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ORIGINAL PAPER

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Characterization of a model system for the study of *Nostoc punctiforme* akinetes

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Abstract *Nostoc punctiforme* is a filamentous cyanobacterium that is capable of dark heterotrophy and cellular differentiation into nitrogen-fixing heterocysts, motile hormogonia, or spore-like akinetes. The study of akinete differentiation at the molecular level has been limited by the asynchronous development and limited number of akinetes formed within a filament. A system in which to study the development and genetic regulation of akinetes was investigated using a *zwf* mutant lacking glucose-6-phosphate dehydrogenase, the initial enzyme of the oxidative pentose phosphate pathway. Upon dark incubation in the presence of fructose, the *zwf*$^-$ strain ceased growth and differentiated into akinete-like cells, whereas the wild-type strain exhibited heterotrophic growth. Dark-induced *zwf* akinetes exhibited periodic acid–Schiff staining characteristics identical to that observed for wild-type akinetes, and synchronous induction of akinetes occurred in treated cultures. Dark-induced *zwf* akinetes exhibited increased resistance to the environmental stresses of desiccation, cold, or treatment with lysozyme relative to vegetative cells of both strains. Transcription of the *avaK* akinete marker gene was strongly induced in developing *zwf* akinetes as shown by Northern blotting and green fluorescent protein transcriptional reporter fusions. ATP levels did not vary significantly between dark incubated strains, indicating that a signal other than energy level may trigger akinete formation. This phenotypic and genetic evidence showing near-synchronous induction of dark-induced *zwf* akinetes indicates that this system will provide a valuable tool for the molecular genetic study of akinete development in *N. punctiforme*.

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Introduction

Nostoc punctiforme is a filamentous cyanobacterium that grows by oxygenic photosynthesis in the light and is capable of switching to a heterotrophic mode of growth in the dark using simple carbohydrates. This strain can also adapt to a range of environmental changes by undergoing three types of cellular differentiation. Lack of combined nitrogen in the medium triggers the formation of nitrogen-fixing heterocysts, regularly spaced along the filament, that provide fixed nitrogen for vegetative cell growth. Perturbations in normal growth conditions can trigger the formation of hormogonial filaments that exhibit gliding motility, likely used to escape harm and promote dispersal in nature. The third type of differentiated cell is the spore-like akinete, which forms under low intensities of light and in stationary cultures still rich in inorganic nutrients (Meeks et al. 2002).

Akinetes are more resistant to environmental stress than are vegetative cells and can withstand long periods of cold or desiccation, but are not as heat resistant as are the endospores of Gram-positive bacteria (Adams and Duggan 1999). The composition of the external akinete polysaccharide layer varies between strains, but has a structure similar to that found in the heterocysts of the same strain in both *Anabaena cylindrica* (Cardemil and Wolk 1979) and *Anabaena variabilis* (Cardemil and Wolk 1981). This similarity is not always conserved, as shown by the starkly different polysaccharide layer from akinetes and heterocysts of *Cylindrospermum licheniforme* (Cardemil and Wolk 1981). Akinetes have also been reported to contain glycolipids characteristic of heterocysts (Soriente et al. 1993).

Internal stores of glycogen and cyanophycin accumulate during akinete formation, and low rates of

photosynthesis and metabolism are maintained (Thiel and Wolk 1983). Akinetes are thought to play a role in the survival from season to season, and may be used as a mechanism to assure long-term survival of the species. In the laboratory, asynchronous akinete formation can be induced by phosphate limitation, with the first akinetes appearing between 7 days and 14 days, following phosphate starvation. The first akinetes in a filament appear midway between heterocysts in *N. punctiforme*, followed by sequential development in both directions toward the heterocysts. If no heterocysts are present, akinetes appear randomly throughout a filament (Meeks et al. 2002).

The *N. punctiforme* genome has been sequenced, and a wide range of genetic tools is available for the study of cell differentiation in this strain (Summers et al. 1995; Cohen et al. 1998; Argueta et al. 2004). However, due to the asynchronous formation of akinetes in a filament, the molecular genetic analysis of this cellular differentiation process has lagged behind that of heterocyst development. Only one akinete marker gene of unknown function, *avaK*, has been identified by using the sequence of a highly expressed protein found in akinetes of *A. cylindrica* (Zhou and Wolk 2002). Several other heterocyst genes have also been found to play a role in akinete formation. The *hepA* gene product is required for correct deposition of the polysaccharide envelope in both heterocysts and akinetes in *A. variabilis*, and the loss of this gene product results in production of malformed akinetes (Leganès 1994). Increased copy numbers of *devR*, encoding a response regulator of a two-component regulatory system involved in polysaccharide biosynthesis (Zhou and Wolk 2003), results in a higher frequency of akinete formation in *N. punctiforme* (Campbell et al. 1996). Mutation of the HetR regulatory protein (Huang et al. 2004), essential for heterocyst formation, has been shown to inhibit granular akinete formation in *Nostoc ellipsosporum* according to visual inspection (Leganés et al. 1994), but does not result in loss of cold-resistance in non-granular akinete-like cells of *N. punctiforme* (Wong and Meeks 2002). These common genetic components support the hypothesis that the developmental processes of akinete and heterocyst formation are related (Wolk et al. 1994).

The aim of this work is to accelerate the molecular genetic analysis of akinete development by demonstrating the utility of a *N. punctiforme* model system for the study of akinete formation. We demonstrate that a *zwf* mutant that lacks the first enzyme, glucose 6-phosphate dehydrogenase (Hagen and Meeks 2001), of the oxidative pentose phosphate pathway (OPP) can be used to study akinete development. Cyanobacteria utilize the OPP pathway extensively in the dark to produce reducing power for growth (Summers et al. 1995). The biochemical and genetic data presented here indicate that a *zwf* mutant forms functional akinetes upon dark incubation in the presence of fructose.

Materials and methods

Strains and culture conditions

N. punctiforme ATCC 29133 and the *zwf* mutant UCD 466 (Hagen and Meeks 2001) were grown photoautotrophically in 125-ml Erlenmeyer flasks containing 50 ml of a fourfold dilution of the nitrogen-free medium (AA medium) described by Allen and Arnon (1955), supplemented with 5 m *M* MOPS buffer (pH 7.5), 2.5 m *M* NH_4Cl, 2.5 m *M* $NaNO_3$, and 2.5 m *M* KNO_3 plus, for plasmid selection, 10 μg ampicillin ml^{-1}. AA medium was solidified with 1% Noble agar for plating. Growth under mixotrophic (fructose in the light) and dark heterotrophic growth conditions was achieved by the addition 50 m *M* fructose to the liquid medium. Cultures were incubated at 25°C on a rotary shaker (120 rpm) with 17–19 μmol photons m^{-2} s^{-1}. Dark conditions were maintained by covering flasks with foil and incubated at room temperature (21–23°C) on a shaker (40 rpm).

Survival experiments

Cultures were cavitated to a mean of approximately two to four cells in length, using a microtip probe of a Daigger GEX600 ultrasonic processor, and allowed to recover in the light for 1 day before darkening. Because experiments using cavitated starting cultures gave similar results to those using uncavitated cultures, but with reduced standard errors, cavitated starting cultures were used in all experiments. To determine colony-forming units (CFUs) in a sample, dilutions of cultures at each time point were spread on solidified media and incubated under photoautotrophic growth conditions. To determine survival after exposure to harsh conditions, CFUs were similarly determined at each time point after subjecting samples of cultures to desiccation, cold, or lysozyme treatment. CFU values obtained after treatment were divided by those obtained before treatment to determine percent survival at each time point. To challenge cultures with harsh environmental conditions, three 1-ml aliquots of cultures were removed at each time point and centrifuged for 5 min at 13,000 *g*. After removal of the supernatant solution, one of the cell pellets was desiccated by incubating overnight at 37°C. Desiccated cells were rehydrated in 1 ml sterile distilled water for 1 h before plating. Another cell pellet was suspended in 1 ml sterile distilled water and placed in the dark at 4°C for 14 days. The third pellet was suspended in 1 ml sterile distilled water containing lysozyme (150 μg/ml) and incubated for 16 h in the dark at room temperature. Lysozyme-treated cells were washed twice with sterile distilled water before dilution and plating. Each filament is defined as a CFU, and filaments averaged 3.4 ± 0.4 ($\pm$ standard error) cells before treatment.

Clumping of these short filaments was not observed. Cells/CFUs were similar before and after treatment within a strain, with the exception of severe filament-length reduction in the wild-type strain, following lysozyme treatment due to cell lysis.

Molecular methods

RNA extraction and Northern blotting were performed as described previously (Summers et al. 1995) from cultures pre-grown under photoautotrophy or mixotrophy for at least four generations, or from mixotrophic cultures at various times following darkening. Probes were generated by random primer biotin labeling (NEN Life Science Products) of a 0.9-kb PCR fragment of *N. punctiforme avaK* generated using primers AATGGCACTGTCAGCGATGT and AGCTATTGGAAGCACAGGTA. Blot hybridization and washing were performed according to the manufacturer's instructions (GeneScreen Plus, NEN). The *avaK*–green fluorescent protein (GFP) reporter vector pSUN8 (Argueta et al. 2004) was transformed into *N. punctiforme* wild-type and UCD 466 strains using electroporation (Summers et al. 1995) to create reporting strains CSUN 100 and CSUN 101, respectively. Hybridization signals and RNA levels in Northern blot analysis were normalized to total ribosomal RNA and corrected for local background using pixel values obtained from the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Purification of akinetes

Akinetes were induced by transferring early stationary phase cultures into an equal volume of AA/4 containing buffered ammonia and nitrate but lacking inorganic phosphate, and incubating for 8–12 weeks at room temperature without shaking under constant illumination at 8–10 μmol photons m^{-2} s^{-1}. Cells were harvested by centrifugation, suspended in an equal volume of sterile distilled water, and incubated overnight with 300 μg/ml lysozyme in the dark at room temperature to lyse remaining vegetative cells. Treated akinetes were washed two times in sterile distilled H_2O before use or stored in sterile water at 4°C.

Staining and microscopy

For the periodic acid–Schiff (PAS) reaction, equal volumes of culture and 1% periodic acid in absolute ethanol were mixed and incubated at room temperature for 5 min. Following centrifugation, the cell pellet was suspended in 20 μl Schiff's reagent (1% basic fuchsin, 1.9% sodium metabisulfite, 15% 1 N HCl) and incubated as before. Following re-centrifugation, the cell pellet was suspended in 0.5% sodium metabisulfite and viewed by bright-field microscopy. Images were obtained using a Nikon Eclipse TE 3000 microscope containing a ×100 oil immersion objective and captured with a Sony DXC-970MD color video camera using Metamorph Imaging, series 5.0, software.

ATP extraction

Exponential mixotrophic cultures of the wild-type and UCD 466 strains were placed in the dark and sampled daily by filtration of 4-ml aliquots through a 25-mm diameter nitrocellulose filter (1.2-μm pore size). The filter and cells were vortexed in 1.27 ml cold 1.4 *M* perchloric acid, 20 m *M* ethylenediaminetetraacetic acid to extract the ATP. Following 30 min incubation on ice, the extract was neutralized by the addition of 0.73 ml 2.5 *M* KOH, 0.2 *M* Tris-base, 85 m *M* K_2SO_4, and stored at −20°C. Samples were diluted at least 1:5 in 20 m *M* $MgSO_4$, 20 m *M* Tris-HCl (pH 7.5), and assayed in a Turner Designs model TD 20/20 luminometer using firefly luciferase (Promega) with an internal standard as per the manufacturer's instructions. Total ATP per sample was normalized to total chlorophyll *a* determined by extraction with 90% methanol (Meeks et al. 1983).

Fluorometry

Aliquots of *avaK*–GFP reporter strains CSUN 100 (in a wild-type background) and CSUN 101 (in a *zwf* background) grown photoautotrophically, mixotrophically, or at various times after a shift to dark heterotrophic growth conditions, were removed from cultures, briefly cavitated to reduce clumping, and washed once in fresh medium before whole-cell GFP assay. Relative changes in fluorescence between strains were obtained from raw fluorescence units obtained from a Turner Design Model TD700 fluorometer calibrated to a solid green standard (Turner Designs, no. 7000-992). Fluorescence was normalized to total chlorophyll extracted (Meeks et al. 1983) from the washed sample. Normalized natural background fluorescence of wild-type and mutant strains at each time point and incubation condition were subtracted from values of the corresponding plasmid-bearing strains. The natural fluorescence of the wild type (5.5 ± 0.3 raw fluorescence units/μg Chla for fructose; 9.9 ± 0.7, mannitol) and UCD 466 mutant (5.5 ± 0.4, fructose; 7.1 ± 0.5, mannitol) strains varied little throughout the course of the experiment. Fluorescence of the wild-type strain bearing the pSUN202 reporter-plasmid (Argueta et al. 2004) lacking a promoter-bearing insert fluoresced (4.8 ± 0.4, fructose; 6.5 ± 0.3, mannitol) at levels similar to that of strains lacking this plasmid.

Results

Survival of non-growing *zwf* mutant in dark heterotrophic conditions

An experiment was conducted to quantitate the viability of wild-type and UCD 466 strains following a switch to the dark (Fig. 1). Without addition of an exogenous carbohydrate carbon source, the wild-type strain was capable of one to two doublings, using internal carbon reserves, whereas the mutant strain did not exhibit such growth. Although both strains showed decreased viability after 4 days in the dark, the mutant strain was more severely affected, and survival decreased to less than 1% after 8 days in the dark. The wild-type strain followed a similar extent of viability loss, with the decline lagging approximately 1–2 days behind that of the mutant strain.

When fructose was present in the medium as an external carbon source, the wild-type strain exhibited dark heterotrophic growth with a doubling time, based on assays of chlorophyll *a*, of 30 h. The *zwf* mutant strain was incapable of dark heterotrophic growth; however, filament viability remained near starting levels for the duration of the experiment.

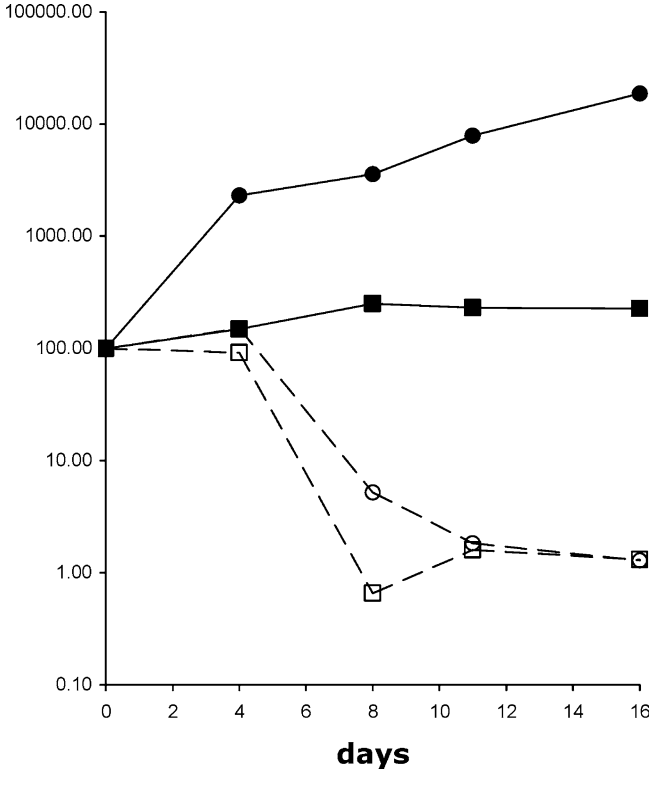

Fig. 1 Survival of *Nostoc punctiforme* and UCD 466 after dark incubation. Cultures of wild-type (*circles*) and UCD 466 (*squares*) strains were grown with (*closed symbols*) or without (*open symbols*) 50 m *M* fructose in the medium and placed in the dark at time 0. Results shown are from a single experiment and plotted as a percent of viable colony-forming units (CFUs) present at the start of dark incubation. The standard error was less than 10% for all CFU determinations

Staining of akinetes

Microscopic examination of cultures containing fructose indicated that although cells from both cultures appeared identical at the start of the experiment, the *zwf* mutant strain formed larger cells resembling akinetes that were discernable at 6 days following darkening (compare Fig. 2c, d). To determine if the larger cells had a polysaccharide layer similar to that found in akinetes, methods for staining of akinete exopolysaccharides were evaluated.

Alcian blue has been shown to stain the outer exopolysaccharide layer of heterocysts of a similar cyanobacterial strain (Gantar et al. 1995; Liu and Golden 2002). However, this strain only lightly stained the outer layer of heterocysts and did not stain akinetes of *N. punctiforme* (data not shown). The PAS reaction resulted in staining of both wild-type akinetes and heterocysts of *N. punctiforme*, but not photoautotrophically grown vegetative cells (Fig. 2e, f). Vegetative cells of the wild-type strain grown mixotrophically (Fig. 2a), or heterotrophically (Fig. 2c) also did not exhibit exopolysaccharide staining. When the *zwf* and wild-type strains were grown under the same conditions, both stained similarly when cultured photoautotrophically (not shown) or mixotrophically (Fig. 2b), but the former strain, unlike the latter, exhibited polysaccharide staining by day 6 of dark incubation under heterotrophic conditions. The intensity of polysaccharide staining did not increase in the *zwf* strain upon further incubation in the dark, and was similar to the staining exhibited by mature wild-type akinetes. Experiments to track the time course of polysaccharide formation in UCD 466 following a shift to dark heterotrophic growth conditions indicated PAS staining was detectable by day 4, and gradually increased to the maximum staining level by day 6 (data not shown). Greater than 95% of all *zwf* strain cells exhibited staining by day 6 in the dark.

Resistance of *N. punctiforme* and UCD 466 to harsh treatment

Preliminary screening to compare the resistance of *N. punctiforme* vegetative cells to that of purified wild-type akinetes was conducted by observing the growth of cells spotted as dilutions on agar plates following treatment with a variety of environmental stresses. The results indicated that survival following exposure to desiccation, cold, and prolonged exposure to lysozyme was similar to that of untreated controls only for purified akinetes. Exposure to UV or heat did not result in significant differences in survival between treated and untreated controls of either akinetes or vegetative cells (data not shown).

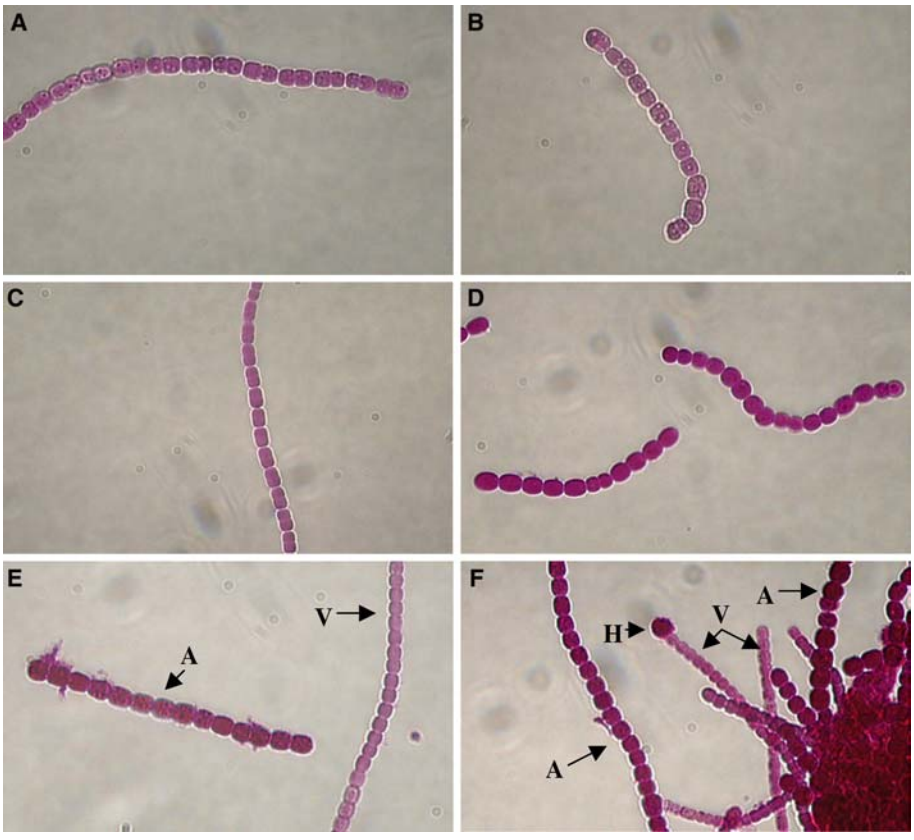

Fig. 2 Periodic acid–Schiff-stained strains of *N. punctiforme*. Wild-type (**a**) and UCD 466 (**b**) strains under mixotrophic growth conditions. Wild-type (**c**) and UCD 466 (**d**) strains under heterotrophic conditions. **e**, **f** Wild-type vegetative cells grown under photoautotrophic growth conditions mixed with wild-type akinetes induced by phosphate limitation and then stained together. *V* Vegetative cell, *A* akinete, *H* heterocyst

To determine the kinetics of dark-induced akinete formation by our model system, samples taken from fructose-grown cultures of wild-type and UCD 466 strains were tested at various times following darkening for resistance to desiccation, cold, and lysozyme treatment. Preliminary screening results were quantified using plate counts of culture dilutions to measure changes in viability following treatment. The results indicate that UCD 466 samples subjected to overnight desiccation at 37°C exhibited increased viability (74% survival) within 3 days following dark induction of akinetes, whereas survival of the wild-type strain fell to less than 15% after 3 days (Fig. 3a). Desiccation survival was maintained above 15% by the UCD 466 strain throughout the remainder of the experiment, whereas survival of the wild-type strain declined from 16% at day 3 to 1% by the end of the experiment. The wild-type and UCD 466 strains showed similar resistance to cold over the first 10 days following darkening. Thereafter, the UCD 466 dark-induced akinetes developed continued to maintain cold resistance, whereas the survival of the wild-type strain declined to 9% after day 10 (Fig. 3b). Overnight treatment with 150 μg/ml of lysozyme was sufficient to kill vegetative cells of the wild-type strain under all growth conditions (Fig. 3c). The mutant strain was unable to survive immediately upon darkening, but survival increased to 10% after 6 days of induction. The increased survival of UCD 466 continued near this higher level for the duration of the experiment, whereas the wild-type strain never gained resistance to lysozyme. The level of resistance demonstrated by UCD 466 dark-induced akinetes for all three treatments was similar to that of wild-type akinetes, which, in preliminary experiments, survived and grew when diluted and spotted after being identically stressed.

Akinete-marker gene expression correlates with stress resistance

The *N. punctiforme* genome encodes a homologue of the AvaK akinete marker protein (gene *NpF5452*) that is 72% identical and 84% similar to that identified in *A. variabilis*. This gene was PCR-amplified from the *N. punctiforme* genome and used as the probe in Northern analysis to test if the UCD 466 resistant cells expressed an RNA characteristics of akinetes. The blots contained RNA from the wild-type and UCD 466 strains harvested photoautotrophically, mixotrophically, and under various times, following a switch to dark heterotrophic conditions (Fig. 4). Results indicate that an approximately 0.9-kb *avaK* transcript is expressed by both strains at low levels under photoautotrophic conditions, and is induced under mixotrophic conditions. Following darkening, transcription decreases slowly over 24 h in the wild-type strain, and is completely absent in UCD 466. By 48 h, *avaK* transcription resumes in both strains. After day 2, constant expression was exhibited by the wild type, whereas transcription of *avaK* is strongly induced in the mutant strain starting at day 2,

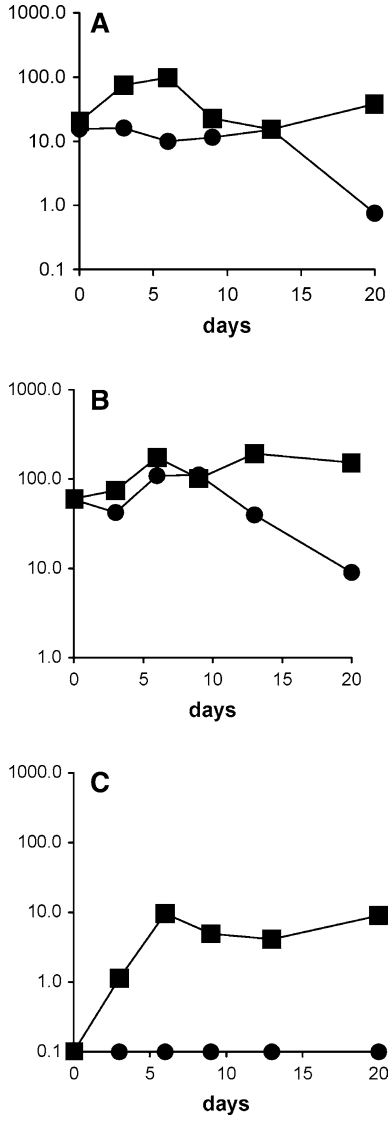

Fig. 3 Resistance of wild-type *N. punctiforme* (*filled circles*) and UCD 466 (*filled squares*) to desiccation (**a**), cold (**b**), and lysozyme (**c**) after various times of dark incubation. Cultures were grown with 50 m *M* fructose in the medium and placed in the dark at time 0. At various times thereafter, samples were removed and subjected to the indicated stresses. Graphs show percent surviving CFUs after treatment relative to those in an unstressed control for each time point. From 0% to 0.1% survival is indicated on the logarithmic scale as 0.1%. Results show the means from duplicate experiments. Standard error was less than 10% for all CFU determinations

and continues at a higher level than that observed in the wild-type strain through day 6.

The plasmid pSUN8 containing the *avaK* promoter driving expression of a promoterless *gfp* transcriptional reporter was demonstrated to successfully report akinete-specific gene expression in the wild-type strain (Argueta et al. 2004). This plasmid was placed into the wild-type and UCD 466 strains and used as an independent measure to compare *avaK* gene expression following a dark shift. Strain-specific autofluorescence was subtracted from all reporter measurements to correct for fluorescence arising from natural pigments. The background autofluorescence was small, showed little variation over the course of the experiment for either strain, and did not increase due to the presence of a promoterless control plasmid.

When the fructose-grown wild-type reporter strain CSUN 100 underwent a dark shift, a small increase in GFP production was observed, peaking at day 2, that was not present in an equimolar mannitol osmotic control. In the fructose-grown mutant reporter strain CSUN 101, GFP expression decreased for the first 2 days, followed by a large increase in gene expression that reached a maximum level at day 6. Similar results were not observed for the mannitol control by either strain, indicating that the early transient induction by the wild type, or later large induction by the mutant is dependent upon the presence of a metabolisable sugar. There is general agreement between the GFP results (Fig. 5) and the Northern blot results (Fig. 4) for the wild-type and mutant strains, with an approximate 0.5- to 1-day lag time for expression of the GFP reporter. The magnitude of transcript differences observed by Northern blotting are muted when assayed by the GFP reporter. For instance, the twofold difference in *avaK*–GFP reporter values for the mutant strain growing photoautotrophically (4.7 ± 0.8 raw fluorescence units/μg Chla) versus mixotrophically (15.8 ± 0.7 shown at time 0, Fig. 5) was much less than the over tenfold difference observed by transcript measurement by Northern blot analysis. Similarly, the 2.5-fold difference in the wild-type strain growing photoautotrophically (4.3 ± 0.8) relative to mixotrophically (10.9 ± 0.4) was less than the fourfold difference observed by Northern blot analysis.

ATP comparison between strains

The UCD 466 strain lacks glucose-6-phosphate dehydrogenase and lacks the ability to produce large amounts of reductant via the OPP pathway that cyanobacteria normally utilize for carbon catabolism. The resulting loss of reducing power has been hypothesized to lower ATP levels due to reduced electron flow through the electron transport chain and resulting proton gradient, causing reduced ATP synthase activity (Summers et al. 1995). To see if lower ATP levels correlate with induction of akinetes in UCD 466, ATP levels were assayed in each strain after a dark shift. The results indicated no significant difference in ATP levels between the mutant and wild-type strains in the light, or for 6 days following darkening (Fig. 6).

Discussion

Several lines of evidence indicate that the UCD 466 strain forms akinetes following a switch from mixotrophic to dark heterotrophic conditions. The Alcian blue staining technique, specific for carbohydrates with

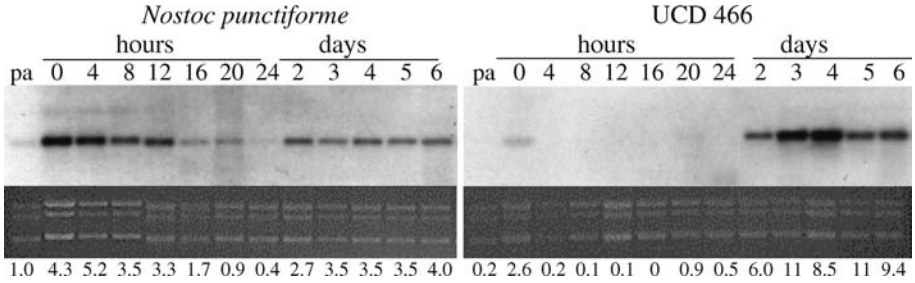

Fig. 4 *avaK* gene expression in wild type and UCD 466. Northern blots containing RNA samples from cultures pre-grown photoautotrophically (*pa*), mixotrophically (time 0), or at various times after switching mixotrophically grown cultures to the dark were probed with *avaK* from *N. punctiforme*. The ribosomal RNA-containing portions of ethidium bromide-stained gels used to generate blots are shown *below the hybridized blot*. Relative *avaK* hybridization values normalized to total RNA are shown *below each lane*

acidic groups (Humason 1967), stained the outer layer of heterocysts but not akinetes of *N. punctiforme*. These results indicate that unlike the identical external polysaccharide structure found in akinetes and heterocysts of *Anabaena* spp. (Cardemil and Wolk 1979, 1981), there may be different polysaccharides associated with each cell type in *N. punctiforme* as has been found for *C. licheniforme* (Cardemil and Wolk 1981). The PAS reaction was able to stain both akinetes and heterocysts successfully. The PAS reaction first breaks and oxidizes carbohydrate α-amino alcohol and/or 1,2 glycol groups of complex carbohydrates to aldehydes using periodic acid, followed by reaction of the aldehyde groups with Schiff reagent to form a purple-red color (Humason 1967). The PAS stain did not react strongly with vegetative cells of either strain when grown in the presence of fructose in the light, implying that akinete polysaccharides are not produced as a result of growth in fructose. Staining of the *zwf* mutant under dark heterotrophy was positive using the PAS technique, but negative using the Alcian blue technique, indicating that the polysaccharide present was similar to that found on akinetes and not on heterocysts. The timing of polysaccharide deposition

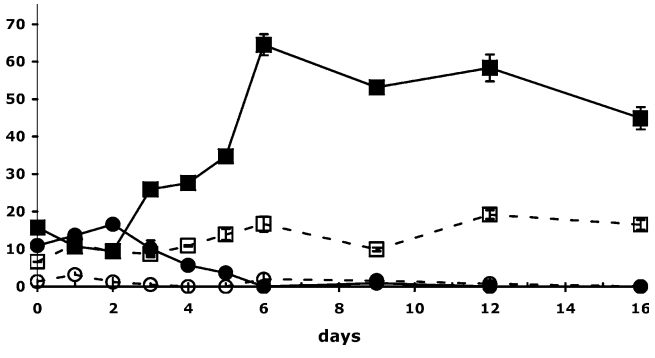

Fig. 5 *avaK*–GFP transcriptional reporter expression in wild-type reporter strain CSUN 100 (*circles*) and UCD 466 reporter strain CSUN 101 (*squares*), following a shift to the dark in the presence of 50 m *M* fructose (*solid symbols*) or mannitol (*open symbols*). Standard error bars smaller than graph symbols may not be visible

starts before day 4 following darkening in the mutant strain, and continues at least until full staining is reached at day 6.

It is interesting to note that the alcoholic, not the aqueous, version of the PAS stain was successful in staining wild-type as well as UCD 466 dark-induced akinetes. The use of alcohol in the protocol reduces loss of complex water-soluble polysaccharides during the staining procedure, indicating that the polysaccharide layer of *N. punctiforme* akinetes may be more soluble than that found in other strains. This could explain why short filaments of germinating akinetes have not been observed emerging from a polysaccharide shell as observed for *Anabaena* sp. strain CA (Adams and Duggan 1999) or *N. spongiaeforme* (Thiel and Wolk 1983), and why akinete formation occurs more frequently when agitation of cultures is reduced or eliminated (unpublished observations).

The exopolysaccharides of *Nostoc commune* have been shown to help stabilize membrane integrity during desiccation (Hill et al. 1997), and may play a similar role in the desiccation resistance of *N. punctiforme* akinetes. Desiccation resistance of dark-induced UCD 466 akinetes, observed by day 3, preceded the earliest appearance of polysaccharides that stained with the PAS procedure by 1 day. The early acquisition of desiccation resistance may indicate that only a small amount of EPS is sufficient for desiccation resistance, or that there may be (an) additional mechanism(s) for desiccation resistance in our strain that requires less time for full expression.

Resistance to desiccation, cold, and lysozyme are gained by dark-induced UCD 466 cells by 6 days following darkening, giving further evidence of akinete formation by our model system. Cold resistance has long been a hallmark of akinete function, but the physiological mechanism or genes associated with this phenotype remains unknown. Lysozyme resistance is assumed to result from protection of the peptidoglycan cell wall by the external exopolysaccharide layer. Thickening of the cell wall has also been observed in mature akinetes of *Nostoc* PCC 7542 (Sutherland et al. 1979) and may also contribute to increased lysozyme resistance. Although it is doubtful that this selection would be found in natural environments, this treatment was included because it has been used by us, and others (Simon 1977), with success in the purification of akinetes from undifferentiated vegetative cells and is a good marker for reduced permeability associated with an intact akinete envelope.

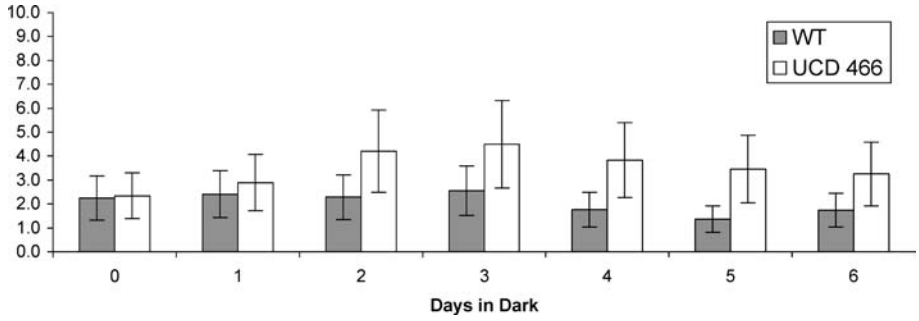

Fig. 6 ATP levels in wild-type (*WT*) and UCD 466 strains following a transfer to the dark in the presence of fructose. ATP was extracted and normalized to chlorophyll daily for 6 days. Results show the mean of triplicate samples in duplicate experiments, ± standard error

At the molecular genetic level, only a single akinete marker gene, *avaK*, has been reported to date using a proteomic approach (Zhou and Wolk 2002). The encoded protein is present in other heterocyst forming filamentous cyanobacteria, and has no known function or conserved motifs. The *N. punctiforme* homologue of this gene has been shown to be highly expressed in akinetes using a GFP fusion (Argueta et al. 2004), and the transcript size indicates that the gene is monocistronic. The Northern results indicate that *avaK* is also transcriptionally induced by fructose (Fig. 4). These results establish a regulatory link between akinete formation and processes associated with carbon metabolism or storage. The role of AvaK in akinete formation/function and growth on fructose are currently being addressed by mutant analysis in our lab. Increased expression of *avaK* at days 3 and 4 of developing UCD 466 dark-induced akinetes relative to wild type, both by Northern analysis (Fig. 4) and GFP reporter (Fig. 5), provides genetic evidence that the model system is actively forming akinetes. It is interesting to note that GFP-reporter levels in a wild-type genetic background drop to nearly undetectable levels in the reporter strain between 2 days and 6 days following darkening (Fig. 5), even though constant transcription is observed relative to rRNA (Fig. 4). We conclude from these results that the growth rate may be sufficient to dilute the GFP reporter to levels masked by the natural fluorescence of chlorophyll. An alternate interpretation may be that actively respiring wild-type cells could have internal oxygen levels below that necessary for proper GFP folding that is required for fluorescence. This may make GFP a less-attractive reporter for genes with low levels of transcription, or for future cyanobacterial studies focusing on heterotrophic growth conditions. *N. punctiforme* is unable to use mannitol as a carbon source for heterotrophic growth. Maintenance of near-starting levels of GFP in the mannitol controls indicates that GFP loss in the wild type is probably not due to degradation of GFP by proteolysis, and that the reporter increase in UCD 466 with fructose is not due simply to accumulation in non-growing cells.

Exogenous carbohydrates are required for dark growth of the wild-type strain and for dark survival of a *zwf* mutant. Internal carbon reserves are usually small under conditions of exponential, photoautotrophic growth. Under mixotrophic growth, glycogen accumulates and can be seen as a large increase of refractile granules in the cytoplasm. Either these increased carbon stores or the lower rate of metabolism caused by incubation at low temperature may explain why the cold survival of mixotrophically grown wild-type cells (Fig. 3b) is higher than that of cultures previously incubated photoautotrophically (Fig. 1; Wong and Meeks 2002). In the absence of a carbon source, the UCD 466 strain dies 1 to 2 days earlier in the dark than the wild-type strain, perhaps by using a less-efficient pathway for dark utilization of its carbon reserves. The glycolytic pathway has been shown to be utilized in cyanobacteria for dark heterotrophic growth (Yang et al. 2002), and is the most likely catabolic path for fructose degradation in a *zwf* mutant. Glycolysis would produce only half the reducing equivalents per hexose as compared to the linear flow through the OPP pathway and lower glycolytic pathway to pyruvate. ATP synthesis per glucose via substrate level phosphorylation would be equal through either linear pathway.

Low light has been a common environmental stimulus triggering akinete formation in a number of cyanobacterial strains, and low energy has been hypothesized to be a possible signal for akinete formation (Adams and Duggan 1999). Our results indicate low energy alone is not responsible, because ATP levels were similar in the mutant strain after dark shift when akinetes were forming (Fig. 6). Because the mutant strain does not grow under dark conditions in the presence of either glucose or fructose, we conclude that ATP produced by substrate level phosphorylation and use of the limited amounts of NADH in oxidative phosphorylation is sufficient to maintain wild-type levels of ATP in the mutant strain. Whereas the actual trigger for akinete differentiation remains unknown, it is interesting to speculate that a metabolic imbalance caused by the *zwf* mutation induces akinete formation in our model system. Support comes from early work on *C. licheniforme*, where addition of the amino acids deriving from the lower glycolytic pathway and tricarboxylic cycle (tryptophan, and to a lesser extent, aspartate, asparagine, phenylalanine, and serine) triggered increased akinete formation (Hirosawa and Wolk 1979). Further experimentation is required to test this hypothesis in our strain.

In summary, the phenotypic and genetic evidence showing near-synchronous induction of dark-induced

UCD 466 akinetes indicates this system will provide a valuable tool for the molecular genetic study of akinete development in *N. punctiforme*.

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