

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

Claudine Franche · Kristina Lindström ·  
Claudine Elmerich

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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*

## 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

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C. Franche  
Equipe Rhizogénèse, UMR DIAPC,  
Institut de Recherche pour le Développement,  
B. P. 64501, 34394 Montpellier Cedex 5, France

K. Lindström  
Department of Applied Chemistry and Microbiology,  
Viikki Biocenter, University of Helsinki,  
P. O. Box 56, 00014 Helsinki, Finland

C. Elmerich (✉)  
Biologie Moléculaire du Gène chez les Extrémophiles,  
Département de Microbiologie, Institut Pasteur,  
25 rue du Dr. Roux,  
75724 Paris Cedex 15, France  
e-mail: elmerich@pasteur.fr

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.

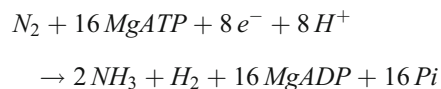
### The nitrogen fixation process

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  $\alpha$  and  $\beta$ , while component 2, also designated Fe protein, is a dimer of 68,000 Da

formed by identical subunits. Two FeMoCo are bound to the  $\alpha$  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  $\alpha$  and  $\beta$  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:



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 *rmfABCDGEF* cluster) to nitrogenase, molybdenum uptake and homeostasis, and oxygen protection and regulation, including respiratory chains adapted to oxygen conditions at

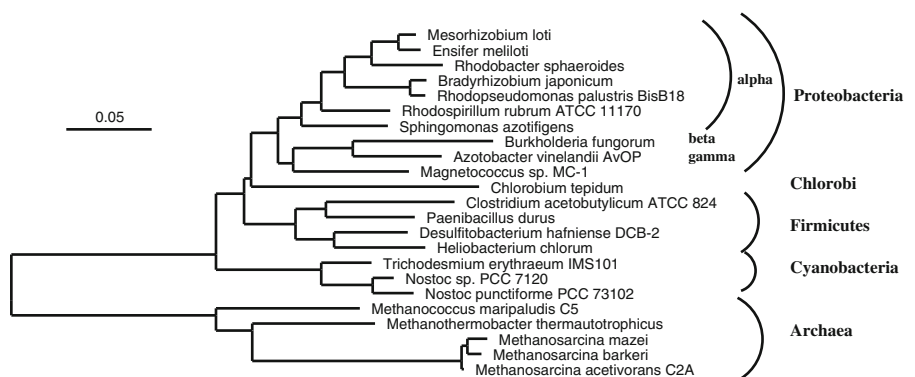
which the nitrogen fixation process can operate (for details see Fischer 1994; Dixon and Kahn 2004; Pedrosa and Elmerich, 2007).

## 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 Winogradsky (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.

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



### 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  $^{15}\text{N}$  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  $^{15}\text{N}$  isotopic gas is impracticable. Instead, a common method is based on differences in the natural abundance of  $^{15}\text{N}$  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

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: Caesalpinoideae, Mimosoideae and Papilionoideae. The Caesalpinoideae has very few nodulating members, whereas most of the important agricultural crops are members of the Papilionoideae. Mimosoideae has recently received attention, since, in many cases, bacteria recovered from their nodules belong to the beta subclass of *Proteobacteria*, while Papilionoideae symbionts belongs to the alpha subclass (see below; Chen et al. 2003, 2005). Only one non-legume, the woody plant *Parasponia* sp., can be

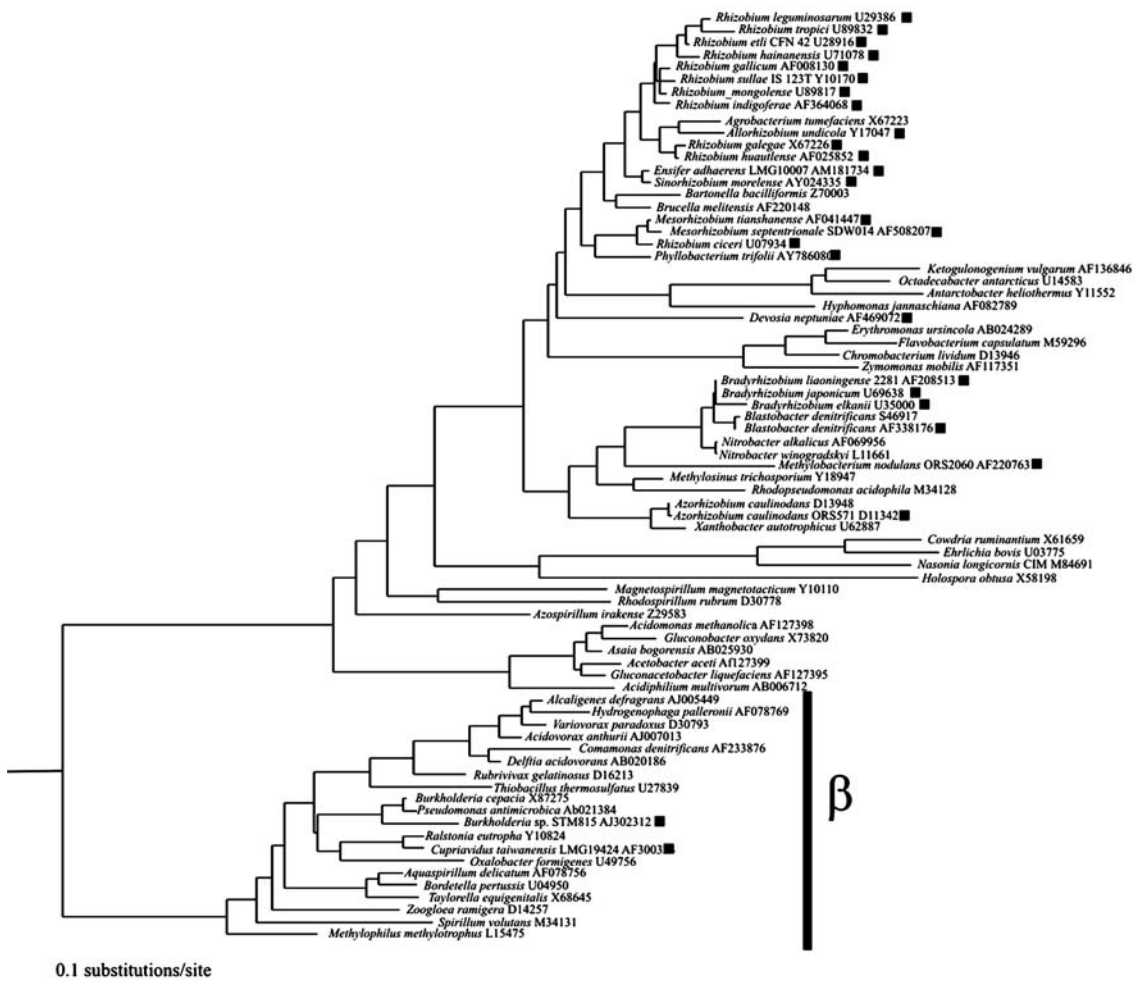
nodulated by rhizobia and utilize nitrogen fixed by the bacteria. Legumes are thought to have been around for 59 Ma, and all leguminous subfamilies evolved 56–50 Ma ago (Sprent and James 2007). In comparison with rhizobia, they are young; the *Sinorhizobium*–*Bradyrhizobium* split was pinpointed to about 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

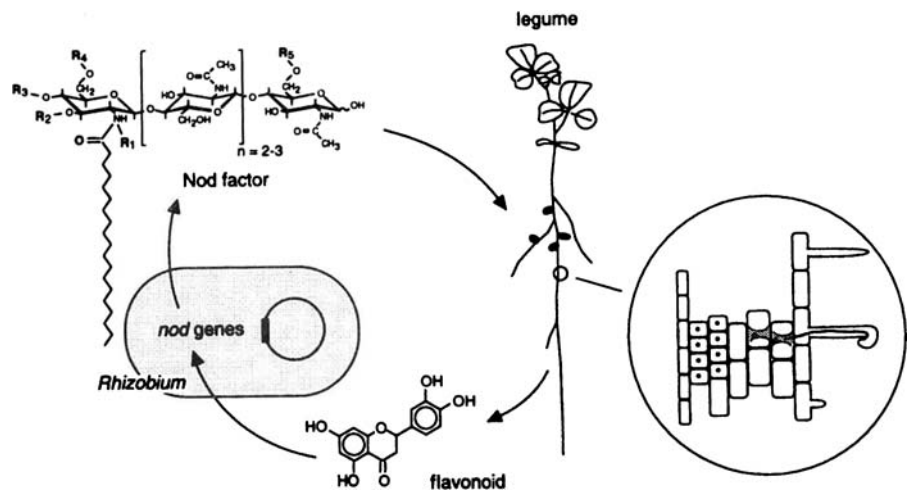
A common genetic determinant for rhizobia is the presence of genes encoding nodulation and nitrogen fixation functions (*nod*, *nol*, *noe*, *nif* and *fix* genes). These genes are often carried on plasmids or other accessory elements, such as symbiotic islands, and properties encoded by them can be 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 blocked 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 (Kajjalainen 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*, *Prosopis* 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, Datisceae 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).

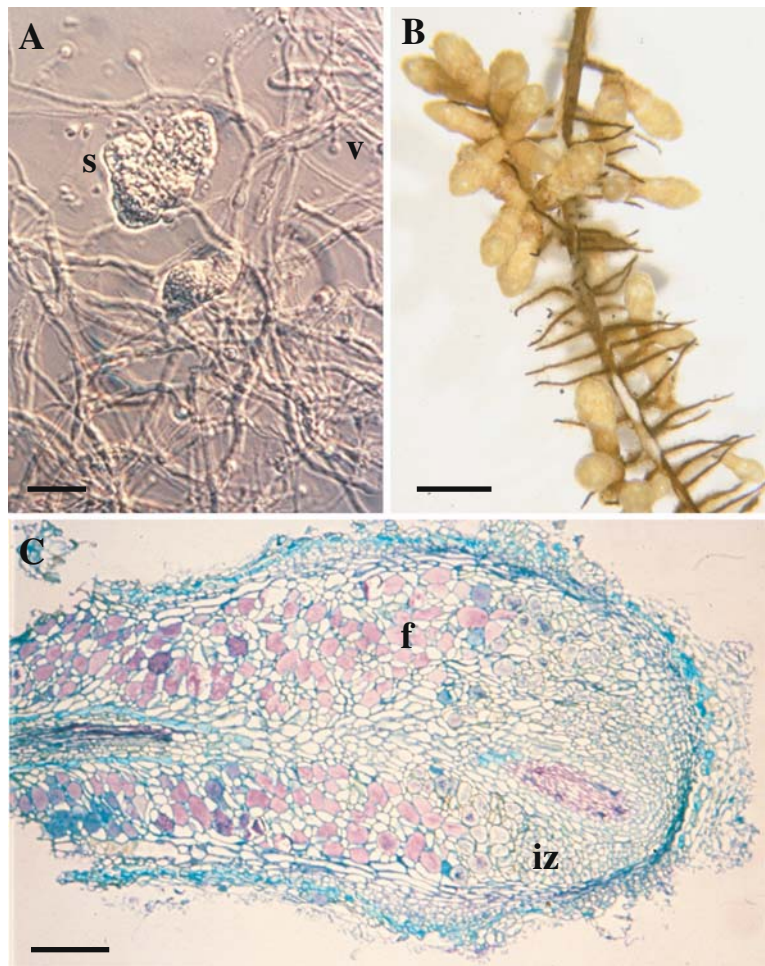
### Frankia

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<sup>-</sup>/Fix<sup>-</sup>), 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*, *Datisceae*, *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  $\mu$ m; B=5 mm; C=200  $\mu$ m



*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 Mbp. Efforts to detect

genes homologous to the *nod* genes of rhizobia in *Frankia* had failed (C er monie 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 (C er monie 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

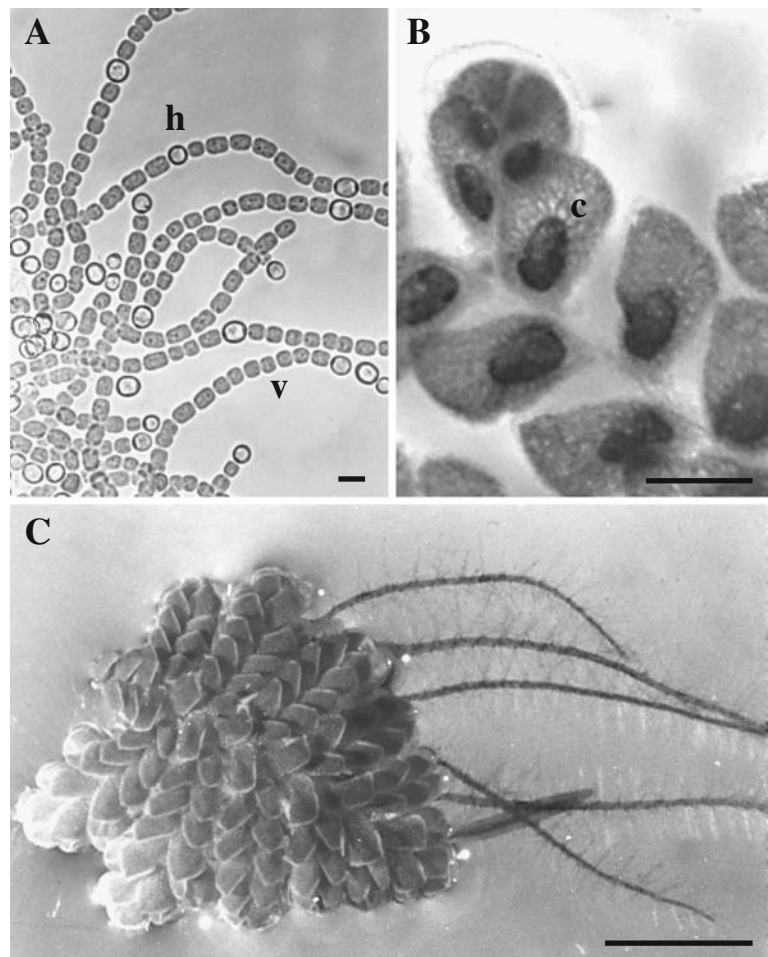


(Fig. 5a) (Rippka et al. 1979) (Fig. 5a). An anaerobic environment compatible with the functioning of nitrogenase in heterocysts is linked to formation of multilayered envelopes external to the outer membrane, elimination of a functional oxygen-producing photosystem II, and additional changes in their 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  $\mu$ m; B=50  $\mu$ m; C=500  $\mu$ m



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  $\text{NH}_4^+$ . 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*, *Derrxia*, several members of *Enterobacteriaceae* (*Klebsiella*, *Pantoea*) 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. doebereineriae*. *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 sys-

tems 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 (Abmuss 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<sub>2</sub> (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<sub>2</sub> 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  $^{15}\text{N}$  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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