

Identification of developmentally regulated proteins in cyanobacterial hormogonia using a proteomic approach

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Abstract

A transient stage in the lifecycle of the cyanobacterial genus *Nostoc* is the development of motile hormogonium filaments, which also function as infection units in cyanobacterial-plant symbioses. As the molecular machinery under-pinning this developmental process is largely unknown, a proteomic study was initiated utilizing 2-D gel electrophoresis coupled to MALDI-TOF mass spectrometry to identify proteins differentially expressed between *Nostoc* PCC 73102 (*Nostoc punctiforme*) vegetative cells and hormogonia. Nine proteins were identified as up-regulated, including a homolog of PilQ, an ABC transporter, a chloroplast outer membrane protein (OEP80) homolog, an S-layer associated multidomain endoglucanase, two proteins without known function, and three photosynthetic proteins; while an 'akinetete-marker' protein homolog was strongly down-regulated. These analyses were complemented with RT-PCR analyses of some corresponding genes and the expression patterns verified the proteome data and revealed that the transcription dynamics were particularly pronounced during early stages of the hormogonial development process. Since the proteins/genes identified as differentially expressed were predominantly surface associated, roles in motility, recognition, adhesion, and communication with host plants are discussed.

Keywords: Cyanobacteria, hormogonia, motility, *Nostoc*, proteomics, signal peptides, surface proteins, symbiosis

1. Introduction

Cyanobacteria (blue-green algae) are photoautotrophic gram-negative bacteria, occupying a wide range of ecological niches. This is particularly true for some members of the genus *Nostoc*, a common terrestrial colonizer (Dodds et al., 1995), which have extended their ecological niche to also include symbioses with a number of plants (Rai et al., 2000, 2002). These plant-associations range from less intimate interactions, such as attachment to moss-plant surfaces (DeLuca et al., 2002; Solheim and Zielke, 2002; Solheim et al., 2004) to highly intricate endosymbioses, such as with the angiosperm *Gunnera* (Bergman et al., 1996; Bergman, 2002).

Although being a prokaryote, *Nostoc* is phenotypically highly plastic with capacity to develop several cell types with varied morphology and functions. Cell differentiation is crucial for enabling diverse and sometimes incompatible functions to be contained within the same organism. For

instance, the photosynthetically competent vegetative cells may turn into nitrogen-fixing heterocysts, or into spore-like resting cells, termed akinetes (Adams and Duggan, 1999; Meeks et al., 2002). Entire vegetative filaments, typically composed of vegetative cells and heterocysts, may also undergo a differentiation process leading to hormogonia, prerequisites for plant infections (Bergman, 2002; Johansson and Bergman, 1992; Rai et al., 2002). This transient life stage usually lasts for 1–2 days, and is characterized by gliding motility, smaller and cylindrically shaped cells, and the absence of heterocysts. Differentiation is initiated in a highly coordinate manner along the vegetative filament with all vegetative cells simultaneously starting to divide. Several rounds of rapid cell division cycles later, without cell enlargement, and accompanied by vegetative filament fragmentation at the heterocyst junctions, hormogonia are formed (Tandeau de Marsac, 1994; Meeks et al., 2002). The repeated cell division cycles in hormogonia are, unlike that of vegetative cells, suggested not to involve DNA replication (Herdman and Rippka, 1988).

Differentiation of hormogonia is induced by various environmental signals. Abiotic signals include transfer of

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vegetative filament cultures into fresh medium, or exposure to specific spectral light qualities, in particular red light, which oxidize the plastoquinone pool (Campbell et al., 1993; Damerval et al., 1991; Rasmussen et al., 1994; Tandeau de Marsac, 1994). Biotic hormogonium inducing factors (HIFs) of unknown chemical identity are produced by host plants. For instance, *Gunnera* plants release a small peptide in the mucilage secreted by stem glands which serve as infection conduits for the cyanobacteria (Rasmussen et al., 1994). HIFs are also produced by the symbiotic host *Anthoceros punctatus* (Campbell and Meeks, 1989) but also by the non-host plant wheat (Gantar et al., 1993). Still, the identification and molecular characterization of plant HIFs remain and their action is unknown. Hormogonium repressing factors (HRFs), i.e. counteracting hormogonium differentiation, have also been identified. HRFs induce expression of hormogonium repressing genes within the *hrmUA* locus of *Nostoc* (Cohen and Meeks, 1997). Also, the DNA binding properties of the HrmR protein has been related to hormogonium differentiation (Campbell et al., 2003). Likewise, the cyanobacterium produces compounds and components involved in the initiation and establishment of symbioses. One such putative signaling compound is the auxin IAA (Sergeeva et al., 2002), since mitotic activities are induced in host cells in presence of compatible hormogonia (Bergman et al., 1996).

Other secreted cyanobacterial molecules are also likely to be involved, as are hormogonial surface structures representing the area of first contact with potential host plants. Although hormogonium formation has crucial roles in cyanobacterial survival and is a prerequisite for the establishment of plant symbiosis, our knowledge about the molecular basis and mechanisms involved in their development are highly restricted. A study of the hormogonium differentiation process was therefore initiated using a proteomic approach to screen for and identify proteins being affected during development of hormogonia, combined with examination of their relative differential transcription levels. As test organism, *Nostoc* PCC 73102 (equivalent to *Nostoc* ATCC 29133 and also designated *Nostoc punctiforme*) was used due to its large phenotypic plasticity reflected in its large genome (~10 Mb), the availability of the sequenced genome (Meeks et al., 2001), and its broad plant host range.

2. Materials and Methods

Biological material and cultivation

Axenic cultures of *Nostoc* PCC 73102 (*Nostoc punctiforme*) were cultivated under sterile conditions in 100 ml flasks containing 25 ml liquid BG-110 medium (Stanier et al., 1971) at 25°C on a shaker at 63 rpm and with continuous white light of 18 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. *Nostoc* PCC 73102 is the original isolate from a cycad

Macrozamia sp. (Rippka et al., 1979), and effectively the same organism as *Nostoc* ATCC 29133 (deposited in the ATCC from the original PCC 73102 culture) whose genome has been sequenced (Meeks et al., 2001).

Hormogonium induction

For induction of hormogonia, seven-day old cyanobacterial cultures were used. The culture density was approximated by measuring the chlorophyll a content (Tandeau de Marsac and Houmard, 1988). The cultures were washed three times in 10 ml fresh BG-110 medium before being diluted into 25 ml of fresh medium and placed under red light (>600 nm) for 24 to 48 h, depending on the various induction time needed for developing mature hormogonia (determined by light microscopy inspection). Prior to harvest, red-light induced cyanobacterial cultures were mixed by using a 1 ml micro-pipette. The hormogonia were mainly found in the supernatants after allowing the vegetative filaments to settle in the culture flasks for a few minutes. The hormogonium containing supernatants were collected and centrifuged for 10 min in a bench top centrifuge (2,800 \times g) at 4°C. From the obtained hormogonial pellets, proteins were extracted as described below for the vegetative cultures.

Protein extraction

To obtain total soluble protein extracts from *Nostoc*, seven day old cultures were used, and are here referred to as vegetative cultures. These cultures were spun in a bench top centrifuge for 5 min (15,800 \times g). The pellets were transferred to a mortar with an extraction buffer [8 M urea, 2% (v/v) 2-mercapto ethanol, 0.5% (v/v) IPG buffer (Amersham Biosciences, Sweden), 2% (v/v) Triton X-100, a trace of bromophenol blue, and 0.28% (w/v) DTT] twice the volume of the pellet. Liquid nitrogen was added repeatedly at least three times to the mortar, and the cells were ground until completely thawed. Cell debris was pelleted by centrifugation using a bench top centrifuge (15,800 \times g) for 10 min at room temperature. The retrieved supernatants were kept at -80°C until use.

2-D gel electrophoresis

Isoelectric focusing of the proteins was performed on an IPGphor (Amersham Biosciences, Sweden). Eighteen cm immobilized gel strips (Amersham Biosciences) with a pH range 4–7 were rehydrated in the extraction buffer (see protein extraction) over night. Prior to the isoelectric focusing, 100 μl protein extracts were loaded onto the gel strips by using application cups positioned close to the acidic end of the strip. The focusing time was adjusted to a total of 40 kVh. The gel strips were then incubated in equilibrated buffer [50 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol [87% (v/v)], 2% (w/v) SDS, a trace of

bromophenol blue, and 1% (v/v) DTT] for 30 min before being positioned at the top of a 10% polyacrylamide gel. The contact between the strip and the gel was sealed by the addition of running buffer solidified by 0.5% (w/v) agarose. After separation (10 mA/gel for 15 min followed by 20 mA/gel until the dye front reached the bottom edge of the gel), protein staining was performed using Coomassie Brilliant Blue R250 (Fluka Chemie AG, Switzerland) or silver staining (Amersham Biosciences, Sweden). Coomassie Blue stained gels obtained from hormogonia and vegetative cells, in at least four independent biological replicates from each cell type, were compared manually. The protein spots that were only present or distinctly upshifted in one or the other of the two cell types were cut out and further processed for identification.

In-gel digestion and MALDI-TOF analysis

In-gel digestion, including reduction with DTT and alkylation with iodoacetamide, was performed according to Shevchenko et al. (2000) using sequence grade modified trypsin (Sigma, Sweden), and analyzed in a Voyager-DE STR mass spectrometer (MALDI-TOF Applied Biosystems, USA). The obtained peptide mass fingerprints were internally calibrated according to known trypsin peaks and compared to databases using the Mascot search engine (<http://www.matrixscience.com>; Perkins et al., 1999). The following search parameters were applied for the Mascot searches: NCBI nr was used as the protein sequence database; taxonomy was set to 'Other Bacteria'; a mass tolerance of 30 ppm was applied without allowing miss cleavages; possible fixed modifications were considered to be alkylation of cysteine by carbamidomethylation. For all peptide mass fingerprints, hits with probability based MOWSE scores >63 were obtained, which indicates that the hits were significant ($p < 0.05$), and that the proteins were successfully identified. In all cases, the identified proteins were represented as the top hits with the highest MOWSE scores, and well separated from the false positive hits.

RT-PCR

Proteins identified from the proteomic analysis (Table 1) were further analyzed using the RT-PCR method. *Nostoc* PCC 73102 was cultivated, and hormogonia were induced by red light, under the same conditions as mentioned above. In a time series, cell samples of 20 mg for each time point were collected by centrifugation. Total RNA was extracted from cell-lysates by using RNeasy Plant Mini Kit (Qiagen, Germany). High specificity primers (Table 2) were designed online using GeneFisher Interactive PCR Primer Design (<http://bibiserv.techfak.uni-bielefeld.de/genefisher>).

For normalization 16S was used, with previously described primers (Wang et al., 2004). RT-PCR was performed using Titan One Tube RT-PCR system (Boehringer Mannheim, Germany) and GenAmp PCR

system 2700 (Applied Biosystems, USA). Each 25 μ l RT-PCR reaction contained: 40 ng total RNA, 20 pmol of each primer, and 0.2 mM of each dNTP. The cycles used were as follows: one cycle (50°C for 30 min), 20–28 cycles (94°C for 30 s, 55°C for 30 s and 68°C for 30 s), one cycle (68°C for 7 min), and a final step at 4°C. Amplification products were separated on 2% agarose gels, stained with ethidium bromide. An optimized number of PCR cycles was used, ranging from 20 to 28 depending on which gene was amplified. The experiment was repeated three independent times, including three independent setups for hormogonium induction, sample collection, RNA extraction, and RT-PCR reaction. The RT-PCR patterns obtained were reproducible and a representative experiment is presented in Fig. 2.

3. Results

Hormogonium enrichment

Hormogonium fractions were obtained after three washes of a vegetative *Nostoc* PCC 73102 culture followed by induction under red light for 24–48 h, both treatments known to stimulate hormogonium differentiation. The resulting cultures were highly enriched in hormogonia, although some vegetative filaments remained. However, after mixing, the larger and heavier vegetative filaments settled, while the hormogonia remained in the culture supernatant. Bright field light microscopy analyses of the supernatant verified the presence of >95% straight non-heterocystous hormogonium filaments. These were harvested by centrifugation. In parallel, a non-induced culture, composed of vegetative filaments with heterocysts, was harvested seven days after inoculation, and served as a hormogonium-free reference material. The Chlorophyll a content was 4–5 μ g/ml, and at this stage, the culture was exponentially growing and dividing as verified by bright field light microscopy. Being grown on nitrogen-depleted BG-110 medium, both terminal and intercalary heterocysts were present and at expected frequencies of 5–10%. In this vegetative culture, no akinetes were seen. However, in stationary cultures that were cultivated for 4–5 weeks without re-inoculation, akinetes started to appear (data not shown). These cells showed the typical characteristics for akinetes in *Nostoc* (Herdman, 1988) i.e. enlarged cells developing from vegetative cells distant from heterocysts, with intracellular cyanophycin granules and becoming unicellular by fragmentation of the filaments.

Protein patterns in hormogonia versus vegetative stage

Total proteins extracted from isolated hormogonia, were compared to proteins in 7-day old vegetative cultures after separation using 2-D gel electrophoresis. The gels from each stage were first stained with the highly sensitive silver stain, visualizing a large number of proteins (data not

Table 1. Proteins identified as up- and down-regulated in hormogonia.

Spot	Accession no.	Locus no.	Protein	Observed		Predicted	
				kD	pI	kD	pI
Up-shifted protein spots in hormogonia							
H6a	ZP_00111132	NpF5008	PilQ	33.2	5.55	83.4	9.41
H6b	ZP_00111132	NpF5008	PilQ	31.8	6.3	83.4	9.41
H3	ZP_00109838	NpF4615	Putative periplasmic binding protein	49.1	5.6	48.1	8.55
H1a	ZP_00107621	NpF2928	Chloroplast outer envelope protein homolog	72.0	4.7	91.5	5.18
H1b	ZP_00107621	NpF2928	Chloroplast outer envelope protein homolog	63.4	4.6	91.5	5.18
H14	ZP_00109598	NpR5769	S-layer associated multidomain endoglucanase	41.4	5.8	59.4	9.54
H4a	ZP_00108176	NpR4212	Hypothetical protein	47.8	5.3	41.8	5.32
H4b	ZP_00108176	NpR4212	Hypothetical protein	47.8	5.3	41.8	5.32
H4c	ZP_00108176	NpR4212	Hypothetical protein	47.8	5.3	41.8	5.32
H15a	ZP_00108177	NpR4213	Hypothetical protein	37.4	5.2	40.1	5.17
H15b	ZP_00108177	NpR4213	Hypothetical protein	37.4	5.2	40.1	5.17
H15c	ZP_00108177	NpR4213	Hypothetical protein	33.7	4.9	40.1	5.17
H8	ZP_00106784	NpF5291	Phycobilisome 30.8 kDa linker polypeptide	29.4	6.3	30.1	8.61
H9a	ZP_00108261	NpF3811	Phycobilisome rod-core linker protein CpcG	28.7	6.2	29.4	6.65
H9b	ZP_00108261	NpF3811	Phycobilisome rod-core linker protein CpcG	19.7	5.7	29.4	6.65
H10a	ZP_00106783	NpF5292	Phycocyanin specific rod linker polypeptide	24.6	6.15	32.0	8.97
H10b	ZP_00106783	NpF5292	Phycocyanin specific rod linker polypeptide	21.5	5.7	32.0	8.97
Down-shifted protein spots in hormogonia							
V9a	ZP_00108348	NpF5452	Akinete-marker protein	42.8	4.4	34.3	4.72
V9b	ZP_00108348	NpF5452	Akinete-marker protein	42.8	4.4	34.3	4.72

The protein names are given according to highly homologous hits in public databases with proteins with known functions, if available. The predicted kD and pI were generated from sequences obtained from the JGI database (<http://img.jgi.doe.gov/>) using the web based Compute pI/Mw prediction tool (http://www.expasy.org/tools/pi_tool.html). The observed molecular masses in kD and pI values are given as approximations from the 2-D gels.

Table 2. Primers used for RT-PCR.

Protein	Forward primer	Reverse primer
H1 (C3)	5'-GTTTATCTCCCGTATTGCTGGC-3'	5'-GATTAGCAGGGTAAACTGCGAC-3'
H3	5'-GGTGAAGATCGCGTACTAACG-3'	5'-CACCCCTTCGCTGGGTCAGTC-3'
H4	5'-CGACAACGCGATGCATCAGAGT-3'	5'-TGGTAATGGGCGATGCCTTTCG-3'
H6	5'-GCTTAGGTGCAGAAAGTCCCGT-3'	5'-CGAGGAGGGCCAATTAAGGTTCAG-3'
H14	5'-CTACCCGGTGCAGTTAATGC-3'	5'-CTTCATCACCCGCAGTGTGTC-3'
H15	5'-CCTAACGAACTGGAAGAAGC-3'	5'-CTACCAAAGCCCTCATAGC-3'
V9	5'-AATTGGTTCGTGCCCGTATCG-3'	5'-AGTCGTCGCTGGCTGATAGG-3'

shown). These gels showed that the total number of protein spots was approximately the same in hormogonia and in the vegetative stage, although some proteins were only present, or strongly up-regulated, during the hormogonial stage. In order to accurately identify the differentially regulated proteins, gels were run with larger quantities of proteins and visualized by the Coomassie stain. Nineteen of the most affected protein spots in the hormogonia gels,

compared to proteins in the vegetative stage gels (Fig. 1), were selected for further analyses using *in-gel* trypsin digestion followed by MALDI-TOF mass spectrometry and database search. Only distinct protein-level changes and those being reproducible between replicate gels were accepted for further analysis, although some additional proteins differed in quantities between individual gels (Fig. 1). In addition, some of the protein spots located close to

each other on the 2-D gels turned out to represent uncharacterized protein modification forms of the same protein (e.g. H4a, H4b and H4c). The occurrence of such multiple spots, or pearl strings, has previously been observed (Huang et al., 2002). Due to these protein modifications, the 19 protein spots selected represented 10 individual proteins. Nine of these were up-shifted in the hormogonial stage, and one highly abundant vegetative stage protein was strongly down-shifted in the hormogonial stage (Table 1).

Identification of developmentally regulated proteins

Following the biochemical identification of the proteins using MALDI-TOF mass spectrometry and database search, the amino acid sequences of the hormogonial up-shifted proteins were subjected to BLAST search in order to find related proteins with known functions (Table 1).

Two of the protein spots (H6a and b) generated peptides matching the same predicted ORF, and were therefore identified as the same protein. The amino acid sequence of this protein shows homology to the slr1277 protein in *Synechocystis* PCC 6803 (38% identity over 647 amino acid residues), designated PilQ (Yoshihara et al., 2001). The deviation detected between the observed and theoretical molecular mass and pI may be explained by a cleavage, resulting in at least two products including the H6a and H6b. Moreover, upstream from *pilQ*, three adjacent ORFs are present in the *Nostoc* PCC 73102 genome being homologous to *pilM*, *pilN*, and *pilO*, all involved in pilus formation. Part of the N-terminal (first 116 amino acids) of *pilQ* showed high similarity (46% identity) to alr2592, a ferrichrome-iron receptor in *Nostoc* PCC 7120, the significance of which is not known. By using a search for pilin Pfam domains in cyanobacteria, an ORF predicted to constitute a structural pilin protein (accession number ZP_00109025, locus number NpF0676) was identified in the *Nostoc* PCC 73102 genome. Relative quantifications using RT-PCR of this hypothetical pilin gene showed a strong up-shift during hormogonium differentiation, similar

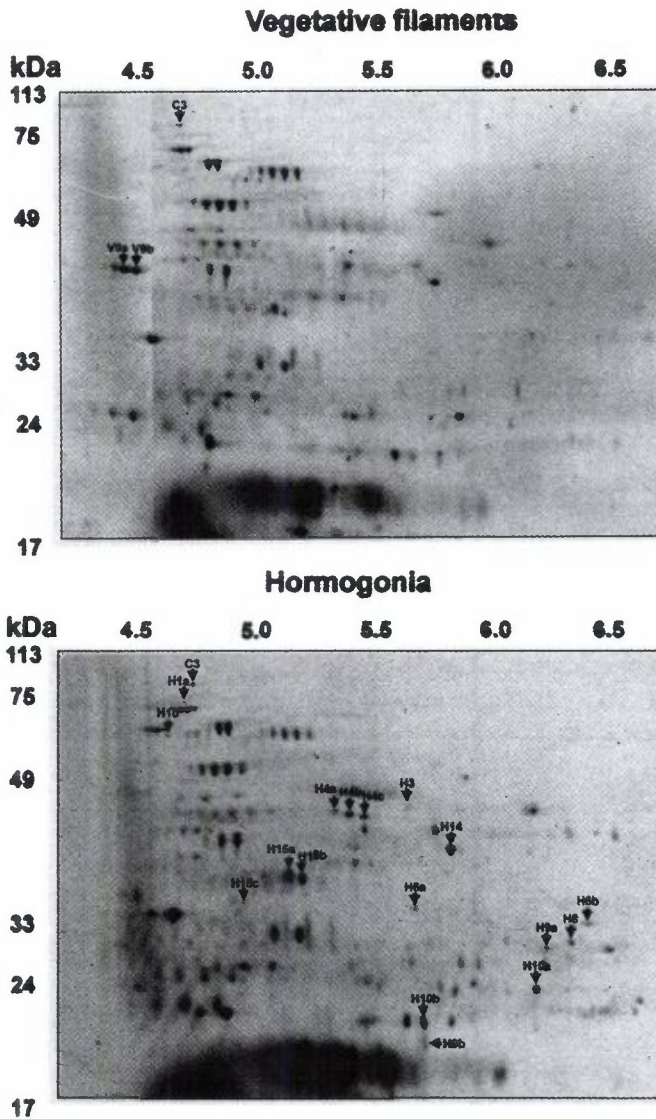


Figure 1. 2-D map of *Nostoc* PCC 73102 proteins obtained from a vegetative reference culture and a purified hormogonial fraction. Proteins identified are marked with arrows and numbers. The numbers correlate with the protein identifications listed in Table 1. The overall protein patterns coincide between the two life-stages, with the exception of nine proteins (17 spots) being up-regulated (marked with H), and one down-regulated (two spots, marked with V) in hormogonia. Gels were stained with Coomassie Brilliant Blue R-250. The pI scale in the first dimension is indicated horizontally. The molecular mass of standard proteins is indicated vertically.

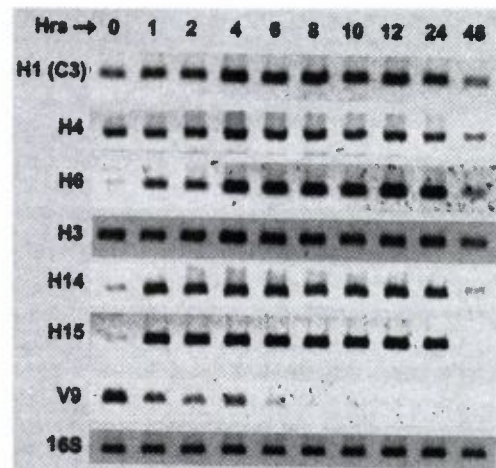


Figure 2. Relative RT-PCR expression analysis of the genes corresponding to the proteins identified during hormogonium differentiation in *Nostoc* PCC 73102, listed in Table 1. The cell samples were harvested at a series of time points after onset of hormogonium induction in red light. Amplification products were separated on 2% (w/v) agarose gels and stained with ethidium bromide. H1, H4, H6, H3, H14, and H15 were identified as being up-shifted and V9 as down-shifted during hormogonium differentiation. The 16S rRNA gene levels (at the bottom) were used to normalize the RNA levels prior to the RT-PCR reactions.

to that of the PilQ homolog (data not shown). Potentially, this protein containing a pilin domain may therefore be a structural pilus component of type IV pili secreted by PilQ in hormogonia of *Nostoc* PCC 73102.

A putative periplasmic binding protein was identified comprising spot H3, which is nearly identical to the substrate binding protein of the urea ABC transporter, UrtA, recently identified in *Anabaena/Nostoc* PCC 7120 (84% identity over 386 amino acid residues) (Valladares et al., 2002). Two protein spots (H1a and b) were identified as a protein homolog to the outer envelope protein OEP80 (accession number NP_568378) located in chloroplasts of *Arabidopsis thaliana* (32% identity over 640 amino acid residues). This protein also appeared in yet another position on the gel (C3), with a molecular mass closer to that predicted (Fig. 1; Table 1). The hormogonium up-shifted H1a and H1b proteins may therefore represent modifications or processing of the protein denoted C3 although the intensity of the latter is approximately equal in hormogonia and vegetative cells. Nevertheless, the transcription of the gene was induced in hormogonia, as shown by RT-PCR (Fig. 2).

Yet another protein, related to the cell surface/extracellular space (H14), was identified as hormogonium up-shifted in *Nostoc* PCC 73102. This inferred protein was homologous to an S-layer multidomain endoglucanase in *Thermoanaerobacterium polysaccharolyticum* (32% identity over 182 amino acid residues), and to other secreted proteins involved in polysaccharide degradation. The corresponding gene occurs as a single ORF in *Nostoc* PCC 73102 with no adjacent genes with the same transcriptional direction.

The protein presented in H4 (H4a, b and c) did not generate any significant BLAST hits to proteins with known function and it lacks any obvious conserved domains in its amino acid sequence, and is therefore designated as being hypothetical. The gene corresponding to the protein spots denoted H15 (spots H15a, b and c) is transcribed in the same direction as the adjacent gene encoding H4. Likewise, this protein shows only low similarity to known proteins. However, a conserved 'chromosome segregation ATPase' (COG1196) domain was detected, but with a moderate E-value (2×10^{-3}).

Three proteins (spots H8; H9a and b; H10a and b), up-shifted in the red-light induced hormogonia, are suggested to be involved in cyanobacterial photosynthesis based on sequence homology to such proteins (Fig. 1; Table 1). H8 is homologous to the phycobilisome 30.8 kDa linker polypeptide in *Fremyella diplosiphon* (67% identity over 268 amino acid residues), H10 is homologous to the phycocyanin specific rod linker polypeptide in unicellular *Synechocystis* PCC 9413 (65% identity over 289 amino acid residues), and H9 is homologous to the phycobilisome rod-core linker protein CpcG in *Synechocystis* PCC 6803 (67% identity over 234 amino acid residues).

One of the most abundant proteins (V9a and V9b) in the

2-D gel analyses of the vegetative filaments was strongly down-shifted in hormogonia of *Nostoc* PCC 73102 (Fig. 1). This protein shows similarities (62% identity over 311 amino acid residues) to the suggested 'akinetete-marker' protein recently identified as highly expressed in akinetes of *Anabaena variabilis* (Zhou and Wolk, 2002). Moreover, this protein contains an uncharacterized domain conserved among bacteria, COG3861 (E-value 4×10^{-27}).

Transcription analysis

To get further insights into the hormogonium developmental process, the expression patterns of the corresponding genes were followed, from early stages of hormogonium differentiation to full maturity (48 h). High specificity primers (Table 2) were designed for the genes corresponding to the six up-shifted non-photosynthetic genes, and the single down-shifted protein. RNA was extracted from the cultures at the start of the red-light hormogonial induction period (Fig. 2, time 0) and at additional time-points during the development of hormogonia (within 48 hours). The RNA was then subjected to reverse transcription (RT) followed by PCR amplification. As RT-PCR is not a truly quantitative method, only relative changes in transcription levels of the individual genes are seen in Fig. 2. The up- (H1–H15) and down- (V9) shifts of the proteins identified in the proteomic analyses were indeed reflected at the gene transcription level. The RT-PCR data also demonstrated that the gene expression patterns varied for the individual genes in specific manners. For some genes the relative expression levels were almost negligible at time zero (approx. vegetative filament cultures) and then increased rapidly during hormogonium differentiation, e.g. the gene corresponding to the putative PilQ protein (H6a and H6b) and the endoglucanase (H14). The expression of V9 showed the opposite pattern, disappearing within 8 hours after hormogonium induction (Fig. 2). In two cases (H3 and H4), the changes in transcription levels were less obvious than changes seen at the protein levels (Figs. 1 and 2). However, most genes showed up-shifts in their expression levels during early stages of the hormogonium development process (<24 h), while expression patterns were again low at 48 h when hormogonia are starting to revert back to become vegetative filaments. As the photosynthetic proteins (H8; H9 and H10) are likely to be up-shifted due to the red light incubation rather than to hormogonium differentiation, these were not studied further.

Signal peptides

As some of the identified proteins (Fig. 1; Table 1) were potentially associated with the periphery of the hormogonium cells, the proteins identified were tested for the occurrence of signal peptides using web-based SignalP software (Nielsen et al., 1997). As seen in Table 3, four of

the proteins contained putative signal peptides (H6, H3, H1 and H14) indicating that these proteins may be secreted, localized in the inner or outer cell membrane, or contained within the periplasm. A deviation between the theoretical and observed molecular mass was observed in three proteins (H6, H1 and H14) containing putative signal peptides (Table 1 and Table 3), which cannot solely be explained by cleavage of the N-terminal signal peptide. Rather, other cleavage events leading to maturation or degradational turnover of the proteins may occur in these proteins.

Table 3. Predicted signal peptide cleavage sites and expected molecular mass of four hormogonium up-shifted proteins.

Protein	Possible cleavage site		Theoretical molecular mass (kD)	
	Position	Sequence	Preprotein	Mature protein
H6	26	VWA-QI	83.4	80.6
H3	28	ACA-NN	48.1	44.8
H1	25	AKA-ET	91.5	89.1
H14	51	VNA-GA	59.4	53.9

4. Discussion

This is the first attempt to identify molecular changes taking place during hormogonium differentiation in cyanobacteria using a proteomic approach. Although soluble and hydrophilic proteins were primarily extracted, four of the nine proteins identified as being up-shifted during hormogonium differentiation are likely to be associated with the periplasm, the cellular outer membrane, or the extracellular space, as indicated by homology to such known proteins. This is also verified by the presence of putative signal peptides in four of the proteins (Table 3).

These data suggest that important molecular events involved in hormogonium differentiation are targeted to changes in the composition of cellular secretion systems, outer membrane proteins, and possibly secretion of proteins. Since proteins involved in modifications of hormogonial surfaces have not been identified before, the proteins presented are likely to represent novel candidates. A homolog of the *pilQ* component in pilus production (spot H6a and b) was up-regulated in hormogonia both at the protein and expression level as shown by proteomics and relative RT-PCR analyses. Pili are thin proteinaceous structures protruding from bacterial cell surfaces. Adjacent on the chromosome of *Nostoc* PCC 73102, and presumably transcribed as one operon, were also homologues to additional genes encoding proteins involved in type IV pili assembly: *PilM*, *PilN*, and *PilO*. This suggests that type IV pili assembly and secretion is up-shifted in hormogonia.

Although pili (fimbriae) have previously been identified in heterocystous cyanobacteria using TEM (Dick and Stewart, 1980; Johansson and Bergman, 1994; Lindblad and Bergman, 1990), this is the first report on the presence of type IV pili proteins in heterocystous cyanobacteria. Type IV pili are known to be involved in motility in unicellular cyanobacteria (Bhaya et al., 2000; Yoshihara et al., 2001), in motility, cell adhesion and/or establishment of plant symbioses in the endophytic bacterium *Azoarcus* sp. (Dörr et al., 1998), as well as in communication between partners in pathogenic systems (Abraham et al., 1998; Källström et al., 1998). Such findings support type IV pili of *Nostoc* PCC 73102 hormogonia to be involved in motility, adhesion, and/or in communication with symbiotic host plant cells.

The function of the ABC transport system being switched on during *Nostoc* hormogonium differentiation (putative periplasmic binding protein H3) may likewise be in adhesion to host cells during early stages of symbiotic interactions. Cyanobacterial filaments entering symbiosis with *Gunnera* appear to adhere to host cells before being engulfed to become endosymbiotic (Johansson and Bergman, 1992). ABC transport systems are also presumed to function in attachment and virulence in *Agrobacterium tumefaciens* (Matthysse et al., 1996) and in adhesion and biofilm formation in *Pseudomonas putida* (Sauer and Camper, 2001). Alternatively, *urtA* and other ABC transporter genes are highly expressed under N-deplete conditions (Valladares et al., 2002), and the up-shift may be a result of conditions experienced by hormogonia growing in N-depleted media and lacking nitrogen-fixing heterocysts.

One of the identified developmentally regulated proteins (H1) in *Nostoc* PCC 73102 is homologous to a protein abundant in the outer envelope of plant chloroplasts, OEP80. This protein is a paralog to OEP75, which is a part of a pore structure in the outer envelope, and is involved in import of proteins into chloroplasts (Perry and Keegstra, 1994). The chloroplast OEP80 is likely to be derived from the ancestral cyanobacterial endosymbiont giving rise to chloroplasts (Inoue and Potter, 2004). The OEP80 protein in *Nostoc* hormogonia displayed molecular masses of 72, 63 and 90 kD (C3), while the vegetative filaments contained a 90 kD OEP80 protein. In addition, a transcriptional induction of the OEP80 gene occurred during the transition from vegetative filaments to hormogonia (Fig. 2). These data suggest that the protein was more highly expressed in hormogonia, although this was not obvious on the 2-D gels (C3 spot). The 90 kD protein of the vegetative filaments may represent the full-length protein, or precursor peptide, while part may be cleaved off during hormogonium differentiation leading to the occurrence of the smaller polypeptides (Fig. 1), and may represent a cell-stage dependent cleavage.

Concurrent with the fragmentation of vegetative filaments at the heterocyst junctions and the abandoning of the parental trichome during hormogonium differentiation,

motility is switched on. Vegetative filaments are surrounded by a thick mucilaginous sheath, which has to be penetrated by the developing hormogonium filaments. The S-layer multidomain endoglucanase (H14) found up-shifted in hormogonia, might be involved in this process. Alternatively, the endoglucanase may be used for penetrating the carbohydrate rich mucilage released through the gland channel of *Gunnera*, through which the hormogonia need to pass to reach the target plant cells further inside the gland (Bergman, 2002).

Both the chromosome segregation ATPase (H15) and one of the hypothetical proteins (H4) were up-shifted in hormogonia and are encoded by genes occurring as succeeding ORFs on the chromosome of *Nostoc* PCC 73102. COGS comparisons suggest their involvement in chromosome segregation and DNA repair. As the chromosome is less active and DNA replication likely to be suppressed during hormogonial differentiation (Herdman and Rippka, 1988), we propose that these proteins may have roles in maintenance of chromosome integrity and suppression of DNA replication in hormogonia.

Three proteins (H8, H9, and H10) up-shifted during hormogonial differentiation are suggested to be associated with the photosynthetic apparatus by their sequences similarity to such proteins. Since hormogonia were induced abiotically by red light, it can not be excluded that the elevated protein expression levels are a response to the changed light quality. For instance, red light is known to induce complementary chromatic adaptations in some cyanobacteria (see Grossman et al., 1994).

The only protein being down-shifted (V9) during hormogonial differentiation showed similarity to an 'akinetate-marker' protein previously described in *A. variabilis* (Zhou and Wolk, 2002). This protein was expressed in the vegetative filaments of *Nostoc* PCC 73102, but below the limit of detection in hormogonia (Fig. 2). Hence, the absence of this protein together with the presence of PilQ in hormogonia could serve as marker proteins for mature hormogonia in *Nostoc* PCC 73102.

In summary, our data clearly demonstrate that several novel proteins/genes are affected during the hormogonial differentiation process. Proteins were both up- and down-shifted during the transition of vegetative filaments into hormogonia, as were the relative expression patterns of the corresponding genes, the latter in particular during early stages of the differentiation process. As most of the up-regulated proteins appear to be associated with the periplasm, outer membrane, or extracellular space, the genetic background for hormogonium differentiation may primarily be surface-oriented. Studies are now required to further characterize these proteins/genes and to clarify their roles.

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