The Mechanism of Symbiotic Nitrogen Fixation

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Chapter 4
The Mechanism of Symbiotic Nitrogen Fixation

Barney A. Geddes and Ivan J. Oresnik

Abstract Nitrogen is a building block of life. Molecular nitrogen is the relatively inert atmospheric form of this element, and it must be fixed into more biologically accessible forms in order to be used for organic processes. In total, approximately 380 teragrams of nitrogen per year are fixed by atmospheric, biological, and industrial nitrogen fixation processes. Whereas the Haber–Bosch process currently accounts for the majority of the reduced nitrogen that is used agriculturally with the world's increasing dependence on agriculture to feed its population, the use of reduced nitrogen derived from energy provided by fossil fuels in not likely to be sustainable. Biological nitrogen fixation is mediated by diazotrophic microorganisms that are capable of fixing atmospheric nitrogen using the enzyme nitrogenase. Much of this is carried out as a symbiotic association between plants and some diazotrophic bacteria. The study of symbiotic nitrogen fixation is an area of research that spans both microbiology and plant biology. Since this is an area that has had a great deal of renewed interest, this chapter reviews what is currently understood about the process of symbiotic nitrogen fixation at the molecular and physiological level from both the plant and bacterial perspective.

4.1 Nitrogen Fixation

Nitrogen is required for the biosynthesis of the basic building blocks of life. Although nitrogen exists in abundance in the earth's atmosphere, its atmospheric form dinitrogen (N_2) is relatively inert. In order to be used for organic processes, nitrogen must be fixed into more biologically accessible forms. There are three common forms of nitrogen fixation. These include atmospheric, biological, and industrial nitrogen fixation. In total, approximately 380 teragrams (1×10^9 kg) of nitrogen per year (Tg N/year) are fixed by these processes (Galloway et al. 2008).

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Most atmospheric nitrogen fixation occurs as a result of lightning. Energy from lightning discharge can drive reactions that form nitrogen compounds from atmospheric N_2 (Noxon 1976). Nitrogen dioxide (NO_2) is the most commonly measured product of these reactions and is deposited by the rainfall associated with thunderstorms (Noxon 1976). Atmospheric nitrogen fixation is estimated to contribute approximately 5 Tg N/year into global nitrogen cycles (Galloway et al. 2008).

Biological nitrogen fixation (BNF) is mediated by diazotrophic microorganisms that are capable of fixing atmospheric nitrogen using the enzyme nitrogenase. Nitrogenase catalyzes the reduction of N_2 to ammonia (NH_3) in the energetically expensive reaction: $N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$. Diazotrophs exist as free-living, associative, or symbiotic microorganisms. Of these, symbiotic diazotrophs are the greatest contributors to BNF, and in turn BNF is the greatest contributor of fixed nitrogen to the nitrogen cycle and was estimated to contribute 230 Tg N/year (Galloway et al. 2008).

Prior to industrial nitrogen fixation, the amount of N that entered the terrestrial N-cycle was limited by atmospheric and biological nitrogen fixation. The discovery of the Haber–Bosch process in the early 1900s and its subsequent industrialization has profoundly increased the quantity of nitrogen that is transformed from atmospheric N_2 to NH_3 (Galloway et al. 2008). The Haber–Bosch process is an energy-intensive process that combines $3H_2$ and N_2 to yield $2NH_3$. It is the result of two reactions: the formation of H_2 and CO_2 from methane (CH_4) and steam (H_2O) using a nickel catalyst and the conversion of N_2 and $3H_2$ to NH_3 using high pressure, heat, and an iron catalyst (Ertl 1991). In 2005, it was estimated that inorganic nitrogen was contributing 121 Tg N/year into global nitrogen cycles (Galloway et al. 2008).

4.2 Agricultural Fertilization

Nitrogen is one of the primary limiting nutrients for plant growth in agriculture. Indeed, the productivity of many ecosystems is controlled by nitrogen availability (Vitousek 2002). For this reason, legume crops have been used in agriculture for thousands of years in crop rotations for their ability to integrate residual nitrogen into agricultural systems. As early as 1838, it was documented that legumes could restore nitrogen to the soil and must be capable of creating it directly. More than 50 years later, it was shown that the active participation of living microorganisms was necessary for the creation of nitrogen by legumes (Smil 2000). These microorganisms are symbiotic diazotrophs, referred to as rhizobia, and have been extensively studied for their ability to fix nitrogen in a symbiotic relationship with legume crops. In modern agriculture, commercially prepared rhizobial inoculants are introduced into the soil during the planting of legume crops to enhance symbiotic nitrogen fixation (SNF). Estimates suggest that currently, 40 Tg N/year is injected into agricultural systems by SNF (Herridge et al. 2008).

The primary source of nitrogen fertilization in modern agriculture is inorganic nitrogen fertilizer synthesized by the Haber–Bosch process. The development of the Haber–Bosch process sparked a "green revolution" that allowed a coincident rapid expansion in agricultural capacity for food production as well as global population following World War I (Galloway et al. 2004). Unfortunately, due to the economic costs associated with inorganic fertilizer use, some countries have been effectively left out of the green revolution and remain limited in their agricultural capacity. Synthetic fertilizers currently account for 121 Tg N/year injected into agricultural systems (Galloway et al. 2008), a rate that has doubled the flux of the terrestrial nitrogen cycle (Röckstrom et al. 2009). This has resulted in a strain on the environment in the form of waterway pollution that has caused eutrophication of water systems and greenhouse gas production (Röckstrom et al. 2009) due to the release of nitrous oxide which occurs when people add synthetic fertilizers to the soil. However, the world's population has now reached a point where its caloric requirement is greater than that which agriculture can provide without utilizing inorganic nitrogen fertilizer.

Because of the environmental and economic costs associated with the use of inorganic nitrogen as a fertilizer, there is growing interest in enhancing the use of SNF in agriculture to help overcome these problems, since SNF is essentially free and environmentally benign.

4.3 Rhizobia

Rhizobia are Gram-negative α- and β-proteobacteria that have acquired the ability to fix atmospheric nitrogen in symbiosis with legumes (Masson-Boivin et al. 2009). Rhizobia are able to elicit the formation of new organs called root nodules on the roots of host plants. Within a microoxic environment provided by the root nodules, rhizobia intracellularly fix atmospheric nitrogen to ammonia that is assimilated by the plant. Two widely distributed sets of genes encode these functions in most rhizobia: the *nod* (nodulation) genes and the *nif* (nitrogen fixation) genes (Masson-Boivin et al. 2009). Rhizobia tend to contain large complex genomes that often include extra replicons called megaplasmids (Jumas-Bilak et al. 1998). Variations to these themes exist. For example, *Frankia* are a group of Gram-positive organisms that have acquired the ability to fix nitrogen in a symbiotic association with actinorhizal plants (Benson and Silvester 1993). Some species of *Bradyrhizobium* that do not contain *nod* genes have been reported to be capable of eliciting nodule formation on host plants (Giraud et al. 2007).

Because of their relevance to agriculture, most research has focused on rhizobia found in the order *Rhizobiales* that nodulate crop and forage legumes. These include *Rhizobium leguminosarum* biovar *viciae* (pea), *trifolii* (clover) and *phaseoli* (kidney bean), *Rhizobium etli* (common bean), *Bradyrhizobium japonicum* (soybean), *Mesorhizobium loti* (*Lotus*), *Sinorhizobium fredii* (soybean), and

Sinorhizobium meliloti (alfalfa). The understanding of symbiosis between rhizobia and legumes as defined in these organisms has become a paradigm of plant–microbe interaction.

4.4 *Sinorhizobium meliloti* Rm1021

Sinorhizobium meliloti belongs to the *Rhizobiaceae* family, of the order *Rhizobiales* in the α-proteobacteria. Along with other well-studied rhizobia, *S. meliloti* is also closely related to the plant and animal pathogens *Agrobacterium* and *Brucella*. *Sinorhizobium meliloti* engages in nitrogen-fixing symbiosis with the agriculturally important forage plant *Medicago sativa* (alfalfa), a model organism for studying legume biology *Medicago truncatula* (barrel medic), as well as other legumes of the genera *Medicago*, *Melilotus* (sweet clover), and *Trigonella*. The parent of the *S. meliloti* strain with which most academic research has been carried out was originally isolated in New South Wales, Australia, in 1937 and designated strain SU47. *Sinorhizobium meliloti* strain Rm1021 is a streptomycin-resistant derivative of *S. meliloti* SU47.

The genome of *S. meliloti* Rm1021 was sequenced relatively early, in 2001, and is composed of a chromosome (3,654,135 bp) and two large megaplasmids called pSymA (1,354,226 bp) and pSymB (1,683,333 bp) (Barnett et al. 2001; Capela et al. 2001; Finan et al. 2001; Galibert et al. 2001). Most essential genes in *S. meliloti* are contained on the chromosome. These include genes for universal biosynthetic pathways, transcription, translation, cell division, and DNA repair (Capela et al. 2001). The essential genes $tRNA^{arg}$, encoding the arginine tRNA, and *engA* which encodes for the GTP-binding protein EngA are encoded on pSymB as well as genes for asparagine and thiamine biosynthesis (Finan et al. 1986; diCenzo et al. 2013). The megaplasmid pSymB also contains many gene clusters involved in the biosynthesis and export of surface polysaccharides and small molecule transport and catabolism. Based on these contents, pSymB has been suggested to play a role in the ability of *S. meliloti* to thrive during saprophytic growth in the diverse environment of the soil (Finan et al. 1986, 2001). The megaplasmid pSymA has been cured from *S. meliloti* and therefore does not encode essential genes (Oresnik et al. 2000). Based on encoding a large portion of the genes involved in symbiosis, including the genes for nodulation and nitrogen fixation, pSymA is thought of as the symbiotic plasmid of *S. meliloti* (Barnett et al. 2001).

The genome of *M. truncatula* has also been sequenced recently (Young et al. 2011). *M. truncatula* has been used as a model legume because it contains a small diploid genome, has a rapid generation time and prolific seed production, and is amenable to genetic transformation. The *S. meliloti–M. truncatula* model (along with the *M. loti–Lotus japonicus*) is emerging as the leading model system for studying rhizobium–legume symbiosis (Capela et al. 2001). The current understanding of rhizobium–legume symbiosis with a focus on the *S. meliloti–Medicago* model is reviewed and will be highlighted in this chapter.

4.5 Rhizobium–Legume Symbiosis

The rhizobium–legume symbiosis is an elaborate process that culminates in the development of root nodules, wherein rhizobia intracellularly fix atmospheric nitrogen that is assimilated by the host plant. To achieve this end result, rhizobia must first infect the legumes through root hair cells on the root surface and traverse intracellular tubules called infection threads (IT) before reaching root inner cortical cells that form the nodule primordium. The rhizobia are then endocytosed and undergo a dramatic developmental differentiation into nitrogen-fixing forms that are referred to as bacteroids (Jones et al. 2007; Oldroyd et al. 2011). This process has been studied extensively in rhizobia. The current understanding of the invasion of *Medicago* species by *S. meliloti* is reviewed in this section.

4.6 Signal Exchange in the Rhizosphere

Invasion of the legume by rhizobia begins with a signal exchange that occurs between the legume and saprophytic, free-living rhizobia in the soil environment surrounding the plant root, referred to as the rhizosphere (Fig. 4.1a). The signal exchange begins with the secretion of inducing molecules such as flavonones and betains by legumes in their root exudate (Gage 2004). The type of inducing molecule is variable among legume species and unique to different rhizobia (Downie 1994). Flavonoids are recognized by rhizobial NodD transcriptional activator proteins, which are members of the LysR family of transcriptional regulators and induce the transcription of downstream *nod* genes. In *S. meliloti*, the flavonoid luteolin, secreted by *M. sativa*, was shown to be responsible for the induction of *nod* genes (Peters et al. 1986). *Sinorhizobium meliloti* NodD1 specifically binds luteolin and induces transcription of a subset of genes that contain a specific nucleic acid motif in their promoter called a nod-box, including the *nod* genes (Fisher and Long 1993). The *nod* genes encode approximately 25 proteins that are involved in the synthesis and export of a lipochitooligosaccharide signaling molecule called Nod factor (NF) (Gage 2004). *Sinorhizobium meliloti* possesses two other NodD variants that are capable of activating *nod* gene expression, NodD2 and NodD3. The NodD2 variant responds to a yet unidentified plant compound (Honma et al. 1990). The NodD3 product is capable of activating *nod* gene expression in the absence of inducing molecules and is integrated into a complex regulatory circuit with the positive regulator SyrM (Kondorosi et al. 1991; Swanson et al. 1993).

Nod factor consists of a 4–5 residue chitin backbone of β-1,4-linked *N*-acetyl-D-glucosamine (GlcNAc) subunits with an N-linked acyl tail attached to the nonreducing end (Gage 2004). Further decorations of the NF backbone as well as the modifications to the lipid tail are variable among rhizobial species, and even individual species are capable of synthesizing an array of different NFs (Perret

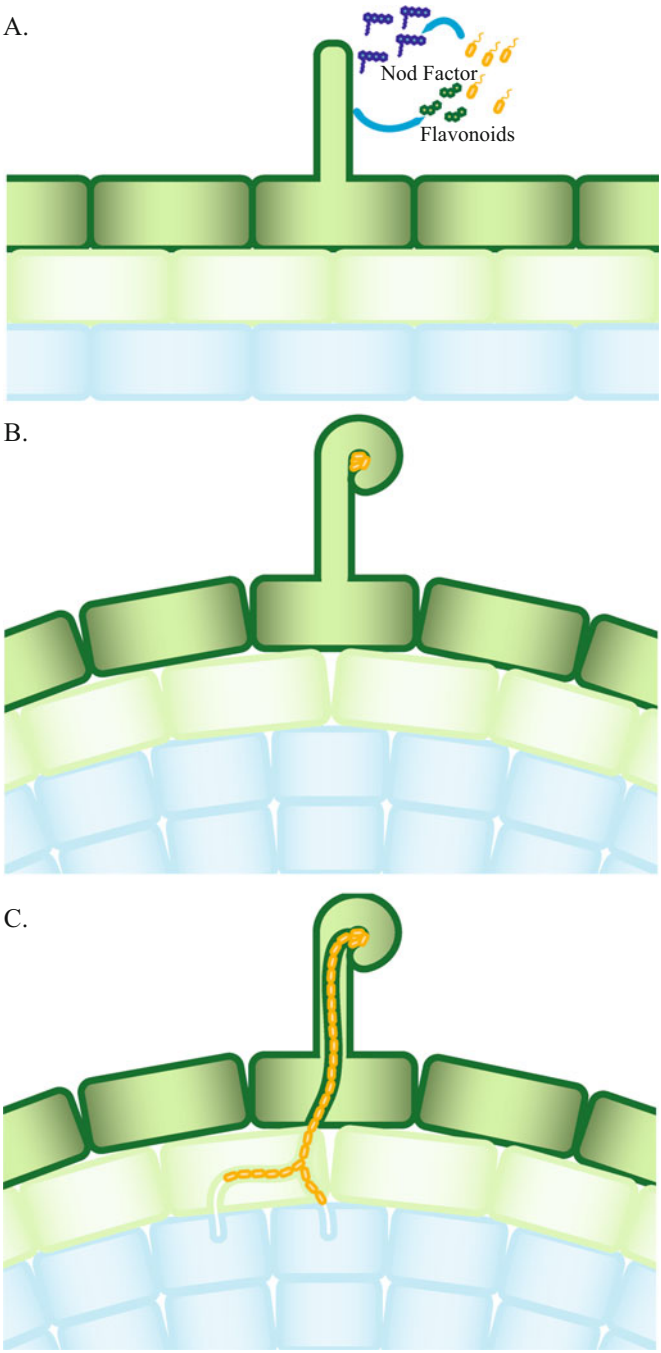

Fig. 4.1 (**a**) *Rhizobium*–legume symbiosis begins with signal exchange in the rhizosphere. Legumes secrete phenolic compounds (flavonoids) that are recognized by rhizobial NodD proteins and trigger the production of the lipochitooligosaccharide, Nod factor, by rhizobia. Nod factor is recognized by legume receptors on the surface of the root hair (*dark green*). (**b**) Nod factor

et al. 2000). The NF synthesized by *S. meliloti* has been purified and shown to be an acetylated, sulfated β-1,4-linked tetrasaccharide of *N*-acetyl-D-glucosamine with a C16 acyl tail (Lerouge et al. 1990). Enzymes for the synthesis of the chitin backbone and attachment of the lipid tail are encoded by the *nodABC* operon in *S. meliloti*, divergently transcribed from *nodD1* (John et al. 1993; Egelhoff et al. 1985; Atkinson et al. 1994; Geremia et al. 1994; Spaink et al. 1994). Enzymes required for acetylation and sulfation of NF are encoded by *nodL* and *nodHPQ*, respectively (Roche et al. 1991; Ardourel et al. 1995). The *nod* genes *nodE* and *nodF* are involved in synthesis of the acyl chain (Demont et al. 1993).

Following its synthesis, NF is perceived by receptor-like kinases on the plasma membrane of the host plant that contain extracellular chitooligosaccharide binding LysM domains (Madsen et al. 2003; Limpens et al. 2005; Radutoiu et al. 2007). In *M. truncatula*, the corresponding receptor-like kinases are MtNFP and MtLYK3 (Amor et al. 2003; Limpens et al. 2003). These LysM receptor-like kinases are involved in NF specificity just as transfer of the LysM receptor-like kinases from *L. japonicus* (*NFRI* and *NFR5*) to *M. truncatula* allowed it to interact with the *L. japonicus* symbiont *M. loti* (Radutoiu et al. 2007). Recognition of NF by LysM receptor-like kinases triggers a calcium (Ca^{+2}) spiking response in the nucleus. It has been shown that *S. meliloti* NF alone was sufficient to trigger this response in *M. sativa* (Ehrhardt et al. 1996).

Nuclear Ca^{2+} spiking is central to a signal transduction pathway that integrates NF perception into physiological responses from the plant. Several components of this pathway in *M. truncatula* were identified using a large mutagenesis screen for mutants that were defective in nodule formation. Mutants of *DMI1*, *DMI2*, and *DMI3* (does not make infections) and *NSP1* were shown to be defective in nodule formation in response to NF (Catoira et al. 2000). It was later shown that the nuclear Ca^{2+} spiking response depends on the receptor-like kinase DMI2 and a ligand gated cation channel on the nuclear envelope DMI1 (Endre et al. 2002; Ané et al. 2004). This Ca^{2+} spiking in the nucleus is decoded by the Ca^{2+}/calmodulin-dependent protein kinase DMI3 (CCaMK) (Lévy et al. 2004). A constitutively active form of CCaMK was able to induce nodule formation in *M. truncatula* in the absence of bacterial elicitation (Gleason et al. 2006). It is known that CCaMK associates with and phosphorylates IPD3 (CYCLOPS) (Horváth et al. 2011) and results in the activation of two GRAS domain transcription factors NSP1 and NSP2. Activated NSP1 and NSP2 form a complex that activates the transcription of early nodulation

Fig. 4.1 (continued) recognition triggers a signaling cascade that leads to morphological changes in the plant, including root hair curling and inner cortical cell division (*blue*). Root hair curling results in the trapping of rhizobia that have colonized the root hair surface in an apoplastic compartment referred to as a curled colonized root hair. Inner cortical cell division gives rise to the nodule primordium. (**c**) Invasion of the legume proceeds by the formation of an intracellular infection thread as a result of a reversal of polar cell growth in the root hair. The infection thread traverses the root hair and multiple cell layers and ramifies before reaching the destination inner cortical cells. There, rhizobia are endocytosed and differentiated into nitrogen-fixing bacteroids

genes (Hirsch et al. 2009), including the transcription factors NIN and ERN1 (Marsh et al. 2007; Middleton et al. 2007). This complex sequence of events affecting signal transduction results in dramatic physiological responses in the plant including root hair curling and the division of inner cortical cells that form the nodule primordium.

4.7 Root Hair Invasion

Asymmetric growth at the root hair tip in response to NF results in root hair curling that traps the rhizobia that are colonizing the root hair surface, forming an apoplastic compartment between two cell walls of the curled colonized root hair (CCRH) (Fig. 4.1b). Rhizobia trapped within the CCRH continue to divide forming colonies called infection foci.

Infection is initiated by localized cell wall degradation at the site of contact between bacteria in the infection foci and the plant cell wall. It is unclear whether the rhizobia or plant are the source of the enzymes responsible for cell wall degradation. Induction of polygalacturonase in *M. sativa* was observed during infection by *S. meliloti* (Muñoz et al. 1998). Bacterial cellulase mutants in *R. leguminosarum* bv. *trifolii* were deficient in the nodulation of clover (Robledo et al. 2008).

Infection proceeds by a reverse of polar growth in the root hair that results in an invagination of the plasma membrane at the site of cell wall degradation. A NF-induced Ca^{2+} influx at the root hair tip that is independent of nuclear calcium spiking has been observed and may be involved in the signaling that leads to the reversal of polar cell growth in *M. truncatula* that results in membrane invagination (Morieri et al. 2013). Root hair Ca^{2+} influx may involve unique NF receptors, as *nodL* mutants of *S. meliloti* that lack the NF acetyl group were impaired for root hair Ca^{2+} influx but not for nuclear Ca^{2+} spiking (Morieri et al. 2013).

The invaginating plasma membrane is lined with new cell wall as it grows inward, forming an IT (Fig. 4.1c). The IT is continually colonized by rhizobia as it traverses the root hair cell. Intracellular IT progression is accompanied by dynamic cytoskeletal rearrangements in the root hair and migration of the nucleus to the growing IT tip (Oldroyd et al. 2011). Upon reaching the base of the root hair cell, localized cell wall degradation allows the IT to continue into the next cell layer. The IT continues to grow and ramify through multiple cell layers until it reaches the inner cortical cells of the root. *Sinorhizobium meliloti* labeled with either green fluorescent protein (GFP) or red fluorescent protein (RFP) were used to visualize the early events of symbiosis between *S. meliloti* and *M. sativa* (Gage et al. 1996; Gage 2002). They demonstrated that active growth of bacteria occurred only at the growing tip of the IT. This resulted in clonal expansion at the tip of the infection thread when inoculated with a mixed culture of *S. meliloti* expressing GFP and RFP such that only a single type of bacterium would enter the nodule (Gage et al. 1996; Gage 2002).

Successful penetration of the IT requires the continued synthesis of NF by *S. meliloti*. A *nodFL* mutant of *S. meliloti* that produces NF that lacks the acetyl group and has a modified acyl tail showed reduced and aberrant IT formation. Concomitantly, ITs that did form were aborted before reaching the base of the root hair (Limpens et al. 2003). A double mutant of *nodFE* that produces NF with a modified acyl tail showed aberrant IT formation in symbiosis with *M. truncatula* with partially depleted *LYK3* (Limpens et al. 2003). Consistent with the role of NF in signaling during IT penetration, plant mutants of various components in the NF-induced Ca^{2+} spiking signaling cascade are defective in the formation of either infection foci (*NIN*, *NSP1*, *NSP2*) or infection threads (*CYCLOPS*, *ERN1*) (Oldroyd et al. 2011).

More recently, it has been shown that nonlegumes are also capable of responding to NF (Liang et al. 2013). In particular, *Arabidopsis*, which is not infected by any rhizobia, is able to respond to NF and suppress its innate immune response (Liang et al. 2013). This suggests that plants other than legumes may have the theoretical capacity to interact with rhizobia (Liang et al. 2013).

Another intriguing class of molecules that must be synthesized by *S. meliloti* in order to penetrate the IT are exopolysaccharides (EPS). *Sinorhizobium meliloti* is capable of producing two main exopolysaccharides: succinoglycan (EPSI) and galactoglucan (EPSII). However, under normal conditions, *S. meliloti* strain Rm1021 only produces succinoglycan. Succinoglycan is a polymer of octosaccharide repeating units composed of one galactose residue and seven glucose residues, with pyruvyl, succinyl, and acetyl modifications. The steps involved in its succinoglycan biosynthesis have been extensively characterized (Reuber and Walker 1993). The repeating unit of galactoglucan is a disaccharide of glucose and galactose, decorated with pyruvyl and acetyl modifications. The genes for succinoglycan and galactoglucan biosynthesis are encoded on pSymB by the *exo* and *exp* loci, respectively (Finan et al. 2001).

A mutant of *exoY*, which encodes the glycosyltransferase responsible for the first step in succinoglycan biosynthesis, is unable to initiate infection thread formation in *M.* sativa (Dickstein et al. 1988; Cheng and Walker 1998). A mutant of *exoH*, which encodes the enzyme responsible for the addition of the succinyl modification, forms aberrant ITs that abort before reaching the base of the root hair (Cheng and Walker 1998). It was hypothesized that low molecular weight (LMW) forms of succinoglycan might be specifically important for invasion because EPSI synthesized in the *exoH* mutant is refractory to cleavage by the endoglycanases ExoK and ExsH into LMW forms (York and Walker 1998). Strains of *S. meliloti* with a functional SinI/ExpR quorum sensing system are capable of synthesis of EPSII under normal conditions. In this background, synthesis of EPSII was able to complement an *exoY* mutant for invasion of *M. sativa* but not *M. truncatula* (Glazebrook and Walker 1989).

It is currently unclear how EPS participates in invasion by *S. meliloti*. A microarray experiment showed increased expression of many genes associated with the plant immune response in *M. truncatula* when inoculated with an *exoY* mutant as compared to wild-type *S. meliloti* (Jones et al. 2008). One hypothesis is

that EPS plays a role in dampening the plant immune response, possibly as a signal that is recognized by the plant. Exopolysaccharides also have been shown to play a role in protection from a variety of stresses, including oxidative stress (Lehman and Long 2013). An oxidative burst is associated with the early stages of *M. sativa* infection by *S. meliloti* (Santos et al. 2001). More recently, it has been shown that the CCRH is an acidic compartment and that EPSI expression is correlated with medium acidification (Geddes et al. 2014). Therefore, EPS may also play a role in tolerating different stresses experienced during infection.

A third class of macromolecule that has been implicated in invasion is the cyclic β-glucans. These are cyclized chains of 17–25 glucose residues, connected by β-1,2 linkages (Spaink 2000). Enzymes that mediate cyclic β-glucan export and synthesis are chromosomally encoded by *ndvA* and *ndvB* (nodule development) (Galibert et al. 2001). Rhizobial *ndv* mutants are impaired in their ability to nodulate *M. sativa* and form small empty pseudonodules (Dylan et al. 1986; Geremia et al. 1987; Dickstein et al. 1988). They have also been reported to be defective in attachment to plant cells, and ITs formed by these mutants abort at early stage (Dylan et al. 1990b). Cyclic β-glucans function in adaption to hypoosmotic conditions in many bacteria, including *S. meliloti*, suggesting that they may play a role in tolerating osmotic stress experienced during invasion (Dylan et al. 1990a; Miller and Wood 1996). However, pseudorevertants in *S. meliloti ndv* mutant backgrounds that regained the ability to effectively nodulate alfalfa remained sensitive to hypoosmotic growth conditions (Dylan et al. 1990b). Therefore, the role of cyclic β-glucans in *S. meliloti* symbiosis remains unclear.

4.8 Bacteroid Differentiation

Rhizobia dividing at the tip of the IT enter inner cortical cells in the nodule primordium by endocytosis. This results in the acquisition of a plant-derived membrane surrounding the rhizobial cell called the symbiosome membrane (SM). The resulting organelle-like structure consisting of a rhizobium cell surrounded by a SM is referred to as a symbiosome. *Medicago truncatula* DMI2 is localized to the infection thread and symbiotic membranes, and knockdown studies have shown that *M. truncatula* with reduced levels of DMI2 failed to release bacteria into symbiosomes (Limpens et al. 2005). The space between the SM and the *S. meliloti* cell wall is referred to as the peribacteroid space. It has been shown that the peribacteroid space in *M. truncatula* is an acidic compartment (Pierre et al. 2013).

Rhizobial lipopolysaccharide (LPS) may be important for interaction with the SM or tolerating conditions of the peribacteroid space. Evidence for this comes from the fact that *S. meliloti* mutants for *bacA* and *lpsB* that express an altered LPS on the cell surface fail to survive the symbiosome and senesce following endocytosis (Glazebrook et al. 1993; Campbell et al. 2002). The protein BacA is important for long-term survival of *S. meliloti* within acidic plant compartments and similarly

important for survival of *Brucella abortus* within acidic compartments of animal cells. Lipopolysaccharide consists of an O-antigen repeating unit, attached to a polysaccharide core that is anchored to the membrane by lipid A. Mutants of *bacA* lack the important ability to modify lipid A with a very long-chain fatty acid (VLCFA) moiety (Ferguson et al. 2004), and symbiosis is unsuccessful without the VLCFA. The lipopolysaccharide core biosynthesis mannosyltransferase gene LpsB is involved with biosynthesis of the LPS core. Mutants of *lpsB* produce a modified LPS core with altered polysaccharide composition (Campbell et al. 2002).

Rhizobia grow and divide along with the SM until the cytoplasm of the infected host cell is packed with thousands of symbiosomes (Udvardi and Poole 2013). Within the symbiosome, *S. meliloti* undergo a dramatic differentiation into their nitrogen-fixing bacteroid form. The *S. meliloti*, along with their host plant cells, undergo several rounds of endoreduplication, yielding chromosome counts of 24 in *S. meliloti* bacteroids. Differentiated bacteroids appear swollen and pleomorphic in shape and show membrane permeability. In *S. meliloti*, this differentiation is said to be terminal; terminally differentiated bacteroids cannot be cultured from nodules (Oldroyd et al. 2011). Terminal differentiation is not universal among legumes, and only takes place in legumes of the inverted repeat-lacking clade (IRLC), which includes *Medicago*, *Pisum sativum* (pea), and *Vicia faba* (faba bean). In other legumes such as *L. japonicas*, bacteroids do not undergo endoreduplication and remain culturable from nodules (Oldroyd et al. 2011). Terminal differentiation is thought to be a plant-dependent trait. This was demonstrated in an experiment where *R. leguminosarum* bv. *viciae* (which undergoes terminal differentiation in symbiosis with its host *P. sativum*) was modified so that it could colonize *L. japonicus* nodules. During nodulation of *L. japonicus*, *R. leguminosarum* bv. *viciae* bacteroids did not undergo endoreduplication and maintained their normal size and shape. Conversely, in another experiment, *R. leguminosarum* bv. *phaseoli* (not terminally differentiated in symbiosis with its host *Phaseolus vulgaris*) was modified to infect *P. sativum* and showed large, branched-bacteroids and endoreduplication. Therefore, some legumes are able to take control of the bacterial cell cycle and impose terminal differentiation upon their rhizobial symbionts (Mergaert et al. 2006).

Terminal differentiation is imposed on rhizobia by small plant peptides that co-localize with bacteroids in the nodule, referred to as nodule cysteine-rich antimicrobial peptides (NCRs). The NCRs are short 60–90 amino acid peptides with conserved cysteine-rich motifs. Treatment of free-living rhizobia with NCR peptides induced rhizobial phenotypes consistent with those observed during terminal differentiation, and these expressed such traits as membrane permeabilization, endoreduplication, and loss of viability (Van de Velde et al. 2010). The treatment with sublethal doses of NCR however had profound effects on cell cycle and general physiology of *S. meliloti* (Farkas et al. 2014).

Microarray analysis showed that over 300 NCR peptides that normally are absent from *Lotus japonicus* are induced in the nodules of *M. truncatula* (Mergaert et al. 2003). Of these, signal peptidase, designated DNF1, is required to target NCRs to the SM, where they induce the changes responsible for terminal

differentiation (Wang et al. 2010). It is important to note that BacA is a membrane component of the ABC (ATP-binding cassette) transporter family, encoded on pSymB. Interestingly, *bacA* mutants show resistance to antimicrobial peptides (Marlow et al. 2009). Although BacA is involved in the transport of VLCFA, VLCFA biosynthetic mutants show unaltered resistance to antimicrobial peptides and form a successful symbiosis, suggesting an alternate role for BacA (Oldroyd et al. 2011). It has been hypothesized that BacA plays a role in the action of NCRs on *S. meliloti* (Marlow et al. 2009). In the broad host range *Sinorhizobium fredii* NGR234, BacA is not required for nodule formation on legume hosts outside of the IRLC. This evidence supports the role of BacA in the terminal differentiation of IRLC legumes, perhaps through interactions with NCRs (Ardissone et al. 2011).

4.9 Nodule Development and Physiology

Nodule organogenesis is mediated by complex hormone signaling, involving the activation of cytokinin and the suppression of polar auxin transport in the root cortex (Oldroyd et al. 2011). Mature nodules are composed of a central infected tissue that contains a mixture of infected and uninfected cells, surrounding by uninfected tissues that connect to the root vascular system (Udvardi and Poole 2013). *Medicago* species form indeterminate nodules that are elliptical in shape and contain a persistent meristem. These indeterminate nodules are organized into several zones: the meristem at the growing tip, an invasion zone that contains undifferentiated rhizobia and is the site of IT penetration into the nodule, an interzone where rhizobia undergo differentiation, a nitrogen fixation zone that contains mature nitrogen-fixing bacteroids, and a senescence zone that contains degraded bacteroids and is absent of nitrogen fixation (Udvardi and Poole 2013).

4.10 Nitrogenase

Rhizobia are classed as obligate aerobes, yet nitrogenase, the enzyme responsible for the reduction of diatomic nitrogen gas to ammonia, is exquisitely sensitive to oxygen with the half life of each component in air being less than 10 min (Burgess 1984). Nitrogenase is a complex metalloenzyme that contains a number of centers composed of Fe, FeS, as well as Mo that are necessary to carry out the transfer of electrons onto diatomic nitrogen. It has been crystallized (Georgiadis et al. 1992; Chan et al. 1993), and although much is understood about the enzyme at the atomic level, it is still an area that is being actively researched and the precise mechanisms for electron transfer are still debated (Hu and Ribbe 2013; Einsle 2014).

The enzyme consists of what is known as the MoFe protein, which is a tetramer with an $\alpha_2\beta_2$ makeup, as well as the Fe protein which is a γ_2 dimer that is encoded by *nifH*. The α component of the MoFe protein is encoded by *nifD*, and the β

component encoded by *nifK*. The Fe protein contains two ATP-binding sites per monomer (four total), and its role is to receive electrons from central metabolism and transfer these electrons to the MoFe protein at the expense of ATP. The role of the MoFe protein is to bind the diatomic nitrogen gas and transfer electrons that allow the reduction of the triple bond in nitrogen.

Since nitrogenase contains many complex metallo-centers, its tertiary structure is dependent on a number of chaperones that are responsible for the correct assembly of each of the structural components. Collectively, many of the genes that encode these chaperones have been genetically characterized as *nif* genes. They have been most extensively studied in free-living diazotrophs such as *Azotobacter vinelandii* as well as *Klebsiella pneumoniae* (Dixon and Kahn 2004). The better-understood chaperones include NifU, NifS, NifB, NifE, NifN, and NifV which are necessary for the assembly of the FeS centers and their proper insertion into NifH (Hu and Ribbe 2013).

The overall generalized mechanism of nitrogen reduction can be summarized as the interplay of two cycles; the Fe protein cycle and the MoFe protein cycle. The Fe protein cycle consists of the Fe protein binding two molecules of ATP while gaining a single electron. The Fe protein then interacts with the MoFe protein resulting in the redox transfer of electrons from the Fe protein to the MoFe protein, which takes place at the expense of two ATP/electrons. The Fe protein is subsequently released and the cycle repeats itself (Fig. 4.2).

The single-electron addition to the MoFe protein repeats itself eight times in what has been described as the MoFe protein cycle. This cycle describes the eight single-electron additions and predicts the points where H_2 and NH_3 are released from the complex (Thornley and Lowe 1985).

4.11 Carbon Flow to the Nodule

The direct energy requirements to reduce nitrogen to ammonia are reductant and ATP. Both reductant and ATP are generated by the bacteria through the metabolism of carbon compounds that are provided by the plant and translocated to the bacteroid. Our current understanding of how carbon is supplied to the nitrogen-fixing symbiont has been shaped from several diverse and complementary approaches utilized in both plant and bacterial studies (Fig. 4.3).

Very early it was shown that there was a correlation between photosynthetic capacity and nitrogenase activity (Allison 1935). A number of years later, pulse-feeding experiments demonstrated that within 3 h of feeding $^{14}CO_2$ to the shoots of a nodulated soybean plant, the label was found in the nodules, predominantly as sucrose, fructose, glucose, and organic acids (Reibach and Streeter 1983). In a similar type of experiment, it was shown that within 4 h of the shoots of a soybean plant being exposed to steady state $^{13}CO_2$ feeding, the CO_2 evolved from the nodules had attained 90 % of the specific activity of the feed gas (Kouchi and

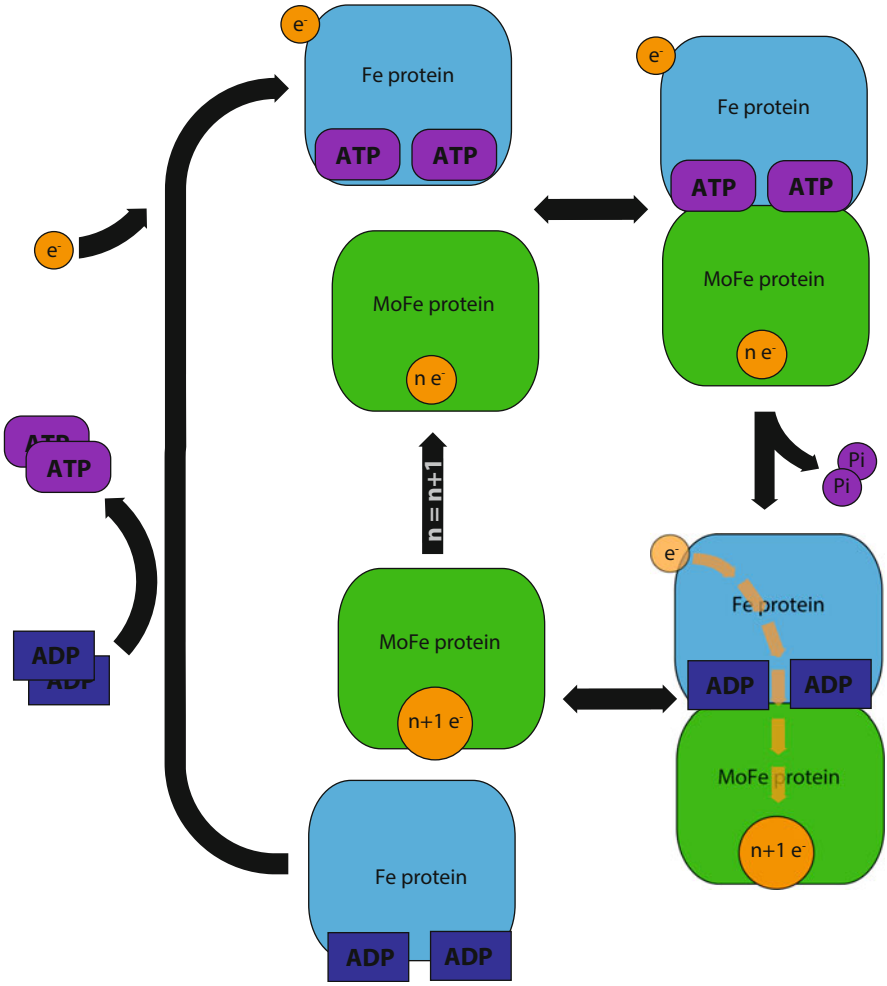

Fig. 4.2 Model of the Fe protein cycle of nitrogenase. Two subunits (one each of the Fe protein and the MoFe protein are shown). Two ATP bind to a reduced Fe protein, exchanging two ADP. The Fe protein then associates with the MoFe protein. Electron transfer from the Fe protein to the MoFe protein is dependent upon ATP hydrolysis to ADP plus the removed phosphate, designated P_i. Following electron transfer from the Fe protein to the MoFe protein, the proteins disassociate. Eight electrons need to be transferred to reduce nitrogen gas into $2NH_3$

Yoneyama 1984a, b, 1985, 1986). Taken together, these data showed that recent photosynthate is translocated to a nitrogen-fixing nodule.

Enzymatic evidence from isolated nodule tissue suggests that sucrose is broken down via glycolysis to phosphoenolpyruvate in the infected plant nodule cells (Vance and Heichel 1991). High levels of phosphoenolpyruvate carboxylase and cytosolic malate dehydrogenase in the infected plant cell cytosol suggest that the phosphoenolpyruvate is converted to oxaloacetate and subsequently to malate

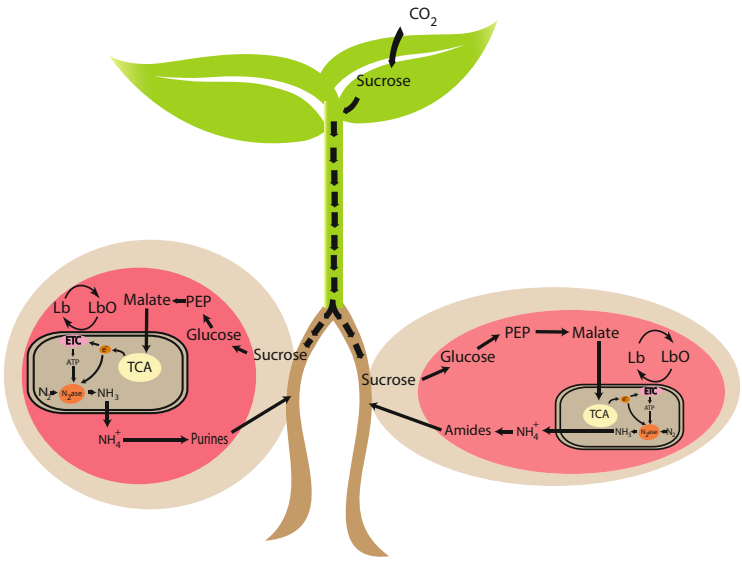

Fig. 4.3 Carbon flow to the nodule and export of nitrogen products to the plant. Nodules are *spherical* (determinate, shown on *left*) or *cylindrical* (indeterminate, shown of *right*) in shape. Indeterminate nodule shape occurs because they contain an apical meristem and continue to grow. Carbon dioxide fixed in the leaves is translocated to the nodules via the phloem as sucrose. Sucrose is broken down into glucose and subsequently to malate in the infected cells (shown in *pink*). Malate is translocated into the symbiosome and is used to generate both ATP and reductant that is needed for nitrogenase. Facilitated diffusion of oxygen to the symbiosome is carried out by leghemoglobin (Lb/LbO, deoxygenated/oxygenated) where the oxygen is used as a terminal electron acceptor. Reduced nitrogen (NH_3) is assimilated by the plant into either purine derivatives (determinate nodules) or amides (indeterminate nodules) and translocated from the nodules via the xylem

which is supplied to actively fixing bacteroids (Miller et al. 1998). Since the symbiosome is surrounded by a plant-derived membrane, the carbon needs to be transported across both the plant and symbiotic membranes to reach the bacterial cytoplasm. Evidence from experiments using gently isolated symbiosomes clearly shows that dicarboxylic acids are actively transported across these membranes (Udvardi et al. 1988, 1990). In a complementary fashion, it was found that bacterial mutants unable to transport dicarboxylic acids were capable of becoming symbiosomes but were unable to fix nitrogen.

4.12 Bacteroid Metabolism

Dicarboxylic acids are widely accepted as the primary carbon sources available to rhizobium bacteroids (Poole and Allaway 2000; Lodwig and Poole 2003; Yurgel and Kahn 2004). With few exceptions, sugar catabolism mutants of various

rhizobium species have been shown to form functional symbioses (Lodwig and Poole 2003). Even the metabolically compromised *S. meliloti* pyruvate carboxylase mutant (*pyc*), which cannot utilize any hexoses, forms a functional symbiosis (Dunn et al. 2001; Geddes and Oresnik 2012). Other pleiotropic mutants unable to grow with numerous hexoses and pentoses as substrate have been isolated which also were capable of forming normal nitrogen-fixing nodules. Thus, it is clear from the literature that the ability to use these classes of sugars is not critical for nodulation.

In contrast to sugar catabolism mutants, strains unable to utilize dicarboxylic acids are unable to form a functional symbiosis. The dicarboxylate transport system in *S. meliloti* consists of three genes: *dctA*, *dctB*, and *dctD*. Both DctB and DctD regulate the expression of *dctA* by sensing dicarboxylates (Yurgel and Kahn 2004). Of these, DctB acts as a dicarboxylate sensor, phosphorylating DctD, which in turn acts as a regulator responsible for activating transcription of *dctA* with the help of the sigma factor RpoN (Ronson et al. 1987; Jiang et al. 1989; Yarosh et al. 1989). The role of DctA is as a dicarboxylate permease which appears to be the sole transport system for malate, succinate, and fumarate in *S. meliloti*. Interestingly, *dct* mutants are generally able to form normal-looking nodules with bacteroids; however, they are unable to fix nitrogen (Finan et al. 1983, 1988; Watson et al. 1988; Yarosh et al. 1989). Although the ability to use dicarboxylates is required for symbiosis, this suggests that dicarboxylates are not necessary during infection and the early stages of nodule development.

To identify which pathways are important in metabolizing dicarboxylates in bacteroids, a strategy was utilized in which mutants were isolated that were able to grow on glucose but were unable to grow on dicarboxylates as a sole carbon source. In addition to dicarboxylate transport mutants, other mutants with lesions in gluconeogenesis were isolated (Finan et al. 1988). In a subsequent study, these mutants were assessed for their ability to fix nitrogen symbiotically (Finan et al. 1991). The results were difficult to interpret since the mutants showed a complex symbiotic phenotype, and it was difficult to reconcile whether the altered nodule development was a direct consequence of the mutation, or due to mutants that were generally compromised, and therefore unable to form an effective symbiosis. Of particular interest, however, was a mutant that lacked phosphoenolpyruvate (PEP) carboxykinase. This mutant did form normal nodules and fix nitrogen; however, the rate of nitrogen fixation was half of what was exhibited by the wild-type strain (Finan et al. 1991). This is a curious finding since PEP carboxykinase activity could not be detected in wild-type alfalfa nodules (Finan et al. 1991).

Since a strain carrying a mutation in the PEP carboxykinase gene could form an effective association, another pathway must exist in bacteroids to generate carbon skeletons larger than four carbons when dicarboxylates are used as a primary carbon source. It was hypothesized that malic enzyme would be important in this regard.

Both fast- and slow-growing genera of Rhizobium have an NAD^+ (Dme)- and an $NADP^+$ (Tme)-dependent malic enzyme (McKay et al. 1988; Copeland et al. 1989; Driscoll and Finan 1993). Using a complex genetic screen, a *S. meliloti* NAD^+ malic enzyme mutant was isolated and shown to be unable to fix nitrogen

symbiotically. The mutant showed normal nodule development to the point that the bacteroid should start fixing nitrogen, resembling the phenotype displayed by a strain carrying a dicarboxylic acid transport mutation (Driscoll and Finan 1993). When a strain carrying a mutation in the gene encoding $NADP^+$ malic enzyme was isolated, however, it was found not be affected in its ability to fix nitrogen symbiotically (Driscoll and Finan 1996, 1997).

Even though rhizobia have both NAD^+- and $NADP^+$-dependent enzymes that carry out the same biochemical activity, and can functionally replace each other in vivo, overexpression of the $NADP^+$ malic enzyme gene was not able to complement NAD^+ malic enzyme mutants for the ability to form a functional symbiosis (Mitsch et al. 2007). The requirement for NAD^+ activity in symbiosis is not currently understood, but it is interesting to note that the *E. coli* NAD^+-dependent malic enzyme activity (dme) was able to complement the symbiotic phenotype of *S. meliloti dme* mutants (Driscoll and Finan 1997).

Other *S. meliloti* mutations affecting the ability to grow on succinate as a sole carbon source are in genes that encode enzymes used in the TCA cycle. A succinate dehydrogenase mutant was isolated, and it was found that it formed nodules, but these nodules did not fix nitrogen, (termed Fix^-) (Gardiol et al. 1982). Other TCA cycle mutations in citrate synthase, isocitrate dehydrogenase, and malate dehydrogenase were also shown to be Fix^- (McDermott and Kahn 1992; Lodwig and Poole 2003; Dymov et al. 2004).

The ammonia produced by nitrogenase in the symbiosome has long been hypothesized to passively diffuse into the acidic peribacteroid space where it is trapped by protonation to NH_4^+ (ammonium) (Day et al. 2001). Ammonium is thought to be further transported across the SM by either ion or aquaporin-like channels, although the molecular identity of an NH_4^+ channel in the SM has yet to be identified (Udvardi and Poole 2013). Ammonium is primarily assimilated by the plant using asparagine and glutamine synthetases to convert ammonia into amino acids (Cordoba et al. 2003; Barsch et al. 2006). Alternately, in a number of nodules that exhibit determinate morphology, the ammonia is assimilated into purine derivatives in the plant fraction of the nodule and subsequently exported to the plant (Sprent 1980).

It has been shown that bacteroids found in some legumes are dependent on the plant for some of their amino acid requirements (Lodwig et al. 2003). Mutations in the broad-specificity amino acid transporters *aap* (amino acid permease) and *bra* (branched-chain amino acid permease) were found to cause *R. leguminosarum* to become Fix^- when both genes were disrupted. When either single gene was disrupted, plants inoculated with the mutants behaved similarly to wild-type inoculated plants. When root xylem amides were measured from plants grown in the presence of $^{15}N_2$ and inoculated with a strain carrying mutations in both *aap* and *bra*, the plants were found to have more labeled asparagine than plants inoculated with the wild type. Based on these data, a new model was proposed: bacteroids secrete both ammonium and an amino acid precursor to asparagine, probably aspartate. In this model, plants synthesize asparagine using ammonium and aspartate secreted by the bacteroids. The plants then supply the bacteroids with

glutamate, some of which is used in a transamination reaction with oxaloacetate to produce aspartate. The aspartate is then exported back to the plant and used as a precursor for asparagine synthesis. The authors proposed two predictions: that mutations in the aspartate transaminase enzyme *aatA* would be Fix^- and that amino acid export mutants would accumulate too much carbon due to the lack of carbon removal via transamination and amino acid export. These predictions appear to be correct. First, it was known that *aatA* mutants are Fix^- in *S. meliloti* (Rastogi and Watson 1991; Watson and Rastogi 1993), and this was subsequently confirmed for *R. leguminosarum* (Lodwig et al. 2003). Secondly, plants inoculated with strains carrying mutations in both *aap* and *bra* were found to contain higher levels of plant starch, and the bacteroids themselves contained granules of the storage polymer, polyhydroxybutyrate (PHB), which the wild type did not contain (Lodwig et al. 2003).

The Aap and Bra transporters were subsequently shown to exhibit broad specificities with respect to the amino acids that could be transported. More recently, it was found that if the solute specificities of these transporters were constrained, it was only the branched-chain amino acids that needed to be provided by the host to the bacteroids (Prell et al. 2009). This phenomenon has been termed symbiotic auxotrophy (Prell et al. 2010). The benefit of this updated model is that it helps reconcile how such a complicated mutualism between plants and bacteria could evolve.

4.13 Diffusion of Oxygen to the Bacteroid

Symbiotic nitrogen fixation has often been thought of as an oxygen paradox (Appleby 1984). The components necessary to enzymatically reduce nitrogen to ammonia are oxygen labile, yet the organisms are generally obligate aerobes and are dependent upon respiration using oxygen as a terminal electron acceptor to generate adequate ATP and a reductant to carry out the process. To be able to carry out symbiotic nitrogen fixation, the plant and the bacterial symbiont have a number of physiological, structural, as well as regulatory features that allow the process to take place.

Nodule structure generally consists of an outer layer of cells that surround a central zone of cells that in turn contain both infected (symbiosome containing) and uninfected cells. By using oxygen electrodes, it has been found that the oxygen concentration of nodules rapidly declined to the point that oxygen was no longer detectable as the probe progressed through the outer cortical layers (Tjepkema and Yocum 1974; Witty et al. 1987). Structural analysis of soybean root nodules has shown that a cell layer exists in the inner cortex with a low proportion of air spaces (Bergersen and Goodchild 1973; Dakora and Atkins 1990; Parsons and Day 1990). On the basis of mathematic models, it was suggested that the central zone of the nodule needs only be surrounded by a thin (45 μm) aqueous barrier of water to provide a sufficient diffusion barrier to oxygen (Sinclair and Goudriaan 1981;

Hunt et al. 1987a; Sheehy et al. 1987). These models all predicted that the oxygen concentration of the central zone should be about 100 nM. More recent mathematical modeling of nodule cells suggests that whereas the cortical barrier may be essential in providing course control, the central zone cell is responsible for the fine control of oxygen concentration, and this may be achieved by the geometry of intracellular spaces in the central zone of the nodule (Thumfort et al. 1994, 2000).

Experiments using flow-through gas exchange measurements were able to show that nodules had the ability to regulate the diffusion of gases (Hunt and Layzell 1993). These studies demonstrated that an undisturbed nodulated root system could respond to the sub- or supra-ambient oxygen concentration changes in a reversible manner that did not lead to the destruction of total nitrogenase activity (Hunt et al. 1987b). Moreover, that when disturbed using a number of physiological perturbations such as increases or decreases of ambient oxygen, nodules would respond by decreasing the nitrogenase activity and increasing nodule resistance to gas diffusion. In related work, this was also corroborated using measurements of the oxygen concentration within undisturbed nodules by measuring the oxygenation of leghemoglobin (see below) (Kuzma et al. 1993b). Taken together, this body of literature shows that under normal conditions, the inner central zone has an oxygen concentration of approximately 20 nM—four orders of magnitude lower than that found in equilibrated water at ambient conditions.

4.14 Aerobic Metabolism in a Microoxic Environment

Continued cellular respiration by bacteroids under low oxygen conditions is facilitated by both plant and bacterial factors. Legumes synthesize nodule-specific proteins called legume hemoglobins (leghemoglobins) that are critical to maintaining the microaerobic environment (Appleby 1984), whereas bacteroids also synthesize a number of enzymes, including a high-affinity terminal oxidase (*fixNOQP*) that is critical for microaerobic respiration. These enzymes as well as the genes encoding nitrogenase are under the control of a complex regulatory circuit that is induced by low oxygen concentrations (Jones et al. 2007).

The synthesis of leghemoglobin proteins results in a pink coloration of nodule tissue. Leghemoglobin consists of a heme moiety that binds oxygen, as well as a globin protein. Both components are synthesized by the host plant (Santana et al. 1998). The role of leghemoglobin is to bind free oxygen and to facilitate its diffusion to sites of oxygen utilization. More recently, RNA interference experiments with the gene encoding leghemoglobin were carried out in *L. japonicus* to directly test the need for leghemoglobin. The reduction of leghemoglobin resulted in higher levels of free oxygen and a complete absence of nitrogenase activity (Ott et al. 2009).

Whereas both the plant and the bacteria consume oxygen, the K_m (binding affinity) associated with the nodule mitochondria has been determined to be 100 nM (Rawsthorne and LaRue 1986), whereas the K_m for oxygen of the terminal

oxidase within the bacteroid is tenfold lower (Bergersen and Turner 1993). It has been suggested that this difference in the affinity for oxygen may lead to the plant fraction of the nodule being oxygen limited under ambient conditions (Oresnik and Layzell 1994).

Rhizobia within the bacteroid are dependent upon oxygen as a terminal electron acceptor. Physiological experiments using either isolated bacteroids or whole nodules have shown that the K_m of the terminal oxidase for oxygen is in the range of 5–10 nM (Kuzma et al. 1993a). This suggests that although the internal oxygen concentration within the nodules is low, it is sufficient to saturate the terminal oxidase and thus be able to carry out oxidative phosphorylation to provide ATP for nitrogenase as well as other bacterial activities. Direct measuring of adenylate pools in soybean nodule tissue that was rapidly frozen and fractioned using nonaqueous centrifugation techniques supports these hypotheses (Oresnik and Layzell 1994; Kuzma et al. 1999).

The high-affinity terminal oxidase has been shown to be encoded by the genes *fixNOQP* (Preisig et al. 1993). These are found in all *Rhizobium*, and in many cases with the faster-growing rhizobia (*Sinorhizobium* and *Rhizobium*), these genes are often duplicated (Renalier et al. 1987; Schlüter et al. 1997). The significance of the duplication, if any, is not known. It has been shown that both gene copies are functional. The expression of these genes is highly regulated by oxygen.

4.15 Genetic Regulation in Response to Low Oxygen in the Bacteroid

Genetic regulation in response to low oxygen concentrations in Rhizobia is carried out by regulatory cascade (Fisher 1994). The overall theme of the regulation is similar; however, the details and nuances of what comprises the regulatory cascade tend to differ with species. The regulatory pathways were primarily elucidated using genetics, followed by more in-depth characterization carried out at the biochemical level. Although a good deal is understood, this remains an active area of research where the details are still being elucidated. The two best-studied systems are those of *S. meliloti* and *B. japonicum* (Fig. 4.4).

In *S. meliloti*, O_2 is sensed by FixL (David et al. 1988), a heme-containing protein that can bind oxygen directly (Gilles-Gonzalez et al. 1991). The FixL protein is part of a two-component system that also includes the transcriptional activator FixJ (Lois et al. 1993). When FixJ is phosphorylated by FixL, FixJ then acts as a transcriptional activator for *fixK* as well as *nifA* (de Philip et al. 1990). The protein FixK is a transcription factor that directly binds promoters of genes necessary for microaerobic growth such as the high-affinity terminal oxidase encoded by *fixNOQP*. The FixK protein also functions as a negative regulator, repressing its own transcription as well as that of *fixT* (Foussard et al. 1997). While in turn, FixT modulates the activity of FixL, thus providing a feedback loop. The components of

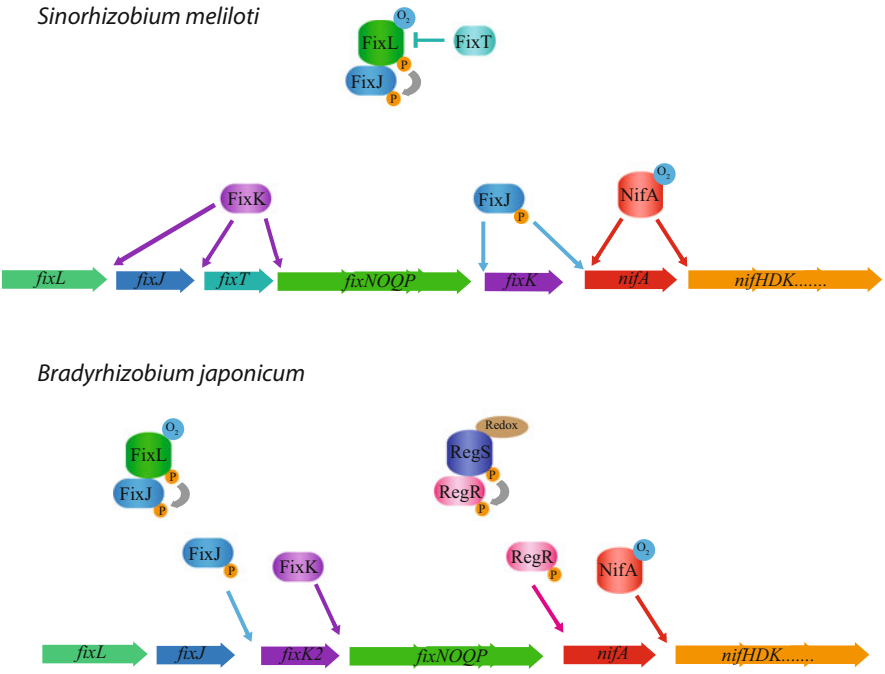

Fig. 4.4 Gene regulation in response to oxygen *Sinorhizobium meliloti* (*top*) or *Bradyrhizobium japonicum* (*bottom*). In both cases, oxygen is directly sensed by FixL, which then leads to the phosphorylation of FixJ. In *S. meliloti*, FixJ directly affects the expression of *fixK* as well as *nifA*. The NifA protein regulates many of the components necessary for the assembly and maintenance of nitrogenase, whereas FixK affects components necessary for feedback regulation of oxygen sensing as well as the genes encoding the high-affinity terminal oxidase (*fixNOQP*). In *B. japonicum* (*bottom*), regulation also occurs by the RegS–RegR system which is believed to be redox responsive

nitrogenase as well as many of the other *nif* genes are directly regulated by NifA (Fisher 1994), and *nifA* expression is also activated by FixJ. In addition, NifA activity has been shown to be directly affected by oxygen (Dixon and Kahn 2004).

Bradyrhizobium japonicum also uses the two-component system FixL/FixJ to sense oxygen (Anthamatten and Hennecke 1991). Unlike *S. meliloti*, FixJ does not regulate *nifA* expression (Fisher 1994). However, FixJ has been shown to directly regulate $fixK_2$, which is one of two *fixK*-like genes found in *B. japonicum* (Anthamatten et al. 1992), and $FixK_2$ is also used to upregulate genes such as *fixNOQP* that are needed to carry out microaerobic respiration (Mesa et al. 2008). The regulation of *nifA* however is carried out by a second two-component redox-sensing system encoded by *regR* and *regS* (Bauer et al. 1998; Dixon and Kahn 2004). It is believed that RegS responds to cellular redox conditions. Activation of RegR by RegS directly affects the transcription of the genes encoded in the operon that includes *nifRA*. The role of NifA in *B. japonicum* is similar to that described for *S. meliloti* in that it also responds to oxygen, and it is necessary for the transcription

of nitrogenase as well as many of the accessory genes that are necessary for its assembly and function (Fisher and Hennecke 1987).

4.16 Concluding Remarks

The study of symbiotic nitrogen fixation is an area of research that spans both microbiology and plant biology. As such, a diverse number of approaches and techniques have been necessary to elucidate what is known today. With the world's increasing dependence on agriculture to feed its population, the use of reduced nitrogen derived from energy provided by fossil fuels is not likely to be sustainable. The key to future work in this area will be the ability to utilize the fundamental knowledge that has been gained by studying the process of symbiotic nitrogen fixation such that it can be translated to something tangible, such as the engineering of this, or of related associations, with other agricultural crops to benefit an increasing world population.

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