Chapter 8
Endophytic Bacteria and Their Role in Legumes Growth Promotion

Tania Taurian, Fernando Ibáñez, Jorge Angelini, María Laura Tonelli, and Adriana Fabra

8.1 Introduction

Plants live in intimate association with microorganisms. Bacteria may exist as free-living organisms in soils, attached to the surface of roots or establishing symbiotic relations with plants, which encompass styles ranging from mutualistic to commensal and parasitic. The rhizosphere (the zone that surrounds the roots of plants) and roots are heavily colonized by microbes since sources of carbon and minerals are very abundant in this zone (Walker et al. 2003). Plants exude high levels of nutrients from their roots such as sugars, amino acids, organic acids, polysaccharides, and proteins (Marschner 1995). In addition to providing a carbon-rich environment, plant roots initiate cross talks with soil microbes by producing molecules that are recognized by microorganisms, which in turn produce signals that initiate colonization (Bais et al. 2006). Consequently, the rhizosphere supports large and active microbial populations capable of exerting beneficial, neutral, or detrimental effects on plant growth.

This chapter will be focused primarily on the positive interactions among legumes and endophytic (*endon* Gr., within; *phyton*, plant) bacteria (defined as microorganisms that inhabit the interior of plant tissues and organs, including nodules). These bacteria can positively influence plant growth through a variety of mechanisms, including fixation of atmospheric nitrogen (Burris and Roberts 1993), increased biotic and abiotic stress tolerance, and other direct and indirect advantages (Kloepper et al. 2004; Timmusk and Wagner 1999). They can also positively interact with plants by producing biofilms or antibiotics that protect them against potential pathogens (Ude et al. 2006) or by degrading plant-produced compounds in soils that would otherwise be allelopathic (Turner and Rice 1975).

T. Taurian • F. Ibáñez • J. Angelini • M.L. Tonelli • A. Fabra (✉)
Dpto. Ciencias Naturales, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto (UNRC), Río Cuarto (Córdoba), Argentina
e-mail: afabra@exa.unrc.edu.ar

D.K. Maheshwari (ed.), *Bacteria in Agrobiology: Plant Probiotics*,
DOI 10.1007/978-3-642-27515-9_8,

The ability to convert atmospheric dinitrogen to ammonia is observed not only in free-living but also in symbiotic diazotrophs, including bacterial species collectively referred to as rhizobia. The interaction between these endophytes and legumes involves widely known molecular mechanisms.

The prospect of manipulating crop rhizosphere bacterial populations by inoculation of those that increase plant growth has shown considerable promise in laboratory and greenhouse studies, but under field conditions, responses have been variable. Progress in our understanding on soil biology and on the ecology and evolution of beneficial microorganisms should increase the environmental benefits of sustainable management practices to achieve better yields and to maintain soil fertility.

8.2 Plant-Growth-Promoting Bacteria

In order to enhance soil fertility and crop productivity, modern agriculture has become heavily dependent on the application of chemical inputs, including fertilizers and agrochemicals (Kiely et al. 2006). Nevertheless, the use of these products has often negatively affected the complex system of biogeochemical cycles (Steinsham et al. 2004). Moreover, it has been demonstrated that the continuous use of these chemical compounds generates environmental problems. Another fact that worsens the negative environmental effects is the low efficiency in the uptake of fertilizers by plants (Barlog and Grzebisz 2004). Therefore, the challenge is to promote more environmental-friendly agricultural practices. In this sense, a wide number of studies have been and still are focused in plant-growth-promoting bacteria as potential supplements of fertilizers, herbicides, fungicides, etc. (Adesemoye and Kloepper 2009). Microbial inoculants are promising components for integral solutions to agro-environmental problems because they promote plant growth by enhancing nutrient availability and uptake, or by holding up the health of the plants (Kloepper et al. 2004; Weller 2007; Adesemoye et al. 2008). The rhizosphere and the phylloplane of plants are habitats for a large number of microorganisms. In particular, the rhizosphere is a main spot of microbial interactions as exudates released by plant roots are an important carbon source for rhizospheric microorganisms. Many members of this microbial community have a neutral effect on the plants, while others have deleterious impact, causing diseases that result in plant death or a major reduction of its fitness and yielding. In contrast, some microorganisms can benefit the plant by promoting its growth, directly or indirectly. Plant-growth-promoting bacteria, term initially defined for rhizobacteria but that later also included bacteria isolated from different plant tissues (aerial and underground), encompasses microorganisms which, under certain conditions, promote plant growth. The use of microorganisms to benefit plant growth and to control plant pests continues being an area of rapid-expanding research. The most studied group of plant-promoting bacteria (PGPB) is the rhizobacteria (PGPR) that colonize the root surface and the portion of soil nearest to the root. Some PGPR can reach interior tissues and generate endophytic populations not only in the roots but also in leaves and stems (Compant et al. 2005). While these rhizobacteria utilize the nutrients that are released from the host for their growth, they also secrete metabolites into the rhizosphere. Over the years, several mechanisms involved in plant growth promotion have been documented. For PGPB to exert beneficial effects on plant growth, they need to be in an intimate relationship with the host plant. The degree of intimacy can vary depending on where and in what extent the beneficial bacteria colonize the host plant (Vessey 2003). The study of plant-associated bacteria is important not only for understanding their role in the interaction with plants but also for biotechnological application in areas as the plant growth promotion (Kuklinsky-Sobral et al. 2004).

It has been suggested that plants establish a communication with PGPB to specifically attract microorganisms for their own ecological and evolutionary benefit (Hardoim et al. 2008). Owing to the complexity of plant–microbe interactions in soil, it is extremely difficult to understand the detailed mechanisms involved in these putative selection processes. However, knowledge of well-studied models as the rhizobia–plant interaction, which indicates the existence of highly evolved species-specific communication systems, could be used as reference when studying novel plant–microbe interactions (Hardoim et al. 2008).

8.2.1 Bacterial Endophytes

Plants constitute an extremely diverse niche for microorganisms. Plant-associated bacteria isolated from rhizoplane and phylloplane are known as epiphytes (Andrews and Harris 2000; Kuklinsky-Sobral et al. 2004). Those isolated and detected inside the tissues by microscopic methods that maintain their ability to infect plants are called endophytes (Azevedo et al. 2000; Kuklinsky-Sobral et al. 2004; Rosenblueth and Martinez Romero 2004; Reinhold-Hurek and Hurek 1998a). There are also some bacterial populations with lifestyles fluctuating between endophytic and epiphytic colonization (Hallman et al. 1997; Kuklinsky-Sobral et al. 2004). By colonizing internal plant tissues, endophytic microorganisms become protected from external biotic and abiotic stresses. Within endophytes, beneficial and pathogenic bacteria can be found. Among the formers, rhizobia are the most studied group which is able to fix atmospheric nitrogen inside nodules (Hardoim et al. 2008). According to their life strategy, endophytic bacteria can be classified as “obligate” or “facultative”. Obligate are strictly dependent on the host plant for their growth and survival, and transmission to other plants occurs vertically or via vectors. On the other hand, the lifecycle of facultative endophytes can be characterized as biphasic, alternating between plants and the environment.

Colonization is an important trait of bacterial endophytes to be ecologically successful. For bacterial colonization, root cracks constitute the main portal of entry. Nevertheless, other ways of internal infection exist, such as wounds caused by microbial or nematode phytopathogens and stomata found in leaf tissue (Hardoim et al. 2008). The sequence of events for the endophytic colonization is similar, at least in the initial phases, to that of the root surface (Hallman et al. 1997). Environmental and genetic factors are presumed to have a role in enabling a specific bacterium to become endophytic (Reinhold-Hurek and Hurek 1998b). Hardoim et al. (2008) proposed the term competent endophytes to describe bacteria having the key machinery required to colonize and persist in the endosphere. On the other hand, opportunistic endophytes are considered as competent rhizosphere colonizers that become endophytic by coincidentally entering root tissue, but lack genes that are essential to their ecological success inside the plant. Additionally, a third group named passenger endophytes has been proposed. It includes bacteria that enter plants purely as a result of chance events since they lack the machinery to either colonize surface or internal tissues (Hardoim et al. 2008). Even when all categories colonize cortical root cells, only competent endophytes are able to systemically spread throughout the entire plant (Dong et al. 2003; Zakria et al. 2007). Capacity of bacteria to colonize plant tissues both externally and internally is a desirable characteristic for seeds inoculation because such bacteria have a greater chance of influencing host development (Kuklinsky-Sobral et al. 2004). It has been described that the roots are the preferential site for epiphytic and endophytic bacteria suggesting that endophytic bacteria may travel upward from the roots into the stem during plant development (Kuklinsky-Sobral et al. 2004).

Phylogenetic diversity of epiphytic and endophytic communities has been studied, and results have shown that both are related, suggesting that endophytes are an evolved state of a previous epiphytic or rhizosphere population (Hallman et al. 1997; Sturz et al. 2000). In many endophytic bacteria–plant interactions, where no specialized structures such as root nodules are formed, the way of infection of the PGPB and their location are not as clearly understood as in legume–rhizobia symbiosis.

8.2.2 Plant Growth Promotion Mechanisms

Plant-growth-promoting bacteria have been widely studied, and several mechanisms of growth promotion have been described. Considering the mode of action, PGPB have been divided into two groups: biocontrol bacteria that indirectly benefit the plant growth and PGPB that directly affect plant growth, seed emergence, or improve crop yields (Bashan and Holguin 1998; Glick et al. 1999). Indirect plant growth promotion occurs when bacteria are able to protect the plant against soilborne diseases by reducing harm caused by pathogens (Lugtenberg and Kamilova 2009). On the other hand, the direct growth promotion occurs when bacteria stimulate plant growth by providing limited nutrients in soil or by promoting enhancement of root biomass conferring a major volume to incorporate soil nutrients. While these rhizobacteria utilize the nutrients that are released from the host for their growth, they also secrete metabolites into the rhizosphere. Several of these metabolites can have a role as signaling compounds that are perceived by neighboring cells within the same microcolony, by other bacteria in the rhizosphere, or by root cells of the host plant (Van Loon 2007; Van Loon and Bakker 2003; Bais et al. 2004; Kiely et al. 2006). The best studied example of signal exchange is rhizobia–legume symbiosis that will be discussed later in this chapter, in which the plant releases flavonoids compounds that induce the bacterium to secrete Nod factors. This symbiosis is a prime example of an intimate relationship between a soil bacterium and its host plant and illustrates the concept behind the term "plant-growth-promoting bacteria" since, in nitrogen-poor environments, the rhizobia promotes legume plant growth by providing a limited nutrient (Van Loon 2007).

8.2.2.1 Indirect Plant Growth Promotion

There are four main groups of plant soilborne pathogens: fungi, nematodes, bacteria, and viruses. In most agricultural ecosystems, soilborne plant pathogens can be a major limitation to reach sustainable yields. The application of microbes to control diseases (biocontrol) is an environmental-friendly approach and is used as complement or alternative of agrochemicals. The term biocontrol is used not only to describe control diseases in living plants but also those occurring during the storage of fruits (also called postharvest control). Microbes able to control pathogen activity may produce secondary metabolites which are released on or near the plant surface. In contrast, the majority of agrochemicals do not reach the plant at all. Moreover, the molecules of biological origin are biodegradable compared with many agrochemicals that are designed to resist microbial degradation (Lugtenberg and Kamilova 2009). Although biocontrol studies usually focus on pathogenic microorganisms, some bacteria are also active against weeds (Flores-Fargas and O'Hara 2006) and insects (Péchy-Tarr et al. 2008; Siddiqui et al. 2005). The control of soilborne diseases by bacteria may result from competition for nutrients, antibiosis, predation, parasitism, and signal interference (Sturz et al. 2000; Van Loon 2007). Such activities are particularly important in the rhizosphere where pathogenic organisms are attracted to plant roots. However, rhizobacteria can reduce the activity of phytopathogens not only through microbial antagonism but also by activating the plant to better defend itself, a phenomenon termed "induced systemic resistance" (ISR) (Van Peer et al. 1991; Van Loon 2007).

Antibiosis

The antibiotics most commonly produced by different biocontrol bacteria include 2,4-diacetylphloroglucinol (DAPG), oomycin A, phenazine-1-carboxylic acid, ammonia, pyrrolnitrin, etc. (Whipps 2001; Verma et al. 2010). A lesser described antibiotic that showed antifungal activity produced by biocontrol bacteria is 2-hydroxymethyl-chroman-4-one, isolated from cultures of an endophytic *Burkholderia* strain

(Kang et al. 2004). An interesting point of this bacterial biocontrol trait is that production of antibiotics is highly influenced by the quantity and quality of available nutrients and is also subjected to quorum sensing (Haas and Keel 2003).

Predation and Parasitism

This biocontrol strategy has been mainly studied for the control of fungal pathogens. Parasitism of pathogenic fungi by biocontrol microorganisms occurs through the production of hydrolytic enzymes that degrade the fungal cell walls. Among them, chitinases and glucanases have been widely studied (Podile and Kishore 2006; Arora et al. 2007).

Competition for Nutrients and Niches

Biocontrol may result not only from direct interaction between the pathogen and the biological control agent but also from their competition for nutrients point of view. Then, competition between bacteria and pathogens for nutrients and niches in the rhizosphere constitutes a biocontrol mechanism. For instance, iron uptake is essential, and under starvation, several microorganisms secrete siderophores to mobilize this metal (Höfte et al. 1993). These molecules are low-molecular-weight compounds of high affinity to iron secreted by microorganisms under iron-limiting conditions (Höfte et al. 1993) that allow its incorporation from the environment. By producing siderophores, PGPB may compete with the pathogen for this nutrient (Duijff et al. 1999; Lugtenberg and Kamilova 2009) or induce systemic resistance in the plant (Leeman et al. 1996).

Induced Systemic Resistance

By this process, treatment with PGPB elicits plant defense as indicated by reduction in the severity or incidence of diseases caused by pathogens that are spatially separated from the inducing agent (Kloepper et al. 2004). This is the consequence of the plant response to compounds released by the PGPB (volatile and no volatile) and implicates a sequence of defense reactions. Many bacterial compounds induce ISR, such as LPS, flagellin, salicylic acid, and siderophores. More recently, cyclic lipopeptides, the antifungal factor Phl, the signal molecule acyl homoserine lactone (AHL), and organic volatile compounds have also been implicated (Lugtenberg and Kamilova 2009). A wide spectrum of ISR activities have been identified to be induced by rhizosphere bacteria. Among them, the activation of defense mechanisms that are also induced by pathogenic microorganisms is activated. Such mechanisms can include production of antimicrobial phytoalexins, synthesis of pathogenesis-related proteins (PRs) (Hammond-Kosack and Jones 1996), enhanced capacity to express these defense responses upon challenge inoculation with a pathogen, phenomenon called "priming" (Conrath et al. 2006; Van Loon 2007). Other ISR responses can involve signal translation, protection against oxidative stress, and generation of structural defenses, such as wall thickening, callose deposition, and accumulation of phenolic compounds (Reymond and Farmer 1998). Plant molecules such as jasmonic acid, ethylene, and salicylic acid play a major role in this defense mechanism. Since its discovery, rhizobacteria-mediated ISR has been documented in at least 15 plant species. Once ISR is induced, plants may remain protected for a considerable part of its lifetime, indicating that this state is rather stable (Van Loon and Bakker 2006; Van Loon et al. 1998; Van Loon 2007).

Other biocontrol mechanisms are interference with activity, survival, germination, and sporulation of pathogen (Lugtenberg and Kamilova 2009). Another less studied indirect growth-promoting activity exerted by PGPB, but not less important, is the promotion or synergism of other beneficial interactions such as legume–rhizobia or plant–fungi symbioses (Vessey 2003).

8.2.2.2 Direct Plant Growth Promotion

Phytohormone production and enhancing plant nutrition are the two main mechanisms by which PGPB directly contribute to plant growth. Enhance of plant nutrition is mainly through increase of the root growth, mineral uptake, and biological nitrogen fixation (BNF).

Phytohormone-Like Molecule Production

Several studies have demonstrated production of compounds chemically and functionally similar to phytohormones. Even when production of these compounds by PGPB has been demonstrated, this growth promotion effect cannot be unequivocally attributed to them (Glick 1995; Vessey 2003; Patten and Glick 2002; Podile and Kishore 2006; Verma et al. 2010). Phytohormone-like molecules found to be produced by PGPB are auxins, cytokinins, gibberellins, abscisic acid, and ethylene (Vessey 2003; Verma et al. 2010). They stimulate density and length of root hairs causing an increase in root surface area and therefore improving the plant potential for water and mineral nutrients uptake from a larger volume of soil (Volkmar and Bremer 1998; Podile and Kishore 2006). Among these growth regulators, auxins are the most studied. These compounds affect plant growth by inducing cell enlargement and division, root development, apical dominance, increase growth rate, photo, and geotropism (Frankerberger and Arshad 1995; Verma et al. 2010). Although cytokinins are produced by several genera of PGPB, few studies have demonstrated their beneficial effects. Similarly, but investigated in a lesser extent, is the case of gibberellins (Gaudin et al. 1994; Gutierrez-Manero et al. 2001; Podile and Kishore 2006). Ethylene is usually considered an inhibitor of plant growth, but at low levels, it can actually promote plant growth in several plant species (Van Loon 2007; Pierik et al. 2006). At moderate levels, it inhibits both root and shoots elongation, and at high levels, it enhances senescence and organ abscission (Abeles et al. 1992; Van Loon 2007). So, the interest is focused in the modulation of this plant growth modulator more than in its production by PGPB. The direct precursor of ethylene in the plant biosynthetic pathway, 1-aminocyclopropane-1-carboxylic acid (ACC), is exuded from plant roots together with other amino acids. PGPB that express the enzyme ACC deaminase, which cleaves ACC into ammonia and α-ketobutyrate, utilize these products as nitrogen and carbon sources, respectively. Under such conditions, re-uptake of ACC and its level in the roots is reduced. As a consequence, ethylene production by the roots is lowered, relieving inhibition of root growth (Glick 2005; Van Loon 2007). A second bacterial mechanism proposed to modulate plant ethylene levels is by inhibiting the enzymes of the ethylene biosynthesis pathway, ACC synthase and/or β-cystathionase (Sugawara et al. 2006; Hardoim et al. 2008). In both mechanisms, the bacteria are more efficient at modulating ethylene levels when they are closer to the plant cells in which ethylene biosynthesis takes place. Bacterial ACC deaminase is not currently known to be excreted from the bacterial cytoplasm (Glick et al. 2007; Hardoim et al. 2008). Hence, the decrease of plant ethylene levels relies on the ability of ACC deaminase expressing bacteria to take up ACC before it is oxidized by the plant's ACC oxidase (Glick et al. 1998; Hardoim et al. 2008). In this context, bacterial endophytes with high locally induced ACC deaminase activity might be excellent plant growth promoters because they ameliorate plant stress by efficiently blocking ethylene production (Cheng et al. 2007; Hardoim et al. 2008).

Volatile Compounds and Other Phytostimulators

Some rhizobacteria, belonging to phylogenetically unrelated genera such as *Bacillus* and *Enterobacter,* promote plant growth by releasing volatile compounds (Ryu et al. 2003; Lugtenberg and Kamilova 2009). Other bacterial cell components or secreted compounds have been proposed to be plant growth stimulators. Within these molecules, the protein pyrroloquinoline quinone (PQQ) has been described as a plant growth promoter in tomato and cucumber plants probably related with its antioxidant activity in plants (Choi et al. 2008). Nevertheless, its role in plant promotion has to be further elucidated since it also has antifungal activity and is able to induce systemic resistance (Lugtenberg and Kamilova 2009).

Increase of Nutrient Availability

Main mineral nutrients required for plant growth are nitrogen, phosphorus, and iron. Numerous PGPB able to increase their availability have been studied, and mechanisms involved in these effects have been determined. Among them, and since nitrogen is the first important nutrient required for plant growth, BNF is the most studied, hence discussed further in this chapter.

Phosphate solubilization and mineralization: Even in phosphorus-rich soils, most of this element is in insoluble form, and only a small proportion (~0.1%) is available to plants (Stevenson and Cole 1999). Additionally, a large percentage of the phosphate fertilizers applied to soils precipitate into insoluble forms thus increasing the phosphorus requirement of the crop (Podile and Kishore 2006). The solubilization of insoluble phosphates in the rhizosphere is one of the most common modes of action of PGPB that enhance nutrient availability to plants (Rodriguez et al. 2006). Phosphate-mineralizing and phosphate-solubilizing bacteria (PMB/PSB) secrete phosphatases and organic acids to convert insoluble phosphates (organic and inorganic) into soluble monobasic and dibasic ions (Rodriguez et al. 2006).

Increased uptake of iron to plants by siderophore-producing bacteria: Given the importance of iron for plants, the ability to produce siderophores is a desirable PGPB trait. Microbial siderophores may stimulate plant growth directly by increasing the availability of iron in the soil surrounding the roots (Kloepper et al. 1980; Verma et al. 2010). Plants, including legumes, demonstrated their ability to use microbial siderophores as a sole source of iron (Jurkevitch et al. 1986; Verma et al. 2010).

Biological nitrogen fixation: The ability to fix atmospheric nitrogen is present in various bacterial species that are either free-living or endophytically associated with plants roots (Dobbelaere et al. 2003). BNF is the most and long time studied plant-growth-promoting effect of soil microorganisms in legumes (Cholaky et al. 1983; Sen and Weaver 1984; Vargas and Ramirez 1989; van Rossum et al. 1993; Castro et al. 1999; Taurian et al. 2002), and mechanisms involved in this symbiotic interaction will be described in the Sect. 8.3. Besides nitrogen-fixing rhizobia, several authors observed that other associated beneficial bacteria exert over this group of plants multiple plant-promoting activities such as phosphate-solubilizing activity, IAA production, and biocontrol properties (siderophore production, antibiosis, etc.) (Pal et al. 2000; Deshwal et al. 2003; Dey et al. 2004; Kishore et al. 2005; Taurian et al. 2008, 2010; Ibañez et al. 2009; Tonelli et al. 2010).

To be efficient in plant growth promotion, the PGPB should remain active under a large range of conditions, such as fluctuating pH, temperature, and concentration of different ions. These requirements are not easy to be fulfilled, which explains why several commercial inoculant products are not successful. In addition, to express beneficial traits, inoculated strains should also be able to compete successfully with other organisms for nutrients from the root and for niches on the root as well as to escape in sufficient numbers from predators (Jousset et al. 2006; Lugtenberg and Kamilova 2009). The increase of our understanding about the mechanisms of plant growth promotion and on the selection procedures of beneficial bacteria will improve the development of PGPB-based inoculants (Lugtenberg and Kamilova 2009).

8.3 The Rhizobia–Legumes Symbiotic Association

Most of the nutrients that plants require for growth are readily available, but a few, as the macronutrient nitrogen, is often limited in soils. Even when molecular nitrogen is the major component of the Earth's atmosphere, it cannot be used directly by biological systems until it is combined with the element hydrogen. This process of reduction of molecular nitrogen is commonly referred to as "nitrogen fixation" and may be accomplished biologically. Biological systems which are able of fixing nitrogen (BNF) are classified as non-symbiotic or symbiotic, depending on the requirement of one or more than one organism, respectively, involved in the process (Burris and Roberts 1993).

Diversity in the metabolic types of free-living microorganisms which are capable of BNF is very wide, including many genera of non-photosynthetic aerobic (*Azotobacter*, *Beijerinckia*) and anaerobic (*Clostridium*) bacteria or photosynthetic cyanobacteria such as *Nostoc* and *Anabaena*. However, the most important contribution to BNF comes from the nitrogen-fixing plant symbiotic association (Bishop and Premakumar 1992).

Nitrogen-fixing plant symbionts belonging to various genera of the order Rhizobiales (collectively called rhizobia) are able to invade legume roots in nitrogen-limiting environments, leading to the formation of a highly specialized organ, the nodule, where bacteria, through the induction of the nitrogenase complex, are able to convert atmospheric dinitrogen into ammonia, which is used by the plant as a nitrogen source.

Nodule formation is a complex process that requires an adequate signal exchange between the plant and the bacteria. Plants secrete flavonoids from the actively growing region of the root. Interaction of these plant signals with rhizobial Nod transcription factors activates the expression of nodulation genes in compatible rhizobial species. Nod gene products synthesize Nod factor, bacterial lipochitooligosaccharide signaling molecules. Plant perception of Nod factors potentiates immediate subcellular changes in the root epidermis and later changes in the root cortex. In the epidermis, Nod signal activates many of the early events involved in the bacterial infection process. The bacteria enter the plant via the root epidermis and induce the reprogramming of root cortical cell division and the formation of a nodule (D'Haeze and Holsters 2002).

In the best studied rhizobia–legume symbiosis, infection occurs through root hairs. The first observable event in this infection process is the curling of the root hair where bacteria become enclosed, the plant cell wall is degraded, the cell membrane is invaginated, and an intracellular structure named infection thread is formed. It is within this structure that bacteria enter the root hair cell and eventually ramify into the root cortex. Simultaneously, the root cortical cells are induced to divide to form the nodule primordium. When the infection thread reaches the cells of the primordium, the bacteria are released into cells via endocytosis, enclosed in vacuole-like structures (symbiosomes) in which they differentiate into bacteroids.

It is within these symbiosomes that the bacteria convert dinitrogen to ammonium (Oldroyd and Downie 2008).

Another mode of rhizobial infection in legumes occurs via natural wounds caused by the splitting of the epidermis and the emergence of young lateral or adventitious roots. It is known as crack entry and has been described in (sub) tropical legumes. In *Sesbania rostrata* (Dreyfus and Dommergues 1981) and *Neptunia* (Subba-Rao et al. 1995), the infection leads to the formation of intercellular infection pockets, which give rise to intracellular infection threads. However, in *Arachis hypogaea, Stylosanthes,* and *Aeschynomene,* structures resembling infection threads have never been observed, and the later penetration of bacteria to the periphery of the nodule primordia occurs intercellularly (Chandler 1978; Fabra et al. 2010).

An intriguing but still not fully understood property of the symbiosis is its host specificity, which is believed to be determined by the recognition of Nod factor structure. However, it has been recently reported that soybean host proteins related with pathogenesis (R proteins) are involved in host specificity. The involvement of R proteins in the control of genotype-specific infection and nodulation reveals a common recognition mechanism implicated in symbiotic and pathogenic plant–bacteria interactions and suggests that establishment of a root nodule symbiosis requires the evasion of plant immune responses triggered by rhizobial elicitors (Yang et al. 2010).

8.3.1 Perception of Nod Factors and Trigger of a Signaling Cascade

In legumes like *Pisum sativum*, *Medicago truncatula*, and *Lotus japonicus* where the rhizobial infection process starts in epidermal root hair cells (Brewin 2004), more than 40 host genes or loci essential for microbial endosymbiosis have been identified so far (Kouchi et al. 2010). NFR1 and NFR5 have been identified as putative Nod factor receptors from *L. japonicus* (Madsen et al. 2003; Radutoiu et al. 2003) and from *Glycine max* (Indrasumunar et al. 2009), as LYK3 and NFP from *M. truncatula* (Limpens et al. 2003; Arrighi et al. 2006), and SYM37 and SYM10 from *P. sativum* (Zhukov et al. 2008). All of them are termed LysM receptor-like kinases (LysM-RLKs) since they have a common structure composed of a single-pass transmembrane domain anchoring to an extracellular lysin motif (LysM) receptor domain and an intracellular kinase domain. At present, however, no structural study has been made on the interactions of LysM domains with specific Nod factor structures.

Another RLK involved in Nod factor signaling, located on the plasma membrane and on the infection thread membrane, has been reported (Limpens et al. 2005). It has leucine-rich repeat (LRR) and serine–threonine kinase domains and is encoded by *M. sativa NORK/PsSYM19/LjSYMRK/MtDMI2/GmNORK* (Endre et al. 2002; Stracke et al. 2002; Mitra et al. 2004; Capoen et al. 2005; Indrasumunar 2007). Activation of the LysM-RLKs seems to be a prerequisite for the activation of this LRR-RLK, and based on downstream responses, the LysM-RLKs may have a specific role in the Nod factor signaling cascade, whereas the LRR-RLK may function more in initiating bacterial infection events (Limpens et al. 2005). In fact, it is predicted that LysM-RLK functions in both Nod factor perception and downstream signal transduction since it is required for the earliest detectable root hair responses, such as Ca^{2+} fluxes, membrane depolarization, and oscillation in cytosolic Ca^{2+} concentrations, known as Ca^{2+} spiking (Endre et al. 2002; Stracke et al. 2002). This signal transduction cascade involves potassium ion channel proteins localized in the nuclear membrane encoded by *MtDMI1, LjCASTOR,* and *LjPOLLUX* (Anè et al. 2004; Imaizumi-Anraku et al. 2005; Riely et al. 2007); nucleoporins encoded by *LjNup133* and *LjNup85* (Kanamori et al. 2006; Saito et al. 2007); and a calcium–calmodulin-dependent protein kinase (CCaMK) encoded by *MtDMI3/PsSYM9* (Levy et al. 2004; Mitra et al. 2004). This later protein acts downstream of Ca^{2+} spiking, while the LRR-RLK, the ion channels, and the nucleoporins seem to act upstream of oscillation in cytosolic Ca^{2+} concentrations. Many transcription factors are activated downstream CCaMK, such as nodulation signaling pathway 1 (NSP1) (Smit et al. 2005), NSP2 (Kalo et al. 2005), Ets2 repressor factor (ERF) required for nodulation (ERN) (Middleton et al. 2007), and nodule inception (NIN) (Schauser et al. 1999; Borisov et al. 2003). It has been suggested that all of them work in combination to regulate the expression of early nodulins in the epidermis (Hirsch et al. 2009). Nodulins are proteins that are coded by plant genes and are necessary for the development of symbiosis in the legume root nodules. According to their time of expression, they can be divided into early and late nodulins.

Simultaneously with this signaling cascade, bacterial infection events are triggered by the activation of the LRR Nod factor receptor. In *M. truncatula,* it has been identified 3-hydroxy-3-methylglutaryl CoA reductase (MtHMGR) as a component of this signaling via, which may be involved in the biosynthesis of cytokinins and brassinosteroids (Kevei et al. 2007). After MtHMGR activation following Nod factor perception in the epidermis, rapid responses are detected in the inner root such as rearrangements in pericycle cells (Timmers et al. 1999), the expression of the nodulin ENOD40 in cortical cells (Asad et al. 1994), and nodule development. For these responses in the inner root after exposing the outer root to Nod factors, a signaling communication seems to be necessary. In root nodule symbiosis, several hormones are reported to be important. Among them, cytokinin has been shown genetically to be essential for nodule organogenesis. *LHK1* in *L. japonicus* and *CRE1 in M. truncatula* (Gonzalez-Rizzo et al. 2006; Tirichine et al. 2007) encode a cytokinin receptor kinase, which functions in the root cortex and is involved only in nodule organogenesis, but not in the infection thread formation. Different studies have shown that downregulation, or loss of function, of this cytokinin receptor results in a decrease in nodule numbers due to the inability of plants to form nodule primordia (Gonzalez-Rizzo et al. 2006; Murray et al. 2007), even when rhizobia infections still take place. This suggests that two temporally and spatially distinct morphogenetic programs are induced after Nod factor/rhizobia perception, one that is activated in epidermis and is related with the bacterial infection, and other initiated at the cortical cells level that is involved in the nodule organogenesis (Ferguson et al. 2010). Even when these processes occur in legumes that are infected through root hairs, in *Aeschynomeme sensitiva*, a legume infected by crack entry, it has been reported that rhizobial infection and nodule organogenesis processes are developed in absence of Nod factors (Giraud et al. 2007). In *A. hypogaea,* the synthesis of Nod factors has been studied (Taurian et al. 2008), and it is also known that they are required for cortical cells division (Ibañez and Fabra 2011) and that the Ca^{2+}/calmodulin-dependent protein kinase is expressed in roots and nodules (Sinharoy and DasGupta 2009).

In summary, on the basis of the resources established for the genome research in model legumes such as *L. japonicus* and *M. truncatula*, which are infected by infection threads formation, a number of host legume genes involved in Nod factors perception and subsequent symbiotic signal transduction have been identified in the past decade. However, this knowledge is still scarce in legumes infected by crack entry.

8.4 Diversity of Bacteria-Nodulating Legumes

Beijerinck in Holland isolated and cultivated by the first time a microorganism from inside nodules of legumes in 1888, which was named *Bacillus radicicola*. Frank (1889) firstly named bacteria isolated inside nodule as *Rhizobium leguminosarum*, and since this date, all bacteria able to nodulate legumes are called rhizobia. However, the taxonomy, and nomenclature of the root nodule bacteria, has been in constant review ever since.

The classification of the first rhizobial species was mainly based on their growth rates on a defined substrate (fast and slow growers) as well as on their legume host specificities (Baldwin and Fred 1929). Nowadays, DNA and protein sequences are widely used to infer phylogenies of rhizobia. However, it is widely accepted that genes easily transferred among species are not useful in taxonomy. In this sense, in 1970s decade, it was reported that symbiotic genes are harbored in plasmid (pSym) in fast and in some intermediate-growing species of rhizobia, whereas they are integrated in the chromosome of intermediate and slow-growing rhizobia, harbored in symbiotic islands (Sullivan et al. 2002; Crossman et al. 2008). Horizontal gene transfer (HGT) of both, pSym and symbiotic islands, has been well documented (Lozano et al. 2010; Ibañez et al. 2010; Sullivan and Ronson 1998). Then, care should be taken when using only symbiotic gene sequences for phylogenetic studies.

In the second edition of Bergey's Manual of Systematic Bacteriology published in 2005, after the analysis of 16S rRNA genes, rhizobia were included in several families within the new order Rhizobiales in the class alpha Proteobacteria (Kuykendall 2005). Taxonomy based on 16S rRNA gene sequence presupposes that genes are inherited in hierarchical manner and that each genome harbors a single copy of this gene or that multiple alleles within a single genome have identical sequences. However, exceptions to this hypothesis have now been described in various taxa (Dreyden and Kaplan 1990; Rainey et al. 1996; Condon et al. 1999; Amann et al. 2000), and therefore, discordance in 16S rRNA phylogeny may also result from HGT and recombination (Ochman et al. 2005).

Van Berkum and coworkers (2003) have reexamined the phylogenetic relationships among rhizobia by comparative analysis of 16S rRNA, 23S rRNA genes, and ITS region within the *rrn* operon sequences. Tree topologies generated with 16S rRNA gene sequences were significantly different to those corresponding to the 23S rRNA and ITS region sequences. For instance, based on 23S rRNA sequences, *Bradyrhizobium elkanii* and *B. japonicum* were placed in a single group, whereas when considering 16S rRNA sequences, they were separated into *Blastobacter denitrificans*, *Rhodopseudomonas palustris*, and *Afipia felis*.

With the current knowledge about the diversity of bacteria able to induce nodule formation on legumes, it became apparent that a common error in the rhizobial taxonomy was to consider the nodulation of legumes as an exclusive ability of rhizobia, and thus, the strains isolated from nodules that do not present the typical colonies on YMA plates were discarded. This situation dramatically changed when scientists started to use 16S rRNA gene sequencing to the identification of nodule isolates. Thus, in the past 9 years, several non-classical rhizobia but also capable of forming nodules and fixing nitrogen in legume roots have been documented and grouped within alpha and beta Proteobacteria, such as *Methylobacterium nodulans*, *Burkholderia* sp., *Blastobacter denitrificans*, *Devosia neptunia*, *Ochrobactrum lupini* and *O. cytisi*, *Phyllobacterium trifolii*, *Ralstonia taiwanensis* (renamed as *Cupriavidus taiwanensis*), *Burkholderia tuberum, B. phymatum*, *B. cepacia*, *B. mimosarum*, *B. nodosa*, and *B. sabiae* (Rivas et al. 2009).

Currently, rhizobial group is constituted by 76 species into 13 genera: *Rhizobium*, *Mesorhizobium*, *Ensifer* (formerly *Sinorhizobium*), *Bradyrhizobium*, *Azorhizobium*, *Methylobacterium*, *Burkholderia*, *Cupriavidus*, *Devosia*, *Herbaspirillum*, *Ochrobactrum*, *Phyllobacterium,* and *Shinella*. However, recent research has shown that there are many other rhizobial species in addition to these. In some cases, these new species have arisen through horizontal gene transfer of symbiotic genes (Weir 2010).

Considering the information generated in the last years, it became clear that the legume symbioses are still poorly understood and that further studies are required especially on symbionts from legumes growing in ecosystems that until now remained unexplored.

8.5 Non-symbiotic Nodule Endophytic Bacteria

Over the years, the term "root nodule bacteria" has been exclusively applied to rhizobia. However, nonsymbiotic endophytic bacteria from several genera have been isolated from legume nodules, and this will be discussed in this section.

8.5.1 Generalities

Traditionally, the term "endophytes" has been restricted to mutualistic or commensal microorganisms found exclusively in regular tissues of the host plant and excluding specific organs such as nodules and galls (Rai et al. 2007). However, in the last years, there was an increase in the number of articles dealing with bacterial endophytes obtained from nodules (specific legume organs). To avoid confusion in the following, the term "nodule endophyte" will be used to refer to nonsymbiotic bacteria that reside inside nodules of legumes but cannot induce nodule formation.

From an ecological perspective, nonsymbiotic colonization of nodules can be understood as a survival and persistence bacterial strategy. The ability to find a new ecological niche within legume nodules could allow bacteria to survive and persist in a challenging environment such as soil. In this sense, the ability of a PGPB to persist and reproduce within nodules is an advantageous and attractive trait, even when the growth promotion effect could not necessarily be performed inside the tissues of the plant. For instance, bacterial phosphate solubilization is a major direct plant-growth-promoting effect that is carried out in the rhizosphere. However, the release of phosphate solubilizing bacteria from senescent nodules ensures the presence of a stable population of this nonsymbiotic PGPB in soils. From other perspective, serious concerns have been raised since nodule can harbor bacteria reported as human pathogens. Bacteria phylogenetically related to *Klebsiella oxytoca*, *Enterobacter cloacae,* and *Pantoea sp.* were obtained from the interior of peanut nodules in Argentina (Ibañez et al. 2009; Taurian et al. 2010); *Salmonella, Erwinia, Klebsiella, Citrobacter, Pantoea, and Enterobacter* were obtained from trunk nodules of *Conzattia multiform* in Mexico (Wang et al. 2006); and *Enterobacter cloacae, Enterobacter kobei, Escherichia vulneris, Pantoea agglomerans,* and *Leclercia adecarboxilata* were isolated from nodules of wild legumes in Algeria (Benhizia et al. 2004). Furthermore, Muresu et al. (2010) indicated that the later collection of nodule endophytes possess virulence determinants such as cytotoxicity, vital stain exclusion and adhesion to epithelia, and displayed complex patterns of antibiotic resistance. Therefore, it becomes evident that the lifecycles of some endophytes are not limited to plant and soil environments and can include stages within animals and humans hosts (Muresu et al. 2010). The existence of this secondary niche for human pathogens is important from a clinical and epidemiological perspective and should be studied carefully.

The presence of bacterial isolates inside nodules belonging to genera not known to include any legume-nodulating member raised questions about their origin. One of the first hypotheses was that nodule endophytes were genuine symbionts that acquired symbiotic genes from conventional symbionts (alpha or beta Proteobacteria) through horizontal gene transfer. According to this presumption, nonsymbiotic nodule endophytes represent potential receptors of symbiotic genes and may be raw material of novel symbiotic bacteria. However, some studies led to the rejection of this hypothesis since the absence of *nod* genes has been demonstrated in peanut nodule endophytes (Ibañez et al. 2009) and spontaneous legumes from Tunisia (Zakhia et al. 2006). Alternatively, and taking into account that the legume *Aeschynomene sensitiva* and *A. indica* are nodulated without Nod factor signaling (Giraud et al. 2007), it could be proposed that nodule endophytes are novel symbiotic bacteria that use an unconventional molecular dialogue to induce nodule formation. Nonetheless, there are evidences against this hypothesis. First, nodule endophytes by themselves are not capable to induce nodulation in the original host legume or in a wide host range legume such as *Macroptilium atropurpureum* (Ibañez et al. 2009; Lei et al. 2008; Zakhia et al. 2006). Second, nodule endophytes are able to colonize nodules previously formed by the compatible rhizobial strain (Ibañez et al. 2009). Third, until now, nodulation without Nod factors is restricted to a few species. In *Arachis hypogaea* L. (peanut), a legume taxonomically related to *Aeschynomene* that is also invaded by crack entry, the requirement of Nod factor for nodule primordia formation has been reported (Ibañez and Fabra 2011). Finally, results from the culture-independent analysis of nodule occupants from native noninoculated legumes revealed that these structures are always co-occupied by a compatible rhizobial strain in viable but not culturable (VBNC) state (Muresu et al. 2008).

8.5.2 Genetic Diversity of Nodule Endophytic Bacteria

Genetic analyses indicated that nodule endophytes exhibit great diversity and represent different bacterial lineages (Table 8.1).

Considering the definition of the term “nodule endophyte,” rhizobia that reside inside nodules of a legume but cannot induce their formation can also be considered within this group.

The traditional strategy used to investigate nodule-associated bacteria involves their isolation and cultivation from internal tissues of surface-sterilized nodules. In the past, isolation procedures focused primarily on cultivable microorganisms. Therefore, culturability of bacteria was a main issue. However, increasing interest in nonculturable endophytic microorganisms has recently led to the application of molecular methods for their identification (Hallmann et al. 2006). The application of a culture-independent approach led to a change in the analysis of bacterial diversity in several ecosystems and could result in a revolution of the concept of nodule endophytes in particular.

Table 8.1 Bacterial taxa described as nodule endophytes

Bacterial taxa	Host legume	Type of nodule	Reference
Agrobacterium	Diverse legumes, including *Phaseolus, Crotalaria, Mimosa*, *Onobrachis,* etc.	Determinate and indeterminate	de Lajudie et al. (1999), Gao et al. (2001), Liu et al. (2005), Mhamdi et al. (2002)
Actinobacteria	Pea	Indeterminate	Tokala et al. (2002)
Bacillus	Soybean *Cajanus cajan*	Determinate	Bai et al. (2002), Rajendran et al. (2008)
Pantoea agglomerans, Enterobacter kobei, Enterobacter cloacae, Leclercia adecarboxylata, Escherichia vulneris, Pseudomonas sp.	*Hedysarum carnosum, Hedysarum spinosissimum* subsp. *capitatum, Hedysarum pallidum*	Indeterminate	Benhizia et al. (2004)
Salmonella, Erwinia, Klebsiella, Citrobacter, Pantoea and Enterobacter	*Conzattia multiflora*	Trunk nodules	Wang et al. (2006)
Phyllobacterium, Sphingomonas, Rhodopseudomonas, Pseudomonas, Microbacterium, Mycobacterium, Bacillus, Paenibacillus	Spontaneous legumes	Determinate and indeterminate	Zakhia et al. (2006)
Agrobacterium and Enterobacteriaceae	Herbaceous legumes		Kan et al. (2007)
Agrobacterium, Phyllobacterium, Ensifer, Shinella, R. tropici, R. leguminosarum	*Vicia*	Indeterminate	Lei et al. (2008)
Pantoea, Serratia, Acinetobacter, Bacillus, Agrobacterium, and *Burkholderia*	Soybean	Determinate	Li et al. (2008)
Enterobacter, Klebsiella and *Pseudomonas*	*Arachis hypogaea* L.	Determinate	Ibañez et al. (2009)
Bacillus megaterium, Brevibacillus chosinensis, Microbacterium trichothecenolyticum	*Medicago sativa*	Indeterminate	Stajković et al. (2009)
Arthrobacter, Bacillus, Dyella, Microbacterium, Staphylococcus	*Lespedeza* sp.	Determinate	Palaniappan et al. (2010)

(continued)

Table 8.1 (continued)

Bacterial taxa	Host legume	Type of nodule	Reference
Micromonospora	*Arachis, Cicer, Glycine, Medicago, Lupinus, Pisum, Trifolium, Lens, Ononis, Ornithopus, Vicia, Mucuna*	Determinate and indeterminate	Cerda Castillo (2008), Trujillo et al. (2010)
Enterobacter, Pseudomonas, Achromobacter, Stenotrophomonas and *Sphingobacter*	*Clitoria ternatea* L.	Determinate	Aeron and Maheshwari (2011)
Acidovorax	*Cajanus cajan* L.	Determinate	Arya and Maheshwari (2009)

8.5.3 Beneficial Effects of Nodule Endophytes

The relationship between endophytes and the host plant could be neutral or beneficial through several mechanisms. Among these, nodule endophytes were mainly analyzed for phosphate solubilization, *nifH* presence and production of organic acids, siderophore, and IAA (Cerda Castillo 2008; Ibañez et al. 2009; Li et al. 2008; Rajendran et al. 2008; Trujillo et al. 2010; Zakhia et al. 2006). The results obtained are variable but indicate that many strains possess PGP activities. However, the expression of these activities inside nodules and their effects on the host legume are still unclear. However, promising effects were observed after co-inoculation of some legumes with genuine symbionts and nodule endophytes. In peanut, co-inoculation of specific symbiont (*Bradyrhizobium* sp.) and nodule endophytes of the genera *Enterobacter* led to a significantly increase in the number of nodules produced. Since it was determined that these *Enterobacter* strains produce IAA (a phytohormone that promotes the formation of lateral roots) and considering that rhizobia invade this legume at the sites of lateral root emergence, it can be speculated that the plants inoculated with these bacteria might have more sites for rhizobial infection (Ibañez et al. 2009). Another interesting cooperative effect between nodule endophytes and rhizobia has been reported (Liu et al. 2010). Co-inoculation with a mixture of *Agrobacterium* sp. II CCBAU21244 and *Sinorhizobium meliloti* induced the formation of nodules in *Wisteria sinensis* and two other woody legumes, which do not establish symbiosis with *S. meliloti* alone. Beneficial effects of co-inoculation with nodule endophytes were also observed in *Medicago* growing under sterile conditions (Stajković et al. 2009), *Cajanus* (Pandey and Maheshwari 2007; Rajendran et al. 2008) and *Lupinus,* and *Phaseolus* (Cerda Castillo 2008). Nevertheless, the mechanisms involved are still unknown. Beyond these results, experiments demonstrate that nodule endophytes constitute a population of bacteria with interesting plant-growth-promoting properties.

Considering that nodule endophytic bacteria constitute a population of microorganisms that directly or indirectly interact with rhizobial symbionts, it is possible to speculate that they could affect the development of an effective nitrogen-fixing symbiosis. However, the fact that bacteria other than rhizobia are present inside nodules has been minimized, and it represents an overlooked phenomena. Further studies are required in order to assess the effects of nonsymbiotic endophytic nodule bacteria.

8.5.4 Perspectives

Regarding nodule endophytic bacteria, there are more questions than certainties. Knowledge of the biological diversity of interactions between legumes and bacteria is still very limited. Extending the study to a greater number of legumes will conduct to a description of new nodule endophytes, possibly with novel PGP traits. In addition, the application of nonculturable approaches will significantly modify the current knowledge of nodule endophytic bacterial diversity.

Another interesting question is whether nodule endophytes only establish association with specific host plants, as occurs in rhizobia–legume symbiosis. Available information is consistent with a scenario in which plant growth promotion by native endophytic bacteria is highly species specific, regardless of whether or not they express general PGP traits (Long et al. 2008). Particular endophytes could often have important, if not essential, roles for plant growth and development. Therefore, it seems likely that plants could select for specific groups of plant-beneficial endophytes. However, the molecular dialogue regulating host specificity is still unknown.

Nodule nonsymbiotic endophytic colonization constitutes a poorly studied phenomenon, and there is still a lot to learn from bacterial ecology and population dynamics. In addition, further study is required to understand the interaction between legume-symbiotic bacteria-nodule endophyte and how it affects plant growth, nodulation, and nitrogen metabolism.

8.6 Conclusions

Information currently available clearly indicates that bacteria other than rhizobia are colonizing inside legume nodules. Many studies have shown that the coexistence of rhizobial and nonrhizobial bacteria in these organs can increase growth of different legumes. It seems to be evident that the ability of endophytic bacteria to reproduce within nodules is an advantageous strategy in their lifecycles. However, little is known about this particular host–endophyte interaction, and many questions need to be answered. In this sense, the requirement of a molecular dialogue between legume and nodule endophytes as the established with rhizobia is unknown. Whatever the mechanisms involved, nodule legume constitute a still unexplored ecological niche for colonization by endophytes other than rhizobia.

Acknowledgment The authors are indebted to UNRC, CONICET, ANPCYT, Ministerio de Ciencia y Tecnología de Córdoba (Argentina) that are currently supporting our research or did so in the past.

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