anabaena variabilis*, which is ~ 94 % identical to the gene *hglB* in *Anabaena* sp. PCC 7120. In order to investigate the role of this *hglB* homolog in the formation of the glycolipid layer of akinetes, we created the mutant DR752 by insertion of a neomycin resistance cassette (CK.3) into the *Ava_2595* gene by double homologous recombination. The genotypic characterization by PCR showed that the DR752 mutant was fully segregated (Figure S1A, Publication 1).

Under standard growth conditions with a combined nitrogen source, strain DR752 showed no difference in growth compared to the wild type (WT). However, the mutant was unable to grow in absence of a source of combined nitrogen (Figure 1A, Publication 1). In contrast to WT, heterocyst differentiation in the mutant was delayed and they appeared mostly at the ends of the filament (Figure S1C, Publication 1). The alcian blue staining demonstrated the deposition of the exo-polysaccharide (HEP) layer around the heterocyst (Figure S2A, Publication 1). In contrast, the heterocyst envelope was not stained by BODIPY, demonstrating the lack of the laminated layer in the mutant (Figure S2B, Publication 1). To investigate, whether the HGLs are synthesized in the mutant DR752 after nitrogen step-down, thin-layer chromatography (TLC) was performed. Our results demonstrated that the mutant could not synthesize HGL during nitrogen starvation condition (Figure 1B, Publication 1), confirming that the *hglB*-gene encodes a protein, involved in the biosynthesis of HGL, as in *Anabaena* sp. PCC 7120. Furthermore, the analysis of the ultrastructure of heterocyst by transmission electron microscopy (TEM) of ultrathin sections revealed that the mutant did not form the laminated layer of heterocyst envelope, explaining why it could not grow diazotrophically, although heterocysts were formed (Figures 1A, C, Publication 1).

By using semi-quantitative reverse transcription polymerase chain reaction (RT-PCR), the nitrogen starvation-induced expression of the *hglB*- and the downstream located *hetN*-gene was studied. Both genes were differentially expressed in the WT strain during nitrogen starvation. The transcript of *hglB* was not detected in the mutant DR752 as expected. The gene *hetN*, downstream of *hglB,* was constitutively expressed in the mutant as the CK.3 cassette used to create the mutant contains a strong promoter without a termination signal (Figure S3, Publication 1). By this, a polar effect of the insertion of CK.3 into the ORF of *hglB* on the *hetN*-gene was excluded.

Without the glycolipid layer, heterocysts cannot create a micro-oxic environment that is essential for the enzyme nitrogenase function (Maldener et al., 2014; Muro-Pastor and Maldener, 2019; Murry and Wolk, 1989). To assess the oxygen condition in the heterocysts, the filaments of WT and *hglB*-mutant were incubated with triphenyl tetrazolium chloride (TTC) *in vivo*. TTC creates dark brown precipitates under micro-oxic conditions (Fay and Kulasooriya, 1972) as observed in the WT heterocysts, while no such precipitates were observed in the mutant heterocysts (Figure 2A, Publication 1). In addition, the acetylene reduction assay was used to measure the nitrogenase activity of mutant and wild-type cultures under oxic and anoxic conditions (Figure 2B, Publication 1). No nitrogenase activity under oxic conditions was detected in the DR752 mutant despite the occurrence of the *nifD* gene rearrangement which is essential to produce a functional enzyme (Figure 2C, Publication 1) (Brusca et al., 1989; Thiel and Pratte, 2014). In contrast, under anoxic conditions, the nitrogenase activity of the mutant was found to be comparable to the WT.

Previously it was shown, by an optimized method for glycolipid analysis from akinetes, that the laminated layers of the akinetes and the heterocysts of *A. variabilis* contain the same glycolipid, 1-α-glucosyl-3,25-hexacosanediol (HG_{26}-diol) (Garg et al., 2022; Perez et al., 2018). However, nothing was known about the synthesis and function of the HGLs in akinetes. Therefore, the *hglB* mutant offered the opportunity to investigate these aspects.

The DR752 mutant showed delayed akinete differentiation, indicating a role of HglB in akinete formation (Figure 3A, Publication 1). In contrast, no effect on germination was observed in the mutant DR752 and, the mutant akinetes germinated similar to the WT akinetes (Figure 3B, Publication 1). Accordingly, we compared the envelopes of WT and mutant DR752 akinetes using BODIPY staining and HGL analysis in lipid extracts (Figure 4A, Publication 1). In line with the BODIPY staining results, in WT akinetes, TLC showed the lipid band at the position similar to

HG_{26}-diol from heterocysts whereas in DR752 mutant akinetes, this specific band was missing (Figure 4B, Publication 1). Furthermore, transmission electron micrographs showed that the laminated layer in the mutant DR752 akinete envelope was absent (Figure 4C, Publication 1). As shown by semi-quantitative RT-PCR study, the increased transcription of the *hglB* gene during akinete differentiation further confirmed our results that HglB is involved in the akinete glycolipids synthesis for envelope (Figure 4D, Publication 1).

As mutant DR752 has no laminated layers in their akinete envelopes, it offered the opportunity to investigate the role of the envelope glycolipids in resistance and response to different stress conditions. The akinetes of WT and mutant DR752 along with the vegetative cells as control were exposed to various stress conditions such as low temperature, freezing, desiccation, freeze-thaw cycles, 10 mM H_2O_2 and lysozyme treatment. The results showed that the akinetes of mutant DR752 were significantly less resistant and suffered a decline in survival efficiency when subjected to these extreme conditions except cold while WT akinetes remained stable and mostly unaffected (Figure 5, Publication 1). Altogether, this study indicates that the glycolipid layer of the akinete envelope has an essential function in survival of akinetes under various stress conditions and points to different roles in heterocysts and akinetes.

6.2 Formation of akinetes is the key strategy for survival in filamentous cyanobacteria and cyanophycin production is not a prerequisite for akinete formation and germination

The development of mature akinetes from vegetative cells involves many structural changes such as increase in cell size, transient accumulation of intracellular granules like cyanophycin and glycogen (Perez et al., 2016; Simon, 1987) and formation of a multi-layered extracellular envelope which is composed of several distinct layers as depicted in Figure 2A (Publication 2) (Perez et al., 2018, 2016). The extracellular envelope mainly consists of outermost polysaccharides and inner glycolipids layer. The glycolipid layer of akinetes envelope can be visualized with fluorescent green dye BODIPY, which stains the lipid layer of envelope as previously described (Figure 2B, Publication 2) (Perez et al., 2018).

Further, we observed the changes that occur during akinete germination in the medium lacking nitrogen. The optimum light and fresh medium were enough to induce germination. Increase in cell size due to cell division inside the akinete envelope was observed within 18–24 h, which led to the rupture of akinete envelope and release of germinating short filament. The germinating filaments can sense the absence of a nitrogen source at the very beginning of cell division as they started to differentiate heterocysts to fulfil the nitrogen requirement of the cells (Figure 2C, Publication 2).

Several cyanobacterial species are known to accumulate cyanophycin in akinetes (Sarma et al., 2004; Sarma and Khattar, 1986). Previous studies showed that in *A. variabilis*, cyanophycin (CP) granules transiently accumulate during akinete differentiation but are degraded again in mature akinetes (Perez et al., 2016). However, their role in akinete differentiation and germination is not known yet. The gene *cphA1*, encoding the cyanophycin synthase, catalyzes the biosynthesis of the nitrogen reserve cyanophycin (multi-L-arginyl-poly-L-aspartic acid) (Berg et al., 2000). Therefore, to elucidate the function of cyanophycin granules in akinete differentiation and germination in *A. variabilis*, we created a mutant of *cphA1* by insertion of a resistance cassette and functionally characterized the resulting strain.

Loss of *cphA1* in the mutant led to the heterocyst formation that lacked cyanophycin polar nodules. However, under standard growth conditions in media supplemented with NO_3^-, no visible differences in growth were observed in *cphA1* mutant compared to the wild type (WT). Moreover, the mutant was able to grow diazotrophically (Figure S1, Publication 2). No accumulation of CP granules was observed in the *cphA1* mutant akinetes after 7 days of phosphate starvation while WT akinetes clearly showed their presence. Interestingly, the *cphA1* mutant was able to differentiate akinetes similar to the WT, despite the absence of CP granules (Figure 3A, Publication 2). When akinetes were induced in low light conditions, the rate and pattern of akinete differentiation in the *cphA1* mutant were found to be similar as compared to the WT (Figure 3B, Publication 2). These results clearly indicate that cyanophycin is not required for the akinete differentiation process. Furthermore, the germination of akinetes from the WT and the mutant showed no difference in efficiency and time scale (Figure 3C, Publication 2), indicating no role for cyanophycin in this process. Altogether, this study showed that cyanophycin production is not crucial for both processes - akinete differentiation as well as germination in *A. variabilis*.

6.3 Changes in cell morphology, envelope structure and cell-cell communication during akinete differentiation and germination.

To elucidate the morphological changes that take place during the transition from vegetative cells to dormant akinetes in *A. variabilis*, analysis using scanning electron microscopy (SEM) was performed. This revealed that mature akinetes had folds or wrinkles on their surface in contrast to vegetative cells which had a smooth and waveless surface (Figure 1, Publication 3). Next, we used focused ion beam (FIB)/SEM tomography to understand the structure of akinetes in more depth. In the tomograms, the multilayered envelope composed of several distinct layers was visible in high resolution, showing multiple folds on the outer surface. Many immature akinetes displayed the intracellular granules mainly consisting of glycogen and cyanophycin which disappear once the akinetes become mature (Figure 2B, C, Publication 3).

As mentioned above (Results section 1), we found that akinete envelope play a crucial role in protecting the cell from various stresses such as cold, freeze, freeze-thaw cycles, desiccation, and lysozyme attack in *A. variabilis* and the mutant with incomplete envelope is highly sensitive to these conditions (Publication 1). To further explore the protective capability of the akinete envelope, their tolerance to osmotic stress was investigated. The treatment of vegetative filaments and akinetes with 40% sucrose for 20 min, resulted in a drastic decrease in the filaments length due to shrinkage of vegetative cells while no effect on akinetes dimensions were observed as analyzed by SEM and bright field microscopy (Figure 3, Publication 3).

Further, we followed the fate of surface structures of mature akinetes during germination by SEM. Mature akinetes were transferred to fresh medium and optimum light conditions to induce germination. We observed an increase in akinete volume followed by initial cell divisions in samples taken between 17 h and 24 h after transfer to germination conditions. This was concomitant with disappearance of wrinkles from the envelope (Figure 4b, S2, Publication 3). Successive cell divisions resulted in the rupture of the akinete envelope and emergence of a short filament at one pole after 48 h (Figure 4c, d, Publication 3). Filaments still attached to the envelope

were observed and development of terminal heterocyst could be seen after 72 h (Figure 4d, 4e, Publication 3).

Cell-cell communication in filamentous cyanobacteria can be studied using fluorescent tracers by FRAP (Mullineaux et al., 2008). We performed FRAP experiments to investigate cell-cell communication during akinete formation and germination and observed a gradual decrease in calcein transfer during akinete differentiation, indicating slower intercellular communication (Figure 5A, Publication 3). Compared to the vegetative cells, 18 days immature akinetes, which were still attached to each other showed 30 % reduced communication. This result indicated that the cells going into the dormant state prepare for a non-communicating single cell stadium with reduced metabolic activity, before they get completely detached from each other as mature akinetes.

When one-month-old akinete culture, containing few filaments with still immature akinetes, was transferred to fresh medium and optimal light conditions, these immature akinetes started to germinate and divide along with the germination of mature akinetes. The FRAP measurements during germination of akinetes especially in these filaments showed a faster recovery of calcein fluorescence after 24 h indicating the regaining of cell-cell communication (Figure 5B, Publication 3). After 48 h, an even faster calcein transfer rate was observed which is necessary to form the multicellular filaments and continued till 72 h, when almost all the filaments achieved normal vegetative growth state (Figure 5B, Publication 3). We also observed that the freshly dividing young vegetative cells during germination had twice as much communication as compared to the vegetative cells in a stationary culture. Altogether, this study indicates the regulation of molecule exchange during akinete differentiation and germination.

7. Discussion and outlook

7.1 The role of HglB in heterocyst and akinete envelope formation

The filament forming cyanobacterium *Anabaena variabilis* forms heterocysts for nitrogen fixation and dormant akinetes for environmental stress survival. These cells have different functions in the life cycle of *A. variabilis* and related species but share several morphological characteristics such as increased size and presence of a thick extracellular envelope consisting of an outermost polysaccharide layer and a glycolipid layer deposited outside the bacterial cell wall.

Heterocysts and akinetes of several species have been suggested to possess a similar envelope structure and identical glycolipids (Adams and Duggan, 1999; Leganés, 1994; Nichols and Adams, 1982; Perez et al., 2018; Soriente et al., 1993). Many genes are known to be involved in heterocyst envelope glycolipids (HGLs) synthesis and their deposition (Fan et al., 2005; Fiedler et al., 1998; Maldener et al., 2003; Nicolaisen et al., 2009; Shvarev et al., 2018; Staron et al., 2011). But, so far, the role of a glycolipid layer, which reduces the entry of oxygen into the heterocysts, was unknown in spore-like akinetes. Also, the information about genes involved in the synthesis and role of this laminated layer in the akinete envelope was missing.

In our study, we have investigated the gene *hglB* and explored its role in the akinete envelope. The *hglB* gene from *Anabaena* sp. PCC 7120 encodes a putative fatty acid synthase/polyketide synthase involved in HGL synthesis (Awai et al., 2009). A mutant in this gene, the transposon mutant P2, is not able to synthesize the HGLs during heterocyst differentiation and thus, unable to grow diazotrophically (Ernst et al., 1992; Fiedler et al., 1998; Maldener et al., 2003). By Blast search, a homologous gene *Ava_2595* was identified in *A. variabilis* and a mutant, DR752 was created. As expected from Fox^- phenotype [unable to fix N_2 under oxic conditions; (Ernst et al., 1992)] of the *hglB*-mutant of *Anabaena* sp. PCC 7120, the DR752 mutant was also not able to grow on N_2 as a sole nitrogen source and formed aberrant heterocysts lacking the HGL layer as well as the lipid in extracts. (Figures 1A, B, C, Publication 1), in line with the suggested function of HglB in HGL synthesis.

To protect the enzyme nitrogenase, the glycolipid layer acts as a barrier to the gases and limits the diffusion of oxygen into heterocysts (Murry and Wolk, 1989; Walsby, 1985), thereby maintaining

the micro-oxic condition. As shown by TTC staining, the mutant DR752 does not provide micro-oxic condition inside heterocysts and cannot sufficiently protect the nitrogenase as no detectable nitrogenase activity was measured in oxic condition (Figures 2A, B, Publication 1). However, in *A. variabilis*, under anoxic conditions, a molybdenum-based nitrogenase is expressed also in the vegetative cells (Thiel et al., 1995; Thiel and Pratte, 2014), which contributes to a significant amount of activity in the mutant under anoxic conditions similar to the wild type (WT). By measuring the activity under oxic and anoxic conditions, we showed that heterocyst specific nitrogenase cannot function in the mutant lacking the HGL layer but this is not caused by inhibition of the genome rearrangement in the *nifD*-gene region (Brusca et al., 1989) (Figure 2C, Publication 1).

The mutation in *hglB* leads to a delay in differentiation of heterocysts and akinetes however, it does not affect the germination of the mutant akinetes (Figure 3, Publication 1). Due to the strong *psbA*- promoter of the CK.3 cassette (Elhai and Wolk, 1988) without termination signal in DR752 mutant, we decided to analyze the polar effect of insertion of the CK.3 cassette on the downstream gene *hetN*, which was shown to be a suppressor gene of heterocyst differentiation in *Anabaena* sp. PCC7120 (Callahan and Buikema, 2001). We observed that the *hetN* gene is constitutively expressed in the DR752 mutant in contrast to the WT, where we observed its upregulation only after nitrogen step-down. Since HetN is not involved in the HGLs synthesis, we believe that its possible involvement can only be in delaying the heterocyst differentiation (As shown in Figure S3, Publication 1).

In electron micrographs of the akinete envelope in *A. variabilis* and *N. punctiforme*, a layer resembling the laminated layer of heterocysts had been observed (Perez et al., 2018). Chemical characterization of this layer showed that it was indeed composed of the same heterocyst glycolipid HG_{26}-diol that forms the heterocyst envelope(Perez et al., 2018). The HGLs are present in a large number of heterocyst-forming cyanobacteria (Bauersachs et al., 2014; Gambacorta et al., 1998; Reddy, 1983; Wörmer et al., 2012), but they were also identified in *Cyanospira rippkae* akinetes (Soriente et al., 1993). This supports the idea that HGLs are not exclusive to heterocysts, but also present in akinetes (Wörmer et al., 2012). Staining and ultrastructure analysis revealed the absence of the laminated layer in the *hglB*-mutant akinetes which is in line with the TLC results (Figure 4, Publication 1). This indicates that the HglB is the enzyme involved in the glycolipid synthesis of

akinete envelope similar to the heterocyst. Presumably, the entire synthesis pathway of glycolipids is identical in heterocysts and akinetes and involves the same enzymes (Awai et al., 2009). We have also followed the expression of the *hglB* gene during akinete differentiation and found increased transcription of this gene during akinete formation. The similar results were obtained during nitrogen step-down where gradual increase in *hglB* expression was observed (Figures 4C, S3, Publication 1). In addition, the akinetes and heterocysts envelopes are composed of the same polysaccharide material (Figure S2, Publication 1) (Cardemil and Wolk, 1979; Perez et al., 2018). These results suggested that the akinete and heterocyst differentiation processes are related to each other and akinetes are the evolutionary precursors of heterocysts (Wolk et al., 1994).

In this study, we also unraveled the role of the glycolipids in protecting the dormant cells against harsh conditions, like freezing, desiccation, oxidative stress and lytic enzymes. In the mutant, lacking the glycolipid layer in the akinete, the cell envelope is less stable and easily gets affected by above mentioned stresses as we observed a severe decline in the survival efficiency in the mutant akinetes (Figure 5, Publication 1). Upon further exposure of the mutant akinetes to freeze-thaw cycles and H_2O_2 treatment and visualization under the bright field microscope, we observed release of soluble blue pigments due to cell lysis. These defective akinetes of mutant lost most of their characteristics for stress-resistance.

The results presented in this study indicate that the glycolipid layer of envelope acts as a solute translocation barrier that excludes gas such as O_2, small molecules such as H_2O_2 and macromolecules such as lysozyme. This remarkably implicates that the nitrogen fixation and dormancy use the same structural component for different functions (Figure 6). Since respiration ceases in old akinetes, they presumably do not need oxygen as electron acceptor in respiration. Hence, it is likely that the HGL layer from akinetes also avoids diffusion of oxygen into them, which needs to be proved in future.

In addition to the chemical and structural identity of the laminated layer of both cell types, our study indicates that akinetes and heterocysts use the same biosynthetic pathway to produce the glycolipid envelope, involving the putative polyketide synthase HglB. So far, this is the only known enzyme involved in the envelope formation of akinetes. However, further genes involved in heterocyst envelope formation, should be studied in future to confirm the common biosynthetic

pathways in heterocysts and akinetes differentiation. Furthermore, known cyanobacterial regulators like NtcA, PII and HetR, need to be investigated for involvement in akinete formation by expression studies and site-directed mutagenesis. Also, further information about developmental program involved in akinete formation, needs to be elucidated in future.

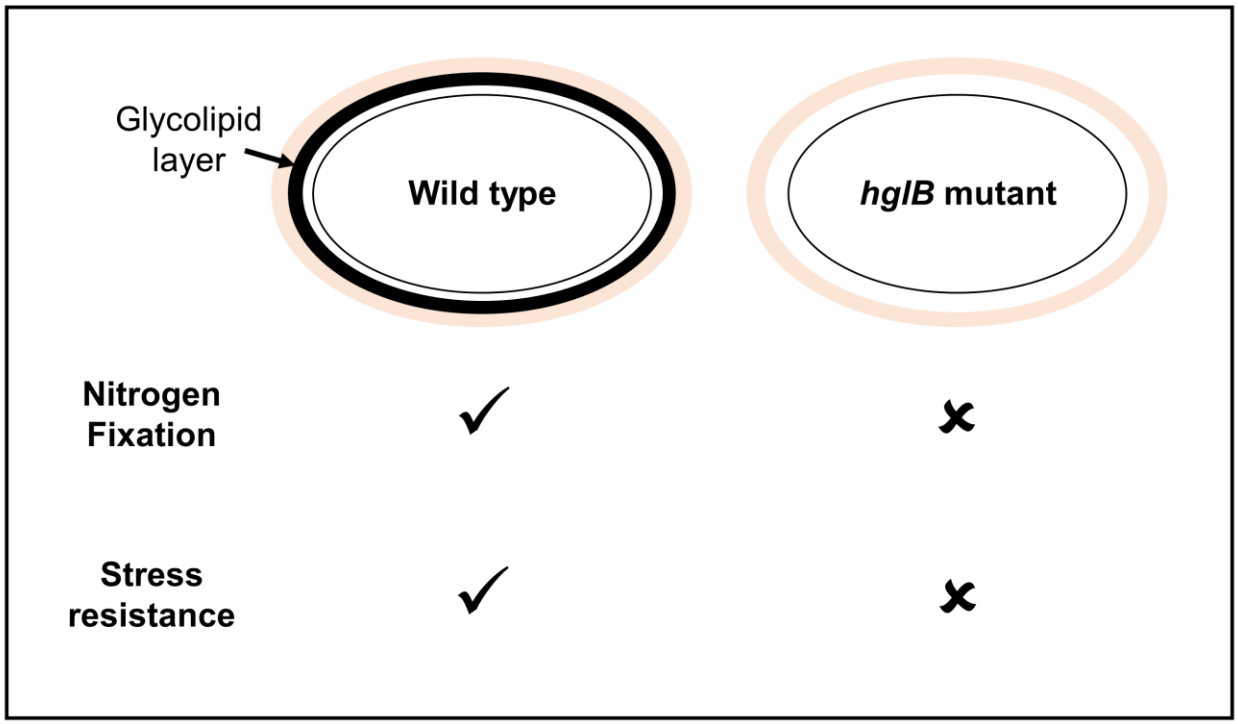

Figure 6. Model depicting the functional role of envelope glycolipid in *A. variabilis*.

7.2 Akinete differentiation and germination process

Compared to our knowledge on heterocyst differentiation, akinete formation and germination is the under-studied area of cell development in filamentous cyanobacteria. Therefore, we analyzed the changes in morphology and physiology of cells in more detail during akinete formation and germination in the model organism *A. variabilis* ATCC 29413. The schematic representation of akinete differentiation and germination process is shown in Figure 7.

Several environmental factors were reported to trigger akinete differentiation in different Nostocales strains (Kaplan-Levy et al., 2010; Maldener et al., 2014). In *A. variabilis*, we found that the transfer of a stationary phase grown liquid culture to low light conditions was enough to

induce akinete differentiation in likely all vegetative cells of the filaments. The exact commitment time for akinete formation is not known yet but in *A. variabilis*, the start of this process was microscopically visible after 3-7 days. The multi-layered thick envelope, characterized by TEM, is required for surviving harsh environmental conditions that ensure the long-term survival of cyanobacterial species (Figures 2A, B, Publication 2).

Furthermore, transfer of akinete culture to fresh medium and increasing the light intensity to normal level was the trigger for germination of akinetes in *A. variabilis*. Germination of akinetes was a fast process and took place within 18-24 h compared to the akinete differentiation, which usually took 3-7 days in low light in *A. variabilis*. We observed the release of 2-4 cell germlings from the ruptured envelope of akinete during germination (Figure 2C, Publication 2). These short filaments kept dividing in presence of sufficient nutrients and light and could form very long filaments containing 80-100 vegetative cells as well. After few days, these germinating filaments had comparable growth and length as the normal filaments. The presence of a nitrogen source was not needed for germination as we observed no difference in germination rate in the medium with or without combined nitrogen. In medium lacking the nitrogen source, cells began to differentiate heterocyst in the freshly germinated filaments which reflects the diazotrophic lifestyle of these cyanobacteria (Figure 2C, Publication 2).

In *A. variabilis* and *A. torulosa*, it has been shown that akinetes accumulate cyanophycin during their development and upon maturation a reduction in cyanophycin amount was observed (Perez et al., 2016; Sarma and Khattar, 1986). Degradation of cyanophycin during germination was also noticed in *A. cylindrica* (Fay, 1969), *Cylindrospermum* (Miller and Lang, 1968), *Nostoc* PCC 6720 (Skill and Smith, 1987) and *A. flos-aquae* (Wildman et al., 1975). However, it was not known whether this transient storage material serves as C- and N- source during dormancy and resuscitation. Our mutational study within *A. variabilis* showed that a cyanophycin-less mutant showed normal akinete development and germination (Figure 3, Publication 2). However, for which processes, akinetes are utilizing cyanophycin still remains unclear. Possibly, during long harsh unfavourable conditions, some structural stability to the akinetes is provided by cyanophycin or these granules are being utilized for thick envelope formation, which further needs to be explored.

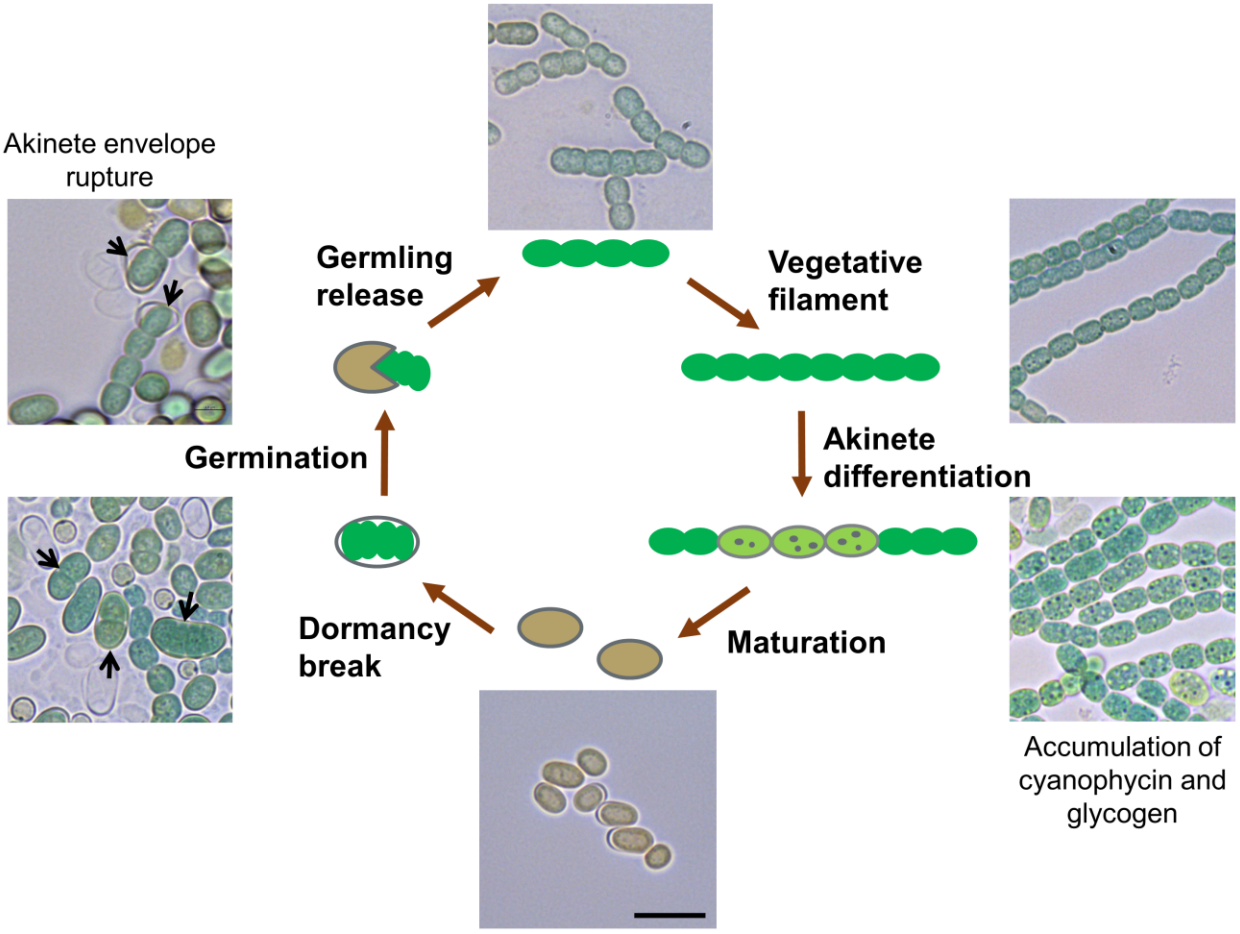

Figure 7. Scheme of akinete differentiation and germination process in *A. variabilis* with the representative bright field micrographs. Black arrows point to the germinating cells. Scale bar, 10 µm.

7.3 Insights into the envelope changes and intercellular communication during akinete differentiation and germination

A key to the success of *A. variabilis* survival in extreme conditions, is their ability to form highly resistant dormant akinetes and return to the normal growth when conditions are favorable again as shown in figures 1 and 7. Akinetes undergo various changes in response to external factors in order to survive and for which, building and maintaining their structure along with modification of shape and physiology is required. In this study, we focused on the aspects of how - after sensing the environmental signals - cells start to change at the structural, cellular, and physiological level during akinete differentiation and germination.

We were able to have a closer look at the akinete differentiation and germination processes using various techniques. First, scanning electron microscopy (SEM) was used to visualize the outer morphology of vegetative filaments and the mature akinetes. SEM images showed the wrinkles on mature akinetes, suggesting the akinete envelope folding (Figure 1, Publication 3). One possibility for wrinkle formation is, if the akinetes dehydrate during their maturation, perhaps due to removal of water similar to the bacterial spores, this would cause a shrinkage of the akinetes and may explain the wrinkled cell wall. As the cell wall is already synthesized during early akinete development, it will be wrinkled during shrinkage of the cells. We assume that these foldings provide the structural flexibility to the akinetes. When turgor pressure increases during germination, these foldings open up and make space for the germinating filament inside the envelope before its rupture.

Further, by using FIB/SEM tomography, we could obtain high-resolution three-dimensional image stacks of the entire akinete cells. With this, we could detect the morphological changes at different time points of akinete differentiation from vegetative cells. We observed the presence of intracellular electron dense granules in immature akinetes and no development of the typical akinete envelope was seen. In mature akinetes, the multilayered envelope was identified (Perez et al., 2018, 2016). In immature akinetes, the accumulation of reserve granules mainly of cyanophycin and glycogen was observed (Figures 2B, C, Publication 3) (Perez et al., 2016). In mature akinetes, these granular structures were not detected. In *A. variabilis*, as mentioned above, we showed that cyanophycin granules are not required for akinete formation and germination however, their direct role needs to be elucidated in future (Publication 2).

The presence of a multilayered envelope structure of akinetes provides the resistance to many environmental extremes. In this thesis, we have shown that the glycolipid layer is crucial for protecting the akinetes from freezing, desiccation, oxidative stress, and lysozyme attack (Publication 1). Further treatment of cells with sucrose showed that akinetes were able to resist osmotic stress and maintain their structure, whereas young vegetative cells turned out to be more susceptible. With 40% sucrose, we could see the shrinkage of most of the vegetative filaments whereas no effect on akinetes was observed (Figure 3, Publication 3). Here, we showed that the akinete envelope protects the cells against osmotic stress and provides structural stability. Another possibility to consider, is a different composition of the akinete cytoplasm compared to vegetative

cells. If the akinete cytoplasm contains less water similar to bacterial spores, then it is less prone to shrinkage. The behavior of vegetative filaments during sucrose treatment showed the interesting pattern of shrinkage as we observed the shrinkage of cells only longitudinally indicative of elasticity along the filament whereas in transversal direction, the wall apparently appeared to be more rigid.

Similarly, SEM analysis was done to visualize morphological and structural changes during germination, emergence, and outgrowth of the short germling. When akinetes start to germinate after sensing the favorable environmental conditions, the first visible changes during germination observed are the increased akinete cell size and disappearance of envelope wrinkles which suggest that akinete envelope is flexible and can adapt to different sizes for providing the structural integrity during germination. Terminally differentiated heterocysts were observed after 48 h indicating that the geminating small filaments can sense the presence/absence of a source of combined nitrogen (Figure 4, Publication 3). For degradation of the spore's peptidoglycan cortex during germination in many bacteria, several spore coat degrading enzymes are known to play redundant roles (Ishikawa et al., 1998; Li et al., 2013; Moriyama et al., 1996). For akinete envelope degradation during germination, no such enzymes have been discovered so far, which should be identified in future.

Here, in this study we have shown for the first time the changes in cell-cell communication during akinete differentiation and germination. We observed a decrease in communication during differentiation that can be explained by approaching the dormant stage by cells, where no exchange of molecules is needed anymore (Figure 5A, Publication 3). In contrast, when akinetes break the dormancy and start to germinate, cells go back to an active metabolism to allow cell division and growth. Therefore, we observed fast exchange of calcein during gemination in the dividing filament of *Anabaena* (Figure 5B, Publication 3). Additionally, we observed that the freshly germinating filaments even communicated faster than standard vegetative cells. In several filamentous cyanobacteria, the hormogonia (short motile filaments) also display a faster communication (Nürnberg et al., 2014). In hormogonia and young filaments, the higher communication rate may help in sensing the favorable light and nutritional conditions and could also play a role in establishing the symbiotic association with plants by providing the motility to hormogonia (Wilde and Mullineaux, 2015). Also, on their way to multicellular filaments, cell-cell

communication restarts enabling the formation of semi patterned heterocysts. The changes in cell-cell communication during akinete differentiation and germination shown in this study lays a stepping-stone for further cellular investigations during these two processes in akinetes. Furthermore, determining the changes in number of nanopores and septal junctions in the septal cell walls during akinete germination and differentiation should also be a focus of future research.

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9. Appendix

9.1 Publication 1

frontiers in Microbiology

ORIGINAL RESEARCH
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The Dual Role of the Glycolipid Envelope in Different Cell Types of the Multicellular Cyanobacterium *Anabaena variabilis* ATCC 29413

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Anabaena variabilis is a filamentous cyanobacterium that is capable to differentiate specialized cells, the heterocysts and akinetes, to survive under different stress conditions. Under nitrogen limited condition, heterocysts provide the filament with nitrogen by fixing N_2. Akinetes are spore-like dormant cells that allow survival during adverse environmental conditions. Both cell types are characterized by the presence of a thick multilayered envelope, including a glycolipid layer. While in the heterocyst this glycolipid layer is required for the maintenance of a microoxic environment and nitrogen fixation, its function in akinetes is completely unknown. Therefore, we constructed a mutant deficient in glycolipid synthesis and investigated the performance of heterocysts and akinetes in that mutant strain. We chose to delete the gene *Ava_2595*, which is homolog to the known *hglB* gene, encoding a putative polyketide synthase previously shown to be involved in heterocyst glycolipid synthesis in *Anabaena* sp. PCC 7120, a species which does not form akinetes. Under the respective conditions, the *Ava_2595* null mutant strain formed aberrant heterocysts and akinete-like cells, in which the specific glycolipid layers were absent. This confirmed firstly that both cell types use a glycolipid of identical chemical composition in their special envelopes and, secondly, that HglB is essential for glycolipid synthesis in both types of differentiated cells. As a consequence, the mutant was not able to fix N_2 and to grow under diazotrophic conditions. Furthermore, the akinetes lacking the glycolipids showed a severely reduced tolerance to stress conditions, but could germinate normally under standard conditions. This demonstrates the importance of the glycolipid layer for the ability of akinetes as spore-like dormant cells to withstand freezing, desiccation, oxidative stress and attack by lytic enzymes. Our study established the dual role of the glycolipid layer in fulfilling different functions in the evolutionary-related specialized cells of cyanobacteria. It also indicates the existence of a common pathway involving HglB for the synthesis of glycolipids in heterocysts and akinetes.

Keywords: cyanobacteria, *Anabaena*, heterocyst, akinete, HglB, glycolipids, stress tolerance, evolution

INTRODUCTION

To cope with various stress conditions, cyanobacteria require adaptation and survival strategies. Cell differentiation is key for successful survival in harsh and changing environmental conditions (Rippka and Herdman, 1985; Flores and Herrero, 2010). The planktonic freshwater filamentous cyanobacterium *Anabaena variabilis* ATCC 29413 can undergo a variety of cellular differentiation processes forming motile hormogonia, nitrogen-fixing heterocysts, and spore-like dormant cells called akinetes, and therefore, serves as a model organism to study the cell differentiation process in prokaryotes (Flores and Herrero, 2010; Maldener et al., 2014). The related species *Anabaena* sp. PCC 7120, which does not form akinetes, is the model organism to study heterocyst differentiation.

In *A. variabilis*, the nitrogen (N_2) fixing heterocysts appear in a semi-regular pattern along the filament in response to insufficient supply of combined nitrogen under aerobic conditions (Fay, 1992; Muro-Pastor and Maldener, 2019). Nitrogen fixation is mediated by the enzyme nitrogenase, which is highly sensitive to oxygen. To protect the nitrogenase, a microoxic environment is maintained in the heterocysts by inactivation of oxygen-evolving photosynthesis, by increased respiration, and by formation of an additional multilayered cell envelope outside the cell wall (Murry et al., 1984; Walsby, 1985; Murry and Wolk, 1989; Valladares et al., 2003, 2007; Magnuson, 2019). The heterocyst envelope is composed of two layers: an inner heterocyst-specific glycolipid (HGL) layer, which acts as a barrier to oxygen and limits its diffusion into the heterocyst, and an outermost heterocyst envelope polysaccharide (HEP) layer, which protects the HGL layer from physical damage (Wolk et al., 1994; Adams, 2000; Maldener et al., 2014; Muro-Pastor and Maldener, 2019).

Akinetes are spore-like non-motile cells that differentiate from the vegetative cells in response to diverse environmental factors including changes in light intensity, temperature, and nutrient deficiency. Akinetes can endure dryness and cold, while being vulnerable to high temperatures unlike spores, which are heat resistant. For this reason, they are just referred to as "spore-like" cells. Akinetes differ from the vegetative cells by their cellular structure, composition, and morphology. Akinetes ensure a longer period of survival to cyanobacteria under harsh and unfavorable conditions due to their resistance to cold and desiccation, and serve a perennation role (Yamamoto, 1976; Kaplan-Levy et al., 2010). Akinete formation is a transient process. When environmental conditions are favorable for growth, akinetes germinate into vegetative cells and start their life cycle over (Maldener et al., 2014). The light, temperature, and nutrient conditions favorable for growth appear to be the major stimulus for akinete germination (Yamamoto, 1976; Van Dok and Hart, 1997; Perez et al., 2016; Sukenik et al., 2018). Compared to akinete differentiation, which takes several days up to weeks, germination was observed to occur much faster in just few hours (Perez et al., 2018).

The process of akinete differentiation is characterized by the transient accumulation of storage compounds (such as glycogen, cyanophycin, lipids and nucleic acids), reduction of metabolic activities and formation of a thick multilayered envelope (Sutherland et al., 1979; Sukenik et al., 2012; Perez et al., 2016, 2018). This specialized envelope is composed of an outermost polysaccharide layer and inner layers of glycolipids, similar in composition to that of the heterocyst envelope (Cardemil and Wolk, 1976, 1981; Reddy, 1983; Soriente et al., 1993; Wolk et al., 1994; Wolk, 1996; Perez et al., 2018; Qiu et al., 2019). Whilst the function of the HGL layer is well understood in heterocysts (Haury and Wolk, 1978; Ernst et al., 1992; Fay, 1992), its role in akinetes and stress survival is unknown. Furthermore, the akinete envelope has some extra laminated layers, the function of which remains unknown (Braune, 1980; Nichols and Adams, 1982; Perez et al., 2018). Due to high similarity between their cell envelopes, akinetes have been supposed to be the evolutionary precursors of heterocysts (Wolk et al., 1994).

Several genes encoding the enzymes responsible for HEP and HGL synthesis in the heterocyst envelope have been identified [summarized in Nicolaisen et al. (2009); Maldener et al. (2014)]. The clustered genes *alr5351–alr5357* (also known as $hglE_A$, *hglD, hglC*, and *hglB)* in *Anabaena* sp. PCC 7120 (Black and Wolk, 1994; Bauer et al., 1997; Awai et al., 2009; Saito and Awai, 2020) and the *hglE* gene in *Nostoc punctiforme* (Campbell et al., 1997) are associated with glycolipid layer formation. The $hglBCDE_A$ gene cluster encodes putative enzymes for the biosynthesis of HGL aglycone (Fan et al., 2005; Awai et al., 2009; Saito and Awai, 2020). The *devBCA* gene cluster has been shown to be required for HGL export in *Anabaena* sp. PCC 7120 and *A. variabilis* (Fiedler et al., 1998a,b; Staron et al., 2011). Similarly, the gene cluster *hgdABC* is essential for proper HGL layer deposition in the heterocyst envelope in *Anabaena* sp. PCC 7120 (Fan et al., 2005; Shvarev et al., 2018).

Several heterocyst genes have also been found associated with akinete formation. For instance, the *hepA* gene, encoding a putative polysaccharide exporter, is essential for correct heterocyst envelope formation and consequently, the *A. variabilis hepA*-mutant forms an abnormal akinete envelope (Leganés, 1994). The overexpression of the heterocyst regulatory gene *devR* results in enhanced akinete differentiation in *N. punctiforme* (Campbell et al., 1996). In *Nostoc ellipsosporum*, deletion of the regulatory gene *hetR* inhibited both heterocyst and akinete differentiation (Leganés et al., 1994). However, a *hetR* mutant of *N. punctiforme* under phosphate starvation conditions could form large akinete-like cold-resistant cells (Wong and Meeks, 2002). These studies indicate a role for heterocyst genes in akinete formation, suggesting a common pathway regulating their differentiation.

The *hglB* gene [also known as *hetM* (Black and Wolk, 1994) or *alr5357*] encodes a putative polyketide synthase required for the synthesis of the glycolipid aglycones. HglB possesses two functional domains, an N-terminal acyl carrier protein (ACP) domain and a C-terminal thioester reductase (TER) domain (Black and Wolk, 1994; Bauer et al., 1997; Awai et al., 2009). The characterization of a *hglB* mutant of *Anabaena* sp. PCC 7120 (Maldener et al., 2003) demonstrated the role of this gene in HGL synthesis during heterocyst differentiation. *A. variabilis* harbors a gene in its genome with high similarity to *hglB*. To investigate the role of this *hglB* homolog in the synthesis of the glycolipid layer of akinetes, we performed a mutational analysis in

A. variabilis. As expected, the HGL layer of heterocysts is absent in the *hglB* mutant of *A. variabilis*. But also, distinct laminated layers are absent in the mutant akinetes. These aberrant akinetes lose their resistance against various stress conditions implying a different function of the glycolipid layer in heterocysts and akinetes. With this study, we were also able to show that envelope formation requires the same biosynthetic pathway in akinetes and heterocysts indicating an evolutionary relationship between both differentiation processes.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Vegetative cultures of *Anabaena variabilis* ATCC 29413 strain FD (Currier and Wolk, 1979; Thiel et al., 2014) and derived mutant strains (**Supplementary Table 1**) were grown photoautotrophically under continuous illumination (17–22 μmol photons m^{-2} s^{-1}) at 28°C with shaking at 120 rpm in standard medium of Allen and Arnon (1955) diluted 4-fold with water (AA/4) and supplemented with 5 mM KNO_3. The solid media remained undiluted with 1.5% (w/v) Difco Agar. For growing the mutant strain, 50 μg ml^{-1} neomycin was added to the medium. To induce heterocyst differentiation, the exponentially growing cultures ($OD_{750\ nm}$ 0.4–0.5) were harvested and washed three times in nitrate-free AA/4 medium, resuspended in same medium equal to the original volume, and cultivated under nitrogen depleted conditions.

Escherichia coli strains were grown in lysogeny broth (LB) medium at 37°C, supplemented with the following antibiotics: 50 μg ml^{-1} kanamycin (Km), 25 μg ml^{-1} chloramphenicol (Cm), 25 μg ml^{-1} streptomycin (Sm) and 100 μg ml^{-1} spectinomycin (Sp), when required. For growth on solid medium, 1.5% (w/v) agar was added. The *E. coli* strain Top 10 was used as a host for plasmid constructions. For triparental conjugation, the *E. coli* strain J53 (bearing the conjugative plasmid RP4), strain HB101 (bearing the helper plasmid pRL528 and the cargo plasmid), and the wild type (WT) *A. variabilis* culture were used (Maldener et al., 1991; **Supplementary Table 1**).

Mutant Construction

To construct the *hglB* mutant in *A. variabilis*, the gene *Ava_2595* (*hglB*) was inactivated by insertion of the neomycin-resistance-conferring cassette (C.K3) into the genome by double-crossover homologous recombination (Elhai and Wolk, 1988). For this, the left- and right-flanking regions of 500 bp from *hglB* were amplified in PCR using primers 1979 and 1980, and 1983 and 1984 (see **Supplementary Table 2** for primers) using genomic DNA as template and high fidelity Q5-polymerase (NEB, Ipswich, MA, United States). The C.K3 cassette was amplified from the plasmid pIM74 (Fiedler et al., 1998b) using primers 1981 and 1982. All PCR products were fused into the PstI digested suicide vector pRL271 (**Supplementary Table 1**) using Gibson assembly (Gibson et al., 2009). The resulting plasmid pIM752 was transferred into WT *A. variabilis* cells via triparental mating followed by the selection of clones on neomycin and 5% sucrose-containing agar plates (Maldener et al., 1991). Several clones were checked for full segregation of the mutated gene by colony PCR using primers 2534 and 2535. One of these genotypically verified and identical mutant clones was chosen for our study and named as DR752.

Akinete Differentiation and Germination

For akinete induction, BG11 medium containing $NaNO_3$ (Rippka et al., 1979) was used, as the differentiation of akinetes was better observed in this medium compared to AA/4. The late-exponentially grown cultures (OD_{750nm} 0.8) were induced to differentiate akinetes by transferring them to low light condition (2–3 μmol photons m^{-2} s^{-1}) with shaking at 50 rpm (Perez et al., 2016).

The germination of mature akinetes, exposed to different stress conditions, was induced by washing and transferring the culture to fresh BG11 media containing $NaNO_3$ and optimal light conditions (Perez et al., 2018). The formation of akinetes and their germination was monitored with a Leica DM 2500 light microscope with an x100/1.3 oil objective, connected to a Leica DFC420C camera (Leica Microsystems GmbH, Wetzlar, Germany).

Staining Procedures for Microscopy: Alcian Blue Staining

To observe the heterocyst envelope polysaccharide layer, Alcian blue staining was performed (Mckinney, 1953). Alcian blue solution [1.5% in H_2O (w/v)] was added to the cell suspension (in a ratio of 1:100) and incubated at room temperature (RT) for 5–10 min.

Triphenyl Tetrazolium Chloride (TTC) Staining

The culture containing heterocysts was mixed with the TTC solution [0.05% of TTC (w/v) in the final mixture] and incubated in dark for 15–30 min at RT (Fay and Kulasooriya, 1972).

BODIPY Staining

In order to visualize the glycolipid layer in the heterocyst and akinete envelope, samples with heterocyst and akinetes were stained with boron-dipyrromethene difluoride (BODIPY) 493/503 (Molecular Probes, Thermo Fisher Scientific, Waltham, MA, United States) as described previously (Perez et al., 2016). After all staining procedures, filaments were placed on the slides covered with 1.5% agarose and observed by light microscopy with a Leica DM 2500 microscope connected to Leica DFC420C camera or with a Leica DM 5500B fluorescence microscope connected to Leica DFC420C camera. The green fluorescence signal was monitored with a BP470 40-nm excitation filter and a BP525 50-nm emission filter.

Transmission Electron Microscopy (TEM)

For electron microscopy studies, individual cultures containing akinetes or heterocysts were fixed with 2.5% glutaraldehyde and post-fixed with 2% potassium permanganate followed by immobilization in agarose. After dehydration by successive increment of the ethanol concentration, the samples were

embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate (Fiedler et al., 1998a), and examined using a Philips Tecnai 10 electron microscope at 80 kHz.

RNA Isolation and Semi-Quantitative RT PCR (Reverse Transcription-PCR)

The total RNA was isolated at different time points after akinete induction (0, 3, 6, 12, and 18 days) or nitrogen step-down (0, 8, and 48 h) from wild-type and mutant DR752 cells, using UPzol reagent (Biotechrabbit, Henningsdorf, Germany) according to the manufacturer's instructions. Briefly, cells were harvested at the different time points, immediately snap-freezed in liquid nitrogen and stored at −80°C till further use. Upon addition of 1 mL UPzol solution, cells were homogenized using glass beads and total RNA was extracted. Concentration and purity of the extracted RNA were estimated using nanophotometer (Implen) and RNA gel-electrophoresis followed by DNase treatment. The genomic DNA contamination was controlled by PCR followed by agarose gel electrophoresis. The DNase-treated RNA was used to generate cDNA using the RT-reaction kit (Applied Biosystems) and 1 μl of this cDNA was used for semi-q RT PCR followed by visualization on an agarose gel. Primers for semi-q RT PCR reactions are listed in **Supplementary Table 2**.

Nitrogenase Activity

Nitrogenase activity was measured with the acetylene reduction method as previously described (Bornikoel et al., 2018; Shvarev et al., 2019). Briefly, after nitrogen step-down and 48 h cultivation under nitrogen-limiting conditions, the cell suspensions (20 μg Chl) were incubated under an atmosphere of 13.3% acetylene in air (oxic conditions) for several hours in flasks sealed with gas-tight caps and shaken in the light at 28°C. To generate anoxic conditions, a solution of 10 μM 3-(3,4-dichlorophenyl)-1, 1-dimethylurea (DCMU) (dissolved in methanol) was added and then, the sealed flasks were degassed, filled with argon and incubated for 1 h followed by incubation with acetylene. From each flask, 1 ml of the gaseous phase was taken, and the amount of ethylene produced was measured using gas chromatography.

Analysis of Heterocyst and Akinete Specific Glycolipids

Thin-layer chromatography (TLC) was performed to analyze the glycolipids composition of heterocysts and akinetes as described (Winkenbach et al., 1972) with minor modifications. First, the chlorophyll *a* (Chl*a*) concentration was measured (Mackinney, 1941). Briefly, Chl*a* was extracted from 1 ml of cultures grown with (BG11) and without nitrogen source ($BG11_0$), and from the cultures induced to form akinetes by low light for 2–4 months by adding methanol to a final concentration of 90% (v/v). The suspension was vortexed for 1 min and the cells were centrifuged at 13,000 rpm for 2 min at RT after incubation for 5 min in darkness followed by the measurement of adsorption of the supernatant at 665 nm. The final Chl*a* concentration was calculated using the formula:

$$\text{Chl } [\mu g/ml] = \text{OD665} \times \text{dilution factor} \times 13.43$$

For TLC, the cells were pelleted at equal Chl*a* concentration and resuspended in a methanol:chloroform mixture (1:1). Afterward, the lipids in the supernatant were concentrated by evaporation in air under a fume hood. The lipids were dissolved in chloroform and applied on an aluminum plate coated with silica gel (Macherey-Nagel, #818033). TLC was run in a mobile phase composed of chloroform:methanol:acetic acid:water in a ratio of 23:4:2.7:1. The lipids were visualized by spraying the TLC plate with 25% sulfuric acid and exposing it to 180°C for 60–120 s.

Drop Assay for Resistance Test

The viability of akinetes (3–4 months old) was tested after exposing them to different stress conditions using an agar spot assay. For this, Chl*a* content of akinetes culture was determined as described in previous section. For inducing the akinetes to different stress conditions, 1 ml of akinete cultures with a concentration of 5 μg Chl*a* ml^{-1} were exposed to either freeze (−20°C) and cold (4°C) conditions or desiccated (centrifuged and pellet dried at 28°C) for 10, 20, and 30 days, respectively. Other stress conditions included the treatment with 10 mM H_2O_2 for 2 h at 28°C, with 300 μg ml^{-1} lysozyme overnight at RT in dark and the freeze (in liquid nitrogen for 5–7 min) and thaw (at RT for 20–30 min) cycles for three or six times. For each stress conditions and time points, various dilutions of akinete samples (in the range of 5 μg ml^{-1}—0.5 μg ml^{-1} Chl*a*) were prepared and 10 μl of each dilution were carefully dropped on agar plates and incubated for 1 week at 28°C under continuous light.

RESULTS

The *hglB* Gene Is Essential for Diazotrophic Growth of *Anabaena variabilis* ATCC 29413

By BLAST search, we identified the homologous *hglB* gene *Ava_2595* in the genome of *A. variabilis*. We created the mutant, which will be denoted DR752 from here onward, by insertion of a neomycin resistance cassette (CK.3) into the *Ava_2595* gene by double homologous recombination (**Supplementary Figure 1A**). The complete segregation of the mutated chromosome version in neomycin resistant clones was confirmed by colony PCR (**Supplementary Figure 1B**) and one correct clone was used for further studies.

Under standard growth conditions in media supplemented with NO_3^-, no significant differences in doubling time (**Figure 1A**), filament length and cell morphology were observed in DR752 mutant compared to the wild type (WT) (not shown). However, the mutant was not able to grow on NO_3^--free medium (**Figure 1A**). In WT filaments, normally distributed heterocysts as well as terminal heterocysts were observed. In contrast, delayed heterocyst differentiation mostly by the terminal cells of the filaments was observed in mutant DR752 (**Supplementary Figure 1C**). After 2 days of nitrogen starvation, deposition of the outer polysaccharide layer in mutant heterocysts was confirmed by Alcian-blue staining, which stains the polysaccharide envelope in blue (**Supplementary Figure 2A**). In contrast, the envelope

glycolipids could not be detected by staining with the fluorescent dye BODIPY (**Supplementary Figure 2B**), which stains the HGL layer of heterocysts and akinetes (Perez et al., 2016).

To investigate whether HGL is being synthesized in the mutant DR752 after nitrogen step-down, we performed thin-layer chromatography (TLC) with the lipids extracted from filaments of the WT and the DR752 strains. In contrast to the WT, the mutant did not show a band with the typical TLC migration property (**Figure 1B**), confirming that the *hglB* gene encodes a protein that is involved in HGL biosynthesis. This is in line with the semi-quantitative RT-PCR results showing that the *hglB* gene is differentially expresses in the WT strain during heterocyst differentiation (**Supplementary Figure 3**). As expected, a transcript of *hglB* could not be detected in the RNA of the mutant. Interestingly, the gene *hetN*, localized downstream of *hglB* is constitutively expressed in the mutant. In the WT, *hetN* was up regulated during nitrogen step-down (**Supplementary Figure 3**). Since the CK.3 cassette contains a strong promoter, but lacks a termination signal, we assume that the *hetN* gene, encoding a suppressor of heterocysts involved in pattern formation, gets under control of this constitutive promoter (Callahan and Buikema, 2001). Overexpression of *hetN* suppresses heterocyst differentiation in *Anabaena* sp. PCC 7120, which could explain the delayed heterocyst formation in the mutant DR752.

Furthermore, we studied the ultrastructure of the mutant heterocysts by transmission electron microscopy (TEM) of ultrathin sections of mutant and WT filaments (**Figure 1C**). As expected, the mutant did not form the laminated layer, explaining why it could not grow diazotrophically under oxic conditions.

To assess the oxygen status in the heterocysts of the mutant, the filaments were incubated with triphenyl tetrazolium chloride (TTC), which forms dark red-brown precipitates in a microoxic environment (Fay and Kulasooriya, 1972). The WT heterocysts showed such precipitates but the DR752 mutant heterocysts did not (**Figure 2A**).

Furthermore, we used the acetylene reduction assay to measure the nitrogenase activity under oxic and anoxic conditions (**Figure 2B**). The DR752 mutant showed no nitrogenase activity under oxic conditions despite the occurrence of the *nifD* gene rearrangement required to produce a functional enzyme (**Figure 2C**; Brusca et al., 1989; Thiel and Pratte, 2014). In contrast, under anoxic conditions, the nitrogenase activity was measurable in the mutant and the WT at a similar level. This indicates that the mutant is not able to provide microoxic conditions suitable for the activity of the nitrogenase, as previously described for the *hglB* mutant of *Anabaena* PCC 7120 (Fox^-, Fix^+ phenotype).

Mutant DR752 Is Affected in Akinete Differentiation

We have previously shown that the laminated layers of *A. variabilis* akinete mostly consist of the heterocyst-specific glycolipid HG_{26}-diol (Perez et al., 2018). However, neither the function of this layer in akinete nor any enzyme for its synthesis was known so far. Hence, we investigated whether the *hglB* mutant from *A. variabilis* was affected in akinete differentiation and function. Stationary phase cultures of the WT and strain DR752 were incubated in low light conditions which trigger akinete formation (Perez et al., 2016). During the first 7 days in low light, WT filaments fragmented, whereas the mutant DR752 retained the long filaments and did not show any early sign of morphological differentiation (**Figure 3A**). After 15 days, approximately half of the WT filaments had turned into mature akinetes showing the typical morphology with the oval shape, thylakoid degradation evidenced by brownish color and a thick envelope (Perez et al., 2016). In contrast, the mutant strain showed only few immature akinetes with greenish color without a defined envelope (**Figure 3A**). After 30 days, most of the cells were mature akinetes in the WT culture. However, in the mutant DR752, approximately one-third of the mature akinetes were formed. This delayed akinete differentiation in DR752 indicates a possible role of HglB in akinete formation. However, upon a prolonged exposure of 2–4 months to low light, most of the vegetative cells in the DR752 mutant had finally differentiated into mature looking akinetes (not shown). Then, we investigated whether the akinetes of DR752 mutant were able to germinate similar to the WT akinetes. Three months after incubation of the filaments in low light, nearly all cells had differentiated to akinetes. After transferring these cultures to normal light, formation of filaments by germinating akinetes was observed. Cell division and resuscitation of the photosynthetic pigments occurred at the same speed in WT and mutant (**Figure 3B**).

HglB Is Involved in the Formation of the Glycolipid Layer of the Akinete Envelope

Heterocysts and akinetes of several species have been suggested to possess a similar envelope structure and identical glycolipids (Nichols and Adams, 1982; Soriente et al., 1993; Leganés, 1994; Adams and Duggan, 1999; Perez et al., 2018). Two laminated layers are visible in electron micrographs of akinetes from *A. variabilis* (**Figure 4C**; see also Perez et al., 2016, 2018), and so far, it was not known, whether both consist of the same glycolipids. Therefore, we first compared the akinete envelopes of the WT with the mutant DR752 using the green fluorescent dye BODIPY, which stains the HGLs of akinetes and heterocysts. After cultivation in low light for 2–4 months, a strong well-defined green-fluorescent signal was detected in the outline of mature akinetes of the WT strain. In contrast, BODIPY did not stain the akinetes from the mutant, indicating the absence of lipid layers (**Figure 4A**).

To investigate the presence of glycolipids in the akinete envelopes of WT and mutant DR752, total lipids were extracted from cultures exposed to low light for 2–4 months and separated by TLC. In the WT extract, a lipid was detected at the position where HG_{26}-ol from heterocyst migrates (**Figure 4B**), consistent with our previous study (Perez et al., 2018). This specific band was not observed in the DR752 mutant akinete culture, confirming that the *hglB* gene is involved in the synthesis of this glycolipid.

Next, we analyzed ultrathin sections of the akinetes of WT and DR752 mutant by TEM to solve the structure of the envelope at a better resolution. In the WT, two distinct

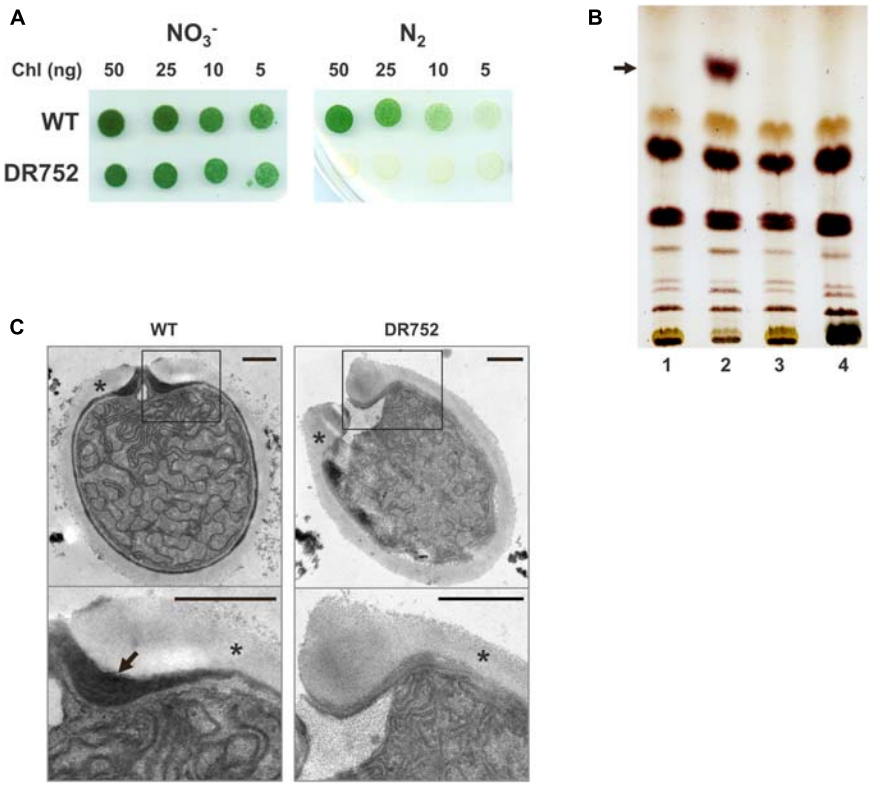

FIGURE 1 | Growth of the mutant strain DR752 and analysis of heterocyst-specific glycolipids in the envelope. **(A)** The growth of *Anabaena variabilis* wild type (WT) and DR752 mutant colonies on solid AA/4 medium ($NO3^-$) and medium lacking a combined nitrogen source (N_2) after 7 days of incubation. **(B)** Thin-layer chromatography of lipid extracts from liquid cultures containing 50 μg of chlorophyll before and after nitrogen step-down for 72 h; 1-WT with nitrate, 2-WT without nitrate, 3-DR752 with nitrate, 4-DR752 without nitrate. The arrow indicates the position of one heterocyst-specific glycolipid. **(C)** Transmission electron micrographs of terminal heterocysts of WT and mutant DR752. The lower panels show the magnified view of the envelope, indicated by squares. Black arrow points toward the laminated layer and star indicates the exopolysaccharide layer. Bars, 0.5 μm.

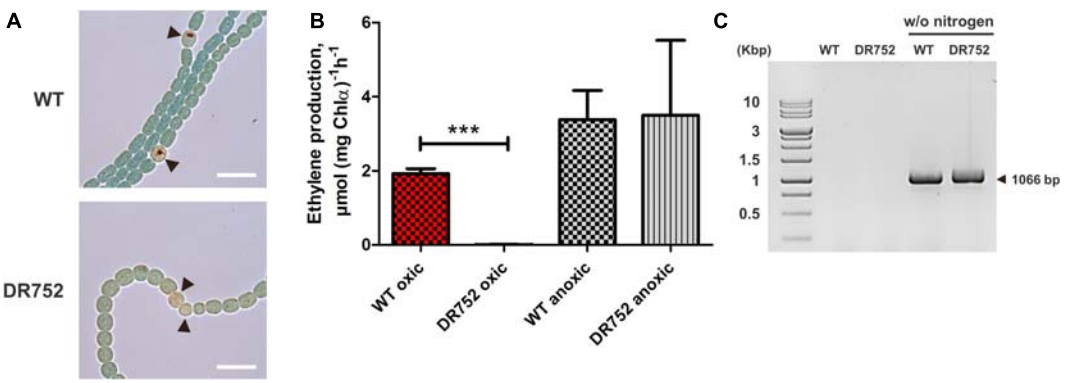

FIGURE 2 | Nitrogenase activity and *nifHDK* operon rearrangement assessment. **(A)** Bright field images of nitrogen-starved filaments of WT and mutant DR752 after incubation with triphenyl tetrazolium chloride (TTC). Black arrowheads point to the heterocysts. TTC reduces to dark formazan crystals under microoxic conditions. Scale bar, 10 μm. **(B)** Assessment of the nitrogenase activity of WT and mutant DR752 under oxic and anoxic conditions. Filaments were incubated for 48 h in nitrogen-free medium before measuring the nitrogenase activity using the acetylene reduction assay. Data are representative of two independent experiments. The histogram shows the mean values ± standard deviation of three experimental replicates. Student's *t*-test *P*-value is indicated as asterisks (***$P < 0.0001$). **(C)** The *nifD* rearrangement in WT and mutant DR752 after 72 h of nitrogen step-down was shown by PCR using primers (Ava903 FW and 919 Rv) left and right to the 11-kb insertion element as listed in **Supplementary Table 2**.

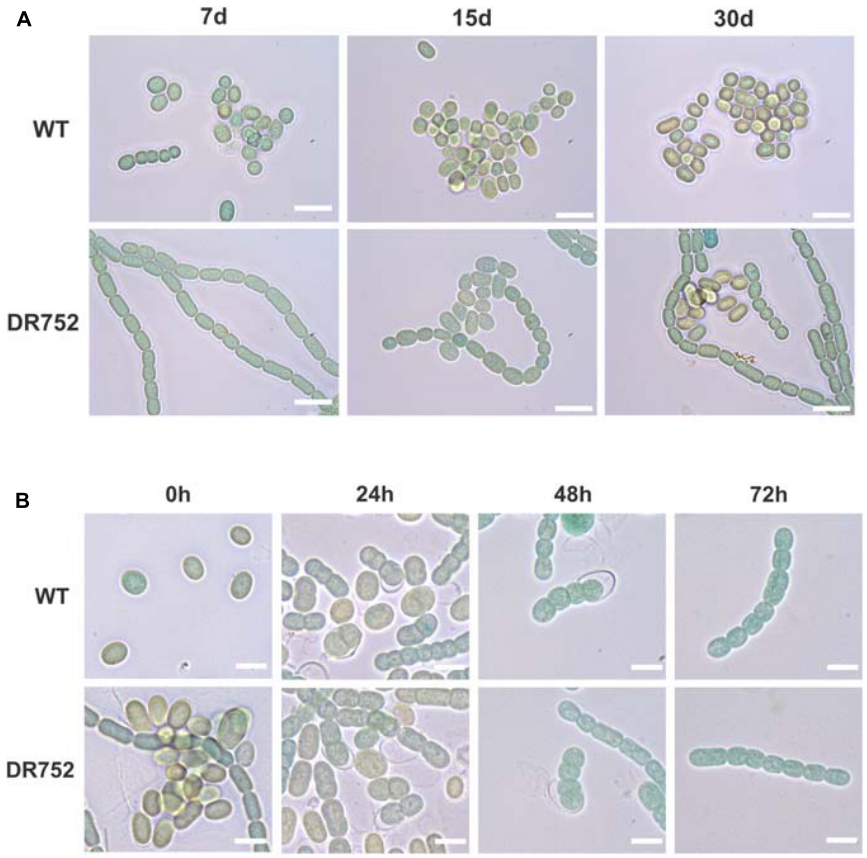

FIGURE 3 | Akinete differentiation and germination process in WT and mutant DR752. **(A)** Bright field micrographs of the WT and mutant DR752 culture after 7, 15, and 30 days of akinetes differentiation in low light. Bars, 10 μm. **(B)** Germination of 3–4 months old mature akinetes was induced by transferring the cultures to normal light conditions and monitored by light microscopy after 0, 24, 48, and 72 h. Bars, 5 μm.

electron dense laminated layers were visible between the outer exopolysaccharide layer and the outer membrane of the gram-negative cell wall [(Perez et al., 2016); **Figure 4C**]. Akinetes of the mutant DR752 showed the outermost polysaccharide layer, but both darkly stained laminated layers were absent. This confirms that the heterocyst lipid HG_{26}-diol is also a component of these laminated layers, and that the HglB is essential for HGL-synthesis in akinetes. This is in line with the increased transcription of the *hglB*-gene during akinete differentiation as shown by semi-quantitative RT-PCR (**Figure 4D**).

The Glycolipid Layer Is Important for Survival of Akinetes Under Various Stress Conditions

Akinetes are highly resistant to various environmental stress factors such as cold, desiccation and nutrient starvation. However, little is known about the molecular basis for such resistance and the role of the special thick envelope (Kaplan-Levy et al., 2010). After having shown that the mutant DR752 has no laminated layers in their akinete envelopes, this mutant offered the opportunity to investigate the importance of the glycolipid layer in resistance and response to different environmental extremes. For this, the akinetes of WT and mutant DR752 along with the WT vegetative cells as control were exposed to various stress conditions for different time points before diluting and plating on non-selective agar plates to determine their survival by colony formation. The WT vegetative cells and the akinetes of WT and mutant DR752 showed similar survival and germination efficiency, respectively being treated (**Figure 5**, left panels). Surprisingly, the same survival efficiency was observed for all types of cells when exposed to low temperature (cold, 4°C) (**Figure 5A**). However, the vegetative filaments were highly susceptible to the other tested stress conditions. The DR752 mutant akinetes were significantly less resistant and suffered a decline in survival efficiency when subjected to freeze and desiccation, compared to WT akinetes (**Figure 5A**). Upon successive freeze-thaw cycles, the mutant DR752 akinetes showed a significant decrease in germination relative to the WT akinetes (**Figure 5B**). Interestingly, the mutant DR752 akinetes were particularly more susceptible and could not germinate upon exposure to six freeze-thaw cycles as compared to three cycles (**Figure 5B**). To determine the effect of oxidative stress, the WT and mutant DR752 akinetes were treated with 10 mM H_2O_2 for 2 h. In these conditions the mutant DR752 akinetes showed decreased resistance and

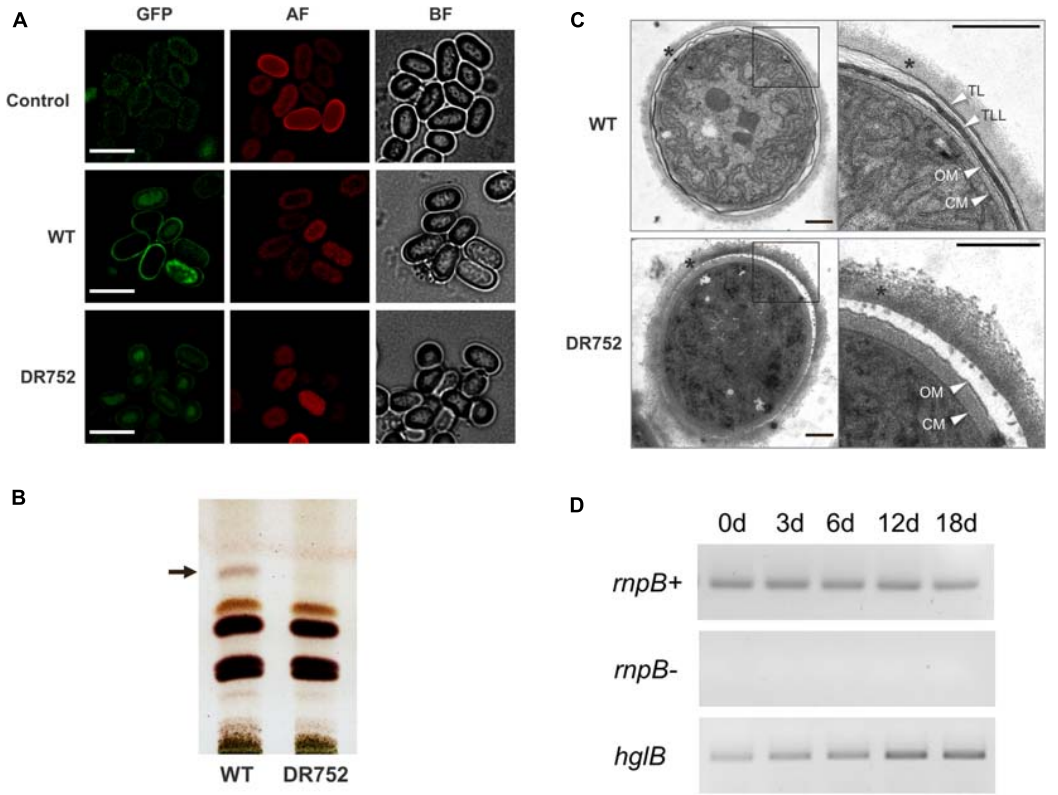

FIGURE 4 | Analysis of glycolipids and expression of *hglB* during akinetes formation. **(A)** Akinete envelope stained with the green fluorescent dye BODIPY. Control, mutant akinetes without BODIPY staining; BF, bright field; GFP, green fluorescent protein filter; AF, autofluorescence red. Bars, 7.5 μm. **(B)** Thin-layer chromatogram of lipid extracts from liquid cultures induced to develop akinetes in low light for 4 months. The position of the glycolipid band corresponding to the heterocyst-specific glycolipid HG_{26}-diol is indicated by an arrow. **(C)** Transmission electron micrographs (TEM) of akinetes of WT and mutant DR752. Right panels show the magnified view of akinete envelope, indicated by square. Star indicates the exopolysaccharide layer. White arrowheads indicate the following layers: TL, thin laminated layer; TLL, thick laminated layer; CM, cell membrane; OM, outer membrane. The empty space between outer membrane and polysaccharide layer is a dehydration artifact of the sample preparation procedure. Bars, 0.5 μm. **(D)** Expression analysis of *hglB* in the WT culture during akinete induction performed by semi-quantitative RT-PCR. *rnpB* (RNase P RNA coding gene) was used as a loading control with (*rnpB*+) and without reverse transcriptase (*rnpB*-). The primers used for semi-quantitative RT-PCR are RT rnpBFw, RT rnpBRv, RT hglBFw, and RT hglBRv; the sequences of the primers are listed in **Supplementary Table 2**.

survival compared to the WT akinetes (**Figure 5B**, right panel). Under these conditions, the mutant akinetes showed cell lysis. Similarly, when treated with 300 μg ml^{-1} lysozyme overnight, survival efficiency of the DR752 mutant akinetes was severely affected and only few colonies could germinate (**Figure 5C**). These results demonstrated that the mutant DR752 akinetes were more stable than the vegetative filaments but compared to the WT akinetes they were less resistant against various environmental stress conditions. Altogether, this study indicates that the glycolipid layer of the akinete envelope has an essential function in survival of akinetes.

DISCUSSION

Many genes are known to be involved in HGL formation in heterocysts of the model organism *Anabaena* sp. PCC 7120. In contrast, nothing is known about formation and role of the laminated layer in the akinete envelope. In this context, we have unraveled a new role of the homologous *hglB* gene in *A. variabilis* in glycolipid layer formation of the akinete envelope. As expected from the previously described phenotype of the *hglB*-mutant of *Anabaena* sp. PCC 7120, the DR752 mutant was also not able to grow on N_2 as a sole nitrogen source and formed aberrant heterocysts lacking the laminated layer (**Figures 1A,C**). Since the DR752 mutant could not synthesize the HGLs (**Figure 1B**), we confirmed the previous assumption that HglB is an enzyme involved in HGL synthesis, even though the enzymatic activity of HglB has not been explored yet (Awai et al., 2009).

Consistent with the lack of the HGL layer, the mutant DR752 was not able to fix N_2 in presence of oxygen, a phenotype described as Fox^- (Ernst et al., 1992; Campbell et al., 1997; Fan et al., 2006), because unable to provide microoxic conditions to protect the nitrogenase (**Figure 2A**). Consistently, nitrogenase activity was observed only under anoxic conditions. It has been shown that under anoxic conditions a second molybdenum-dependent nitrogenase is expressed also in vegetative cells of *A. variabilis* (Thiel et al., 1995, 2014), and is likely to contribute to a significant amount of activity under anoxic condition in the mutant as in the WT (**Figure 2B**). These observations support

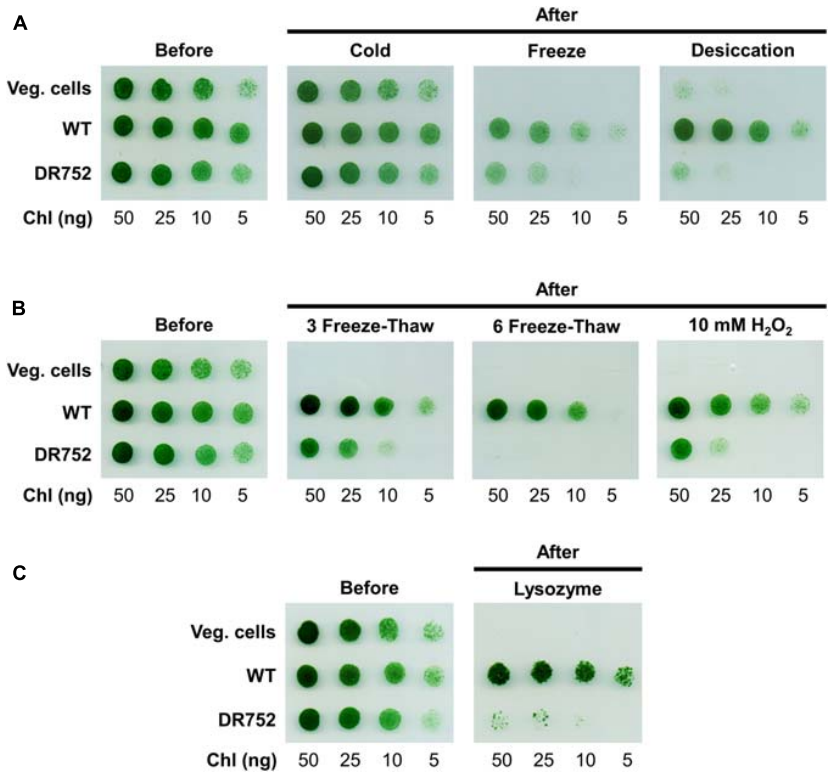

FIGURE 5 | Viability of WT vegetative filaments and the akinetes of WT and mutant DR752 exposed to different stress conditions. Vegetative cells (Veg. cells) and 3–4 months akinete-induced culture of WT and mutant DR752 were exposed to/treated with **(A)** cold, freeze and desiccation for 20 days, **(B)** three and six freeze-thaw cycles or 10 mM H_2O_2 for 2 h and **(C)** 300 μg ml^{-1} lysozyme for overnight. Serial dilution and plating were performed on agar plates before and after treatments and incubated for 7 days to allow germination and pseudo colony formation.

the non-functionality of the mutant heterocyst owing to the gas-permeability of cell envelope. The genome rearrangement within the *nifD*-gene (Brusca et al., 1989) is obviously not under control of oxygen, and can take place also in the mutant after withdrawal of a nitrogen source (**Figure 2C**).

Like heterocyst differentiation, akinete differentiation was delayed in the mutant and mature looking akinetes were detected much later compared to the WT (**Figure 3A**). Obviously, the state of the envelope formation has an influence on the maturation process of akinetes, but how this is sensed and influences the downstream signaling cascade needs to be further explored. However, an immediate downstream gene *hetN*, which was shown as a suppressor gene of heterocyst differentiation in *Anabaena* sp. PCC 7120 (Callahan and Buikema, 2001), was found to be constitutively expressed in DR752 mutant in contrast to the WT, where we observed its upregulation only after nitrogen step-down (**Supplementary Figure 3**). This may be due to the strong *psbA*- promoter of the CK.3 cassette (Elhai and Wolk, 1988) in the DR752 mutant, which might cause polar effects on the downstream genes. However, the *hetN* gene is not involved in glycolipid synthesis and we believe that its possible involvement can only be in delaying the heterocyst differentiation observed in the mutant. Whether *hetN* overexpression is also responsible for delayed akinete differentiation needs to be investigated in future.

For heterocyst envelope formation, it is well known that the envelope builds up in a highly regulated and ordered process [reviewed in: (Maldener et al., 2014; Flores et al., 2019)]. Electron micrographs and chemical characterization of the akinete envelope in *A. variabilis* and *N. punctiforme* showed that the laminated layer is composed of the heterocyst glycolipid HG_{26}-diol (Perez et al., 2018). The HGLs are found in a plethora of heterocyst-forming cyanobacteria (Reddy, 1983; Gambacorta et al., 1998; Wörmer et al., 2012; Bauersachs et al., 2014), but they were also identified in akinetes of *Cyanospira rippkae* (Soriente et al., 1993), consistent with the perception that HGLs are not exclusive to heterocyst, but also found in akinetes (Wörmer et al., 2012). In addition, the outermost akinete envelope consists of the same polysaccharide material that comprises the HEP layer of the heterocyst envelope (Cardemil and Wolk, 1979; Leganés, 1994; Perez et al., 2018) thus, emphasizing the evolutionary and developmental relationships between these two specialized cells (Wolk et al., 1994).

In addition to the chemical and structural identity of the laminated layer of both cell types, our study indicates that akinetes and heterocysts use an overlapping biosynthetic pathway to produce the glycolipid envelope, involving the putative polyketide synthase HglB. To our knowledge, *hglB* (Ava_ 2595) from *A. variabilis* is the first known gene involved in the envelope formation of akinetes. In this respect, it would be

interesting to analyze akinete formation of the previously described *hglE* mutant of *N. punctiforme*, which is lacking the HGLs in heterocysts and is affected in diazotrophic growth (Campbell et al., 1997).

To illustrate the importance of the glycolipid layers of the akinete cell envelope in stress tolerance and survival, we exposed the akinetes of WT and the *hglB*-mutant to unfavorable conditions. The significantly reduced resistance to freezing in the mutant, especially when using multiple freeze-thaw cycles, could be the consequence of a reduced cell wall stability (**Figures 5A,B**). Since, water expands when forming ice, the cell must withstand extreme pressure. In the mutant lacking the glycolipid layers the cell envelope is likely less stable. Indeed, the mutant akinetes and the vegetative cells lysed more easily upon freezing (visible by blue color of the medium; not shown), making them less viable in sub-zero environments. While the WT akinetes survived desiccation easily, the germination efficiency of the mutant was severely reduced (**Figure 5A**). This emphasizes the importance of the HGLs for resistance to desiccation, suggesting that the glycolipid layers prevent water efflux during dry periods. In bacteria, it is reported that desiccation causes hyperosmotic stress characterized by water loss, membrane disorganization and accumulation of reactive oxygen species and ultimately leads to the cell death (Potts, 1994; Greffe and Michiels, 2020). The reduced resistance to exogenous H_2O_2 could be explained by a thinner physical protective envelope in the mutant akinetes, which makes them more susceptible to damage by reactive oxygen species. The akinetes of the mutant are found to be less stable against any tested stress conditions, except the exposure to cold. Although the mutant develops defective akinetes, they are nevertheless more resistant to extreme environmental influences than vegetative cells due to the presence of the extracellular polysaccharide layer and intracellular adaptations (Argueta and Summers, 2005; Sukenik et al., 2015; Perez et al., 2016). Expectedly, the vegetative cells, which do not have an extra protecting cell envelope, were less resistant to any of the stress factors applied and only able to withstand cold for 20 days of incubation, conditions, which do not mimic cold winter seasons. In nature, cyanobacteria live in community with eukaryotic predators and other competing bacteria, which secrete proteases and cell wall lysing enzymes. Lysozyme, is a peptidoglycan degrading muramidase, which destroys the cell walls of bacteria (Ragland and Criss, 2017). The thick envelope of akinetes is capable to protect the dormant cells from lysozyme (Jensen and Sicko, 1971; Argueta and Summers, 2005) but the fully developed envelope with the glycolipid layers is a prerequisite for this (**Figure 5C**). Altogether, this study highlights the role of glycolipids in protecting the akinetes in harsh environments with changing conditions.

In summary, this study has established the diverse role of *hglB* in akinete and heterocyst envelope formation in *A. variabilis*. Additionally, we showed that glycolipid layers are necessary to protect the akinetes from various stress factors. The sharing of similar envelope-structures indicates that the developmental processes for akinetes and heterocysts occur in a similar way but fulfill different functions in these two specialized cell types of multicellular cyanobacteria. Further genes known to be involved in heterocyst envelope formation should be studied in the future to confirm the common biosynthetic pathways in heterocyst and akinetes and to learn more about their function in survival of dormant cells. It would also be interesting to understand the upstream divergent signaling pathways and to which point they converge.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

RG designed and performed the experiments, interpreted the data, wrote most of the manuscript, drafted the work, made manuscript revisions, and gave final approval. IM designed and supervised the research, wrote part of the manuscript, made substantial contributions to the design of the work, and made interpretation of data for the work. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.645028/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplemental Material

Table S1. Strains and plasmids used in this study.

Strain or plasmid	Source or reference
A. variabilis wild type	(Currier and Wolk, 1979; Thiel et al., 2014)
DR752	This study
pIM74	(Fiedler et al., 1998a)
pIM752	This study
Top10	Invitrogen
HB101 (pRL528)	(Wolk et al., 1984)
J53 (RP4)	(Wolk et al., 1984)
pRL271	(Black et al., 1993)

Table S2. Oligonucleotides used in this study in PCR.

All primers were purchased from Sigma-Aldrich.

Primer name	Sequence (5' to 3')
1979	GAAAGCTTGCATGCCTGCAATGAGTC TAAAACAAAATTATAG
1980	CATTGAGATCCTCTAGACGTACCAAA CAATATACATCAG
1981	GTATATTGTTTGGTACGTCTAGAGGA TCTCAATGAATATTG
1982	CATCAAATTGATAAAGTTCTAGAGGA TCCCCGGTGGGCGAAG
1983	CACCGGGGATCCTCTAGAACTTTATC AATTTGATGGTTAAG
1984	CAACGTTGTTGCCATTGCTCAAGCCA AGGTCAAGAACCCAG
Ava903 Fw	GTCCTCGTTTGGAAGGTAACAC
919 Rv	GAAGATACTGCGGAGCAAGGC
RT rnpBFw	GACCAGACTTGCTGGATAAC
RT rnpBRv	AAGACTCAAAATCCAAAATT
RT hglBFw	ACAGCAAACTCAGGCTGATG
RT hglBRv	CGCCAAAGTCTGGAACTGTG
RT hetNFw	ACAGGCAACGGTAGTTTGTG

RT hetNRv	AGGGCCGACAATATCTTGAG
2534	GGTAATGGGCTGTGGGCTTG
2535	CGCGTCTTCACCCGATTCAT

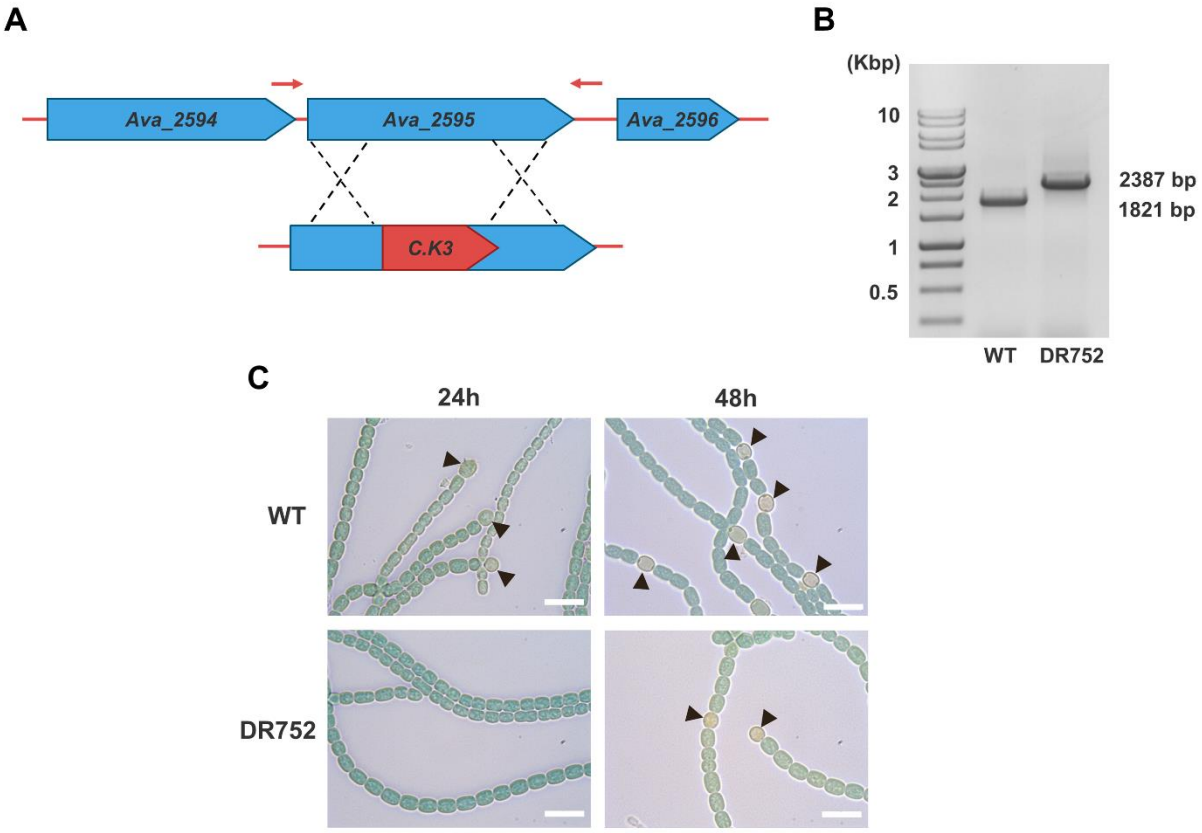

Figure S1 Construction and characterization of the *Anabaena variabilis* DR752 mutant. **(A)** Schematic of the strategy for the construction of mutant DR752. The *hglB* gene was disrupted by insertion of the neomycin resistance cassette (C.K3) into the genome by homologous recombination. Arrows indicate the location of primers used to verify complete segregation of the DR752 strain. **(B)** Confirmation of full segregation of the mutant by PCR. The amplification product of the wild type (WT) sequence is 1821 bp. After insertion, the sequence is 2387 bp. **(C)** Light micrographs of WT and mutant DR752 showing the heterocyst differentiation after 24 and 48 h of nitrogen step-down. Heterocysts are indicated by arrowheads. Bars, 10 µm.

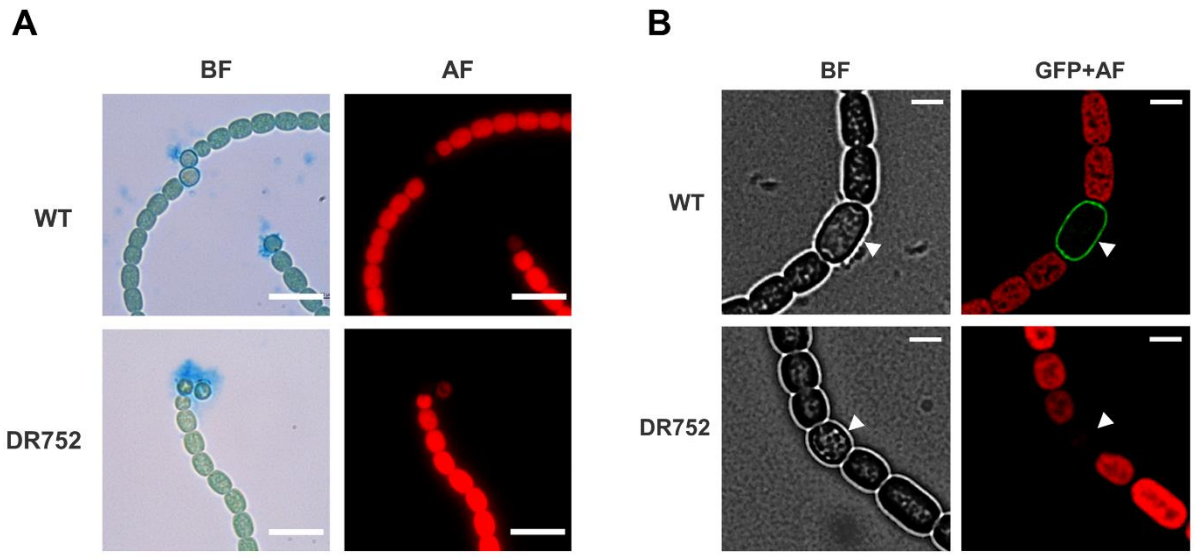

Figure S2 Alcian blue and BODIPY staining of the envelope of heterocyst**. (A)** Heterocysts envelope of WT and mutant DR752 stained with the Alcian blue dye (which stains exo-polysaccharides in blue) showed the presence of the polysaccharide layer. BF-bright field, AF-Autofluorescence. Bars, 10 µm. **(B)** Micrographs of filaments with heterocysts envelope stained with the green fluorescent dye BODIPY which binds to the neutral lipids. BF-bright field, GFP-green fluorescent protein filter, AF-Autofluorescence red. Heterocysts are indicated by white arrowheads. Bars, 3 µm.

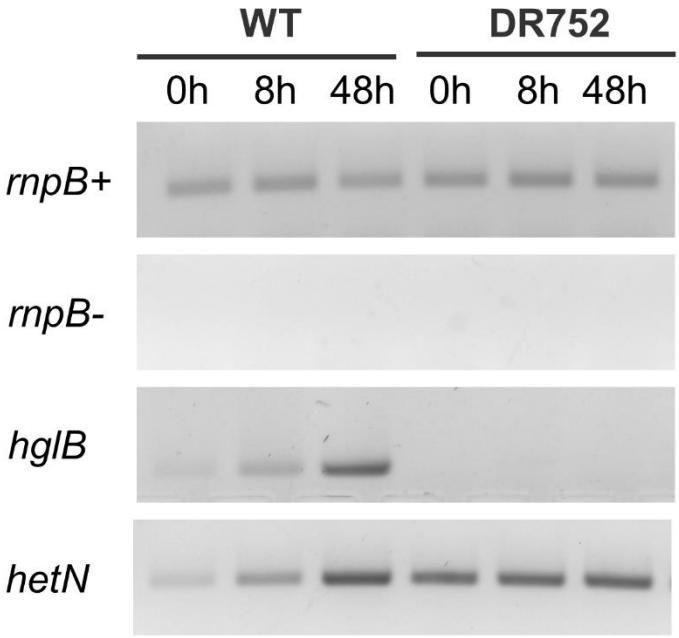

Figure S3 Analysis of gene expression during heterocyst differentiation. Time-dependent expression analysis of genes in the WT and mutant DR752 cultures during nitrogen starvation as assessed by semi-quantitative RT PCR. *rnpB* (ribonuclease B) was used as a loading control with (*rnpB*+) and without reverse transcriptase (*rnpB*-). Primers used for semi-quantitative RT PCR are listed in Table S2 in the supplemental material.

9.2 Publication 2

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The Formation of Spore-Like Akinetes: A Survival Strategy of Filamentous Cyanobacteria

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Keywords
Cyanobacteria · *Anabaena* · Akinete · Heterocyst · Germination · Envelope · Stress resistance

Abstract
Some cyanobacteria of the order Nostocales can form akinetes, spore-like dormant cells resistant to various unfavorable environmental fluctuations. Akinetes are larger than vegetative cells and contain large quantities of reserve products, mainly glycogen and the nitrogen storage polypeptide polymer cyanophycin. Akinetes are enveloped in a thick protective coat containing a multilayered structure and are able to germinate into new vegetative cells under suitable growth conditions. Here, we summarize the significant morphological and physiological changes that occur during akinete differentiation and germination and present our investigation of the physiological function of the storage polymer cyanophycin in these cellular processes. We show that the cyanophycin production is not required for formation and germination of the akinetes in the filamentous cyanobacterium *Anabaena variabilis* ATCC 29413.

Introduction

Cell Differentiation in Filamentous Cyanobacteria

The ability to differentiate specialized cells during the adaptation to environmental changes is a hallmark of filamentous cyanobacteria, which represent original multicellular organisms. Well-studied representatives of these filamentous cyanobacteria belong to the order Nostocales, including *Anabaena variabilis* ATCC 29413 and *Nostoc punctiforme* PCC 73102. Under favorable conditions, aquatic forms can grow in filaments of hundreds of cells, which are directly connected by cell-cell junctions [Flores et al., 2016, 2019; Kieninger et al., 2019]. These allow communication between cells all along the filament and, as a consequence, a rapid response to stress signals with a complete division of labor between different cell types [Weiss et al., 2019]. For instance, upon nitrogen starvation, about 10% of semi-randomly spaced cells can differentiate into nitrogen-fixing heterocysts, which support the filaments with nitrogen, presumably through the septal junctions. Another example of specialized cell type is represented by motile short filaments – the hormogonia – which enable dispersal of the cyanobacteria by float-

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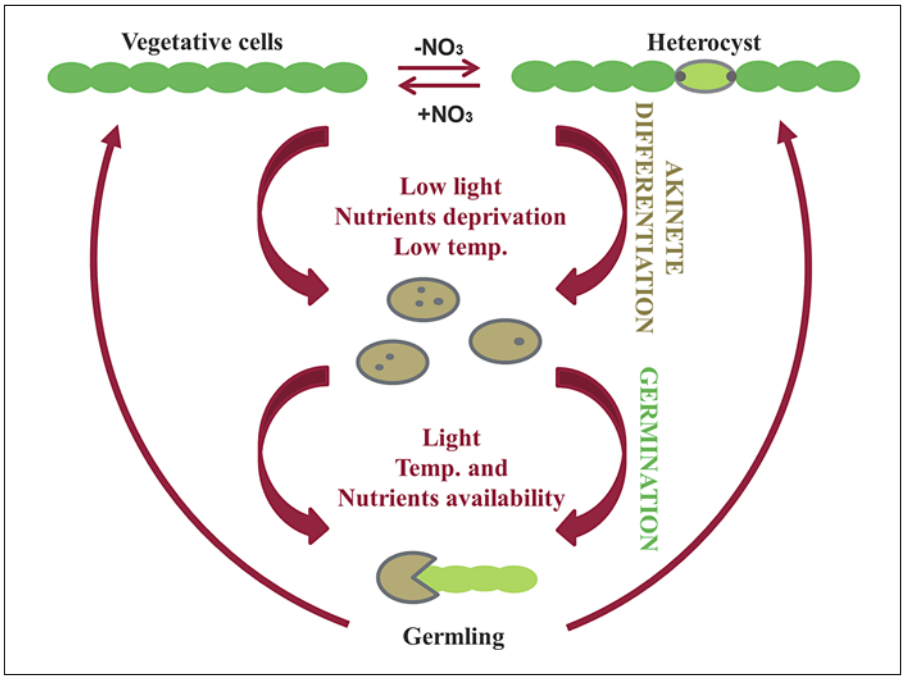

Fig. 1. Schematic representation of the life cycle of cyanobacteria of the order Nostocales. Under favorable growth conditions, vegetative cells can successively grow and divide forming long filaments. In absence of combined nitrogen sources, some vegetative cells differentiate into heterocysts, which can fix atmospheric nitrogen. Akinetes are formed under unfavorable conditions, such as nutrient starvation, low light, and low temperature. Akinetes are resting cells, which can survive under harsh environmental conditions. Finally, if the conditions are sufficiently favorable, the wall of akinetes ruptures, allowing germination and re-growth of the filaments.

ing or gliding [Meeks et al., 2002]. Finally, many species form spore-like resting cells, called akinetes, when the environment temporarily changes to unfavorable conditions like cold winters in temperate zones [Kaplan-Levy et al., 2010]. Akinetes are transient cells which differentiate from vegetative cells to enable these bacteria to withstand harsh environmental conditions [Maldener et al., 2014; Sukenik et al., 2018]. Several environmental factors have been reported to trigger the differentiation of akinetes in a species-specific way, including light intensity, light quality, temperature, and nutrient deficiency (Fig. 1) [Sukenik et al., 2013; Maldener et al., 2014].

The Akinete Structure

Akinetes differ from vegetative cells by their cellular composition and ultra-structure and are usually larger (sometimes up to 10-fold) than vegetative cells [Adams and Duggan, 1999]. During akinete differentiation, the cells transiently accumulate storage compounds, such as carbon in the form of glycogen, nitrogen in the form of cyanophycin globules (co-polymers of arginine and aspartate), and nucleic acids [Sutherland et al., 1985; Simon, 1987; Sarma et al., 2004; Kaplan-Levy et al., 2010; Sukenik et al., 2012]. In contrast, mature akinetes drastically reduce their metabolic activities [Perez et al., 2016].

Akinetes are surrounded by a thickened cell wall and a multilayered extracellular envelope. The distinct layers of the envelope include the outermost polysaccharide layer, similar to the homogeneous polysaccharide layer of the heterocyst envelope, the mucilaginous layer, and the glycolipids layer, which is identical in composition to that of the heterocyst envelope and absent in vegetative cells [Cardemil and Wolk, 1981; Nichols and Adams, 1982; Soriente et al., 1993; Wolk et al., 1994; Perez et al., 2018].

Interestingly, the reserve storage materials accumulated during akinete differentiation are degraded again upon akinete maturation [Perez et al., 2016]. This suggests that large amounts of reserve material are gradually consumed for the formation of the thick and complex akinete envelope during maturation and the extended period of dormancy.

Akinete formation is a transient differentiation process; when the environmental conditions are appropriate for growth, the akinetes can germinate, releasing small filaments from the envelope that resume the vegetative cell cycle [Kaplan-Levy et al., 2010]. The presence of light, moderate temperature, and nutrient conditions favorable for growth are the major stimuli for akinete germination (Fig. 1) [Yamamoto, 1976; Rai and Pandey, 1981; Huber, 1985; Fay, 1988; Adams and Duggan, 1999; Kaplan-Levy et al., 2010]. The presence of light is required for akinete germination in *Anabaena circinalis* [Van Dok and Hart, 1997], *A. variabilis* [Perez et al., 2018], and *Aphanizomenon flos-aquae* [Karlsson-Elfgren and Brunberg, 2004; Karlsson-Elfgren et al., 2004]. In addition, the germination of akinetes was shown to be dependent on light intensity and did not occur in the dark or in the presence of

the photosynthesis inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) [Yamamoto, 1976; Braune, 1979].

During germination in response to medium light, cell division starts inside the envelope of *A. variabilis* akinetes, with the energy for cell division initially supplied by the respiration of glycogen and subsequently by photosynthesis [Perez et al., 2018]. In *Anabaena cylindrica*, oxygen and light are required for germination, indicating that respiration of reserve material and photosynthesis provide the energy and carbon for this process [Yamamoto, 1976]. The germination of akinetes in *A. circinalis* requires light, as mentioned previously, but also phosphate [Van Dok and Hart, 1997]. Hence, the energy for akinete germination comes from the photosynthetic activity in this species, and respiration determines the rate of germination in a temperature-dependent manner [Kezhi et al., 1985; Fay, 1988].

Genes Required for Akinete Differentiation

Many putative transcriptional regulators of heterocyst differentiation are also involved in akinete differentiation. The overexpression of the heterocyst regulatory gene *devR,* encoding a response regulator of a two-component system involved in polysaccharide biosynthesis [Zhou and Wolk, 2003], results in enhanced akinete differentiation in *N. punctiforme* [Campbell et al., 1996]. The transcriptional regulatory gene *hetR* was shown to be downregulated in akinetes of *N. punctiforme*, and a *hetR* mutant strain formed cold-resistant akinete-like cells [Wong and Meeks, 2002]. However, *hetR* was shown to be essential for both heterocyst and akinete formation in *Nostoc ellipsosporum* [Leganés et al., 1994]. In addition, the *hepA* gene product is required for the formation of the polysaccharide layer in heterocysts [Wolk et al., 1994] as well as normal envelopes in akinetes of *A. variabilis* [Leganés, 1994]. A sigma/anti-sigma factor pair Npun_F4153 SigG/Npun_F4154 SapG was shown to be involved in the stress resistance mechanisms of akinetes. The transcription of *sigG* increases in heterocysts and akinetes, and its regulon includes genes that are mainly involved in cell envelope formation [Bell et al., 2017]. Recently, the *A. variabilis hglB* gene, involved in glycolipid synthesis of the heterocyst envelope, was shown to be also required for envelope formation in akinetes [Garg and Maldener, 2021]. So far, only one gene, *avaK*, has been identified as akinete marker gene in *A. variabilis* [Zhou and Wolk, 2002], but its cellular function is unknown yet. However, its homologous gene product AcaK43 has recently been found to be abundant in heterocysts of *A. cylindrica* [Qiu et al., 2020]. The presence of common genetic components suggests that akinetes and heterocysts share similar developmental processes but offers no clear evidence of a common regulatory pathway controlling their differentiation [Wolk et al., 1994].

Role of Akinetes in Stress Resistance and Survival

Akinetes have an ecological advantage as they can survive long enough for the environmental conditions to become favorable again for growth and to produce vegetative cyanobacteria cells. Akinetes are resistant to cold and desiccation, thereby allowing perennation or longer periods of survival [Hori et al., 2003; Kaplan-Levy et al., 2010]. They play a key role in the survival under stress conditions [Sukenik et al., 2012].

While initially reported to be sensitive to heat [Adams and Duggan, 1999], akinetes of *Nostoc* sp. HK-01 were recently shown to be heat tolerant as a result of the accumulation of betaine, glucosylglycerol, and glycine [Kimura et al., 2017]. Akinetes are highly resistant to dry conditions and a wide range of temperatures [Yamamoto, 1976; Kaplan-Levy et al., 2010; Kimura et al., 2015]. They have been reported to survive 5–7 years of desiccation and retain the capacity to germinate after storage in the darkness for 5 years at 27°C [Yamamoto, 1975; Sili et al., 1994] or months of cold (4°C) dark conditions [Sutherland et al., 1979] and have been isolated from sediments as old as 64 years [Livingstone and Jaworski, 1980].

In *A. cylindrica*, akinetes showed high tolerance to severe drying processes and were able to germinate after heat-drying in an oven at 60°C for 50 h and drying under sunlight for 10 h, suggesting that they are highly resistant [Hori et al., 2003]. In some cyanobacteria species, immature pre-akinetes are more tolerant to abiotic stress factors, such as osmotic stress [Kaplan et al., 2013; Pichrtová et al., 2014a], freezing [Trumhová et al., 2019], and desiccation [Pichrtová et al., 2014b]. Recently, we have also reported that the akinete envelope glycolipids are necessary to protect the akinetes from various stress conditions, such as freezing, desiccation, oxidative stress, and lysozyme attack, in *A. variabilis* [Garg and Maldener, 2021].

The Mature Akinete and Its Germination in A. variabilis

As akinetes are an important aspect of the cyanobacterial life cycle, more investigations are needed to understand their biology, differentiation process, and germination. Therefore, akinetes of *A. variabilis* were induced by exposure to low light as described previously [Perez et al., 2016]. We observed that during the differentiation of aki-

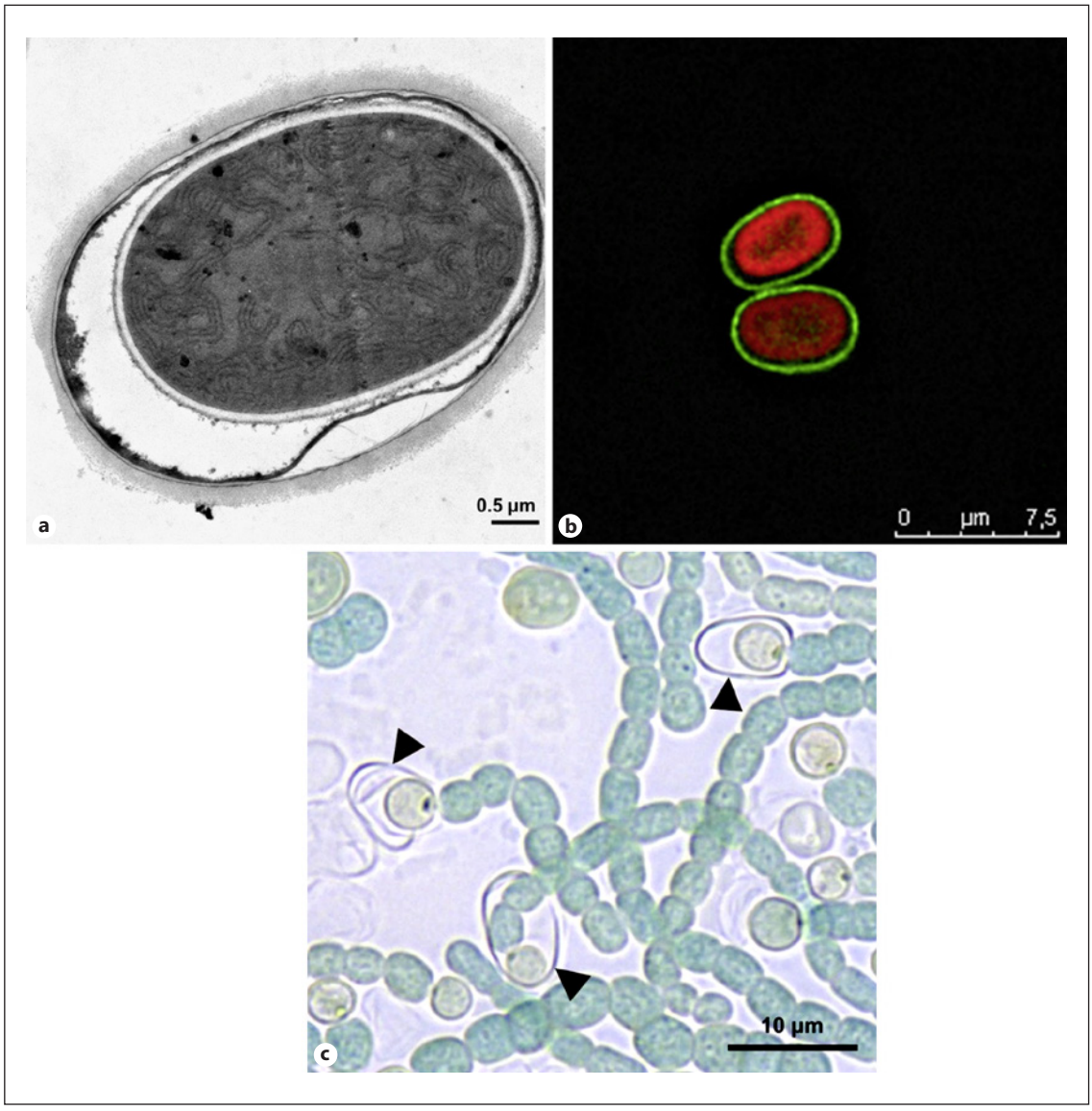

Fig. 2. Structural analysis of the akinete envelope and germination in *A. variabilis*. **a** Transmission electron micrographs of mature akinete exposed to low light for 30 days. A multilayered envelope surrounding the cell is visible. **b** In vivo visualization of the lipid layer of akinetes with the green fluorescent dye BODIPY. Shown are the overlaid images of BODIPY green fluorescence and red autofluorescence. **c** Bright-field micrograph showing germinating akinetes in medium lacking a source of nitrogen. Clearly, heterocyst differentiation gets induced at a very early stage of germination. Black arrowheads indicate the remaining akinete envelope after rupture.

netes from vegetative cells, many structural changes occurred, such as an increase in cell size and transient accumulation of intracellular granules like cyanophycin and glycogen [Simon, 1987; Perez et al., 2016]. Furthermore, a multilayered extracellular envelope built up, mainly consisting of an outermost polysaccharide layer and an inner glycolipid layer, as depicted in Figure 2a [see also Perez et al., 2016, 2018]. The glycolipid layer of akinetes could also be visualized with the fluorescent green dye BODIPY, which stains the lipid layer in living cells as previously described (Fig. 2b) [Perez et al., 2018].

Further, we analyzed the changes that take place during the akinete germination process. Two-month-old akinetes of *A. variabilis* were transferred to optimum light and fresh medium to induce germination. Akinete germination started with the increase in cell size, and the first cell division

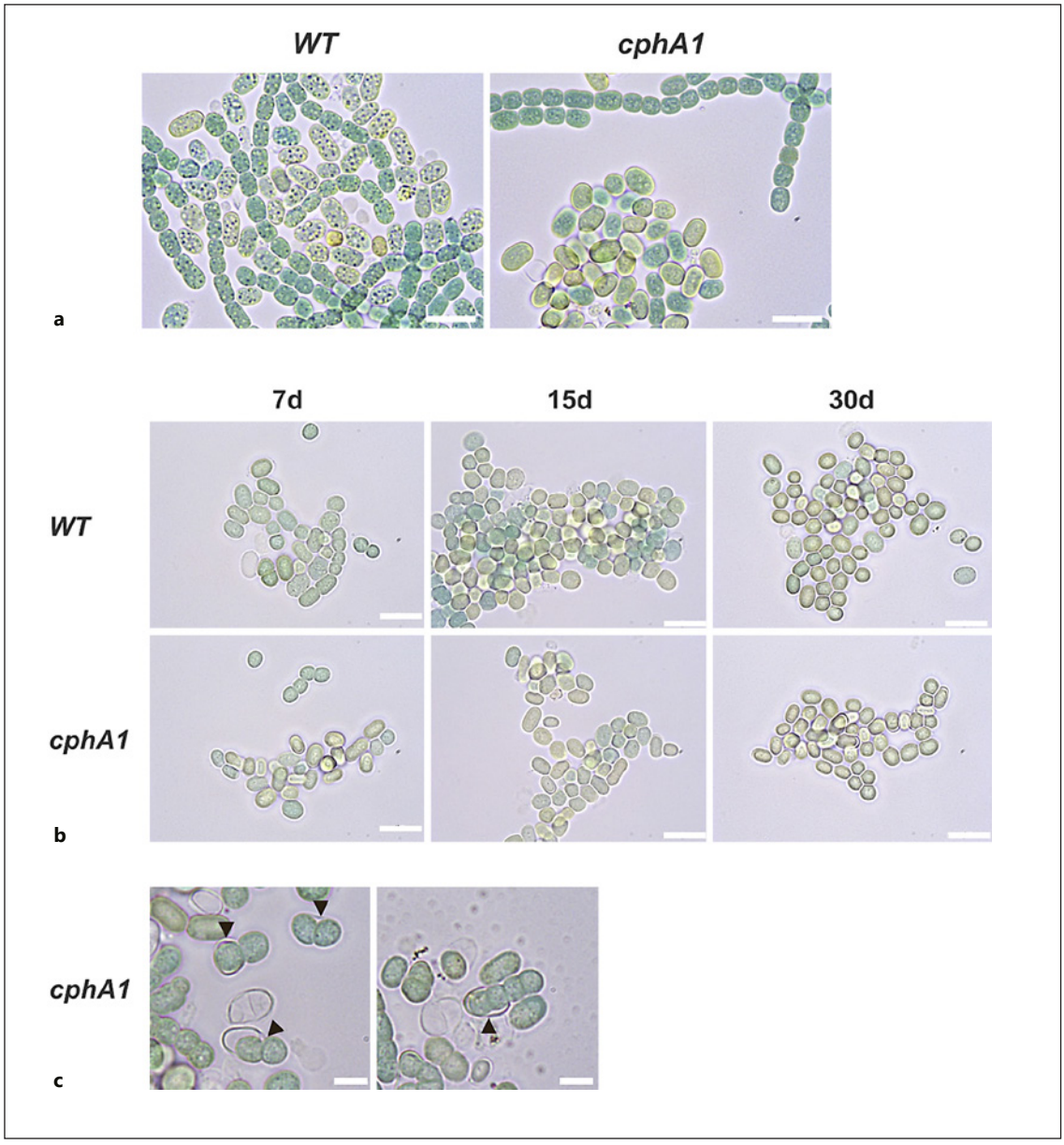

Fig. 3. Akinete differentiation and germination in the *A. variabilis* WT and *cphA1* mutant. **a** Bright-field images of WT and mutant *cphA1* akinete cultures induced by 7 days of phosphate starvation. Bars, 10 μm. **b** Akinete differentiation process in WT and mutant *cphA1* strains was induced by exposure to low-light condition and monitored by light microscopy after 7 days, 15 days, and 30 days. Bars, 10 μm. **c** Bright-field micrograph showing akinete germination in *cphA1* mutant. Germination of mature akinetes was induced by transferring the cultures to fresh medium and normal light conditions. Black arrowheads indicate the germinating akinetes. Bars, 5 μm. WT, wild type.

inside the akinete envelope was observed within 18–24 h. Successive cell divisions increased the pressure inside the envelope, which eventually ruptured and released the short filament. When using fresh nitrogen-free medium, the terminal cell began to differentiate into a heterocyst soon after the first cell division (Fig. 2c). This result clearly indicated that the germinating small filament can sense the presence/absence of a nitrogen source at the very beginning of cell division. The heterocyst presumably performs nitrogen fixation to support the growing filament with amino acids, since the nitrogen storage compounds were used up during maturation of the dormant cell [see also Perez et al., 2018].

Role of Cyanophycin in Akinete Differentiation and Germination

As mentioned earlier, several cyanobacterial species are known to accumulate intracellular reserve material, such as glycogen and cyanophycin, during akinete differentiation. In *Anabaena torulosa*, akinetes accumulate cyanophycin during their development and decrease the amount of it when mature [Sarma and Khattar, 1986]. Similarly, *Aphanizomenon ovalisporum* also accumulates cyanophycin during the formation of akinetes induced by potassium starvation [Sukenik et al., 2015]. A previous study by Leganés et al. [1998] showed that cyanophycin granule formation is necessary for the function of heterocysts and akinetes in *N. ellipsosporum*. However, vegetative cells also accumulate glycogen and cyanophycin in the stationary growth phase and under stress conditions [Lawry and Simon, 1982; Herdman, 1987], indicating that the accumulation of reserve material is not restricted to akinete formation. Moreover, unicellular cyanobacteria, with no capacity for differentiation, accumulate storage compounds in response to starvation conditions [Doello et al., 2018; Watzer and Forchhammer, 2018]. Hence, the accumulation of reserve material is considered a general response to starvation to regulate nutrient homeostasis [Forchhammer and Schwarz, 2019].

Cyanophycin is a dynamic carbon/nitrogen storage polymer widespread among cyanobacteria and a few heterotrophic bacteria. The accumulation of cyanophycin granules in akinetes was observed for many Nostocales species [Sarma and Khattar, 1986; Sarma et al., 2004]. During germination, the degradation of cyanophycin was observed in *Cylindrospermum* sp. [Miller and Lang, 1968], *A. flos-aquae* [Wildman et al., 1975], and *A. cylindrica* [Fay, 1969]. Mutation of the arginine biosynthesis gene, *argL*, in *N. ellipsosporum* [Leganés et al., 1998] and incubation of *A. cylindrica* with the arginine analog, canavanine [Nichols and Adams, 1982], resulted in the production of cyanophycin-lacking akinetes that were unable to germinate. This suggested the requirement of cyanophycin accumulation for the germination of akinetes, but not for its formation.

Results

Characterization of a cphA Mutant

To determine whether the cyanophycin granules that accumulate during akinete differentiation in *A. variabilis* are required for akinete formation and germination, we created an *A. variabilis* mutant void of cyanophycin synthesis. For this, the gene *cphA1* encoding the cyanophycin synthetase was interrupted by insertion of a neomycin resistance cassette by double homologous recombination (see Materials and Methods). CphA catalyzes the biosynthesis of the nitrogen reserve cyanophycin (multi-L-arginyl-poly-L-aspartic acid) [Berg et al., 2000]. Under standard growth conditions in media supplemented with NO_3^-, no visible differences in cell morphology and filament length were observed in *cphA1* mutant compared to the wild type (WT) (not shown). Moreover, the mutant grew similar to the WT strain on medium containing or lacking the combined nitrogen source (online suppl. Fig. S1A; see www.karger.com/doi/10.1159/000517443 for all online suppl. material). We also observed that the mutant heterocysts lacked cyanophycin polar nodules, consistent with a previous study by Ziegler et al. [2001] (online suppl. Fig. S1B).

In the *cphA1* mutant, cyanophycin granules were also absent in akinetes after 7 days of phosphate starvation (Fig. 3a). In contrast to akinete induction by low light (see below), this nutrient limitation results in better synchronization. The WT akinetes clearly displayed cyanophycin granules under the same conditions. Despite the absence of cyanophycin granules, the *cphA1* mutant was able to differentiate akinetes similar to the WT.

The rate of akinete formation was also investigated in a low-light condition, which is reported as a better inducing factor for akinete differentiation in *A. variabilis* [Perez et al., 2016]. The akinete development process and the rate of differentiation were similar to the WT (Fig. 3b). Also, the envelope ultra-structure was like the WT in transmission electron micrographs (data not shown). These results clearly indicate that cyanophycin is not required for akinete differentiation in *A. variabilis*.

Furthermore, we investigated the germination of akinetes of the *cphA1* mutant to determine the role of cyanophycin granules in germination and to check the viability of the mutant akinetes. Germination of mutant *cphA1* akinetes was induced as described above for the WT. We observed a similar germination rate and pattern for the mutant akinetes as for the WT (Fig. 3c). Altogether, these results indicate that the differentiation of akinetes in *A. variabilis* does not require cyanophycin and the akinetes lacking cyanophycin granules can germinate under the applied conditions.

Materials and Methods

Strains and Growth Conditions

A. variabilis ATCC 29413 strain FD [Currier and Wolk, 1979; Thiel et al., 2014] and derived mutant strains were cultivated pho-

toautotrophically under continuous illumination (17–22 μmol photons m^{-2} s^{-1}) in 100 mL Erlenmeyer flasks at 28°C with shaking at 120 rpm in standard medium of Allen and Arnon [1955] diluted 4-fold with water (AA/4) and supplemented with 5 mM KNO_3. The solid media remained undiluted with 1.5% (w/v) Difco Agar. The mutant strain was grown in the medium supplemented with 50 μg mL^{-1} neomycin.

To induce heterocyst differentiation, exponentially growing cultures were washed 3 times in nitrate-free AA/4 medium, resuspended in the same medium equal to the original volume, and cultivated under nitrogen-depleted growth conditions. Chlorophyll *a* (Chl*a*) content of culture was determined as reported previously [Mackinney, 1941].

Escherichia coli strains were grown in lysogeny broth medium at 37°C, supplemented with the following antibiotics: 50 μg mL^{-1} kanamycin (Km), 25 μg mL^{-1} chloramphenicol (Cm), 25 μg mL^{-1} streptomycin (Sm), when required. For growth on solid medium, 1.5% (w/v) agar was added. The *E. coli* strain Top10 was used as a host in plasmid constructions. For triparental mating, the *E. coli* strain J53 (bearing the conjugative plasmid RP4), strain HB101 (bearing the helper plasmid pRL528 and the cargo plasmid), and the WT *A. variabilis* strain were used [Maldener et al., 1991] (online suppl. Table S1).

Mutant Construction

To construct the *cphA1* mutant in *A. variabilis*, the gene *Ava_1814* (*cphA1*) was interrupted by insertion of the neomycin resistance-conferring cassette (C.K3t4) into the genome by double-crossover homologous recombination. For this, the left- and right-flanking regions of *cphA1* were amplified by PCR using primers 2015/2016 and 2019/2020 (see online suppl. Table S2 for primers) using genomic DNA as a template and high-fidelity Q5-polymerase (NEB, Ipswich, MA, USA). The C.K3t4 cassette was constructed by the modification of the previously described cassette C.K3 [Elhai and Wolk, 1988]. Briefly, the C.K3 cassette was fused to the C-terminus of the bacteriophage T4 gene *32* [Krisch and Allet, 1982] bearing a transcriptional terminator and a translation tandem stop codon. The C.K3t4 cassette was amplified using primers 2017 and 2018. All PCR products were fused into the *Xho*I digested suicide vector pRL277 (online suppl. Table S1) using Gibson assembly [Gibson et al., 2009]. The resulting plasmid pIM764 was transferred into *A. variabilis* WT cells using triparental mating followed by the selection of clones on neomycin and 5% sucrose-containing agar plates [Maldener et al., 1991]. The segregation of the mutant colony was analyzed by colony PCR and the strain was named *cphA1* mutant.

Akinete Differentiation and Germination

Akinete differentiation was induced in late exponentially grown cultures by either low light or phosphate starvation. Low-light conditions (2–3 μmol photons m^{-2} s^{-1}) were maintained by covering flasks with paper towels. For phosphate starvation, filaments were washed 3 times with AA/4 medium without inorganic phosphate and then transferred to AA/4 medium supplemented with 5 mM MOPS buffer (pH 7.5), 2.5 mM NH_4Cl, 2.5 mM $NaNO_3$, and 2.5 mM KNO_3, but lacking inorganic phosphate as described previously [Perez et al., 2016]. All induced cultures were maintained at 28°C with shaking at 50 rpm.

The germination of mature akinetes was induced by washing and transferring the akinete culture to either BG11 medium containing $NaNO_3$ or $BG11_0$ medium lacking combined nitrogen and optimal light conditions [Perez et al., 2018].

The akinetes differentiation and germination process was observed by Leica DM 2500 light microscope with a ×100/1.3 oil objective, connected to a Leica DFC420C camera (Leica Microsystems GmbH, Wetzlar, Germany).

Transmission Electron Microscopy

For electron microscopy, the akinete cells were fixed with 2.5% glutaraldehyde followed by postfixation with 2% potassium permanganate and immobilization in agarose. Upon dehydration by successive increment of the ethanol concentration, the samples were embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate [Fiedler et al., 1998] and examined with a Philips Tecnai 10 electron microscope at 80 kHz.

BODIPY Staining

To visualize the glycolipid layer in akinetes envelope, samples of akinete culture were stained with boron-dipyrromethene difluoride (BODIPY) 493/503 (Molecular Probes, Thermo Fisher Scientific, Waltham, MA, USA) as described previously [Perez et al., 2016]. After staining, filaments were placed on the slides covered with 1.5% agarose and observed by light microscopy with a Leica DM 2500 microscope connected to a Leica DFC420C camera or with a Leica DM 5500B fluorescence microscope connected to a Leica DFC420C camera. The green fluorescence signal was monitored with a BP470 40-nm excitation filter and a BP525 50-nm emission filter.

Conclusions

The aim of our studies was to understand the changes in morphology and physiology in more detail during the formation and germination of akinetes in the model organism *A. variabilis* ATCC 29413 and discern the long-term survival strategy of these spore-like cells. Various environmental signals were reported to trigger akinete differentiation and germination. However, these processes are still not well understood at the cellular and molecular level [Adams and Duggan, 1999; Kaplan-Levy et al., 2010; Maldener et al., 2014].

A. variabilis akinetes have a multi-layered envelope required for surviving harsh environmental conditions. We have shown recently that this reliable barrier between the dormant akinete cell and the harsh conditions is determined by the chemical composition and fine structure of the akinete envelope, especially the presence of a glycolipid layer [Perez et al., 2018; Garg and Maldener, 2021].

Like akinete differentiation, the germination of mature akinetes of *A. variabilis* is a highly asynchronous process. Various environmental stimuli, such as moderate temperature, increased light intensity, sediment resus-

pension, and nutrition, can trigger akinete germination [Huber, 1985; Van Dok and Hart, 1997; Karlsson-Elfgren and Brunberg, 2004; Perez et al., 2016].

The presence of a nitrogen source was not needed for germination and reflects the diazotrophic lifestyle of these cyanobacteria, which form heterocysts in the freshly germinated filaments.

We clarified the biological function of cyanophycin in akinetes differentiation and germination in *A. variabilis* and showed that cyanophycin provides the WT cyanobacterium with no advantage over the non-cyanophycin-producing mutant (online suppl. Fig. S1) consistent with the previous report [Ziegler et al., 2001]. It remains unknown for which processes cyanophycin is being utilized in akinetes. It might be possible that cyanophycin provides some structural stability to the akinetes during long harsh unfavorable conditions, which needs to be further elucidated.

The availability of the data from fully sequenced genomes of several Nostocales species and implementation of various molecular and genomic tools ensure the advancement of a better understanding of the dormancy phenomenon in cyanobacteria. Additionally, to promote the understanding of akinete differentiation, survival, and germination, further elaboration of methods for mutational analysis and cellular characterization of mutants specifically impaired in the cellular function of akinete is needed.

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Statement of Ethics

No ethical approval was sought as neither human or animal participation was involved.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

R.G. designed and performed the experiments, interpreted the data, and wrote most parts of the manuscript. I.M. designed and supervised the experiments, wrote part of the manuscript, revised the work critically for important intellectual content, and gave the final approval. All authors made substantial contributions to the design of the work and performed acquisition, analysis, and interpretation of data for the work. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

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SUPPLEMENTAL MATERIAL

Table S1. Strains and plasmids used in this study.

Strain or plasmid	Source or reference
A. variabilis wild type	(Currier and Wolk, 1979; Thiel et al., 2014)
CK.3	(Elhai & Wolk 1988)
pIM764	This study
Top10	Invitrogen
HB101 (pRL528)	(Wolk et al., 1984)
J53 (RP4)	(Wolk et al., 1984)
pRL277	(Black et al., 1993)

Table S2. Oligonucleotides used in this study in PCR.

All primers were purchased from Sigma-Aldrich.

Primer name	Sequence (5' to 3')
2015	GATGAATGGCAGAAATTCGATATCTAGATCATGAGAATCCTCAAGATCCAG
2016	CAATATTCATTGAGATCCTCTAGAAAGCTTATCACGCCATAAATCTTTCAGG
2017	ATACAAGACCTGAAAGATTTATGGCGTGATAAGCTTTCTAGAGGATCTCAATG
2018	TGAGCATAGTCAACTAAGGCGTGGTAGTTGCAAGCTTTATGCTTGTAAACCG
2019	TTCACAAAACGGTTTACAAGCATAAAGCTTGCAACTACCACGCCTTAGTTGAC
2020	CTGGCGGACGGGAAGTATCCAGCTCGACTACAGCAAAGTATTAATTACAGAAGAAG

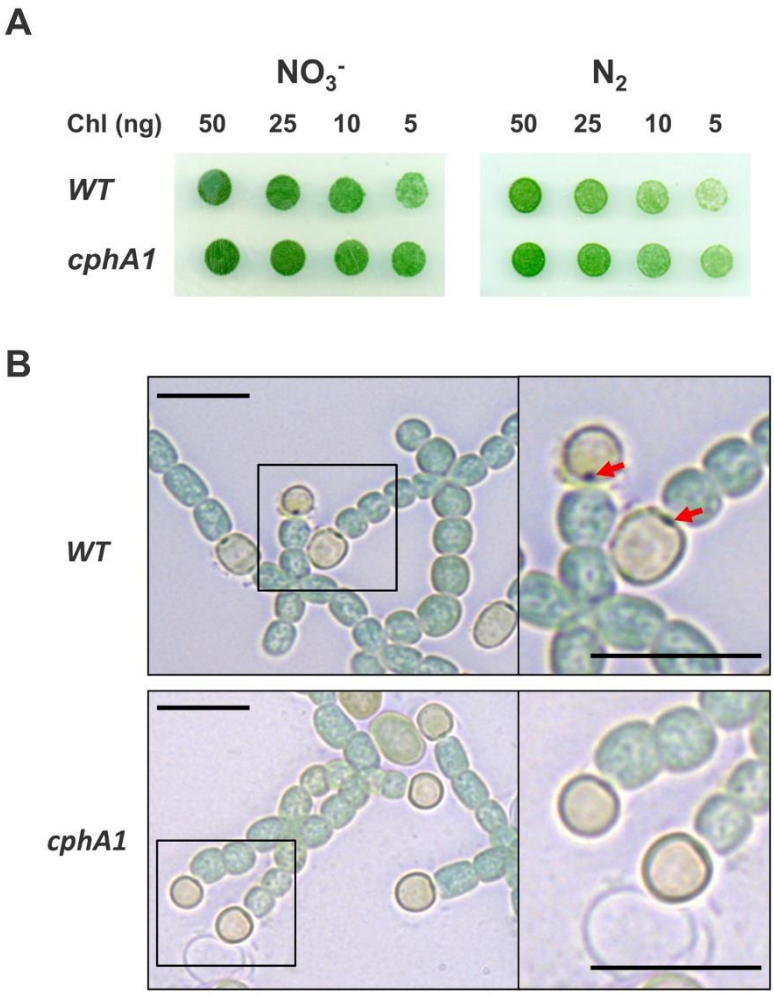

Figure S1. Growth and heterocyst differentiation. **A** The growth of *A. variabilis* WT and *cphA1* mutant filaments spotted on solid AA/4 medium ($NO3^{-}$) and medium lacking a combined nitrogen source (N_2) after 7 days of incubation. **B** Light micrographs of WT and mutant *cphA1* showing the heterocyst differentiation after 72 h of nitrogen step-down. Right panels show the magnified view of heterocyst, indicated by square. Red arrow indicates the cyanophycin polar nodule. Bars, 10 µm.

9.3 Publication 3

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Article

Changes in Envelope Structure and Cell–Cell Communication during Akinete Differentiation and Germination in Filamentous Cyanobacterium *Trichormus variabilis* ATCC 29413

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Abstract: Planktonic freshwater filamentous cyanobacterium *Trichormus variabilis* ATCC 29413 (previously known as *Anabaena variabilis*) can differentiate heterocysts and akinetes to survive under different stress conditions. Whilst heterocysts enable diazotrophic growth, akinetes are spore-like resting cells that make the survival of the species possible under adverse growth conditions. Under suitable environmental conditions, they germinate to produce new vegetative filaments. Several morphological and physiological changes occur during akinete formation and germination. Here, using scanning electron microscopy (SEM), we found that the mature akinetes had a wrinkled envelope, and the surface of the envelope smoothened as the cell size increased during germination. Thereupon, the akinete envelope ruptured to release the short emerging filament. Focused ion beam–scanning electron microscopy (FIB/SEM) tomography of immature akinetes revealed the presence of cytoplasmic granules, presumably consisting of cyanophycin or glycogen. In addition, the akinete envelope architecture of different layers, the exopolysaccharide and glycolipid layers, could be visualized. We found that this multilayered envelope helped to withstand osmotic stress and to maintain the structural integrity. Furthermore, by fluorescence recovery after photobleaching (FRAP) measurements, using the fluorescent tracer calcein, we found that intercellular communication decreased during akinete formation as compared with the vegetative cells. In contrast, freshly germinating filaments restored cell communication.

Keywords: cyanobacteria; *Trichormus variabilis*; akinetes; FIB/SEM tomography; cell-cell communication; FRAP

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1. Introduction

The filaments of the multicellular cyanobacterium *Trichormus variabilis* ATCC 29413 consist of hundreds of vegetative cells that have the potential to differentiate into specialized cells, heterocysts and akinetes. The semi-regularly spaced heterocysts enable the fixation of atmospheric nitrogen (N_2), if no other combined nitrogen sources are available, and provide organic nitrogen to the vegetative cells [1,2]. Akinetes are spore-like nonmotile single cells that develop from the vegetative cells in response to diverse environmental factors, including light intensity, temperature and nutrient deficiency [3–6]. They are resistant to different types of biotic and abiotic stresses, compared with vegetative cells [4,7].

Akinete differentiation from vegetative cells involves the development of overall-different structures, which includes cell enlargement, granulation and, similar to heterocysts, development of a thickened multilayered envelope surrounding the cells [8–11].

Akinetes contain abundant reserves of nitrogen, stored in cyanophycin bodies, and carbon, in glycogen granules [4,12–14]. However, it was recently shown that the production of cyanophycin granules is not crucial for akinete development and germination in *T. variabilis* [6]. Although the akinete stage is considered dormant, minimum metabolic activities were observed in mature akinetes [9,15]. The content of DNA and RNA in akinetes of species *Nostoc* PCC 7524 were similar to those of vegetative cells [16]. The transient resistant akinete structure allows cyanobacteria to survive harsh environmental situations, and they can undergo germination after favorable conditions return [1,4]. Light, moderate temperature and nutrient conditions suitable for growth appear to be the major stimuli for akinete germination [4,5,7–9,17–20].

The akinete envelope is composed of several distinct layers consisting of exopolysaccharides and glycolipids [9,10,21,22]. Heterocyst glycolipids (HGs), present in many heterocyst-forming cyanobacteria, were also identified in the akinetes of *Cyanospira rippkae* [23–26]. In *T. variabilis*, a lipid layer was detected in the akinete envelope, which was composed of the same glycolipids (HG_{26}-diol) that formed the heterocyst envelope [22]. Furthermore, the *hglB* gene is responsible for the synthesis of this lipid for both heterocyst and akinete envelopes [27].

Akinetes are highly resistant to various stress factors, such as darkness, osmotic stress, desiccation, freezing and a wide range of temperatures [4,16,17,28–33]. In consequence, akinetes can survive buried in sediments for several decades [34]. The akinetes of *Nostoc* sp. HK-01 have tolerance to dry heat due to the accumulation of compatible solute glucosylglycerol, betaine and glycine [35,36]. Therefore, akinete formation is considered a key strategy for survival under extreme conditions and is responsible for perennation in several orders of cyanophycean.

The multicellular lifestyle including cell differentiation and division of labor along the filaments in heterocyst-forming cyanobacteria requires mechanisms of cell–cell communication. Cells must perceive signals and communicate with each other through direct cell–cell connections, the septal junctions, which allow the intercellular exchange of metabolites and regulatory compounds to be performed [37–39]. The septal junctions are gated proteinaceous complexes that resemble metazoan gap junctions [40]. In the septal-cell walls, an array of nanopores (circular perforations) drilled by AmiC-type amidases were discovered [41–44]. These nanopores are the framework for the formation of septal-junction complexes between adjacent cells [43]. The gating of septal junctions depends on environmental conditions, as shown by measuring the cell–cell-communication rates under stress conditions using fluorescence recovery after photobleaching (FRAP) measurements [39,40].

Since environmental signals trigger several vegetative cells in the trichomes of *T. variabilis* to differentiate into akinetes, it can be assumed that regulatory molecules are exchanged among cells during akinete induction. During differentiation, structural, cellular and physiological changes occur, leading to a complete separation of the cell. After sensing unfavorable conditions, the cells of the filament start to build an extra envelope, which, finally, surrounds each individual cell. At the same time, developing akinetes reduce their metabolic activities and require less communication with each other. Exactly the opposite happens when akinetes start to germinate; on their way to multicellular filaments, cell–cell communication restarts, enabling the formation of semi-patterned heterocysts. However, nothing is known about the dynamics of cell–cell communication during akinete differentiation and germination. Here, we addressed the question of whether the exchange of metabolites and signaling molecules correlates to their physiological conditions during these two processes. We used the fluorescent marker calcein to follow the molecule exchange during akinete differentiation and germination. Furthermore, we demonstrated, by scanning electron microscopy and FIB/SEM tomography, several changes in the cell structure occurring during akinete formation and germination to provide a basis for understanding the survival strategy of akinetes in the model organism, *T. variabilis* ATCC 29413.

2. Materials and Methods

2.1. Strains and Growth Conditions

Trichormus variabilis ATCC 29413 strain FD [45,46] was cultivated photoautotrophically under continuous illumination (17–22 µmol photons m^{-2} s^{-1}) in Erlenmeyer flasks at 28 °C with shaking at 120 rpm in BG11 medium containing $NaNO_3$ [47] or on medium solidified with 1.5% (*w*/*v*) Difco Agar.

2.2. Akinete Induction and Germination

Akinete differentiation was induced in the late phase of an exponentially growing culture by transferring the filaments to low-light conditions (2–3 µmol photons m^{-2} s^{-1}) by covering the culture flasks with paper towels [9]. Akinete-induced cultures were maintained at 28 °C with gentle shaking at 50 rpm.

The germination of mature akinetes that had been kept in low light from two to three months (hereafter called 2–3-month-old akinetes) was induced by washing and transferring the culture to either BG11 medium (containing $NaNO_3$) or $BG11_0$ medium lacking combined nitrogen and optimal light conditions [22].

Akinete differentiation and germination was observed using a Leica DM 2500 light microscope with an 100x/1.3 oil objective, connected to a Leica DFC420C camera (Leica Microsystems GmbH, Wetzlar, Germany).

2.3. Osmotic-Stress Resistance

From a liquid culture of vegetative filaments and a suspension of 2–3-month-old akinetes, 1 mL was centrifuged, and the supernatants were removed. After the addition of 200 µL of 40% sucrose, the cells were incubated at room temperature (RT) for 20 min, then immediately visualized under a bright-field microscope. For SEM, cells were fixed after sucrose treatment with 4% formaldehyde and 2.5% glutaraldehyde in 1x PBS for 2 h at RT followed by overnight incubation at 4 °C. Cells were washed in PBS, post-fixed with 1% OsO_4 on ice for 1 h and transferred onto poly-L-lysine-coated coverslips. After gradual dehydration in ethanol and critical-point drying (CPD300; Leica), the samples were sputter-coated with a 3 nm platinum layer (CCU-010; Safematic) and examined with a Hitachi Regulus SU 8230 field emission scanning electron microscope (Hitachi High Technologies, Tokyo, Japan) at an accelerating voltage of 5 kV.

2.4. Fluorescent Recovery after Photobleaching (FRAP) Assay

For FRAP, the samples were collected during akinete differentiation and germination at different time points. The loading of *T. variabilis* vegetative cells and akinetes with calcein and the FRAP measurements were performed as previously described [37,48]. Briefly, cells were washed three times and resuspended in 500 µL of fresh BG11 medium; this was followed by the addition of 10 µL of calcein acetoxymethylester (1 mg/mL in DMSO) and incubation in the dark for 2.5 h at 28 °C with gentle agitation. The samples were washed again three times with BG11 medium, then further incubated for 1.5 h with gentle shaking in the dark. The cell suspensions were spotted onto BG11 agar and covered with a cover slip. All FRAP measurements were performed at RT with a Zeiss LSM 800 confocal microscope using a 63x/1.4 oil-immersion objective and ZEN 2.3 (blue edition) software as described previously [44]. The 488 nm line of a 10 mW laser at 0.2% intensity was used as the excitation source. Chlorophyll *a* auto-fluorescence (emission detection: 650–700 nm) and calcein fluorescence (emission detection: 400–530 nm) were imaged simultaneously using a 191 µm confocal pinhole (corresponding to 4.49 airy units) resulting in a point spread in the Z-direction of about 3 mm.

For imaging, the following settings were used: laser intensity, 0.2%; frame size, 36.2 × 36.2 µm; pixel size, 0.07 µm; pixel dwell time, 1.52 µs; averaging, 1x line average. After capturing five initial prebleach images, the laser intensity was increased by a factor of at least 10 for bleaching the fluorescence of a region of interest by a 'fast-bleach' option. A sequence of images at 1 s intervals for 30–60 s was taken to record the recovery of the

fluorescence signal in the bleached cell. The images were processed using the 'Time series analyzer V3' ImageJ plugin for measuring the fluorescence intensity of a FRAP sequence, and data were processed with GraphPad PRISM v6.01 for Windows (GraphPad Software, La Jolla, San Diego, CA, USA). The fluorescence recovery rate constant *R* of a bleached cell was calculated as described previously using the formula $C_B = C_0 + C_R\ (1 - e^{-2Rt})$, where C_B is the fluorescence of the bleached cell, C_0 is the fluorescence immediately after the bleach and tending towards ($C_0 + C_R$) after fluorescence recovery, C_R is the fluorescence during recovery, *t* is time, and *R* is the recovery rate constant due to the molecular exchange of the tracer with neighboring cells [42,48].

2.5. EM Preparation and FIB/SEM Imaging

A culture that had been incubated for akinete differentiation for two months contained mature akinetes and was induced to germinate. Samples of 500 µL were collected before germination and at different time points during germination. The cells were fixed with 2.5% glutaraldehyde in cacodylate buffer (2 mM $MgCl_2$, 50 mM cacodylate; pH 7.0) for 30 min at RT followed by overnight incubation at 4 °C. The cells were postfixed with 1% (*v*/*v*) OsO_4 and 1% (*w*/*v*) $K_4[Fe(CN)_6]$ in cacodylate buffer for 30 min, washed 3 times in ddH_2O, incubated with 1% (*w*/*v*) thiocarbohydrazide in ddH_2O for 30 min, washed 3 times with ddH_2O, then postfixed with 1% OsO_4 in ddH_2O for 30 min. The samples were rinsed 3 times with ddH_2O before being dehydrated in a graded series of acetone containing a step of 1% uranyl acetate in 20% acetone for 30 min, infiltrated and ultrathin-embedded on glass slides, then imaged by focused ion beam–scanning electron microscopy (FIB/SEM) tomography as described previously [49].

2.6. Three-Dimensional Reconstruction and Visualization

The datasets were aligned using Amira™ version 2019.2 (Thermo Fisher Scientific, Waltham, MA, USA) with the module *align slices*. The FIB/SEM-image stacks were segmented and 3D-reconstructed or processed with a direct volume rendering algorithm (VOLREN) for immediate visualization.

3. Results

3.1. Morphological Changes Associated with Akinete Formation

The transition from dividing vegetative cells to dormant akinetes is associated with morphological changes and the accumulation of storage compounds [9]. Under standard laboratory growth conditions, akinete differentiation was induced in stationary-phase cultures of *T. variabilis* by transferring the filaments to low-light conditions and maintaining them under low light for 2 months. The cultures of *T. variabilis* began to differentiate akinetes after 3–7 days of incubation, and 95 % of the cells had differentiated into akinetes after 60 days. Akinete differentiation was characterized by the visual change in color of the cultures from blue–green to yellow–brown and by the fragmentation of the filaments into oval-shaped mature akinetes (Figure S1A) [9].

To elucidate the morphological changes during akinete differentiation, scanning electron microscopy was performed. As shown in Figure 1, the surface of the vegetative cells was smooth and waveless (Figure 1A), while mature akinetes had wrinkles or folds on their surface (Figure 1B). To understand the structure of akinetes in more depth, focused ion beam (FIB)/SEM tomography [50] was used, where akinetes were cut in sections first vertically, then horizontally (Figure 2A). The cross-sections created by FIB milling were further analyzed by FIB/SEM tomography. The akinetes showed, without exception, typical envelopes and cellular structures (Figure 2B,C). A thick envelope coat formed around the cell, consisting of several layers that were folded in multiple places on the outer surface (Figure 2C). This multilayered envelope composed of several distinct layers was also shown by previous studies using transmission electron microscopy [9]. Notably, the FIB/SEM images depicted that the folds or wrinkles were only present in the envelope. Many immature akinetes also showed intracellular granules, which disappeared once the akinete reached its

mature stage (Figures 2B,C and S1B); this is consistent with a previous study that showed that younger akinetes (18 days) accumulated glycogen and cyanophycin granules, which disappeared in older akinetes (30 days) [9].

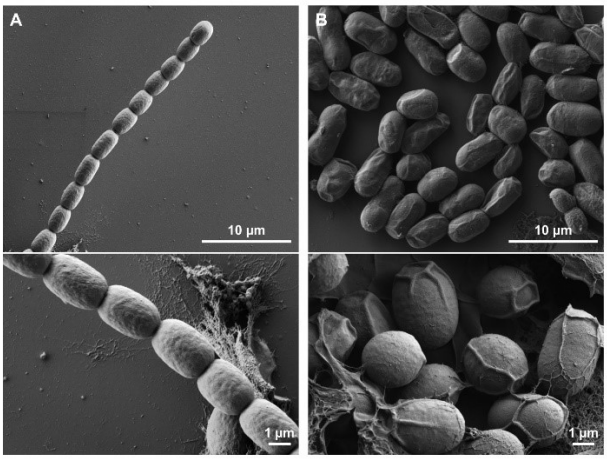

Figure 1. Scanning electron micrographs of *Trichormus variabilis*. (**A**) Young vegetative filament. (**B**) Two-month-old mature akinetes induced under low-light conditions.

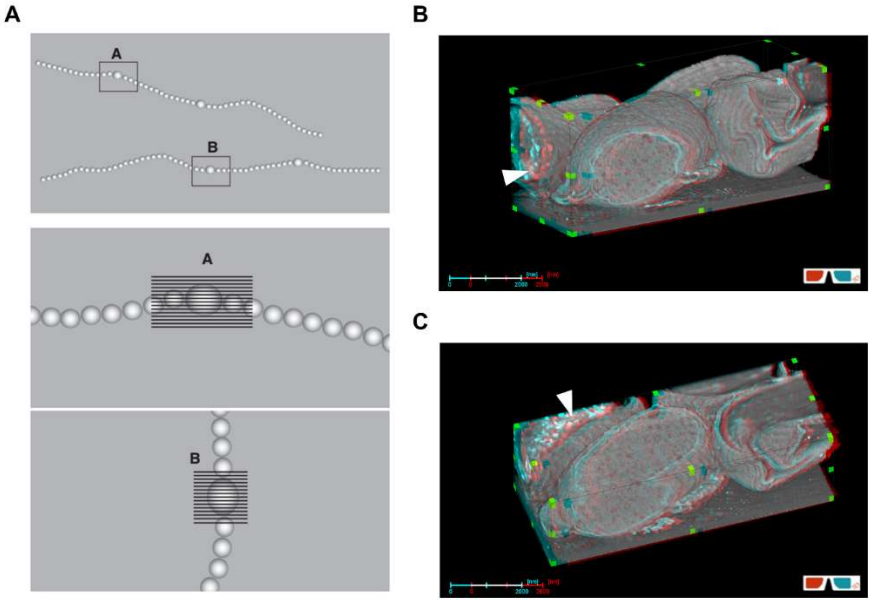

Figure 2. Three-dimensional visualization of akinetes from FIB/SEM tomogram of *T. variabilis*. (**A**) Illustration of FIB-milling process with *T. variabilis* filament after flat embedding in SEM. Box denotes the targeted cells for FIB-miling; shaded parts indicate horizontal or vertical cross-sectioning of cells. (**B**,**C**) SEM images of two-month-old akinete after longitudinal (**B**), then transversal (**C**) FIB milling. White arrowheads indicate the cytoplasmic granules in immature akinete. Use of 3D glasses is recommended.

3.2. Tolerance of Akinetes against Sucrose Treatment

Akinetes are the key for survival under harsh environmental conditions, where they must face various drastic changes. To understand the protective capability of akinetes, the limit of tolerance of akinetes against osmotic stress was investigated. For this, vegetative filaments and akinetes were treated with 40% sucrose and monitored under a bright-field microscope for 20 min. The results showed a drastic decrease in the filament length due to shrinkage of the vegetative cells, while no effects on akinete dimensions were observed (Figure 3A). The length of approximately 90–100 cells in the filaments and akinetes was measured using ImageJ, showing that the akinetes were able to maintain their structure

and size of individual cells during sucrose treatment; however, the vegetative cells in the filaments showed a 30 % shrinkage longitudinally, from a mean length of 4.62 ± 0.16 µm to 3.23 ± 0.09 µm (Figure 3B), while no effects on cell width were observed. We also observed normal germination of akinetes after sucrose treatment (not shown).

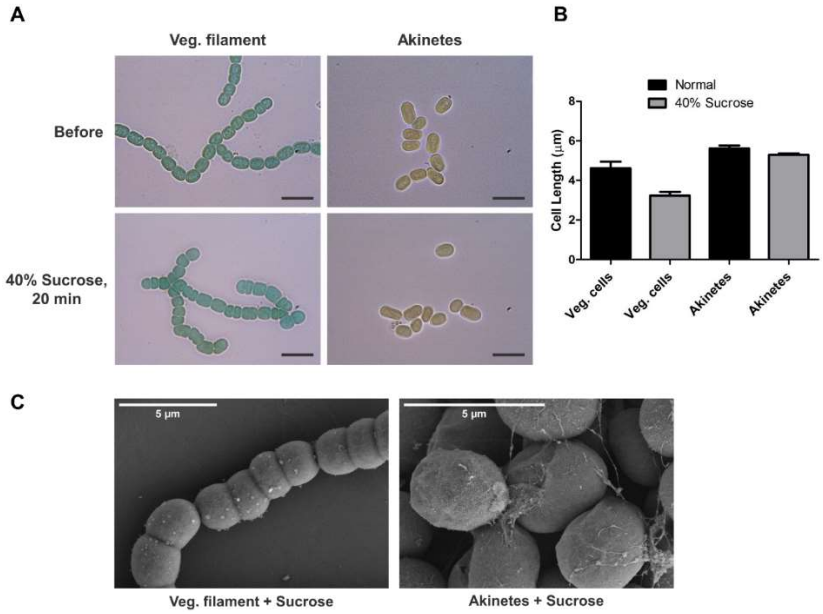

Figure 3. Akinetes are resistant to osmotic stress. (**A**) Bright-field images of vegetative filaments and akinetes treated with 40% sucrose for 20 min. Scale bar, 10 µm. (**B**) Cell-length measurement using ImageJ before and after sucrose treatment of vegetative cells and akinetes. (**C**) SEM images of vegetative filaments and akinetes after treatment with 40% sucrose.

Next, we analyzed the morphological changes in the cells after sucrose treatment by SEM (Figure 3C). The length of the vegetative cells treated with sucrose was reduced, resulting in shorter filaments than those of the untreated ones (Figures 1A and 3C). However, the surface of the vegetative cells was not affected by the sucrose treatment. Akinetes showed no obvious changes in their dimensions nor morphologies (Figures 1B and 3C), consistently with the bright-field micrographs. Our results indicate that the akinete envelope kept its structure; this may help in the maintenance of structural integrity, which is confirmed by the normal germination efficiency of the akinetes that we observed after sucrose treatment.

3.3. Analysis of Akinete Germination Process by SEM

The remarkable surface structure of the mature akinetes (Figure 1) with wrinkles and thick raised folds prompted us to investigate these surface structures during germination. The germination of akinetes was induced by transferring mature akinetes to fresh medium and optimum light conditions. The beginning of germination could be recognized by the increased volume of the akinetes, and the first cell division was visible between 17 h and 24 h. After 24 h, the increase in akinete size led to the disappearance of the wrinkles from the envelope (Figures 4b and S2). Then, the akinete coat ruptured, and the small developing filament penetrated the akinete envelope mostly at one pole (Figure 4c). The release of two–four-celled germlings by lysis of the akinete coat could be seen within 48 h (Figure 4d). Akinetes did not have a hole or any kind of aperture in their envelope surface (Figure S2), as it was observed for the coat of many bacterial spores [51]. After 72 h, the normal growing filaments were also seen to be still attached to the envelope of the akinetes (Figure 4d,e). Since media lacking a nitrogen source were used, we could also follow terminal-heterocyst formation during germination and heterocyst development, which was visible after approximately 48 h (Figure 4e).

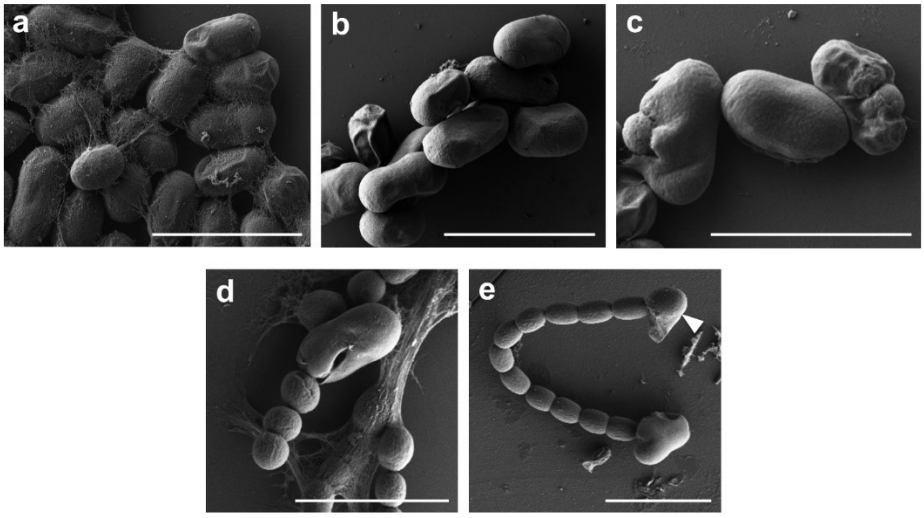

Figure 4. SEM analysis of akinete germination and cellular growth of *T. variabilis* at different time intervals. (**a**) Mature akinetes before germination; (**b**) akinete germination on day 1; (**c**) germinated filament inside the envelope on day 2; (**d**) cellular growth and trichome development on day 3, with attached envelope; (**e**) growing filament with terminal heterocyst differentiation in $BG11_0$ medium on day 3. White arrowhead points to the terminally differentiated heterocyst. Scale bar, 10 μm.

3.4. Intercellular Communication during Differentiation and Germination of Akinetes

In previous studies, FRAP analyses showed that filamentous cyanobacteria exchange fluorescent tracers between the cytoplasm of the cells along filaments [37]. In addition, the transfer of tracers was observed between heterocysts and vegetative cells [37,42,52]. This indicates cell–cell communication along filaments and between heterocysts and vegetative cells.

We performed FRAP experiments to investigate cell–cell communication during akinete formation and germination. Using the diffusible fluorophore calcein, we followed the ability of molecule transfer in filaments during akinete differentiation. After sensing low-light conditions, the program of akinete differentiation started in *T. variabilis*. At the beginning, the immature akinetes remained attached to each other; then, they completely detached after maturation and stopped direct cell–cell communication. The time course of ceased molecule exchange between maturing akinetes was followed using FRAP measurements performed at different time points up to 18 days of akinete differentiation. Our results show a gradual decrease in calcein transfer, indicating slower intercellular intracellular communication during akinete differentiation (Figure 5A). Compared with the beginning of the experiment, 18-day pre-akinetes had reduced communication by approximately 30%. This indicates that cells preparing for dormancy are metabolically less active and do not need to communicate.

In one month old akinete cultures, we observed few filaments that mostly contained immature akinetes which had not yet detached from each other. When these akinetes were transferred to fresh medium and optimal light conditions, nearly all of them started to germinate and divide along with the germination of mature akinetes. So, we performed FRAP measurements during the germination of akinetes especially in these filaments and investigated the transfer of calcein in the dividing filaments. We observed that, after 24 h of germination, the immature akinete-containing filaments resumed greening, started to divide fast and showed a faster transfer of calcein, indicating the regaining of cell–cell communication (Figure 5B). After 48 h, an even faster calcein transfer rate was observed because dividing cells became metabolically active; this continued till 72 h, when almost all the filaments reached the normal vegetative growing state (Figure 5B). Furthermore, we observed that the freshly dividing young filaments after germination (48 h/72 h, Figure 5B) had twice as much communication as compared with the vegetative cells in the stationary culture that had been used at the onset of the experiment (Figure 5A). These results indicate

that molecule exchange is regulated during these two processes and depends on the physiological conditions.

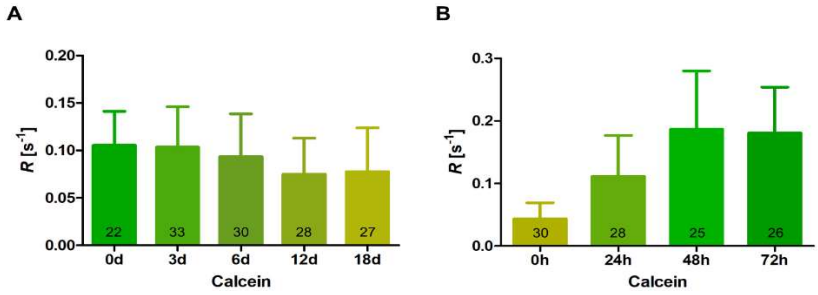

Figure 5. Fluorescence recovery after photobleaching (FRAP) analysis of the intercellular exchange of calcein during akinete differentiation and germination. (**A**) Cell–cell communication during akinete differentiation in low light over a span of 18 days. Numbers in bars indicate the number of analyzed cells (n) from different filaments subjected to FRAP analysis. Data are mean ± SD from the results obtained from three independent cultures. (**B**) Regaining of cell–cell communication during akinete germination up to 72 h in fresh medium and optimum light conditions. Numbers within the bars indicate number of analyzed cells (n) from different filaments subjected to FRAP analysis. Data are mean ± SD from three independent cultures.

4. Discussion

Bacterial cells can adapt to changing external conditions by various mechanisms, including morphological and physiological changes, to maintain their cellular structure. A key feature that contributes to the success of *T. variabilis*' survival under extreme conditions is their ability to form highly resistant akinetes. When environmental conditions become favorable, the dormancy of akinetes is broken and germination occurs, giving rise to the filaments of vegetative cells.

With the availability of various techniques, we were able to have a closer look at the akinete differentiation and germination processes. We used scanning electron microscopy (SEM) to visualize the surface, shape and size of vegetative filaments and mature akinetes. The SEM images showed wrinkles on mature akinetes, suggesting akinete-envelope folding (Figure 1). We assume that these wrinkles provide the structural flexibility of these cells. When turgor pressure increased during germination, the wrinkles smoothed out, providing space for the emerging filament inside the envelope before rupture.

By using FIB/SEM tomography, we could obtain high-resolution-image stacks of the entire akinete cells in 3D, which allowed us to detect morphological changes at different time points during differentiation from vegetative cells and maturation. Intracellular electron-dense granules were still present in immature akinetes, and the typical akinete envelope was not developed yet. The envelope of mature akinetes consists of several layers [9,22]. The accumulation of reserve granules observed in immature akinetes was mainly of glycogen and cyanophycin granules (Figure 2B,C) [9]. The direct role of cyanophycin granules is still unknown in *T. variabilis*, but it was found that they are not required for akinete formation and germination [6].

In accordance with our study, akinetes were able to resist osmotic stress and maintain their structure during sucrose treatment, whereas young vegetative cells turned out to be more susceptible (Figure 3A). Akinetes are highly resistant to many environmental stresses, owing in part to the presence of a multilayered envelope structure. Recently, we found that the glycolipid layer of the akinete envelope is crucial for protecting the akinetes from freezing, desiccation and lytic attacks [27]. It was suggested that the exopolysaccharide layer present in akinetes plays a role in membrane stabilization during desiccation [53]. For maintaining rigidity of the akinete envelope and to provide stress tolerance, the presence of hapanoids was also found to be necessary [54]. Our results demonstrate the importance of the akinete envelope in providing structural stability under osmotic stress. This adaptation is crucial for surviving the harsh environmental conditions prevailing in nature. Another

possibility to consider is a reduced water content of the cytoplasm of akinetes compared to vegetative cells, which would be similar to bacterial endospores [55]. By this, the akinetes were less prone to shrinkage during osmotic stress.

The vegetative cells are surrounded by outer and inner membranes, which are separated by the periplasmic space and a peptidoglycan (PG) layer. The behavior of vegetative filaments during sucrose treatment was interesting, as we observed the shrinkage of cells only longitudinally, which is indicative of the architecture of peptidoglycans. Apparently, in the transversal direction, the wall was much more rigid, whereas it was elastic in the longitudinal direction (Figure 3A).

SEM was used to visualize structural changes during the germination, emergence and outgrowth of the short filament. In *T. variabilis*, we observed that an increase in light intensity rapidly triggered the germination of akinetes [22], which was similarly reported in previous studies on the germination of akinetes in *Anabaena cylindrica* and *Anabaena variabilis* Kutzing [17,21]. We observed that the first cell division occurred inside the akinetes upon transfer from dark or low light to medium light (ca. 20 µmol photons m^{-2} s^{-1}) (Figure 4b). During germination, as the cell size increased, we observed the disappearing of the envelope folds; this suggests that akinete envelope can adapt to different sizes and provide the structural integrity after the initial cell divisions at the beginning of germination. After reaching a certain size, it finally ruptured to release the short filament (Figure 4b). After 48 h, terminally differentiated heterocysts were observed, indicating that, from the very beginning of cell division, the presence/absence of a source of combined nitrogen could be sensed by the small germinating filament (Figure 4e).

In many bacteria, spore-coat-degrading enzymes such as CwlJ and SleB are known to play redundant roles in the degradation of the spore peptidoglycan cortex during germination [56–58]. However, so far, no such enzymes were discovered for akinete-envelope degradation during germination. So, it would be of future interest to identify the envelope-degrading enzymes in cyanobacteria.

In our study, we also measured cell–cell communication during akinete differentiation and germination. Decreased communication during differentiation can be expected, as cells go into a dormant stage where they do not need to exchange molecules (Figure 5A). In contrast, with resuscitation, cells need to return to an active metabolism to allow cell division and growth to be performed. In the emerging filament of *T. variabilis*, rapid cell divisions occurred, and cells concomitantly started cell–cell communication (Figure 5B). This is particularly essential when heterocysts are formed, and the nutrient status of the cells has to be communicated to assure the typical heterocyst pattern in the filaments. Additionally, the freshly germinating filaments communicated even faster than standard vegetative cells (Figure 5A,B). Faster communication was also observed in hormogonia, short motile filaments, which have functions in dispersal and symbiotic association in several filamentous cyanobacteria [59]. The higher communication rate in young filaments and hormogonia could be important to sense the favorable light and nutritional conditions and to provide the motility necessary for hormogonia to establish symbiotic associations with plants [60]. The development of cell–cell-communication machinery during akinete germination, including nanopore formation in the freshly formed septal peptidoglycan layer and the building of septal-junction complexes during the transition from the unicellular to the multicellular organism, should be a focus in future research on akinete germination.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/life12030429/s1, Figure S1: Two-month-old akinetes of *T. variabilis*. (A) Light micrographs showing akinetes induced under low-light conditions. Images of bright field (left) and red auto-fluorescence (right). Bar, 25 µm. (B) FIB/SEM images of akinetes after FIB milling and 3D visualization (volume rendering). Intracellular granules are present in immature akinetes (right) and absent in mature akinetes (left), Figure S2: Germinating akinete showed increase in cellular size and random rupture of envelope during germination. White arrowheads indicate breaks and distortion of the akinete envelope.

Author Contributions: I.M., K.F. and R.G. conceived and designed the experiments; R.G., M.L., J.B., K.H. and G.W. performed the experiments; R.G., I.M., K.F. and G.W. analyzed the data; R.G. and I.M. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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life

Supplementary Material of Changes in Envelope Structure and Cell–Cell Communication during Akinete Differentiation and Germination in Filamentous Cyanobacterium *Trichormus variabilis* ATCC 29413

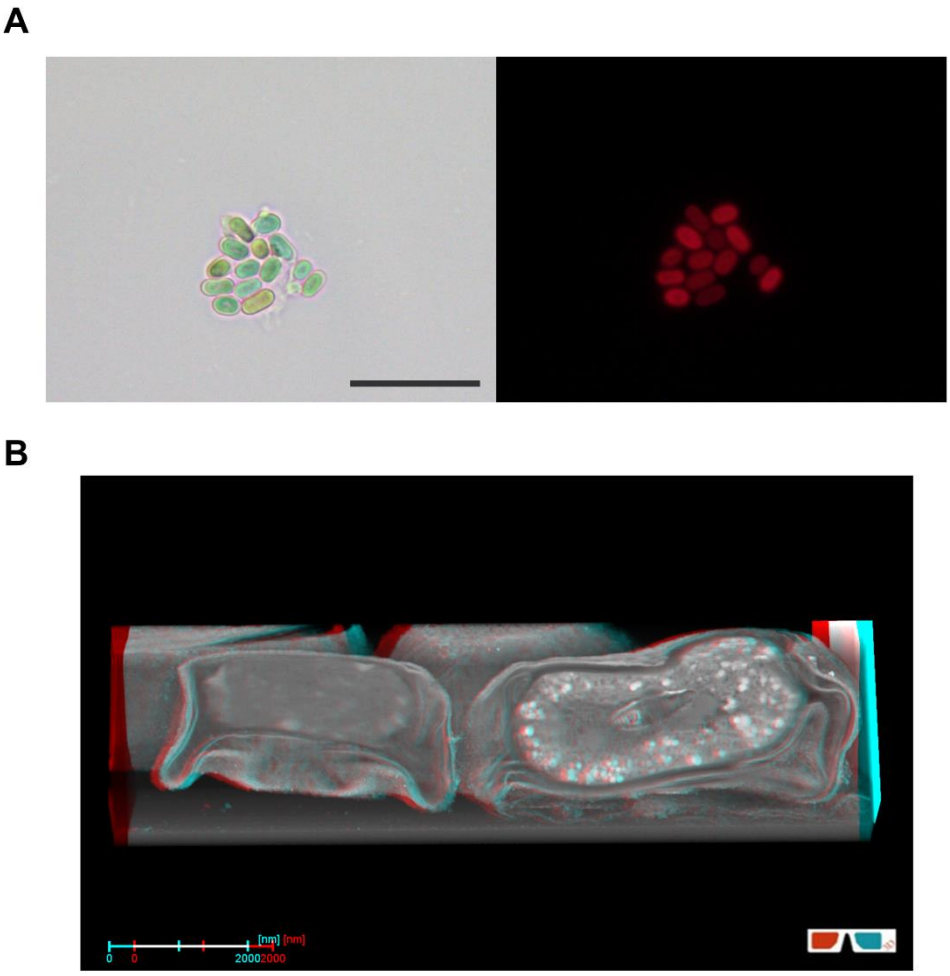

Figure S1. Two months old akinetes of *A. variabilis*. (**A**) Light micrographs showing akinetes induced under low light condition. Images of bright field (left), red auto-fluorescence (right). Bar, 25 µm. (**B**) FIB/SEM images of akinetes after the FIB milling and 3D visualization (volume rendering). Intracellular granules are present in immature akinete (right) and absent in mature akinete (left).

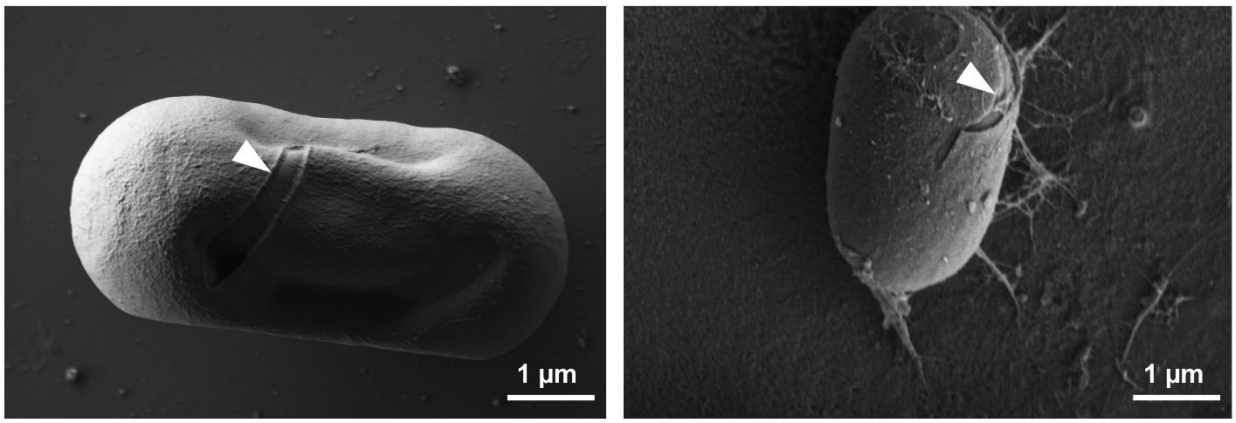

Figure S2. Germinating akinete showed increase in cellular size and rupture of envelope randomly during germination. White arrowheads indicate breaks and distortion of the akinete envelope.

9.4 Publication 4

bio-protocol

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Analysis of Heterocyst and Akinete Specific Glycolipids in Cyanobacteria Using Thin-layer Chromatography

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Abstract

Several filamentous cyanobacteria like *Nostoc* differentiate specialized cells in response to changes in environmental factors, such as low light or nutrient starvation. These specialized cells are termed heterocysts and akinetes. Under conditions of nitrogen limitation, nitrogen-fixing heterocysts form in a semi-regular pattern and provide the filament with organic nitrogen compounds. Akinetes are spore-like dormant cells, which allow survival during adverse unfavorable conditions. Both cell types possess multilayered thick envelopes mainly composed of an outermost polysaccharide layer and inner layers of glycolipids, that are important for stress adaptation. To study these envelope glycolipids, a method for the isolation, separation and analysis of lipids from heterocysts and akinetes is essential. The present protocol describes a method involving the extraction of lipids from cyanobacteria using solvents and their separation and visualization on silica plates, to render analysis simple and easy. This protocol is relevant for studying mutants that are defective in glycolipid layer formation and for the comparison of glycolipid composition of heterocysts and akinetes under different environmental stresses.

Keywords: Cyanobacteria, Heterocyst, Akinete, Envelope, Glycolipids, Thin layer chromatography

This protocol was validated in: Front Microbiol (2021), DOI: 10.3389/fmicb.2021.645028

Background

Filamentous cyanobacteria, such as *Anabaena variabilis* ATCC 29413 and *Nostoc punctiforme* ATCC 29133, are widely used as model organisms for the study of multicellularity and their capability to differentiate heterocysts and akinetes. Under favorable conditions, these photosynthetic Gram-negative bacteria grow in the form of filaments, which are composed of hundreds of vegetative cells. In response to insufficient supply of organically combined nitrogen, about 10% of semi-randomly spaced cells can differentiate into nitrogen-fixing heterocysts, which provide the filaments with nitrogen (Figure 1A) (Fay, 1992; Muro-Pastor and Maldener, 2019). Akinetes are spore-like resting cells that differentiate from the vegetative cells in response to diverse environmental conditions, including changes in light intensity and quality, temperature, and nutrient deficiency (Figure 1B).

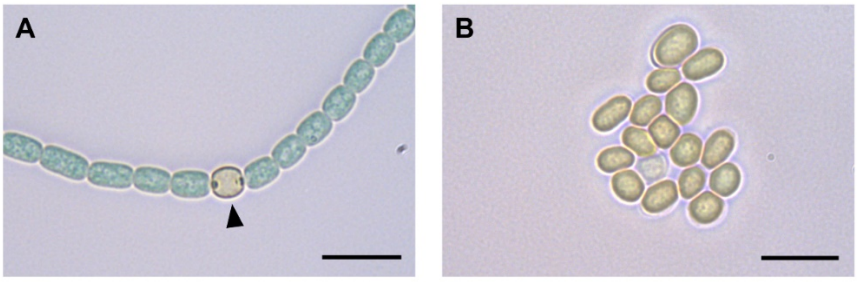

Figure 1. Light micrographs showing cell differentiation in *A. variabilis*.
(A) Vegetative filament containing heterocyst, indicated by black arrowhead. (B) Akinetes induced under low light condition. Scale bars, 10 µm.

Both cell types—heterocysts and akinetes—are characterized by the presence of a thick multilayered envelope, mainly composed of exopolysaccharides and glycolipids (HGLs) (Maldener *et al*., 2014; Perez *et al*., 2016; Garg and Maldener, 2021b). The glycolipids, which form laminated layers in the heterocyst and akinete envelopes, are of great interest due to their different functions in these two cell types. In heterocysts, they act as an oxygen diffusion barrier to protect the oxygen labile enzyme nitrogenase from oxygen (Fay, 1992; Wolk *et al*., 1994), while in akinetes, glycolipids protect the cells from various stress factors, such as freezing, desiccation, and lysozyme attack (Garg and Maldener, 2021a).

Mutants with aberrantly formed heterocyst envelopes are unable to grow without alternative sources of combined nitrogen. Many of those lose the ability to synthesize HGLs or to transport them beyond the cell wall to the heterocyst envelope (Ernst *et al*., 1992; Fiedler *et al*., 1998; Fan *et al*., 2005). The akinete envelope shares some structural similarities with the heterocyst envelope, suggesting that akinetes are the evolutionary ancestors of heterocysts (Wolk *et al*., 1994; Perez *et al*., 2018). To better understand this evolutionary relationship, a prerequisite is the elucidation of the composition of these special cells' envelopes. Additionally, mutants showing phenotypes related to survival under environmental stress or starvation could be checked for the correct laminated layer composition using the protocol presented here.

Here, we use the thin-layer chromatography (TLC) protocol that was previously established by Nichols and Wood in 1968; with this method, they identified glycolipids, which are specific for the heterocyst forming cyanobacteria (Nichols and Wood, 1968). Later, the Wolk group showed that these glycolipids constitute the laminated layer in the envelope of heterocysts (Winkenbach *et al*., 1972). The two major heterocyst specific glycolipids in *Anabaena* species are characterized as 1-α-glucosyl-3,25-hexacosanediol (HG_{26}-diol) and its 3-ketotautomer (HG_{26}-keto-ol). Their separation via TLC is dependent on their polarity. We utilized this method with a few modifications for the analysis of the akinete specific glycolipids (Perez *et al*., 2018; Garg and Maldener, 2021a). We present a reproducible and reliable TLC protocol to analyze the glycolipids present in the heterocyst and akinete envelopes. This protocol includes the easy extraction of lipids using regular solvents, such as methanol and chloroform, and their clear separation and visualization on the TLC plate, which can be scanned directly. Furthermore, this method allows the extraction of specific glycolipids from the TLC plate in a manner that subsequent analysis of their chemical composition by HPLC-MS analysis is possible, as previously described (Perez *et al*., 2018; Shvarev *et al*., 2018).

Materials and Reagents

1. 50 mL of cyanobacterial liquid cultures containing heterocysts or akinetes
2. Pipette tips (10 µL, 200 µL, 1,000 µL) (LTS, RAININ, Mettler Toledo. Gießen)
3. 1.5 mL microcentrifuge tubes
4. 50 mL conical tubes
5. Soft graphite pencil
6. 30 cm ruler
7. Paper tape (masking tape)
8. Methanol ≥99.9% (HPLC grade) (Sigma, Merck, Darmstadt)
9. Chloroform (HPLC grade) (Roth, Karlsruhe)
10. Aluminium plate coated with silica gel (Macherey-Nagel, catalog number: 818033)
11. Acetic acid (Sigma, Merck, Darmstadt)
12. MilliQ water
13. 25% Sulfuric acid (H_2SO_4) (Carl Roth, catalog number: 0967.1)

Equipment

1. Pipettes (P1000, P200, P20 and P10) (RAININ, Mettler Toledo)
2. 100 mL Erlenmeyer flasks
3. 50 mL glass beaker
4. Centrifuges:
   Centrifuge (Eppendorf, 5417C) for microcentrifuge tubes
   Centrifuge (Eppendorf, Centrifuge 5804 R) for conical tubes
5. Vortex Genie® 2 (Scientific Industries, Inc., catalog number: 6235684)
6. Fume hood
7. Hair dryer
8. Incubator or oven for TLC plate development (This can be any incubator that reaches the desired temperature of 180°C)
9. Flat-bed Scanner (*e.g.*, EPSON Perfection V550 Photo)
10. Developing Chamber for TLC (Macherey-Nagel GmbH & Co. KG, catalog number: 9003500)
11. TLC sprayer with Erlenmeyer flask (*e.g.*, DWK Life Sciences Kimble™ Kontes™ Reagent Sprayers Head Only)

Software

1. Scanner software (*e.g.*, EPSON Scan)

Procedure

A. Cell culture

The protocol requires cyanobacterial cultures grown in the presence of NO_3^- (filaments with vegetative cells only) and without NO_3^- (filaments containing heterocysts), or the cultures that were induced to form akinetes (Perez *et al*., 2016).

B. Thin-layer chromatography (TLC)

Day 1. Sample preparation

1. Take 3 × 1 mL of each culture and estimate the chlorophyll *a* concentration as described (Zavřel *et al*., 2015).

   Note: To obtain a good visible lipid pattern in the TLC plates, the sample should contain 20–50 µg Chl a.

2. Centrifuge the estimated volume of culture at 3,000 × *g* and room temperature (RT) for 10 min (the cells were pelleted at equal Chl *a* amount in each sample).
3. Add 30 mL of methanol:chloroform (1:1) to the pellet and mix well with a vortex mixer. This step will result in the extraction of lipids.

   Note: Always work under the fume hood when using organic solvents.

4. Centrifuge at 3,000 × *g* for 10 min and transfer the supernatant to a 50 mL glass flask.
5. Let it evaporate overnight under a fume hood. You may accelerate evaporation by applying a stream of nitrogen vapor. This would be necessary if detailed analysis is intended, such as precise identification and quantification of different glycolipid species by high performance liquid chromatography coupled to mass spectrometry (HPLC-MS).

Day 2

1. Sample collection
   a. Add 200 µL of chloroform to the dried extract in the glass flask, dissolve the pellet by pipetting, collect the lipids and transfer to 1.5-mL microcentrifuge tubes. Repeat this step one more time to collect all the extract from the flask (This step should be done quickly because the chloroform evaporates very fast).
   b. Let the sample dry in the fume hood until a volume of 20–40 µL remains in the tube.
2. Preparation of developing chamber
   a. Always prewash the chamber with methanol before doing an experiment.
   b. Mix the solvents (chloroform:methanol:acetic acid:water) in the chamber, at a ratio of 23:4:2.7:1 (170 mL of chloroform, 30 mL of methanol, 20 mL of acetic acid, and 7.4 mL of MilliQ water).
   c. Close the lid and seal the tank with paper tape. Do not move the chamber.
   d. Before using the solvent system (solvent mixture), equilibrate the chamber with it for 1 h.
3. Spotting of samples and development of the TLC

   Note: This can be done while the chamber is being equilibrated.

   a. Using a soft pencil, mark the origin line (the line at which you will spot the samples) at 2 cm and the development line at 15 cm (predetermined solvent front height) from the bottom of the TLC plate.

      Notes:
      i. Always handle the TLC plate with gloves on. Never touch the surface of the plate even with gloves.
      ii. Mark only the outer edges of the TLC plate, because the pencil may interfere with the samples and solvent.

b. Use a micropipette (P20) to spot 5–10 µL of the samples on the origin line, at a distance of at least 1 cm from each other.
c. After spotting, use a hair drier in low heat mode to dry the spots.
d. Repeat steps b. and c. until the entire sample is used up and dried.
e. Quickly place the TLC plate in the chamber to prevent solvent vapor elusion from the chamber.
f. Let the samples run until they reach the development line (~1.5 h).
g. Take the plate out from the chamber, and let it dry under the fume hood for 10–20 min (do not use a hair drier).

Note: Always remove the remainder of the solvent from the chamber and transfer it to the organic waste.

h. When the plate is completely dry, spray the whole plate with H_2SO_4 (25%) with a TLC sprayer, from a distance of 5–10 cm under the fume hood (spraying close to the plate can obfuscate the result).

Note: Use lab apron, gloves, and surgical mask.

i. Allow the plate to dry under the fume hood for a short time (2–5 min).
j. Place the plate into an oven at 180°C for 60–120 s (until you see the bands).
k. Use a flat-bed scanner to record the result (see Figure 2).

Data analysis

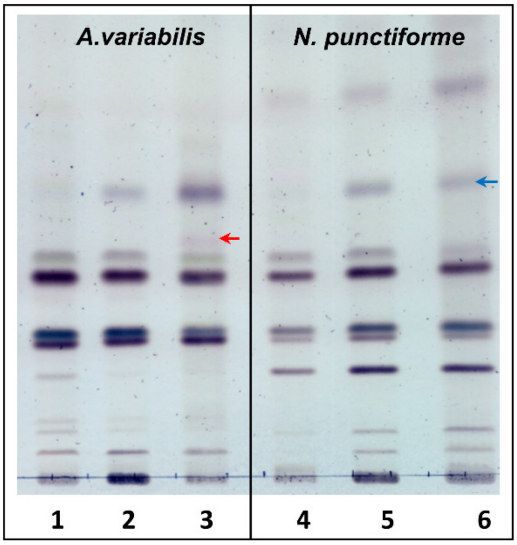

Figure 2. Thin-layer chromatogram of lipid extracts from different cell extracts of *A. variabilis* (1–3) and *N. punctiforme* (4–6).
(1, 4) Vegetative filaments grown with nitrate. (2, 5) Heterocysts containing filaments grown without nitrate. (3) Akinetes induced by low light, or (6) phosphate starvation for 2 months. The blue arrow indicates one heterocyst-specific glycolipid (HG_{26}-diol). The red arrow indicates an unknown lipid in the akinete extract of *A. variabilis*. Figure adapted from Perez *et al.* (2018).

Acknowledgments

This protocol is an adapted version of the method described by Nichols and Wood (1968) and Winkenbach *et al.* (1972).
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Competing interests

We declare no conflict of interests.

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