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REVIEW ARTICLE

Nitrogen-fixing bacteria associated with leguminous and non-leguminous plants

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Abstract Nitrogen is generally considered one of the major limiting nutrients in plant growth. The biological process responsible for reduction of molecular nitrogen into ammonia is referred to as nitrogen fixation. A wide diversity of nitrogen-fixing bacterial species belonging to most phyla of the *Bacteria* domain have the capacity to colonize the rhizosphere and to interact with plants. Leguminous and actinorhizal plants can obtain their nitrogen by association with rhizobia or *Frankia* via differentiation on their respective host plants of a specialized organ, the root nodule. Other symbiotic associations involve heterocystous cyanobacteria, while increasing numbers of nitrogen-fixing species have been identified as colonizing the root surface and, in some cases, the root interior of a variety of cereal crops and pasture grasses. Basic and advanced aspects of these associations are covered in this review.

Keywords Nitrogen fixation · Symbiosis · Rhizobia · *Frankia* · Cyanobacteria · *Azospirillum*

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Introduction

Fixed nitrogen is a limiting nutrient in most environments, with the main reserve of nitrogen in the biosphere being molecular nitrogen from the atmosphere. Molecular nitrogen cannot be directly assimilated by plants, but it becomes available through the biological nitrogen fixation process that only prokaryotic cells have developed. Proliferation of bacteria in soil adhering to the root surface was discovered toward the end of the nineteenth century, at the same time as the discovery of nitrogen fixation. The term "rhizosphere" was then coined by Hiltner in 1901 to designate soil immediately surrounding roots under the influence of the plant (see Rovira 1991).

For many years, a limited number of bacterial species were believed to be nitrogen fixers (Postgate 1981), but in the last 30 years nitrogen fixation has been shown to be a property with representatives in most of the phyla of *Bacteria* and also in methanogenic *Archaea* (Young 1992). The property of symbiotically fixing nitrogen within nodules of vascular plants is found in two major groups of bacteria

not phylogenetically related: rhizobia (*Alpha-proteobacteria*) that associate essentially with leguminous plants belonging to one superfamily of angiosperms (Fabaceae), (Sprent 2001) and *Frankia* (in *Actinobacteria*) that associate with a broader spectrum of plants from eight families (Huss-Danell 1997; Vessey et al. 2004). Another important group of nitrogen-fixing bacteria is that of the cyanobacteria, found in association with a large variety of higher and lower plants, fungi and algae (Meeks and Elhai 2002). Associative symbiosis refers to a wide variety of nitrogen-fixing species that colonize the root surface of non-leguminous plants, without formation of differentiated structures (Elmerich and Newton 2007). Among these, the frequent isolation of bacteria from surface-sterilized root led to identification of a new category, nitrogen-fixing endophytes (Döbereiner 1992; Reinhold-Hurek and Hurek 1998; Baldani and Baldani 2005).

The present paper presents an overview of the general biology of the different types of nitrogen-fixing associations and of basic mechanisms by which bacteria can interact with the root system of their host plant. It also reports the phylogeny and particular physiology of bacteria involved in these associations, as well as the relative agronomic importance of the different systems.

The nitrogen fixation process

Enzymatic conversion of molecular nitrogen to ammonia is catalyzed by nitrogenase, an oxygen-labile enzyme complex highly conserved in free-living and symbiotic diazotrophs. The most common form of nitrogenase, referred to as Mo-nitrogenase or conventional nitrogenase, contains a prosthetic group with molybdenum, FeMoCo. Some bacteria, such as *Azotobacter* and several photosynthetic nitrogen fixers (including some cyanobacteria), carry additional forms of nitrogenase whose cofactor contains vanadium (V-nitrogenase) or only iron (Fe-nitrogenase) (Rubio and Ludden 2005; Newton 2007). We shall limit this chapter to the conventional enzyme.

The nitrogenase enzyme, which has been purified from different sources, is composed of two metalloproteins. Component 1, also designated MoFe protein, is a tetramer of 220,000 Da composed of two non-identical subunits α and β, while component 2, also designated Fe protein, is a dimer of 68,000 Da formed by identical subunits. Two FeMoCo are bound to the α subunits of the MoFe protein. In addition, there are two other prosthetic groups containing 4Fe-4S clusters. 'P-clusters' are covalently bound to cysteine residues of MoFe protein bridging α and β subunits. The third type of Fe-S group is linked to the Fe protein (Zheng et al. 1998; Hu et al. 2007; Newton 2007; Rubio and Ludden 2008).

Nitrogen reduction is a very complex mechanism not as yet fully elucidated. The result of net reduction of molecular nitrogen to ammonia is generally accounted for by the following equation:

$$N_2 + 16\,MgATP + 8\,e^- + 8\,H^+ \rightarrow 2\,NH_3 + H_2 + 16\,MgADP + 16\,Pi$$

The genetics of nitrogen fixation was initially elucidated in *Klebsiella oxytoca* strain M5a1 (first identified as *K. pneumoniae*). In that strain, *nif* genes necessary for synthesis of a functional nitrogenase are clustered in a 24 kb region (Arnold et al. 1988). This is the most compact organization of *nif* genes ever described. The three structural genes coding for Mo nitrogenase polypeptides are *nifD* and *nifK* for Mo protein subunits and *nifH* for Fe protein. Full assembly of nitrogenase requires products of other *nif* genes involved in synthesis of FeMoCo (*nifB, nifQ, nifE, nifN, nifX, nifU, nifS, nifV, nifY* also *nifH*) and in assembly of iron sulfur clusters (*nifS* and *nifU*) and maturation of the nitrogenase components (*nifW* and *nifZ*) (Zheng et al., 1998; Rubio and Ludden, 2005; Hu et al. 2007; Rubio and Ludden, 2008). In addition, *Klebsiella* contains genes required for electron transport to nitrogenase (*nifF* and *nifJ*), as well as the regulatory *nifLA* genes controlling expression of the *nif* cluster (Merrick and Edwards 1995; Dixon and Kahn 2004). It is now established that a core of *nif* genes (*nifH*, *nifD nifK*, *nifY, nifB, nifQ, nifE, nifN, nifX, nifU, nifS, nifV, nifW, nifZ)* required for nitrogenase synthesis and catalysis is conserved in all diazotrophs. Depending on the system, other genes are required for in vivo nitrogenase activity, such as those coding for components of physiological electron transport chains (flavodoxin, ferredoxin and the NADH-ubiquinone oxidoreductase (NQR) encoded by the *rnfABCDGEF* cluster) to nitrogenase, molybdenum uptake and homeostasis, and oxygen protection and regulation, including respiratory chains adapted to oxygen conditions at

which the nitrogen fixation process can operate (for details see Fischer 1994; Dixon and Kahn 2004; Pedrosa and Elmerich, 2007).

Soil and rhizosphere microbiology

Discovery of nitrogen fixers

The discovery of nitrogen fixation was attributed to the German scientists Hellriegel and Wilfarth, who in 1886 reported that legumes bearing root nodules could use gaseous (molecular) nitrogen. Shortly afterwards, in 1888, Beijerinck, a Dutch microbiologist, succeeded in isolating a bacterial strain from root nodules. This isolate happened to be a *Rhizobium leguminosarum* strain. Beijerinck (in 1901) and Lipman (in 1903) were responsible for isolation of *Azotobacter* spp., while Winodgradsky (in 1901) isolated the first strain of *Clostridium pasteurianum* (see Stewart 1969). Discovery of nitrogen fixation in blue-green algae (now classified as cyanobacteria) was established much later (Stewart 1969). By 1960, the nitrogen fixation capacities of free-living soil bacteria had been established for only a dozen genera. This was a long way from our present knowledge of the distribution of nitrogen fixation ability in most phyla of the *Bacteria* domain (Postgate 1981; Balandreau 1983; Young 1992; Henson et al. 2004; Lindström and Martínez-Romero 2007; Schmid and Hartmann 2007) (Fig. 1). Indeed, identification of new nitrogen-fixing genera and species has long been hampered by technical limitations due to both the unavailability of proper tools for taxonomy and phylogeny and the difficulty in proving nitrogen-fixing capacity.

Measurement of nitrogen fixation

Measurement of the net gain in fixed nitrogen by nitrogen balance was greatly facilitated, at the end of the 19th century, when Kjeldahl, a Danish scientist, introduced an analytical method for determination of total nitrogen (Bergersen 1980). The technique proved to be useful for quantifying nitrogen in different ecosystems. Development of ^{15}N isotopic tracer techniques (in the 1940's) enabled scientists to more effectively demonstrate that a gain in nitrogen resulted from nitrogen fixation (Burris and Miller 1941). Under field conditions, the use of ^{15}N isotopic gas is impracticable. Instead, a common method is based on differences in the natural abundance of ^{15}N contained in mineral sources of nitrogen compared to atmospheric nitrogen (Boddey et al. 2000; 2001). It enables determining the proportion of plant N derived from atmospheric nitrogen (%Ndfa). A major achievement for rapid determination of nitrogenase activity was the development of an assay using the acetylene reduction technique (Hardy et al. 1973), which could be applied not only to pure cultures and cellular extracts, but also to excised roots, soil cores and greenhouse experiments (Bergersen 1980). This technique makes use of the ability of nitrogenase to reduce alternative substrates such as acetylene into ethylene, which can be determined by gas chromatography. Nonetheless, the nitrogen fixation capacity of isolated bacteria is often difficult to prove, e. g. a majority of root nodule bacteria (*Frankia* and rhizobia) are unable to grow at the expense of molecular nitrogen ex planta, and a large number of bacterial isolates from non-symbiotic systems do not express nitrogenase at a high rate in the free-living state. Thus, detection of nitrogenase activity by

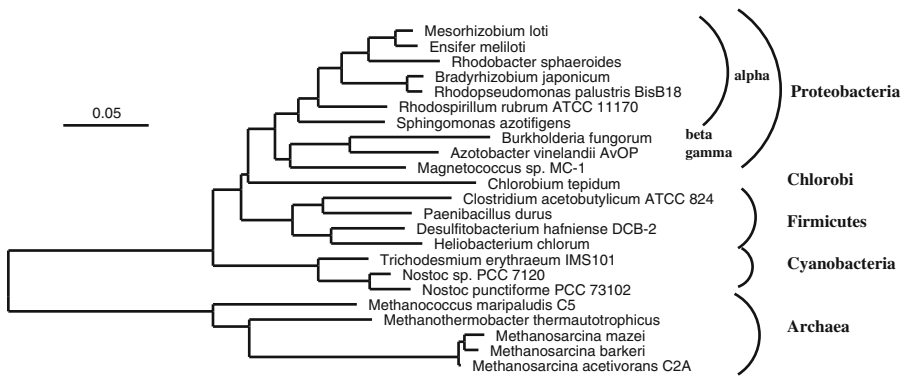

Fig. 1 Phylogenetic 16S tree with prokaryotes carrying *nif* genes (by courtesy of German Jurgens)

acetylene reduction test is sometimes ineffectual and inconclusive.

Amplification of *nif* DNA from environmental samples and community analyses

An alternative means of identifying nitrogen fixers became popular with the development of *nif* gene cloning and sequencing and of DNA amplification by polymerase chain reaction (PCR). This led to the demonstration of the presence of *nif* DNA in putative nitrogen-fixing isolates by PCR amplification, followed by nucleotide sequencing of the amplicon. A series of oligonucleotides such as universal *nifH* primers initially developed by Zehr and McReynolds (1989) were designed to amplify *nifH* fragments from environmental samples. This is of importance in ecological surveys of nitrogen fixers in the soil of rhizospheres. Another advantage is that it enables assessment of the biodiversity of bacteria without strain isolation, taking into account the non-culturable population (Ueda et al. 1995; Elbeltagy et al. 2001; Hamelin et al 2002; Roesch et al. 2008). Strain-specific probes based on conserved sequences of 16S rRNA genes were also developed (see e. g. Stoffels et al. 2001) to follow populations of a particular nitrogen-fixing species in environmental samples.

Rhizobium-legume symbiosis

Host plants

Many leguminous plant species can enter into a symbiotic relationship with root-nodule bacteria, collectively referred to as rhizobia. The legumes belong to the order Fabales, family Leguminosae (alternatively Fabaceae), in eurosid clade I (Doyle and Luckow 2003). Traditionally, three main subfamilies are distinguished: Caesalpinoidae, Mimosoidae and Papilionidae. The Caesalpinoidae has very few nodulating members, whereas most of the important agricultural crops are members of the Papilionidae. Mimosoidae has recently received attention, since, in many cases, bacteria recovered from their nodules belong to the beta subclass of *Proteobacteria*, while Papilionidae symbionts belongs to the alpha subclass (see below; Chen et al. 2003, 2005). Only one non-legume, the woody plant *Parasponia* sp., can be nodulated by rhizobia and utilize nitrogen fixed by the bacteria. Legumes are thought to have been around for 59 Ma, and all leguminous subfamilies evolved 56–50 Ma ago (Sprent and James 2007). In comparison with rhizobia, they are young; the *Sinorhizobium–Bradyrhizobium* split was pinpointed to about 500 Ma ago (Turner and Young 2000), implying that rhizobia were around before there were any legumes to nodulate.

Alpha and beta rhizobia

The rhizobia are Gram-negative and belong to the large and important *Proteobacteria* division (Fig. 2). The alpha-proteobacterial genera *Agrobacterium, Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium, Sinorhizobium, Devosia, Methylobacterium, Ochrobactrum* and *Phyllobacterium* all harbor nodule-forming bacteria, and so do the beta-proteobacterial *Burkholderia* and *Cupriavidus* (Lindström and Martínez-Romero 2007; http://edzna.ccg.unam.mx/rhizobial-taxonomy). The taxonomic classification of rhizobia follows standard procedures and is based on the phylogeny of housekeeping genes and whole-genome similarities (Lindström et al. 2006). Since nodulation functions did not evolve until long after bacterial housekeeping properties, it is thus not always possible to distinguish nodule formers by their names. Only genera in which nodulating bacteria were first discovered have "rhizobium" in their names, whereas e.g. *Burkholderia* (former *Pseudomonas*) species were first recognized through other properties.

Nodulation genes and *nod* factors

A common genetic determinant for rhizobia is the presence of genes encoding nodulation and nitrogen fixation functions (*nod, nol, noe, nif* and *fix* genes). These genes are often carried on plasmids or other accessory elements, such as symbiotic islands, and properties encoded by them can be easily lost or gained (for a recent review, see MacLean et al. 2007). The *nod, nol* and *noe* gene products are involved in production of a nodulation signal, the Nod factor, which is a lipo-chitooligosaccharide. Initiation of nodule formation on compatible host plants results from a molecular dialogue between the host and the bacteria (Fig. 3) (Dénarié et al. 1993; Schultze and

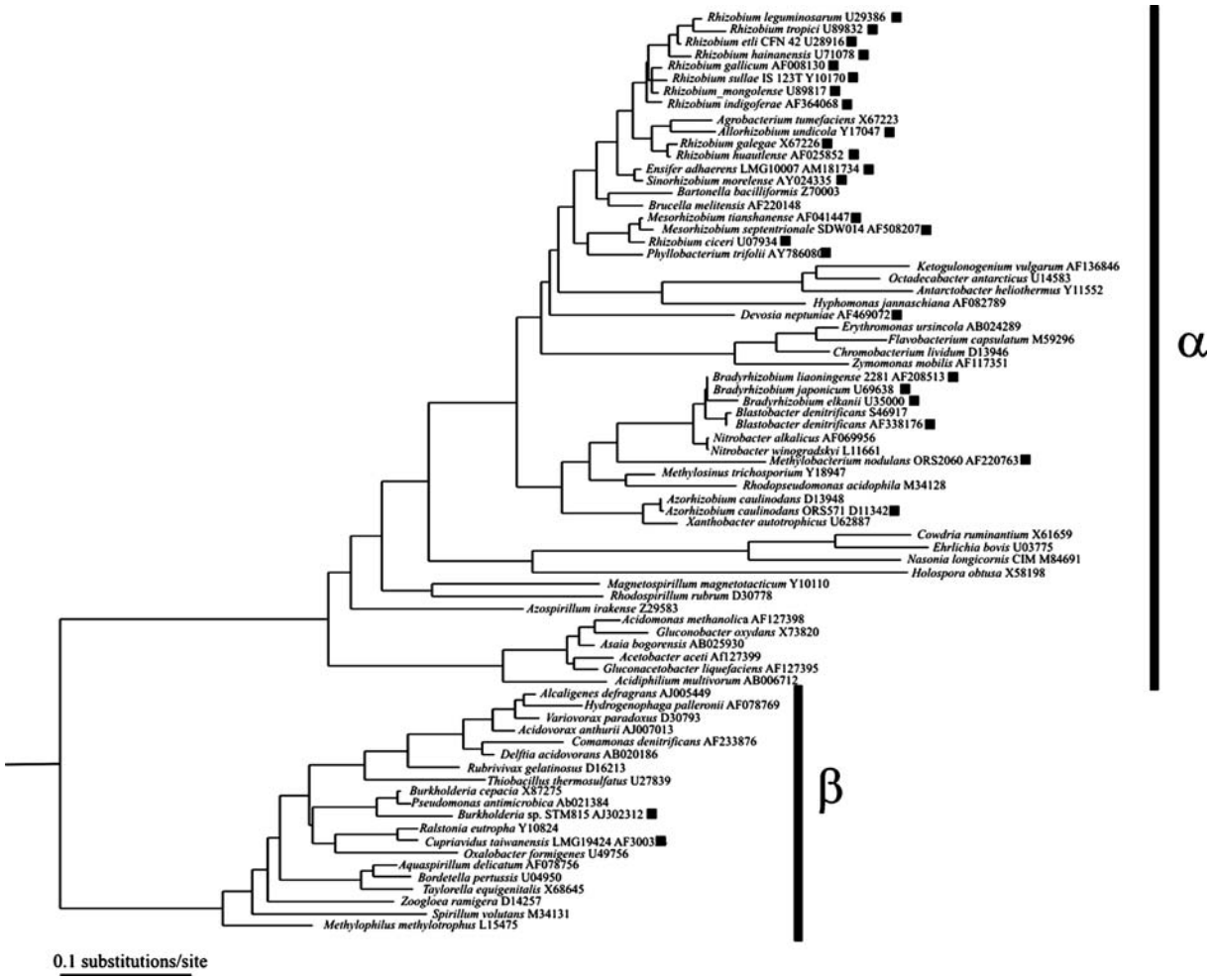

Fig. 2 Phylogeny of rhizobia. A maximum likelihood tree based on *rrs* genes from 75 taxa from alpha- and beta-subdivisions of *Proteobacteria.* Representatives of species capable of forming nodules are marked with a *black box*. (Reprinted from Dresler-Nurmi et al. 2007, with permission)

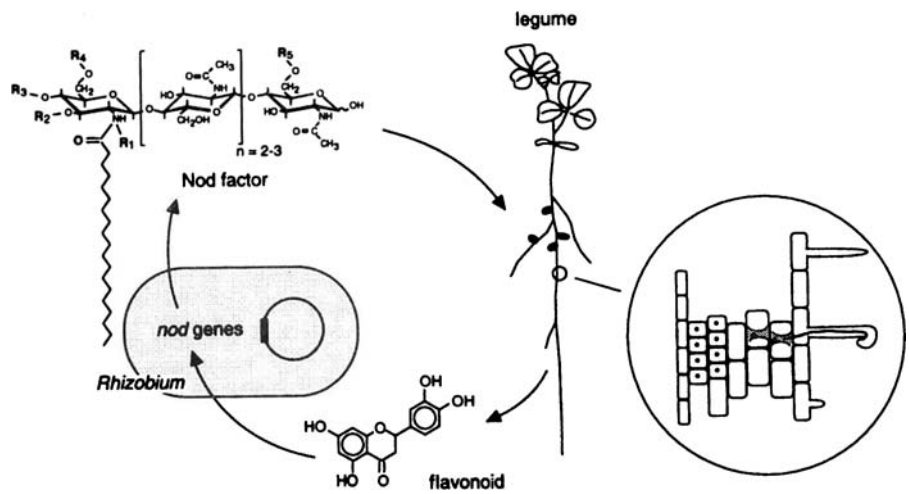

Fig. 3 Signal exchange in Rhizobium-plant symbiosis. Flavonoids produced by the host plant induce rhizobial *nod* genes. This leads to production of Nod factors. The insert shows an infection thread passing the root cortex toward a cluster of dividing cells that will become a root promordium. (Reprinted from Schultze and Kondorosi 1998, with permission)

Kondorosi 1998; Perret et al. 2000; Spaink 2000). The host plants produce flavonoids (and related secondary metabolites) in the rhizosphere. These signals can be perceived by a specific bacterial receptor, NodD, which acts as a transcriptional activator of other nodulation genes. The core of the Nod factor molecule is encoded by canonical *nodA, nodB* and *nodC* whereas, for example, *nodFE* are involved in polyunsaturation of the fatty acyl group attached to the core molecule (Yang et al. 1999). Other nodulation genes encode enzymes which add a variety of substituents to the core, as in the case of Nod factors produced by *Azorhizobium caulinodans* (Mergaert et al. 1993). The Nod factor acts as an elicitor of root nodule formation by the plant by triggering a developmental program leading to construction of the root nodule and entry of rhizobia into the nodule (Long 2001; Geurts and Bisseling 2002; Gage 2004). It is an important host specificity determinant (Spaink 2000).

Recently, the Nod factor paradigm was challenged by Giraud et al. (2007), who discovered that certain photosynthetic, stem- and root-nodulating bradyrhizobia do not possess canonical *nodABC* genes but use other mechanisms for signalling to the plant. Their experiments led them to hypothesize that a purine derivative might play a role in triggering nodule formation instead of the Nod factor. This points to the complexity of the symbiotic system and shows that bacteria have employed diverse strategies to gain entry into the roots.

The infection process and nodule organogenesis

The textbook example of rhizobial infection is via plant root hairs which, prior to the infection process, respond to the presence of compatible rhizobia by deformation (shepherd's crooks, cauliflower structures, etc.). At the deformation stage, the plant perceives the rhizobial signal and initiates a developmental program aimed at formation of symbiotically nitrogen-fixing nodules (Dénarié et al. 1996). A set of plant genes, initially called nodulins, is specifically activated in response to nodulation factor perception (Geurts and Bisseling 2002).

The first receptors of Nod Factors are LysM-type receptor kinases named NFR1 and NFR5 in the model legume *Lotus japonicus* and LYK3 and NFP in *Medicago truncatula*. Different legumes mutants bocked in early step of nodulation, referred as *DMI* (does not make infection) were found to be also blocked in colonization with arbuscular mycorrhiza fungi (AM) suggesting common signalling pathways. The LRR receptor like kinase SYMRK or NORK is central for signal transduction and conserved among both nitrogen fixing and AM symbioses. Calcium spiking is observed in the early steps of root hair infection suggesting that calcium plays a role of secondary messenger in the infection process. Further steps in the signalling cascade lead to induction of cortical cell division (Limpens and Bisseling 2003; Kinkema et al. 2006; Oldroyd and Downie 2008).

A nodule meristem is thus formed within the root while the rhizobia enter through a plant-derived infection thread—a tube formed to facilitate rhizobia entry to the deeper layers. The infection threads grow transcellularly and finally, rhizobia wrapped into a plant-derived membrane, now called symbiosome membrane, are delivered into plant cells (Fig. 3). Nodules are either of an indeterminate type with an apical meristem, or they are determinate, meaning that the peripherally located meristem stops functioning after nodule completion (Foucher and Kondorosi 2000). In some nodules, all plant cells are infected with rhizobia, whereas in other nodule types, there are interstitial cells without symbiosomes. All mimosoid legumes and over half of the papilionid legumes represent this latter infection and nodulation type (Sprent and James 2007).

In their interesting and speculative review, however, Sprent and James (2007) present a tentative scheme for evolution of different nodule structures in which other infection and nodulation types are presented. Caesalpinoid legumes, which are seldom nodulated, display symbiosis in which the bacteria are retained within infection threads throughout symbiosis. Another evolutionarily interesting nodulation mode is present in many agronomically important crop plants as well, namely, infection through cracks in the root ("crack entry"). This type is represented in e.g. *Lupinus* and *Arachis.* Interestingly, in the case of the aquatic legumes (e.g. *Sesbania rostrata*) infection occurred through root hair curling except under flooding conditions where the mode of infection was by crack entry (Goormachtig et al. 2004).

Nodule physiology

During nodule formation, host tissues develop to form a specialized tissue that maintains an environment in

which nitrogen fixation can occur. Functioning of the nodule was recently reviewed by White et al. (2007). In the nodule, specialized organelle-like forms of bacteria called bacteroids are engulfed in plant-derived membranes, forming symbiosomes. The reduction in dinitrogen inside the nodule requires energy, which is provided by the plant. Photosynthate in the form of sucrose is transported to the nodule, whereas dicarboxylic acids further provide the bacteroids with carbon and energy through the symbiosome membrane. For generation of energy through respiration, a high flux but a low internal concentration of oxygen is achieved with the aid of leghemoglobin.

Ammonia produced in the bacteroid needs to be transported to the plant through the symbiosome membrane. In addition to ammonia, alanine is transported. An amino acid flux back through the symbiosome membrane has also been proposed to be involved in the transport mechanism (Prell and Poole 2006). Ammonia is further assimilated into glutamine or asparagine in the plant cytosol. In determinate nodules, these are further converted into ureides in uninfected cells adjacent to the infected ones. In indeterminate nodules, this does not occur, and all plant cells are normally infected. The study of symbiosome biochemistry is impaired by technical difficulties involved when intact but isolated symbiosomes are used.

Host specificity and effectiveness

The origin of nodulation genes in bacteria is still unknown, but by studying infection and nodulation modes in extant legumes, information about the evolution of nodulation can be deduced. Some plant species can be infected by rhizobia representing different bacterial genera, whereas other species are extremely restrictive, accepting only a very narrow range of symbionts. The composition of the root exudates, on one hand, and the structure of the Nod factor, on the other, confer host specificity to the symbiotic interaction (Perret et al. 2000). The presence of type III secretion systems was discovered in several rhizobial genomes (Marie et al. 2001). Nodulation outer proteins (Nops) were identified as protein secreted by this apparatus and Nops were reported to play a role in nodulation efficiency and in some cases host specificity (Marie et al. 2001; Ausmees et al 2004; Cooper 2007).

The plant genus *Galega*, with representatives *G. orientalis* and *G. officinalis* (in the Hologalegina group of the Papilionidae; Doyle and Luckow 2003) are nodulated only by the rhizobial species *Rhizobium galegae* (Lipsanen and Lindström 1988). Host specificity of the symbiotic interaction is manifested at two levels. *Galega* plant root exudate induces *R. galegae* strains in a very specific interaction with NodD1 (Räsänen et al. 1991, Suominen et al. 2003). The Nod factor produced by the bacteria in response to exudate induction carries a unique mixture of molecules with polyunsaturated fatty acyl chains and an acyl group on the penultimate chito-oligosaccharide residue (Yang et al. 1999). These features result in very specific symbiosis. Bacteria producing polyunsaturated acyl chain substituents are taxonomically diverse, but all nodulate closely related legumes in the Hologalegina group (Suominen et al. 2001; Yang et al. 1999).

Host specificity is also expressed at the level of nitrogen fixation. Strains effective (fixing nitrogen) on *G. orientalis* will be ineffective (non-fixing) on *G. officinalis* and *vice versa*. It is interesting, though, that the plant cannot distinguish between effective and ineffective bacteria, but accepts both types in mixed inoculation experiments (Tas et al. 1996). Based on these phenotypic features, which are also reflected in the genomic makeup (Kaijalainen and Lindström 1989), two biovars orientalis and officinalis we distinguished (Radeva et al. 2001).

Rhizobium leguminosarum is a classical example of a rhizobial species having biovars (Laguerre et al. 1996; Mutch and Young 2004). The biovars confer host specificity for nodulation: *R. leguminosarum* biovar viciae typically nodulates peas (*Pisum* sp.) and vetches (*Vicia* sp.), biovar phaseoli beans (*Phaseolus* sp.) and biovar trifolii clovers (*Trifolium* sp.). In these biovars, the core genomes are similar (one species), whereas symbiotic genes are carried on plasmids which can be interchanged (Johnston et al. 1978; Laguerre et al. 1992; Young et al. 2006).

Sinorhizobium sp. strain NGR 234 represents another extreme. This strain was found to form nodules on 112 plant genera. This is due to a very versatile NodD protein induced by a variety of root extracts, to the capacity to produce a mixture of diverse Nod factors and to synthesis of Nops proteins by a type III secretion system (Freiberg et al. 1997; Pueppke and Broughton 1999; Ausmees et al 2004).

Our own studies (Dresler-Nurmi et al. unpublished) of 293 strains of bacteria isolated from the plant species *Calliandra calothyrsus* growing in different parts of the world revealed that several rhizobial species and even genera could nodulate this plant. Isolates from the Central American gene center of the plant were more diverse than those from countries into which the plant had been introduced. However, when the *nodA* gene of 70 isolates was sequenced and compared, they all grouped together and were closely related to the *nodA* gene of *R. tropici*. Thus, *Calliandra* is a promiscuous plant which, when introduced into a new site, seems to nodulate with local indigenous rhizobia with this *nodA* type. Other studies have shown that plants from the Mimosoidae (tribe Ingae, species *Calliandra calothyrsus* and tribe Mimosae, species *Mimosa diplotricha, Prosopsis* sp., *Leucaena leucocephala*) as well as the Papilionidae (tribe *Robinieae,* species *Gliricidia sepium*) form one cross-inoculation group (rhizobial isolates from one plant can nodulate all other plants in the group).

In the case of plant-rhizobium combinations which result in nodules with a proper structure, but without nitrogen-fixation activity, the bacteria act as parasites, probably attracted by the carbon and energy provided by the plant. In the case of *Rhizobium galegae,* the plant seemed not to be able to discriminate between effective and ineffective symbiotic partners, but both were equally competitive and the plant starved to death because of the absence of combined nitrogen (Tas et al. 1996). From a practical point of view, competition and effectiveness are, at the moment, among the most challenging questions for the scientific community.

Actinorhizal symbiosis

Host plants

Actinorhizal plants represent about 200 species distributed among 24 genera in eight angiosperm families (Huss-Danell 1997). Almost all genera are nodulated by Frankia in the Casuarinaceae, Coriariaceae, Eleagnaceae, Datisticaceae and Myricaceae families, whereas nodulation occurs occasionally in Betulaceae, Rhamnaceae and Rosaceae (Benson and Clawson 2000). This wide distribution contrasts with rhizobial symbiosis that, with the exception of *Parasponia* in the Ulmaceae, is limited to the Fabaceae family. All actinorhizal plants are woody trees or shrubs except for *Datisca*, a genus of flowering plants. These perennial dicotyledon angiosperms are distributed worldwide, from cold high latitudes with strong seasonal influences to warm tropical regions with no pronounced difference between seasons. Examples of well-known genera include *Alnus* (alder), *Eleagnus* (autumn olive), *Hippophae* (sea buckthorn) and *Casuarina* (beef wood).

Frankia

The genus *Frankia* comprises high mol% G+C Gram-positive genera belonging to the family *Frankiaceae* in the order *Actinomycetales* (Normand et al. 1996). The closest relative to *Frankia* among actinomycetes is the non-sporulating, rod-shaped cellulolytic thermophile *Acidothermus cellulolyticus*. Unlike rhizobia obtained in pure culture toward the end of the 19th century, the first successful isolation of an effective *Frankia* strain was only achieved from nodules of *Comptonia peregrina* in 1978 (Callaham et al. 1978). *Frankia* is a filamentous bacterium forming hyphal colonies without an aerial mycelium and characterized by a slow growth rate. One striking feature is its ability to differentiate two unique developmental structures that are critical to its survival: vesicles and spores (Fig. 4a) (Lechevalier 1994). Vesicles are the site for actinorhizal nitrogen fixation, while spores contained in multilocular sporangia are the reproductive structures of *Frankia*.

Over 200 strains of *Frankia* have been isolated from 20 plant genera. They are closely phylogenetically related and there is no evidence of the presence of nodulating ability in related actinobacteria (Clawson et al. 2004). Trees generated from sequence alignments of 16S rDNA, nitrogen fixation and glutamine synthetase genes generally yield three major closely related clades with respect to the nodulated host plants (Benson and Clawson 2007). A fourth contains related "*Frankia*-like" actinomycetes (Nod^-/Fix^-), unable to fix nitrogen or to induce nodules (Benson and Clawson 2000). *Frankia* from Clade I include strains nodulating members of the hamamelid families *Betulaceae*, *Casuarinaceae* and *Myricaceae*. *Frankia* from Clade II are typically associated with members of the *Coriariaceae*, *Datiscaceae*, *Rosaceae* and

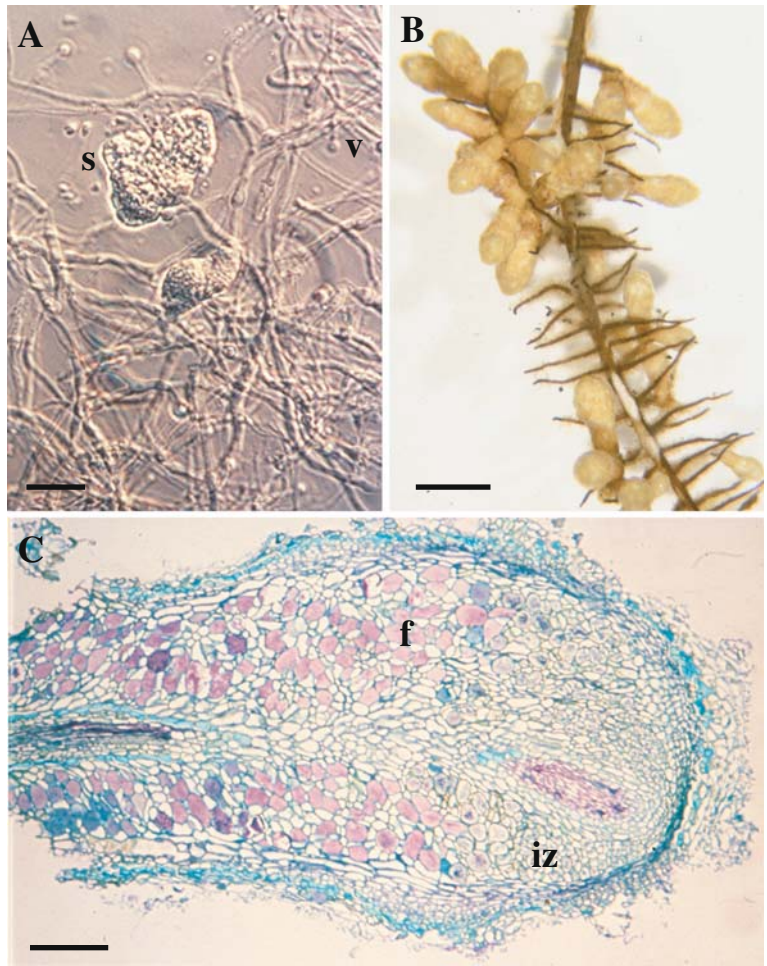

Fig. 4 *Frankia* and actinorhizal nodules **a** *Frankia* in pure culture; nitrogen-fixing vesicles (v) and sporangia (s) can be observed. **b** Actinorhizal multilobed nodules on the root system of the actinorhizal plant *Allocasuarina verticillata*. **c** Pseudolongitudinal section of a nodular lobe from *A. verticillata*; the nitrogen-fixing zone contains large cells filled with *Frankia* (f), and the infection zone (iz) is located in the apex of the nodular lobe. Bars: A= 10 μm; B=5 mm; C=200 μm

Ceanothus of the *Rhamnaceae*. This Clade II is characterized by low diversity, supporting a recent origin for symbiosis in this lineage. Clade III is still poorly known; strains appear to nodulate most *Ceanothus* sp. and also appear in *Myricaceae*, *Eleagnaceae*, *Rosaceae*, *Betulaceae* and *Gymnostoma* of the *Casuarinaceae* family (Benson and Clawson 2007).

Compared to that in rhizobia, the development of molecular genetic tools in *Frankia* has been difficult to implement mainly due to the relatively slow growth rate of filamentous hyphae; in most cases, genetic transformation, mutagenesis and functional complementation failed to provide conclusive results (Lavire and Cournoyer 2003; Normand and Mullin 2008). It was hypothesized that the absence of DNA-mediated transformation could result either from lack of gene expression, DNA restriction or the use of an inappropriate replicon. Hence, for some time, genetic analysis of *Frankia* has been mainly based on gene cloning via hybridization to genes from other organisms, phylogenetic analyses of selected gene sequences and isolation and characterization of plasmids (Simonet et al. 1990; Wall, 2000). Several groups have focused their research on the development of appropriate *Frankia* cloning vectors from native plasmids (John et al. 2001; Lavire et al. 2001; Xu et al. 2002). Studies on codon usage and *Frankia* promoter recognition have also been initiated (Cournoyer and Normand 1994; McEwan and Gatherer 1999; Bock et al. 2001). The genomics era together with the use of molecular biology methods have led to significant progress, including mRNA transcript analyses and overexpression of *Frankia* proteins in *Escherichia coli*. In 2007, the complete genome sequence of three *Frankia* strains was established (Normand et al. 2007). Their sizes varied from 5.4 Mbp to 9 Mpb. Efforts to detect

genes homologous to the *nod* genes of rhizobia in *Frankia* had failed (Cérémonie et al. 1998) until preliminary analysis of the *Frankia* genome revealed disperse putative *nod*-like genes. However, these do not appear to be organized in clusters as in rhizobia, and the key *nodA* gene is absent.

Infection process

Two modes of infection of actinorhizal plants by *Frankia* have been described: intracellular root hair infection and intercellular root invasion (Wall and Berry 2008). Similarly to the situation encountered in rhizobia, the mode of infection depends on the host plant. Intracellular infection via root hairs (e.g. of *Casuarina, Alnus, Myrica*) starts with root hair curling following signal exchange between *Frankia* and the host plant. The signalling molecule pathway has not yet been identified, despite investigations in several laboratories (Prin and Rougier 1987; van Ghelue et al. 1997). However, preliminary characterization of a *Frankia* molecule capable of inducing root hair curling in host plants indicates that it differs from Nod factors in rhizobia (Cérémonie et al. 1999), consistent with the absence of *nod* genes in *Frankia* genome. After invagination of growing filaments of *Frankia* in the curled root hairs, infection proceeds intracellularly in the root cortex. *Frankia* hyphae become encapsulated by a cell wall deposit that is believed to consist of xylans, cellulose and pectins of host origin (Berg 1990, 1999). At the same time, limited cell divisions occur in the cortex near the invading root hair, leading to formation of a small external protuberance called the prenodule (Berry and Sunell 1990). Infection threads consist of lines of encapsulated *Frankia* hyphae progressing intracellularly toward the mitotically active zone and finally invading most cells of the prenodule. As the prenodule develops, cell divisions are induced in the pericycle located opposite the protoxylem pole, giving rise to another nodule primordium. In fact, actinorhizal prenodules do not evolve into nodules and the distantly induced primordium constitutes the nodule. The actual function of the prenodule was investigated in *Casuarina glauca*. A study of symbiosis-related gene expression coupled with cellular modification (cell wall lignification) indicated that prenodules displayed the same characteristics as nodules and hence could be considered very simple symbiotic organs (Laplaze et al. 2000, 2008). Thus, sequential differentiation of prenodules and then nodules constitutes a major difference from the situation in legumes, where cortical cell divisions lead to formation of a unique nodule primordium evolving into a mature nodule. The prenodule might thus be a parallel symbiotic organ of its own or the remaining form of a common nodule ancestor for legumes and actinorhizal plants.

Prenodule formation does not occur in the intercellular root invasion process (e.g. *Discaria, Ceanothus, Elaeagnus*, *Hyppophae*). *Frankia* hyphae penetrate between two adjacent rhizoderm cells and progress apoplastically through cortical cells within an electron-dense matrix secreted into the intercellular spaces (Miller and Baker 1985; Liu and Berry 1991; Wall and Berry 2008). Once the nodule primordium has developed from the pericycle, intracellular penetration by *Frankia* and formation of infection threads is initiated acropetally in developing cortical cells of the nodule lobe primordium, following a pattern similar to that described in plant species invaded through root-hairs.

Nodule development and functioning

For both intracellular and intercellular modes of infection by *Frankia*, an apical meristem is responsible for primordium growth towards the root surface in regions not infected by *Frankia*. As previously indicated, the nodule primordium does not incorporate the prenodule, but becomes infected by hyphae arising from the prenodule. Further development of the primordium gives rise to an indeterminate actinorhizal nodule lobe with a central vascular bundle surrounded by an endoderm, an expanded cortex containing *Frankia*-infected cells and a periderm (Fig. 4b,c). New lobes arise continuously to form a coralloid nodule. Some species like *Casuarina* or *Myrica* develop a so-called root nodule at the apex of each lobe (Duhoux et al. 1996). This root nodule lacks root hairs, has a reduced root cap and displays negative geotropism. It might be involved in diffusion of gas, especially oxygen, in and out of the nodule lobe.

Based on cytological and gene expression studies, four zones are recognized in mature actinorhizal nodules (Duhoux et al. 1996; Laplaze et al. 2008). Zone I is the apical meristem free of *Frankia*. Adjacent to the meristem is zone II, an infection zone

in which some of the young cortical cells resulting from the meristem activity are infected by *Frankia*. The encapsulated bacterium starts to proliferate and the plant cells enlarge. Zone III is the fixation zone containing both infected and uninfected cortical cells. Infected cells are hypertrophied and are filled with *Frankia* filaments that differentiate vesicles where nitrogen fixation takes place. The appearance and shape of these vesicles is controlled by the plant. In some species like *Casuarina*, infected cells have a lignified cell wall and there is no vesicle differentiation. Uninfected cells are smaller and, in some species, contain amyloplast and phenolic compounds and might be involved in nitrogen and carbon metabolism. Finally, a basal senescence zone (zone IV) is observed in old nodules; plant cells and bacteria degenerate and the nitrogenase is switched off. More recently, a second level of compartmentalization was described in *Casuarina glauca* nodules based on accumulation of flavans, which occurs in uninfected cells in the endodermis and cortex. These cells form layers that delimit *Frankia*-infected compartments in the nodule lobe and may play a role in restricting bacterial infection to certain zones of the nodule (Laplaze et al. 1999).

Molecular biology and actinorhizal nodule and plant gene expression

During differentiation of the actinorhizal nodule, a set of genes called actinorhizal genes is activated in the developing nodule. Heterologous probing and differential screening of nodule cDNA libraries with root and nodule-specific cDNA resulted in isolation and characterization of more than 25 nodule-specific or nodule-enhanced plant genes in several actinorhizal plants, including *Alnus*, *Datisca, Eleagnus* and *Casuarina* (Pawlowski and Bisseling 1996; Laplaze et al. 2008; Pawlowski and Sprent, 2008). One of the earliest symbiotic genes characterized thus far is *cg12*, which encodes a subtilisin-like protease expressed in *Frankia*-infected root hairs of *C. glauca* (Svistoonoff et al. 2003). A homologue of the receptor-like kinase gene SymRK found in legumes was also recently shown to be necessary for actinorhizal nodule formation in the tree *Casuarina glauca* (Gherbi et al. 2008a).

Recently emerging tools should contribute to increasing our knowledge of the molecular mechanisms of actinorhizal symbiosis over the next few years. One is the development of the first genomic platform for the study of plant gene expression in actinorhizal symbiosis (Hocher et al. 2006). This was recently applied to a *C. glauca* gene nodule library and it revealed increased expression of genes involved in primary metabolism, protein synthesis, cell division and defense. The second tool is the use of hairpin RNA to achieve post-transcriptional gene silencing in *C. glauca*, providing a versatile approach to assessing gene function during the nodulation process induced by *Frankia* (Gherbi et al. 2008b). Expressed sequence tags (ESTs) that exhibit homology with the early symbiotic genes *DMI2* and *DMI3* (Catoira et al. 2000) from legumes involved in the Nod factor transduction pathway are currently being characterized using this RNA interference approach.

Associations with cyanobacteria

Cyanobacteria and symbiosis

Cyanobacteria are widely distributed in aquatic and terrestrial environments. Long regarded as algae because they performed oxygenic photosynthesis, they are now classified into the domain of *Bacteria*, in five Sections based mostly on morphological criteria (Rippka et al. 1979). Indeed, cyanobacteria constitute the largest and most diverse group of Gram-negative prokaryotes. While nitrogen fixation is found both in unicellular and filamentous species, associations with plants are essentially limited to heterocystous cyanobacteria *Nostocales*, primarily of the genus *Nostoc* and *Anabaena.* Besides vascular plants, there exist a wide variety of non-vascular lower plant belonging to bryophytes, including liverworts and hornworts, algae and fungi, that develop associations with cyanobacteria, as well as many marine eukaryotes (Rai 1990; Bergman et al. 1996; Rai et al. 2000, 2002). We shall limit this review to associations with gymnosperms (Cycads), angiosperms (*Gunnera*) and pteridophytes (*Azolla*).

Differentiation of heterocysts, nitrogen-fixing specialized cells

Some filamentous cyanobacteria from Sections IV and V are able to differentiate specialized cells called heterocysts under nitrogen limitation conditions

(Fig. 5a) (Rippka et al. 1979) (Fig. 5a). An anaerobic environment compatible with the functioning of nitrogenase in heterocysts is linked to formation of multilayered envelopes external to the outer membrane, elimination of a functional oxygen-producing photosystem II, and additional changes in their physiology not detailed here (Buikema and Haselkorn 1993). Nitrogen fixed by heterocysts is exported to vegetative cells of the filaments; in return, vegetative cells provide heterocysts with carbohydrates derived from their photosynthetic activity. This interdependence ensures filament growth under conditions of nitrogen limitation.

Initial information on heterocyst differentiation came essentially from the study of *Anabaena* sp. strain PCC 7120, a strain not known to associate with plants (Buikema and Haselkorn 1993). Heterocysts develop within about 24 h from vegetative cells located at semi-regular intervals in the filaments (Fig. 5a). The signalling pathway that leads to initiation of heterocyst differentiation at a particular location in the filament is complex, and the number of genes identified as being involved in the developmental process is regularly increasing (Ehira et al. 2003; Golden and Yoon 2003; Zhang et al. 2006; Xu et al. 2008). Two of them, *hetR* and *ntcA,* have a critical function in initial steps of heterocyst differentiation (Buikema and Haselkorn 1991; Wei et al. 1994; Shi et al. 2006), while *patS* is involved in heterocyst spacing (Yoon and Golden 1998). HetR is a transcriptional regulator with autoprotease activity, which functions as a master switch in heterocyst differentiation. NtcA is a global nitrogen regulator that belongs to the CRP (cAMP receptor proteins) superfamily and which acts as a sensor of nitrogen deprivation in response to internal concentrations of 2-oxoglutarate. NtcA plays a role in control of *hetR* expression under N deprivation consistent with the

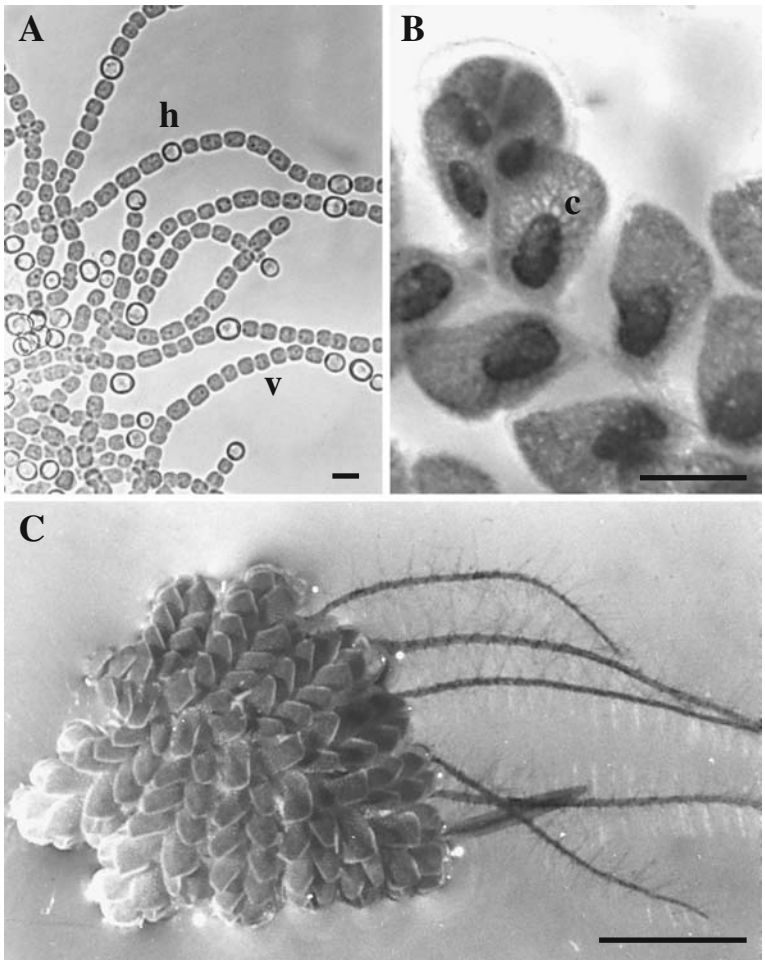

Fig. 5 Free-living *Anabaena* and *Azolla* **a** Free-living *Anabaena* strain cultured in medium deprived of nitrogen; heterocysts (h) can be observed among vegetative cells (v). **b** Frond of *Azolla pinnata* digested by cellulase and pectinase; cavities (c) filled with symbiotic *Anabaena azollae* are visible. **c**: Frond of *A. pinnata*. Bars: A=10 μm; B=50 μm; C=500 μm

fact that *ntcA* mutants cannot form heterocysts. PatS is a small diffusible peptide inhibitor of heterocyst differentiation, probably by inhibiting the HetR transcription activation function (Huang et al. 2004). Thus, the semiregular pattern of heterocyst formation may derive from the autoregulatory activity of HetR and diffusion of the PatS peptide (Xu et al. 2008). HetN, a protein similar to ketoacyl reductase, is also thought to downregulate expression of *hetR* (Callahan and Buikema 2001; Borthakur et al. 2005).

The percentage of heterocysts in filaments grown in the free-living state is in the range of 5 to 10% of cells, whereas it reaches 30 to 40% of cells within the filaments hosted by the plant and, in the particular case of *Gunnera,* up to 60–80% (Meeks and Elhai 2002; Wang et al. 2004). This reflects a direct correlation between the efficiency of nitrogen fixation and heterocyst frequency. Whereas *Anabaena azollae* appears to be an obligate symbiont, other symbiotic cyanobacteria can be grown in free-living culture and retain their ability to infect their host plant. Thus, properties of mutants impaired in heterocyst differentiation can be assayed in the host plant. Mutants of *ntcA*, *hetR* and *hetF* have been obtained in *N. punctiforme* strain PCC 92293, an isolate from *Gunnera,* which can also infect *Anthoceros punctatus*. None could differentiate heterocysts, similarly to what was found with corresponding mutants of non-symbiotic strain PCC 7120 (Wong and Meeks 2001, 2002). Both *hetF* and *hetR* mutants can infect *A. punctatus* with a frequency similar to that of the wild type, but are unable to support growth of the plant because of their inability to develop heterocysts and fix dinitrogen. The *ntcA* mutant failed to infect *Anthoceros* because it is also impaired in formation of hormogonia, which is the "infection unit" (see below).

Differentiation of hormogonia, the "infection unit"

Hormogonia are filaments, motile by gliding, formed by cells with reduced metabolic activity, of smaller size than vegetative cells produced by the genus *Nostoc* (Rippka et al. 1979). Because of their motility, they provide a means of dispersing cyanobacteria and play a critical role in establishment of symbiosis with host plants by enabling access to plant structures that will house symbiotic colonies. Hormogonia differentiation results from division of vegetative filaments without growth. Their formation is induced in response to different stress conditions at the free-living state and in response to host plant factors called HIF (for hormogonia-inducing factor) in symbiosis. After 48 h, hormogonia can regenerate vegetative filaments and differentiate heterocysts (Campbell and Meeks 1989).

Inactivation of the *N. punctiforme* ATCC 29133 *hrmA* gene by transposon mutagenesis results in a higher frequency of hormogonia formation in response to HIF of *A. punctatus* (Cohen and Meeks 1997). The *hrmA* gene is part of an *hrmRIUA* operon that has high sequence similarity to sugar uronate metabolism operons of other bacteria. *Nostoc hrmA* mutants are unable to survive in long-term coculture with *Anthoceros* due to their continued formation of hormogonia. In contrast, wild-type filaments of *Nostoc* spp., after an initial burst of HIF-induced hormogonia formation, show a period of immunity to HIF enabling growth and nitrogen fixation.

Symbiotic associations with vascular plants

Gunnera, a genus of about 40 species, is the only angiosperm with which the cyanobacterium referred to as *Nostoc punctiforme* is associated (Rasmussen and Svenning 2001). Specialized stem glands, susceptible to infection by the cyanobacterium *Nostoc*, are formed at the base of petioles (Bergman 2002). Acidic mucilage, containing HIF (Rasmussen et al. 1994), secreted from the stem glands, induces differentiation of vegetative *Nostoc* filaments into motile hormogonia, which move into channels present in the glands. Then, compatible *Nostoc* strains induce divisions in the host cells lining the channel and *Nostoc* cells are subsequently taken up into plant cells. Once intracellular, a high frequency of differentiation of vegetative cells into heterocysts occurs and nitrogen is fixed at a high rate (Rasmussen et al. 1996).

Cycads are the only gymnosperms that fix dinitrogen, including 90 species in 9 genera (Costa and Lindblad 2002). They develop specialized lateral roots, so-called coralloid roots, in which a cell layer has been prepared for infecting cyanobacteria. Following an unknown infection process, the endophyte invades a mucilage-filled space in the outer cortex of the root nodule. Morphological features of the cyanobacterium change as the root nodules mature, with heterocyst frequency increasing with age, while vegetative cell enlargement also occurs with age. Large amounts of phenolic compounds are found in

the cortical cells surrounding the zone of cyanobacteria. It has been suggested that these compounds may inhibit growth of other organisms and contribute to containment of the symbiont.

Azolla species are native to Asia, Africa and the Americas, and have been dispersed by man and by natural pathways to various parts of the world. Some are strictly tropical or subtropical, while others grow and thrive in lakes, swamps and streams, and other small bodies of water under either temperate or tropical climates. The genus *Azolla* includes seven species that have been grouped into two sections, *Euazolla* and *Rhizosperma*, based on the structure of their sporocarps. A view of *Azolla pinnata* is shown in Fig. 5c. Symbiosis between the aquatic fern *Azolla* and *A. azollae* is of particular interest because it is the only plant-prokaryote symbiosis known to persist throughout the reproductive cycle of the host plant (Lumpkin and Plucknett 1980; Nierzwicki-Bauer 1990; Lechno-Yossef and Nierzwicki-Bauer 2002). During vegetative growth, the symbiont is located in a distinct leaf cavity at the base of the dorsal lobe of the leaves (Fig. 5b). Vegetative maintenance of the association depends on retention of *A. azollae* filaments, morphologically similar to hormogonia, at the apical meristem of fronds. The directed movement of the cyanobacterium within the host is accomplished by specialized plant epidermal trichomes. It has been hypothesized that the specific surface properties of hormogonia, which differ from those of vegetative filaments, enable recognition by trichomes, so that only generative hormogonium cells serve as inocula for new cavities developing at the apex of the frond. The leaf cavity of *Azolla* can also host other bacteria together with the symbiotic *Anabaena;* the function of these bacteria remains unknown. The cavity is surrounded by mucilage and completely lined by an envelope. As leaf maturation occurs, the non-heterocystous hormogonia of the youngest leaves develop into heterocystous filaments located at the periphery of the cavity. Finally, the symbiotic cavities respond to the presence of cyanobacteria by elaborating long, finger-like cells that may serve to increase the surface area for nutrient exchange. Nitrogen is released from the cyanobiont almost exclusively as $NH4^+$. During sexual reproduction, the cyanobiont colony survives in the indusium cap of the megaspore. In *Azolla*, deoxyanthocyanins produced by the aquatic fern also contribute to induction of *hrmA* expression (Cohen et al. 2002). This result suggests that in *Azolla,* appropriate localization of phenolic compounds could function in plant-mediated mechanisms for repressing hormogonium formation after penetration of cyanobacteria into the host plant.

Associative and endophytic nitrogen fixers

Nitrogen-fixing plant-growth-promoting rhizobacteria

Nitrogen-fixing bacteria that contribute to plant growth stimulation or to disease prevention and suppression are referred to as plant growth-promoting rhizobacteria (PGPR). Their isolation from the roots of forage grasses and cereal crops and many other plants, in both natural and cultivated ecosystems, has been extensively performed (Döbereiner and Pedrosa 1987; Okon and Labandera-Gonzales 1994; Baldani and Baldani 2005). This led to identifying two groups with respect to the degree of association with the host plant: rhizospheric and endophytic colonizers. Classical microbiological techniques involving cultivation of bacteria identified soil bacteria belonging to genera such as *Azospirillum*, *Azotobacter*, *Alcaligenes*, *Bacillus, Beijerinckia, Campylobacter, Derxia,* several members of *Enterobacteriaceae* (*Klebsiella, Pantoae*) and *Pseudomonas stutzeri* (Rennie 1980; Balandreau 1983; Elmerich et al. 1992; Yan et al. 2008). As most of these strains were isolated from surface-sterilized root samples, this suggests that proportions of these cells are protected from sterilizing agents, but it may also reflect some colonization of the root tissues. Other isolates, such as those of *Azoarcus* (Hurek and Reinhold-Hurek 2003), *Burkholderia* (Caballero-Mellado et al. 2004), *Herbaspirillum* and *Gluconacetobacter* (Baldani and Baldani 2005), or *K. pneumoniae* strain 342 (Chelius and Triplett 2000), happened to belong to endophytes. The complete genome of *Azoarcus* and *P. stutzeri* A1501 was established (Krause et al. 2006; Yan et al. 2008) genomes projects of *Azospirillum, Azotobacter, Herbaspirillum* and *Gluconacetobacter* are under completion.

Colonization of the root system: *Azospirillum* as a model bacterium

Colonization of the root surface has been best studied in *Azospirillum. Spirillum*-like bacteria were first

isolated by Beijerinck in 1923; they were rediscovered by Becking in 1963 and by Johanna Döbereiner's team in Brazil in the 1970's (Von Bülow and Döbereiner 1975). The *Azospirillum* genus was described by Tarrand et al. (1978); it belongs to the *Alphaproteobacteria* phylum and 7 species were recognized in 2007 (Schmid and Hartmann 2007): *A. brasilense, A. lipoferum, A. amazonense, A. irakense*, *A. halopraeferens*, *A. largimobile* and *A. doebereinerae. Azospirillum* species display an extremely wide ecological distribution and are associated in nature with a wide diversity of plants, including those of agronomic importance such as wheat, rice, sorghum and maize and several non-gramineous species (Döbereiner and Pedrosa 1987). These bacteria are aerobic non-fermentative chemoorganotrophs, vibrioid to S-shaped, containing polyhydroxyalkanoate granules (PHA). In liquid medium motility is ensured by a polar flagellum. In some species (e. g. *A. brasilense, A. lipoferum, A. amazonense*), lateral flagellation enables swarming on a solid surface. Another important property of azospirilla is the ability to differentiate resistant forms which are non-motile ovoid cyst-like cells, much larger than vegetative cells and surrounded by a thick capsule (Lamm and Neyra 1981). Cyst formation is concomitant with cell aggregation into macroscopic flocs occurring in some culture conditions. Encapsulated azospirilla display greater resistance to desiccation and heat.

Cyst formation and production of siderophores and bacteriocins (spirilobactin) (Tapia-Hernández et al. 1990) are likely to play a role in survival of these bacteria under unfavorable conditions and in competition with other members of the soil microflora. Bacterial motility as well as chemotactic responses towards root exudates are involved in the initial step of the root colonization process (Vande Broek et al. 1998). Attachment to the root system is mediated by the polar flagellum and is followed by irreversible anchoring of the bacteria (Steenhoudt and Vanderleyden 2000). The polar flagellum is glycosylated and binds wheat root, whereas the lateral flagella are not essential during the adsorption phase. As *rpoN* controls flagellar biogenesis, *rpoN* mutants are impaired in colonization. An operon carrying chemotaxis genes (*che*) was identified in *A. brasilense* (Hauwaerts et al. 2002). However, *cheB* and *cheR* mutants were only partially impaired in their chemotactic response, suggesting multiple chemotaxis systems in this bacteria (Stephens et al. 2006). Several genes governing motility in *A. brasilense* Sp7 have been mapped onto a 90 MDa plasmid, while other genes are located on the chromosome. The complete nucleotide sequencing of this plasmid was established (Vanbleu et al. 2004). The plasmid also carries several genes governing surface polysaccharides that might play some role in the colonization process. The structural gene for major outer membrane protein, *omaA,* was characterized and found to encode an adhesin with high affinity to roots (Burdman et al. 2001). A transcriptional regulator of the LuxR-UhpA family, *flcA,* controlling differentiation into cysts and flocculation, was also described as playing a role in surface colonization (Pereg-Gerk et al. 1998).

Electron micrographs of colonized roots revealed that azospirilla were anchored to roots by fibrillar material, probably similar to the fibrillar material produced during flocculation. Bacteria colonize the rhizoplane and are found in high numbers upon emergence of lateral roots and also near the root cap (De Oliveira Pinheiro et al. 2002). The degree of invasion of plant tissues differs between strains. In an early report, using fluorescent antibody staining techniques, it was found that *Azospirillum* colonizes the intercellular spaces between the epidermis and the cortex of the root (Schank et al. 1979). Other techniques involving bacteria carrying *gus* and *lacZ* fusions were helpful not only at locating the bacteria on the root surface, but also for assaying *nif* gene expression during the colonization process (Vande Broek et al. 1993; Arsène et al. 1994). The fluorescent *in situ* hybridization (FISH) technique developed for phylogenetic purposes, coupled with the use of confocal laser scanning microscopy (CLSM), enabled better *in situ* localization of bacteria on the root (Aßmuss et al. 1995). In particular, it established that endophytic colonization of some *Azospirillum* strain such as *A. brasilense* Sp245 was found in the intercellular spaces of the root epidermis (Rothballer et al. 2003). Attempts to identify genes expressed at early stages of the interaction with the host plants are in progress (Pothier et al. 2007).

Nitrogen-fixing endophytes

An increasing number of reports describe the occurrence of nitrogen-fixing bacteria within plant tissues of a host plant that does not show diseases symptoms,

with the most studied genera being *Azoarcus* sp, *Gluconacetobacter* and *Herbaspirillum* (Hurek and Reinhold-Hurek, 2003, Lery et al. 2008). Endophytes multiply and spread within plant tissues without causing damage. Early steps in infection may be similar to those reported with rhizospheric bacteria, initially involving surface colonization at the site of emergence of root hairs (Hurek and Reinhold-Hurek, 2003). In the case of *Azoarcus,* type IV pili were found to be essential for that process and hydrolytic enzymes, or endoglucanases, are involved in tissue penetration (Dörr et al. 1998; Krause et al. 2006). The concentration of bacteria recovered after sterilization of the root system can reach up to 10^8 CFU per g of dry weight. Another characteristic is systemic spreading of bacteria, which can be found in plant xylem vessels and in shoots, as described in the case of sugar cane infection with *G. diazotrophicus* (James and Olivares 1998) and in the case of infection of the C4-gramineous plant *Miscanthus sinensis* by *H. frisingense* (Rothballer et al. 2008). Bacteria are located mostly in intercellular spaces, but intracellular location is also seen in dead cells (Hurek and Reinhold-Hurek 2003). The main difference from rhizospheric bacteria (that can also be found, in some cases, in the first layers of the root cortex), is the fact that endophytes do not persist in the soil. Therefore, the frontier between rhizospheric and endophytic systems is not truly strict.

Factors involved in plant growth promotion and crop protection

Colonization of the plant root affects both the morphology and physiology of the host plant. A typical response after inoculation with *Azospirillum* is enhanced proliferation of lateral roots and root hairs. In general, this is accompanied by changes in root physiology, such as increased mineral and water uptake, increased root respiration, delay in leaf senescence and increased dry weight (Okon 1985; Dobbelaere and Okon 2007). The plant growth promotion effect was tentatively attributed to production of auxin-like compounds such as indole-3-acetic acid (IAA), commonly produced by soil bacteria. In general, biosynthesis of IAA uses tryptophan (Trp) as a precursor, and several pathways for conversion of Trp into IAA have been described (Costacurta and Vanderleyden 1995; Baca and Elmerich 2007). The indole acetamide route common in phytopathogenic bacteria is not present in nitrogen-fixing root colonizers, including *Azospirillum*. Instead, many soil bacteria possess the indole pyruvate route, involving an indole-3-pyruvate decarboxylase encoded by *ipdC* (Koga et al. 1991). *Azospirillum* insertion mutants in *ipdC* still produce IAA, and strain Sp7 contains two alternative pathways (Carreño-Lopez et al. 2000). Indeed, it is difficult to distinguish between hormones produced by the plant and bacteria. Elevated IAA pools in plants colonized with *Azospirillum* was reported, but it could not be concluded as to whether this increase was linked to hydrolysis of IAA-conjugates produced by the plant, to bacterial production, or to both (Fallik et al. 1989). Other plant hormones, such as gibberellins and cytokinins, are also produced by many soil bacteria, but as yet, little is known concerning the involvement of these compounds in the plant response to inoculation (Baca and Elmerich 2007). In contrast, the role of bacteria in preventing ethylene synthesis has been better studied. Ethylene is a plant hormone that prevents root elongation. Many soil bacteria encode a deaminase (*acdS* gene) which degrades the direct precursor of ethylene, i.e. 1-aminocyclopropane-1-carboxylic acid (ACC) (Glick 2005), including strains of *A. lipoferum* (Prigent-Combaret et al. 2008), *P. stutzeri* A1501 (Yan et al. 2008) and *H. frisingense* (Rothballer et al. 2008). Introduction of *acds* gene into *A. brasilense* resulted in increased root elongation in some plants (Holguin and Glick 2001). Production of N-acylhomoserine lactone that may be involved in plant growth promotion was also observed in some cases (Boyer et al. 2008; Rothballer et al. 2008).

To date, there is still limited information related to the role of nitrogen-fixing bacteria as biocontrol agents. Production of antimicrobial metabolites as well as hydrolytic enzymes has been reported, but their role in crop protection remains to be established. A particular strain of *A. brasilense* was reported to be antagonistic of the parasitic weed *Striga*, that affect many tropical cereals by preventing *Striga* seed germination (Miché et al. 2000).

Nitrogen fixation and crop productivity

Biological nitrogen fixation represents, annually, up to 100 million tons of N for terrestrial ecosystems,

and from 30 to 300 million tons for marine ecosystems. In addition, 20 million tons result from chemical fixation due to atmospheric phenomena (Mosier 2002). The first industrial production of rhizobium inoculant began by the end of the 19th century. However, to sustain production of cereal crops, legumes and other plants of agricultural importance, the supply of nitrogenous chemical fertilizers has been regularly increasing since the Second World War. According to an FAO report, production of N fertilizer for 2007 was 130 million tons of N, and this should further increase in the coming years (FAO 2008). This extensive use has certain drawbacks. A proportion of added fertilizer is lost as a result of denitrification and leaching of soil by rainfall and irrigation. In addition, leaching leads to water pollution caused by eutrophication. As a consequence, extending application of biological nitrogen fixation by any means is an important issue.

Benefits from legumes and actinorhizal plants

Because the concentration of fixed nitrogen is a limiting factor for growth, nitrogen fixers have a selective advantage that enables them to adapt to the most extreme conditions and to colonize diverse ecological niches. Indeed, nitrogen-fixing symbiotic microorganisms play an important role in the life of plants, ensuring not only their nutrition, but also their defense against pathogens and pests, and adaptation to various environmental stress.

Legumes are often considered to be the major nitrogen-fixing systems, as they may derive up to 90% of their nitrogen from N_2 (e.g. faba bean, lupin, soybean, groundnut). The rate of fixation, in the range of 200 to 300 kg N/ha/crop, can be attained for most species, but this largely depends on cultivars and culture conditions (Peoples et al 1995). Among grain legumes, soybean represents more than 50% of the world oilseed production.

Many actinorhizal plants, but also legumes, are capable of sustaining a mycorrhizal association as well, thus forming tripartite symbiosis and enhancing the success of these plants under poor soil conditions. Due to these properties, actinorhizal species can grow and improve soil fertility in disturbed sites and are used in recolonization and reclaiming of eroded areas, sand dunes, moraines and areas of industrial waste and road cuts, and are planted following fires, volcanic eruptions and logging (Wheeler and Miller 1990). In addition, some actinorhizal species can grow well under a range of environmental stresses such as high salinity, heavy metal and extreme pH (Dawson 1990). Actinorhizal plant nitrogen fixation rates are comparable to those found in legumes (Torrey and Tjepkema 1979; Dawson 1983). Alders, in particular, are known to be beneficial at improving nutrient-poor soils (Wheeler and Miller 1990; Myrold and Huss-Danell 2003). The annual input of N from N_2 fixation in alder stands ranges from 20 to 300 kg N/ha, depending on stand age, stand density and site conditions (Wheeler and Miller 1990). As much as 85–100% of foliar N in a speckled alder stand is estimated to have been derived from the atmosphere (Hurd et al. 2001).

Benefits of association with cyanobacteria

The rate of dinitrogen fixation in plant-associated cyanobacteria is much greater than that of the same free-living strains, and it correlates broadly with the increased heterocyst frequency observed in symbiosis. In the *Anthoceros* association, dinitrogen fixation is 4- to 35-fold higher than that of the free-living *Nostoc*. In *Anthoceros* and in *Blasia*, fixed dinitrogen is released to the plant as ammonia and it has been shown that as little as 20% of dinitrogen is retained by the cyanobiont (Adams 2002). Thus far, nitrogen-fixing *Azolla*-cyanobacterial symbiosis is the only one of economic importance to farming systems. Symbiosis has for centuries been used to produce green manure plants, especially in China, Vietnam, and Southeast Asia (Lumpkin and Plucknett 1982), and there is no doubt of the beneficial effect of *Azolla* in terms of increasing rice grain yield. The entire nitrogen requirement of *Azolla* is fulfilled by the cyanobacteria and *Azolla* can accumulate 2-4 or more kg N/ha/day. The fern grows rapidly and can double its biomass in 3 days under optimal conditions. These unique properties have made it possible for rice production to replace part of the chemical fertilizer with *Azolla*-cyanobacteria symbiosis (Peters and Meeks 1989; Liu and Zheng 1992). The nitrogen fertilizer fixed by *Azolla* becomes available to rice after the *Azolla* mat is incorporated into soil and its nitrogen begins to be released through decomposition. Furthermore, it has been shown that the presence of an *Azolla* mat on the surface of water significantly

reduces volatilization of nitrogen fertilizers (Vlek et al. 1995). Though *Azolla* use as a rice fertilizer is currently declining, it is still used at the farm level in China, India, Senegal, the Philippines, Colombia, Bolivia and Brazil. However, the widespread use of *Azolla* is limited by environmental factors such as high temperature, insects and disease. Furthermore, genetic improvement in symbiosis remains difficult (van Hove and Lejeune 2002).

Benefits of association with grasses

Taken together, legumes occupy about 10% of cultivated land, while cereals represent almost 50% (Peoples et al. 1995). Nitrogen fixation with cereal crops is often considered negligible in comparison to rates of symbiotic nitrogen fixation attained by root nodulated plants. Hence, the possibility of increasing nitrogen fixation with cereal crops by inoculation with wild type or engineered nitrogen-fixing bacteria is an extremely challenging project. Inoculation of cereals and vegetables with *Azotobacter* and bacilli, called "bacterization", was in common use in Russia in the 1950s and beneficial effects were reported (Macura 1966). Whether or not the benefit is due to nitrogen fixation depends on the systems, plant cultivars, soil and many other parameters. It was long known that some crops like sugar cane could grow with little addition of nitrogen fertilizer in Brazil, and many other examples, including rice and wheat, have been reported (van Berkum and Bohlool 1980; Boddey and Döbereiner 1982). In particular, *Paspalum notatum* cv. batatais that specifically associates with *Azotobacter paspali* was reported to fix from 15 to 90 kg N/ha/year (Döbereiner et al. 1972). At present, sugar cane is a good example of a crop that can benefit from nitrogen fixation, since certain cultivars can derive more than 150 kg N/ha/year from BNF (Boddey et al. 2001). Thus, some crops naturally benefit from the nitrogen fixation capacity of associated bacteria. In their survey of 20 years of field inoculation worldwide, Okon and Labandera-Gonzales (1994) concluded that significant increases in yields, from 5 to 30%, could be achieved by inoculation with *Azospirillum*, in particular when the use of chemical N fertilizer was low. However, they believed that the growth-promoting effect was probably linked to phytohormone production rather than nitrogen fixation. Recently, Rodrigues et al. (2008) reported significant nitrogen fixation, determined by the ^{15}N isotope dilution method, after inoculation of rice with certain strains of *A. amazonense*. Plant growth promotion due to nitrogen fixation of endophytes was also demonstrated (Sevilla et al. 2001; Hurek et al. 2002). This reinforces the view that research on a rhizosphere association might be of great value for agriculture.

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References

Aßmus B, Hutzler P, Kirchhof G, Amann R, Lawrence JR, Hartmann A (1995) In situ localization of *Azospirillum brasilense* in the rhizosphere of wheat with fluorescently labeled, rRNA-targeted oligonucleotide probes and scanning confocal laser microscopy. Appl Environ Microbiol 61:1013–1019

Adams DG (2002) Symbioses with hornworts and liverworts. In: Rai AN, Bergman B, Rasmussen U (eds) Cyanobacteria in symbiosis. Kluwer academic, Dordrecht, pp 117–136

Arnold W, Rump A, Klipp W, Priefer UB, Pühler A (1988) Nucleotide sequence of a 24,206-base-pair DNA fragment carrying the entire nitrogen fixation gene cluster of *Klebsiella pneumoniae*. J Mol Biol 203:715–738 doi:10.1016/0022-2836(88)90205-7

Arsène F, Katupitiya S, Kennedy IR, Elmerich C (1994) Use of *lacZ* fusions to study the expression of *nif* genes of *Azospirillum brasilense* in association with plants. Mol Plant Microbe Interact 7:748–757

Ausmees N, Kobayashi H, Deakin WJ, Marie C, Krishnan HB, Broughton WJ, Perret X (2004) Characterization of NopP, a type III secreted effector of *Rhizobium* sp. strain NGR234. J Bacteriol 18:4774–4780 doi:10.1128/JB.186.14.4774-4780.2004

Baca BE, Elmerich C (2007) Microbial production of plant hormones. In: Elmerich C, Newton WE (eds) Associative and endophytic nitrogen-fixing bacteria and cyanobacterial associations. Springer, Dordrecht, pp 113–143

Balandreau J (1983) Microbiology of the association. Can J Microbiol 29:851–859

Baldani JI, Baldani VLD (2005) History on the biological nitrogen fixation research in graminaceous plants: special emphasis on the Brazilian experience. An Acad Bras Cienc 77:549–579 doi:10.1590/S0001-37652005000300014

Benson DR, Clawson ML (2007) Recent advances in the biogeography and genecology of symbiotic *Frankia* and its host plants. Physiol Plant 130:318–330 doi:10.1111/j.1399-3054.2007.00934.x

Benson DR, Clawson ML (2000) Evolution of the actinorhizal plant symbioses. In: Triplett EW (ed) Prokaryotic nitrogen

fixation: A model system for analysis of biological process. Horizon Scientific Press, Wymondham, UK, pp 207–224
Berg RH (1990) Cellulose and xylans in the interface capsule in symbiotic cells of actinorhizae. Protoplasma 159:35–43 doi:10.1007/BF01326633
Berg RH (1999) *Frankia* forms infection threads. Can J Bot 77:1327–1333 doi:10.1139/cjb-77-9-1327
Bergersen F (1980) Methods for evaluating biological nitrogen fixation. Willey and Sons, Chichester
Bergman B (2002) *Nostoc-Gunnera* symbiosis. In: Rai AN, Bergman B, Rasmussen U (eds) Cyanobacteria in symbiosis. Kluwer academic, Dordrecht, pp 207–232
Bergman B, Matveyev A, Rasmussen U (1996) Chemical signalling in cyanobacterial-plant symbioses. Trends Plant Sci 1:191–197 doi:10.1016/1360-1385(96)10021-2
Berry MA, Sunell AL (1990) The infection process and nodule development. In: Schwintzer RC, Tjepkema JD (eds) The Biological of *Frankia* and actinorhizal plants. Academic press Inc, San Diego, pp 61–88
Bock JV, Battershell T, Wiggington J, John TR, Johnson JD (2001) *Frankia* sequences exhibiting RNA polymerase promoter activity. Microbiology 147:499–506
Boddey RM, Döbereiner J (1982) Association of *Azospirillum* and other diazotrophs with tropical gramineae. In: Non symbiotic nitrogen fixation and organic matter in the tropics. Indian Society of Soil Science, New Delhi, pp 28–47
Boddey RM, Peoples MB, Palmer B, Dart PJ (2000) The use of ^{15}N natural abundance technique to quantify biological nitrogen fixation by woody perennials. Nutr Cycl Agroecosyst 57:235–270 doi:10.1023/A:1009890514844
Boddey RM, Polidoro JC, Resende AS, Alves BJR, Urquiaga S (2001) Use of the ^{15}N natural abundance technique for the quantification of the contribution of N_2 fixation to sugar cane and other grasses. Aust J Plant Physiol 28:889–895
Borthakur PB, Orozco CC, Young-Robbins SS, Haselkorn R, Callahan SM (2005) Inactivation of *patS* and *hetN* causes lethal levels of heterocyst differentiation in the filamentous cyanobacterium *Anabaena* sp PCC 7120. Mol Microbiol 57:111–113 doi:10.1111/j.1365-2958.2005.04678.x
Boyer M, Bally R, Perrotto S, Chaintreuil C, Wisniewski-Dyé F (2008) A quorum quenching approach to identify quorum-sensing-regulated functions in *Azospirillum lipoferum*. Res Microbiol ▪▪▪:159 doi:10.1016/j.resmic.2008.08.003
Buikema WJ, Haselkorn R (1991) Characterization of a gene controlling heterocyst differentiation in the cyanobacterium *Anabaena* sp PCC 7120. Genes Dev 5:321–330 doi:10.1101/gad.5.2.321
Buikema WJ, Haselkorn R (1993) Molecular genetics of cyanobacterial development. Annu Rev Plant Physiol 44:33–52 doi:10.1146/annurev.pp.44.060193.000341
Burdman S, Dulguerova G, Okon Y, Jurkevitch E (2001) Purification of the major outer membrane protein of *Azospirillum brasilense*, its affinity to plant roots and its involvement in cell aggregation. Mol Plant Microbe Interact 14:555–561 doi:10.1094/MPMI.2001.14.4.555
Burris RH, Miller CE (1941) Application of ^{15}N to the study of biological nitrogen fixation. Science 93:114–115 doi:10.1126/science.93.2405.114
Caballero-Mellado J, Martínez-Aguilar L, Paredes-Valdez G, Estrada-de los Santos P (2004) *Burkholderia unamae* sp nov, an N2-fixing rhizospheric and endophytic species. Int J Syst Evol Microbiol 5:1165–1172 doi:10.1099/ijs.0.02951-0
Callaham D, DelTredici P, Torrey JG (1978) Isolation and cultivation in vitro of the actinomycete causing root nodulation in *Comptonia*. Science 199:899–902 doi:10.1126/science.199.4331.899
Callahan SM, Buikema WJ (2001) The role of HetN in maintenance of the heterocyst pattern in *Anabaena* sp PCC 7120. Mol Microbiol 40:941–950 doi:10.1046/j.1365-2958.2001.02437.x
Campbell EL, Meeks JC (1989) Characteristics of hormogonia formation by symbiotic *Nostoc* spp in response to the presence of *Anthoceros punctatus* or its extracellular products. Appl Environ Microbiol 55:125–131
Carreño-Lopez R, Campos-Reales NB, Elmerich C, Baca BE (2000) Physiological evidence for differently regulated tryptophan-dependent pathways for indole-3-acetic acid synthesis in *Azospirillum brasilense*. Mol Gen Genet 264:521–530 doi:10.1007/s004380000340
Catoira R, Galera C, de Billy F, Penmetsa RV, Journet EP, Maillet F, Rosenberg C, Cook D, Dénarié J (2000) Four genes of *Medicago truncatula* controlling components of a Nod factor transduction pathway. Plant Cell 12:1647–1666
Cérémonie H, Cournoyer B, Maillet F, Normand P, Fernandez MP (1998) Genetic complementation of rhizobial nod mutants with *Frankia* DNA: artefact or realty? Mol Gen Genet 260:115–119 doi:10.1007/s004380050877
Cérémonie H, Debellé F, Fernandez MP (1999) Structural and functional comparison of Frankia root hair deforming factor and rhizobia Nod factor. Can J Bot 77:1293–1301 doi:10.1139/cjb-77-9-1293
Chelius MK, Triplett EW (2000) Immunolocalization of dinitrogenase reductase produced by *Klebsiella pneumoniae* in association with *Zea mays* L. Appl Environ Microbiol 66:783–787 doi:10.1128/AEM.66.2.783-787.2000
Chen WM, James EK, Chou JH, Sheu SY, Yang SZ, Sprent JI (2005) Beta-rhizobia from *Mimosa pigra*, a newly discovered invasive plant in Taiwan. New Phytol 168:661–675 doi:10.1111/j.1469-8137.2005.01533.x
Chen WM, Moulin L, Bontemps C, Vandamme P, Béna G, Boivin-Masson C (2003) Legumes symbiotic nitrogen fixation by beta-proteobacteria is widespread in nature. J Bacteriol 185:7266–7272 doi:10.1128/JB.185.24.7266-7272.2003
Clawson ML, Bourret A, Benson DR (2004) Assessing the phylogeny of *Frankia*-related plant nitrogen-fixing root nodule symbioses with *Frankia* 16SRNA and glutamine synthetase gene sequences. Mol Phylogenet Evol 31:131–138 doi:10.1016/j.ympev.2003.08.001
Cohen MF, Meeks JC (1997) A hormogonium regulating locus, *hrmUA*, of the cyanobacterium *Nostoc punctiforme* strain ATCC29133 and its response to an extract of a symbiotic plant partner *Anthoceros punctatus*. Mol Plant Microbe Interact 10:280–289 doi:10.1094/MPMI.1997.10.2.280
Cohen MF, Sakihama Y, Takagi YC, Ichiba T, Yamasaki H (2002) Synergistic effect of deoxyanthocyanins from symbiotic fern *Azolla* spp on *hrmA* gene induction in the cyanobacterium *Nostoc punctiforme*. Mol Plant Microbe Interact 9:875–882 doi:10.1094/MPMI.2002.15.9.875
Cooper JE (2007) Early interactions between legumes and rhizobia: disclosing complexity in a molecular dialogue. J

Appl Microbiol 103:1355–1365 doi:10.1111/j.1365-2672.2007.03366.x
Costa JL, Lindblad P (2002) Cyanobacteria in symbiosis in cycads. In: Rai AN, Bergman B, Rasmussen U (eds) Cyanobacteria in symbiosis. Kluwer academic, Dordrecht, pp 195–206
Costacurta A, Vanderleyden J (1995) Synthesis of phytohormones by plant-associated bacteria. Crit Rev Microbiol 21:1–18 doi:10.3109/10408419509113531
Cournoyer B, Normand P (1994) Gene expression in *Frankia*: characterization of promoters. Microbiology 78:229–236
Dawson JO (1983) Dinitrogen fixation in forest ecosystems. Can J Microbiol 29:979–992
Dawson JO (1990) Interaction among actinorhizal and associated plant species. In: Schwintzer RC, Tjepkema JD (eds) The Biological of *Frankia* and actinorhizal plants. Academic press Inc, San Diego, pp 299–316
De Oliveira Pinheiro R, Boddey LH, James EK, Sprent JI, Boddey RM (2002) Adsorption and anchoring of *Azospirillum* strains to roots of wheat. Plant Soil 246:151–166 doi:10.1023/A:1020645203084
Dénarié J, Debellé F, Promé JC (1996) *Rhizobium* lipochitooligosaccharide nodulation factors: signalling molecules mediating regcognition and morphogenosis. Annu Rev Biochem 65:503–535 doi:10.1146/annurev.bi.65.070196.002443
Dénarié J, Debellé F, Truchet G, Promé JC (1993) *Rhizobium* and legume nodulation: A molecular dialogue. In: Palacios R, Mora J, Newton WE (eds) New horizons in nitrogen fixation. Kluwer, Dordrecht, pp 19–30
Dixon R, Kahn D (2004) Genetic regulation of biological nitrogen fixation. Nat Rev Microbiol 2:621–631 doi:10.1038/nrmicro954
Dobbelaere S, Okon Y (2007) The plant growth promoting effect and plant response. In: Elmerich C, Newton WE (eds) Associative and endophytic nitrogen-fixing bacteria and cyanobacterial associations. Springer, Dordrecht, pp 145–170
Döbereiner J, Day JM, Dart PJ (1972) Nitrogenase activity and oxygen sensitivity of the *Paspalum notatum-Azotobacter paspali* association. J Gen Microbiol 71:103–116
Döbereiner J, Pedrosa FO (1987) Nitrogen-fixing bacteria in non-leguminous crop plants. Science Tech, Madison and Springer Verlag, Berlin
Döbereiner J (1992) History and new perspectives of diazotrophs in association with non-leguminous plants. Symbiosis 13:1–13
Dörr J, Hurek T, Reinhold-Hurek B (1998) Type IV pili are involved in plant-microbe and fungus-microbe interactions. Mol Microbiol 30:7–17 doi:10.1046/j.1365-2958.1998.01010.x
Doyle JJ, Luckow MA (2003) The rest of the iceberg. Legume diversity and evolution in a phylogenetic context. Plant Physiol 1331:900–910 doi:10.1104/pp.102.018150
Dresler-Nurmi A, Fewer D, Räsänen LA, Lindström K (2007) The diversity and evolution of rhizobia. In: Pawlowski K (ed) Prokaryotic endosymbionts in plants. Springer Verlag doi:101007/7171
Duhoux E, Diouf D, Gherbi H, Franche C, Ahée J, Bogusz D (1996) Le nodule actinorhizien. Acta Bot Gallica 143:593–608
Ehira S, Ohmori M, Sato N (2003) Genome-wide expression analysis of the responses to nitrogen deprivation in the heterocyst-forming cyanobacterium *Anabaena* sp strain PCC 7120. DNA Res 10:97–113 doi:10.1093/dnares/10.3.97
Elbeltagy A, Nishioka K, Sato T, Suzuki H, Ye B, Hamada T, Isawa T, Mitsui H, Minamisawa K (2001) Endophytic colonization and in planta nitrogen fixation by a *Herbaspirillum* sp isolated from wild rice species. Appl Environ Microbiol 67:5285–5293 doi:10.1128/AEM.67.11.5285-5293.2001
Elmerich C, Newton WE (2007) Associative and Endophytic Nitrogen-fixing Bacteria and Cyanobacterial Associations. Springer, The Netherlands
Elmerich C, Zimmer W, Vieille C (1992) Associative nitrogen-fixing bacteria. In: Stacey G, Burris RH, Evans HJ (eds) Biological nitrogen fixation. Chapman and Hall Inc, New York, pp 212–258
Fallik E, Okon Y, Epstein E, Goldman A, Fischer M (1989) Identification and quantification of IAA and IBA in *Azospirillum brasilense*-inoculated maize roots. Soil Biol Biochem 21:147–153 doi:10.1016/0038-0717(89)90024-2
FAO (2008) Current world fertilizer trends and outlook 2011/2012. Food and agricultural organization of the United Nations, Rome
Fischer HM (1994) Genetic regulation of nitrogen fixation in rhizobia. Microbiol Rev 58:352–386
Freiberg C, Fellay R, Bairoch A, Broughton WJ, Rosenthal A, Perret X (1997) Molecular basis of symbiosis between *Rhizobium* and legumes. Nature 387:394–401 doi:10.1038/387394a0
Foucher C, Kondorosi E (2000) Cell cycle regulation in the course of nodule organogenesis in Medicago. Plant Mol Biol 43:773–786 doi:10.1023/A:1006405029600
Gage DJ (2004) Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. Microbiol Mol Biol Rev 68:280–300 doi:10.1128/MMBR.68.2.280-300.2004
Geurts R, Bisseling T (2002) *Rhizobium* nod factor perception and signalling. Plant Cell 14(Suppl):S239–S249
Gherbi H, Markmann K, Svistoonoff S, Estevan J, Autran D, Giczey G, Auguy F, Péret B, Laplaze L, Franche C, Parniske M, Bogusz D (2008a) SymRK defines a common genetic basis for plant root endosymbioses with arbuscular mycorrhiza fungi, rhizobia, and Frankiabacteria. Proc Natl Acad Sci USA 105:4928–4932 doi:10.1073/pnas. 0710618105
Gherbi H, Nambiar-Veetil M, Zhong C, Félix J, Autran D, Girardin R, Auguy F, Bogusz D, Franche C (2008b) Post-transcriptional gene silencing in the root system of the actinorhizal tree *Allocasuarina verticillata*. Mol Plant Microbe Interact 21:518–524 doi:10.1094/MPMI-21-5-0518
Giraud E, Moulin L, Vallenet D, Barbe V, Cytryn E, Avarre JC, Jaubert M, Simon D, Cartieaux F, Prin Y, Bena G, Hannibal L, Fardoux J, Kojadinovic M, Vuillet L, Lajus A, Cruveiller S, Rouy Z, Mangenot S, Segurens B, Dossat C, Franck WL, Chang WS, Saunders E, Bruce D, Richardson P, Normand P, Dreyfus B, Pignol D, Stacey G, Emerich D, Verméglio A, Médigue C, Sadowsky M (2007) Legumes symbioses: absence of *nod* genes in photosynthetic bradyrhizobia. Science 316:1307–1312 doi:10.1126/science.1139548
Glick BR (2005) Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase. FEMS Microbiol Lett 251:1–7 doi:10.1016/j.femsle.2005.07.030

Golden JW, Yoon HS (2003) Heterocyst development in *Anabaena*. Curr Opin Microbiol 6:557–563 doi:10.1016/j.mib.2003.10.004

Goormachtig S, Capoen W, Holsters M (2004) Rhizobium infection : lessons from the versatile nodulation behaviours of water tolerant legumes. Trends Plant Sci 9:518–522 doi:10.1016/j.tplants.2004.09.005

Hamelin J, Fromin N, Tarnawski S, Teyssier-Cuvelle S, Aragno M (2002) *nifH* gene diversity in the bacterial community associated with the rhizosphere of *Molinia coerulea*, an oligonitrophilic perennial grass. Environ Microbiol 4:477–481 doi:10.1046/j.1462-2920.2002.00319.x

Hardy RWF, Burns RC, Holsten RD (1973) Application of the acetylene-ethylene reduction assay for measurement of nitrogen fixation. Soil Biol Biochem 5:47–81 doi:10.1016/0038-0717(73)90093-X

Hauwaerts D, Alexandre G, Das SK, Vanderleyden J, Zhulin IB (2002) A major chemotaxis gene cluster in *Azospirillum brasilense* and relationships between chemotaxis operons in α-proteobacteria. FEMS Microbiol Lett 208:61–67

Henson BJ, Watson LE, Barnum SR (2004) The evolutionary history of nitrogen fixation, as assessed by *nifD*. J Mol Evol 58:390–399 doi:10.1007/s00239-003-2560-0

Hocher V, Auguy F, Argout X, Laplaze L, Franche C, Bogusz D (2006) Expressed sequence-tag analysis in *Casuarina glauca* actinorhizal nodule and root. New Phytol 169:681–688 doi:10.1111/j.1469-8137.2006.01644.x

Holguin G, Glick BR (2001) Expression of the ACC deaminase gene from *Enterobacter cloacae* UW4 in *Azospirillum brasilense*. Microb Ecol 41:281–288

Hu Y, Fay AW, Lee CC, Ribbe MW (2007) P-cluster maturation on nitrogenase MoFe protein. Proc Natl Acad Sci USA 104:10424–10429 doi:10.1073/pnas.0704297104

Huang X, Dong Y, Zhao J (2004) HetR homodimer is a DNA binding protein required for heterocyst differentiation, and the DNA-binding activity is inhibited by PatS. Proc Natl Acad Sci USA 101:4848–4853 doi:10.1073/pnas. 0400429101

Hurd TM, Raynal DJ, Schwintzer CR (2001) Symbiotic N2 fixation of *Alnus incana* spp *rugosa* in shrub wetlands of the Adirondack mountains, New York, USA. Oecologia 126:94–103 doi:10.1007/s004420000500

Hurek T, Reinhold-Hurek B (2003) *Azoarcus* sp strain BH72 as a model for nitrogen-fixing grass endophytes. J Biotechnol 106:169–178 doi:10.1016/j.jbiotec.2003.07.010

Hurek T, Handlley LL, Reinhold-Hurek B, Piché Y (2002) *Azoarcus* grass endophytes contribute fixed nitrogen to the plant in an unculturable state. Mol Plant Microbe Interact 15:233–242 doi:10.1094/MPMI.2002.15.3.233

Huss-Danell K (1997) Actinorhizal symbioses and their N2 fixation. New Phytol 136:375–405 doi:10.1046/j.1469-8137.1997.00755.x

James EK, Olivares FL (1998) Infection and colonisation of sugarcane and other graminaceous plants by endophytic diazotrophs. Crit Rev Plant Sci 17:77–119 doi:10.1016/S0735-2689(98)00357-8

John TR, Rice JM, Johnson JD (2001) Analysis of pFQ12, a 224-kb *Frankia* plasmid. Can J Microbiol 47:608–617 doi:10.1139/cjm-47-7-608

Johnston AWB, Beynon JL, Buchanan-Wollaston AV, Setchell SM, Hirsch PR, Beringer JE (1978) High frequency transfer of nodulating ability between strains and species of *Rhizobium*. Nature 276:634–636 doi:10.1038/276634a0

Kaijalainen S, Lindström K (1989) Restriction fragment length polymorphism analysis of *Rhizobium galegae* strains. J Bacteriol 171:5561–5566

Kinkema M, Scott PT, Gresshoff PM (2006) Legume nodulation: successful symbiosis through short and long-distance signalling. Funct Plant Biol 33:1–15 doi:10.1071/FP06056

Koga J, Adachi T, Hidaka H (1991) Molecular cloning of the gene for indolepyruvate decarboxylase from *Enterobacter cloacae*. Mol Gen Genet 226:10–16 doi:10.1007/BF00273581

Krause A, Ramakumar A, Bartels D, Battistoni F, Bekel T, Boch J, Böhm M, Friedrich F, Hurek T, Krause L, Linke B, McHardy AC, Sarkar A, Schneiker S, Syed AA, Thauer R, Vorhölter FJ, Weidner S, Pühler A, Reinhold-Hurek B, Kaiser O, Goesmann A (2006) Complete genome of the mutualistic, N(2)-fixing grass endophyte *Azoarcus* sp. strain BH72. Nat Biotechnol 24:1385–1391 doi:10.1038/nbt1243

Laguerre G, Géniaux E, Mazurier SI, Rodriguez Casartelli R, Amarger N (1992) Conformity and diversity among field isolates of *Rhizobium leguminosarum* bv. *viciae*, bv. *trifolii*, and bv. *phaseoli* revealed by DNA hybridization using chromosome and plasmid probes. Can J Microbiol 39:412–419

Laguerre G, Mavingui P, Allard MR, Charnay MP, Louvrier P, Mazurier SI, Rigottier-Gois L, Amarger N (1996) Typing of rhizobia by PCR DNA fingerprinting and PCR restriction fragment length polymorphism analysis of chromosomal and symbiotic gene regions: application to *Rizobium leguminosarum* and its different biovars. Appl Environ Microbiol 62:2029–2036

Lamm RB, Neyra CA (1981) Characterization and cyst production of *Azospirilla* isolated from selected grasses growing in New Jersey and New York. Can J Microbiol 27:1320–1325

Laplaze L, Duhoux E, Franche C, Frutz T, Svistoonoff S, Bogusz D (2000) *Casuarina glauca* prenodule cells display the same differentiation as the corresponding nodule cells. Mol Plant Microbe Interact 13:107–112 doi:10.1094/MPMI.2000.13.1.107

Laplaze L, Gherbi H, Frutz T, Pawlowski K, Franche C, Macheix J-J, Auguy F, Bogusz D, Duhoux E (1999) Flavan-containing cells delimit *Frankia*-infected compartments in *Casuarina glauca* nodules. Plant Physiol 121:113–122 doi:10.1104/pp.121.1.113

Laplaze L, Svistoonoff S, Santi C, Auguy F, Franche C, Bogusz D (2008) Molecular biology of actinorhizal symbioses. In: Pawlowski K, Newton WE (eds) Nitrogen-fixing actinorhizal symbioses. Springer, Dordrecht, pp 235–259

Lavire C, Cournoyer B (2003) Progress on the genetics of the N2-fixing actinorhizal symbiont *Frankia*. Plant Soil 254:125–137 doi:10.1023/A:1024915300030

Lavire C, Louis D, Perriere G, Briolay J, Normand P, Cournoyer B (2001) Analysis of pFQ31, a 8551-bp cryptic plasmid from three symbiotic nitrogen-fixing actinomycete. *Frankia*. FEMS Microbiol Lett 197:111–116 doi:10.1111/j.1574-6968.2001.tb10591.x

Lechevalier MP (1994) Taxonomy of the genus *Frankia* (Actinomycetales). Int J Syst Bacteriol 44:1–8

Lechno-Yossef S, Nierzwicki-Bauer SA (2002) *Azolla-Anabaena azollae* symbiosis. In: Rai AN, Bergman B, Rasmussen U (eds) Cyanobacteria in symbiosis. Kluwer academic publishers, Dordrecht, pp 153–178

Lery LMS, von Krüger WMA, Viana FC, Teixeira KRS, Bisch PM (2008) a comparative proteomic analysis of *Gluconacetobacter diazotrophicus* PAL5 at exponential and stationary phases of cultures in the presence of high and low levels of inorganic nitrogen compound. Biochim Biophys Acta

Limpens E, Bisseling T (2003) Signaling in symbiosis. Curr Opin Plant Biol 6:343–350 doi:10.1016/S1369-5266(03)00068-2

Lindström K, Martínez-Romero E (2007) International committee on systematics of prokaryotes subcommittee on the taxonomy of *Agrobacterium* and *Rhizobium*: minutes of the meeting, 23-24 July 2006, Århus, Denmark. Int J Syst Evol Microbiol 57:1365–1366 doi:10.1099/ijs.0.65255-0

Lindström K, Kokko-Gonzales P, Terefework Z, Räsänen LA (2006) Differentiation of nitrogen-fixing legume root nodule bacteria. In: Cooper JE, Rao JR (eds) Molecular techniques for soil and rhizosphere microorganisms. CABI Publishing, Wallingford, pp 236–258

Lipsanen P, Lindström K (1988) Infection and root nodule structure in the *Rhizobium galegae* sp nov—*Galega* sp symbiosis. Symbiosis 6:81–96

Liu CC, Zheng WW (1992) Nitrogen fixation of *Azolla* and its utilization in agriculture in China. In: Hong GF (ed) Nitrogen fixation and its research in China. Springer-Verlag, Berlin, pp 526–537

Liu Q, Berry AM (1991) The infection process and nodule initiation in the *Frankia-Ceanothus* root nodule symbiosis. Protoplasma 163:82–92 doi:10.1007/BF01323332

Long SR (2001) Gene and signals in the *Rhizobium*-legume symbiosis. Plant Physiol 125:69–72 doi:10.1104/pp.125.1.69

Lumpkin TA, Plucknett DL (1980) *Azolla*: botany, physiology, and use as a green manure. Econ Bot 34:111–153

Lumpkin TA, Plucknett DL (1982) *Azolla* as a green manure: use and management in crop production. Westview, Boulder, Colorado

MacLean AM, Finan TM, Sadowsky MJ (2007) Genomes of the symbiotic nitrogen-fixing bacteria of legumes. Plant Physiol 144:615–622 doi:10.1104/pp.107.101634

Macura J (1966) Rapport général. Ann Inst Pasteur (Paris) 111 (suppl 3):9–38

Marie C, Broughton WJ, Deakin WJ (2001) *Rhizobium* type III secretion systems: legume charmers or alarmers? Curr Opin Plant Biol 4:336–342 doi:10.1016/S1369-5266(00)00182-5

McEwan NR, Gatherer D (1999) Codon indices as a predictor of gene functionality in a *Frankia* operon. Can J Bot 77:1287–1292 doi:10.1139/cjb-77-9-1287

Meeks JC, Elhai J (2002) Regulation of cellular differentiation in filamentous cyanobacteria in free-living and plant-associated symbiotic growth states. Microbiol Mol Biol Rev 66:94–121 doi:10.1128/MMBR.66.1.94-121.2002

Mergaert P, Van Montagu M, Promé JC, Holsters M (1993) Three unusual modifications, a D-arabinosyl, an N-methyl, and a carbamoyl group, are present on the Nod factors of *Azorhizobium caulinodans* strain ORS571. Proc Natl Acad Sci USA 90:1551–1555 doi:10.1073/pnas.90.4.1551

Merrick MJ, Edwards RA (1995) Nitrogen control in bacteria. Microbiol Rev 59:604–622

Miché L, Bouillant ML, Rohr R, Salle G, Bally R (2000) Physiological and cytological studies on the inhibition of Striga seed germination by the plant growth-promoting bacterium *Azospirillum brasilense*. Eur J Plant Pathol 106:347–351 doi:10.1023/A:1008734609069

Miller IM, Baker DD (1985) The initiation, development and structure of root nodules in *Eleagnus angustifolia L* (*Eleagnaceae*). Protoplasma 128:107–119 doi:10.1007/BF01276333

Mosier AR (2002) Environmental challenges associated with needed increases in global nitrogen fixation. Nutr Cycl Agroecosyst 63:101–116 doi:10.1023/A:1021101423341

Mutch LA, Young JPW (2004) Diversity and specificity of *Rhizobium leguminosarum* biovar *viciae* on wild and cultivated legumes. Mol Ecol 13:2435–2444 doi:10.1111/j.1365-294X.2004.02259.x

Myrold DD, Huss-Danell K (2003) Alder and lupine enhance nitrogen cycling in a degraded forest soil in Northern Sweden. Plant Soil 254:47–56 doi:10.1023/A:1024951115548

Newton WE (2007) Physiology, biochemistry and molecular biology of nitrogen fixation. In: Bothe H, Ferguson SJ, Newton WE (eds) Biology of the nitrogen cycle. Elsevier, Amsterdam, pp 109–130

Nierzwicki-Bauer SA (1990) *Azolla-Anabaena* symbiosis. In: Rai AN (ed) Handbook of symbiotic cyanobacteria. CRC, Boca Raton, pp 119–136

Normand P, Mullin BC (2008) Prospects for the study of a ubiquitous actinomycete, *Frankia*, and its host plants. In: Pawlowski K, Newton WE (eds) Nitrogen-fixing actinorhizal symbioses. Springer, Dordrecht, pp 289–303

Normand P, Lapierre P, Tisa LS, Gogarten JP, Alloisio N, Bagnarol E, Bassi CA, Berry AM, Bickhart DM, Choisne N, Couloux A, Cournoyer B, Cruveiller S, Daubin V, Demange N, Francino MP, Goltsman E, Huang Y, Martinez M, Mastronunzio JE, Mullin BC, Nieman J, Pujic P, Rawnsley T, Rouy Z, Schenowitz C, Sellstedt A, Tvares F, Tomkins JP, Vallenet D, Valverde C, Wall L, Wang Y, Médigue C, Benson DR (2007) Genome characteristics of facultatively symbiotic *Frankia* sp strains reflect host range and host plant biogeography. Genome Res 17:7–15 doi:10.1101/gr.5798407

Normand P, Orso S, Cournoyer B, Jeannin P, Chapelon C, Dawson J, Evtushenko L, Misra AK (1996) Molecular phylogeny of the genus *Frankia* and related genera and emendation of family *Frankiaceae*. Int J Syst Bacteriol 46:1–9

Okon Y (1985) *Azospirillum* as a potential inoculant for agriculture. Trends Biotechnol 3:223–228 doi:10.1016/0167-7799(85)90012-5

Okon Y, Labandera-Gonzales CA (1994) Agronomic applications of *Azospirillum*: an evaluation of 20 years worldwide field inoculation. Soil Biol Biochem 26:1591–1601 doi:10.1016/0038-0717(94)90311-5

Oldroyd GED, Downie JA (2008) Coordinating nodule morphogenesis with rhizobial infection in Legumes. Annu Rev Plant Biol 59:519–546 doi:10.1146/annurev.arplant.59.032607.092839

Pawlowski K, Bisseling T (1996) Rhizobial and actinorhizal symbioses: what are the shared features? Plant Cell 6:1899–1913

Pawlowski K, Sprent JI (2008) Comparison between actinorhizal symbiosis and legume symbiosis. In: Pawlowski K, Newton WE (eds) Nitrogen-fixing actinorhizal symbioses. Springer, Dordrecht, pp 261–288

Pedrosa FO, Elmerich C (2007) Regulation of nitrogen fixation and ammonium assimilatiuon in associated and endophytic nitrogen fixing bacteria. In: Elmerich C, Newton WE (eds) Associative and endophytic nitrogen-fixing bacteria and cyanobacterial associations. Springer, The Netherlands, pp 41–71

Peoples MB, Herridge DF, Ladha JK (1995) Biological nitrogen fixation: an efficient source of nitrogen for sustainable agricultural production? Plant Soil 174:2–28

Pereg-Gerk L, Paquelin A, Gounon A, Kennedy IR, Elmerich C (1998) A transcriptional regulator of the LuxR-UhpA family, FlcA, controls flocculation and wheat root surface colonization by *Azospirillum brasilense* Sp7. Mol Plant Microbe Interact 11:177–187 doi:10.1094/MPMI.1998.11.3.177

Perret X, Staehelin C, Broughton WJ (2000) Molecular basis of symbiotic promiscuity. Microbiol Mol Biol Rev 64:180–201 doi:10.1128/MMBR.64.1.180-201.2000

Peters GA, Meeks JC (1989) The *Azolla-Anabaena* symbiosis: basic biology. Annu Rev Plant Physiol Plant Mol Biol 40:193–210 doi:10.1146/annurev.pp.40.060189.001205

Postgate J (1981) Microbiology of the free-living nitrogen-fixing bacteria, excluding cyanobacteria. In: Gibson AH, Newton WE (eds) Current perspectives in nitrogen fixation. Elsevier/North-Holland Biomedical, Amsterdam, pp 217–228

Pothier JF, Wisniewski-Dyé F, Weiss-Gayet M, Moënne-Loccoz Y, Prigent-Combaret C (2007) Promoter-trap identification of wheat seed extract-induced genes in the plant-growth-promoting rhizobacterium *Azospirillum brasilense* Sp245. Microbiology 153:3608–3622 doi:10.1099/mic.0.2007/009381-0

Prell J, Poole P (2006) Metabolic changes of rhizobia in legume nodules. Trends Microbiol 14:161–168 doi:10.1016/j.tim.2006.02.005

Prigent-Combaret C, Blaha D, Pothier F, Vial L, Poirier M-A, Wisniewski-Dyé F, Moënne-Loccoz Y (2008) FEMS Microbiol Ecol 65:220-219 doi:10.1111/j.1574-6941.2008.00545.x

Prin Y, Rougier M (1987) Preinfection events in the establishment of *Alnus-Frankia* symbiosis: study of the root hair deformation step. Plant Physiol 6:99–106

Pueppke SG, Broughton WJ (1999) *Rhizobium* sp strain NGR234 and *R fredii* USDA257 share exceptionally broad, nested host ranges. Mol Plant Microbe Interact 12:293–318 doi:10.1094/MPMI.1999.12.4.293

Radeva G, Jurgens G, Niemi M, Nick G, Suominen L, Lindström K (2001) Description of two biovars in the *Rhizobium galegae* species: biovar orientalis and biovar officinalis. Syst Appl Microbiol 24:195–205 doi:10.1078/0723-2020-00029

Rai AN (1990) Handbook of symbiotic cyanobacteria. CRC Press, Boca Raton, Florida, USA

Rai AN, Bergman B, Rasmussen U (2002) Cyanobacteria in symbiosis. Kluwer Academic, Dordrecht

Rai AN, Söderbäck E, Bergman B (2000) Cyanobacterium-plant symbiosis. New Phytol 147:449–481 doi:10.1046/j.1469-8137.2000.00720.x

Räsänen LA, Heikkilä-Kallio U, Suominen L, Lipsanen P, Lindström K (1991) Expression of common nodulation genes of *Rhizobium galegae* in various backgrounds. Mol Plant Microbe Interact 4:535–544

Rasmussen U, Svenning MM (2001) Characterization of genotypic methods of symbiotic *Nostoc* strains isolated from five species of *Gunnera*. Arch Microbiol 176:204–210 doi:10.1007/s002030100313

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

Rasmussen U, Johansson C, Renglin A, Petersson C, Bergman B (1996) A molecular characterization of the *Gunnera-Nostoc* symbiosis: comparison with *Rhizobium*- and *Agrobacterium*-plant interactions. New Phytol 133:391–398 doi:10.1111/j.1469-8137.1996.tb01906.x

Reinhold-Hurek B, Hurek T (1998) Life in grasses, diazotrophic endophytes. Trends Microbiol 6:139–144 doi:10.1016/S0966-842X(98)01229-3

Rennie RJ (1980) Dinitrogen-fixing bacteria: computer-assisted identification of soil isolates. Can J Microbiol 26:1275–1283

Rippka R, Deruelles J, Waterbury JB, Herdman M, Stanier RY (1979) Genetic assignments, strain histories and properties of pure cultures of cyanobacteria. J Gen Microbiol 111:1–61

Rodrigues EP, Rodrigues LS, Martinez de Oliveira AL, Baldani VLD, dos Santos Teixeira KR, Urquiaga S, Reis VM (2008) *Azospirillum amazonense* inoculation: effect on growth yield and N_2 fixation of rice (*Oryza sativa* L.). Plant Soil doi:10.1007/s11104-00079476-1

Roesch LFW, Camargo FAO, Bento FM, Triplett EW (2008) Biodiversity of diazotrophs within the soil, root and stem of field grown maize. Plant Soil 302:91–104 doi:10.1007/s11104-007-9458-3

Rothballer M, Eckert B, Schmid M, Fekete A, Scholter M, Lehner A, Pollmann S, Hartmann A (2008) Endophytic root colonization of gramineous plants by *Herbaspirillum frisingense*. FEMS Microbiol Ecol 66:85–95 doi:10.1111/j.1574-6941.2008.00582.x

Rothballer M, Schmid M, Hartmann A (2003) *In situ* localization and PGPR-effect of *Azospirillum brasilense* colonizing roots of different wheat varieties. Symbiosis 34:261–279

Rovira AD (1991) Rhizosphere research, 85 years of progress and frustration. In: Keister DL, Cregan PB (eds) The rhizosphere and plant growth. Kluwer Academic, The Netherlands, pp 3–13

Rubio LM, Ludden PW (2005) Maturation of nitrogenase: a biochemical puzzle. J Bacteriol 187:405–414 doi:10.1128/JB.187.2.405-414.2005

Rubio LM, Ludden PW (2008) Biosynthesis of the iron-molybdenum cofactor of nitrogenase. Annu Rev Microbiol 62:93–111 doi:10.1146/annurev.micro.62.081307.162737

Schank SC, Smith RL, Weiser GC, Zuberer DA, Bouton JH, Quesenberry KH, Tyler ME, Milam JR, Littel RC (1979) Fluorescent antibody technique to identify *Azospirillum brasilense* associated with roots of grasses. Soil Biol Biochem 11:287–295 doi:10.1016/0038-0717(79)90074-9

Schmid M, Hartmann A (2007) Molecular phylogeny and ecology of root associated diazotrophic α and β Proteo-

bacteria. In: Elmerich C, Newton WE (eds) Associative and endophytic nitrogen-fixing bacteria and cyanobacterial associations. Springer, Dordrecht, pp 41–71

Schultze M, Kondorosi A (1998) Regulation of symbiotic root nodule development. Annu Rev Genet 32:33–57 doi:10.1146/annurev.genet.32.1.33

Sevilla M, Burris RH, Gunapala N, Kennedy C (2001) Comparison of benefit to sugar cane plant growth and $^{15}N_2$ incorporation following inoculation of sterile plants with *Acetobacter diazotrophicus* wild-type and Nif$^-$-mutant strains. Mol Plant Microbe Interact 14:359–366 doi:10.1094/MPMI.2001.14.3.358

Shi Y, Zhao W, Zhang W, Ye Z, Zhao J (2006) Regulation of intracellular free calcium concentration during heterocyst differentiation by HetR and NtcA in *Anabaena* sp Pcc7120. Proc Natl Acad Sci USA 103:11334–11339 doi:10.1073/pnas.0602839103

Simonet P, Normand P, Hirch M, Akkermans ADL (1990) The genetics of the *Frankia*-actinorhizal symbiosis. In: Gresshoff PM (ed) Molecular biology of symbiotic nitrogen fixation. CRC, Bocaraton, USA, pp 77–109

Spaink HP (2000) Root nodulation and infection factors produced by rhizobial bacteria. Annu Rev Microbiol 54:257–288 doi:10.1146/annurev.micro.54.1.257

Sprent JI (2001) Nodulation in legumes. Royal Botanic Gardens, Kew, UK

Sprent JI, James EK (2007) Legume evolution: where do nodules and mycorrhizas fit in? Plant Physiol 144:575–581 doi:10.1104/pp.107.096156

Steenhoudt O, Vanderleyden J (2000) *Azospirillum*, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. FEMS Microbiol Rev 24:487–506 doi:10.1111/j.1574-6976.2000.tb00552.x

Stephens BB, Loar SN, Alexandre G (2006) Role of CheB and CheR in the complex chemotactic and aerotactic pathway of *Azospirillum brasilense*. J Bacteriol 188:4759–4768 doi:10.1128/JB.00267-06

Stewart WDP (1969) Biological and ecological aspects of nitrogen fixation by free-living microorganisms. Proc Roy Soc B (London) 172:367–388

Stoffels M, Castellanos T, Hartmann A (2001) Design and application of new 16S-rRNA-targeted oligonucleotide probes for the *Azospirillum-Skermanella-Rhodocista*-cluster. Syst Appl Microbiol 24:83–97 doi:10.1078/0723-2020-00011

Suominen L, Lortet G, Roos C, Paulin L, Lindström K (2001) Identification and structure of the *Rhizobium galegae* common nodulation genes: evidence for horizontal gene transfer. Mol Biol Evol 18:906–916

Suominen L, Luukkainen R, Lindström K (2003) Activation of the *nodA* promoter by the *nodD* genes of *Rhizobium galegae* induced by synthetic flavonoids or *Galega orientalis* root exudates. FEMS Microbiol Lett 19:225–232 doi:10.1016/S0378-1097(02)01206-5

Svistoonoff S, Laplaze L, Auguy F, Runions J, Duponnois R, Haseloff J, Franche C, Bogusz D (2003) *cg12* expression is specifically linked to infection of root hairs and cortical cells during *Casuarina glauca* and *Allocasuarina verticillata* actinorhizal nodule development. Mol Plant Microbe Interact 16:600–607 doi:10.1094/MPMI.2003.16.7.600

Tapia-Hernández A, Mascarúa-Esperza MA, Caballero-Mellado J (1990) Production of bacteriocins and siderophore-like activity by *Azospirillum brasilense*. Microbios 64:73–83

Tarrand JJ, Krieg NR, Döbereiner J (1978) A taxonomic study of the *Spirillum lipoferum* group with description a new genus, *Azospirillum* gen nov and two species, *Azospirillum lipoferum* (Beijerinck) comb nov and *Azospirillum brasilense* sp nov. Can J Microbiol 24:967–980

Tas E, Leinonen P, Saano A, Piippola S, Kaijalainen S, Räsänen LA, Hakola S, Lindström K (1996) Assessment of the competitiveness of rhizobia infecting *Galega orientalis* using plant yield, nodulation, and strain identification by PCR and antibiotic resistance. Appl Environ Microbiol 62:529–535

Torrey JG, Tjepkema JD (1979) Symbiotic nitrogen fixation in actinomycete-nodulated plants. Bot Gaz 140(Suppl):i–ii doi:10.1086/337026

Turner SL, Young JPW (2000) The glutamine synthetases of rhizobia: phylogenetics and evolutionary implications. Mol Biol Evol 17:309–319

Ueda T, Suga Y, Yahiro N, Matsuguchi T (1995) Remarkable N_2-fixing bacterial diversity detected in rice roots by molecular evolutionary analysis of *nifH* gene sequences. J Bacteriol 177:1414–1417

van Berkum P, Bohlool BB (1980) Evaluation of nitrogen fixation by bacteria in association with roots of tropical grasses. Microbiol Rev 44:491–517

van Ghelue M, Lovaas E, Ringo E, Solheim B (1997) Early interaction between *Alnus glutinosa* and *Frankia* strain Arl3. Production and specificity of root hair deformation factors. Physiol Plant 9:579–587 doi:10.1111/j.1399-3054.1997.tb05360.x

Van Hove C, Lejeune A (2002) Applied aspects of *Azolla-Anabaena* symbiosis. In: Rai AN, Bergman B, Rasmussen U (eds) Cyanobacteria in symbiosis. Kluwer academic, Dordrecht, pp 179–194

Vanbleu E, Marchal K, Lambrecht M, Mathys J, Vanderleyden J (2004) Annotation of the pRhico plasmid of *Azospirillum brasilense* reveals its role in determining the outer surface composition. FEMS Microbiol Lett 232:165–172 doi:10.1016/S0378-1097(04)00046-1

Vande Broek A, Lambrecht M, Vanderleyden J (1998) Bacterial chemotactic motility is important for the initiation of wheat root colonization by *Azospirillum brasilense*. Microbiology 144:2599–2606

Vande Broek A, Michiels J, Van Gool AP, Vanderleyden J (1993) Spatial-temporal colonization patterns of *Azospirillum brasilense* on the wheat root surface and expression of the bacterial *nifH* gene during association. Mol Plant Microbe Interact 6:592–600

Vessey JK, Pawlowski K, Bergman B (2004) Root-based N_2-fixing symbioses: legumes, actinorhizal plants, *Parasponia* and cycads. Plant Soil 266:205–230 doi:10.1007/s11104-005-0871-1

Vlek PLG, Diakite MY, Mueller H (1995) The role of *Azolla* in curbing ammonia volatilization from flooded rice systems. Fert Res 42:165–174 doi:10.1007/BF00750511

Von Bülow JFW, Döbereiner J (1975) Potential for nitrogen fixation in maize genotypes in Brazil. Proc Natl Acad Sci USA 72:2389–2393 doi:10.1073/pnas.72.6.2389

Wall LG (2000) The actinorhizal symbiosis. J Plant Growth Regul 19:167–182

Wall LG, Berry AM (2008) Early interactions, infection and nodulation in actinorhizal symbiosis. In: Pawlowski K, Newton WE (eds) Nitrogen-fixing actinorhizal symbioses. Springer, Dordrecht, pp 147–166

Wang CM, Ekman M, Bergman B (2004) Expression of cyanobacterial genes involved in heterocyst differentiation and dinitrogen fixation along a plant symbiosis development profile. Mol Plant Microbe Interact 17:436–443 doi:10.1094/MPMI.2004.17.4.436

Wei TF, Ramasubramanian TS, Golden JW (1994) *Anabaena* sp strain PCC 7120 *ntcA* gene required for growth on nitrate and heterocyst development. J Bacteriol 176:4473–4482

Wheeler CT, Miller IM (1990) Current potential uses of actinorhizal plants in Europe. In: Schwintzer RC, Tjepkema JD (eds) The biological of *Frankia* and actinorhizal plants. Academic press Inc, San Diego, pp 365–389

White J, Prell J, James EK, Poole P (2007) Nutrient sharing between symbionts. Plant Physiol 144:604–614 doi:10.1104/pp.107.097741

Wong FCY, Meeks JC (2001) The *hetF* gene product is essential to heterocyst differentiation and affects HetR function in the cyanobacterium *Nostoc punctiforme*. J Bacteriol 183:2654–2661 doi:10.1128/JB.183.8.2654-2661.2001

Wong FCY, Meeks JC (2002) Establishment of a functional symbiosis between the cyanobacterium *Nostoc punctiforme* and the bryophyte *Anthoceros punctatus* requires genes involved in nitrogen control and initiation of heterocyst differentiation. Microbiology 148:315–323

Xu X, Elhai J, Wolk CP (2008) Transcriptional and developmental responses by *Anabaena* to deprivation of fixed nitrogen. In: Herrero A, Flores E (eds) Cyanobacteria: Molecular biology, genomics and evolution. Horizon Scientific, Norwich, pp 383–422

Xu XD, Kong RQ, de Bruijn FJ, He SY, Murry MA, Newman T, Wolk P (2002) DNA sequence and genetic characterization of plasmid pFQ11 from *Frankia alni* strain CpI1. FEMS Microbiol Lett 207:103–107 doi:10.1111/j.1574-6968.2002.tb11036.x

Yan Y, Yang J, Dou Y, Chen M, Ping S, Peng J, Lu W, Zhang W, Yao Z, Li H, Liu W, He S, Geng L, Zhang X, Yang F, Yu H, Zhan Y, Li D, Lin Z, Wang Y, Elmerich C, Lin M, Jin Q (2008) Nitrogen fixation island and rhizosphere competence traits in the genome of root-associated *Pseudomonas stutzeri* A1501. Proc Natl Acad Sci USA 105:7564–7569 doi:10.1073/pnas.0801093105

Yang GP, Debellé F, Savagnac A, Ferro M, Schiltz O, Maillet F, Promé D, Treilhou M, Vialas C, Lindström K, Dénarié J, Promé JC (1999) Structure of the *Mesorhizobium huakuii* and *Rhizobium galegae* Nod factors: a cluster of phylogenetically related legumes are nodulated by rhizobia producing Nod factors with alpha,beta-unsaturated N-acyl substitutions. Mol Microbiol 34:227–237 doi:10.1046/j.1365-2958.1999.01582.x

Yoon HS, Golden JW (1998) Heterocyst pattern formation controlled by a diffusible peptide. Science 282:935–938 doi:10.1126/science.282.5390.935

Young P (1992) Phylogenetic classification of nitrogen-fixing organisms. In: Stacey G, Burris RH, Evans HJ (eds) Biological nitrogen fixation. Chapman and Hall Inc, New York, pp 43–86

Young JPW, Crossman LC, Johnston AWB, Thomson NR, Ghazoui ZF, Hull KH, Wexler M, Curson ARJ, Todd JD, Poole PS, Mauchline TH, East AK, Quail MA, Churcher C, Arrowsmith C, Cherevach I, Chillingworth T, Clarke K, Cronin A, Davis P, Fraser A, Hance Z, Hauser H, Jagels K, Moule S, Mungall K, Norbertczak H, Rabbinowitsch E, Sanders M, Simmonds M, Whitehead S, Parkhill J (2006) The genome of *Rhizobium leguminosarum* has recognizable core and accessory components. Genome Biol 7:R34 doi:10.1186/gb-2006-7-4-r34

Zehr JP, McReynolds LA (1989) Use of degenerate oligonucleotides for amplification of the *nifH* gene from the marine cyanobacterium *Trichodesmium* spp. Appl Environ Microbiol 55:2522–2526

Zhang CC, Laurent S, Sakr S, Peng L, Bedu S (2006) Heterocyst differentiation and pattern formation in cyanobacteria: a chorus of signals. Mol Microbiol 59:367–375 doi:10.1111/j.1365-2958.2005.04979.x

Zheng L, Cash DL, Flint DH, Dean DR (1998) Assembly of iron-sulfur clusters. Identification of an iscSUA-hscBA-fdx gene cluster from *Azotobacter vinelandii*. J Biol Chem 273:13264–1327272 doi:10.1074/jbc.273.21.13264