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Isolation of Host Plant Induced cDNAs from *Nostoc* sp. Strain PCC 9229 Forming Symbiosis with the Angiosperm *Gunnera* spp.

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Abstract

The identification of three genes differentially expressed in a symbiotically competent cyanobacterium during an early stage of its infection of the angiosperm *Gunnera* is reported. The symbiotic isolate *Nostoc* sp. PCC 9229 was treated for 6 and 16 hours with the carbohydrate rich mucilage excreted by *Gunnera* stem glands, the infection organ known to possess multiple induction potential. Subtractive unique cDNA libraries were constructed. The cyanobacterial clones identified after screening the libraries were tentatively termed *hieA*, *hieB* and *hieC*. The clones were specifically expressed in response to the secreted *Gunnera* gland mucilage as shown by RT-PCR. Analysis of the deduced amino acid sequences suggest that *hieA* encodes a putative precursor of a pheromone-like signalling peptide; *hieB* encodes an outer membrane or secreted glycoprotein, possibly involved in cell-identification, and the product of *hieC* is probably involved in adaptation to the acidic environment offered by the mucilage. The nucleotide sequences are deposited in GenBank (accession numbers AF291436, AF293349, AF292396). The potential role of these clones in symbiosis is discussed.

Keywords: *Nostoc, Gunnera*, symbiosis, infection, subtractive hybridisation

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1. Introduction

Cyanobacteria form a large and ancient group of phototrophic bacteria capable of oxygenic photosynthesis. They are also progenitors of plastids of modern algae and plants, the result of symbiotic events in the past (Margulis, 1970; Douglas, 1994). At present, only a few plants, but from all major taxa, form symbioses with nitrogen-fixing cyanobacteria, known as cyanobionts (Bergman et al., 1996; Rai et al., 2000). The cyanobionts primarily belong to group IV, i.e. filamentous cyanobacteria forming heterocysts – cells specialised on nitrogen fixation (Rippka et al., 1979). The cyanobionts inhabit a variety of organs and tissues in the various host plants. They often occupy extracellular cavities, as in bryophytes (*Blasia* and *Anthoceros* spp.), in the water fern *Azolla* and in cycads, but may also persist intracellularly as in a fungus (*Geosiphon*), some diatoms (*Hemiaulus* and *Rhizosolenia*) and one angiosperm, *Gunnera* spp. (Bergman et al., 1992b; Rai et al., 2000).

Most symbiotically competent strains of cyanobacteria are quite promiscuous in their host range, hence one specific symbiotically competent *Nostoc* may infect different and phylogenetically distant host plants (Enderlin and Meeks, 1983; Bergman et al., 1996; Nilsson et al., 2000). Similarly, one host plant may accept different *Nostoc* strains. An exception is the water fern *Azolla* in which the cyanobacterial diversity is limited (Zheng et al., 1999) as the cyanobacterium is carried over from one host generation to the next via its sporocarps (see Rai et al., 2000). The fact that *Nostoc* species are common world-wide in terrestrial and aquatic habitats probably enables plant hosts to find suitable micro-symbionts at almost any geographical location (Rasmussen and Svenning, 1998; Costa et al., 1999).

On contact with host plants the cyanobacteria are induced to differentiate into hormogonia, i.e. small-celled and motile filaments (Campbell and Meeks, 1989; Bergman et al., 1992b). The hormogonia are attracted by the plant and move towards the site of infection (Rasmussen et al., 1994; Knight and Adams, 1996; Meeks, 1998; Rai et al., 2000). The inducers and attractants appear to be low molecular weight proteins and/or carbohydrates (Campbell and Meeks, 1989; Rasmussen et al., 1994), though Gantar et al. (1993) suggest the presence of a high molecular weight hormogonia-inducing factor in the wheat root exudates. The involvement of other compounds, such as flavonoids is another possibility (Bergman et al., 1996). On completion of the infection the hormogonia eventually re-differentiate into the vegetative state. This stage is often characterised by a several fold higher frequency of heterocysts, than under free-living conditions (Bergman et al., 1992b).

Molecular mechanisms involved in recognition of the host by cyanobacteria and host-induced differentiation events in *Nostoc* spp. are largely unknown. Cohen and Meeks (1997) addressed this problem by using transposon

mutagenesis of a symbiotically competent cyanobacterium (*Nostoc punctiforme* ATCC 29133). Mutants defective in hormogonia formation were tested for symbiotic competence allowing identification of the hormogonia-regulating locus *hrmUA*, the expression of which is enhanced in the presence of the host plant, the hornwort *Anthoceros punctatus*.

In this paper we present data obtained by using an alternative approach: the isolation of host-induced gene sequences by subtractive hybridisation of commonly expressed RNA/DNA sequences. Libraries were constructed from *Nostoc* mRNAs induced by the mucilage excreted by the symbiotic stem glands of *Gunnera* (Bergman et al., 1996). The mucilage is a carbohydrate-rich, acidic substance covering young non-infected *Gunnera* glands (Bergman et al., 1996). It functions as the first contact between the pro- and eukaryotic partners, and causes dramatic changes in cell differentiation and behaviour of *Gunnera*-compatible cyanobionts (Rasmussen et al., 1994). Due to these properties we decided to use gland mucilage as a tool for identifying genes in symbiotically competent *Nostoc* PCC 9229 differentially expressed during early phases of the *Gunnera* infection process. Three cDNA clones were identified and the characters of these and their putative role in symbiosis are discussed.

2. Materials and Methods

Cyanobacterial strain and growth conditions

The cyanobacterium *Nostoc* sp. strain PCC 9229, originally isolated from *Gunnera manicata*, was cultivated in BG-11_0 medium (Stanier et al., 1971) under continuous light with shaking as described previously (Johansson and Bergman, 1992). The carbohydrate rich non-sterile mucilage which appeared to be not axenic, from naturally grown and actively excreting *Gunnera manicata* was collected and generously provided by Prof. W.B. Silvester (University of Waikato, Hamilton, New Zealand). Treatment of *Nostoc* cultures with the natural *Gunnera* mucilage was as in Viterbo et al. (1999). The cyanobacterial cultures were washed three times in BG-11_0 at 4°C after treatment with the mucilage for 6 and 16 hours, frozen in liquid nitrogen and kept at –80°C until RNA extractions were performed.

DNA and RNA extraction and hybridisation

Total DNA from *Nostoc* sp. PCC 9229 was extracted according to Dzelzkalns et al. (1988), digested with *Eco*RI and *Hind*III, separated on a 1% agarose gel and blotted onto Zeta-Probe GT nylon membrane (Bio-Rad). The DNA probes were labelled with (P^{32})dCTP (Amersham Pharmacia) using the DECAprime-

kit (Ambion). Hybridisations were performed according to the manufacturer's instructions and the membranes were washed at high stringency (0.1x SSC, 0.1% SDS).

Total RNA was extracted from *Nostoc* PCC 9229 as in Viterbo et al. (1999) and 30 µg of each sample was separated on formaldehyde containing gels according to Sambrook et al. (1989). Blotting onto Zeta-Probe membrane and hybridisations were performed according to the manufacturer's instructions. The amount of the extracted nucleic acids was quantified with RNA/DNA Calculator Gene Quant II (Pharmacia Biotech).

Construction of subtractive cDNA libraries

Ten µg of total RNA from the *Nostoc* cultures induced with the mucilage for 6 and 16 hours were reverse transcribed with Avian Myeloblastosis Virus reverse transcriptase (Boehringer Mannheim) using random hexamer primers (Boehringer Mannheim) in the presence of RNasin (Promega) at 42°C for 1.5 hours to produce the corresponding single stranded cDNA. The mixtures were then extracted with phenol/chloroform and precipitated with 0.1 volumes of 7.5 M $(NH_4)OAc$ and 0.6 volumes isopropanol, followed by a wash with 70% ethanol. The pellets were resuspended in 50 µl TE buffer, boiled for 1 minute, cooled on ice and treated with RNAse A and RNAse H (Boehringer Mannheim) at a concentration of 0.5 mg/ml each for 1 hour at 37°C. RNAse was removed by phenol extraction followed by re-extraction of the organic phase with TE and back-extraction with chloroform. The aqueous phases were combined, precipitated and washed as above.

The subtraction was performed by resuspending the single-stranded cDNA in 30 µl hybridisation buffer (40 mM PIPES, pH 6.4; 1 mM EDTA, pH 8.0; 0.4 M NaCl; 80% formamide) and by adding 100 µg in 5 µl total RNA extracted from non-induced *Nostoc*. The mixtures were heated to 85°C for 10 minutes and kept at 37°C overnight with RNasin, precipitated and washed as above. The pellets were resuspended in water and the second DNA strand was synthesised with 10 U Klenow DNA polymerase (Pharmacia Biotech), dNTPs and hexamer primers in the presence of RNasin at 37°C for 30 minutes. Then 10 U of *E. coli* DNA ligase (Pharmacia Biotech) and 2 µl of 0.5 mM NAD^+ were added. Incubation was continued for another hour and was followed by phenol/chloroform extraction, precipitation and washing as above. The pellets were resuspended in 1x T4 DNA polymerase buffer. One U T4 DNA polymerase (Pharmacia Biotech) and 1 µl of 10 mM dNTP were added and incubated at 37°C for 1 hour, followed by 30 minutes incubation with RNAse A and phenol/chloroform extraction. The DNA was precipitated and washed.

The blunt-ended, double-stranded cDNA was resuspended in ligation buffer

and ligated using T4 DNA ligase (Boehringer Mannheim) into the *Hinc*II site of Bluescript KS+ vector (Stratagene). The libraries thus obtained were used to transform *E. coli* XL-Blue (Stratagene) by electroporation with a BTX Electro Cell Manipulator 600 (Biotechnologies & Experimental Research Inc.).

Sequencing and analyses

Sequencing was performed with a BigDye sequencing kit and ABI Prism machine (Perkin-Elmer). The standard T7 and T3 primers (Stratagene) and primers designed according to the sequences obtained here, were used. Preliminary sequence data of *Nostoc punctiforme* ATCC 29133 was obtained from The DOE Joint Genome Institute (JGI) at http://spider.jgi-psf.org/JGI_microbial/html. Sequences from *Nostoc* sp. PCC 7120 and *Synechocystis* sp. PCC 6803 were obtained from Kazusa DNA Research Institute at http://www.kazusa.or.jp/cyano/cyano.html. Similarity searches were performed using BLAST (Altschul et al., 1997) provided at the sites listed above or included in the Biology Workbench 3.2 package from San Diego Supercomputer Center at http://workbench.sdsc.edu. Multiple sequence alignments were done using CLUSTAL W programme (Thompson et al., 1994).

Secondary screening and colony hybridisation

Randomly chosen clones which did not generate a signal after Southern blot hybridisation with total DNA from *Nostoc* sp. PCC 9229 were sequenced. Eight of these randomly chosen clones were then mixed and used as probe against the rest of the clones. The *E. coli* colonies containing plasmids with cDNA inserts were grown overnight on nitrocellulose membranes NC 45 (Schleicher and Schuel) on the selective LB medium (Sambrook et al., 1989). The hybridisation was performed according to the manufacturer's instructions. The silent clones were then taken for further Southern blot analyses.

Reverse transcription-PCR

Reverse transcription PCR was performed using TITAN One Tube RT-PCR System (Boehringer Mannheim) and a Perkin Elmer GeneAmp 2400 Thermocycler. The reactions were mixed according to the manufacturer's instructions. The equivalent of 1 µg of total RNA was used in each reaction. All RNA samples were DNase treated. Amplification of a fragment of 16S RNA served as a loading control. The primers used for amplification of fragments of respective clones and of the loading control are listed in Table 1. The RT-PCR was repeated three to four times for each clone and RNA batch.

Table 1. Primers used for reverse transcription PCR and loading control.

Gene	Name	Sequence	Reference
hieA	ART-F	5' CCGGCTGCCTAATACAGTTG	This study
	ART-R	5' GCCGCAAAAATATCTGACAC	
hieB	BRT-F	5' GCCCCTAGCAGAGTAATGTG	This study
	BRT-R	5' AGGCGAATTACTAGATTTAC	
hieC	CRT-F	5' CGCCGCATTGATGAGGTAGG	This study
	CRT-R	5' GCGCAACGAGGGTAGTATTC	
16S	CC	5' TGTAAACGACGGCCAGTCCAG - ACTCCTACGGGAGGCAGC	Rudi et al., 1997
	CG	5' CGCGTTAGCTACGGCACGGCTCGG	

3. Results

Subtractive cDNA libraries, construction and screening

To construct subtractive *Nostoc* cDNA libraries total RNA from the *Nostoc* cultures, initially induced with the host plant mucilage for 6 and 16 hours respectively, were reverse transcribed to produce the corresponding single stranded cDNAs. Subtraction was then performed by mixing the single-stranded cDNA and total RNA extracted from a non-induced *Nostoc* PCC 9229 culture. The second DNA strand was then synthesised and the double stranded cDNAs ligated into the vector and introduced into *E. coli* by electroporation.

The subsequent screening of portions of the libraries from mucilage induced *Nostoc* sp. PCC 9229 resulted in approximately one thousand clones. This was the case with clones from both the 6 and 16 h *Nostoc* cDNA library. The size of the inserts identified varied between 300 bp and 3.5 kb, though most of them were in the range from 500 to 700 bp. As the *Gunnera* mucilage used for the induction of the cyanobacterium was not sterile, being inhabited by its microflora as is typical for natural *Gunnera* mucilage (Bergman et al., 1992a), the cyanobacterial origin of the identified cDNAs required a further verification by Southern blot analyses. Southern blot analysis of total DNA from *Nostoc* sp. PCC 9229 probed with the cloned cDNA fragments, followed by sequencing of the positive clones were then used to select relevant cDNAs. However, the first 30 clones tested generated no signal and sequencing of these clones proved that most of them represented eukcaryotic rRNA. A secondary screening was therefore introduced in which some of these clones were used as

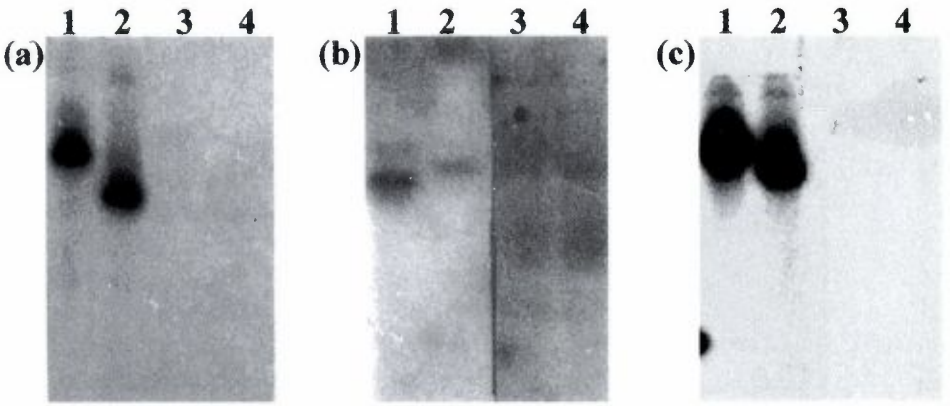

Figure 1. Southern-blot analyses of three cDNA *Nostoc* PCC 9229 clones. Lanes 1 and 2 – DNA from symbiotically competent *Nostoc* sp. PCC 9229 digested with *Eco*RI and *Hind*III respectively; lanes 3 and 4 – DNA from the symbiotically incompetent and non-hormogonia-forming *Nostoc/Anabaena* sp. PCC 7120 probed with (a) *hieA*; (b) *hieB*; (c) *hieC*.

probes in colony hybridisation against the rest of the cDNA clones. Negative clones (totally 69) were examined by Southern blotting. In this way, numerous irrelevant cDNA clones were dismissed. None of the tested cDNAs represented bacterial, chloroplastal or mitochondrial rRNA, which indicates that the subtraction went successfully (data not shown).

Six of the totally 69 clones were confirmed by Southern blot analysis to belong to *Nostoc* sp. PCC 9229 (Fig. 1). Three of these were selected for a further characterisation and were tentatively named *hieA*, *hieB* and *hieC*, where *hie* stands for "host induced expression". The sequences were deposited in the GenBank at the National Centre for Biotechnological Information, NCBI (accession numbers: AF291436, AF293349, AF292396). The three remaining clones also represented *hieA*.

Expression of the three clones was first analysed by northern blot hybridisation. No signals were detected suggesting that if the transcripts were present, they must have been at low levels. However, by using RT-PCR a distinct differential expression of all three clones could be confirmed (Fig. 2).

Characterisation of cloned cDNAs

The cDNA clones of *hieA* were obtained repeatedly from both the 6 h and 16 h libraries, with the longest cDNA being 497 bp. The cDNA contained a full length ORF of 34 amino acids. Southern blot analyses confirmed the presence of

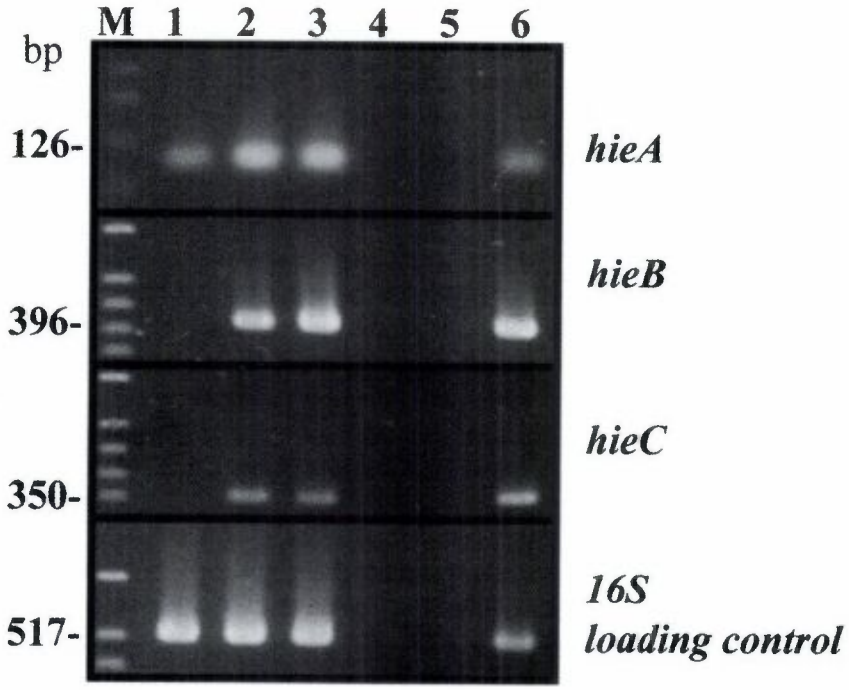

Figure 2. Reverse transcription PCR-analyses of the expression of *hieA*, *hieB* and *hieC* in *Nostoc* sp. PCC 9229 in response to mucilage secreted from the infection organ (stem glands) of the host-plant *Gunnera manicata* Lamb. Lane 1 – culture not exposed to the mucilage; lane 2 – expression after 6 hours; lane 3 – expression after 16 hours exposure to the mucilage; lane 4 – no reverse transcription PCR; lane 5 – blank (water) control; lane 6 – PCR with DNA from *Nostoc* sp. PCC 9229. The primers used are listed in Table 1.

Figure 3. Alignment of the deduced amino acid sequences of HieA from *Nostoc* sp. PCC 9229 and its homologue from *Nostoc punctiforme* ATCC 29133. Identical residues are shaded by black.

hieA in *Nostoc* sp. PCC 9229 (Fig. 1). The RT-PCR assays showed that *hieA* was also expressed in free-living *Nostoc* sp. PCC 9229 but that the expression was enhanced upon treatment with the host mucilage. Analysis of the

hydropathy profile of the encoded 34 aa protein by Kyte-Doolittle suggested a hydrophilic C-terminus. As seen in Fig. 3, the genome of the symbiotic isolate *Nostoc punctiforme* ATCC 29133 contains a similar short ORF. However, the genome of the non-symbiotic and non-hormogonia-forming *Nostoc* sp. PCC 7120 lacks *hieA*.

The *hieB* clone was isolated as a large 5'-partial cDNA fragment of 2744 bp and was clearly differentially expressed in response to treatment with *Gunnera* mucilage (Fig. 2). The deduced amino acid sequence is 880 aa long. No significant homology was found with protein sequences available in the public databases. The C-terminus of the protein shows similarities with a number of putative outer-membrane proteins in the unicellular *Synechocystis* sp. PCC 6803 and filamentous *Nostoc punctiforme* ATCC 29133 and *Nostoc* sp. PCC 7120. Similarities are also found to hemolysin-like secreted proteins from bacteria (data not shown), even though HieB lacks the typical Ca^{2+}-binding motif, GGXGXD (Ludwig et al., 1988), common in these proteins. The N-terminus of HieB consists of several tandem repeats of 72 amino acids. The partial sequence obtained contains 5 perfect and one imperfect repeat and each repeat has an N-glycosylation site NXT/S (Bause, 1983).

hieC was found in a 1089 bp long cDNA isolated from the 16 h library. It contains a full length ORF of 278 aa. The corresponding transcript was detected only in *Nostoc* treated with the mucilage (Fig. 2). Analysis of the hydropathy profile of the predicted protein by Kyte-Doolittle suggests that the clone represents an integral membrane protein. Similarity searches with Blast indicated similarity to putative transmembrane proteins sharing a conserved C-terminus (*Synechocystis* sp. PCC 6803, GenBank accession number BAA10758; *E. coli*, P75770; *Sinorhizobium meliloti*, LpiA, AAF20821). A homology search in the genomes of *Nostoc punctiforme* ATCC 29133 and *Nostoc* sp. PCC 7120 showed that HieC is highly conserved in these strains (73% and 59% identity, respectively). The aligned C-termini are shown in Fig. 4.

4. Discussion

Construction of subtractive cDNA libraries is a proven and effective way of obtaining differentially expressed clones, in particular from eukaryotic systems (see for example Hedrick at al., 1984). We adjusted the method to a prokcaryotic organism using random hexamer primers. Unfortunately we underestimated the risk of contaminating clones from associated organisms. In fact, most of the clones obtained originated from the microflora of the mucilage, stressing the importance of only using strictly axenic systems. In our case, the introduction of a step of secondary screening partly solved this problem.

During the early stages of the infection process in the *Gunnera-Nostoc*

Figure 4. Alignment of the deduced amino acid sequences of the C-terminus of HieC from *Nostoc* PCC 9229 and homologous C-termini from different bacteria. N 9229 – HieC from *Nostoc* sp. PCC 9229 (GenBank accession number AF292396); N 29133 – HieC homologue from *Nostoc punctiforme* ATCC 29133; S 6803 – a hypotetical transmembrane protein from *Synechocystis* sp. PCC 6803 (BAA10758); LpiA – a low pH induced protein from *Sinorhizobium meliloti* (AFF20821); *E. coli* – a hypothetical transmembrane protein from *Echerichia coli* (P75770). Identical residues are shaded by black, conserved by dark grey, similar amino acids by light grey.

symbiosis symbiotically competent *Nostoc* encounters a completely different biotic and abiotic environment. For instance, the mucilage secreted by the glands offers an acidic and carbohydrate enriched environment (Rasmussen et al., 1994). This in turn elicits physiological, developmental and behavioural changes, such as stimulated growth, hormogonia formation and negative phototaxis (Rai et al., 2000). Thus, we expected to isolate genes not only responsible for the actual cyanobacteria/host cross-talk, but also genes that underpin the complex differentiation process noted: a rapid change from the stage of free-living vegetative filaments with heterocysts, into the transitional motile hormogonial stage and adjustment to the new environment. The three cDNA clones isolated were therefore expected to make up a diverse representation of these events.

HieA is a short peptide with a hydrophilic C-terminus, a feature typical of bacterial signal peptides-pheromones. The final product of such proteins is likely to be a processed and exported peptide, which serves as a cell-cell signal and inducer of competence and life-cycle stages in bacteria (Håvarstein and Morrison, 1999; Lazazzera et al., 1999; Perego, 1999). Recently, the first short signal peptide involved in cell-cell signaling in a cyanobacterium was discovered (Yoon and Golden, 1998). This PatS peptide is apparently involved in establishing the regular pattern of heterocysts along the *Anabaena* sp. PCC 7120 filaments. A key differentiation event in the life cycle of *Nostoc* spp. and during early stages of the plant infection process in the *Nostoc-Gunnera* and other symbioses is hormogonia formation. This life cycle stage, normally induced by specific adverse external conditions, is induced by mucilage secreted from the infection organs, the glands, of the host plant (Rai et al., 2000). This motile stage is however always transient and typically lasts 16–24 hours in cyanobacteria cultivated under free-living conditions (Tandeau de Marsac, 1994). In bacteria, a shift from one life cycle stage to another is often caused by accumulation of low molecular compounds (quorum-sensing). Examples of such peptides are pheromones in Gram-positive bacteria and homoserine lactones (HSL) in Gram-negative bacteria (Perego, 1999; Fuqua and Greenberg, 1998). It is known that treatment of *Nostoc* with *Gunnera* mucilage prolongs the motile hormogonial stage. This suggests that the plant produces a putative hormogonia inducing factor carried by the mucilage (Bergman et al., 1996) or interferes with the synthesis or recognition of inhibitors of cyanobacterial hormogonia formation. Our current hypothesis is that *min*A encodes a precursor of a pheromone-like peptide, which is involved in regulation of hormogonia differentiation. This hypothesis is partly supported by the fact that the genome of symbiotically competent *Nostoc punctiforme* ATCC 29133, but not that of non-symbiotic *Nostoc* sp. PCC 7120, contains a homologous ORF (Fig. 3). Interestingly, cyanobacterial host plants may not only induce hormogonia differentiation, but also enhance transcription of genes probably involved in

repression of this motile stage, such as *hrmUA* and *sigH* (Cohen and Meeks, 1997; Campbell et al., 1998). It therefore seems that the host plant may have a global impact on the life cycle regulation of the cyanobacterium, and that the latter tries to compensate for the interference by the plant.

The second clone, *hieB*, shows some similarities to putative extracellular glycoproteins from the cyanobacterium *Synechocystis* sp. PCC 6803 and some bacteria (data not shown). These proteins belong to a wide group of the hemolysin type, which includes the motility related glycoproteins oscillin and SwmA, identified previously in cyanobacteria (Hoiczyk and Baumeister, 1997; Brahamsha, 1996). HieB also shares features typical for these proteins. For instance, it lacks cysteine residues and is rich in glycine.

However, the true function of HieB can not be deduced from such comparison but it appears likely that HieB is a secreted or outer membrane glycoprotein. Through their life cycle cyanobacteria change features of their cell surfaces. For example, in *Nostoc punctiforme*, forming an intracellular symbiosis with the fungus *Geosiphon*, the lectin binding profile differs during the different developmental stages (Schussler et al., 1997).

It is also known that a symbiotic bacterium, *Rhizobium leguminosarum*, secretes a hemolysin-like protein, NodO, which is required for successful nodulation (Scheu et al., 1992). Moreover, it has been shown that symbiotically competent cyanobacteria secret another type of cell identity proteins, the arabinogalactan-like proteins (AGP). The identity and role of these proteoglucans in cyanobacteria is currently being investigated (Rasmussen, Bateman and Bergman, pers. comm.). Whether *hieB* is involved in determining cell identity or involved in extracellular enzymatic activities or motility now needs to be elucidated.

The function of the HieC homologues identified is not known, but it shares homologies with the C-terminus of LpiA from *Sinorhizobium meliloti*, a low pH inducible protein required for survival in low pH environments (Reeve et al., unpublished). Interestingly, the mucilage secreted by *Gunnera* has a pH about 4–5 (Bergman et al., 1992a), a pH far too low for most cyanobacteria, which are typically acido-phobic. The possibility therefore exists that HieC is required for survival in the hostile conditions offered by the mucilage secreted by the host plant, and that HieC is a key-factor involved in rendering cyanobacteria symbiotically competent.

The work presented here represents an attempt to decipher and understand molecular communication in the unique *Nostoc-Gunnera* interaction. As *Nostoc* strains infecting *Gunnera* are quite promiscuous and readily infect other host plants (Rai, 2000), and since the cyanobiont often ends up in some mucilaginous material in many of the plant symbioses (Rai et al., 2000), the gene clones identified here may have a general role in cyanobacterial-plant interactions.

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REFERENCES

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**: 3389-3402.

Bause, E. 1983. Structural requirements of N-glycosylation of proteins. *Biochemical Journal* **209**: 331-336.

Bergman, B., Johansson, C., and Söderbäck, E. 1992a. The *Nostoc-Gunnera* symbiosis. *New Phytologist* **122**: 379-400.

Bergman, B., Rai, A.N., Johansson, C., and Söderbäck, E. 1992b. Cyanobacterial-plant symbioses. *Symbiosis* **14**: 61-81.

Bergman, B., Matveyev, A., and Rasmussen, U. 1996. Chemical signalling in cyanobacterial-plant symbioses. *Trends in Plant Science* **1**: 191-197.

Brahamsha, B. 1996. An abundant cell-surface polypeptide is required for swimming by the nonflagellated marine cyanobacterium *Synechococcus*. *Proceedings of National Academy of Science (USA)* **93**: 6504-6509.

Campbell, E.L., Brahamsha, B., and Meeks, J.C. 1998. Mutation of an alternative sigma factor in the cyanobacterium *Nostoc punctiforme* results in increased infection of its symbiotic plant partner, *Anthoceros punctatus*. *Journal of Bacteriology* **180**: 4938-4941.

Campbell, E.L. and Meeks, J.C. 1989. Characterisation of hormogonia formation by symbiotic *Nostoc* spp. in response to the presence of *Anthoceros punctatus* or its extracellular products. *Applied and Environmental Microbiology* **55**: 125-131.

Cohen, M.F. and Meeks, J.C. 1997. A hormogonium regulating locus, hrm UA, of the cyanobacterium *Nostoc punctiforme* strain ATCC 29133 and its response to an extract of a symbiotic partner *Anthoceros punctatus*. *Molecular Plant-Microbe Interactions* **10**: 280-289.

Costa, J.-L., Paulsrud, P., and Lindblad, P. 1999. Cyanobiont diversity within coralloid roots of selected cycad species. *FEMS Microbial Ecology* **28**: 85-91.

Douglas, S. 1994. Chloroplast origins and evolution. In: *The Molecular Biology of Cyanobacteria*. D.A. Bryant, ed. Kluwer Academic Publishers, Dordrecht, Boston, London, pp. 91-118.

Dzelskans,V.A., Szekeres, M., and Mulligan, B.J. 1988. The molecular biology of cyanobacteria. In: *Plant Molecular Biology. A Practical Approach*. C.H. Shawn, ed. IRL Press, Oxford, Washington, DC, pp. 227-299.

Enderlin, C.S. and Meeks, J.C. 1983. Pure culture and reconstitution of the *Anthoceros-Nostoc* symbiotic association. *Planta* **158**: 157-165.

Fuqua, C. and Greenberg, E.P. 1998. Self perception in bacteria: quorum sensing with acylated homoserine lactones. *Current Opinion in Micribiology* **1**: 183-189.

Gantar, M., Kerby, N.W., and Rowell, P. 1993. Colonization of wheat (*Triticum vulgare* L.) by N_2-fixing cyanobacteria: III. The role of a hormogonia-promoting factor. *New Phytologist* **124**: 505-513.

Hedrick, S.M., Cohen, D.I., Nielsen, E.A., and Davis, M.M. 1984. Isolation of cDNA clones encoding T cell-specific membrane associated proteins. *Nature* **308**: 149-153.

Hoiczyk, E. and Baumeister, W. 1997. Oscillin, an extracellular, Ca^{2+}-binding glycoprotein essential for the gliding motility of cyanobacteria. *Molecular Microbiology* **26**: 699-708.

Håvarstein, L.S. and Morrison, D.A. 1999. Quorum sensing and peptide pheromones in streptococcal competence for genetic transformation. In: *Cell-Cell Signalling in Bacteria*. G.M. Dunny and S.C. Winans, eds. ASM Press, Washington, pp. 9-26.

Johansson, C. and Bergman, B. 1992. Early events during the establishment of the *Gunnera-Nostoc* symbiosis. *Planta* **188**: 403-413.

Johansson, C. and Bergman, B. 1994. Reconstitution of the symbiosis of *Gunnera manicata* Linden: cyanobacterial specificity. *New Phytologist* **126**: 643-652.

Knight, C.D. and Adams, D.G. 1996. A method for studying chemotaxis in nitrogen fixing cyanobacterium-plant symbioses. *Physiological and Molecular Plant Pathology* **49**: 73-77.

Lazazzera, B.A., Palmer, T., Quisel, J., and Grossman, A.D. 1999. Cell-density control of gene expression and development in *Bacillus subtilis*. In: *Cell-Cell Signalling in Bacteria*. G.M. Dunny and S.C. Winans, eds. ASM Press, Washington, pp. 27-46.

Ludwig, A., Jarchau, T., Benz, R., and Goebel, W. 1988. The repeat domain of *Escherichia coli* haemolysin (HlyA) is responsible for its Ca^{2+} dependent binding to erythrocytes. *Molecular and General Genetics* **214**: 553-561.

Margulis, L., ed. 1970. *Origin of Eucaryotic Cells*. Yale University Press.

Meeks, J.C. 1998. Symbiosis between nitrogen-fixing cyanobacteria and plants. *Bioscience* **48**: 266-277.

Nilsson, M., Bergman, B., and Rasmussen, U. 2000. Cyanobacterial diversity in geographically related and distant host plants of the genus *Gunnera*. *Archives of Microbiology* **173**: 97-102.

Perego, M. 1999. Self-signalling by Phr peptides modulates *Bacillus subtilis* development. In: *Cell-Cell Signalling in Bacteria*. G.M. Dunny and S.C. Winans, eds. ASM Press, Washington, pp. 243-258.

Rai, A.N., Söderbäck, E., and Bergman, B. 2000. Cyanobacterium-plant symbioses. *New Phytologist* **147**: 449-481.

Rasmussen, U., Johansson, C., and Bergman, B. 1994. Early communication in the *Gunnera-Nostoc* symbiosis: plant induced cell differentiation and protein synthesis in the cyanobacterium. *Molecular Plant-Microbe Interactions* **6**: 696-702.

Rasmussen, U. and Svenning, M.M. 1998. Fingerprinting of cyanobacteria based on PCR with primers derived from short and long tandemly repeated repetitive sequences. *Applied and Environmental Microbiology* **64**: 265-272.

Reeve, W.G., Tiwari, R.P., Dilworth, M.J., and Glenn, A.R. 1999. *Sinorhizobium meliloti* activates lpiA after exposure to low pH. (Unpublished)

Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M., and Stanier, R.Y. 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *Journal of General Microbiology* **111**: 1-61.

Rudi, K., Skulberg, O.M., Larsen, F., and Jacobsen, K.S. 1997. Strain characterization and classification of oxyphotobacteria in clone cultures on the basis of 16S rRNA sequences, from the variable regions V6, V7, and V8. *Applied and Environmental Microbiology* **63**: 2593-2599.

Sambrook, J., Fritsch, E.F., and Maniaitis, T. 1989. *Molecular Cloning, a Laboratory Manual.* Cold Spring Harbor Laboratory Press, NY.

Scheu, A.K., Economou, A., Hong, G.F., Ghelani, S., Johnston, A.W., and Downie, J.A. 1992. Secretion of the *Rhizobium leguminosarum* nodulation protein NodO by haemolysin-type systems. *Molecular Microbiology* **6**: 231-238.

Schussler, A., Meyer, T., Gehrig, H., and Kluge, M. 1997. Variation of lectin binding sites in extracellular glycoconjugates during the life cycle of *Nostoc punctiforme*, a potentially endosymbiotic cyanobacterium. *European Journal of Phycologyogy* **32**: 233-239.

Stanier, R.Y., Kunisawa, R., Mandel, M., and Cohen-Bazire, G. 1971. Purification and properties of unicellular blueeu-green algae (order Chroococcales). *Bacteriological Reviews* **35**: 171-205.

Tandeau de Marsac, N. 1994. Differentiation of hormogonia and relationship with other biological processes. In: *The Molecular Biology of Cyanobacteria.* D.A. Bryant, ed. Kluwer Academic Publishers, pp. 825-842.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673-4680.

Viterbo, A., Matveyev, A., Rasmussen, U., and Bergman, B. 1999. Characterization of a nodM/glmS homologous gene in the symbiotic cyanobacterium *Nostoc* PCC 9229. *Symbiosis* **26**: 237-246.

Yoon, H-S. and Golden, J.W. 1998. Heterocyst pattern formation controlled by a diffusible peptide. *Science* **282**: 935-938.

Zheng, WW., Nilsson, M., Bergman B., and Rasmussen, U. 1999. Genetic diversity and classification cyanobacteria in different *Azolla* species by the use of PCR fingerprinting. *Theoretical and Applied Genetics* **99**: 1187-1193.