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SPECIAL ISSUE REVIEW PAPER

Cyanobacteria–bryophyte symbioses

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Abstract

Cyanobacteria are a large group of photosynthetic prokaryotes of enormous environmental importance, being responsible for a large proportion of global CO_2 and N_2 fixation. They form symbiotic associations with a wide range of eukaryotic hosts including plants, fungi, sponges, and protists. The cyanobacterial symbionts are often filamentous and fix N_2 in specialized cells known as heterocysts, enabling them to provide the host with fixed nitrogen and, in the case of non-photosynthetic hosts, with fixed carbon. The best studied cyanobacterial symbioses are those with plants, in which the cyanobacteria can infect the roots, stems, leaves, and, in the case of the liverworts and hornworts, the subject of this review, the thallus. The symbionts are usually *Nostoc* spp. that gain entry to the host by means of specialized motile filaments known as hormogonia. The host plant releases chemical signals that stimulate hormogonia formation and, by chemoattraction, guide the hormogonia to the point of entry into the plant tissue. Inside the symbiotic cavity, host signals inhibit further hormogonia formation and stimulate heterocyst development and dinitrogen fixation. The cyanobionts undergo morphological and physiological changes, including reduced growth rate and CO_2 fixation, and enhanced N_2 fixation, and release to the plant much of the dinitrogen fixed. This short review summarizes knowledge of the cyanobacterial symbioses with liverworts and hornworts, with particular emphasis on the importance of pili and gliding motility for the symbiotic competence of hormogonia.

Key words: Bryophyte, cyanobacteria, gliding motility, pili, symbiosis.

Introduction

Cyanobacteria form a wide variety of symbiotic associations with eukaryotic hosts including plants, fungi, sponges, and protists (for reviews, see Adams, 2000; Rai *et al*., 2000, 2002; Adams *et al*., 2006; Bergman *et al*., 2007). The cyanobacterial symbionts (cyanobionts) generally supply their hosts with fixed nitrogen, although they can also provide fixed carbon to non-photosynthetic hosts. The major plant hosts are bryophytes, cycads, the angiosperm *Gunnera*, the water-fern *Azolla*, and fungi (to form lichens). Although all cyanobacteria are photoautotrophs, many are also facultative heterotrophs and so are not restricted to the areas of the plant that receive light, and can be found in roots, stems, leaves, and thalli. This review will concentrate on the cyanobacteria–bryophyte symbioses, focusing in particular on the importance of pili and gliding motility in plant infection. For comprehensive reviews on the cyanobacteria–bryophyte symbiosis, see Adams (2002), Meeks and Elhai (2002), and Meeks (2003). Space constraints have meant that, in reviewing the literature, we have had to concentrate on the more recent articles and reviews, rather than referring to the original literature.

Plant cyanobionts all have two major characteristics in common: (i) the ability to differentiate both specialized nitrogen-fixing cells known as heterocysts (for reviews, see Adams and Duggan, 1999; Meeks and Elhai, 2002; Golden and Yoon, 2003; Zhang *et al*., 2006); and (ii) short, motile filaments known as hormogonia, which lack heterocysts and provide a means of dispersal for otherwise immotile cyanobacteria (Campbell and Meeks, 1989; Meeks, 1990, 1998; Johansson and Bergman, 1994; Bergman *et al*., 1996). Heterocysts usually occur singly in a semi-regular spacing within filaments of vegetative cells (Golden and Yoon, 2003; Zhang *et al*., 2006). The infective agents in most plant symbioses are hormogonia and some, perhaps all, plants produce chemical signals that trigger their formation and chemoattractants that guide them into the plant tissue.

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The symbiotic partners

Bryophytes

Bryophytes are small, non-vascular land plants encompassing the liverworts (Hepaticae), the hornworts (Anthocerotae), and the mosses (Musci), a relatively small number of which are able to form epiphytic or endophytic associations with cyanobacteria (Adams, 2002; Meeks, 2003). In the case of the mosses, the cyanobacteria grow mostly epiphytically (Solheim and Zielke, 2002; Gentili *et al*., 2005), the exception being two *Sphagnum* species in which the cyanobacteria occupy water-filled, hyaline cells, which are thought to provide some protection from the acidic bog environment (Solheim and Zielke, 2002). Similar protection for cyanobacteria on the moss leaf surface is provided by the secretion of alkaline substances (Belnap, 2001). These nitrogen-fixing cyanobacterial associations with mosses often supply most of the combined nitrogen in local ecosystems in the Arctic, the Antarctic, and boreal forest regions (Zielke *et al*., 2002, 2005). This may be particularly significant in boreal forests where the feather moss *Pleurozium schreberi* (with its epiphytic cyanobacteria) can provide 80% of the ground cover (DeLuca *et al*., 2002, 2007; Zackrisson *et al*., 2004; Gentili *et al*., 2005).

Cyanobacterial associations with liverworts are rare, being found in only four of the >340 liverwort genera, two of the associations (*Marchantia* and *Porella*) being epiphytic and two (*Blasia* and *Cavicularia*) endophytic (Meeks, 1990). By contrast, in the hornworts, of which there are presently 13 genera described (Duff *et al*., 2007), endophytic associations are ubiquitous (Renzaglia *et al*., 2007). In nature, liverworts and hornworts exist as a flattened gametophyte thallus a few centimetres in length and symbiotic colonies are visible as small, dark spots (Fig. 1A). The endophytic associations with hornworts, such as *Anthoceros* and *Phaeoceros*, and liverworts such as *Blasia*, are particularly suited to laboratory

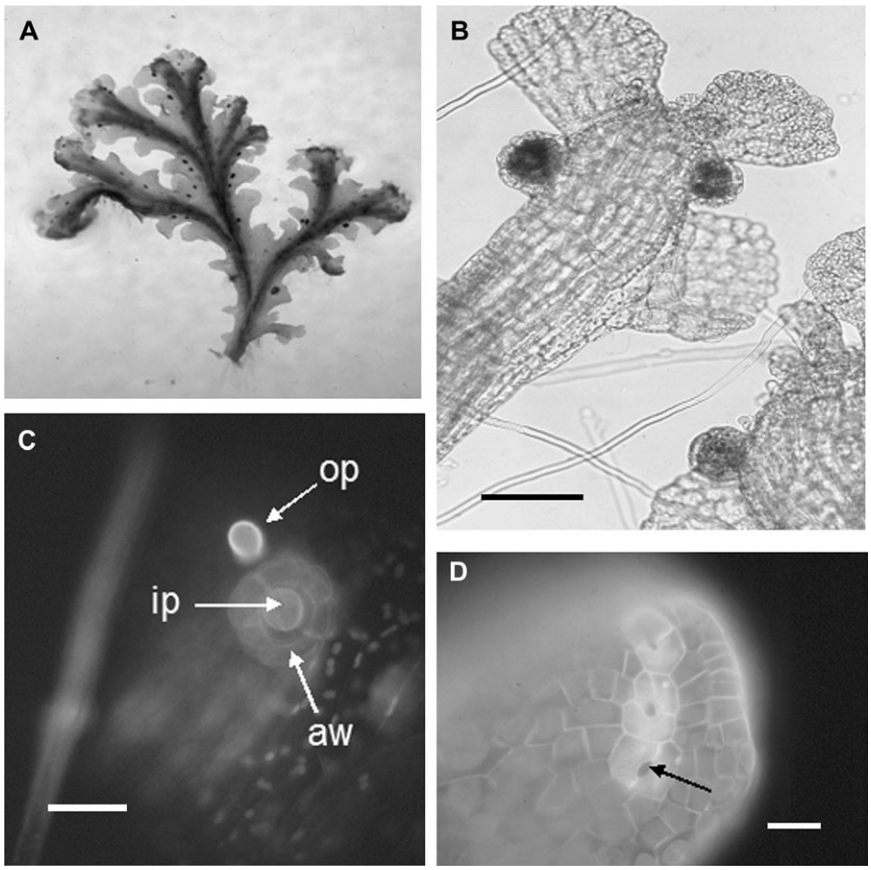

Fig. 1. (A) The liverwort *Blasia pusilla* showing dark colonies of *Nostoc*, ~0.5–1 mm in diameter, bordering the midrib of the thallus. (B) *Blasia pusilla* thallus infected in the laboratory with two different cyanobacteria. The two colonies in the top half of the image contain a different cyanobacterium to the colony at the bottom. (C) Fluorescence micrograph of a *Blasia pusilla* auricle, stained with calcofluor, showing the inner (ip) and outer (op) papillae. In infected auricles the cyanobacteria occupy the space (which appears dark in this micrograph) between the inner papilla and the auricle wall (aw). (D) Fluorescence micrograph of the hornwort *Phaeoceros* sp., stained with calcofluor, showing the slit-like entrances (one of which is arrowed) to the slime cavities within the thallus, which become infected by cyanobacteria. Scale bars represent 30 μm in (C) and (D), and 120 μm in (B). Photographs (C) and (D) courtesy of S Babic. (A), (C), and (D) reproduced with kind permission of Springer Science and Business Media from Adams (2000); (B) reproduced with permission of *New Phytologist*, Copyright 1999, from Adams and Duggan (1999).

experimentation because the host plant can be freed of its symbiotic cyanobacteria and grown in shaken liquid culture, and the symbiosis re-established with the original or with novel cyanobacteria (Fig. 1B; Adams, 2002; Meeks, 1998, 2003).

Bryophyte 'symbiotic' structures: Mature cyanobacterial colonies are up to 0.5 mm in diameter and occupy dome-shaped structures known as auricles on the ventral surface of the thallus of the liverwort *Blasia* (Fig. 1C), or slime cavities within the thallus of hornworts such as *Anthoceros* and *Phaeoceros* (Fig. 1D). These structures are not unique to the symbiotic state, but are also present in plants not infected with cyanobacteria. The hornwort slime cavity is connected to the ventral surface of the thallus via stomata-like pores (Meeks, 1998, 2003; Adams, 2002). By contrast, in the hornwort *Leiosporoceros dussii*, the cyanobacteria are found in mucillage-filled 'canals' which run parallel to the main axis of the thallus (Villarreal *et al*., 2005; Villarreal and Renzaglia, 2006). These canals are formed by the separation of plant cell walls along their middle lamellae and, as the canals elongate they bifurcate to form an integrated network throughout the plant (Villarreal and Renzaglia, 2006). This unique arrangement of canals enables the *Nostoc* to spread throughout the *Leiosporoceros dussii* thallus without the repeated infections required for the discrete colonies in other hornworts and liverworts.

The cyanobacteria

In the great majority of cases, plant cyanobionts are members of the genus *Nostoc*, which is commonly found free-living in nature (Dodds *et al*., 1995; Rai *et al*., 2002). This is equally true of the liverworts and hornworts in which the major cyanobionts are *Nostoc* spp. (West and Adams, 1997; Costa *et al*., 2001). However, in the laboratory, other hormogonium-developing cyanobacterial genera, such as *Calothrix* and *Chlorogloeopsis*, may infect liverworts (West and Adams, 1997).

Hormogonia: For free-living cyanobacteria to locate and enter the symbiotic cavities of host plants they must be motile. Cyanobacteria move by a process known as gliding, which requires contact with a surface. The mechanism of gliding is unknown, but as motility is so vital to plant infection it will be dealt with separately in the final two sections of this review. Members of the genus *Nostoc* are primarily non-motile, but a characteristic of the genus is the ability to produce specialized motile filaments known as hormogonia which serve as a means of dispersal as well as plant infection (Bergman *et al*., 1996; Meeks, 1998, 2003; Adams, 2000; Meeks and Elhai, 2002). Hormogonia development is triggered by a variety of environmental factors, including plant-derived chemical signals (see the section on hormogonia induction). For a successful symbiosis to be established with the plant host the cyanobiont must also have the capacity to develop nitrogen-fixing heterocysts, although mutants capable of forming hormogonia, but unable to form heterocysts, are still able to infect *Anthoceros punctatus* but cannot establish a fully functional, nitrogen-fixing symbiosis (Wong and Meeks, 2002).

The development of hormogonia in heterocystous cyanobacteria results from a round of rapid, synchronous cell divisions which result in a decrease in cell size (Meeks and Elhai, 2002; Meeks *et al*., 2002). This is followed by fragmentation of the filament at the heterocyst–vegetative cell junctions, releasing short, motile hormogonia. Hormogonia lack heterocysts and are a temporary stage in the *Nostoc* life-cycle, soon returning to vegetative growth and developing heterocysts once more. For hormogonia to locate the symbiotic tissue of a plant host they must attach to the surface and both extracellular polysaccharides and pili (fimbriae) are thought to play a role in this process (Adams, 2000). Type IV pili are required for gliding in some unicellular cyanobacteria (Bhaya, 2004), and the cell surface of hormogonia of the symbiotically competent *Nostoc punctiforme* is covered with pili (Duggan *et al*., 2007). The importance of pili in the symbiotic competence and motility of hormogonia will be discussed in detail in the final section of this review.

Recent microarray data have revealed over 1800 differentially transcribed genes in hormogonia 24 h post-induction, some 5-fold greater than the number transcriptionally active in akinete (spore)-forming or dinitrogen-fixing cultures (Campbell *et al*., 2007). Of these, 944 genes are up-regulated, with the majority of these encoding proteins for signal transduction and transcriptional regulation. Genes encoding putative chemotaxis proteins and pilus biogenesis genes are also up-regulated in hormogonia (Meeks *et al*., 2001; Klint *et al*., 2006; Campbell *et al*., 2007). The hormogonium appears genetically rich in terms of potential for sensory and regulatory perception and has evolved to sense and respond to a variety of environmental signals, such as light quality and quantity, chemicals released from potential host plants, or favourable environments that will support subsequent growth. The genes involved in these sensory pathways remain to be characterized and genes central to the hormogonia differentiation process itself remain to be identified.

The infection process

Hormogonia induction

Plant hosts increase the likelihood of infection by cyanobacteria by both stimulating the formation of

hormogonia in potential cyanobionts and by guiding the hormogonia to the symbiotic tissues by chemotaxis. Hormogonia formation is stimulated by hormogonia-inducing factors (HIFs). HIF production has been found in the hornwort *Anthoceros punctatus* (Meeks, 2003), as well as cycads and the angiosperm *Gunnera* (Rasmussen *et al*., 1994; Ow *et al*., 1999). *Anthoceros punctatus* HIF is a small, heat-labile product released by the hornwort when starved of combined nitrogen (Meeks and Elhai, 2002; Meeks, 2003). The liverwort *Blasia* also releases HIF when nitrogen-starved (Adams, 2002). *Nostoc punctiforme* mutants with increased sensitivity to *Anthoceros* HIF, also show a greater initial frequency of infection of the hornwort than the wild type (Cohen and Meeks, 1997). Mutation of *ntcA*, which encodes the global transcriptional regulator, NtcA (Herrero *et al*., 2004) results in a reduction in the frequency of hormogonia induced by HIF in *N. punctiforme*, and the hormogonia that are produced do not infect *Anthoceros* (Wong and Meeks, 2002).

The efficiency of infection of the liverwort *Blasia* by *N. punctiforme* is influenced by mutations in the gene *cyaC*, which encodes adenylate cyclase, the enzyme responsible for the biosynthesis of the intracellular messenger adenosine 3′,5′-cyclic monophosphate (cAMP). Cyanobacteria possess multiple adenylate cyclases, of which CyaC is the most complex, consisting of seven domains (Cann, 2003). A *N. punctiforme* mutant containing an antibiotic resistance cassette towards the 5′ region of *cyaC* (within the second of the two GAF domains encoded by the gene), infects *Blasia* at ~25% of the wild-type frequency (Chapman *et al*., 2004). By contrast, a mutant carrying an insertion of the same cassette in the 5′ region of *cyaC*, encoding the putative catalytic domain, infects *Blasia* at a 3–4-fold higher frequency than the wild type (Chapman *et al*., 2004; Chapman, 2006). In the presence of *Blasia*, both mutants form similar frequencies of hormogonia to the wild type, but ~12 h earlier than the wild type. Transfer of wild-type cells from light growth conditions to the dark produced a rapid increase in cAMP, consistent with its role as a signalling molecule. By contrast, the *cyaC* mutants do not show any corresponding increase in cellular cAMP levels following transfer from the light to the dark and cellular cAMP levels are only ~25% of the wild type. The *Nostoc* genome contains additional putative adenylate cyclases which may account for the small amounts of cAMP present in the mutant strains. The similar cAMP levels in these two mutants with contrasting infection phenotypes, implies that cAMP *per se* is not involved in symbiotic competency. The reason for these different phenotypes remains unclear; both mutants are motile and there is no apparent difference in hormogonia piliation compared with the wild type. It may be that unrecognized differences in the behaviour of the mutant hormogonia, in response to plant signals, are responsible for their infection phenotypes.

Further evidence that hormogonia behaviour, as well as frequency, has an influence on plant infection comes from work on the *N. punctiforme* gene *sigH*, which encodes an alternative sigma subunit of RNA polymerase, and transcription of which is induced by *Anthoceros* HIF (Campbell *et al*., 1998; Meeks, 2003). Inactivation of *sigH* has no effect on the frequency of HIF-induced hormogonia, but the hormogonia are 3–5-fold more efficient than the wild type at infecting *A. punctatus* (Meeks and Elhai, 2002; Meeks, 2003).

Chemotaxis

Hormogonia production alone is not sufficient to guarantee establishment of symbiosis since non-infective *Nostoc* strains are also capable of forming motile hormogonia in the presence of the angiosperm *Gunnera* (Johansson and Bergman, 1994). Clearly, hormogonia must possess specific characteristics that enable them to infect a host successfully. One such characteristic is likely to be the ability to respond chemotactically to plant-derived chemicals and this is especially important in plants such as *Gunnera* and cycads in which the symbiotic tissue does not receive light and, as a consequence, the hormogonia must overcome their natural positive phototaxis. The importance of chemotaxis is reflected in the genome of the symbiotically competent cyanobacterium *N. punctiforme* which has three to five copies each of the genes encoding homologues of the chemotaxis-related proteins CheA, CheB, CheW, CheD, and CheR (Meeks *et al*., 2001). When the liverwort *Blasia* is starved of combined nitrogen it releases both HIF (Babic, 1996) and a very effective chemoattractant (Knight and Adams, 1996; Watts *et al*., 1999). Non-host plants such as *Trifolium repens* (Nilsson *et al*., 2006) and germinating wheat seeds (Knight and Adams, 1996; Watts *et al*., 1999) can also release hormogonia chemoattractants.

Invasion of the plant

Once hormogonia enter *Blasia* auricles (and presumably the slime cavities of hornworts), they lose motility and differentiate heterocysts (Kimura and Nakano, 1990). The infection of hornworts via the stomata-like opening to the slime cavity has interesting parallels with the likely method of entry of cyanobacteria into the primitive, extinct land plant *Aglaophyton major*. This symbiosis is only known from fossil evidence, but an *Archaeothrix*-type filamentous cyanobacterium is thought to have entered the plant via stomatal pores (Taylor and Krings, 2005). The cyanobacteria are thought to have initially colonized the substomatal chambers and then spread throughout the outer cortical tissue, where they can be seen in fossil specimens of the plant. This is somewhat

similar to the infection process in the extant hornwort *Leiosporoceros dussii* in which the cyanobacteria are found in mucilage-filled 'canals' (see section on bryophyte symbiotic structures; Villarreal *et al*., 2005; Villarreal and Renzaglia, 2006).

Although the development of heterocysts is essential for functional, nitrogen-fixing symbiotic colonies, *Nostoc* mutants unable to form heterocysts can still infect the hornwort *A. punctatus*. Strains carrying mutations in *hetR* (Wong and Meeks, 2002), which is the primary activator of heterocyst development (Golden and Yoon, 2003; Zhang *et al*., 2006), and *hetF* (Wong and Meeks, 2002), which is involved in the control of *hetR* transcription and the localization of HetR to developing heterocysts (Wong and Meeks, 2001), infect *Anthoceros* at wild-type frequency but fail to support the growth of the hornwort (Wong and Meeks, 2002). By contrast, a strain carrying a mutation in *ntcA*, which encodes the global nitrogen regulator NtcA (Flores and Herrero, 2005), does not infect the hornwort, despite producing motile hormogonia, albeit at a reduced level to the wild type (Wong and Meeks, 2002).

Post-infection changes

Once the cyanobacterium has entered the host plant a number of morphological, developmental, and physiological changes occur (Table 1). The development of hormogonia is repressed, whereas the development of heterocysts is greatly stimulated. The rate of cell division is reduced, ensuring that the cyanobiont does not outgrow the host. The rate of CO_2 fixation is greatly reduced, whereas nitrogen fixation is stimulated and ammonium assimilation down-regulated.

Table 1. *Summary of morphological and physiological changes in cyanobacteria symbiotically associated with hornworts and the liverwort* Blasia

The table was compiled from the following references: Steinberg and Meeks (1989), Meeks (1990), Rai (1990), and Bergman *et al*. (1992). n.d.=not determined, although values are likely to be similar to hornwort data.

	Hornworts	Liverworts (*Blasia*)
Plant structure infected	Slime cavities	Auricles
Cyanobiont	*Nostoc*	*Nostoc*
Location of cyanobiont	Intercellular	Intercellular
Heterocyst frequency[a]	30–50%	30–50%
Nitrogenase specific activity	23.5	n.d.
Glutamine synthetase:		
Amount of protein[b]	~86%	n.d.
Specific activity[b]	~38%	n.d.
Form of combined N released	NH_4^+	NH_4^+
Light-dependent CO_2 fixation	12%	n.d.
Rubisco:		
Amount of protein[c]	100%	n.d.
Specific activity[c]	12%	n.d.

[a] Heterocyst frequencies are expressed as a percentage of total cells. Values for free-living cyanobacteria are typically 4–10%.
[b] Values are for the symbiont as a percentage of the same cyanobacterium in the free-living state.
[c] Values are expressed as a percentage of those for the same free-living cyanobacterium.

Hormogonia repression

Once inside the host plant the hormogonia revert to vegetative growth, differentiating heterocysts and fixing nitrogen. In the hornwort *A. punctatus* (and presumably also liverworts) further hormogonia formation within the symbiotic colony is prevented by a water-soluble hormogonium repressing factor (HRF) released into the colony by the plant (Meeks and Elhai, 2002; Meeks, 2003). HRFs induce expression of the hormogonium-repressing genes located in the *hrmUA* locus of *N. punctiforme* (Cohen *et al*., 1994; Cohen and Meeks, 1997). Regulation is achieved through the sugar-binding transcriptional repressor HrmR, which prevents any new rounds of hormogonium development (Campbell *et al*., 2003).

CO_2 fixation

Light-dependent CO_2 fixation of the *Nostoc* cyanobiont of *Anthoceros*, immediately after isolation from the hornwort, is only 12% that of the free-living cyanobacterium (Table 1). However, the level of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) protein, the major enzyme involved in the fixation of CO_2 in cyanobacteria, is little different in free-living or *Anthoceros*-associated *Nostoc*, implying that activity is regulated by an unknown post-translational modification of the protein (Meeks and Elhai, 2002; Meeks, 2003). The reduced carbon-fixing capacity of the cyanobiont is compensated by sugars derived from the host (Meeks and Elhai, 2002; Adams, 2002; Meeks, 2003).

N_2 fixation

The nitrogen fixation rates for cyanobacteria symbiotically associated with bryophytes are several-fold higher than for the same free-living cyanobacteria (Table 1). This increase is due to a greatly elevated heterocyst frequency, which may be 6–10-fold higher than in the free-living state (Table 1). As little as 20% of the nitrogen fixed is retained by the cyanobiont, the remainder being transferred as ammonia to the host (Adams, 2002; Meeks and Elhai, 2002). The primary route of ammonia assimilation in cyanobacteria is the GS-GOGAT (glutamine synthetase–glutamate synthase) pathway (Meeks and Elhai, 2002). The level of GS protein in *Anthoceros*-associated *Noctoc* is similar to that in free-living cyanobacteria, but GS activity is reduced (Table 1) implying that activity is regulated by an unknown, and presumably plant-regulated, post-translational modification of the enzyme (Meeks and Elhai, 2002; Meeks, 2003).

Morphological changes to cyanobiont and host

Cyanobiont growth rate must match that of the host plant if the association is to be stable. Indeed, the growth of *Nostoc* symbiotically associated with *Anthoceros* can be 5–10-fold slower than in the free-living state (Meeks, 2003; see Table 1). The host can also regulate the biomass and nitrogen fixation of the symbiotic colonies to maintain a constant rate of nitrogen fixation per unit of plant tissue, but the mechanism by which this is achieved is unknown (Meeks, 2003).

As is the case with all cyanobacteria–plant symbioses, cyanobacteria in symbiosis with hornworts and liverworts exhibit greatly elevated heterocyst frequencies compared with the free-living state (Table 1; Meeks, 2003). This enhances the colony's dinitrogen-fixing capacity to the benefit of the host, although it seems likely that at least some of these extra heterocysts are non-functional (Meeks and Elhai, 2002). Within the symbiotic colony, heterocysts become difficult to recognize because they often lose some of the morphological characteristics that distinguish them from vegetative cells, including their regular shape and thickened cell walls (Adams, 2002; Meeks and Elhai, 2002). The vegetative cells also show altered morphology in symbiosis, being enlarged and irregular in shape compared with their free-living counterparts (Meeks and Elhai, 2002).

Although cyanobacteria occupy liverwort and hornwort structures present prior to the infection, limited morphological changes do occur to these structures post-infection. In both *Blasia* and *Anthoceros*, the walls of the symbiotic cavity elaborate branched, multicellular filaments that penetrate the colony, facilitating the exchange of nutrients between the colony and the plant (Adams, 2002). Indeed, the filaments in *Blasia*, which show ultrastructural differences to those in *Anthoceros*, possess some of the characteristics of transfer cells (Duckett *et al*., 1977). What induces these morphological changes is not known, although cyanobacteria do produce the phytohormone auxin, indole-3-acetic acid (Sergeeva *et al*., 2002) and other compounds known to be involved in plant development (Liaimer and Bergman, 2004). Plant host genes equivalent to the nodulation genes of legumes have so far not been identified in any of the plant hosts in cyanobacterial symbioses (Udvardi *et al*., 2005).

Cyanobacterial motility

For cyanobacteria to locate and invade the relevant plant host tissue, the capacity for motility, coupled with chemotaxis, is essential. Of the many motile cyanobacteria the vast majority move by gliding, which requires attachment to a surface. The exception is a few unicellular *Synechococcus* strains that can swim by a mechanism in which thrust is thought to be generated, not by flagella, but by the extracellular glycoprotein SwmA (McCarren and Brahamsha, 2005; McCarren *et al*., 2005). The form of gliding employed by the unicellular *Synechocystis* sp. strain PCC 6803 is sometimes referred to as twitching motility, and is powered by type IV pili on the cell surface (Bhaya *et al*., 1999, 2000; Yoshihara and Ikeuchi, 2004). In filamentous strains, such as members of the Oscillatoriaceae, forward movement is often accompanied by rotation of the filament about its long axis (Halfen, 1973), although the mechanism of gliding is unknown. An early model for gliding in these filamentous strains was developed by Halfen and Castenholz (1971), based on both their own observations and the theories of Jarosch (1963). They proposed that the thrust for gliding might be provided by contraction of 6–9 nm diameter protein fibrils, situated between the peptidoglycan layer and the outer membrane. The helical arrangement of these fibrils could account for the rotation of filaments as they glide.

More recently, Adams *et al*. (1999) observed an array of parallel fibrils, in the same location as those observed by Halfen and Castenholz (1971), between the peptidoglycan and the outer membrane, but of a much greater diameter (25–30 nm, rather than 6–9 nm). Penetration of the outer membrane between the rows of fibrils results in corrugations in the outer surface of the cells. This corrugated appearance can be visualized by field emission scanning electron microscopy (FEGSEM; Fig. 2) and by atomic force microscopy (AFM; Fig. 3). In a re-iteration of the model of Halfen and Castenholz (Halfen and Castenholz, 1971; Halfen, 1973), Read *et al*. (2007) have proposed that rhythmical waves passing along the 25–30 nm fibrillar array could be transmitted via the outer membrane and, by interaction with the substrate on which the cyanobacterium rests, drive the filament in the opposite direction.

An alternative model, which involves slime extrusion, has been developed by Hoiczyk and Baumeister (1995) who identified a layer of helically arranged 10–12 nm diameter fibrils of a calcium-binding protein, which they called oscillin, in several filamentous cyanobacteria (Hoiczyk and Baumeister, 1997). These fibrils are external to the outer membrane and rest on a tetragonal S-layer (Hoiczyk and Baumeister, 1995). Hoiczyk and Baumeister (1997, 1998) proposed that gliding was powered by the extrusion of slime from the junctional pores that form a ring around each cell septum. They concluded that the helically arranged oscillin fibrils acted like a screw thread, resulting in rotation of the trichome. A mutant of *Phormidium uncinatum* unable to extrude slime and lacking both the S-layer and oscillin fibrils is immotile, implying that one, or more, of these components is essential for motility (Hoiczyk and Baumeister, 1997). At present there is no conclusive evidence to support either the slime extrusion or the surface wave models for force generation in gliding. The development of AFM scanning of live

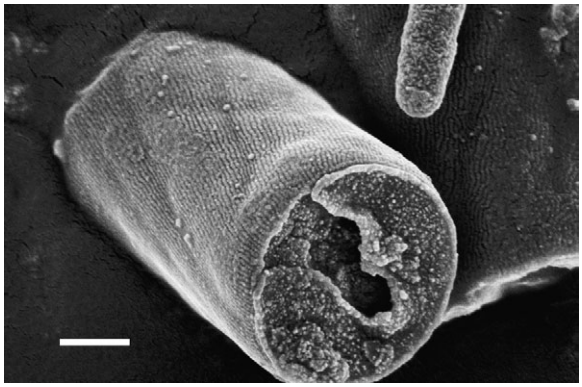

Fig. 2. *Oscillatoria* sp. strain A2 cell surface visualized by FEGSEM. The single cell visible in the centre of the micrograph would have originally been part of a filament hundreds of cells in length. The broken end of the cell, where it was attached to the remainder of the filament, can be seen to the bottom right. The array of 25–30 nm fibrils in the cell wall results in the parallel corrugations visible on the cell surface; the array runs at an angle to the long axis of the filament. The end of a contaminating bacterium can be seen to the upper right of the micrograph. The scale bar represents 500 nm. Reproduced with permission from Read *et al.* (2007; copyright © 2007, American Society for Microbiology).

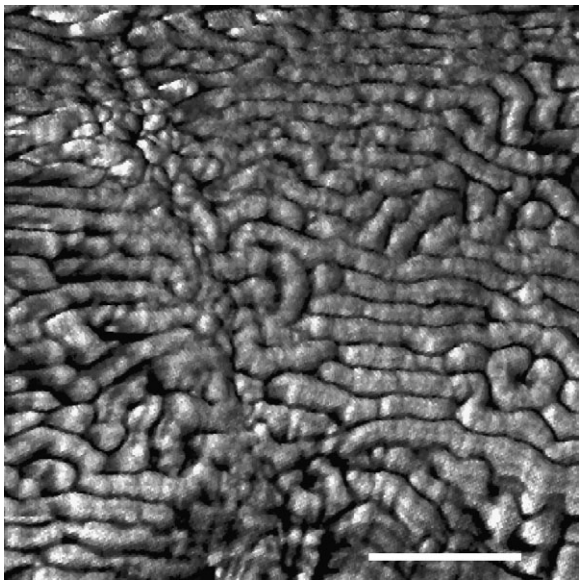

Fig. 3. *Oscillatoria* sp. strain A2 cell surface visualized by AFM. The scan was performed in air on a sample dried onto a glass slide. A cell septum runs from the top left to the bottom middle of the micrograph. There is some disruption to the parallel array of the 25–30 nm fibrils, probably caused by the drying process. The scale bar represents 200 nm. Reproduced with permission from Read *et al.* (2007); copyright © 2007, American Society for Microbiology.

cyanobacterial filaments under liquid may soon make it possible to visualize surface waves, should they exist.

Hormogonia and pili

The mechanism of gliding in non-heterocystous filamentous cyanobacteria, such as members of the Oscillatoriaceae, remains obscure, but what of the heterocystous cyanobacteria that are potential cyanobionts of plants? These cyanobacteria are capable of transforming immotile filaments into motile hormogonia, but what is known about the mechanism of hormogonia motility?

The external surface of a wide range of bacteria is covered with filamentous structures known as Type IV pili (Tfp) which function in numerous processes including motility, adhesion, pathogenesis, and DNA uptake (Wolfgang *et al*., 2000; Mattick, 2002; Nudleman and Kaiser, 2004; Burrows, 2005). *Nostoc punctiforme* vegetative filaments are non-motile and appear to lack these structures (Fig. 4A), whereas the cell surface of motile *N. punctiforme* hormogonia has abundant Tfp-like appendages (Fig. 4B), produced during differentiation (Doherty and Adams, 1999). The pili are arranged peritrichously, extend up to 10 μm from the cell surface, and have diameters of 7–10 nm. Tfp are composed primarily of a single protein, pilin (PilA), which is synthesized as a precursor (prepilin) and undergoes endoproteolytic cleavage, followed by methylation of the N-terminal residue, prior to assembly into the filament. Both endoproteolytic cleavage and *N*-methylation are carried out by the prepilin peptidase (PilD), which is known to process a wide range of other prepilin-like proteins (Strom *et al*., 1991; Paranjpye *et al*., 1998; Lammertyn and Anne, 2004). Motility associated with Tfp involves cycles of pilus extension, surface attachment of the pilus tip, and retraction of the pilus filaments to transport the cell toward the point of adhesion (Merz *et al*., 2000; Skerker and Berg, 2001; Merz and Forest, 2002). Pilus retraction generates considerable force (Merz *et al*., 2000; Maier *et al*., 2002) and is thought to occur via filament disassembly mediated by the hexameric ATPase nucleotide-binding protein, PilT (Skerker and Berg, 2001; Morand *et al*., 2004). PilT mutants are unable to retract their pili, leading to hyperpiliation and loss of twitching motility (e.g. Whitchurch *et al*., 1991; Wolfgang *et al*., 1998; Merz *et al*., 2000).

To date, cyanobacterial Tfp systems have been demonstrated in *Synechocystis* PCC 6803 (Bhaya *et al*., 1999, 2000, 2001; Yoshihara *et al*., 2001, 2002) and *Microcystis aeruginosa* PCC 7806 (Nakasugi and Neilan, 2005; Nakasugi *et al*., 2007), together with a report of Tfp-like genes in the genome of the thermophillic cyanobacterium *Thermosynechococcus elongates* BP-1 (Iwai *et al*., 2004). Additionally, Tfp-like genes can be found in the cyanobacterial genomes of *Anabaena* sp. PCC 7120, *Gloeobacter violaceus* PCC 7421, *Prochlorococcus marinus* MIT9313, *Synechococcus* sp. WH8102, *Synechococcus elongatus* PCC 6301, and *Synechococcus* sp. CC9311 (http://bacteria.kazusa.or.jp/cyano/). The genome of the symbiotically competent *N. punctiforme* (http://genome.jgi-psf.org/finished_microbes/nospu/nospu.home.html) also contains open reading frames (ORFs) that are predicted to

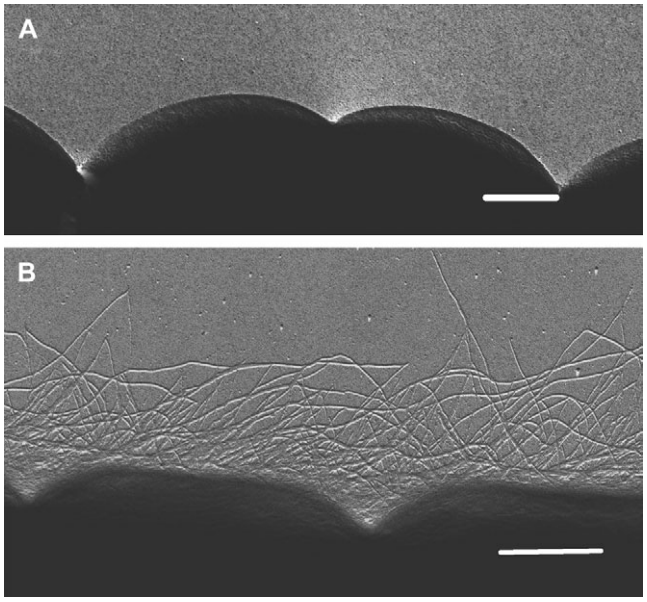

Fig. 4. Comparison of the cell surfaces of *N. punctiforme* wild-type vegetative filaments and hormogonia by transmission electron microscopy (TEM). (A) Wild-type vegetative filaments which do not possess pili, and (B) wild-type hormogonia with abundant pili. Scale bars represent 1 μm. For electron microscopy, platinum wire (2 cm×0.2 mm) was evaporated onto the surface of each sample using an Edwards 306A high vacuum coating unit and samples viewed on a JEOL1200EX transmission electron microscope at 80 kV. Reproduced with permission from Duggan *et al.* (2007; copyright © 2007, American Society for Microbiology).

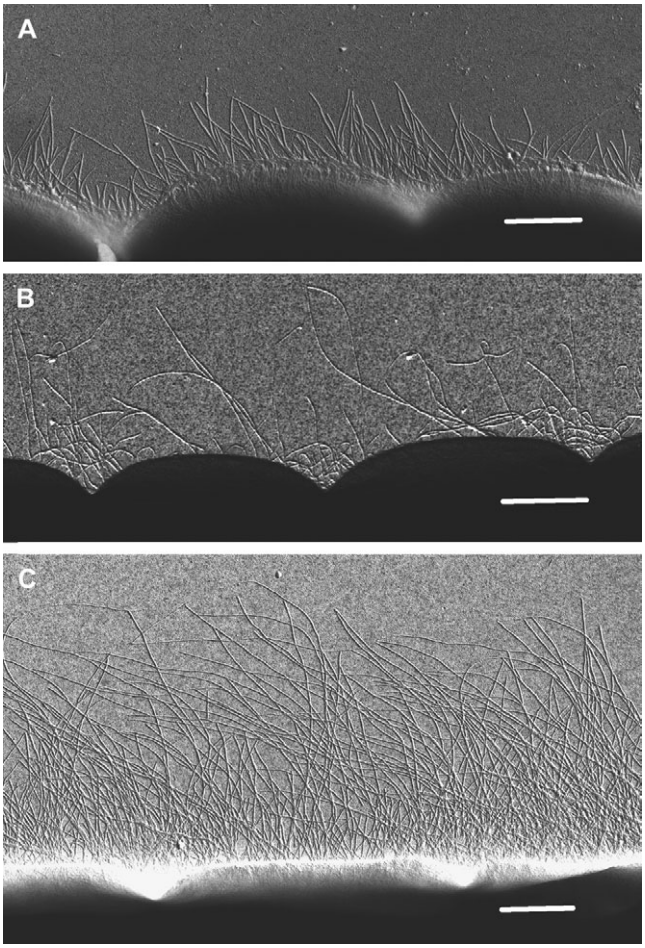

Fig. 5. Comparison of the cell surface of *pil* mutants of *N. punctiforme* visualized by TEM. (A) Hormogonia of the *NpR2800* (*pilD* homologue) mutant which produces short pili. (B) Hormogonia of the *NpF2507 (pilT* homologue) mutant which produces few pili. (C) Hormogonia of the *NpR0117* (*pilT* homologue) mutant which is hyperpiliated. Scale bars represent 1 μm. Reproduced with permission from Duggan *et al.* (2007; copyright © 2007, American Society for Microbiology). For TEM details, see the legend to Fig. 4.

encode proteins with similarity to Tfp biogenesis proteins, including two *pilT*-like genes. Inactivation of one of these *pilT*-like genes (*NpR0117)* results in a hyperpiliated (overproduction of pili) phenotype (Fig. 5C) that is typical of mutations within *pilT* in other bacteria (Duggan *et al.*, 2007). Mutation of the second *Nostoc pilT*-like *NpF2507* results in slightly less-abundant pili than the wild type, although their length and diameter appear to be similar to those of the wild type (Fig. 5B). The *NpR0117* (hyperpiliated) mutant infects the host plant *Blasia pusilla* at 9.5% of the frequency of the wild type, implying that normal surface piliation is required for effective establishment of symbiosis. The role of *pilT*-like *NpF2507* is less clear. NpF2507 mostly resembles PilT2 from *Synechocystis* PCC 6803, which is dispensable for motility but is believed to function in a signalling pathway that regulates the positive phototaxis of this cyanobacterium (Bhaya *et al.*, 2000). The *NpF2507* mutant infects *Blasia* at an initial frequency 50% that of the wild type, but reaches a level equivalent to that of the wild type after 28 d, 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 of the *Synechocystis* PilT2, remains to be investigated.

Disruption of *N. punctiforme pilD*-like *NpR2800* gives 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 pilus diameter (7–10 nm) is similar to that of the wild type (Fig. 5A; Duggan *et al.*, 2007). In other bacteria, mutations in *pilD* abolish motility and produce a non-piliated phenotype (e.g. Lory and Strom, 1997; Bhaya *et al.*, 2000; Friedrich *et al.*, 2002). The truncated pilus phenotype observed in the *Nostoc pilD* mutant is unusual and may represent partially retracted or partially extended pili. With the *pilD*-like *NpR2800* mutant the frequency of *Blasia* infection is only 2.4% of that of the wild type. Many of the symbiotic filaments are brown in colour, implying that they are dead or dying and that the mutant is unable to grow effectively within the host symbiotic cavity. The *Nostoc pilD*-like gene may therefore be required for some aspect of symbiotic growth.

Nostoc punctiforme has at least five ORFs whose predicted products contain features that characterize the pilus structural subunit (PilA). Of the three *Nostoc pilA*-like ORFs investigated by Duggan *et al*. (2007), complete segregation was only achieved with *pilA*-like ORF *NpF0069*. The pili expressed on the surface of the hormogonia produced by this mutant were indistinguishable from those of the wild type, yet the mutant infected plant tissue at a frequency only 38% that of the wild type. It is possible that *pilA*-like *NpF0069* encodes a minor pilin (Alm and Mattick, 1996; Winther-Larsen *et al*., 2005) that may support the activity of the major pilin in some aspect of motility and/or adhesion and therefore contribute to symbiotic competency.

It is clear that mutation of some *pil* genes affects the symbiotic competence of hormogonia, but what effect do these mutations have on motility? The hormogonia of the wild-type strain employed in the study of Duggan *et al*. (2007) were inconsistently motile and when motility was present the hormogonia progressed slowly at rates of 0.7 μm s^{-1} to 1.7 μm s^{-1}. Slight twitching of filaments, but without forward progression, was detected in the *pilA*-like *NpF0069* and *pilT*-like *NpF2507* mutants, whereas motility was not observed in strains inactivated in *pilD*-like *NpR2800* and *pilT*-like *NpR0117*. Given the inconsistencies in the motile behaviour of the wild-type strain, the possibility that the lack of motility associated with the *NpR2800* and *NpR0117* mutants is the result of mutation in the *pilD*-like ORF and the *pilT*-like ORF, respectively, remains to be demonstrated. Nevertheless, the data confirm that Tfp are required for effective establishment of *Nostoc–Blasia* symbiosis and, most likely, other cyanobacteria–plant symbioses.

Concluding remarks

Although current understanding of the molecular basis for many of the interactions involved in cyanobacteria–plant symbioses is poor, continuing advances in molecular genetic techniques for symbiotically competent cyanobacteria should lead to more rapid advances in this area. For example, recently obtained *N. punctiforme* microarray data has revealed the remarkable complexity of hormogonium differentiation, with over 1800 genes being differentially transcribed during the process (Campbell *et al*., 2007). Despite the importance of motility and chemotaxis in plant infection by hormogonia, neither process is well understood. However, the availability of the genome sequence of the symbiotically competent *N. punctiforme* (http://genome.jgi-psf.org/finished_microbes/nospu/nospu.home.html) and the *N. punctiforme* microarray, should lead to significant advances in this area. Certainly such advances will be needed if the goal of developing novel associations between cyanobacteria and crop plants is to be realized.

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