Clear differences in metabolic and morphological adaptations of akinetes of two *Nostocales* living in different habitats

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Akinetes are resting spore-like cells formed by some heterocyst-forming filamentous cyanobacteria for surviving long periods of unfavourable conditions. We studied the development of akinetes in two model strains of cyanobacterial cell differentiation, the planktonic freshwater *Anabaena variabilis* ATCC 29413 and the terrestrial or symbiotic *Nostoc punctiforme* ATCC 29133, in response to low light and phosphate starvation. The best trigger of akinete differentiation of *A. variabilis* was low light; that of *N. punctiforme* was phosphate starvation. Light and electron microscopy revealed that akinetes of both species differed from vegetative cells by their larger size, different cell morphology and large number of intracellular granules. *A. variabilis* akinetes had a multilayer envelope; those of *N. punctiforme* had a simpler envelope. During akinete development of *A. variabilis*, the amount of the storage compounds cyanophycin and glycogen increased transiently, whereas in *N. punctiforme*, cyanophycin and lipid droplets increased transiently. Photosynthesis and respiration decreased during akinete differentiation in both species, and remained at a low level in mature akinetes. The clear differences in the metabolic and morphological adaptations of akinetes of the two species could be related to their different lifestyles. The results pave the way for genetic and functional studies of akinete differentiation in these species.

**INTRODUCTION**

Cyanobacteria of the order *Nostocales* are multicellular organisms that grow as filaments of hundreds of cells, some of which can differentiate to carry out specialized functions (Flores & Herrero, 2010; Maldener et al., 2014). Such differentiated cells include nitrogen-fixing heterocysts, motile hormogonia and larger resting cells called akinetes. These resting cells represent a survival strategy of cyanobacteria under unfavourable environmental conditions that do not support growth. Akinetes differentiate from vegetative cells in the filament in response to diverse environmental changes. Depending on the cyanobacterium, they develop adjacent to heterocysts or midway between them. However, in the presence of a combined nitrogen source they can differentiate from any vegetative cell in the filament and are often found in clusters. Under suitable conditions they germinate again to produce new filaments (Adams & Duggan, 1999). Although they do not resemble endospores in structure and differentiation process, and are not heat resistant, they are resistant to cold and desiccation, and are referred to as spore-like cells. Their ability to overwinter and survive dry periods allows persistence of filamentous cyanobacteria in habitats with fluctuating conditions (Adams et al., 1981; Kaplan-Levy et al., 2010; Maldener et al., 2014).

In contrast to the profound knowledge on heterocyst differentiation gained during the past few decades with the use of molecular genetics (Kumar et al., 2010), much less is known about the process of akinete differentiation. Heterocysts are formed in response to nitrogen deficiency. Their differentiation from vegetative cells in different cyanobacterial species is similar. By contrast, akinete formation amongst different species differs greatly with respect to environmental signal, morphology, cell envelope structure and general survival strategy (Adams & Duggan, 1999; Maldener et al., 2014).

Akinetes of a few cyanobacterial species have been described at the morphological and physiological levels (for recent reviews, see Kaplan-Levy et al., 2010; Maldener...
et al., 2014). Akinetes of some species have a multilayered extracellular envelope (Cardemil & Wolk, 1981; Nichols & Adams, 1982). In general, mature akinetes have substantially decreased metabolic activities, e.g. carbon dioxide fixation in *Anabaena cylindrica* and *Nostoc* spp. strain PCC 7524 (Kaplan-Levy et al., 2010). The respiration of akinetes is often initially elevated but decreases as akinetes age, presumably as part of the maturation process (Kaplan-Levy et al., 2010). In *Nostoc spongiaeforme*, mature akinetes are not completely dormant but retain a low level of metabolic activity (Thiel & Wolk, 1983). Photosynthetic activity is substantially decreased in isolated mature akinetes of *Aphanizomenon ovalisporum* and *Anabaena cylindrica*, and absent in *Anabaena variabilis* (Bjorn et al., 1983; Fay, 1969; Sukenik et al., 2007, 2015).

A conspicuous feature of akinete differentiation is the accumulation of reserve materials, such as glycogen and cyanophycin. Cyanophycin is a product of non-translational peptide synthesis and serves as a nitrogen storage polymer (multi-i-arginine-poly-l-aspartic acid) (Shi & Carmichael, 1997; Simon, 1971, 1973; Sutherland et al., 1979). However, glycogen and cyanophycin also accumulate in vegetative cells in the stationary growth phase and under stress conditions (Herdman, 1987; Lawry & Simon, 1982).

The study of akinete differentiation at the molecular level is now feasible as several complete genome sequences of akinete-forming cyanobacteria are available (Meeks et al., 2015) and plasmids can be transferred by conjugation in formerly inaccessible strains, such as the complex multicellular lifestyle model organisms *Nostoc punctiforme* ATCC 29133 and *Anabaena variabilis* ATCC 29413, both facultative heterotrophs that form akinetes (e.g. Cohen et al., 1994; Maldener et al., 1991; Meeks & Elhai, 2002). However, only a few genes involved in akinete formation have been so far identified (e.g. Argueta et al., 2006; Campbell et al., 1996; Leganés et al., 1994, 1998; Lehner et al., 2011; Zhou & Wolk, 2002; see also review by Maldener et al., 2014). Microarray analysis of a zwf mutant of *N. punctiforme* that is able to differentiate akinetes with similar phenotype characteristics to WT akinetes in a few days (Argueta & Summers, 2005) revealed the upregulation of 255 genes, 41 % of which encode known proteins (Campbell et al., 2007). To study the function of the genes putatively involved in akinete differentiation in *Anabaena variabilis* and *N. punctiforme*, it would be necessary to construct mutants and compare their phenotype with that of the WT strains. Before this can be done, the morphology and metabolic activities of akinetes of both of these model species need to be elucidated. To fill this gap in our knowledge, we investigated the morphological and physiological changes that take place during akinete formation in these species. Our results on the differences in signals, storage compounds and envelope structure of the two species, and on the lower metabolic activities of mature akinetes of both species, form a solid base for future studies of genes involved in akinete function and formation.

### METHODS

#### Strains and growth conditions.

Cultures of vegetative filaments of *Anabaena variabilis* ATCC 29413 strain FD (obtained from Peter Wolk, MSU-DOE Plant Research Laboratory, East Lansing, MI, USA) (Currier & Wolk, 1979; Thiel et al., 2014) and *N. punctiforme* ATCC 29133 (obtained from Jack Meeks, University of California, Davis, CA, USA) were grown in Allen and Arnon liquid medium (Allen & Arnon, 1955) diluted fourfold with water (AA/4), and supplemented with 5 mM MOPS (pH 7.5), 2.5 mM NH₄Cl, 2.5 mM NaNO₃ and 2.5 mM KNO₃. Cultures were incubated under continuous illumination (17–22 μmol photons m⁻² s⁻¹) at 28 °C with shaking at 120 r.p.m.

#### Induction of akinete differentiation.

For both strains, akinete differentiation was induced in stationary-phase cultures by either low light or phosphate starvation. Low-light conditions (2 μmol photons m⁻² s⁻¹) were maintained by covering flasks with paper towels. Phosphate starvation was brought about by washing filaments three times with AA/4 medium without inorganic phosphate, and transferring to AA/4 medium supplemented with 5 mM MOPS buffer (pH 7.5), 2.5 mM NH₄Cl, 2.5 mM NaNO₃ and 2.5 mM KNO₃, but lacking inorganic phosphate. Phosphate starvation was also performed with nutrient enrichment by adding 5 mM fructose or 10 mM KNO₃. All induced cultures were maintained at 25 °C with shaking at 50 r.p.m. Low-temperature induction was achieved by transferring the cultures to 15 °C with shaking. The cells were checked by light microscopy periodically for up to 60 days.

#### Electron microscopy.

For electron microscopy studies, *Anabaena variabilis* cultures were induced to form akinetes by exposing to low light for 20–60 days and *N. punctiforme* cultures were induced by phosphate starvation for 32–60 days. Filaments of vegetative and differentiated cells were fixed and post-fixed with glutaraldehyde and potassium permanganate, and ultrathin sections were stained with uranyl acetate and lead citrate (Fiedler et al., 1998), and then examined with a Philips Tecnai 10 electron microscope at 80 kHz.

#### Cyanophycin extraction and quantification.

Cyanophycin was extracted from cells in triplicate according to Watzer et al. (2015) with the following modifications: aliquots (25 ml) of liquid cultures were taken at 1, 18, 30 and 60 days after akinete differentiation was induced by low light or phosphate starvation. After fixing, the cells were harvested by centrifugation at 25000 g, resuspended in 1 ml 0.1 M HCl and incubated for 2 h with shaking at 25 °C. The pH was adjusted to 7.5 by Tris/HCl pH 9.0 and ultracentrifuged at 25000 g for 15 min. The pellet was resuspended in 1 ml 0.1 M HCl and incubated for 2 h with constant shaking (1400 r.p.m.) at 60 °C; the suspension was centrifuged at 25000 g for 20 min. To precipitate cyanophycin, 1 ml 1 M Tris/HCl pH 9.0 was added to the clear supernatant. The mixture was incubated for 90 min at 4 °C and centrifuged at 25000 g for 30 min at 4 °C. The supernatant was discarded and the cyanophycin pellet was dissolved in 1 ml 0.1 M HCl. Cyanophycin was quantified in the samples and in a standard curve generated with isolated cyanophycin (Watzer et al., 2105) by determining the arginine concentration using the Sakaguchi reaction according to Messineo (1966).

#### Sakaguchi staining of cyanophycin.

To visualize cyanophycin granules by light microscopy, cultures were sampled at different times as akinetes developed, and stained according to Watzer et al. (2015), except that cells were fixed by resuspending in 1 ml PBS buffer containing 4 % (w/v) paraformaldehyde and incubated for 60 min at 4 °C. Cyanophycin in cells and akinetes was observed using a Leica DM 2500 light microscope with a ×100/1.3 oil objective and connected to a Leica DFC420C camera.
Glycogen determination. Glycogen was extracted from triplicate aliquots (50 ml) of cultures of both species taken 0, 18 and 30 days after akinete formation was induced by phosphate starvation. The samples were centrifuged at 4000 g for 10 min at room temperature, lyophilized and the pellets boiled in water brought to pH 9 with ammonia. After centrifugation, the cell pellet was washed with 80 % ethanol (v/v), resuspended in 400 μl H2O and autoclaved at 120 °C for 1 h. α-Amyloglucosidase was added to the supernatant, and glycogen of the samples and a standard curve was determined (Curatti et al., 2008).

BODIPY staining of neutral lipid structures. Akinete differentiation by N. punctiforme was induced for 30 days by phosphate starvation. Vegetative cells and akinetes were then stained with BODIPY 493/503 (Molecular Probes) to visualize neutral lipid structures using the following protocol. A 1 ml aliquot of culture was centrifuged twice at 4000 g for 10 min at room temperature, and the cells were resuspended in 1 ml PBS buffer (pH 7.5), centrifuged and resuspended in 1 ml PBS buffer containing 4 % (w/v) paraformaldehyde. The mixture was incubated for 1 h at room temperature, and the cells were centrifuged and resuspended in 0.5 ml PBS buffer (pH 7.5) and 1 μl 50 ng BODIPY ml⁻¹ in DMSO was added. The cell suspension was incubated in the dark for 15 min at room temperature, centrifuged and resuspended in 100 μl PBS buffer (pH 7.5). Cells and akinetes were placed on a slide covered with 1 % agarose gel. Cells were examined by light microscopy with a Leica DM 5500B microscope connected to a Leica DFC420C camera. To capture fluorescence or phase-contrast images, a Leica monochromator DFC360 FX camera was used. A BP470 40 nm excitation filter and a BP525 50 nm emission filter were used to detect BODIPY fluorescence. For 3D deconvolution, a series of images derived from different focal planes (z-stack) was provided and the scatter was recalculated by deconvolution using the built-in function of the Leica ASL software. Images were recoloured by the Leica ASF software based on the filter used and intensity levels were adjusted using Adobe Photoshop CS3.

Neisser staining of polyphosphate bodies. Vegetative cells and akinetes from cultures grown under low light for 18 and 60 days to induce akinete differentiation were Neisser stained (Seki et al., 2014). Polyphosphate bodies stained dark purple–black (Sukenik et al., 2012). Cells were viewed by light microscopy as described for cyanophycin staining.

Photosynthetic oxygen evolution measurement. Photosynthetic oxygen evolution was measured in vivo using a Clark-type oxygen electrode (Hansatech DW1). Light (55 μmol photons m⁻² s⁻¹) was provided from a high-intensity white light source (Hansatech L2). Oxygen evolution was measured at constant and controlled temperature (20 °C) from 2 ml aliquots in triplicate of both strains after induction of akinete differentiation by phosphate starvation for 0, 18, 30 and 60 days. The activity of photosystem II (PSII) was measured using a Hill reaction according to Dai et al. (2014) with a light intensity of 1500–2000 μmol photons m⁻² s⁻¹. As artificial electron acceptors, 2,5-dimethyl-p-benzoquinone and potassium ferricyanide were used. Respiratory activity was determined by measuring oxygen consumption in the dark. Chlorophyll a in cultures was determined by the method of Mackinney (1941).

RESULTS AND DISCUSSION

Triggers of akinete differentiation

Akinetes were commonly observed in stationary-phase cultures of Anabaena variabilis (Fig. 1c) and N. punctiforme (Fig. 1d) in standard medium. They could be identified easily under light microscopy by their larger size and intracellular granules (compare Fig. 1a, b with c–f). To study the physiological and morphological changes that occur during akinete formation, it was necessary to determine the best conditions for synchronized differentiation and to obtain cultures of both strains with as close to 100 % akinetes as possible. For this purpose, we tested various conditions to induce akinete formation in stationary-phase cultures: low temperature, low light and phosphate starvation without and with nutrient enrichment, but always in the presence of a combined nitrogen source. Akinete differentiation was induced efficiently in stationary-phase cultures of Anabaena variabilis by low light and resulted in a culture with 95 % akinetes after 10 days of induction. After 30 days of phosphate starvation, by contrast, cultures contained only ~70 % mature akinetes (Fig. S1, available in the online Supplementary Material). In stationary-phase cultures of N. punctiforme, phosphate starvation was most efficient and led to 70 % akinetes after 18 days (Fig. S2). Low temperature and other conditions tested (see Methods) were less effective, resulting in ~50 % akinetes during the same induction time.

Light intensity is a major trigger known to induce akinete development (Adams & Duggan, 1999), but other
environmental factors also trigger akinete differentiation in different species of Nostocales (Kaplan-Levy et al., 2010). Here, we showed that low light was the best inducer of akinete formation in Anabaena variabilis and confirmed the earlier finding (Argueta & Summers, 2005; Argueta et al., 2006) that phosphate starvation was the best inducer in N. punctiforme. The different inducing conditions also had an effect on the time course of akinete development. The triggers are apparently species specific, as the major trigger in Anabaena cylindrica is phosphate starvation (Wolk, 1965) and the major trigger in Nostoc spp. strain PCC 7524 is not phosphate limitation, but rather phosphate excess (Sutherland et al., 1979).

In contrast to heterocysts, which appear after nitrogen limitation under laboratory conditions within one generation (24 h), akinetes in N. punctiforme develop much slower and it is not possible to obtain completely synchronized cultures after a few days (Argueta & Summers, 2005). However, after 2 months, cultures of Anabaena variabilis and N. punctiforme consisted of up to 100 % fully matured akinetes (Fig. 1e, f). As phosphate starvation triggered akinete formation well for both strains and led to large numbers of akinetes in cultures incubated for 60 days, we used this inducer in several of the following experiments, as indicated. As light limitation was the best trigger of akinete formation in Anabaena variabilis, this condition was also used in some of the following experiments, as indicated.

**Morphological changes during akinete formation**

Akinetes developed within groups of adjacent vegetative cells of both Anabaena variabilis and N. punctiforme (Fig. 1c–f). The spore-like cells were distinguished from vegetative cells by their larger size (Anabaena variabilis: 7.45 × 4.24 μm; N. punctiforme: 8.12 × 5.7 μm), large number of intracellular granules (see below) and different cell shape. The large, oval-shaped akinetes of Anabaena variabilis were detached from the filaments and were surrounded by an envelope (Figs 1e and S1). Most akinetes of N. punctiforme remained attached to each other, even after the septum between the enlarged spherical cells was constricted, but an envelope was not visible under the light microscope (Figs 1d, f and S2).

We analysed the ultrastructure of akinetes of both species by preparing samples at different stages after akinete formation was induced by low light (Anabaena variabilis) or phosphate starvation (N. punctiforme). The ultrastructure of immature akinetes of Anabaena variabilis 20 days after induction (Fig. 2b) clearly differed from that of vegetative cells, in which polyhedral carboxysomes and the typical arrangement of inner thylakoid membranes were observed (Fig. 2a). The large granules in the akinetes consist of cyanophycin (nitrogen storage) and a large number of smaller glycogen granules (carbon storage) were observed (see below). The typical thylakoid organization was already disordered, carboxysomes were lacking and an extra envelope surrounding the Gram-negative cell wall had begun to develop. By contrast, mature akinetes (Fig. 2c, d) did not contain large cyanophycin granules, and their envelope was thicker and composed of five distinct layers as described by Braune (1980).

We also analysed the ultrastructure of akinetes of N. punctiforme in cultures starved of phosphate for 32 days (Fig. 3b, c) and 60 days (Fig. 3d). Vegetative cells of N. punctiforme, similar to those of Anabaena variabilis, contained polyhedral carboxysomes and had the typical arrangement of inner thylakoid membranes (Fig. 3a). Different morphologies of akinetes, representing different maturation stages, were observed at both time points after induction, which indicated that they developed asynchronously in the culture. The immature akinete contained glycogen granules, cyanophycin granules, carboxysomes and lipid droplets (see below), and thylakoid membranes were rearranged (Fig. 3c). In mature akinetes, carboxysomes, cyanophycin granules and lipid droplets were lacking, thylakoid membranes were pronouncedly rearranged, and a thick envelope was present. The envelope showed some structural similarities to the heterocyst-specific exopolysaccharide envelope as was reported for Anabaena cylindrica and Anabaena variabilis (Argueta & Summers, 2005;
Cardemil & Wolk, 1981) (Fig. 3d). In rare cases, a second layer, similar to the laminated glycolipid layer of heterocysts, was visible between the outermost thick envelope and the cell wall in akinetes of *N. punctiforme* (Maldener et al., 2014; Soriente et al., 1993).

To the best of our knowledge our study provides the first ultrastructural characterization of akinetes of *Anabaena variabilis* and *N. punctiforme* in different stages of maturation. We designate akinetes at the stage of development represented by the presence of granules, reordering of thylakoid membranes and presence of an initial or no akinete envelope as immature. We consider mature akinetes as those with a thick envelope. Akinetes of *N. punctiforme* strain PCC 7524 have also been described as those that first accumulate cyanophycin in the immature stage and then develop a multilayered extracellular envelope. A similar envelope has also been described for *Cylindrospermum* spp. and *Anabaena cylindrica* (Cardemil & Wolk, 1981; Nichols & Adams, 1982). It is not clear why planktonic species such as *Anabaena variabilis* form a multilayer envelope. One reason could be that the thick envelope enables survival under the high hydrostatic pressure that occurs during sedimentation of the developing akinete in the water column in wintertime.

### Intracellular storage compounds of akinetes

To verify that the large granules observed in immature akinetes of *Anabaena variabilis* and *N. punctiforme* were composed of cyanophycin, we used two approaches. First, cyanophycin was extracted from cultures exposed to low light or starved of phosphate, but in presence of combined nitrogen source for 1, 18, 30 and 60 days, and its concentration was determined using the Sakaguchi reaction. Cyanophycin in *Anabaena variabilis* starved of phosphate for 1 day was not detectable, and the content was much higher after 18 and 30 days of phosphate starvation, i.e. during induction of akinete formation, and was much lower after 60 days of phosphate starvation; exposure to low light also increased the cyanophycin content, but was not as effective (Fig. 4a). Cyanophycin in *N. punctiforme* starved of phosphate for 1 day was also not detectable, and the content was much higher after 18 days of phosphate starvation and decreased after 30 and 60 days of starvation; as in *Anabaena variabilis*, exposure to low light also increased the cyanophycin content, but was not as effective (Fig. 4d). In both cyanobacterial species, the granules in akinetes induced by phosphate starvation were much larger than those induced by low light (Figs S1 and S2). To correlate the cyanophycin determined in the chemical reaction with the large granules observed in micrographs, we stained cyanophycin *in situ* using the Sakaguchi reaction (Watzer et al., 2015). The results of the staining were in keeping with the measurements in cell extracts (Fig. 4a, d). The numerous large granules in akinetes of cultures starved of phosphate stained dark red (*Anabaena variabilis*: 6.0 ± 1.5 cyanophycin granules per akinete, *n*=10; *N. punctiforme*: 6.0 ± 3.0 cyanophycin granules per akinete, *n*=10) (Fig. 4c, f). In both species, fewer granules were observed in cultures exposed to low light and they were less intensively stained (*Anabaena variabilis*: 3.0 ± 2.1 cyanophycin granules per akinete, *n*=10; *N. punctiforme*: 2.0 ± 1.6 cyanophycin granules per akinete, *n*=0) (Fig. 4b, e).

Similarly, akinetes of *Anabaena torulosa* accumulate cyanophycin during development and decrease the amount when mature (Sarma & Khattar, 1986), and akinetes of *Aphanizomenon ovalisporum* accumulate cyanophycin during formation induced by potassium starvation (Suknen et al., 2015). In a previous study, Leganés et al. (1998) showed that cyanophycin granule formation is necessary for the function of heterocysts and akinetes in *Nostoc ellipso sporum*. The function of cyanophycin in *Anabaena variabilis* and *N. punctiforme* needs to be investigated in the future by mutational analyses.

Many small granules in akinetes at different stages of differentiation were observed in transmission electron micrographs. To test whether these granules consisted of glycogen, we extracted glycogen from the cultures after 0, 18 and 30 days of phosphate starvation, and enzymically determined it using α-amylglucosidase. The amount of glycogen per cell dry weight in akinetes of *Anabaena*...
variabilis cultures starved of phosphate for 18 days increased by 150 % compared with unstarved cultures; the levels after 30 days of phosphate starvation were almost as low as in unstarved cultures (Fig. 5). By contrast, the amount of glycogen per cell dry weight in akinetes in N. punctiforme cultures was nearly the same in unstarved cultures and those starved of phosphate for 18 or 30 days (Fig. 5). Glycogen accumulation during akinete development has also been observed in other species of Anabaena and in Nostoc spp. strain PCC 7524 (Sarma & Khattar, 1986; Sutherland et al., 1979).

To investigate the presence of lipoid structures in akinetes of N. punctiforme, vegetative cells and akinetes in cultures starved of phosphate for 30 days were stained with the fluorescent BODIPY stain, which is specific for neutral lipids. Stained intracellular lipid droplets were then visualized by fluorescence microscopy. In vegetative cells of N. punctiforme, green fluorescent spots (1.0 ± 1.2 lipid droplets per cell; n=10) corresponding to lipid droplets were identified (Fig. 6a). Such spherical droplets accumulated in immature akinetes (8.0 ± 5.0 lipid droplets per cell; n=10) and were rarely present in mature akinetes (1.0 ± 1.0 lipid droplets per cell; n=10; Fig. 6b). Such lipid droplets have recently been described as carbon storage compounds of N. punctiforme (Peramuna & Summers, 2014). Hence, it is likely that lipid droplets present in early akinetes also serve as a carbon reserve. BODIPY staining did not reveal any lipid droplets in Anabaena variabilis vegetative cells or akinetes (not shown).

The presence of polyphosphate bodies during akinete differentiation was investigated by exposing cultures of Anabaena variabilis and N. punctiforme to low light for 0, 18 and 60 days. Cells were then stained with Neisser stain and observed by bright-field microscopy. Vegetative cells of both species contained dark purple spots corresponding to polyphosphate bodies (Fig. 7a, d). By contrast, polyphosphate bodies were rarely observed in immature akinetes (Fig. 7b, e) and were absent in mature akinetes (Fig. 7c, f).

Fig. 5. Glycogen concentration during akinete development. The glycogen concentration in cell extracts of cultures of Anabaena variabilis and N. punctiforme starved of phosphate to induce akinete formation was measured using α-amylglucosidase at the times indicated. The data are the means of at least three independent samples ±SD.

Fig. 4. (a–f) Cyanophycin accumulation during akinete development. Cellular cyanophycin concentration of cultures of (a) Anabaena variabilis and (d) N. punctiforme exposed to low light or starved of phosphate at 1, 18, 30 or 60 days. The data are the means of at least three independent samples ±SD. Bright-field images of Sakaguchi-stained akinetes of (b, c) Anabaena variabilis and (e, f) N. punctiforme. Akinete formation was induced by (b, e) low light or (c, f) phosphate starvation for 18 days. Arrows, examples of individual stained granules. ND, not detected. Scale bars, 10 μm.
This supports the assumption that polyphosphate bodies are used to increase the pools of nucleic acids in akinetes (Sukenik et al., 2012), but could also be transferred to phospholipids.

Our measurements of storage compounds indicated that Anabaena variabilis produces glycogen and cyanophycin as reserve material, and N. punctiforme produces cyanophycin and lipid droplets, whilst glycogen levels did not change significantly (Table S1). In comparison with vegetative cells of both species, the amount of reserve materials was elevated in immature akinetes. Upon maturation, these storage compounds were lower or had disappeared completely. This implies that during the entire differentiation process, large amounts of carbon, nitrogen and energy are stored, which are then probably consumed to generate the thicker and complex akinete envelope. In this context, it has been suggested that in some cyanobacteria that do not form akinetes, excess glycogen is used to build extracellular mucilage under growth-limiting conditions (Stal, 2012).

Changes in photosynthetic and respiratory activity during akinete development

We measured metabolic activities of cultures of both strains induced to form akinetes by phosphate starvation for 18, 30 or 60 days and in stationary-phase cultures (day 0). The photosynthetic oxygen evolution of cultures was measured in vivo using a Clark-type oxygen electrode, which is an accurate method for measuring photosynthetic activity in comparison with the pulse-amplitude modulated fluorometer measurement. In cultures of both Anabaena variabilis (Fig. 8a) and N. punctiforme (Fig. 8d), oxygen evolution after 18 days of phosphate starvation was lower than that of vegetative cells, and decreased further after 30 days to <30 % of that of unstarved cultures. (Fig. 8a, d). In comparison, mature akinetes of Aphanizomenon ovalisporum retain only residual photosynthetic activity measured by chlorophyll fluorescence and nano-metric-scale secondary ion MS analysis (Sukenik et al., 2007, 2015).

To investigate solely PSII activity and not the entire electron transport chain, oxygen evolution was measured at high light intensity in the presence of an artificial electron acceptor system (Hill reaction). In cultures of Anabaena variabilis starved of phosphate for 18, 30 or 60 days, PSII-dependent oxygen evolution was ~50 % of that of...
vegetative cells (Fig. 8b). N. punctiforme cultures starved of phosphate maintained almost the same level of PSII activity as vegetative cells in the stationary phase (Fig. 8e). Hence, PSII functions properly in both Anabaena variabilis and N. punctiforme. This is in contrast to the suggestion that mature akinetes of Anabaena variabilis lack a functional PSII, as determined by fluorescent measurements (Bjorn et al., 1983).

We also determined the respiratory activity by measuring oxygen consumption in the dark. In cultures of Anabaena variabilis, respiration was lower after 18 days of phosphate starvation than in unstarved cultures, and was even lower after 30 and 60 days of starvation (Fig. 8c). By contrast, in cultures of N. punctiforme, respiration after 18, 30 and 60 days of phosphate starvation was much lower than in unstarved cultures (Fig. 8f).

To the best of our knowledge, this is the first characterization of PSII and respiration activities during akinete differentiation in Anabaena variabilis and N. punctiforme. The cultures maintained residual photosynthetic and respiratory activities after 60 days of akinete formation. It cannot be excluded that some of this residual activity was from some germinating akinetes, which were sometimes detected in very low numbers in the cultures...
(not shown). The adaptation of metabolic activities seems to be species specific, as the photosynthetic activity of *Anabaena cylindrica* decreases gradually as akinetes mature, but respiration increases (Fay, 1969); however, isolated mature akinetes of *N. spongiforme* photosynthesize and respire less than vegetative cells (Thiel & Wolk, 1983). Our results indicated that all metabolic activities in *Anabaena variabilis* and *N. punctiforme* greatly decrease as akinetes develop, and that reducing power and ATP are not consumed in anabolic reactions.

To the best of our knowledge, our study provides the first detailed characterization of akinete differentiation in the two model cyanobacterial species *Anabaena variabilis* and *N. punctiforme*, and shows the marked differences in morphology, reserve materials and metabolic activity that take place during akinete differentiation (Table S1). According to the taxonomical studies by Rippka et al. (1979), both species belong to subsection IV, which form unbranched filaments that are able to fix nitrogen by the formation of heterocysts. In the 16S phylogenetic tree as well as in the genome-based study, the species cluster in different groups (Shih et al., 2013; Thiel et al., 2014). *Anabaena variabilis* ATCC 29413 is closely related to *Nostoc* spp. strain PCC 7120 and other *Nostoc* species. *N. punctiforme* is less related to this group, and its genome sequence clusters together with *Microchaete* spp. strain PCC 7126 and *Calothrix* spp. strain PCC 7507 (Shih et al., 2013). The species are diverse with respect to the ecological niches they occupy. *Anabaena variabilis* ATCC 29413 is a planktonic freshwater organism isolated from the Mississippi and misinterpreted as *Anabaena flos-aquae* (Moore & Tischer, 1965; Thiel et al., 2014). For this lifestyle, the akinetes may require a lipid envelope layer to survive at the bottom of sediments. By contrast, *N. punctiforme* lives mainly in terrestrial habitats and in symbiotic interactions with eukaryotic organisms, such as mosses, lichens and higher plants (Meeks & Elhai, 2002). In this terrestrial habitat, *Nostoc* species experience cycles of dry periods. These two environments produce different stress conditions that may require specific adaptation mechanisms. Future mutagenesis approaches in genes related to the cell envelope and metabolism of akinetes will show which of the cellular and metabolic modifications are essential for akinete function and germination of these diverse species.

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