Akinetes: Dormant Cells of Cyanobacteria

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Chapter 2
Akinetes: Dormant Cells of Cyanobacteria

Ruth N. Kaplan-Levy, Ora Hadas, Michael L. Summers, Jacqueline Rücker, and Assaf Sukenik

Abstract Cyanobacteria are an ancient and morphologically diverse group of photosynthetic prokaryotes, which were the first to evolve oxygenic photosynthesis. Cyanobacteria are widely distributed in diversed environments. In the case of members of the orders Nostocales and Stigonematales, their persistence and success were attributed to their ability to form specialized cells: heterocysts, capable of fixing atmospheric nitrogen and spore-like cells, the akinetes. This review focuses on akinetes of Nostocales, emphasizing environmental triggers and cellular responses involved in differentiation, maturation, dormancy, and germination of these resting cells. Morphological and structural changes, variation in akinete composition, and metabolism are summarized. Special attention is given to the genetic regulation of the differentiation process in an attempt to close gaps in our understanding of the dormancy phenomenon in cyanobacteria and to identify open questions for future research.

2.1 Introduction

The cyanobacteria comprise a very diverse group of photoautotrophic oxygenic prokaryotic organisms. They are found all over the world: in seas, soils, glaciers, deserts, and hot springs, but most species reside in freshwater in both benthic and

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pelagic habitats (van den Hoek et al. 1998; Adhikary 1996). In open freshwater environments, they can become extremely dominant forming dense blooms. Based on their life strategy, pelagic cyanobacteria can be classified into the following categories (1) species that lack specialized resting cells, for example, members of the orders Chroococcales and Oscillatoriales and (2) species that form specialized resting cells, for example, members of orders Nostocales and Stigonematales. The ability to form resting cells enables these species to survive harsh environmental conditions while dormant in bottom sediments. As environmental conditions improve, vegetative cells germinate from the resting spores and float due to newly formed gas vesicles, thus assisting in dispersal throughout the water column. Dormancy and floating features of these species are responsible for their domination in many water bodies. Within a short period of time, they bloom and influence the phytoplankton composition in a seasonally repetitive pattern. This annual life cycle of planktonic Nostocales is illustrated in Fig. 2.1, using *Aphanizomenon ovalisporum* as the representative Nostocales species.

The resting cells of Nostocales and Stigonematales species are called akinetes (from the Greek "akinetos" – motionless). These are spore-like, thick-walled, nonmotile cells that differentiate from vegetative cells and serve a perennating role. Akinetes are larger and have a thicker wall than vegetative cells and contain large amounts of food reserves and DNA. The akinete shape differs among species

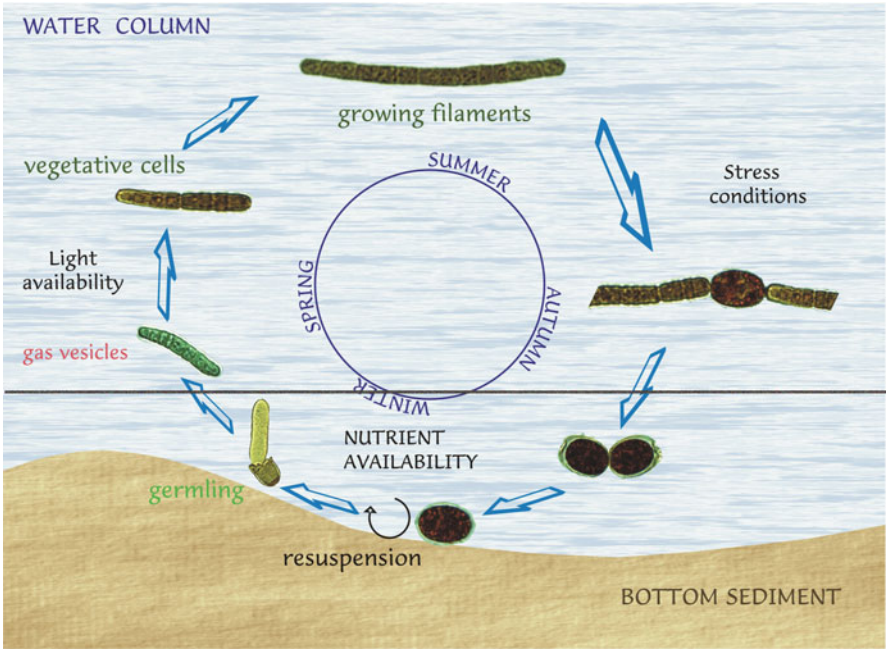

Fig. 2.1 Life cycle of the cyanobacterium *Aphanizomenon ovalisporum* (Nostocales). Adopted from Hense and Beckmann (2006)

from sphere to oblate spheroid and their distribution and position within a filament (trichome) is used as a taxonomic feature. Differentiation, maturation, dormancy, and germination of akinetes in Nostocales share some features with other prokaryotes (endospore in *Bacillus* – see Errington 2003) and some eukaryotes (spore in yeast – see Hohmann et al. 2010, cyst in Protists – see Corliss 2001). Here we present an updated overview on physiological, ecological, and molecular aspects of cyanobacterial akinetes. The reader is referred to earlier review papers of value by Nichols and Adams (1982), Herdman (1987), and by Adams and Duggan (1999).

2.2 Structure, Composition, and Metabolism of Akinetes

Akinetes are larger (sometimes by up to tenfold) than vegetative cells or nitrogen-fixing cells – heterocysts (Adams and Duggan 1999). Akinetes are surrounded by a thickened cell wall and a multilayered extracellular envelope (Nichols and Adams 1982; Herdman 1987, 1988), composed of glucose-rich carbohydrate and amino compounds as shown for *Anabaena cylindrica* by Cardemil and Wolk (1976, 1979). During differentiation, akinetes accumulate both glycogen and granules of cyanophycin (Simon 1987).

The position of akinetes along the trichome varies among cyanobacterial species and strains, where in some cases heterocysts were reported to influence their location (Wolk 1966). Akinetes develop immediately adjacent to heterocysts in *A. cylindrica* but several cells away from the heterocyst in *Anabaena circinalis* and in some other planktonic species (Fay et al. 1984; Li et al. 1997). In most cases, akinetes develop in strings, showing a gradient of decreasing maturity away from the first to develop. Adams and Duggan (1999) explained the akinete placement in relation to heterocysts by the need to accumulate large amounts of cyanophycin.

Akinetes undergo various metabolic and morphological changes during their development and maturation. Metabolic activities of akinetes such as CO_2 fixation showed reduced rates in *A. cylindrica* and *Nostoc* PCC 7524 (Fay 1969a; Sutherland et al. 1985a; Rao et al. 1987; Rai et al. 1985), whereas the rate of respiration was often elevated (Yamamoto 1976; Herdman 1987), presumably in relation to the maturation process but lost in older akinetes (Chauvat et al. 1982). Isolated akinetes of *Nostoc spongiaeforma* respired in the dark, evolved oxygen in the light and retained residual capability to synthesize proteins and lipids (Thiel and Wolk 1983). While developing akinetes of *A. cylindrica* are metabolically active, they have significantly decreased activity as they mature (Fay 1969a). Mature akinetes of *A. cylindrica* were reported to have little chlorophyll and no functional photosystem I (PSI) (Fay 1969b). However, the pigment content of akinetes of a different isolate of *A. cylindrica* was similar to that of the vegetative cells (Wolk and Simon 1969). Akinetes of *Anabaena doliolum* lost both chlorophyll and phycocyanin following incubation in the dark for several weeks (Singh and Sunita 1974). In vivo fluorescence measurements of *A. variabilis* akinetes suggested that the akinetes lacked a functional Photosystem II (PSII), although the reaction center chlorophyll was

present (Bjorn et al. 1983). Using transmission electron microscopy and immunocytological labeling, the 32 kDa – PsbA protein (D1 polypeptide) of PSII was detected in akinetes (and other cell types) of the cyanobionts within leaf cavities of *Azolla caroliniana* Willd (Braun-Howland and Nierzwicki-Bauer 1990). In a recent study, Sukenik et al. (2007) demonstrated changes in the photosynthetic activities of individual vegetative cells and akinetes in trichomes of *A. ovalisporum* during akinete formation, whereas mature isolated akinetes retained only residual photosynthetic capacity. In mature akinetes of *A. ovalisporum*, the phycobilisome antenna was reduced in size and apparently detached from the reaction centers. Similarly, the disappearance of phycocyanin from *A. cylindrica* akinetes was reported by Fay (1969b) in accordance with observations on diminishing photosynthetic activity in isolated akinetes (Fay 1969a). The stoichiometric ratio of PSI to PSII in *A. ovalisporum* akinetes remained more or less the same as in vegetative cells and the cellular content of PsbA (D1) and PsaC proteins per cell volume remained fairly stable (Sukenik et al. 2007). Furthermore, preliminary immunoblotting experiments indicated the presence of the RubisCO large subunit in *A. ovalisporum* akinetes (Sukenik unpublished). Thus, it was concluded that in *A. ovalisporum* the reduction in the phycobilisome pool in mature akinetes is targeted at minimizing absorption of light energy to diminish potential damage to reaction centers during dormancy. The presence of reaction center complexes in mature akinetes ensures a prompt recruitment of photosynthesis upon germination, to energize essential cellular processes (Sukenik et al. 2009).

Akinetes accumulate both glycogen and cyanophycin, a nonribosomally produced reserve polymer composed of an aspartate backbone with arginine side groups (Simon 1987). In akinetes of *Nostoc* PCC 7524, the mean cellular content of cyanophycin was eightfold higher than in vegetative cells (Sutherland et al. 1979). However, accumulation of cyanophycin was not specific for akinete development, vegetative cells also accumulated glycogen and cyanophycin when entering the stationary phase (Herdman 1987). Incubation of *A. cylindrica* with the arginine analogue, canavanine (Nichols and Adams 1982), and mutation of the arginine biosynthesis gene, *argL*, in *Nostoc ellipsosporum* (Leganés et al. 1998), resulted in the production of akinetes lacking cyanophycin, suggesting that cyanophycin accumulation is not essential for the formation of akinetes. The mean cellular content of RNA, DNA, and protein was similar in vegetative cells and akinetes of *Nostoc* PCC 7524 (Sutherland et al. 1979), whereas the akinetes of *A. cylindrica* contained the same amount of RNA, but more than twice as much DNA, and ten times as much protein as vegetative cells (Simon 1977). These high values are probably a consequence of the increased size of the *A. cylindrica* akinetes, which were up to ten times the volume of the vegetative cells (Fay 1969b). In *A. ovalisporum*, DAPI staining demonstrated the accumulation of nucleic acids in developed akinetes. The intensity and localization of the DAPI signal indicate a homogeneous dispersion of nucleic acids in the entire akinete volume and the absence of polyphosphate bodies (Sukenik et al. 2009). Polyphosphate bodies were rare in mature akinetes of *Nostoc* PCC 7524, although they were commonly present in vegetative cells during akinete differentiation (Sutherland et al 1979).

2.3 Factors that Influence Akinete Differentiation

Various environmental factors were reported as triggers for differentiation of akinetes in different cyanobacterial species and strains (Table 2.1). The major, although not the only, trigger for akinete development is light intensities (Adams and Duggan 1999). For example, in *Nostoc* PCC 7524 cultivated in the presence of excess inorganic nutrients, akinetes differentiated as light availability was reduced by 90% or more of the incident light, due to the culture self shading (Sutherland et al 1979). However, high light intensities triggered the formation of akinete in *Cylindrospermopsis raciborskii* (Moore et al. 2005).

Light quality also plays a role in the control of akinete formation. In *Gloeotrichia*, akinete differentiation was stimulated by green rather than white light. As green light is the dominant spectral component during bloom conditions, this could also explain observations by Rother and Fay (1977) that akinete differentiation in natural populations is frequently associated with the development of surface blooms. Similar observations were recently reported by Thompson et al. (2009) for the toxic cyanobacterium *A. circinalis*, red or green irradiance were much more effective for akinete production than blue light. For cells grown under a predominantly red, white, or green irradiance, even short exposures to blue light substantially reduced the number of akinetes, suggesting that blue light inhibits akinete formation.

Limitation of phosphate has been implicated as a trigger for akinete development (Nichols and Adams 1982; Herdman 1987, 1988) and increasing numbers of akinetes were found during phosphorus deficiency (Sinclair and Whitton 1977). In *A. circinalis*, phosphate limitation appeared to be the major trigger, whereas limitations for N, inorganic C, iron, trace elements, or light had no effect on the development of akinetes (van Dok and Hart 1996). In *N. punctiforme*, akinetes were induced within 2 weeks starvation for phosphate (Meeks et al. 2002). However, phosphorus was required to allow full development of akinetes in *C. raciborskii* (Moore et al. 2003, 2005) and in *A. circinalis* (Fay et al. 1984).

In addition to phosphate, other nutrients and abiotic conditions are also known to affect the formation of akinetes. Deficiencies in Mg, Ca, Fe, and S, for example, led to a decrease in the number of akinetes in *Gloeotrichia ghosei*, while in a range of planktonic *Anabaena* isolates, temperature was important for triggering akinete differentiation (Li et al. 1997). In *A. doliolum* (Rao et al. 1987) and *Anabaena torulosa* (Sarma and Khattar 1993) a critical C:N ratio appeared to be important. In *C. raciborskii*, the formation of akinetes was triggered by an initial temperature shock, by the frequency of temperature fluctuations, and by high light intensity (Moore et al. 2005). Recently it was reported that deprivation of potassium ions (K^+) triggered the formation of akinetes in the cyanobacterium *A. ovalisporum*. A burst of akinete formation was observed within 1–2 weeks after the induction (K^+ depletion) was imposed (Sukenik et al. 2009). K^+-deficiency was found to induce akinete formation also in *Nostoc spongiaeforme* and in *N. punctiforme* (Sukenik and Summers unpublished). K^+-deficiency stimulus seems to induce a secondary signal, apparently related to cellular osmo-regulation and desiccation

Table 2.1 Summary of environmental conditions and factors that influence differentiation and germination of akinetes in various cyanobacterial species (Order Nostocales)

Environmental variable	Cyanobacterial species	Reference	Observation
A. Differentiation of akinetes			
Light intensity[1]	*Anabaena circinalis*	Fay et al. (1984)	Light limitation induced akinete formation
	Nostoc PCC 7524	Sutherland et al. (1979)	
	Anabaena cylindrica	Nichols et al. (1980), Fay (1969a)	
	Aphanizomenon flos-aquae	Rother and Fay (1977)	
	Cylindrospermopsis raciborskii	Moore et al. (2005)	Increase in light intensity resulted in an increase in akinete concentration
Light quality	*Gloeotrichia echinulata*	Wyman and Fay (1986)	Akinete differentiation was stimulated by green light
	Anabaena circinalis	Thompson et al. (2009)	Red light enhanced differentiation, short exposure to blue light inhibited akinete production
Phosphate[2]	*Anabaena cylindrica*	Nichols and Adams (1982)	Phosphate limitation has been implicated as a trigger
	Cylindrospermum licheniforme	Fisher and Wolk (1976)	
	Anabaena circinalis	van Dok and Hart (1996)	
	Gleotrichia ghosei	Sinclair and Whitton (1977)	
	Anabaena circinalis, *Nostoc* PCC 7524	Fay et al. (1984)	Phosphate stimulated akinete differentiation
	Nostoc PCC 7524	Sutherland et al. (1979)	Akinetes were never produced in the absence of phosphate
Temperature[3]	*Anabaena* spp.	Li et al. (1997)	Important for triggering of akinete differentiation in a range of strains
	Cylindrospermopsis raciborskii	Moore et al. (2005)	Maximum akinete concentrations were observed in cultures that experienced multiple diurnal temperature fluctuations with a magnitude of 10°C (25°C–15°C)
C:N ratio	*Anabaena doliolum*.	Rao et al. (1987)	Critical C:N ratio appeared to be important
	Anabaena torulosa.	Sarma and Khattar (1993)	
K^+ ion[4]	*Aphanizomenon ovalisporum*	Sukenik et al. (2007)	Depletion of potassium from the medium triggered akinete formation
	Nostoc spongiaeforme, *Nostoc punctiforme*	Sukenik and Summers (unpubl.)	
B. Germination of akinetes			
Light[5]	*Nodularia spumigena*	Huber (1985)	Low light was sufficient for germination
	Anabaena cylindrica	Yamamoto (1976)	Light was essential, germination did not occur in dark
	Anabaena circinalis	van Dok and Hart (1997)	

(*continued*)

Table 2.1 (continued)

Environmental variable	Cyanobacterial species	Reference	Observation
	Anabaena, Aphanizomenon	Karlsson-Elfgren and Brunberg (2004)	
	Gleotrichia echinulata	Karlsson-Elfgren et al. (2004)	
Light quality[5]	*Cylindrospermopsis raciborskii*	Wiedner et al. (2007)	Red light supported germination
	Nodularia spumigena	Huber (1985)	
	Anabaena variabilis	Braune (1979)	
	Anabaena doliolum, Fischrella mucicola	Kaushik and Kumar (1970)	Germination occurred also in non-photosynthetic light
Phosphate[5]	*Anabaena circinalis*	Thompson et al. (2009)	Phosphate was required for germination
	Anabaena circinalis	van Dok and Hart (1997)	
	Nodularia spumigena	Huber (1985)	
Temperature[5]	*Anabaena circinalis*	Fay (1988)	Incubation in high temperatures (37-45 °C) imposed reduction in germination rate
	Anabaenopsis arnoldii	Reddy (1983), Pandey and Talpasayi (1981)	High germination rate at around optimal temperature for growth
	Nostoc spumigena, Anabaena vaginicola	Rai and Pandey (1981)	
	Aphanizomenon ovalisporum	Hadas (unpubl.)	
	Cylindrospermopsis raciborskii	Wiedner et al. (2007)	
Sediment mixing and resuspension[6]	*Gleotrichia echinulata*	Ståhl-Delbanco and Hansson (2002), Karlsson-Elfgren et al. (2004)	Germination is enhanced by mixing of bottom sediment
	Anabaena, Aphanizomenon	Karlsson-Elfgren and Brunberg (2004)	
	Aphanizomenon ovalisporum	Hadas et al. (1999)	
	Anabaena circinalis	Baker and Bellifemine (2000)	
	Anabaena sp., *A. solitaria*, and *A. lemmermannii*	Rengefors et al. (2004)	
	Anabaena cylindrica	Yamamoto (1976)	
Oxygen	*Nostoc PCC 7524*	Chauvat et al. (1982)	Oxygen was essential for germination
	Anabaena circinalis	Kezhi et al. (1985)	

[1]The response to light intensity is species dependent
[2]In some species phosphate deficiency triggers akinete formation while in others a basal level of phosphate is required for akinete development
[3]Different temperature optima for different species. Temperature fluctuations play a role in some species
[4]K^+ -deficiency may be involved in secondary internal signals
[5]In most cases, conditions that support growth of vegetative cultures are required for germination
[6]Observations are mainly from lakes and water reservoirs

that leads to the induction of akinete formation. Adams and Duggan (1999) postulated that the diverse stimuli, reported to affect akinete formation, induce a common physiological trigger – perhaps decreased cell division or low energy – which results in akinete development. Argueta and Summers (2005) speculated that a metabolic imbalance triggers akinete formation as a *zwf* mutant of *N. spongiaeforme*, lacking the first enzyme of the oxidative pentose phosphate pathway, formed functional akinetes during dark incubation in the presence of fructose. The collective observations on the environmental stimuli that trigger akinete formation in different cyanobacterial species and strains (Table 2.1) are mostly consistent with cellular energy limitation and cessation of cell division as primary signals.

2.4 Factors Influencing Akinete Germination

Germination of akinetes is a complex coordinated metabolic process triggered by various ambient conditions such as temperature, increased light availability (day length and penetration to sediments), and by sediment resuspension induced by turbulence in proximity to the bottom sediments (Reynolds 1972; Karlsson-Elfgren et al. 2004) as specified in Table 2.1. *Light* – This was identified as a significant factor triggering germination of akinetes. In *A. cylindrica*, germination was dependent on light intensity and did not take place in the dark or in the presence of DCMU (Yamamoto 1976). However, in *Nodularia spumigena* very low light intensities (0.5 μmol photon m^{-2} s^{-1}) were enough to initiate germination. Akinetes were not able to germinate in the dark (Huber 1985; Rengefors et al. 2004) even under heterotrophic conditions; however, supply of suitable organic carbon may result in germination (van Dok and Hart 1997). The most active spectral range for germination was between 620 and 630 nm, coinciding with the maximum light absorption by C-phycocyanin (Nichols and Adams 1982). Light and phosphate were required for germination of *A. circinalis* (van Dok and Hart 1997) and *N. spumigena* (Huber 1985). Light was an important trigger for the recruitment of *Gloeotrichia echinulata* in Lake Erken but not as important for *Anabaena and Aphanizomenon* in Lake Limmaren, Sweden. In all species, light was correlated to the scale of recruitment via germination (Karlsson-Elfgren and Brunberg 2004; Karlsson-Elfgren et al. 2004). Dilution of an akinete-containing culture with a fresh medium stimulated germination, apparently due to increased light intensity (Herdman 1988; Adams and Duggan 1999). The process of germination may be photoperiodic (day-length) dependent and germination would occur only after maturation perioda was completed (Karlsson-Elfgren et al. 2004). *Temperature* – The tolerance of akinetes to temperature extremes vary among species. *A. fertilissima* akinetes when pretreated at high (37–45°C) or low (0–7°C) temperatures for 48 h showed no effect on germination, while a reduced germination rate was observed in akinetes of *Anabaenopsis arnoldii*, *N. spumigena*, and *A. vaginicola* when incubated in extreme temperatures (Reddy 1983; Pandey and Talpasayi 1981; Rai and Pandey 1981). Unlike bacterial and fungal spores, germination of akinetes of *A. cylindrica* was not stimulated by

heat shock (Yamamoto 1976). Akinetes of *A. ovalisporum* isolated from Lake Kinneret (Israel) and grown in cultures, germinated within a temperature range of 18–25°C but germination yield was low and unsynchronized (Hadas unpublished). Based on a field study in a shallow lake in northern Germany, Wiedner et al. (2007) suggested a germination temperature for *C. raciborskii* of 15–17°C, but Tingwey et al. (personal communication) found germination down to 13°C in an experimental set up with sediment from the same lake. *Sediment mixing and resuspension* – In *G. echinulata*, the process of recruitment from bottom sediments via akinete germination was influenced by high temperature and light, and significantly enhanced by mixing of bottom sediment imposed by bioturbation and physical processes (Ståhl-Delbanco and Hansson 2002; Karlsson-Elfgren et al. 2004). In Lake Kinneret, the benthic boundary layer and the sediment water interface are subject to turbulence processes, whereas sediments in the littoral zone are resuspended due to wave breaks, thus possibly affecting recruitment of akinetes. Bottom sediments collected from Lake Kinneret and incubated under control conditions in N-free BG11 medium yielded many filaments of *A. ovalisporum*, pointing to the role of akinetes in the establishment of a new population (Hadas et al. 1999). It is possible that shallow wetlands, shallow lakes, and littoral zones of deep lakes provide a conducive environment for germination of akinetes due to continuous resuspension of akinetes from the sediments and their exposure to an appropriate level of light (Baker and Bellifemine 2000; Rengefors et al. 2004) and oxygen, which are crucial for germination (Fay 1988). *Salinity* – When *N. spumigena* akinetes were pretreated at low (Pandey and Talpasayi 1981) or high concentrations of sodium chloride (Huber 1985), germination rate was reduced. The appearance of *A. circinalis* germlings increased with increased salinity up to 2.5 g l^{-1} with 26.9% germination and decreased to 0.2% at 5 g l^{-1}. No germination was observed at 10 g l^{-1} salinity (Baker and Bellifemine 2000). *Nutrients* – Addition of organic compounds such as sucrose and a supply of oxygen increased the efficiency of germination in *Nostoc* PCC 7524. Under these conditions all akinetes germinated, although slowly, indicating that successful germination required respiration and cyclic photophosphorylation (Chauvat et al. 1982). Germination of akinetes of *A. cylindrica* was completely inhibited by DCMU (Yamamoto 1976). Accumulated cyanophycin served as a source of nitrogen required for protein synthesis in the early stages of germination in *A. variabilis* (Braune and Doehler 1996). Degradation of cyanophycin during germination was observed in *Cylindrospermum* (Miller and Lang 1968), *A. flos-aquae* (Wildman et al. 1975), *A. cylindrica* (Fay 1969a), and *Nostoc* PCC 6720 (Skill and Smith 1987), whereas in *Nostoc* PCC 7524 other intracellular storage compounds were consumed (Sutherland et al. 1985a). The involvement of hydrolytic enzymes that degrade cyanophycin during germination was postulated (Braune 1979).

The environmental stimuli that trigger akinete germination in different cyanobacterial species (Table 2.1) generally correspond to the conditions that support growth of vegetative cultures. In addition, sediment mixing and resuspension play an important role in germination as they relocate the akinetes from the bottom sediment into the water column and photic zone.

2.5 The Germination Process

Germination of akinetes begins with cell division that occurs inside the akinete's envelope as described for *Nostoc* PCC 2574 (Sutherland et al. 1985b; Herdman 1988) and other cyanobacterial species (Moore et al 2004; Hori 2003; Baker and Bellifemine 2000; Braune 1980). Expansion of the cells results in an increase in turgor pressure which, consequently leads to a disruption of the envelope and emergence of the germling from the akinete's envelope. The open envelope may remain associated with the developing filament for some time. Morphological changes of the germling eventually leads to a fully developed young trichome (Moore et al. 2004). The germination process of *A. ovalisporum* akinetes, demonstrated in Fig. 2.2, begins with reorganization of cellular material (Fig. 2.2b) followed by elongation and division of the spore-like cell and opening of the akinete envelope on either terminal side of its slightly longer axis (Fig. 2.2c–g). It is unclear whether the envelope disruption is assisted by enzymatic activity or if it occurs merely due to the increased internal pressure resulting from the expanded cells. Finally, the germling, comprised of several cells, emerges from the akinete (Fig. 2.2 h). In some strains such as *N. punctiforme*, during germination the entire akinete wall may dissolve and, hence, not be microscopically visible (Adams and Duggan 1999; Meeks et al. 2002). Akinetes of *A. ovalisporum* isolated from Lake Kinneret did not germinate synchronously and the germination frequency was low.

Germination of *Cyanospira* akinetes was accompanied by de novo synthesis of proteins which took place prior to the first cell division (Sili et al. 1994). In *A. circinalis*, photosynthetic activity provided the energy for akinete germination (Kezhi et al. 1985), but the rate of germination was determined by the respiratory oxygen uptake of the akinetes, in a temperature-dependent manner (Fay 1988).

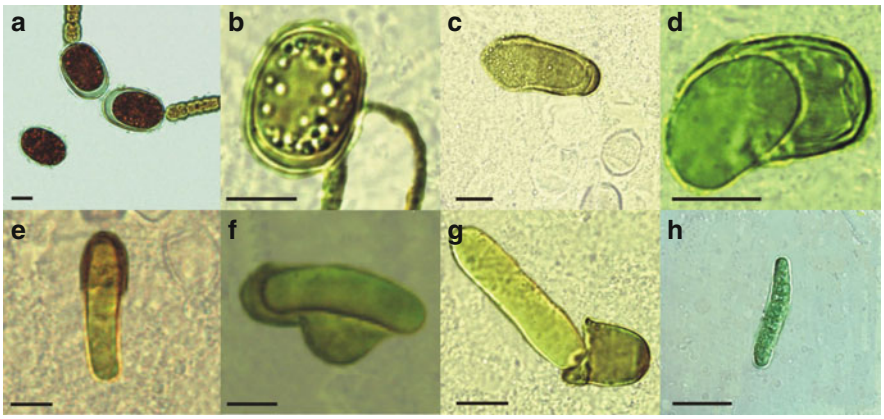

Fig. 2.2 Germination stages in akinetes of *A. ovalisporum*. (**a**) A free akinete and two connected akinetes within a filament of vegetative cells; (**b**) A matured granulated akinete; (**c**) The envelope of the akinete is opened (ruptured); (**d**)–(**g**) A germling emerging from the envelope; (**h**) A young filament; *Black horizontal scale bar* indicates 10 μm

An additional stage (although not an obligatory one) in the germination process is the development of gas vacuoles that support successful flotation of germlings and trichomes in the water column as depicted in Fig. 2.1. In *G. echinulata*, the newly formed filaments float 2–4 days after germination (Karlsson-Elfgren et al. 2004).

2.6 Ecological Functions of Akinetes

Variable harsh conditions imposed in laboratory studies showed that akinetes are resistant to low temperatures and desiccation, but not to heat, with the exception of *A. cylindrica* which germinate after drying at 60°C or under sunlight (Hori et al. 2003). Extended survival was reported for akinetes of *A. cylindrica* surviving in the dark and dry state for 5 years, whereas vegetative cells survived no longer than 2 weeks under similar conditions (Yamamoto 1975). Akinetes of *Nostoc* PCC 7524 survived in the dark at 4°C for 15 months, whereas vegetative cells lost viability within 7 days (Sutherland et al. 1979). Akinetes of *Aphanizomenon* and *Anabaena,* 18 and 64 years old, respectively, found in the sediment of Rostherne Mere (England) were viable and successfully germinated (Livingstone and Jaworski 1980). Thus, akinetes do not only have a temporary resting function, but may also ensure the long-term survival of a species giving it an ecological advantage. The term temporary resting means the overwintering and survival through dry periods. In temperate climatic zones, where the vegetative cells die in autumn, akinetes are a key factor in the annual life cycle of Nostocales (Fig. 2.1). A good example is the life cycle regulations of *C. raciborskii* in North German lakes where the time of germination was temperature mediated but further growth was mainly controlled by underwater light supply (Wiedner et al. 2007). Using a simple mathematical model it was demonstrated that temperature is the most important variable determining the population size: the earlier the germination took place in spring a larger population was recorded the next summer. *C. raciborskii* population size determines the annual input of akinetes to the sediment (Rücker et al. 2009 submitted). Consequently, interannual variations in pelagic populations were reflected by a varying number of akinetes deposited in the sediment, representing different inoculum sizes for the proceeding growing season. Although the akinete "seed bank" in the sediments of lakes is important for the recolonization of the pelagic zone, the contribution of akinetes toward the bloom success of next year's population of Nostocales seems to be rather small: 0.62% in Green Lake, OR (Barbiero and Welch 1992), 8% in Agency Lake, OR (Barbiero and Kann 1994), and 0.003–0.05% in Lake Limmaren, Sweden (Karlsson-Elfgren and Brunberg 2004). However, small deposits of akinetes may be sufficient for later colonization. For instance, *C. raciborskii* population size in a shallow German lake was more dependent on abiotic conditions after germination than on the inoculum size (Rücker et al. 2009 submitted).

Besides their role in survival, akinetes have the ability to serve as dispersal units. The most dramatic change in geographic distribution could be observed for the originally tropical cyanobacterium *C. raciborskii*, which spread from tropical to

temperate regions on all continents except Antarctica during recent decades (Padisák 1997). Two hypotheses have been put forward to explain these changes in biogeography (a) the species spread to temperate regions due to increasing water temperatures associated with climate change and (b) selected ecotypes with lower temperature and light requirements have spread northward. Wiedner et al. (2007) assumed that an earlier rise in water temperature associated with climate change has promoted the species expansion. Transport of akinetes by migratory birds as a possible means of dispersal (Padisák 1998) may increase the chances of akinete-producing strains to be spread. The possible role of akinetes as a prerequisite for spreading of *Anabaena bergii* and *Aphanizomenon aphanizomenoides*, which invaded lakes of northern Germany was also hypothesized by Stüken et al. (2006)

The robust shells of akinetes are useful microfossil indicators, which may contribute to the reconstruction of earlier phytoplankton composition and trophic state of water bodies (van Geel 1986; van Geel et al. 1994). The invasion of *C. raciborskii* to north German lakes in the last 10–20 years could be proved by the detection of akinete shells in the upper part of sediment cores of two shallow lakes (Rücker et al. unpublished data). Since akinetes may stay viable in deeper sediment layers for a long time, providing an interesting tool for studying genetic variability of ancient Nostocales populations, or perhaps even physiological studies if they could be germinated and induced to grow in the laboratory.

2.7 Genes Involved in Akinete Differentiation

While the formation of akinetes presents a relatively simple model for cellular differentiation, the elucidation of the molecular mechanism regulating and involved in this process lagged until recently. Many studies were carried out using filamentous cyanobacteria to decipher the differentiation of nitrogen-fixing cells, heterocysts, from photosynthetically active vegetative cells but only few attempts focused on the differentiation of the dormant forms (Meeks et al. 2002). Heterocysts were used as a preferred model, mainly for their simple cell differentiation triggered by deprivation of fixed nitrogen. The advanced data accumulated on heterocyst formation suggest that these cells and akinetes share some commonalities in the molecular pathway of cell differentiation (Zhang et al. 2006). Four genes were found to be involved in both differentiating cells. One of these genes is *hepA*. A mutation in this gene resulted in alterations of akinete and heterocysts envelopes in *Anabaena variabilis* (Leganés 1994). This gene encodes for an ABC transporter required for the deposition of polysaccharides in the envelope of both cell types. The second gene, also implicated in polysaccharide synthesis is *devR*. *devR* encodes for a response regulator of a two-component system. When this gene was overexpressed in *Nostoc punctiforme*, an increase in akinete differentiating cells was observed (Campbell et al. 1996). The third gene found to be involved in both heterocysts and akinetes differentiation is *hetR*, which encodes for a DNA-binding protease. This gene when mutated by a transposon insertion in *N. ellipsosporum* resulted in a failure of cells to differentiate

either to heterocysts or akinetes. Further analysis using the luciferase reporter gene, showed that *hetR* was expressed in akinetes (Leganés et al. 1994). In *Nostoc punctiforme*, a *hetR* mutant was capable of producing akinete-like cells. These akinetes-like cells lacked the granular characteristics found in the wild type. Both types of akinetes, however, mutant and wild type, had similar viability upon low-temperature treatment following phosphate starvation, when compared to vegetative cells. Therefore, it was suggested that although involved in the process, *hetR* is not essential for akinete differentiation (Wong and Meeks 2002). Another gene affecting akinete and heterocyst development in *Nostoc ellipsosporum* is *argL*, which encodes for an *N*-acetylglutamate semialdehyde dehydrogenase, an enzyme involved in L-arginine biosynthesis. A mutation caused by a transposon insertion in *argL* of *N. elipsosporum* resulted in smaller than wild type akinetes, which lack cyanophycin granules and failed to germinate (Leganés et al. 1998).

The study of akinete differentiation at the molecular level has been limited by the asynchronous development and restricted number of akinetes formed within a filament and by the lack of a marker gene for developing or mature akinetes. The first akinete marker was identified in *Anabaena variabilis* (Zhou and Wolk 2002) representing a breakthrough in the study dormant cells development in cyanobacteria. Separation of total protein extract by SDS-PAGE showed the presence of a 43-kDa protein in akinetes. This protein was designated AvaK. *avaK* was highly expressed in akinetes but to a small degree in vegetative cells as was demonstrated by GFP fusion in this strain (Zhou and Wolk 2002) and in *N. punctiforme* (Argueta et al. 2004). The deduced protein sequence of AvaK shows the existence of a PRC barrel domain in its N-terminal region, a domain implicated in RNA metabolism (Anantharaman and Aravind 2002); however, the function of this gene remains unknown. Synchronized differentiation of akinetes was reported in the *zwf* mutant of *Nostoc punctiforme* (Argueta and Summers 2005), which lacks the activity of glucose-6-phosphate dehydrogenase, the first enzyme of the oxidative pentose phosphate pathway (Summers et al. 1995). In this mutant, vegetative cells differentiate into akinetes synchronously, following dark incubation of cultures with fructose as an external carbon source. It was, therefore, chosen as a preferred strain for studies of akinete development (Argueta and Summers 2005).

The identification of an akinete marker, together with a reliable system that gives synchronous akinete differentiation allowed the application of high-throughput technology to study akinete formation in cyanobacteria. Argueta et al. (2006) reported the detection of three novel genes involved in akinete differentiation. These genes were detected by differential display and confirmed with quantitative RT-PCR and promoter fusions to a reporter gene (GFP) to demonstrate cell-type-specific gene expression. The genes were designated (a) *aet* (Npun_F0062) an akinete expressed transporter encoding an ABC transporter with high similarity to the *E. coli* MsbA a lipopolysaccharide transporter. (b) *aapN* (Npun_F5999) – an akinete aminopeptidase belonging to the M28 peptidase family. (c) *hap* (Npun_R4070) a hormogonium/akinete-expressed protease homologous to the β-subunit group of the M16 zinc-dependent proteases complex.

Sequencing of the *N. punctiforme* genome allowed the production of an open reading frame (ORF) microarray. This was then used to compare the global gene expression of *N. punctiforme* cultures with heterocyst and during the differentiation of hormogonia and *zwf* akinetes (Campbell et al. 2007). In that study, a single time point, 3 days into the akinete differentiation process was tested. During that time window, 255 genes were up-regulated, 41% of which encoded for characterized proteins. The global gene expression 3 days after induction, showed an increase in transcript levels of four transcription regulators: two transcription factors members of the Crp family and two sigma factors. There was an increase in genes involved in cell envelope metabolism, such as *amiC*, encoding for an enzyme that biodegrades peptidoglycan linker bonds. The expression of *nblA* gene that encodes for a phycobilisome degradation protein increased as well (Campbell et al. 2007). It is postulated that the increase in the expression of *nblA* facilitates the degradation of phycobilisome antenna in maturating akinetes as reported by Sukenik et al (2007). The *avaK* orthologous gene encoding the akinete marker was up-regulated as well in a 3-days-old akinete induced culture of *N. punctiforme* (Campbell et al. 2007). *A. ovalisporum* genes orthologous to *avaK*, *aet*, and *nblA* were highly expressed in isolated akinetes as compared to their expression level in vegetative cells of an exponentially grown culture as shown by a semiquantitative RT-PCR experiment (Fig. 2.3). These results are consistent with the expression of *avaK* in *A. variabilis* (Zhou and Wolk 2002), with *N. punctiforme* differential display results for *aet* (Argueta et al. 2006), and with microarray results from *zwf* akinetes for *nblA* and *avaK* (Campbell et al. 2007).

Transcript levels of *patA* and the CHF class protease – *hetF* genes increased during heterocyst differentiation. Transcript levels of both genes were also increased in the akinete-forming culture (Campbell et al. 2007), suggesting a common regulatory pathway for differentiation of these two cell types. During heterocyst development, the expression pattern of the cell differentiation regulatory protein *hetR* was

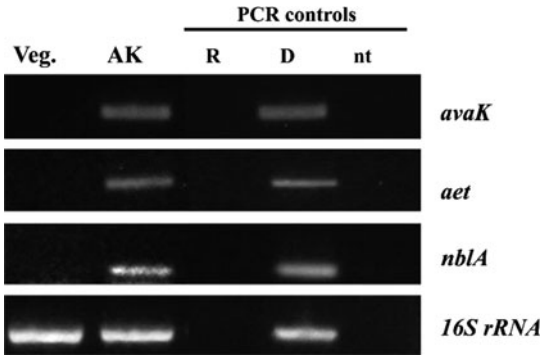

Fig. 2.3 SQ-RT-PCR (semiquantitative reverse transcriptase-PCR) of akinete marker genes in *Aphanizomenon ovalisporum* using specific primers for the *A. ovalisporum* orthologous genes to *avaK*, *aet*, and *nblA*. RNA was extracted from an exponentially grown culture lacking akinetes (Veg.) and from isolated akinetes (AK). Negative controls contained only RNA as template (R) or no template (nt). The positive control contained genomic DNA (D) as template in the PCR reaction

similar to that of *ntcA*, suggesting a mutual dependency in the expression of these two genes (Muro-Pastor et al. 2002). HetF was found to be essential for proper regulation of HetR both in the transcription and posttranslational level (Risser and Callahan 2008). The gene *hetF* is constitutively expressed in both vegetative cells and heterocysts (Wong and Meeks 2001). In heterocysts, PatA facilitates HetF activity to regulate the levels of HetR in an unknown manner (Risser and Callahan 2008). Interestingly, in *A. ovalisporum* cultures induced to form akinetes, the expression of *patA* was observed only after 3 weeks of induction, and its transcript was preferentially found in mature akinetes (Kaplan-Levy unpublished). *devR* encodes a small protein similar to the receiver domain of two-component regulatory systems that was implicated in heterocyst cell envelope formation and required for normal nitrogen fixation in *N. punctiforme* (Campbell et al. 1996). The presence of a complementing *devR* gene on a multicopy plasmid resulted in induction of akinete formation under noninducing conditions, implicating it in a phosphorelay system involved directly, or indirectly, through crosstalk, with development of heterocysts and akinetes. In akinete induced cultures of *A. ovalisporum*, the transcript levels of *devR* increased in a similar manner to that of *hetR* and *hetF*, with high levels in the isolated akinetes (Kaplan-Levy unpublished). It is suggested that the HetR regulatory pathway is involved also in the akinete differentiation process. However, unlike in heterocysts differentiation, we postulate that this pathway is activated at later stages of akinete differentiation, leading to akinete maturation. In heterocysts, the HetR pathway leads to activation of several processes (a) the synthesis of a polysaccharide envelope a process in which DevR is implicated; (b) deposition of a glycolipid layer (possibly via expression of *aet*); (c) cell division is stopped; and (d) cessation of oxygenic photosynthetic activity (Zhao and Wolk 2007). The first three processes are also essential for the formation and maturation of akinetes.

2.8 Similarity of Akinetes to Dormant Forms of Other Prokaryotes

Other types of prokaryotes form specialized differentiated resting cells in response to nutritional stress. These cells display less metabolic activity than their vegetative counterparts and do not divide. As is found for akinetes, differentiated resting cells are commonly more resistant to environmental stress, and exhibit an altered morphology relative to vegetative cells.

Endospores, so termed due to spore formation within an existing cell, are among the most resistant dormant cells. They are commonly found among the gram-positive *Bacillus*, *Clostridia*, and the thermophilic genus *Thermoactinomyces* (Cross 1968). Endospore development begins with an asymmetric septation within a single cell. The larger compartment, destined to become the "mother cell," engulfs the smaller cell destined to become the endospore. Each cell type contributes materials to the endospore envelope to create a thickened multilayered protective envelope, while the nucleoid of the endospore is condensed and

protected by interactions with newly synthesized proteins and compounds. Lysis of the mother cell releases the mature endospore, which is resistant to boiling, radiation, and chemical attack (Setlow 2000). Akinetes do not undergo internal septation and engulfment, instead create the dormant form by deposition of additional protective layers around an existing cell. Endospores exhibit no detectable metabolism or ATP, a characteristic that also separates them from akinetes, and spores of streptomyces and myxobacteria (Setlow 2000). The timing and gene regulation involved in septation, engulfment, and deposition of endospore envelope layers between the mother cell and developing endospore has been extensively studied, and used as a model for comparison with other prokaryotic developmental systems. It is controlled by the sequential action of different compartment-specific sigma factors and signaling by two-component regulatory systems (Kroos 2007).

Another type of dormant cells are cysts, such as those formed by *Azotobacter* and *Rhodospirillum*. In *Azotobacter vinelandii*, the differentiating cell accumulates poly-β-hydroxybutyrate (PHB) and forms a large sphere surrounded by a thick multilayered covering consisting of an inner layer containing carbohydrates and lipids, and an outer layer composed of lipopolysaccharides and lipoproteins (Pope and Wyss 1970). Like akinetes, *A. vinelandii* cysts are minimally resistant to heat, are resistant to desiccation, and can be observed to germinate from ruptured cyst envelopes (Socilifsky and Wyss 1962). Akinetes also accumulate storage material, albeit in the form of glycogen and cyanophycin. Cysts of the anoxygenic photosynthetic bacterium *Rhodospirillum centenum* contain multiple cells per cyst, but show many similarities to cyst formation in *A. vinelandii* (Berleman and Bauer 2004).

Streptomyces and myxobacteria also contain well-studied examples of bacteria that differentiate into spores. Streptomyces are the most complex type of gram-positive actinomycetes that grow as a mycelium of branching hyphal filaments. The best studied is *Streptomyces coelicolor* that produces a series of aerial spores from long hyphae growing up from the colony upon nutrient depletion, similar to the sporulation and dispersal strategy used by molds (Wildermuth 1970). Although superficial similarity exists between *S. coelicolor* spore formation and that of akinetes in strains exhibiting contiguous stretches of maturing akinetes within a filament, cyanobacterial akinete formation does not physically resemble this process.

In gram-negative myxobacteria such as *Myxococcus xanthus*, large numbers of spores are formed within fruiting bodies. Fruiting body formation occurs on solid substrates when large numbers of motile myxobacteria sense a nutritional downshift (Dworkin 1996). By comparison, cyanobacterial akinetes form individually within non-motile filaments and are not enclosed in a larger structure. Any cell–cell signaling would be limited to adjacent cells and those in close proximity on other filaments. In myxobacteria, only a small proportion of cells is destined to become spores in a fruiting body, whereas in some cyanobacteria as differentiation proceeds down a filament, all the vegetative cells can eventually convert to akinetes (e.g., *Nostoc* strains). In other strains, differentiation into akinetes occurs not progressively along a filament but simultaneously along long sequences of cells (Sarma and Khattar 1993). Akinetes are similar to spores of *Streptomyces* and *Myxococcus*, but unlike endospores they are not resistance to extreme heat (Setlow 2000).

Both *M. xanthus* and *Streptomyces* spores contain large amounts of the disaccharide trehalose, which has been implicated in their desiccation protection (Cruze-Martin et al. 1989; McBride and Ensign 1987). Trehalose and sucrose have been shown to be induced in cyanobacteria found in desert crusts (Hershkovitz et al. 1991). Extracellular polysaccharides of desiccation-resistant cyanobacteria in combination with trehalose or sucrose have been shown to stabilize membrane structure (Hill et al. 1997), which could account for an alternative mechanism of desiccation resistance for these sugars, in addition to their role as "chemical chaperones" within the cytoplasm (Crowe et al. 1998). A smaller but significant induction of sucrose by desiccation stress was found in *Anabaena* 7120, although very little trehalose accumulation was observed (Higo et al. 2006), indicating a plausible explanation for the range of desiccation resistance observed among cyanobacteria. The increased amount of polysaccharides in the envelope of akinetes (Cardemil and Wolk 1981; Wolk et al. 1994) could play a role similar to the external polysaccharides of desiccation-resistant cyanobacterial species; however, the presence and role of these polymers remain largely unexplored.

As in *Bacillus* endospore formation, regulation of sporulation in streptomyces and myxobacteria has been linked to regulatory cascades that include interactions between sigma factors and members of two-component regulatory systems (Chater 2000; Kroos 2007). Research into the regulation of akinete formation is still in its infancy. However, the identification of two alternative sigma factors and a subset of two-component regulatory systems up-regulated in *zwf* akinetes (Campbell et al. 2007) provide hints that similar regulatory cascades may be involved in akinete formation.

2.9 Conclusions and Future Prospects

Differentiation of vegetative cells to dormant forms (akinetes) in cyanobacteria that belong to the orders Nostocales and Stigonematales, and the role of akinetes in the life history and the success of cyanobacteria in nature have been studied since 1856 (see Herdman 1987). Structural changes and metabolic variations during akinete formation and maturation were described for a wide range of species and strains. Nevertheless, understanding of mechanisms that trigger akinete formation via the conversion of a vegetative cell within a filament into a resting cell was hindered until recently. Identification of environmental triggers that induce akinete formation, the availability of mutants that form akinetes under well-defined conditions and above all, development of advanced genomic tools that have been implemented to study the regulation and differentiation of cyanobacteria, have allowed progress in understanding the akinete differentiation process. Significant progress in analysis of mechanisms that lead to akinete maturation has been made recently. Further identification of signal perception and transduction as well as characterization of cellular processes and metabolic activities leading to the formation of akinetes are expected in the near future.

Numerous genes, including regulatory genes that are involved specifically in maturation have been identified, and microarray experiments have demonstrated that many genes are activated at different times during akinete induction and maturation. The involvement of regulatory cascade and transcription factors, primarily associated with the formation of heterocysts, were also identified in the akinete induction process. These findings clearly support an early notion (Wolk et al. 1994) that heterocysts may have evolved from akinetes.

Implementation of various molecular techniques and data from fully sequenced genomes of several Nostocales species ensure a rapid advancement toward a better understanding of the dormancy phenomenon in cyanobacteria. Akinete transcriptomic, proteomic, and metabolomic data is rapidly accumulating and the mechanism of dormancy in cyanobacteria is emerging as a heterogeneous process, as has been found in other prokaryotes, protists, and higher organisms. However, many questions are yet to be resolved: How are external signals that initiate germination perceived by an akinete and how are they processed to resume a fully active dividing vegetative cell? What are the factors that determine which vegetative cells along a filament will differentiate into akinetes? How are cellular and regulatory processes integrated into the environmental phenomenon of seasonal repetitive blooms? And finally, can we learn from akinete formation and dormancy processes about long-term preservation of eukaryotic cells under permissive temperatures and other environmental conditions?

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