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Symbiosis between the cyanobacterium *Nostoc* and the liverwort *Blasia* requires a CheR-type MCP methyltransferase

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Abstract In response to environmental change, the cyanobacterium *Nostoc punctiforme* ATCC 29133 produces highly adapted filaments known as hormogonia that have gliding motility and serve as the agents of infection in symbioses with plants. Hormogonia sense and respond to unidentified plant-derived chemical signals that attract and guide them towards the symbiotic tissues of the host. There is increasing evidence to suggest that their interaction with host plants is regulated by chemotaxis-related signal transduction systems. The genome of *N. punctiforme* contains multiple sets of chemotaxis (*che*)-like genes. In this study we characterize the large *che5* locus of *N. punctiforme*. Disruption of NpR0248, which encodes a putative CheR methyltransferase, results in loss of motility and significantly impairs symbiotic competency with the liverwort *Blasia pusilla* when compared with the parent strain. Our results suggest that chemotaxis-like elements regulate hormogonia function and hence symbiotic competency in this system.

Keywords Symbiosis · Cyanobacteria · *Nostoc* · Liverwort

1 Introduction

Filamentous cyanobacteria of the genus *Nostoc* form symbiotic associations with a wide range of plants, including liverworts and hornworts (Adams et al. 2012; Adams and Duggan 2008, 2011; Bergman et al. 2008). In response to certain environmental stimuli *Nostoc* develops small-celled, motile filaments known as hormogonia, which are essential for infection of the host plant (reviewed by Bergman et al. 2007; Meeks and Elhai 2002; Meeks 2009; Adams and Duggan 2011). Indeed, host plants release hormogonium-inducing factors (Bergman et al. 1996; Campbell and Meeks 1989; Cohen and Meeks 1997; Knight and Adams 1996; Rasmussen et al. 1994; Watts et al. 1999). Hormogonia formation involves the up-regulation of 944 genes, the majority of which appear to be involved in signal transduction and transcriptional activation (Campbell et al. 2007). The hormogonium is therefore highly adapted to sense and respond rapidly to its environment and is specialised for motility, dispersal and the initiation of symbiosis with host plants (e.g. Adams and Duggan 2008; Bergman et al. 2008; Meeks 2009). The surface of *Nostoc punctiforme* hormogonia is covered with pili (fimbriae) that are important for the infection process and may be involved in motility, host recognition and surface attachment (Duggan et al. 2007).

Work in our laboratory (Knight and Adams 1996; Watts et al. 1999) as well as by others (Nilsson et al. 2006; see also Adams and Duggan 2011) has shown that plant-derived signals likely act as chemoattractants, guiding hormogonia towards the sites of infection within the host plant. However, the molecular elements regulating this process have yet to be characterised. One of the best studied chemotaxis systems is the regulation of flagellar-based motility in *Escherichia coli* (Sourjik 2004; Wadhams and Armitage 2004). Transmembrane chemoreceptors (MCPs, methyl-accepting chemotaxis proteins) perceive extracellular stimuli, producing signals that are transmitted to their cytoplasmic domains. These domains regulate an associated two-component phosphotransfer signal transduction system, CheA-CheY (with a linker protein CheW) that controls flagellar rotation by binding to the flagellar motor protein FliM. Additional components of the chemotaxis machinery

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include CheB, a methylesterase, and CheR, a methyltransferase, that modulate the methylation status of MCPs, which affects their sensitivity. CheB, activated through phosphorylation of CheA, removes the methyl groups added to MCPs by CheR, leading to an adaptive response that enables the bacterium to respond to new stimuli in a background containing constant levels of chemoattractants and/or repellents (Bren and Eisenbach 2000; Clausznitzer et al. 2010). Chemotaxis-like genes are widespread among bacteria, and many are known to be involved in signalling pathways that regulate functions distinct from their well established role in the regulation of chemotaxis (reviewed by Kirby 2009). Some examples include the regulation of pili gene expression in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (Bhaya et al. 2001; Chung et al. 2001), virulence gene expression in *Vibrio cholerae* (Lee et al. 2001), biofilm formation (Tran et al. 2008; see also Kirby 2009), fibril biogenesis and expression of developmental genes in *Myxococcus xanthus* (Yang et al. 2000; Kirby and Zusman 2003).

N. punctiforme hormogonia exhibit both chemotactic and phototactic behaviour. Directed motility likely requires a signal transduction system to detect the appropriate attractant in the environment and communicate the information to a motility apparatus. In the *N. punctiforme* genome sequence (completed by the Joint Genome Institute and available at http://www.jgi.doe.gov; Meeks et al. 2001) there are five loci (designated *che1-5*) containing ORFs exhibiting sequence similarity to known chemotaxis (*che*) genes from other bacterial species. Unlike the other loci, the *che5* locus, containing seven *che*-like ORFs (*NpR0244-NpR0250*), appears to have been acquired by horizontal gene transfer (Wuichet and Zhulin 2003). This locus lacks the *cheY*-like genes found in the other 4 loci, but contains genes encoding a response regulator receiver modulated CheB methylesterase (NpR0244) and a tetratricopeptide repeat (TPR)-containing CheR-type MCP methyltransferase (NpR0248). The TPR domain is a structural motif found in a wide range of proteins and mediates protein-protein interactions (Blatch and Lassle 1999). Genes encoding two other CheR proteins and three additional CheB-like proteins have been found elsewhere in the *N. punctiforme* genome. The additional CheB proteins may have different physiological roles as they lack the response regulator receiver domain that is receptive to the phosphoryl group transfer occurring in phospho-relay systems. In this study we characterize the large *che5* locus of *N. punctiforme* showing that the CheR-like NpR0248 protein is required for hormogonia motility and for symbiotic competency, using the liverwort *Blasia pusilla* as host.

2 Methods

Strains and growth conditions N. punctiforme American Type Culture Collection, ATCC 29133 (Pasteur Culture Collection, PCC 73102; a kind gift of Jack Meeks) was originally isolated from a symbiotic association with the cycad *Macrozamia* (Rippka et al. 1979). *N. punctiforme* and its derivatives were grown in BG11 medium (which contains 17.5 mM $NaNO_3$ as combined nitrogen source; Rippka et al. 1979) with shaking at 27 °C with continuous irradiance of 23 $\mu mol m^{-2} s^{-1}$. Recombinant *N. punctiforme* 29133 were selected for resistance to, and maintained in, the presence of 25 $\mu g ml^{-1}$ neomycin (Nm). *E. coli* strains DH5α (plasmid construction) and HB101 (conjugation experiments) were routinely grown in Luria-Bertani medium (Sambrook et al. 1989) supplemented, as appropriate, with antibiotics, ampicillin (Ap; 100 $\mu g ml^{-1}$), kanamycin (Km 50 $\mu g ml^{-1}$) and chloramphenicol (Cm; 170 $\mu g ml^{-1}$). The liverwort *Blasia pusilla* was grown in liquid BG11 medium with shaking at 100 rpm, at 20 °C under a 12 h/12 h light/dark cycle (average light irradiance of 20 $\mu mol m^{-2} s^{-1}$).

Red light induction of hormogonia formation N. punctiforme filaments recovered by centrifugation were washed three times in $BG11_0$ medium (BG11 lacking combined nitrogen) and resuspended in twice the original culture volume of $BG11_0$. Flasks were covered with red cellophane and incubated for 18 h at 27 °C with shaking and a continuous irradiance of 50 $\mu mol m^{-2} s^{-1}$ to allow hormogonia to develop.

Reconstitution of symbiotic associations Nitrogen starved (by growing for 10–14 days in $BG11_0$) *B. pusilla* tissue (2–2.5 cm^2 in diameter and 0.8–0.85 g wet wt) was co-cultured with mutant and wild-type *N. punctiforme* (at a total chlorophyll *a* content of 100 μg) as described previously (Duggan et al. 2007). In the *Blasia* symbiosis the cyanobacteria occupy dome-shaped structures known as auricles on the surface of the thallus. The number of symbiotic colonies (i.e. infected auricles) was determined microscopically after 14 and 28 days of co-culture and the frequency of infection expressed as a percentage of the total number of auricles counted (at least 400 auricles were counted for each determination).

Phototaxis assays Phototactic movement of hormogonia was determined on 0.2 % (w/v) agar-solidified BG11 plates incubated at ambient temperature (24 °C) in a dark room. The plates were stacked on top of one another, placed inside a cardboard box and illuminated, through a vertical 1 cm wide slit in the wall of the box, with continuous unidirectional light (9.8 $\mu mol m^{-2} s^{-1}$) provided by a single vertical Thorn EMI 18 W PLUSLUX 3000 warm white strip lamp.

Insertional mutagenesis To inactivate *cheR*-like *NpR0248*, a *N. punctiforme* DNA fragment of 1281-bp containing the entire target gene was amplified by PCR (HotStar HiFidelity

Polymerase kit, Qiagen) using *cheR* primers (Table 1) and cloned into pGEM®-T Easy vector (Promega). A 1229-bp fragment containing a neomycin phosphotransferase gene cassette (*npt*), conferring Nm^r and Km^r was PCR-amplified from plasmid pRL1063a (Wolk et al. 1991) using *npt* primers containing *Nhe*I restriction sites (underlined in Table 1). The *npt*-containing amplimer was trimmed with *Nhe*I and introduced into the corresponding site (nucleotide position 990) within *NpR0248*. The resulting construct was removed from pGEM®-T Easy by digestion with *Not*I and cloned into the corresponding site of pBluescript SK. Finally, the construct was removed from pBluescript as a *Sac*I-*Xho*I fragment and introduced into the corresponding sites of the suicide vector pRL271 (Cai and Wolk 1990). The inactivated *cheR* allele was introduced into wild-type *N. punctiforme* 29133 using a conjugal-helper *E. coli* strain (strain HB101 containing pRK2013/pRL528) and double recombinants were selected on Nm (25 $\mu g ml^{-1}$) and 5 % (w/v) sucrose as described by Cohen et al. (1994). Mutants were confirmed by PCR analysis of genomic DNA isolated from putative double recombinants using a primer pair (Table 1) that flanks the *Nhe*I site used for insertion of the *npt* cassette within *NpR0248*. Southern blot analysis was also performed, using suicide vector pRL271 and *npt* DNA sequences as probes, employing the DIG-High Prime labelling (DIG-11-dUTP) and colourimetric detection kit (Roche Molecular Biochemicals) according to the manufacturer's protocols.

Examination of pili by electron microscopy Hormogonia were centrifuged and the pellets washed 6× with sterile distilled water. Pellets were suspended in 1 ml of water and 10 µl samples were added to Formvar-coated copper grids. After air-drying, platinum wire (2 cm× 0.2 mm) was evaporated onto the surface of the sample at an angle of 19°. Evaporation was carried out using an Edwards 306A high vacuum coating unit and samples were viewed using a JEOL1200EX transmission electron microscope at 80 kV.

RNA isolation Total RNA was extracted from both hormogonia (induced as described above) and vegetative cell pellets (recovered from 100 ml cultures grown in BG11 medium as described above). Cell pellets were washed twice with TES buffer (10 mM Tris–HCl, 0.1 mM EDTA, 0.5 M NaCl, pH 8.0) and resuspended in 10 ml of RLT buffer, a guanidine-isothiocyanate-containing lysis buffer (supplemented with β–mercaptoethanol) provided with the RNeasy Kit (Qiagen). An equal volume of phenol-chloroform (24:1; pH 4.7), 0.2 % (final concentration) SDS and 10 ml of acid-washed glass beads (diameter 425–600 µm, Sigma G-8772, Sigma-Aldrich) were added. The cells were broken by vortexing at maximum speed using four cycles of 1 min on and 1 min off (on ice). After centrifugation the RNA partitioned in the aqueous phase was purified using the spin columns provided by the RNeasy kit in accordance with the manufacturer's instructions. The procedure included an on-column RNase-free DNase treatment step. Purified RNA was subjected to a second DNase incubation using Turbo RNase-free DNase (Ambion; Applied Biosystems) according to the manufacturer's instructions.

Reverse transcription (RT)-PCR Prior to RT-PCR analysis samples of RNA were used in PCR with each of the primer pairs (Table 1) to confirm that contaminating genomic DNA had been effectively removed and therefore eliminate the possibility of any false-positive reactions. *N. punctiforme* genomic DNA (25 ng) was included as a template for a positive PCR control, as well as in RT-PCR reactions, to verify the thermocycling conditions and primer specificity. Negative controls lacking template were also included in all reactions. RT-PCR was performed using *cheR*-like (*NpR0248*) gene-specific primers (Table 1). A primer set (*rnpB*; Table 1) for the constitutively expressed RNase P RNA gene (Vioque 1997) was used as internal loading control. RT-PCR was performed using One-Step RT-PCR kit (Qiagen) as described by the manufacturer. PCR

Table 1 Primers used in mutant construction and RT-PCR

Target gene	Forward primer	Reverse primer	Product size (bp)
Mutant construction/analysis:			
cheR	ATGGTATTATCTGCGATCGCAAC	CTAAGCTTCTGTTCTAAGACTGG	1281
npt[a]	GTGCCAACATAGCTAGCCAGTATA	CATGGATGCGCGGCTAGCCGCTGC	1229
Diagnostic	AACCACCGCTTCCAAAATTGGAA	CTAAGCTTCTGTTCTAAGACTGG	634/1863[b]
RT PCR primers:			
rnpB	GCAATAGCAACCATACAGAC	CATAAGCCGGGTTCTGTTCT	457
NpR0248 (*cheR*)	ATACCTGCAATCAGAAGC	AACATTGCTCTGCTTGGT	506

[a] NheI restriction enzyme sites generated within each primer are underlined

[b] Wild-type amplimer of 634-bp and a 1863-bp amplimer expected with a double recombinant

products were electrophoresed at 70 V for 2 h on 1 % (w/v) agarose and stained with ethidium bromide (0.5 $\mu g ml^{-1}$).

3 Results and discussion

3.1 Organisation and predicted proteins of the *che5* locus

The *N. punctiforme che5* locus spans approximately 9.8 kb and contains seven predicted open reading frames encoding chemosensory (Che) pathway-like proteins, ordered *cheB-cheA-cheW-mcp-cheR-mcp-cheW* (Fig. 1) as annotated in the *N. punctiforme* genomic database (http://genome.jgi-psf.org/finished_microbes/nospu/nospu.home.html). The gene order and elements found in the *che5* locus are different from those found in any other cyanobacterial genome, leading to the suggestion that the operon was acquired by lateral transfer (Wuichet and Zhulin 2003). The *che5* organisation (and sequence similarity) is analogous to the *Myxococcus xanthus che3* cluster regulating fruiting body formation (Kirby and Zusman 2003) and the *Pseudomonas fluorescens* Wsp chemosensory operon involved in the regulation of cellulose biosynthesis and biofilm formation (Spiers and Rainey 2005; Bantinaki et al. 2007; Navazo et al. 2009; Barahona et al. 2010).

cheB (NpR0244) The *N. punctiforme che5*-encoded CheB-like methylesterase (NpR0244) contains two predicted domains: an N-terminal response regulatory domain and a C-terminal methyl esterase catalytic domain (Table 2). In the chemotaxis system of the enteric bacteria *E. coli* and *Salmonella enterica*, phosphorylated CheB catalyzes deamidation of specific glutamine residues in the cytoplasmic region of the chemoreceptors, and demethylation of specific methylglutamate residues within the cytoplasmic domains of methyl-accepting chemotaxis proteins that have been methylated by CheR (an *S*-adenosylmethionine-dependent methyltransferase). The opposing activities of CheB and CheR regulate the methylation state of the receptors, and provide an adaptive response that permits the bacterium to respond to new stimuli in a background containing constant levels of chemoattractants and/or repellents (Clausznitzer et al. 2010). The response regulator domain of the *N. punctiforme* CheB-like methyl esterase contains several aspartate residues (Asp-8, Asp-34, Asp-45, Asp-48, Asp-53 and Asp-60) that may be involved in phosphorelay systems. The major active-site aspartate residues considered important in enteric systems are Asp-10 and Asp-11, as well as the phosphorylation site, Asp-56 (Stock et al. 2000). The C-terminal domain of CheB is a methylesterase with the major active sites consisting of serine (Ser-164), histidine (His-190), and aspartate (Asp-286) residues (Djordjevic et al. 1998). In *N. punctiforme* the C-terminal domain of NpR0244 contains the methylesterase active site Ser-164 as well as a histidine residue at position 192 and an aspartate residue at 285.

cheA (NpR0245) The *N. punctiforme* CheA-like *che5* locus member NpR0245 (Table 2) has a C-terminal response receiver domain and shows the most structural homology to the hybrid CheA-histidine kinase protein found in the *M. xanthus che3* operon (Kirby and Zusman 2003; Willett and Kirby 2011), and the *Pseudomonas* WspE protein (Bantinaki et al. 2007). The CheA-like domain of NpR0245 may have been acquired from outside the cyanobacterial lineage and has four distinct regions: a histidine-containing phosphotransfer (HPt) domain that includes the residue His-48 involved in autophosphorylation of the kinase (Hess et al. 1988); the signal transducing histidine kinase homodimeric domain; the kinase (catalytic) domain (resembling the two ATPases, Gyrase B and Hsp90); and a domain involved in receptor-mediated regulation (which contains the CheW and receptor binding domains).

cheW (NpR0246 and NpR0250) and mcp (NpR0247 and NpR0249) There are two predicted CheW proteins in the *N. puntiforme che5* locus (NpR0246 and NpR0250; Fig. 1 and Table 2). NpR0246 is a putative ortholog of the *Pseudomonas* chemotaxis-related protein WspD involved in the regulation of acetylated cellulose polymer production (Bantinaki et al. 2007). There are also two putative

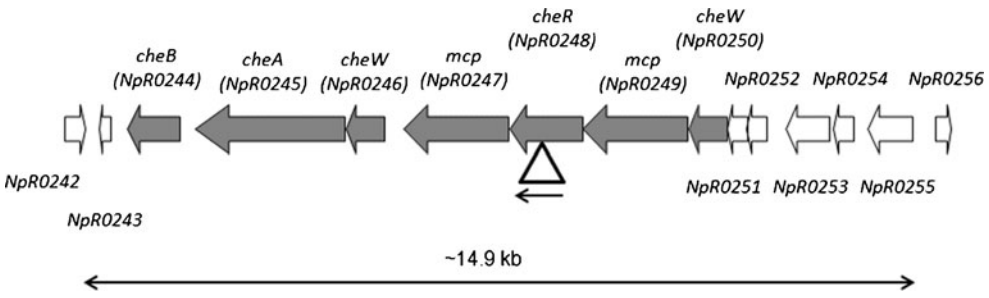

Fig. 1 Schematic diagram of the chemotaxis-like *orf5* locus in the genome of *N. punctiforme* wild type. The flanking ORFs transcribed in the same direction form part of the same operon. The position and orientation of the *npt* (neomycin phosphotransferase gene) in the *cheR* mutant is indicated by the *triangle* and *arrow* respectively; insertion was at nucleotide position 990. The figure is not drawn to scale

Table 2 Chemosensory-like characteristics of the *N. punctiforme* ATCC 29133 *che5* operon proteins

Protein (Homologue)	Size (aa[a])	Predicted function/definition	Pfam domain	*E*-value
NpR0244 (CheB)	354	Chemotaxis-specific methylesterase	Response_reg	3.5e–14
			CheB methylesterase	1.4e–58
NpR0245 (CheA)	747	CheA signal transduction histidine kinase	HPt	1.3e–14
			H-kinase_dim	0.0012
			HATPase_c	3.4e–22
			CheW	6.7e–18
			Response_reg	2.8e–28
NpR0246 (CheW)	247	CheW protein	CheW	5e–21
NpR0247 (MCP)	568	Methyl-accepting chemotaxis sensory transducer	TarH	6.8e–12
			Four helix bundle motif (4HB_MCP_2)	1.7e–17
			HAMP	5.8e–08
			TPR_MLP1_2	0.025
			MCPsignal	0.098
			MCPsignal	1.8e–41
NpR0248 (CheR)	426	TPR repeat-containing CheR-type MCP methyltransferase	CheR	2e–47
			SAM binding	
			Methyltransf_18	3.6e–08
			Methyltransf_12	6.8e–06
			Methyltransf_11	1.5e–06
			TPR_1 (Tetratricopeptide repeat)	0.00017
			TPR_2	0.0064
NpR0249 (MCP)	531	Methyl-accepting chemotaxis sensory transducer	HAMP	1.5e–12
			VPS28	0.051
			MCPsignal	0.94
			MCPsignal	1.5e–40
			TPR_MLP1_2	0.39
NpR0250 (CheW)	192	CheW Protein	CheW-like domain	1.9e–26

[a] Number of amino acids

membrane-associated methyl-accepting chemotaxis proteins (MCP): NpR0247 and NpR0249 (Table 2). The N-terminal (periplasmic) sensory ligand-binding domain of NpR0247 includes the four-helix bundle motif (4HB_MCP_2) always found between two predicted transmembrane helices, suggesting that it detects only extracellular signals. In most cases the domain is associated with a cytoplasmic HAMP domain, which is common to many prokaryotic signalling proteins (including histidine kinases, adenylyl cyclases, chemotaxis receptors, and phosphatases). HAMP domains link extracellular sensory parts of proteins with their intracellular signaling domains and provide a method for signal transduction (Hulko et al. 2006). As well as the HAMP domain, NpR0247 has the conserved (C-terminal) MCP signalling domain that is typical of chemotactic transducers (see Wuichet et al. 2007). NpR0249 also shows typical MCP-like domain architecture, including an N-terminal sensory ligand binding domain, the conserved HAMP domain and a C-terminal domain with two transmembrane domains and the conserved methyl-accepting protein signaling domain (summarized in Table 2). Both of the MCP-like members of the *che5* locus, in common with all MCP-like receptor proteins in the *N. punctiforme* genome, lack the pentapeptide motif (NWETF or NWESF) found at the extreme C-terminal end of some transmembrane receptors. The motif is a binding site for CheR and CheB-P and was shown to strongly enhance methylation, demethylation and deamidation in enteric bacteria (e.g. Barnakov et al. 1998, 1999; Li and Hazelbauer 2005).

cheR (NpR0248) The *N. punctiforme* putative CheR (NpR0248) possesses a C-terminal TPR-containing domain. The TPR is found in a wide variety of proteins and has been shown to mediate protein-protein interactions and the assembly of multiprotein complexes (D'Andrea and Regan 2003). There is evidence to suggest that TPR or other protein-protein interaction motifs present in pentapeptide-independent CheR proteins may mediate interaction with

other chemotaxis proteins (Bustamante et al. 2004; Perez and Stock 2007). *N. punctiforme* CheR (NpR0248) likely has methyltransferase activity; the predicted protein has features similar to chemotaxis CheR proteins, including a conserved N-terminal CheR-type methyltransferase domain (members of this family are SAM-dependent methyltransferases). The chemotaxis-like domain features of the predicted *che5* locus proteins are summarised in Table 2.

3.2 Characterisation of the *NpR0248* (*cheR*) mutant

Transcription of cheR in hormogonia Hormogonia are the motile phase in the *Nostoc* life cycle and there is evidence that they are directed to host plant symbiotic tissue by chemotaxis (Knight and Adams 1996; Nilsson et al. 2006; Watts et al. 1999; Duggan and Adams 2009; Adams and Duggan 2008, 2011). We therefore thought that chemosensory components might be expressed preferentially in hormogonia. However, RT-PCR using the primers NpR0248 (*cheR*) (Table 1) and RNA isolated from vegetative filaments and hormogonia of both the wild-type and the *cheR* mutant, revealed only a slight increase in transcripts in the WT hormogonia compared with WT vegetative filaments (Fig. 2). This finding is in agreement with DNA microarray and Northern hybridisation studies (Christman et al. 2011). The RT-PCR primers NpR0248 (*cheR*) flank the site at which the *npt* gene cassette was inserted within *cheR*. The *npt* gene is highly expressed in cyanobacteria from the strong *psbA* promoter derived from the chloroplast genome of the higher plant *Amaranthus hybridus* (Wolk et al. 1991). As expected, transcripts were not observed with the *cheR* mutant, confirming that the gene had been effectively interrupted (Fig. 2). Sequence analysis of the construct used to introduce the inactivated allele into WT *N. punctiforme* confirmed that the *npt* gene was oriented in the direction of *cheR* transcription; thus, any necessary co-transcribed downstream genes would be transcribed from the strong *psbA* promoter inserted in the *cheR* gene.

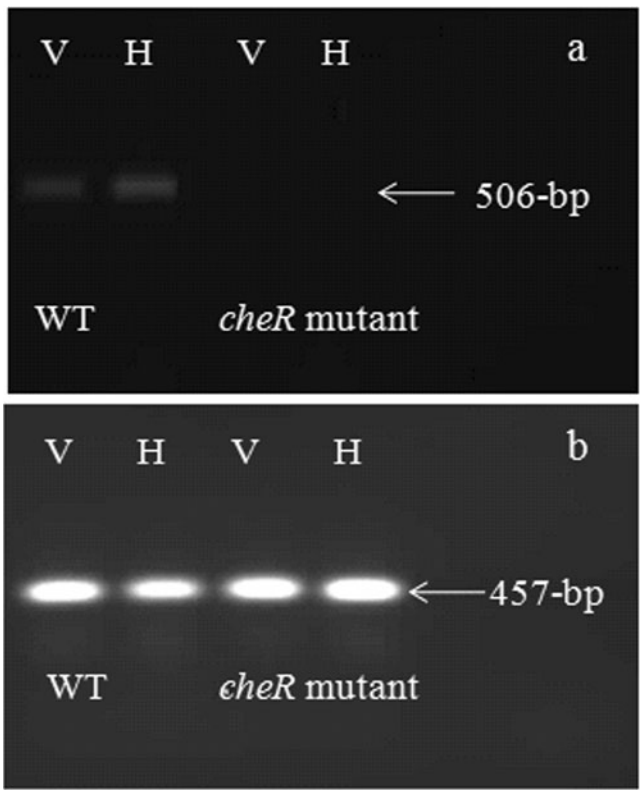

Fig. 2 RT-PCR comparisons of *NpR0248* (*cheR*) expression in hormogonia (H) and vegetative filaments (V) of WT and mutant NpR0248 (*cheR*) *N. punctiforme* (panel **a**). The *rnpB* gene (panel **b**) was used as a control for equal amounts of RNA

Symbiotic competency and motility Hormogonia are essential for the establishment of symbiosis with plants, although hormogonia formation alone does not guarantee a successful infection, implying that several factors contribute to symbiotic competency (Campbell and Meeks 1989; Johansson and Bergman 1994; see also: Enderlin and Meeks 1983; Rasmussen et al. 1994). Efficient hormogonia formation and rapid migration of these filaments appear to be critical to the successful establishment of artificial associations with rice (Nilsson et al. 2005). Our research has shown that functional pili expressed on the hormogonium surface (Duggan et al. 2007), as well as differences in hormogonia behaviour, possibly involving responses to plant signals (Chapman et al. 2008), are involved in symbiotic competency. Similarly, host-cyanobacterial recognition processes and evasion of the host's natural defences may also be involved in the infection process.

Is NpR0248 (cheR) required for host plant infection? To investigate the importance of chemotaxis-like gene *NpR0248* (*cheR*) in the establishment of symbiosis, the frequency of infection was determined after co-culture of the mutant and wild-type strains with the symbiotic partner *B. pusilla* (Table 3). The numbers of symbiotic colonies detected in the auricles (symbiotic cavities located on the ventral surface of the *Blasia* thallus, Fig. 3a, b) were estimated over a 28 d co-culture period (Table 3). With wild-type *N. punctiforme* there was a steady increase in the frequency of infection, reaching 74 % of auricles after 14 days, and a mean value of almost 84 % after 28 days of co-culture. By contrast, with the *NpR0248* (*cheR* mutant), the mean percentage of colonised auricles was just 0.39 % after 28 days of co-culture.

Are other che5 operon genes required for host infection? We also mutated *che5* operon genes *NpR0244* and *NpR0247*

Table 3 Mean infection frequencies of *B. pusilla* tissue following 14 and 28 days co-culture with the wild-type and the *cheR* (*NpR0248*) mutant strain of *N. punctiforme* 29133

Nostoc 29133 strain	Infection frequency (%)[a]			
	14 days (n)	SD	28 days (n)	SD
Wild-type *Nostoc* 29133	74.20 (14)	6.43	83.81 (14)	10.17
NpR0248 (*cheR* mutant)	0.56 (11)	0.30	0.39 (11)	0.34

n represents the number of co-culture replicates

SD is standard deviation

[a] Values are the mean number of infected auricles expressed as a percentage of the total number of auricles counted for each determination

by insertion of an antibiotic resistance cassette, orientated in the same direction as the gene targeted, at nucleotide position 382 in *cheB*-like *NpR0244* and nucleotide position 659 in *mcp*-like *NpR0247*. However, these mutants were constructed in a spontaneous variant of the original isolate of *N. punctiforme* ATCC 29133. The variant strain grows quickly in a dispersed "smooth" state compared with the original "clumpy" isolate used to construct the *NpR0248* (CheR) mutant described here (Fig. 3c, d). The "smooth" variant does not form hormogonia well, is 5-10-fold less symbiotically competent and displays less robust photo- and chemotactic responses than the original "clumpy" strain. Much lower frequencies of infection were observed with the mutants, the *cheB* mutant being approximately 4-fold lower, and the *mcp* mutant 37-fold lower, than the parent wild-type strain (Duggan and Adams 2009). Thus, genes *NpR0248* (CheR), *NpR0244* (CheB) and *NpR0247* (MCP) of the *che5* operon are required for efficient infection.

Motility of the cheR mutant Microscopic examination of aliquots of culture medium removed after 24 h of co-culture of the wild-type *N. punctiforme* with the host plant *Blasia* revealed motile hormogonia. Although the *cheR* mutant also differentiated hormogonia, these filaments were not visibly motile. Hormogonia induction by a combination of starvation for combined nitrogen and incubation under red light was found to yield 90–100 % conversion of filaments to hormogonia in both wild-type and the *cheR*-like *NpR0248* mutant; thus CheR was not

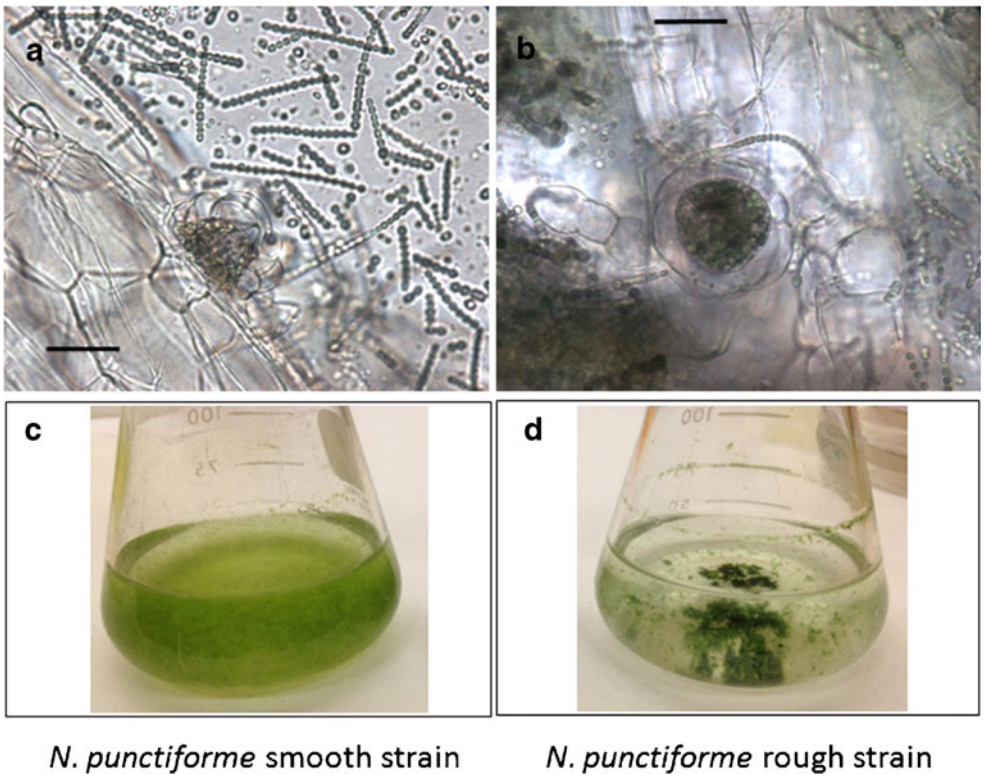

Fig. 3 Infection of *B. pusilla* with **a** the smooth strain and **b** the rough strain of *N. punctiforme.* A single infected auricle can be seen at the centre of each image. Also illustrated are the cultural morphologies of liquid-grown cultures of the smooth strain (**c**), and the rough strain (**d**) of *N. punctiforme.* Bars in a and b represent 60 μm

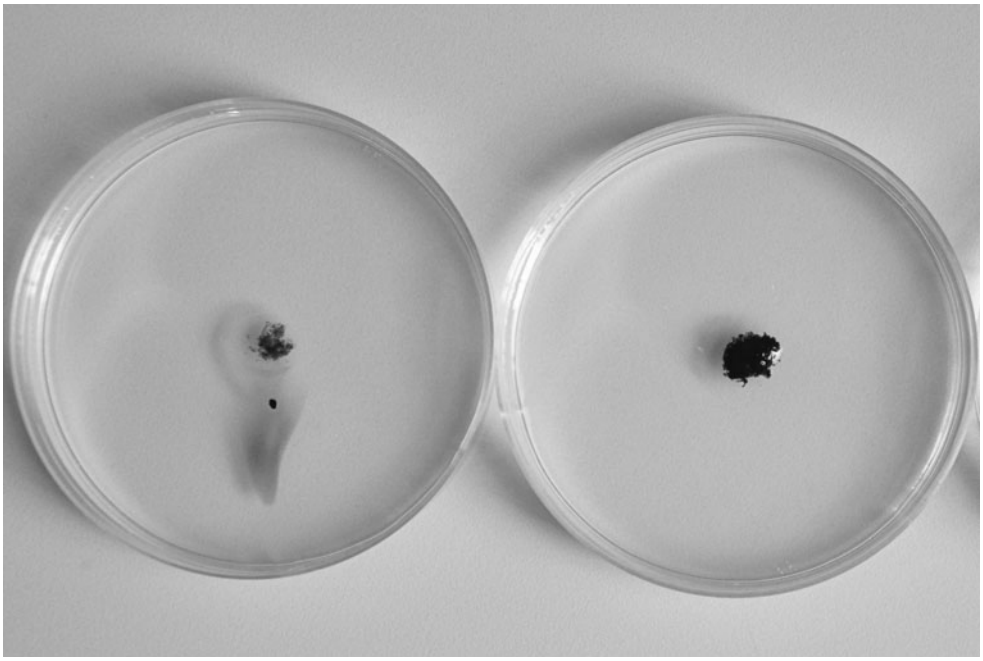

Fig. 4 Phototaxis of the *N. punctiforme* WT (shown to the left of the image) and mutant *NpR0248* (*cheR;* right hand image) filaments. Hormogonia were applied to the surface of 0.2 % agar and incubated in front of a unidirectional light source for 24 h. The migration of wild-type hormogonia is visible as a fuzzy mass. Petri dishes are approximately 9 cm in diameter. The directional light was from the bottom of the picture

required for differentiation of hormogonia. However, motile hormogonia were observed only in the parent wild-type strain.

In addition to being motile, hormogonia need to be directed to the host symbiotic tissue. Directed motility, most probably governed by a chemotaxis-like transduction system, likely helps to guide the hormogonia to the specialised sites of infection within the host plant. Motility and chemotaxis may therefore be required for the efficient establishment of symbiosis. In some symbiotic systems the sites of infection are deep within the host tissue where they receive little or no light, and the attraction of hormogonia to these sites must therefore overcome the natural positive phototaxis of hormogonia. Hormogonia from wild-type *N. punctiforme* showed clear phototaxis on agar plates, progressing as a visible 'comet' of filaments extending in some cases up to 3 cm from the original inoculation site (Fig. 4). By contrast, the *cheR* mutant showed no phototactic movement (Fig. 4), implying that some aspect of motility is impaired in this strain.

Tactic signals in diverse bacteria and archaea are transduced to at least three different types of motility apparatus, the flagellum, the archaeal flagellum, and the type IV pili that underlie gliding motility in some cyanobacteria and other bacteria. The pilus-like structures expressed on the surface of hormogonia are believed to be their motility apparatus (Duggan et al. 2007). Pilus biosynthesis and

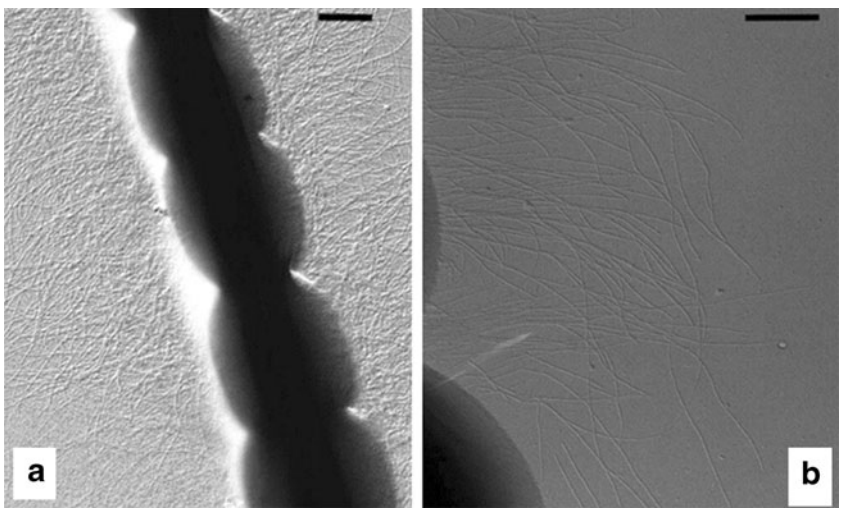

Fig. 5 Transmission electron microscopy of pilus-like appendages on the cell surface of hormogonia produced by *N. punctiforme* wild-type (**a**) and the *NpR0248* (*cheR*) mutant (**b**). Scale bars represent 1 μ (**a**) and 500 nm (**b**)

phototaxis in the unicellular cyanobacterium *Synechocystis* appear to be regulated by chemotaxis-like elements in two different loci (Bhaya et al. 2001), raising the possibility that the *N. punctiforme che* mutants described here might be altered in pilus structure or function. Electron microscopy revealed that the hormogonia of the *N. punctiforme NpR0248* (*cheR*) mutant expressed pili, but they were less abundant than in the wild-type strain (Fig. 5). In other bacteria the pilus structural protein, pilin, is encoded by the gene *pilA*; there are several *pilA*-like genes in the *N. punctiforme* genome, although the gene encoding the pilin monomer is not known (Duggan et al. 2007). Inactivation of *pilA*-like *NpF0069* (designated *pilA* in the *N. punctiforme* genomic data base: http://genome.jgi-psf.org/finished_microbes/nospu/nospu.home.html) reduces symbiotic competency, although pili are still expressed (Duggan et al. 2007). Similarly a *N. punctiforme* mutant inactivated in *pilA*-like *NpF0676* is non-motile and unable to infect the host plant but also retains some pili (Duggan and Adams, unpublished data) implying that there may be two different types of pili expressed on the surface of *N. punctiforme* hormogonia.

3.3 Concluding comments

The lack of phototaxis and very low symbiotic competency of the NpR0248 (*cheR*) mutant could be due solely to a loss of pilus function, and hence motility, or could be a result of disruption of signal transduction pathways involved in directed motility. Further experimental work is necessary to distinguish between these possibilities. The sequence similarity between the *N. punctiforme* NpR0248 (CheR) and other CheR proteins implies the involvement of a methylation-dependent system regulating motility in *N. punctiforme*. Indeed, there is evidence that protein methylation in *N. punctiforme* is widespread (Anderson et al. 2006). It remains to be seen if this system involves the MCP-like receptors within the *che5* operon or, indeed, any of the other MCP-like receptors in the *N. punctiforme* genome. Methylation reactions may also involve the orphan CheR proteins, although a *N. punctiforme* mutant disrupted in one of these proteins, CheR-like ORF NpF3533 is, in terms of morphology, growth, motility and symbiotic competency, indistinguishable from the wild-type (data not shown). By contrast, our attempts to disrupt the other orphan CheR-like ORF NpF0263 have been unsuccessful, implying that this ORF may be essential for growth.

This report represents the first account of a chemosensory-like signal transduction system regulating motility and symbiotic competence in a filamentous cyanobacterium. Further investigation is needed to identify the other components involved in this system and confirm the involvement of methylation in this process.

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