Establishment of a functional symbiosis between the cyanobacterium *Nostoc punctiforme* and the bryophyte *Anthoceros punctatus* requires genes involved in nitrogen control and initiation of heterocyst differentiation

Francis C. Y. Wong and John C. Meeks

Three mutant strains (*ntcA*, *hetR*, *hetF*) of the cyanobacterium *Nostoc punctiforme* unable to differentiate heterocysts were characterized and examined for their ability to form a symbiotic association with the bryophyte *Anthoceros punctatus*. Previously unknown characteristics of the *N. punctiforme* *hetR* mutant include differentiation of chilling-resistant akinetes, while vegetative cells of the *ntcA* mutant randomly lysed, yielding short filaments, following ammonium deprivation. Strains with mutations in *hetF* and *hetR* infected *A. punctatus* with similar frequency to that of wild-type *N. punctiforme* but did not support growth of the plant partner. These results confirm that the infection of *A. punctatus* by hormogonia leading to the establishment of an association is physiologically uncoupled from the development of a functional diazotrophic association. They also indicate that heterocyst regulatory elements downstream from HetR and HetF are required in both free-living and symbiotic heterocyst differentiation and nitrogenase expression. A strain with a mutation in the global nitrogen regulator *ntcA* did not infect *A. punctatus* despite its ability to differentiate hormogonia at a low frequency. When complemented with one or more copies of *ntcA*, the mutant strain infected *A. punctatus* at a similar frequency as the wild-type and supported growth of the plant partner in the absence of combined nitrogen. These results established a connection between the presence of a functional copy of *ntcA* and the magnitude of hormogonium differentiation, and the behaviour of the formed hormogonia.

**Keywords:** cellular differentiation, cyanobacteria, nitrogen control, nitrogen fixation

**INTRODUCTION**

The oxygenic phototrophic cyanobacterium *Nostoc punctiforme* and related members of the unbranched filamentous order Nostocales have exceptionally diverse morphological, physiological and ecological properties (Castenholz & Waterbury, 1989). *N. punctiforme* vegetative cells have three developmental alternatives in response to environmental changes. The first and most well-known developmental alternative is the patterned differentiation of about 8% of the vegetative cells into nitrogen-fixing heterocysts when combined nitrogen is limited (Wolk, 2000; Wolk et al., 1994). Second, under conditions of energy limitation such as low light intensity or phosphate starvation, vegetative cells can differentiate into spore-like akinetes for survival through unfavourable growth conditions (Adams & Duggan, 1999). Akinetes are suggested to be the evolutionary precursors of heterocysts (Wolk et al., 1994). Third,
following the sensing of a positive or negative stress signal for growth, all vegetative cells of a filament can divide uncoupled from biomass increase and DNA replication to form small-celled, relatively short, motile filaments called hormogonia, which lack heterocysts and function in short-distance dispersal (Tandeau de Marsac, 1994). The completion of a hormogonium developmental cycle is marked by the resumption of cellular growth and DNA replication, and by differentiation of heterocysts when nitrogen is limited.

Many Nostoc strains, including N. punctiforme, also form diazotrophic symbiotic associations with a variety of terrestrial plants (Meeks, 1998). Differentiation and behaviour of hormogonia and heterocysts are altered in the symbiotic growth state. Diazotrophic cyanobacteria–plant symbioses have similarities to associations of rhizobia with leguminous plants. In both cases, the plant partners provide the symbionts with reduced carbon in exchange for fixed nitrogen (Meeks et al., 1985; Steinberg & Meeks, 1991; Vance et al., 1998). However, cyanobacteria–plant interactions are primarily unidirectional from plant to cyanobacterial partner (Meeks, 1998), in contrast to the complex signal exchanges between both partners that define the rhizobia–legume plant associations (Oke & Long, 1999; Schultz & Kondorosi, 1998).

We are interested in identifying the interactions between the bryophyte hornwort Anthoceros punctatus and N. punctiforme leading to the control of cyanobacterial growth, metabolism and cellular differentiation in the formation of a stable symbiotic association. Analogous to the characterizations of rhizobia–plant associations, the N. punctiforme–A. punctatus interactions can be defined in two stages. In the first, or infective, stage, the symbiotic association is established following invasion of pre-existing cavities in A. punctatus gametophyte tissue by motile hormogonia of N. punctiforme. The plant partner both stimulates the formation of hormogonia (Campbell & Meeks, 1989), and produces signals that affect the chemotactic motility of the hormogonia (Knight & Adams, 1996). A. punctatus also influences the behaviour of hormogonia through regulation of as yet unidentified genes (Campbell et al., 1998) and activates repressive mechanisms in N. punctiforme that block subsequent hormogonium formation once the cavities have been colonized (Cohen & Meeks, 1997). The second stage of interaction results in the formation of a functional, effective, diazotrophic association. The functional symbiotic growth state is characterized by negative regulation of N. punctiforme growth and metabolism (Meeks, 1998), and an increase in the frequency of heterocyst differentiation (Enderlin & Meeks, 1983) and the rate of nitrogen fixation (Steinberg & Meeks, 1989), coupled with the release of fixed nitrogen as ammonium (Joseph & Meeks, 1987; Meeks et al., 1985). The enhanced frequency of symbiotic heterocyst differentiation appears to be independent of direct nitrogen control (Campbell & Meeks, 1992).

These observations imply that there is a set of Nostoc spp. genes that respond to the initial plant signals affecting hormogonium formation and behaviour, leading to the establishment of the cyanobacterial colony in the plant tissue. A second separate set of genes is present in symbiotically competent Nostoc strains and responds to the growth conditions in the plant, resulting in enhanced heterocyst differentiation and nitrogen fixation, as well as other physiological changes in the symbiotic growth state. Therefore, we hypothesize that heterocyst differentiation and nitrogen fixation are dispensable during the initial establishment of cyanobacterial–plant symbiosis. This hypothetical condition is analogous to naturally occurring non-effective rhizobia–legume associations that are more parasitic than mutualistic in nature (Denison, 2000).

In the present study, we characterized three mutant N. punctiforme strains unable to differentiate heterocysts (Het−) and examined their ability to form a symbiotic association with the plant partner A. punctatus. The three mutants have defects in ntcA, betR and betF. ntcA encodes a DNA-binding protein in the Crp family of bacterial transcriptional regulators. NtcA functions as a nitrogen-dependent global regulator in cyanobacteria and controls the transcription of a number of genes expressed in the entire course of heterocyst development (Herrero et al., 2001; Fiedler et al., 2001). Transcription of betR is indirectly dependent on ntcA. betR is temporally the first heterocyst-specific gene induced in the developmental cascade and is considered to be the primary activator of heterocyst differentiation (Wolk, 2000). HetR exhibits a serine-dependent autoprotease activity (Zhou et al., 1998), regulates its own transcription (Black et al., 1993; Buikema & Haselkorn, 2001), and is sufficient to induce heterocyst differentiation when ectopically expressed under nitrogen-replete conditions (Buikema & Haselkorn, 2001). Transcription of betR is enhanced in specific cells following combined nitrogen deprivation (N step down), and HetR accumulates in heterocysts (Black et al., 1993; Wong & Meeks, 2001). HetF is an ancillary positive activator of heterocyst differentiation that functions in the enhanced transcription of betR and the localization of HetR to differentiating cells (Wong & Meeks, 2001).

The results of this study provide evidence that the differentiation and behaviour of hormogonia are necessary and sufficient in the initial establishment of symbiosis, and that currently known genes essential for the initiation of heterocyst differentiation in the free-living growth state are also required for symbiosis-dependent heterocyst differentiation.

**METHODS**

**Bacterial cultures and media.** Wild-type and mutant strains of N. punctiforme (Table 1) were grown in liquid Allen and Arnon medium diluted fourfold (AA/4) and on solidified AA medium plates as previously described (Cohen et al., 1994). When necessary, the medium was supplemented with 2.5 mM NH₄Cl buffered with 5 mM MOPS (pH 7.8). Neomycin at 25 µg ml⁻¹, ampicillin at 10 µg ml⁻¹, and erythromycin at...
Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference</th>
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<tr>
<td><strong>N. punctiforme</strong> strains</td>
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<tr>
<td>ATCC 29133</td>
<td>Wild-type</td>
<td>Rippka &amp; Herdman (1992)</td>
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<tr>
<td>UCD 415</td>
<td>betR::Tn5-1063</td>
<td>This study</td>
</tr>
<tr>
<td>UCD 416</td>
<td>betF::Tn5-1063</td>
<td>Wong &amp; Meeks (2001)</td>
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<tr>
<td>UCD 438</td>
<td>Wild-type with pSCR30 integrated into the chromosome; wild-type ntcA and ntcA:: Ω-npt</td>
<td>This study</td>
</tr>
<tr>
<td>UCD 444</td>
<td>ntcA:: Ω-npt</td>
<td>This study</td>
</tr>
<tr>
<td>UCD 454</td>
<td>UCD 444 carrying pSCR57</td>
<td>This study</td>
</tr>
<tr>
<td>UCD 463</td>
<td>Wild-type with pSCR74 integrated into the chromosome; wild-type hetR and hetR::Tn5-1063</td>
<td>This study</td>
</tr>
<tr>
<td>UCD 464</td>
<td>betR::Tn5-1063, reconstructed</td>
<td>This study</td>
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<tr>
<td><strong>E. coli strain</strong></td>
<td></td>
<td></td>
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<tr>
<td>DH5α-MCR</td>
<td>Methylation-dependent restriction-defective derivative of strain DH5α; used for general cloning</td>
<td>Gibco-BRL</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pBluescript KS(+)</td>
<td>Cloning vector; Ap'</td>
<td>Stratagene</td>
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<tr>
<td>pAM996</td>
<td>4·3 kb region containing Anabaena 7120 ntcA in pBS; Ap'</td>
<td>Wei et al. (1993)</td>
</tr>
<tr>
<td>pRL271</td>
<td>Conjugatable non-replicating cyanobacterial vector containing sacB; Em' Cm'</td>
<td>Cai &amp; Wolk (1990)</td>
</tr>
<tr>
<td>pSCR9</td>
<td>Ω-npt cassette source vector; Nm' / Km'</td>
<td>Cohen &amp; Meeks (1997)</td>
</tr>
<tr>
<td>pSCR25</td>
<td>Interposon plasmid recovered from EcoRI-digested genomic DNA of strain UCD 415; Nm'</td>
<td>This study</td>
</tr>
<tr>
<td>pSCR27</td>
<td>4·8 kb AccI fragment subcloned from cosmid clone 5E11 into pBS hybridized to ntcA probe; Ap'</td>
<td>This study</td>
</tr>
<tr>
<td>pSCR28</td>
<td>1·2 kb HindIII–EcoRV fragment subcloned from pSCR27 into pBS containing ntcA as the only ORF; Ap'</td>
<td>This study</td>
</tr>
<tr>
<td>pSCR29</td>
<td>Ω-npt cassette ligated into internal MfeI site of ntcA in pSCR 27; Ap'</td>
<td>This study</td>
</tr>
<tr>
<td>pSCR30</td>
<td>6·6 kb SpeI–Xhol insert from pSCR29 ligated into corresponding sites in pRL271; Em' Cm' Nm'/Km'</td>
<td>This study</td>
</tr>
<tr>
<td>pSCR57</td>
<td>1·2 kb SalI–EcoRI insert from pSCR28 cloned into the same sites of pSCR202</td>
<td>This study</td>
</tr>
<tr>
<td>pSCR74</td>
<td>EcoRI-digested tnp-inactivated pSCR25 cloned into Ecl136II-digested pRL271; Nm' Sm' Cm' Em'</td>
<td>This study</td>
</tr>
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</table>

15 µg ml⁻¹ were used for the selection and maintenance of recombinant N. punctiforme strains.

Escherichia coli strain DH5α-MCR (Table 1) and derivatives were grown in Luria–Bertani (LB) broth supplemented with antibiotics at the following concentrations: kanamycin at 25 µg ml⁻¹; ampicillin at 100 µg ml⁻¹; and chloramphenicol at 30 µg ml⁻¹.

Plant cultures and reconstitution of symbiosis. A. punctatus gametophyte tissue was grown in basal medium (Enderlin & Meeks, 1983) buffered with 5 mM MES (pH 6·4) and supplemented with 0·5% (w/v) glucose. To reconstitute the symbiotic associations, A. punctatus tissue was first nitrogen-starved in glucose-supplemented basal medium minus ammonium nitrate for 7–10 d. Mutant and wild-type strains of N. punctiforme at a total chlorophyll a content of 60 µg were then combined with the nitrogen-starved A. punctatus tissue in the same conditioned medium. The percentage of hormogonium filaments in the population was scored microscopically 24–48 h after addition of cells to the plant tissue and was estimated as one of four levels as previously described (Campbell & Meeks, 1989): no differentiation (0%), low (5–15%), intermediate (40–60%), and high (90–100%). Symbiotic colonies were counted with the aid of a dissecting microscope after co-culturing for 2 weeks. For some reconstitution experiments involving the ntcA mutant strain UCD
444 and the corresponding wild-type control, the conditioned medium was supplemented with 2.5 mM NH₄Cl after the cultures were established. To test whether the betR or betF mutant symbiotic colonies established by the first round of infection were viable, the reconstitution medium was supplemented with 15 mM nitrate (equimolar sodium and potassium salt) after the initial 2 week co-culture period. Nitrogenase activity of the symbiotic associations was measured by acetylene reduction assay as previously described (Enderlin & Meeks, 1983).

**Akinete viability assay.** To specifically induce akinetes, wild-type and betR mutant strains of *N. punctiforme* cultures were incubated in AA/4 medium minus phosphate for 2 weeks. Akinete differentiation was confirmed by light microscopy. The cultures were then incubated for 10 d in the dark at 4°C without shaking. Filaments of each culture typically shortened to about 10–20 cells per filament after incubation. Equal numbers of c.f.u. (a c.f.u. being defined as an individual colony) formed by contract using ABI Prism Sequencers and the dye reagents from Amersham Scientific. The probe for *N. punctiforme* was generated by PCR using primers PntcA1 (5’-TTCATGTTTGTGTCGTAAATCTTC-3’) and PntcA2 (5’-GGTTATGGACCTGTAATCTTC-3’). DNA hybridization analyses were performed with GeneScreen Plus membrane in 50% formamide hybridization buffers as previously described (Summers et al., 1995).

**Isolation and characterization of hetF and betR mutant strains.** Methods for transposon mutagenesis of *N. punctiforme* with Tn5-1063 and the corresponding wild-type control, the conditioned medium was supplemented with 15 mM nitrate (equimolar sodium and potassium salt) after the initial 2 week co-culture period. Nitrogenase activity of the symbiotic associations was measured by acetylene reduction assay as previously described (Enderlin & Meeks, 1983).

**Molecular biology procedures.** Plasmids used in this study are shown in Table 1. Preparations and manipulations of plasmid DNA from *E. coli* were done using standard methods (Sambrook et al., 1989). Large-scale plasmid purifications from *E. coli* were carried out using a commercial kit (Qiagen). DNA restriction and modification enzymes were obtained from New England Biolabs or Gibco-BRL and used according to the manufacturers’ instructions. Total cyanobacterial genomic and plasmid DNA were isolated as previously described (Cohen et al., 1994). DNA sequencing was performed by contract using ABI Prism Sequencers and the dye reagents from Amersham Scientific. The probe for *N. punctiforme* was generated by PCR using primers PntcA1 (5’-GTATTTCTGCAAGATGCGACC-3’) and PntcA2 (5’-GGTTTATGGACCTGTAATCTTC-3’). DNA hybridization analyses were performed with GeneScreen Plus membrane in 50% formamide hybridization buffers as previously described (Summers et al., 1995).

**Isolation and characterization of hetF and betR mutant strains.** Methods for transposon mutagenesis of *N. punctiforme* with Tn5-1063 and the corresponding wild-type control, the conditioned medium was supplemented with 15 mM nitrate (equimolar sodium and potassium salt) after the initial 2 week co-culture period. Nitrogenase activity of the symbiotic associations was measured by acetylene reduction assay as previously described (Enderlin & Meeks, 1983).

**RESULTS AND DISCUSSION**

**Phenotypes of hetF and betR mutant strains in the free-living growth state.**

Both betF (strain UCD 416) and betR (strain UCD 415) mutant strains of *N. punctiforme* were derived by transposon mutagenesis using Tn5-1063 and selected by screening of colony bleaching upon N step-down (Fig. 1 and Wong & Meeks, 2001). Both mutants grew normally as vegetative cell filaments when supplemented with nitrate or ammonium. When deprived of combined nitrogen, neither strain showed morphological evidence for the initiation of heterocyst differentiation. Although

strain UCD 463, in which pSCR74 was integrated into the chromosome, was isolated by neomycin selection. Strain UCD 464, in which pRL271 and the wild-type betR were eliminated via a second recombination event, was selected for by the presence of neomycin and 5% (w/v) sucrose.

**Construction and complementation of the ntcA mutant.** *N. punctiforme* ntcA was identified in a 4.8 kb AccI fragment of the genomic cosmid SE11 (Cohen et al., 1994) by Southern hybridization using a 760 bp probe containing *Anabaena* sp. strain PCC 7120 *ntcA* from the plasmid pAM996 (Wei et al., 1993). The fragment was cloned into pBluescript KS (+) (pBS) to form pSCR27. A strongly hybridizing 1.2 kb EcoRV–HindIII fragment of pSCR27 was subcloned into pBS to form pSCR28, whose insert sequence contains the complete ntcA gene. To construct ntcA mutant strain UCD 444, ntcA was inactivated in pSCR27 by insertion of an omega neomycin phosphotransferase gene cassette (Ω-ntp); from the plasmid pSCR9 into an internal MfeI site to form pSCR29 (Fig. 1). The insert in pSCR29 was excised with SpeI and Xhol and was ligated into XhoI-digested pRL271 to form pSCR30, which was then co-transformed into wild-type *N. punctiforme* via triparental mating (Cohen et al., 1994). Strain UCD 438, in which pSCR30 was integrated into the genome, was selected by the presence of neomycin and maintained in medium containing erythromycin and neomycin. Strain UCD 444, in which pRL271 and the wild-type ntcA were eliminated via a second recombination event, was selected for by the presence of neomycin and 5% (w/v) sucrose. Restriction mapping followed by Southern hybridization confirmed elimination of the wild-type gene and replacement with the introduction of the ntcA gene (data not shown). To complement the ntcA mutation in *trans*, the 1.2 kb insert from pSCR28 was excised with Sall and EcoRI and ligated to the same sites of the *N. punctiforme* replicating plasmid pSCR202, yielding pSCR57. *N. punctiforme* maintains 15 copies of pSCR202-based plasmids per chromosome (Summers et al., 1995). pSCR57 was then transformed by electroporation into strain UCD 444 according to a previously described protocol (Summers et al., 1995), yielding strain UCD 462.

**Nucleotide sequence analysis and accession numbers.** Protein sequence alignments were carried out using the GAP program in SeqWeb v.1.2 (Genetics Computer Group). Similarity searches were carried out using the BLAST program (Altschul et al., 1997) against the databases available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST) and the Joint Genome Institute website (http://www.jgi.doe.gov) for the unfinished *N. punctiforme* genome sequence. The GenBank accession numbers for the coding regions of *ntcA*, betR and betF are AY038370, AF318069 and AF288130, respectively.
vegetative cells rapidly bleached to a yellow-brown colour, the filaments remained intact (Fig. 2a) and viable for at least 1 month in liquid medium without combined nitrogen, as evidenced by a regreening of the cultures following the addition of combined nitrogen.

Complementation in trans and reconstruction of the hetF mutant was previously reported (Wong & Meeks, 2001). The hetR mutation in strain UCD 415 was complemented both with the wild-type gene in trans, and with a full-length HetR–green fluorescent protein fusion (data not shown). The N. punctiforme HetR sequence shares 94% and 88% identity with those of Nostoc sp. strain PCC 9229 and Anabaena PCC 7120, respectively. The N. punctiforme hetR gene has similar transcriptional characteristics as Anabaena PCC 7120 betR (Wong & Meeks, 2001). The hetR mutation was reconstructed as strain UCD 464, which exhibited the same Het” phenotype as strain UCD 415. All Tn5-1063-induced mutants of N. punctiforme characterized to date show fidelity to the transposition events (Campbell et al., 1996, 1997; Cohen & Meeks, 1997; Wong & Meeks, 2001), in contrast to results with Anabaena PCC 7120 (Ernst et al., 1992; Liang et al., 1992), where secondary mutations and not the transposition events can cause the mutant phenotype.

When starved for phosphate in the presence of nitrate, hetR mutant strains UCD 415 and UCD 464 formed large cells similar in overall structure to akinetes, but lacking their characteristic granular appearance (Fig. 2c, d). hetR mutants of Nostoc ellipsosporum were reported to be defective in both heterocyst and akinete differentiation (Léganes et al., 1994), suggesting a common developmental pathway for the two cell types, bifurcating downstream of HetR (Wolk, 2000). Akinetes of N. punctiforme are more resistant to prolonged incubation at low temperature than are vegetative cells (Michael Summers, personal communication). To test the chilling resistance of the hetR mutant akinete-like cells, exponentially growing wild-type vegetative filaments, wild-type akinetes and strain UCD 464 akinete-like cells were assayed for viability after incubation for 10 d in the dark at 4 °C. The plating efficiency of the exponential-phase culture was 0–1%, while cultures of the wild-type induced akinetes and strain UCD 464

Fig. 1. Physical maps of the 1183 bp region containing ntcA and the 3776 bp region containing hetR. Orientations of the open reading frames are indicated by arrows. The inverted triangles show either Tn5-1063 or Ω-npt insertions as indicated. Selected restriction sites are shown: EcoRI (EI), EcoRV (EV), HindIII (H), MfeI (M) and PvuI (P).

Fig. 2. Photomicrographs of filaments from wild-type and mutant strains of N. punctiforme. Intact vegetative cell filament of hetR mutant strain UCD 415 (a) and fragmenting filaments of ntcA mutant strain UCD 444 (b) observed after 36–48 h in medium free of combined nitrogen. Wild-type akinetes (c) and hetR mutant strain UCD 464 akinete-like cells (d) observed after a 2 week incubation in medium without phosphate. Notice the lack of granular texture in the akinete-like cells in (d) in contrast to (c). Bar, 10 μm.
Phenotype of the ntcA mutant strain in the free-living growth state

The \textit{N. punctiforme} ntcA mutant strain UCD 444 was constructed by replacing the wild-type \textit{ntcA} with one interrupted by an \textit{Ω-npt} cassette (Fig. 1). Similar to \textit{Anabaena} PCC 7120 \textit{ntcA} mutants (Frias \textit{et al.}, 1994; Wei \textit{et al.}, 1994), strain UCD 444 failed to grow on nitrate and initiate heterocyst differentiation. These defects are consistent with the loss of a positive global regulator of nitrogen assimilation. Strain UCD 444 carrying multiple copies of \textit{ntcA} (strain UCD 462) differentiated heterocysts at the wild-type frequency and spacing pattern. This was in contrast to the over-production of heterocysts that occurred when the copy number of \textit{betF} or \textit{hetR} was elevated in \textit{trans} (Wong & Meeks, 2001), and is consistent with the role of NtcA in general nitrogen regulation and not in the specific activation of heterocyst differentiation.

When shifted to medium without ammonium, vegetative cells of strain UCD 444 randomly lysed, so that the filaments became short, debris accumulated in the medium (Fig. 2b), and the culture remained a light green colour. This behaviour is in contrast to the rapid bleeding and non-lytic response of the \textit{betF} and \textit{hetR} mutants to nitrogen starvation. A similar non-bleaching and lytic response has been reported for a \textit{ntcA} mutant strain of the unicellular, non-diazotrophic \textit{Synechococcus} sp. strain PCC 7942 (Sauer \textit{et al.}, 1999) and may indicate a common signalling pathway shared between chlorosis and induction of heterocyst differentiation.

Symbiotic phenotypes of the \textit{hetF} and \textit{hetR} mutants

\textit{betF} mutant strain UCD 416 and \textit{hetR} mutant strain UCD 464 differentiated an intermediate level (40–60\%) of motile hormogonia in co-cultures with nitrogen-starved \textit{A. punctatus} tissue, which secretes an as yet unidentified hormogonium-inducing factor (HIF) into the nutrient-limited growth medium (Campbell & Meeks, 1989). These strains infected \textit{A. punctatus} tissue at a frequency similar to that of wild-type \textit{N. punctiforme} during co-culture in the absence of combined nitrogen (Table 2). However, neither strain supported the \textit{N}_{2}-dependent growth of the plant partner. The symbiotic colonies, which in the wild-type strain are dark green, remained yellow-brown, and the plant tissue slowly lost pigmentation (Fig. 3a). No nitrogenase activity, as analysed by the acetylene reduction assay, was detected from these bleached colonies (data not shown). Viability of the symbiotic colonies of the two mutants and of \textit{A. punctatus} could be restored by the addition of 15 mM nitrate, as evidenced by the dark greening of the colonies and the plant tissue (Fig. 3b). These results confirm our hypothesis that the infection of \textit{A. punctatus} by hormogonia leading to the initial establishment of an association is physiologically uncoupled from the development of a functional diazotrophic association. Whether such non-effective cyanobacterial associations occur in the natural habitat of \textit{A. punctatus} is unknown.

We previously established that \textit{N. punctiforme} Fox\textsuperscript{-}mutants with defects in the heterocyst wall could infect \textit{A. punctatus}, fix nitrogen, and support dinitrogen-dependent growth of the plant partner (Campbell \textit{et al.}, 1996, 1997). A conclusion from those studies was that the oxygen-sensitive mutant phenotype can be physiologically complemented by the nearly anoxic environment of the symbiotic cavity (Campbell & Meeks, 1992). Nevertheless, heterocyst differentiation, even if defective, remained a prerequisite for symbiotic nitrogen expression. The results with the \textit{betR} and \textit{hetF} mutants verify the requirement for heterocyst differentiation in nitrogen expression (Elhai & Wolk, 1990). They are also consistent with the lack of a gene cluster encoding a vegetative-cell nitrogenase in the genome sequence of \textit{N. punctiforme} (http://www.jgi.doe.gov). Such a vegetative-cell nitrogenase functions under microoxic conditions in some heterocyst-forming cyanobacteria, such as \textit{Anabaena variabilis} (Thiel \textit{et al.}, 1995).

Table 2. Infection frequencies of wild-type and mutant strains of \textit{N. punctiforme}

<table>
<thead>
<tr>
<th>Strain</th>
<th>Infection frequency*</th>
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<tbody>
<tr>
<td>ATCC 29133 (wild-type)</td>
<td>0.28 ± 0.05 (5); 0.13 ± 0.01 (2)†</td>
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<tr>
<td>UCD 416 (betF)</td>
<td>0.26 ± 0.06 (3)</td>
</tr>
<tr>
<td>UCD 464 (hetR)</td>
<td>0.36 ± 0.04 (4)</td>
</tr>
<tr>
<td>UCD 444 (ntcA)</td>
<td>0.36 ± 0.05 (3)</td>
</tr>
<tr>
<td>UCD 438 (ntcA')</td>
<td>0.37 ± 0.07 (5)</td>
</tr>
<tr>
<td>UCD 462 (ntcA')</td>
<td>0.37 ± 0.07 (5)</td>
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</table>

* Values are the mean number ± st of symbiotic colonies per mg dry weight of tissue per unit of \textit{N. punctiforme} cells added (as µg chlorophyll a). Symbioses were reconstituted in the absence of combined nitrogen unless otherwise noted. Number of repetitions is indicated in parentheses.

† Reconstitutions in the presence of 2.5 mM ammonium chloride.
The nitrogen-independent enhanced frequency of heterocysts observed in symbiosis is hypothetically a consequence of a symbiosis-specific signalling system responding to plant-produced signals (Campbell & Meeks, 1992). This system is predicted to supersede the nitrogen signalling system which responds to nitrogen limitation and initiates heterocyst differentiation in the free-living growth state (Meeks, 1998). The nature of the symbiotic signal and where it might enter the developmental cascade is unknown. Based on the results with the betR and betF mutants, both free-living and symbiotic heterocyst differentiation share a common developmental pathway. Because a functional NtcA is required for the transcription of genes expressed throughout heterocyst development (Herrero et al., 2001; Fiedler et al., 2001), we speculate that the symbiotic signal should enter the developmental cascade at or prior to ntcA. Presently, no genes nor enzyme activities are known to be involved in the initiation of heterocyst differentiation cascade prior to ntcA.

Symbiotic phenotype of the ntcA mutant

In contrast to the betF and betR mutants, ntcA mutant strain UCD 444 responded with markedly less intensity than the wild-type in the differentiation of hormogonia in co-cultures with A. punctatus. The low level (5–15%) of hormogonia formed had the typical morphology and gliding properties of wild-type hormogonia. However, strain UCD 444 failed to infect A. punctatus tissue in multiple trials of co-cultures in the absence or presence of 2–5 mM ammonium chloride (Table 2). Ammonium was added periodically during these experiments to ensure viability of the ntcA mutant and increase the probability of an infection event. Such a supplementation was not necessary with the infective betR and betF mutant strains. Epiphytic growth of strain UCD 444 was present on the gametophyte tissue at the end of the 2 week co-culture period, indicating that the ammonium level was sufficient to maintain viability. Sufficient time (up to 1 month) was allowed in the co-culture experiments with strain UCD 444 that any delayed infection and formation of small colonies in the absence or presence of ammonium would have been detected microscopically. When ammonium was not included in the co-cultures, strain UCD 444 lysed as it did in nitrogen-limited culture in the absence of A. punctatus. Wild-type Nostoc strains, including N. punctiforme, can infect A. punctatus in the presence of 1–5 mM ammonium, but at a lower frequency (Table 2; Enderlin & Meeks, 1983). The colonies established in the presence of ammonium are small and have low nitrogenase activity.

The non-infective phenotype of strain UCD 444 was complemented by the presence of one or more copies of ntcA. Both strain UCD 438, which carries a wild-type and a mutant copy of ntcA in the genome, and strain UCD 462, which is strain UCD 444 carrying a wild-type copy of ntcA on a multicopy replicating plasmid (pSCR57), yielded wild-type infection frequencies and supported growth of the A. punctatus association in the absence of combined nitrogen (Table 2). Thus, the non-infective phenotype of strain UCD 444 can be ascribed to the mutation of ntcA and not to an unknown and undetected genetic lesion. However, the lack of infection by the ntcA mutant precludes any attempts to define a regulatory role for NtcA in the enhanced frequency of symbiotic heterocyst differentiation.

The infection-defective phenotype of ntcA mutant strain UCD 444 was unexpected. NtcA is a relatively well-characterized global nitrogen regulator in cyanobacteria (Herrero et al., 2001). There is no previous evidence suggesting that hormogonium development is regulated by nitrogen control. Hormogonium differentiation induced by HIF is insensitive to the absence or presence of ammonium (Campbell & Meeks, 1989), and hormogonia will infect A. punctatus in the presence of nitrate or ammonium (Table 2; Enderlin & Meeks, 1983). Therefore, the reduced frequency of hormogonium differentiation in strain UCD 444 does not extrapolate to the failure of the formed hormogonia to infect A.
**punctoratu**: the expected number of infections would be lower, but should still be greater than zero. Nevertheless, the results establish a connection between a functional copy of ntcA, the extent of hormogonium differentiation and the behaviour of the formed hormogonia.

There is precedence for both low and highly infective hormogonia from natural Nostoc isolates and mutant strains. *Nostoc* sp. strain ATCC 27896 (PCC 6320) converts essentially 100% of its filaments into motile hormogonia in the presence of HIF, but the hormogonia have a low and delayed infection frequency of *A. punctatus* (Meeks, 1998). When reisolated from *A. punctatus* tissue and again co-cultured, the same rare, delayed infection is observed. Thus, defective infection appears to be a genetic property of the strain, perhaps resulting from insensitivity to chemotactic signals. Conversely, mutation of a gene encoding an alternative group 2 sigma subunit of RNA polymerase (sigH) in *N. punctiforme* results in a strain that has a sixfold higher infection frequency of *A. punctatus* than the wild-type (Campbell *et al*., 1998). The sigH mutant does not differentiate more hormogonia, rather it is the behaviour of the hormogonia that appears to have changed. Collectively, these results indicate that hormogonia differentiation and behaviour in infection of *A. punctatus* tissue involves more complex regulatory circuits than is superficially apparent. Moreover, NtcA appears to be involved in these circuits, suggesting that NtcA may have regulatory roles apart from nitrogen assimilation.

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