

Molecular Analysis of Genes in *Nostoc punctiforme* Involved in Pilus Biogenesis and Plant Infection[∇]

Paula S. Duggan, Priscila Gottardello,[†] and David G. Adams*

Institute of Integrative and Comparative Biology, Faculty of Biological Sciences, University of Leeds, Leeds, LS2 9JT, United Kingdom

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Hormogonia are the infective agents in many cyanobacterium-plant symbioses. Pilus-like appendages are expressed on the hormogonium surface, and mutations in *pil*-like genes altered surface piliation and reduced symbiotic competency. This is the first molecular evidence that pilus biogenesis in a filamentous cyanobacterium requires a type IV pilus system.

Cyanobacteria, such as *Nostoc* spp., differentiate specialized filaments, known as hormogonia, which serve as the infective units in the establishment of symbioses with plants (1, 31) and exhibit a surface-dependent form of motility known as gliding. Social gliding of myxobacteria (30) and the twitching motility observed in a wide range of gram-negative bacteria, including the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 (7, 9, 48, 63), are associated with surface-expressed, retractile appendages known as type IV pili (Tfp). While there is evidence that hormogonia possess pilus-like structures that are expressed during their formation (14) and transcripts hybridizing to the pilus retraction gene, *pilT*, increase in abundance within the first few hours of hormogonium formation (16), direct evidence that these structures underlie their motility is still lacking. Similarly, questions remain over the contribution of pili to establishment of cyanobacterium-plant symbioses. Dick and Stewart (15) noted that pilus-like structures were important in the specificity of the *Nostoc-Peltigera* symbioses, but Johansson and Bergman (20) noted that they appeared to be of little significance in the establishment of *Nostoc-Gunnera* symbioses.

Now that the genome sequence for *Nostoc punctiforme* ATCC 29133 (PCC 73102) is available (http://genome.jgi-psf.org/finished_microbes/nospu/nospu.home.html), it is clear that it contains open reading frames (ORFs) that are predicted to encode proteins with similarity to Tfp biogenesis proteins. To address the possibility that hormogonia require a Tfp system, we inactivated six of the *pil*-like ORFs and examined the mutant phenotypes.

Identification of *N. punctiforme pil*-like genes. Of 15 ORFs identified (Table 1), 6 were chosen for disruption analysis. The predicted amino acid sequence of the protein encoded by the *pilT*-like ORF NpR0117 is most similar (75% identity; 88% similarity) to PilT1 from *Synechocystis* strain PCC 6803

(Slr0161) and shares 54% identity (69% similarity) with PilT from *Pseudomonas aeruginosa* (GenBank accession no. M55524). The PilT-like NpF2507 protein mostly resembles PilT2 of *Synechocystis* (74% identity; 86% similarity). Both PilT-like proteins possess the conserved Walker box motifs characteristic of nucleotide binding proteins (Fig. 1). Walker box A, in ATP/GTP-binding consensus sequence, [AG]-X₄-G-K-[ST], where X is a variable (PROSITE accession no. PDOC00017), is glycine rich (54, 57). Walker box B is also present in some of the bacterial type II secretion pathway (GSP) proteins (40, 54). The NpF2507 protein has a proline-rich N-terminal extension of about 74 amino acids that is also present in PilT2 of *Synechocystis* strain PCC 6803 (7). *pilT*-like NpR0117 is clustered with two other *Nostoc pil*-like genes *pilB* (NpR0118) and *pilC* (NpR0116), in the order *pilB*, *pilT*, and *pilC*. In *Synechocystis* strain PCC 6803, *pilC* is clustered with *pilT* but not with a *pilB* homologue (63), while *pilC* is downstream of *pilB*, and *pilT* is found at a different locus in *P. aeruginosa* (29).

The NpR2800 protein resembles prepilin peptidases (PilD proteins), which process pilin and a number of other prepilin-like proteins (3, 38) (data not shown). NpR2800 shares most sequence similarity (48%; 63% identity) with the *Synechocystis* strain PCC 6803 prepilin peptidase (Slr1120) and contains at least six transmembrane helices, suggesting that the protein may be an integral membrane protein. The highly conserved two-pair cysteine signature, which is required for both proteolysis and methylation activities (25), is also present (data not shown).

The *N. punctiforme* genome has at least five ORFs encoding proteins containing features that characterize the pilus subunit (PilA), a unique leader peptide sequence, and high sequence conservation in the N-terminal region of the mature protein (data not shown). Other significant similarities include conserved Gly residue at position -1 that is essential for cleavage of the leader sequence, a conserved Phe (+1 position of the mature protein) and a Glu residue (+5 position of the mature protein). It is worth noting that pseudopilins, which are components of the GSP (18, 21, 37, 45, 51), are also synthesized as precursors and share extensive sequence similarity with PilA (18, 25).

Mutants were constructed by the insertion of an omega

* Corresponding author. Mailing address: Institute of Integrative and Comparative Biology, Garstang Building, Faculty of Biological Sciences, University of Leeds, Leeds, LS2 9JT, United Kingdom. Phone: 44(0)113 3435588. Fax: 44(0)113 3434311. E-mail: d.g.adams@leeds.ac.uk.

[†] Present address: The Institute of Pharmaceutical Innovation, University of Bradford, Bradford, BD7 1DP, United Kingdom.

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TABLE 1. List of putative pilus biogenesis genes in the *Nostoc punctiforme* ATCC 29133 genome^a

<i>N. punctiforme</i> gene	No. of aa residues encoded	Annotation (<i>Nostoc</i> genome)	Homologous gene in <i>P. aeruginosa</i> / <i>Synechocystis</i> strain PCC 6803 ^b	Proposed function/product ^c	Mutant phenotype ^c	References
NpR0117*	375	<i>pilT</i>	<i>pilT</i> *	Pilus retraction	Hyperpilated, nonmotile	7, 8, 39, 40, 47, 57
NpF2507*	425	<i>pilT</i>	<i>pilT</i> *	Pilus retraction/phototaxis ^d	Hyperpilated, nonmotile/negative phototaxis ^d	7, 8, 39, 40, 47, 57
NpR2800*	293	Prepilin peptidase gene <i>pulO</i>	<i>pilD</i> *	Prepilin peptidase	Loss of motility and Tfp	3, 7, 25, 38, 49, 50, 51, 63
NpF0676*	188	Tfp assembly protein <i>pilE</i>	<i>pilA</i>	Prepilin	Loss of motility and Tfp	6, 7, 28, 42, 47, 63
NpF0067	226	<i>pulG</i> pseudopilin	<i>pilA</i>	Prepilin	Loss of motility and Tfp	6, 7, 28, 42, 47, 63
NpF0068*	322	Hypothetical	<i>pilA</i> *	Prepilin	Loss of motility and Tfp	6, 7, 28, 42, 47, 63
NpF0069*	232	Tfp assembly protein FimT gene	<i>pilA</i> *	Prepilin	Loss of motility and Tfp	6, 7, 28, 42, 47, 63
NpF0073	185	<i>pulG</i> pseudopilin gene	<i>pilA</i> *	Prepilin	Loss of motility and Tfp	6, 7, 28, 42, 47, 63
NpR0118	668	<i>pilB</i>	<i>pilB</i>	Pilus extension	Loss of motility and Tfp	28, 36, 52, 57, 63
NpF2747	371	<i>pilB</i>	<i>pilB</i>	Pilus extension	Loss of motility and Tfp	28, 36, 52, 57, 63
NpR0116	411	GSP member <i>pulF</i>	<i>pilC</i>	Counteracts PilT-mediated pilus retraction	Loss of motility and Tfp	36, 39
NpF5005	368	<i>pilM</i>	<i>PilM</i>	Unknown	Loss of motility and Tfp	27, 63
NpF5006	255	<i>pilN</i>	<i>pilN</i>	Localization/stability of Tfp	Loss of motility and Tfp	27, 63
NpF5007	258	<i>pilO</i>	<i>pilO</i>	Localization/stability of Tfp	Loss of motility and Tfp	27, 63
NpF5008	792	<i>pilQ</i>	<i>pilQ</i>	Pore-forming secretin	Loss of motility and Tfp	26, 63

^a *, ORFs chosen for characterization in the present study.

^b *Nostoc pil*-related genes were identified by searching the protein database derived from the genome of *N. punctiforme* with deduced amino acid sequences of the known *P. aeruginosa* and *Synechocystis* strain PCC 6803 *pil* proteins.

^c The proposed functions and mutant phenotypes are based primarily on that established for *P. aeruginosa* but is also based on that for *Synechocystis*, unless otherwise noted.

^d Phototaxis is a proposed function/phenotype for *Synechocystis* only.

neomycin phosphotransferase gene into each ORF and the introduction of inactivated alleles into wild-type *N. punctiforme* as described previously (13). Complete segregation was achieved with all the mutants, except *pilA*-like ORFs NpF0068 and NpF0676.

Pilus structure on the cell surface of hormogonia. In agreement with previous observations (15, 20, 53), the surface of wild-type *N. punctiforme* hormogonia possesses peritrichously arranged pilus-like structures (Fig. 2B). Individually, these structures have a fairly uniform diameter of 7 to 10 nm, whereas the length is variable, extending up to 10 μ m from the cell surface. We were able to discern only one *Nostoc* pilus

morphotype, whereas others have determined that *Synechocystis* strain PCC 6803 has two morphologically distinct pilus types (7, 63). By contrast, the vegetative trichomes of the immotile parent filaments are largely devoid of pili (Fig. 2A). The pili of the *pilA*-like NpF0069 mutant were, in terms of abundance,

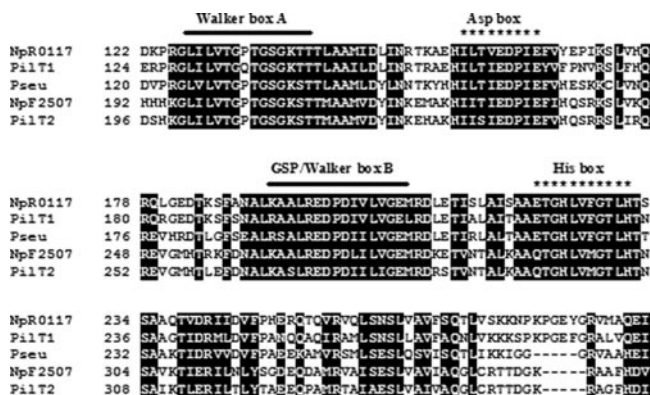


FIG. 1. Conserved motifs of the *N. punctiforme* PilT proteins (NpR0117 and NpF2507 proteins), *P. aeruginosa* PilT (Pseu), *Synechocystis* strain PCC 6803 PilT1, and *Synechocystis* strain PCC 6803 PilT2. Black boxes indicate identical or conserved residues in all five of the sequences. Protein sequences were aligned using the program ClustalX. The Walker box motifs (thick lines), the Asp box and the His box (asterisks) are indicated. Note that only the central regions of the PilT sequences are shown.

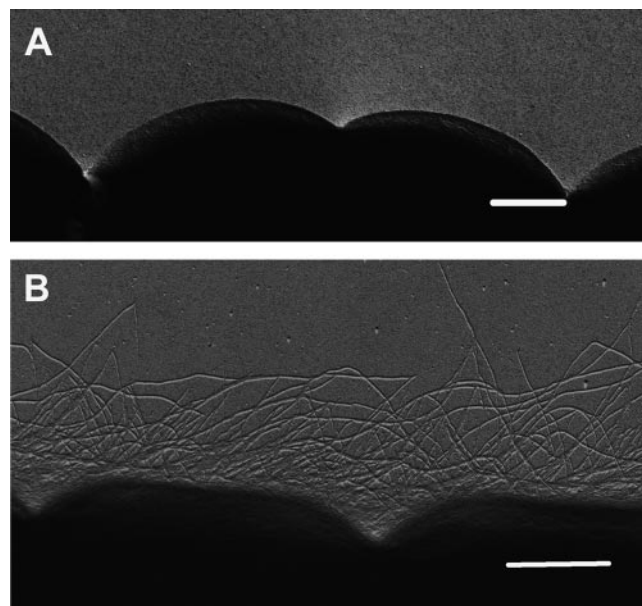


FIG. 2. Comparison of the cell surfaces of wild-type vegetative filaments and hormogonia. (A) Wild-type vegetative filaments; (B) wild-type hormogonia. Scale bars represent 1 μ m. For electron microscopy, platinum wire (2 cm by 0.2 mm) was evaporated onto the surface of each sample by using an Edwards 306A high-vacuum coating unit and samples were viewed on a JEOL1200EX transmission electron microscope at 80 kV.

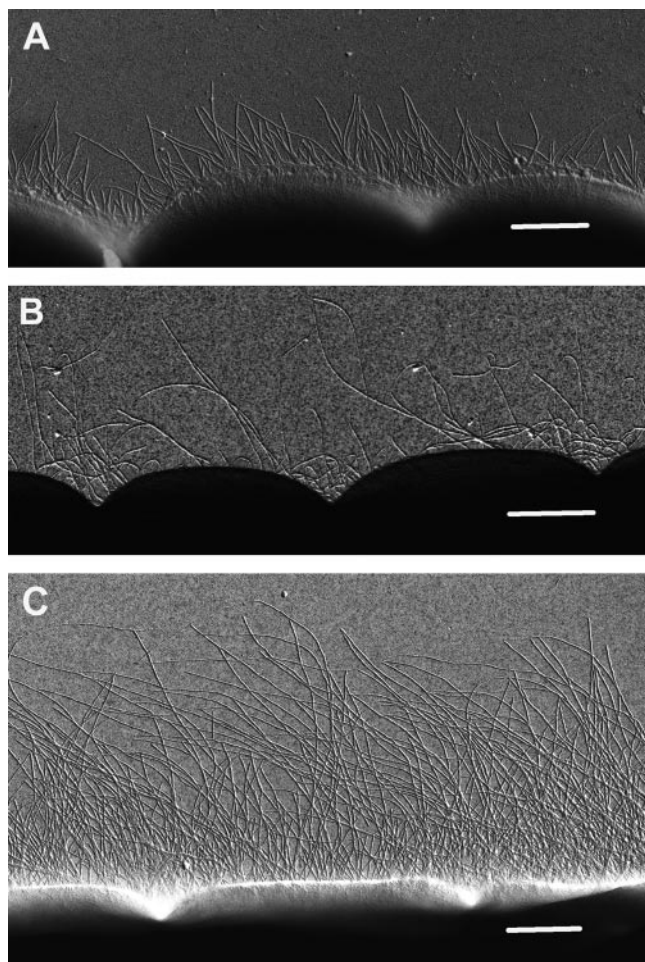


FIG. 3. Presence of pilus-like appendages on the cell surface of hormogonia produced by *pil* mutants of *N. punctiforme*. (A) Hormogonia of the NpR2800 mutant (*pilD* homologue); (B) hormogonia of the NpF2507 mutant (*pilT* homologue); (C) hormogonia of the NpR0117 mutant (*pilT* homologue). Scale bars represent 1 μm . At least 50 filaments were observed for each mutant phenotype, and the electron micrographs shown are representative of each strain.

distribution, and structure, indistinguishable from those expressed on the surface of wild-type hormogonia, implying that the gene is not responsible for the structural monomer of the *Nostoc* pilus-like appendages (data not shown).

The phenotype of the *pilT*-like NpR0117 mutant is unmistakably the hyperpiliated phenotype (i.e., overproducing pili) (Fig. 3C) that is a typical result of mutations within *pilT* in other bacteria (7, 8, 32, 57, 59, 61). The mutation of the second *pilT*-like NpF2507 mutant resulted in pili that were slightly less abundant than those in the wild type, although their structure, in terms of length and diameter, appeared to be similar to that of the wild type (Fig. 3B). Remarkably, disruption of *pilD*-like NpR2800 gave rise to much shorter pili (up to 2.5 μm in length) than those of the wild type (up to 10 μm in length), although the pilus diameter (7 to 10 nm) was similar to that of the wild type (Fig. 3A). In other bacteria, mutations in *pilD* abolish motility and produce a nonpiliated phenotype (see, e.g., references 7, 19, and 25). The bald phenotype of the *Synechocystis* strain PCC 6803 *pilD* mutant is of special interest

in that it implies that both of its pilus morphotypes are processed by the same prepilin peptidase (7). Searches of the *N. punctiforme* genomic sequences did not reveal any other PilD-like prepilin peptidases. The possibility of functional homologs, however, cannot be excluded. The truncated pilus phenotype reported here is unusual and may represent partially retracted or partially extended pili.

Symbiotic competencies of wild-type and mutant *N. punctiforme* strains. The importance of cyanobacteria in their associations with plants is evidenced by the fact that host plants synthesize hormogonium-inducing factors (4, 10, 12, 22, 46, 55) and that both natural host plants and nonhost plants induce chemoattractive behavior in hormogonia (22, 34, 55). There is evidence, however, that hormogonium production per se is not sufficient for the establishment of symbiosis, because Johansson and Bergman (20) noted that noninfective *Nostoc* strains were also capable of forming motile hormogonia in the presence of the angiosperm *Gunnera* spp. (see also references 17 and 46). Fast hormogonium formation and rapid migration of these filaments appear critical to the successful establishment of artificial associations (33). Several factors therefore contribute to symbiotic competency. Tfp also function in adhesion (44). Indeed, adhesion, rather than motility, has been shown to be necessary for the initiation of biofilm formation in *P. aeruginosa* (11). Our wild-type hormogonia glided at rates of 0.7 $\mu\text{m s}^{-1}$ to 1.7 $\mu\text{m s}^{-1}$ (although it should be noted that motility was not always present in the wild type). Slight twitching of filaments, without forward progression, was detected by light microscopy in the NpF0069 and NpF2507 mutants. Motility in strains with NpR2800 and NpR0117 inactivated was not observed. To investigate the importance of functional pili in the establishment of symbiosis, the frequency of infection was estimated after coculture with the host bryophyte *Blasia pusilla* (Table 2) (22). Reconstitution of symbiotic associations was performed as described previously (60).

The wild-type-piliation phenotype observed with the *pilA*-like NpF0069 mutant implies that the gene is not required for the biosynthesis of the pilus-like appendages. It was therefore surprising that the NpF0069 mutant infected plant tissue at frequencies lower than those of the wild type, both during the early stages of the infection process and after a protracted

TABLE 2. Mean infection frequencies of wild-type and mutant strains of *N. punctiforme* estimated after 14 and 28 days of coculture with *Blasia*

<i>N. punctiforme</i> strain (putative homologue) ^a	14 days				28 days			
	No. of coculture replicates	Infection frequency (%) ^b		No. of coculture replicates	Infection frequency (%) ^b			
		Mean	SD		Mean	SD		
Wild-type <i>N. punctiforme</i>	28	12.03	7.8	32	15.95	12		
NpF0069 mutant (<i>pilA</i>)	7	3.49	1.12	8	6.00	2.72		
NpF2507 mutant (<i>pilT</i>)	23	6.00	4.35	18	14.13	8.35		
NpR0117 mutant (<i>pilT</i>)	17	0.48	0.56	18	1.52	1.03		
NpR2800 mutant (<i>pilD</i>)	20	0.68	0.4	20	0.39	0.37		

^a The putative homologue of each ORF is indicated in parentheses.

^b Values are expressed as the mean number of infected auricles (the symbiotic cavities of *Blasia*) expressed as a percentage of the total number of auricles counted for each determination (a minimum of 400 auricles were counted for each determination).

period of coculture (Table 2). Some mutations in the *P. aeruginosa pilA* gene give rise to strains that are capable of assembling surface pili but have impaired motility and adherence (reviewed in reference 9). Further work is therefore required to fully discount the possibility that NpF0069 encodes the *Nostoc* pilin monomer. Alternatively, the protein encoded by *pilA*-like NpF0069 may represent a minor pilin (2, 58) that may support the activity of the major pilin in some aspect of motility and/or adhesion and therefore contribute to symbiotic competency. *Synechocystis* strain PCC 6803 has at least 10 *pilA*-like genes (5, 7, 62, 63), some of which are critical for motility and therefore probably represent components of the Tfp machinery.

For the *pilD*-like NpR2800 mutant, the frequency of infection after 14 days was 0.68%, decreasing to 0.31% after 28 days. Many of the symbiotic filaments were brown, implying that they were dead or dying and that the mutant was unable to grow effectively once it had reached the host symbiotic cavity. It is well established that prepilin peptidases, as well as processing pilin precursor, are also involved in the extracellular secretion of proteins (23, 24, 41, 50). *pilD* mutants are therefore pleiotrophic. Indeed, there is evidence to suggest that the *Synechocystis* strain PCC 6803 *pilD* gene is also critical for normal growth (63). The death of symbiotically associated *Nostoc* NpR2800 filaments opens the intriguing possibility that the *Nostoc pilD*-like gene may be required for some aspect of symbiotic growth.

Because wild-type hormogonia were occasionally immotile, we were unable to demonstrate irrefutably that the apparent loss of motility associated with the NpR0117 mutant resulted from mutation in the *pilT*-like ORF. Questions therefore remain as to whether motility, adhesion, or both function in the establishment of symbioses. Nevertheless, the NpR0117 (hyperpiliated) mutant infected *Blasia* tissue at a considerably lower frequency than the wild type (Table 2), implying that normal surface piliation is required for effective establishment of symbioses. Both *N. punctiforme* PilT-like proteins resemble other PilT proteins and share sequence homology with components of the GSP found in gram-negative bacteria (37, 43). Both proteins, however, lack the tetracysteine motif that is characteristic of GSP members and is also found in the DNA competence PilF protein (56) and the Tfp biogenesis protein PilB (35), indicating that the *Nostoc* PilT-like proteins can be included in the PilT family. Because the NpR0117 product resembles other PilT proteins and inactivation of the gene resulted in a hyperpiliated strain with a reduced capacity for infection, we suggest that NpR0117 at least is a component of the *Nostoc* piliation system and encodes the functional homologue of the pilus retraction protein PilT. The role of *pilT*-like NpF2507 is less clear. The NpF2507 protein mostly resembles PilT2 from *Synechocystis* strain PCC 6803, which is dispensable for motility but is believed to function in a signaling pathway that regulates the positive phototaxis of this cyanobacterium (7). The NpF2507 mutant infected *Blasia* tissue at an initial frequency 50% that of the wild type but reached a level equivalent to that of the wild type after 28 days (Table 2), implying a possible role during the early stages of the infection process. Whether NpF2507 is required for the direction of hormogonia motility (possibly involving chemotaxis-like regulatory elements), as may be the case with *Synechocystis* PilT2, remains to

be investigated. Surprisingly, many of the plant structures that host cyanobacterial colonies are in regions that receive little or no light, suggesting that the chemoattractants released by the host plant override the elements that regulate phototactic behavior.

In conclusion, we report that *N. punctiforme* has a type IV piliation system and that inactivation of the *pilT*- and *pilD*-like components (NpR0117 and NpR2800, respectively) specifically altered the surface piliation levels and reduced symbiotic competency. We speculate that hormogonia motility is driven by type IV pili and that these structures contribute to the establishment of *Nostoc-Blasia* symbioses.

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