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Review article.

How Many *Rhizobium* Genes, in Addition to *nod*, *nif/fix*, and *exo*, are Needed for Nodule Development and Function?

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Abstract

The establishment of a nodule during the *Rhizobium*-legume symbiosis requires the induction of new developmental programs within each partner. During the initial interaction between *Rhizobium* and its host legume, operons of bacterial genes are induced to perceive plant-secreted signals and, in turn, to synthesize the bacterium's own secreted nodulation signals. However, a number of rhizobial genes which are required for free-living conditions as well as for legume infection have been identified. Mutations in these genes affect the establishment and/or effectiveness of the nitrogen-fixing symbiosis. As the rhizobia encounter different zones of the developing nodule, some genes play very specific roles in bacteroid function, whereas others appear to be part of regulons involved in general cell maintenance. In this review, we catalog these genes and describe their involvement, either direct or indirect, in the symbiosis.

Keywords: "housekeeping" genes, symbiotic phenotype, nodulation, nitrogen fixation, bacteroid development.

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1. Introduction

The establishment of the symbiotic interaction between leguminous plants and rhizobia (*Rhizobium*, *Bradyrhizobium*, and *Azorhizobium*) involves the activation of new developmental programs within each partner (for reviews, see Hirsch, 1992; Long, 1996). The initial developmental changes in the plant occur within a zone of the root containing emerging root hairs. Within this region, the quiescent cells of the root cortex are programmed to dedifferentiate, divide, and become reorganized into a home for the bacteria, a nodule. Concurrently, free-living, motile bacteria undergo morphological and biochemical changes in the interior of the nodule and differentiate into non-dividing cells known as bacteroids. Bacteroids are capable of converting atmospheric nitrogen into ammonia which is assimilated into amino acids and transported into plant cells. In return, the bacteroids are supplied with fixed carbon that is derived from photosynthate produced in the aerial portions of the plant.

The associations between the partners of the symbiosis are often highly specific and occur only in nitrogen-deficient soils. Recognition of a specific host is based on the perception by both the bacteria and the plant of signals that are released into the rhizosphere, the environment surrounding the root. In addition to bacterially-encoded recognition signals known as Nod factors, which are produced to initiate nodule development, there is increasing evidence that other gene products help prepare the bacteria for entry into their hosts. Expression of specific genes may be related to rhizobial position within the root and state of differentiation. Mutations in some of these genes have revealed important regulatory pathways in nodule development and nitrogen fixation.

Stages and zones of nodule development

In the *Rhizobium*-legume interaction, the developmental changes that result in the induction of a nitrogen-fixing nodule can be characterized by four major stages: 1) the infection of the plant by the bacteria; 2) the organization of the newly divided plant cells into a nodule; 3) the maintenance of nitrogen fixation; and 4) the senescence of the nodule tissues (for review, see Hirsch, 1992). On legumes, such as bean and soybean, mature nodules are spherical and lack a discrete meristem. Such so-called determinate nodules reach their final size by cell expansion after an initial period of meristematic activity rather than by continued cell division from a distal meristem. The nodules formed on alfalfa, pea, vetch, and clover, in contrast, are elongate and often club-shaped. These nodules, described as indeterminate, recapitulate in space the temporal

phases of nodule development described by the four major stages. Thus, there is a gradient of differentiation from the distal to the proximal end of the nodule (Fig. 1).

The meristem, zone I according to the terminology of Vasse et al. (1990), is a region of actively dividing, small, isodiametric cells and is free of bacteria (Fig. 1, arrow 1). Behind the nodule meristem, an area designated as zone II, are cells filled with the invading bacteria, encased within infection threads (stage 1 rhizobia). In interzone II/III, adjacent to zone II, the bacteria are released from the infection threads (stage 2 rhizobia) and colonize the host cells, which have increased in size. Within the interzone, the transition from vegetative to elongate bacteroid forms occurs abruptly so that adjacent cells exhibit dramatic changes in the degree of rhizobial differentiation (Fig. 1, arrow 2). Interzone II/III is characterized by having cells filled with amyloplasts (Vasse et al., 1990). Nitrogen fixation (stage 3 rhizobia) occurs within the cells of zone III, in the central portion of the nodule. This region consists of both highly vacuolated plant cells which are devoid of bacteroids as well as cells packed with membrane-bound bacteroids (Fig. 1, arrow 3). Finally, proximal to the point of attachment to the plant root, are the oldest tissues of the nodule, zone IV, or the senescent zone, in which both symbiotic partners senesce (stage 4 rhizobia). The transition from zone III to zone IV is often also abrupt; a cell filled with elongate bacteroids can be adjacent to one with degenerate bacteroids (Fig. 1, arrow 4). The senescent zone cells contain amyloplasts and vegetative rhizobia which are not surrounded by plant membrane (Fig. 1, arrow 5). The rhizobia are released from infection threads that have proliferated throughout the nodule (Fig. 1, arrow 4). Eventually, as the nodule dies, the bacteria return to the soil.

Rhizobium genes important for nodulation

During the development of the nodule, the rhizobia are exposed to at least three different environments: first, the rhizosphere, then, the infection thread, and finally, the peribacteroid compartment. Several regulons of genes that are expressed specifically in each of these environments have been identified by transposon mutagenesis (for reviews, see Long, 1989; Dénarié et al., 1992). Many of these genes are located on very large symbiotic plasmids (pSym) (for a review, see Mercado-Blanco and Toro, 1996). In *R. meliloti*, the nodulation (*nod/nol/noe*) genes are involved in the early events of the bacterial-host interactions, including the production of Nod factor signals; they are located on one of two megaplasmids (pSyma). Mutations in these genes prevent or delay the bacteria from infecting the plant (stage 1) (van Rhijn and Vanderleyden, 1995; Long, 1996). Experiments involving fusions

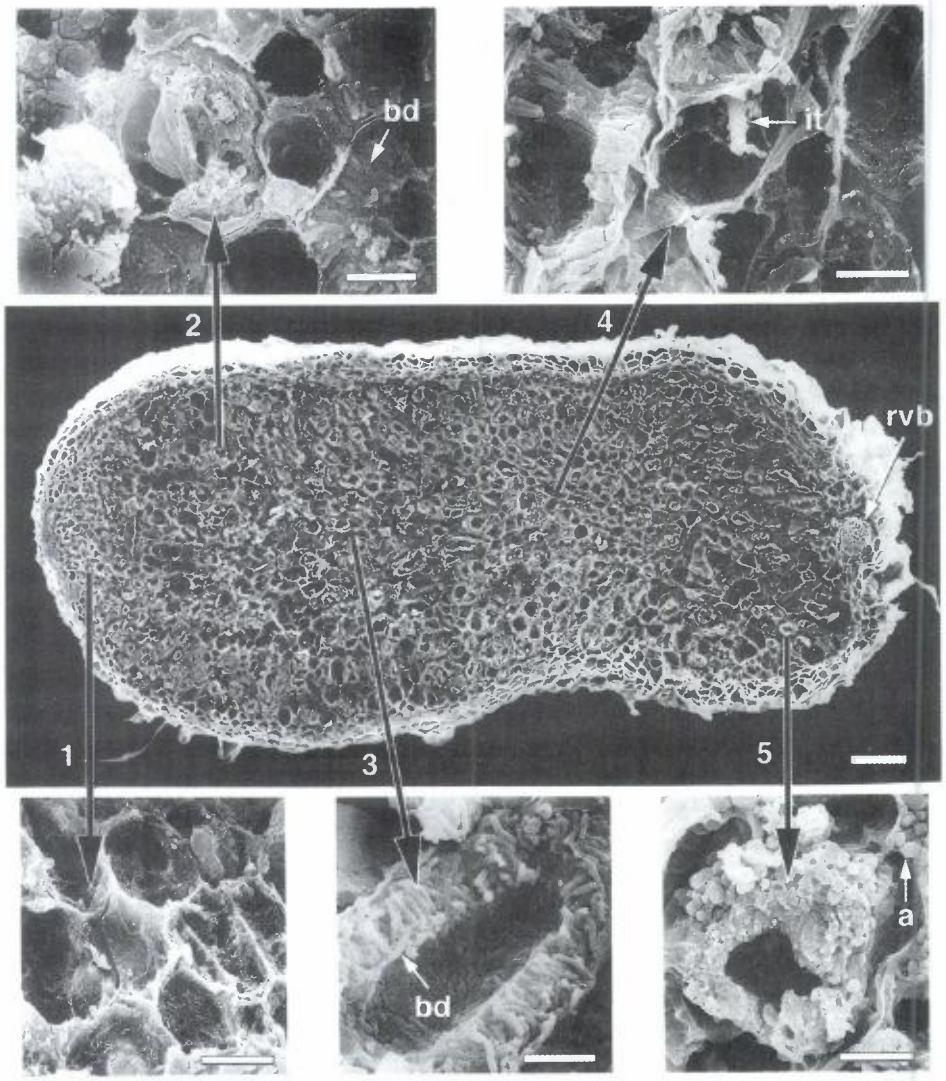

Figure 1. Scanning electron micrograph of a mature alfalfa nodule. The connection to the parent root is indicated by rvb (root vascular bundle). Bar = 100 μm. Arrow 1 points to the meristematic zone (zone I). No bacteria are present within these small, isodiametric cells. Bar = 10 μm. Arrow 2 points to the proximal end of the invasion zone (zone II). Bacteria are released into the expanded host cells and differentiate into bacteroids (bd). Bar = 10 μm. Arrow 3 indicates the nitrogen-fixing zone (zone III). Host cells are filled with bacteroids. Bar = 10 μm. Arrow 4 points to the transition between the nitrogen fixation zone and the senescent zone (zone IV). Infection threads (it) are evident in host cells in which the bacteroids have degenerated. Arrow 5 indicates the oldest region of the nodule. Amyloplasts (a) and senescing rhizobia fill the cells. Bar = 10 μm. Zones I–IV designations are described in the text and in Vasse et al. (1990).

between *nod* and reporter genes have shown that the *nod* genes are not expressed in bacteroids and that their expression is limited to zone II (Sharma and Signer, 1990).

Also found on pSyma are genes required for nitrogen fixation (*nif* and *fix*) which are expressed in the mature nodule (stage 3; zone III). These genes are required for the synthesis of nitrogenase which converts N_2 to ammonium and for exporting fixed nitrogen into the peribacteroid space (respiration and transport complexes) (Long, 1989; Dénarié et al., 1992). In addition, regulons involved in the production of acidic exopolysaccharide, lipopolysaccharide, and capsular polysaccharide surface components (*exo/exp*, *lps*, and *rkp*) are required for bacterial invasion (stages 1 and 2) (Petrovics et al., 1993; Leigh and Walker, 1994; Becker et al., 1997; Kiss et al., 1997). Located on pSymb and the chromosome, these genes, function primarily during adhesion of the bacteria to the root hair and the development of infection threads within zone II and interzone II/III. Mutations result in the abortion of infection threads and the lack of development of a discrete meristem in indeterminate nodules. Consequently, the nodules are small, do not contain bacteria, and are Fix^- (Finan et al., 1985; Yang et al., 1992).

Other gene products influencing nodule formation and nitrogen fixation either directly or indirectly have been identified as part of the repertoire required for symbiosis. Comprising surface constituents, metabolic pathway enzymes, and global regulatory systems, these gene products generally allow rhizobia to adapt to different environments. With the prospect that eventually the entire rhizobial genome will be sequenced, we thought it useful to review the contribution of various *Rhizobium* and *Bradyrhizobium* genes, exclusive of *nod/nol/noe*, *nif/fix* and *exo/exp/lps/rkp* to the symbiotic interaction. For reviews on the genes which are directly involved, see Leigh and Walker (1994), van Rhijn and Vanderleyden (1995), Long (1996), and Dénarié et al. (1996). In the next section, we review recent literature describing those "other" genes which, if mutated, influence either the nodulation or nitrogen-fixing capability of the rhizobia. To guide our discussion of these other genes, we will focus on the three different environments that the bacteria inhabit: 1) the rhizosphere, 2) the infection thread niche, and 3) the peribacteroid membrane compartment (symbiosome).

2. The Rhizosphere

Rhizobia in the rhizosphere must compete with the other organisms in the soil for nutrients and access to legume roots. Numerous genes are involved in maintaining the saprophytic competence of rhizobia in the soil (see Mercado-

Blanco and Toro, 1996). In the next section, we focus on those rhizosphere-expressed genes which, when mutated, result in rhizobia with an attenuated symbiotic phenotype. However, we include descriptions of some genes which are expressed at relatively high levels in the rhizosphere or which may otherwise influence nodule development. Rhizobia with mutations in these genes may not exhibit a symbiotic phenotype in the laboratory. In other cases, a mutant gene has not been identified.

Rhizosphere-expressed genes

rhi

In *R. leguminosarum* bv. *viciae*, *rhi* (for rhizosphere-expressed) genes are located within the *nod/nol/noe* operon (Economou et al., 1989). Several observations, including repression of *rhi* expression by *nod/nol/noe*-inducing compounds, suggest an interaction between these two regulons (Economou et al., 1989). Moreover, nodulation defects are only detected in certain *nod rhi* double mutants (Cubo et al., 1992). The significance of these interactions, however, is not understood.

The function of the proteins encoded by *rhiABC* is thought to be involved in the recognition of a plant-made metabolite (Cubo et al., 1992). In the rhizosphere of pea roots or in stationary-phase laboratory cultures, RhiA is the most prominent single protein found within the cell (Dibb et al., 1984). *rhiA* expression appears to be specific for this environment because RhiA protein is not detected within bacteroids. Expression is regulated by RhiR, a homolog of the LuxR family of transcriptional regulators that require a homoserine lactone co-factor for activation (Cubo et al., 1992). Indeed, a homoserine lactone derivative, referred to as RLAI (*R. leguminosarum* autoinducer), is required for RhiR activation of *rhiABC* (Gray et al., 1996). Schripsema et al. (1996) identified the same molecule as *small*, a previously identified anti-microbial compound or bacteriocin that is produced in many *R. leguminosarum* bv. *viciae* and bv. *trifolii* strains (van Brussel et al., 1985). Homoserine lactones present in culture filtrates from several *R. leguminosarum* strains leads to the inhibition of growth and the entry of the cells into stationary phase growth (Gray et al., 1996). In addition to inducing transcription, such autoinducers appear to be involved in sensing cell density in other bacteria (Schripsema et al., 1996). Homoserine lactone derivatives from *R. meliloti* culture filtrates, distinct from RLAI, appear unable to activate *R. leguminosarum's rhiA*, suggesting that, in *R. meliloti*, there may be additional genes, perhaps also specifically expressed in the rhizosphere, which require activation by homoserine lactone derivatives (Pearson et al., 1994; Gray et al., 1996). RLAI/*small* synthesis or regulation also requires gene products from two

plasmid loci: *rps* (repression production *small*) and *sbs* (*small* bacteriocin sensitivity) (Wijffelman et al., 1983; van Brussel et al., 1985). The loss of either of these loci or *small* appears to affect rhizosphere-specific functions and has no effect on subsequent infection.

Amino acid auxotrophs

Several amino acid metabolic pathways have been shown to be involved in *Rhizobium*'s ability to nodulate its host. One such *R. meliloti* mutant induces normal root hair deformation on alfalfa roots (Hac^+) but only small, uninfected nodules (Hirsch et al., 1982). This mutation is thought to be due to a Tn*5* insertion in the *ilvC* gene (Aquilar and Grasso, 1991). *ilvC* encodes acetohydroxy acid isomeroreductase, the second enzyme in the biosynthetic pathways for isoleucine and valine. Although certain pathway intermediates overcome IlvC auxotrophy, they do not restore nodulation ability. In contrast, *E. coli ilvC* restores prototrophy as well as a nodulation phenotype to the mutant. The inability of these *R. meliloti ilvC* mutants to nodulate was shown to be due to the incapability of the nodulation genes, *nodABC*, to be activated by NodD in response to flavonoids. This effect is apparently specific for *ilvC* because a different mutation, expected to be in *ilvD*, shows wild-type levels of *nodABC* expression (Aquilar and Grasso, 1991). However, the Hac^+ phenotype originally described by Hirsch et al. (1982) is difficult to reconcile with the lack of activation of *nodABC*. To date, the *ilvC-nod/nol/noe* association is not understood.

The loss of the ability to utilize proline by free-living *R. meliloti* also results in poor nodulation of alfalfa (Jiménez-Zordo et al., 1995). In *R. meliloti*, a Tn*5* insertion within *putA*, the gene encoding the bifunctional enzyme proline dehydrogenase/pyrroline-5-carboxylate dehydrogenase affects bacterial colonization of alfalfa roots, suggesting that proline is a key nutrient during nodulation and growth in the rhizosphere (Jiménez-Zordo et al., 1995, 1996). Proline or other proline-containing compounds secreted by roots, such as the betaine stachydrine, may also behave as signaling molecules; alfalfa root extracts induce *putA-lacZ* expression (Jiménez-Zordo et al., 1996). Stachydrine catabolism is also required for optimal nodulation of alfalfa (Goldman et al., 1994). Interestingly, *putA-lacZ* fusions are expressed in developing nodules – from zones I to interzone II/III – from the crook of the curled root hair to the release of bacteria from the infection threads. An unexpected result from these studies was the lack of β–galactosidase expression in bacteroids; proline had long been hypothesized to be utilized in these differentiated cells. The bacteroids, however, appear capable of normal nitrogen fixation and the nodules are Fix^+ (Jiménez-Zordo et al., 1996). Similarly, although early nodulation defects of *putA* mutants of *B. japonicum* have not been described, the

mature nodules elicited from such mutants are also Fix$^+$ (Straub et al., 1996). Therefore, *putA* appears to respond to signals in the rhizosphere and within infection threads prior to bacteroid differentiation.

Motility and chemotaxis

Motility has long been considered to be important for a successful interaction between rhizobia and their hosts. Rhizobia in the rhizosphere are chemotactically attracted to certain molecules such as flavonoids, phenolics, certain sugars, and amino acids which are exuded from the plant's roots (Parke et al., 1985; Robinson and Bauer, 1993; Phillips et al., 1994; Vande Broek and Vanderleyden, 1995). The ability to swim toward specific stimuli has led to the proposal of two chemotaxis pathways in *R. meliloti* – one specifically toward food and the other responding to root-released signals (Bergman et al., 1988). Greck et al. (1995) have further speculated that the nutrient-directed pathway, responding to amino acids, requires two novel ORFs whereas the other pathway, which is specific for plant signals, acts via CheW-CheA-CheY-CheR-CheB. Recently, homologs of *E. coli* chemoreceptors (*mcpA*, *mcp3*, and *rlvCP*), namely, methyl-accepting chemotaxis proteins (MCP) and signaling component homologs, have been identified in rhizobia (Greck et al., 1995; Brito et al., 1996; Schmitt et al., 1997; Yost and Hynes, 1997; Freiberg et al., 1997). *mcpA* from *R. leguminosarum* bv. *viciae* and two *mcp*-like genes from *Rhizobium* sp. NGR234 are located on symbiotic plasmids (Brito et al., 1996; Freiberg et al., 1997).

R. meliloti chemotaxis mutants exhibit a range of motility defects from non-motile to random or tumbling movement to normal smooth swimming patterns (Bergman et al., 1988). Two mutants with random or tumbling motion have been complemented with *flaA*, a flagellin gene (Bergman et al., 1991). The genes defective in the other mutants have not yet been identified, although at least three operons of flagellar assembly and motility have been sequenced: *flaABDC*, *flgC-fliE-flgGAI-orf6-flgH-fliLP*, *motA*, and *orf14-motBCD* (Platzer and Schmitt, 1995, 1996a, 1996b; Schmitt and Sterr, 1997).

Despite the role of motility in chemotaxis, the ability to swim is not obligatory for infection of the root. Non-motile mutants, it appears, are still Nod$^+$, but they elicit fewer nodules than their motile counterparts when the two are inoculated together in equal numbers (Hunter and Fahring, 1980). Mutations in *fliP*, which is located upstream of *flaAB* in *R. meliloti* and which encodes a protein showing some similarity to gene products involved in pathogenicity, result in non-motile bacteria lacking flagella (Finan et al., 1995). However, there were no differences in nodulation or nitrogen-fixation ability when *fliP* mutants were compared to wild-type rhizobia. Thus, although motility functions cannot be considered essential for nodulation, at

least under laboratory conditions, it is possible that non-motile or non-chemotactic rhizobia are not as competitive in the field.

Nodulation competitiveness

The term nodulation competitiveness is used to indicate that some rhizobial species are better adapted to the rhizosphere environment and, therefore, "outcompete" other strains to infect their host's roots (Triplett, 1990). Numerous criteria have been used to measure this attribute – some specific for rhizobial survival in the rhizosphere and others based on the interaction between rhizobia and its host. For example, some studies focus on the early infection process, measuring root colonization and co-inoculation infectivity of different rhizobial strains. Alternatively, some groups monitor nodule function, waiting until nodules develop before determining the timing and overall nodulation ability as numbers, dry weight, and nitrogen-fixing capacity of nodules.

Survival in the rhizosphere is manifested by rhizobia's ability to acquire nutrients and endure environmental stress (see Miller and Wood, 1996). One key factor in survival is active cell growth. Lupwayi et al. (1996) have shown that *R. meliloti* in logarithmic growth infect host roots more readily than do stationary phase cells. In addition to growth phase, specialized pathways for the catabolism of rhizosphere-specific compounds as sources of carbon and nitrogen have been isolated that enhance survival. Genes encoding enzymes for these pathways are found on both the symbiotic and cryptic (nonsymbiotic) plasmids (for review, see Mercado-Blanco and Toro, 1996).

Anti-microbial compounds secreted from rhizobia to kill or restrict growth of other rhizosphere bacteria also contribute to competitiveness. In addition to the bacteriocin RLAI/*small*, a less potent bacteriocin from *R. leguminosarum* bv. *viciae*, known as *medium*, is encoded on one of the other plasmids, but its role in the rhizosphere has not been characterized to date (van Brussel et al., 1985). Recently, genes similar to *Escherichia coli*'s microcin biosynthetic and uptake genes have been identified in *Rhizobium* sp. NGR234 and *R. meliloti* (Freiberg et al., 1997; Ichige and Walker, 1997). Also, genes involved in the production and resistance to the peptide antibiotic trifolitoxin (*tfx* and *tfuA*) have been isolated from *R. leguminosarum* bv. *trifolii*; these genes confer increased nodulation competitiveness (Breil et al., 1993, 1996; Robleto et al., 1997).

In *R. meliloti* and *B. japonicum*, mutations in *nfe*, so-called for nodule formation efficiency, cause a delay in nodulation on alfalfa and soybean roots, respectively (Soto et al., 1993; Chun and Stacey, 1994). Once infected, however, the resulting nodules are normal in dry weight and number as well as acetylene reduction ability. In *B. japonicum*, a Tn*5* insertion within *nfeC* delays

nodulation from 6.5 to 13 days (Chun and Stacey, 1994). Although the function of the *B. japonicum's nfeC* gene product has not been determined, the regulation of these genes implies that the gene products are involved in several stages in the developing nodule. *nfeC* is regulated by two promoters, one of which contains the –24/–12 consensus of σ^{54}-regulated promoters and is expressed exclusively in bacteroids and not under anaerobic conditions in free-living bacteria (Chun and Stacey, 1994). The *R. meliloti nfe1/nfeA* also encodes a gene of unknown function and appears to be regulated by a σ^{54}-regulated promoter as well (Sanjuan and Olivares, 1991). There appears to be additional regulation by NifA, the regulator of *nif* gene expression, to ensure bacteroid expression (Soto et al., 1993). Transcriptionally linked to *nfe2/nfeB* gene in *R. meliloti*, and present in *R. etli*, is a third gene involved in nodulation efficiency, *nfeD* (Soto et al., 1993, 1994; Borthakur and Gao, 1996). The *nfeD* gene product shows similarity to an *Agrobacterium tumefaciens* enzyme, ornithine cyclodeaminase (OCD), that converts ornithine into proline. OCD catalyzes the reaction prior to the catabolism of proline by PutA. Therefore, it is not surprising that both *nfeD* and *putA* are involved in optimizing nodulation competitiveness (Soto et al., 1994; Jiménez-Zurdo et al., 1996).

Other genes involved in nodulation competition, encoding unknown functions, have been identified in *R. fredii*, *R. etli*, and *B. japonicum* (Sadowsky et al., 1988; Bhagwat and Keister, 1992; Michiels et al., 1995a; Bittinger et al., 1997). Each of these genes – host-inducible gene in *R. fredii*, *orf3* and *rosR* (encoding a putative transcriptional regulator) in *R. etli*, and two *B. japonicum* genes isolated by subtractive hybridization – appears to be important in the rhizosphere in optimizing the rhizobia's response to their hosts.

Attachment and adhesion

Attachment of rhizobia to plant roots has been proposed to be a two-step process, involving an initial binding of the bacteria to the plant cell wall followed by anchoring via bacterial appendages to plant lectin receptors (for reviews, see Smit et al., 1992; Vande Broek and Vanderleyden, 1995). An adhesin involved in the first step of binding appears to be common among the Rhizobiaceae (Smit et al., 1989). Termed rhicadhesin, for rhizobial calcium-dependent adhesin, this adhesin is released from *R. leguminosarum* bv. *viciae* cells grown under Ca^{2+}-limiting conditions. Application of purified rhicadhesin to pea roots inhibits attachment of *R. leguminosarum* bv. *viciae* cells (Smit et al., 1989). The genes encoding rhicadhesin have not been identified to date, so that the role of this protein at other stages of the symbiosis has not been determined. Recently, a glycoprotein receptor for rhicadhesin, as well as a hexapeptide containing the eukaryotic integrin

binding sequence for arginine-glycine-aspartic acid (R-G-D), have been identified as the possible attachment sites for rhizobia on pea roots (Swart et al., 1994).

Another potential rhizobial adhesion protein has been identified in *B. japonicum*. This protein, a carbohydrate-binding lectin, designated BJ38, is localized as a tuft at one pole of *B. japonicum* (Loh et al., 1993). Mutants lacking BJ38 on their surfaces, N4 and N6, show reduced binding to cultured soybean cells and soybean roots. Confocal microscopy experiments indicate that the lectin is found at the point of contact of the bacteria with cultured cells, supporting arguments for adhesion of BJ38 to soybean surface molecules. Interestingly, purified BJ38 binds preferentially within the zone of emerging root hairs on soybean roots (Ho et al., 1994). BJ38 does appear to be directly involved in nodulation because, although both N4 and N6 infect roots, they are less efficient in doing so than wild-type cells.

For the second step in attachment, Smit et al. (1992) suggested that pili (or fimbriae) and cellulose fibrils anchor rhizobia to the plant surface. Pili have been shown to contribute to non-specific attachment of rhizobia to root hairs. The addition of antibodies to the pilin proteins leads to a loss of *B. japonicum's* ability to attach to soybean roots (Vesper and Bauer, 1986). However, no pilin genes have been identified in rhizobia, so that the role of these proteins in attachment has not been clearly delineated.

Hydrolytic enzyme activity

Hydrolytic enzyme production has long been assumed to be involved in the initial penetration of the rhizobia into the host root hair. Whether or not such enzymes are produced by the rhizobia or by the plant or both is still not clear (see Mateos et al., 1992). In various transmission electron microscopic analyses of rhizobial invasion of root hairs, a localized degradation of the cell wall has been observed (see references in van Spronsen et al., 1994). Pectinolytic and cellulolytic enzyme activities have been identified from *R. leguminosarum* bv. *trifolii*, although the genes encoding these activities have not been identified (Mateos et al., 1992; Fennington and Hughes, 1990). Interestingly, when *pelB* from *Erwinia chrysanthemi* was used to probe *R. meliloti* chromosomal DNA, the hybridizing DNA fragment showed high sequence homology to *murD* from *Escherichia coli* and no significant homology to *pelB* (Leach et al., 1994). *murD* and a neighboring ORF, *mraY*, encode UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate synthetase and a transferase, two enzymes which are involved in cell wall synthesis, not degradation, in *E. coli*. Hydrolytic enzymes that are involved in rhizobial invasion, therefore, remain to be isolated and their role in symbiosis remains to be determined.

Candidate genes encoding a transport or secretion system for extracellular enzymes have been isolated from *R. leguminosarum* bv. *trifolii*, *R. leguminosarum* bv. *viciae*, and *R. meliloti* (Król and Skorupska, 1997; Finnie et al., 1997; York and Walker, 1997). Two genes, *prsDE* for protein secretion, appear to be involved in formation of an ABC-type (ATP-binding cassette) transporter system. Both PrsD and PrsE have extensive homology with *E. chrysanthemi* Prt and *Pseudomonas aeruginosa* Apr transporter proteins required for protease export. Indeed, *R. leguminosarum* bv. *viciae* is capable of secreting PrtB protease, as well as numerous Ca^{2+}-binding proteins, including NodO, presumably through a complex of PrsD and PrsE (Crank and Downie, 1994; Finnie et al., 1997; Scheu et al., 1992). Cultures of *prsDE* mutants are extremely mucoidy, suggesting that these gene products are also involved in the secretion of extracellular endoglycanases (Finnie et al., 1997; York and Walker, 1997; Król and Skorupska, 1997). Despite altering the profile of secreted proteins and exopolysaccharide composition of free-living cells, the loss of *prsDE* does not affect infection. However, symbiotic defects were detected in bacteroids of *R. leguminosarum* bv. *viciae* and *R. leguminosarum* bv. *trifolii*, but not in *R. meliloti*; mutants of the former elicited Fix^- nodules on pea, vetch, and clover (Finnie et al., 1997; Król and Skorupska, 1997; York and Walker, 1997). Because the *prsD* mutants act like wild-type cells during infection, the authors argue that this transporter is unlikely to be involved in exopolysaccharide transport *in nodulo* but may be involved in secretion of other proteins that are required for bacteroid function and nitrogen fixation.

3. Infection Thread Niche

Rhizobia with mutations in genes that are expressed in this environment will most likely induce nodules that are uninfected or partially infected.

Genes expressed in the infected zone

ndv

ndv (for nodule development) mutations in rhizobia have pleiotrophic affects, including loss of motility, increased resistance to bacteriophage infection, increased sensitivity to certain antibiotics and hypoosmotic conditions, as well as induction of ineffective nodules on host plants (for a review, see Breedveld and Miller, 1994). To date, four *ndv* loci have been identified: *ndvA*, *ndvB*, and *ndvC* are involved in the production of cyclic β-glucans, polysaccharides that were thought to be unique to Rhizobiaceae but have recently been isolated from the mammalian pathogen *Brucella*; and *ndvF*

is involved in phosphonate uptake (Briones et al., 1997; Breedveld and Miller, 1994; Bhagwat et al., 1996; Charles et al., 1991; Bardin et al., 1996). An association between polysaccharide biosynthesis and phosphonate uptake loci has been shown for *mucS*, a putative regulatory gene located within the *exp* gene cluster (Astete and Leigh, 1996). Whereas *ndv* genes are considered homologs of *Agrobacterium chv* genes, two rhizobial *chv* genes, *chvI* and *chvG*, have also been identified (Osterås et al., 1995b). ChvI and ChvG, appear to be essential for growth and comprise a sensor/response regulator pair. To date, however, the role of these genes has not been determined in either *Agrobacterium* or rhizobia.

NdvB is suspected to be a large, multifunctional membrane protein involved in cyclic β-glucan synthesis, whereas NdvA is required to transport these sugars into the periplasm (Breedveld and Miller, 1994). It has been estimated that cyclic β-glucans can account for 5–20% of total cellular dry weight with levels of β-glucan within bacteroids similar to amounts found in free-living rhizobia (Breedveld and Miller, 1994). This observation suggests that like the *exo/exp/lps* polysaccharide constituents on the cell surface, cyclic β-glucans are also involved in nodule development. For example, nodulation by *ndvC* mutants is significantly delayed; the 8–10 day delay may be due to reduced attachment to roots, as is seen with *R. meliloti ndvA* mutants (Dylan et al., 1990). The resulting nodules are small, devoid of infection threads or viable bacteroids, and Fix^-, a characteristic of all *ndv* mutations (Bhagwat et al., 1996; Breedveld and Miller, 1994). Infection threads in *ndv* nodules abort during infection; in soybean nodules, they appear to be absent (Dunlap et al., 1996). Recently, another gene, *cgmA*, involved in modification of cyclic β-glucans has been isolated from *R. meliloti*. Its symbiotic phenotype, however, has not yet been described (Wang and Miller, 1997).

Detailed characterization of the *R. meliloti ndvF* locus indicates that genes within this region are homologous to phosphonate transport genes (*phn*) in *E. coli* (Charles et al., 1991; Bardin et al., 1996). Four genes, *phoCDET*, encoded at this locus on pSymb, comprise the inducible integral membrane and periplasmic binding-proteins of an ABC-type transport system for P_i and, possibly, phosphonate uptake (Bardin et al., 1996). *ndvF/phoCDET* mutants are unable to assimilate phosphonate compounds from the medium and, thus, do not grow. In contrast, when cultured in a medium containing glycerol-3-phosphate or aminoethylphosphonate, the mutants grow, suggesting the use of distinct transport systems in *R. meliloti* (Bardin et al., 1996). Two other operons involved in phosphonate utilization and regulation, *phnGHIJK* and *phoUB*, have also been isolated and sequenced from *R. meliloti* (McLean et al., 1992, 1997). The contribution of these genes to bacterial growth has not yet been determined, however. Symbiotic defects attributed to *ndvF/phoCDET*

mutations include delayed nodule development and nodules devoid of bacteroids, which suggest a block in development within zone II such that bacteria are not released from the infection threads (Charles et al., 1991). The symbiotic phenotypes may result from the inability of the mutants to acquire P_i and grow *in planta* or the alteration of surface components. Second site mutations in *ndvF/phoCDET* affect colony morphology and cell surface moieties (Oresnik et al., 1994).

Auxotrophy

Many auxotrophies do not gravely affect the fate of the symbiosis. For example, riboflavin, histidine, and leucine auxotrophs induce ineffective nodules on their hosts, but each can be restored to wild-type by the addition of the corresponding vitamin or amino acid to an inoculated plant (Beringer et al., 1980; Kerppola and Kahn, 1988). The nodules induced on alfalfa by *R. meliloti* leucine auxotrophs, for example, exhibited a typical nodule structure with a meristem and peripheral vasculature, but were completely devoid of bacteria (Truchet et al., 1980; Hoying et al., 1990). On the contrary, the loss of nucleotide biosynthetic pathways cannot always be compensated for by the exogenous application of precursor compounds. Whereas pyrimidine auxotrophs are rarely symbiotically defective, purine auxotrophs of a number of *Rhizobium* sp. elicit small, irregularly shaped nodules on their respective host plants (Beringer et al., 1980; VandenBosch et al., 1985; Noel et al., 1988; Djordjevic et al., 1988; Kerppola and Kahn, 1988; Swamynathan and Singh, 1992; Newman et al., 1994). For *R. meliloti* purine auxotrophs, root hairs curl normally, infection threads are formed, but the nodules are Fix$^-$ (Dickstein et al., 1991; Swamynathan and Singh 1992). The nodules induced by *R. etli* and *Rhizobium* sp. NGR234 Pur$^-$ strains, in contrast, appear more as bumps than true nodules, in that they lack infection threads and more closely resemble roots in their structure and protein profiles (VandenBosch et al., 1985; Djordjevic et al., 1996). Attempts to overcome the purine deficiency with exogenously applied adenine or IMP (inosine monophosphate) to *R. meliloti* auxotrophs did restore growth on minimal medium, but neither compound nor AICAR (5-aminoimidazole-4-carboxamide riboside) restored the nodulation phenotype to the wild-type condition (Swamynathan and Singh, 1992). In contrast, AICAR addition (and inosine at ten times higher concentration) enhanced nodule development by the *R. etli* Pur$^-$ and *Rhizobium* sp. NGR234 *purMN* and *purYQL* mutants (Newman et al., 1995; Djordjevic et al., 1996). Nevertheless, although infection threads developed and penetrated into the central tissues of bean nodules, bacteria were not released from the threads (Newman et al., 1995).

Several amino acid auxotrophies have dramatic effects on bacteroid development. For example, *B. japonicum* glycine auxotrophs, due to a Tn*5* insertion into *glyA*, elicit numerous, tiny, Fix$^-$ nodules along the length of the soybean root (Rossbach and Hennecke, 1991). Moreover, no infection threads were detected in the nodules even 3 weeks after inoculation. *glyA* encodes a serine hydroxymethyltransferase (SHMT) that is responsible for the biosynthesis of glycine from serine. Despite the symbiotic defect, the phenotype is leaky, suggesting that these bacteria have an additional pathway for glycine biosynthesis. Other biosynthetic pathways may be affected as well (Noel et al., 1988).

In *R. meliloti*, tryptophan biosynthetic genes are encoded by six genes arranged in three operons (Bae et al., 1989). Only the loss of one of these genes, *trpE(G)*, a fusion between the *trpE* and *trpG* coding sequences, leads to symbiotic defects. Rhizobia mutated in genes encoding enzymes required later in the pathway elicit Fix$^+$ nodules on alfalfa (Barsomian et al., 1992). TrpE(G), or anthranilate synthase, is responsible for conversion of chorismate to anthranilate in the first step of the pathway. *trpE(G)* mutants elicit two types of nodules on alfalfa. Type A nodules are oversized, about 30% as frequent as type B, and have a small pink zone proximal to the plant, making them capable of limited nitrogen fixation. In contrast, type B nodules are Fix$^-$. Both nodule types show an enlargement of zone II of the nodule, contain normal infection threads and numerous amyloplast-containing cells in interzone II/III. Type A nodules have a normal-sized zone III, which is packed with bacteroids. Interestingly, mutations in *aro*, aromatic amino acid biosynthesis genes, also lead to Fix$^-$ nodules on alfalfa, suggesting that anthranilate, but not tryptophan, is required during nitrogen fixation (Barsomian et al., 1992). The authors speculate that the block in development at the onset of bacteroid differentiation may be determined by the distance of the rhizobia in the nodule to the parent root. They propose that a root factor, perhaps anthranilate itself, may be supplied only to the nodule tissues near the root and consequently, these develop normally and are Fix$^+$.

Mutations in the chromosomal copy of the glucosamine synthase gene (*glmS*) in *R. leguminosarum* bv. *viciae* affect several stages of infection of pea roots (Marie et al., 1994). First, in the rhizosphere, *glmS* mutants appear to produce limiting amounts of glucosamine that cause a reduction of surface lipopolysaccharide production, despite the presence of a homologous gene, *nodM*, involved in Nod factor production. Once *nodM* is induced by pea root exudates, however, glucosamine auxotrophy is overcome. Yet, as the bacteria are released from the infection threads, *nodM* is no longer expressed and the subsequent development of the bacteroids is abnormal, resulting in low levels of nitrogen fixation. The differentiated cells appear enlarged, highly

vacuolated, and senesce rapidly. The abnormal LPS profiles of *glmS* mutants may contribute to the rapid senescence of bacteroids.

ropA

RopA is an outer membrane protein from *R. leguminosarum* bv. *viciae* and bv. *trifolii* whose expression is abruptly down-regulated from zone II to interzone II/III in pea nodules (de Maagd et al., 1994; Roest et al., 1995a). *ropA* transcripts and proteins are restricted to this region of the nodule, where the bacteria are enclosed within infection threads. To date, a *ropA* mutant has not been described. *ropA* encodes one of two proteins that are covalently linked to the peptidoglycan layer. Together, RopA and a porin homolog belong to the outer membrane protein antigen group III isolated from free-living *R. leguminosarum* bv. *viciae* and shown by immunohistochemistry to be found in very low amounts in bacteroid cell envelopes (de Maagd et al., 1989; Chevalier and Delamarche, 1992). An outer membrane protein of antigen group II, RopB, also shows reduced levels in bacteroids (Roest et al., 1995b). *In vitro*, the only conditions that have been determined to decrease *ropA* expression are high calcium concentrations. Although this gene does not appear to be regulated by NifA, the authors speculate that *in vivo* regulation of *ropA* may involve a sudden drop in oxygen levels between zones II and III (de Maagd et al., 1994). The actual signals for repression are not known, however.

bacA

R. meliloti bacA mutants behave normally in infection threads but lyse upon release into plant cells, resulting in the production of small, empty, Fix^- nodules on alfalfa (Glazebrook et al., 1993). *bacA* encodes a putative seven transmembrane spanning receptor of the inner membrane that is functionally similar to *E. coli*'s *sbmA*, a gene implicated in uptake of several aminoglycoside antibiotics, including microcins (Glazebrook et al., 1993; Ichige and Walker, 1997). Susceptibility of *bacA* mutants to ethanol and detergents also suggests the importance in maintaining membrane integrity as the bacteria enter a new environment of the plant cell. Ichige and Walker (1997) note that *bacA* mutations lead to resistance to rifampicin, viomycin, and kanamycin/neomycin antibiotics, perhaps by altering membrane physiology and the uptake of these antibiotics. Such defects in cellular membranes may account for the symbiotic phenotypes observed upon inoculation with a number of antibiotic resistant mutants (for an example, see Reddy et al., 1992).

Recent studies of *R. meliloti bacA* mutants suggest that signaling between the bacteria and the host may be crucial for proper differentiation of bacteroids (Ichige and Walker, 1997). Interestingly, *sbmA* is capable of complementing all *bacA* defects except the Fix^- phenotype. Only when the *E.*

coli gene is expressed under the 168 bp of the *bacA* promoter are pink nodules produced, implying that the gene must be correctly induced *in planta* for bacteroids to differentiate. It is likely that the transcriptional regulators of *bacA* are key players in preparing the bacteria for the next environment they encounter. The attempts to identify these regulators led to identification of the *E. coli degP* homolog transcribed downstream of *bacA* (Glazebrook et al., 1996). Homologous to a periplasmic endopeptidase found in intracellular mammalian pathogens, the *degP* gene product is not essential for symbiosis in *R. meliloti*. However, Tn*5* insertions within an *orf* just upstream of *degP* result in white, ineffective nodules on alfalfa. Based on its phenotype and the proximity of *degP* to *cyc* genes, the authors speculate that this gene may encode a component of a respiratory chain.

orf74

A mutation in *orf74* from *B. japonicum* leads to a severe defect in nodule development and the formation of Fix^- nodules on soybean (Weidenhaupt et al., 1995). The nodulation phenotype has been characterized as $Nod^{+/-}$ due to the presence of necrotic bumps on roots. Within the bumps, there are few, apparently degraded bacteroids that are surrounded by plant cells filled with amyloplasts, a phenotype reminiscent of mutant *nifA*-induced nodules. Although the gene product encoded by *orf74* has not yet been identified, there is some homology to *orf17* from *Bacillus subtilis*. Indeed, *orf17* can partially complement the *orf74* mutation, leading to the formation of about half as many nodules as wild-type-infected *B. japonicum*. Additionally, *orf17* also restores the growth defect in *orf74* mutants as well as the overproduction of "slime", confirming that *orf74* is expressed in free-living cells and in bacteroids. The nodulation phenotype and the presence of *orf74* transcripts in non-symbiotic *B. japonicum* suggest that this gene may encode a regulatory factor that is involved in general cell maintenance.

4. The Peribacteroid Membrane Compartment

Rhizobial mutants in the genes described below will most likely induce nodules in which bacterial release occurs and perhaps even exhibit bacteroid differentiation. However, no nitrogen is fixed, and the bacteroids degenerate prematurely.

Functions required for bacteroid development and maintenance

Global regulation

Sigma factors play an essential role in transcriptional activation in

prokaryotes to ensure the expression of specific sets of genes at different stages of bacterial growth or in response to external stimuli. The sigma factor required for the transcription of most genes during logarithmic phased growth of most general metabolic functions is *rpoD* (sigma-70, σ^{70}). Homologs for *rpoD* as well as several alternative sigma factors, *rpoS*, *rpoH*, and *rpoN*, have been identified in a number of rhizobia. Each sigma factor probably plays its unique role at different stages of infection and nodule development.

Rhizobial *rpoD* homologs, designated *sigA*, have been identified in *R. meliloti*, *R. etli*, and *B. japonicum* (Rushing and Long, 1995; Luka et al., 1996; Beck et al., 1997). *In vitro* transcription studies using promoter fragments of the rRNA (*rrn*) and the chaperonin ($groEL_4$) operons show that *B. japonicum* SigA recognizes components of the canonical –35/–10 promoter elements found in RpoD-regulated genes (Beck et al., 1997). Recognition of *rrn* implies that, like RpoD, SigA is required under all growth conditions for normal housekeeping functions. Moreover, because $groESL_4$-*lacZ* fusions are detected at high levels in bacteroids, albeit at lesser levels than fusions to the $groEL_3$ gene, *sigA* must be expressed in nodules (Fischer et al., 1993). Defects in *sigA* may therefore be lethal, because no symbiotic phenotypes have been specifically identified.

Homologs of *E. coli*'s *rpoS* (σ^s), a sigma factor gene that is induced as cells enter stationary phase growth, have been detected by DNA hybridization analysis in *R. meliloti* (Miksch and Dobrowolski, 1995). In accordance that such a factor would replace RpoD in non-dividing cells, there is evidence in *R. etli* that *sigA* is down-regulated as cells enter stationary phase growth (Luka et al., 1996). Although the *R. meliloti* gene has not been isolated, RpoS-regulated reporter genes from *E. coli* are expressed in *R. meliloti* as cells exit exponential growth and enter stationary phase, implying functional complementation (Miksch and Dobrowolski, 1995). It is quite possible that such a gene product is activated *in planta* within zone II in bacteria trapped with the infection threads. Using fluorescently-tagged *R. meliloti*, Gage et al. (1996) have shown that only the bacteria at the tip of the growing infection thread are actively dividing.

Recently, in *B. japonicum*, a family of the heat shock sigma factor gene *rpoH* was identified (Narberhaus et al., 1996, 1997). The family comprises three members: $rpoH_1$, located within the same operon as the chaperonin genes $groESL_1$ and three heat shock proteins, *hspA* and *hspBC*; $rpoH_2$, an essential gene; and $rpoH_3$, found downstream of an uncharacterized sensor/response regulator pair of genes (*ragAB*, for $rpoH_3$-associated gene) (Narberhaus et al., 1996, 1997). All three *B. japonicum rpoH* homologs are functionally equivalent but show distinct differences in temperature-sensitive growth (Narberhaus et al., 1997). Although the loss of either $rpoH_1$ or $rpoH_3$ has no effect on nodulation, $rpoH_2$ mutants have not been isolated and the role of this gene

during symbiosis is not yet determined. Interestingly, *R. meliloti* mutants lacking *suhR*, a gene capable of suppressing the *E. coli rpoH* mutant phenotype, show no symbiotic defects (Bent and Signer, 1990). However, assessment of *B. japonicum* heat shock *dna* mutants indicate that DnaJ is not involved in nitrogen fixation, but that DnaK may be essential for growth and nodulation (Minder et al., 1997). In *R. meliloti*, *dnaK* and *dnaJ* mutant phenotypes have not been described (Falah and Gupta, 1994).

Perhaps, the most important sigma factor to be involved in nitrogen regulation and bacterial differentiation is σ^{54} (*rpoN/ntrA/glnF)* (Gussin et al., 1986). Required both in free-living and symbiotic rhizobia, σ^{54}-regulated genes are characterized by a conserved motif, CTGGYAYR-N_4-TTGCA, located at –24 to –12 from the start of the gene. Mutations in *R. meliloti's rpoN* result in Fix$^-$ alfalfa nodules (Ronson et al., 1987b; Shatters et al., 1989). Similarly, inoculation of cowpea and siratro roots (both form determinate nodules) with an *rpoN* mutant of *Rhizobium* sp. NGR234 elicits a Fix$^-$ phenotype (van Slooten et al., 1990). However, the Fix$^-$ phenotype of the determinate nodules differs from that of the ineffective alfalfa nodules in that no peribacteroid membranes were observed around the differentiated bacteroids. The symbiotic phenotype of the *R. etli rpoN* mutant has not yet been described (Michiels et al., 1995b).

B. japonicum contains two genes encoding σ^{54} (*rpoN*$_1$ and *rpoN*$_2$) (Kullik et al., 1991). In a series of experiments involving single and double *rpoN* mutations, Kullik et al. (1991) determined that *rpoN*$_1$ is regulated in response to oxygen, and *rpoN*$_2$ is negatively autoregulated. Moreover, *B. japonicum* cells lacking both of these genes induced twice as many nodules on soybean, but the nodules were Fix$^-$. These results suggest that each *rpoN* gene has a specialized function during different stages of bacteroid development.

groEL. Rhizobia are unusual in that they have multiple copies of the chaperonin genes *groES* and *groEL* (Fischer et al., 1993; Rusanganwa and Gupta, 1993; Wallington and Lund, 1994; Ogawa and Long, 1995). This finding strongly suggests that these bacteria utilize the different chaperonins in response to specific environmental conditions, some of which include nodulation and nitrogen fixation. GroEL appear to be involved in *nif* gene expression and nitrogenase assembly in other nitrogen-fixing bacteria as well (Govezensky et al., 1991). In *R. meliloti*, Ogawa and Long (1995) reported the presence of two *groEL* genes, a chromosomally-encoded locus, designated *groESLc*, and a second locus found on pSyma. A third *groEL* gene has been identified on pSymb by Rusanganwa and Gupta (1993). GroELc appears to be involved during nodulation, assisting the regulatory proteins NodD1 and NodD3 in their binding to *nod* promoters. It had previously been isolated as a contaminant protein in NodD1 preparations (Fisher et al., 1988). Additionally, *groELc*

mutants showed a delay and a decrease in nodulation effectiveness on alfalfa; nodules elicited by these mutants were Fix$^-$ (Ogawa and Long, 1995).

In *B. japonicum*, GroEL is also suspected to play a role in nitrogen fixation. Fischer et al. (1993) believe that this chaperonin is involved in the assembly of *B. japonicum nif* and *fix* gene products, because one of five *groEL* genes (*groEL*$_3$) is co-regulated with the transcriptional activator gene *nifA*. *groEL*$_3$ is also induced upon oxygen limitation, the first example of this type of regulation for this class of genes other than heat shock. Interestingly, three of the five *groESL* genes respond to heat shock; a σ^{32}-like promoter element upstream has been found upstream of *groESL*$_1$ (Babst et al., 1996).

sra. A small, 216 nt RNA that is crucial to nodule development has also been isolated (Ebeling et al., 1991). Identified in *B. japonicum* and *R. meliloti*, the gene encoding this RNA (*sra* for symbiotic ribonucleic acid) appears to be essential for bacterial colonization of the nodule as well as effective nitrogen fixation. A Tn*5* insertion into *B. japonicum sra* leads to a slight decrease in growth rate. Although the role of this RNA in bacteroid function has not yet been determined, it is expressed *in planta* as well as in cultures of *B. japonicum* grown in anaerobic or aerobic conditions (Ebeling et al.,1991; Weidenhaupt et al., 1995). Because *nif* and *fix* genes are expressed under similar conditions in *B. japonicum*, Ebeling et al. (1991) suggested that *sra* may play a more general role in maintaining the cell's competency to fix nitrogen instead of being involved directly during the bacterial-host interaction.

ftsZ. As rhizobia are released from infection threads, some developing bacteroids lose their rod-like shape and become elongate (Fig. 1); others may form swellings, buds, or branches. These shapes allow the no-longer-dividing bacteroids to increase their cell mass and membrane surface area. Latch and Margolin (1997) suggested that the development of *R. meliloti* bacteroid forms results from a block in the cell cycle that can be mimicked by overexpression of *ftsZ*. While overexpression of *ftsZ1* induced some budding forms in culture, high levels of a second gene, *ftsZ2*, led to even more exaggerated branching phenotypes. In wild-type *R. meliloti*, however, FtsZ2 cannot be detected, indicating that it may not be essential for growth under free-living conditions (Margolin and Long, 1994). The effect that *ftsZ2* overexpression has on cell morphology suggests that this gene plays a role in bacteroid development during nodulation.

Nitrogen regulation

Nitrogen fixation by rhizobia occurs in conditions when both ammonium and O_2 are limiting. In addition to their ability to assimilate nitrogen, many rhizobia, free-living or symbiotic, contain enzymes for denitrification and the production of N_2. Pathways involving other forms of nitrogen, i.e., nitrous

oxide (N_2O) dissimilatory reduction (encoded by *nos*), nitrate reduction, as well as nitrite reduction appear not to be essential; mutations in these genes do not affect nodule development or symbiotic nitrogen fixation (Holloway et al., 1996; Chan and Wheatcroft, 1993; O'Hara and Daniel, 1985). Denitrification, the generation of N_2, also does not appear to augment or enhance nitrogen fixation. Rather, this process is thought to help bacteroid survival by keeping the concentrations of toxic substrates, such as nitrite and nitric oxide, low (Holloway et al., 1996). The nitrite reductase gene, *nirK*, from a strain (HCNT1) of *R. "hedysari"* that denitrifies nitrite has been isolated (Toffanin et al., 1996). Although mutations in *nirK* uncouple nitrite reduction and energy conservation, they have no effect on infection or nodulation. In contrast, mutations resulting in defects in nitrogen reductase activity in exponentially growing *R. meliloti*, referred to as "respiratory" nitrate reduction (Rnr^-) phenotype, yield Fix^- nodules on alfalfa. The lesion in these mutants has recently been linked to components of the respiration cytochrome complexes in *R. meliloti* and *B. japonicum* (see below) (Kereszt et al., 1995; Bott et al., 1995).

Nitrogen metabolism is regulated by several pathways in rhizobia: a global nitrogen regulatory system, *ntr*, involved in transcriptional regulation; a detection system to sense the ratio of glutamine to α-ketoglutarate levels; and a microaerobic environment for nitrogen fixation (for reviews, see Kennedy et al., 1994; Merrick and Edwards, 1995; Soupéne et al., 1995). Ntr transcriptional control occurs predominantly in free-living rhizobia. For example, *R. meliloti* mutants lacking a nitrogen-sensitive regulator, designated NtrR, are unable to sense availability of fixed nitrogen, and subsequently nodulate alfalfa more abundantly than wild-type rhizobia in the presence of ammonium (Dusha et al., 1989). The gene encoding this transcription factor has not yet been characterized. In addition to transcriptional regulation involving the alternative sigma factor NtrA/RpoN/σ^{54}, *ntr*-regulated gene expression requires activation by the transcriptional activator NtrC. Mutations in *ntrC* had only transient effects on *R. meliloti* infection, generally delaying nodulation on alfalfa and normal nitrogen fixation capacity (Ronson et al., 1987b). In *B. japonicum* mutants, the loss of NtrC does not elicit a symbiotic defect, as demonstrated by continued *glnII* expression and effective nitrogen fixation (Martin et al., 1988). *ntrC* in *R. etli* appears to be cotranscribed with two upstream ORFs, one uncharacterized and the other *ntrB* (Patriarca et al., 1993). In free-living rhizobia, a β-galactosidase fusion to all three genes, *ORF1-ntrBC-lacZ*, is expressed only when cells are actively dividing (Patriarca et al., 1996). In determinate nodules of bean, the fusion is down-regulated in mature bacteroids (Patriarca et al., 1996). These bacteroids are located in the central region of the nodule where *ntr* expression is expected to be low because rhizobial nitrogen fixation is regulated mainly by O_2 rather than

by nitrogen levels. The *ORF1-ntrBC-lacZ* construct is expressed only in young bacteroids in cells peripheral to the central tissue of the nodule. Introduction of this construct into *R. leguminosarum* bv. *viciae* and subsequent inoculation of vetch indicated that gene expression occurs within infection threads and abruptly stops with the arrest of cell division, suggesting that this site represents a region low in oxygen.

In rhizobia, the ratio of glutamine to α-ketoglutarate levels is indicative of available intracellular nitrogen stores. When N is low (α-ketoglutarate > glutamine), the product of *glnD* is activated; *glnD* encodes a uridyl transferase/uridyl-removing enzyme. The product of the *glnB* gene, P_{II} protein, is then uridylated by the *glnD* gene product. This uridylation reaction leads to the deadenylation of GS (glutamine synthase), thereby activating it, such that GS becomes involved in glutamine biosynthesis. In addition to this role in nitrogen metabolism, P_{II} is required for nodule development but not for effective nitrogen fixation (Arcondéguy et al., 1997). Nodules induced by *R. meliloti glnB* mutants showed multiple defects, including deformed root hairs, inefficient infection, and numerous aborted infection threads. These defects delay the onset of successful infection and lead to a heterogenous mixture of nodules on alfalfa roots, ranging from small bumps to pink, Fix^+ nodules, indicative of high nitrogenase activity. As a result, in *R. meliloti*, P_{II} links nitrogen metabolism with nodule inititation.

Rhizobia may contain as many as three GS enzymes that are regulated differently depending on the environment (de Bruijn et al., 1989). *glnA*, *glnII*, and *glnT* (found in *R. meliloti* and *R. etli*) encode GSI, GSII, and GSIII activities, respectively, and are expressed at low levels in nodules; no *glnII* transcripts are detectable in bacteroids (de Bruijn et al., 1989; Arcondéguy et al., 1996; Shatters et al., 1993; Chiurazzi et al., 1992). Only the *B. japonicum glnA glnII* double mutant has been reported to be symbiotically defective, suggesting that GS is not essential for nodule development among all rhizobia (Carlson et al., 1985).

Carbon-related metabolism

Rhizobia utilize amino acids as a carbon source under both free-living and symbiotic conditions. For example, genes homolgous to an ABC transport system for oligopeptide uptake (*oppBCDF*) have been identified on the symbiotic plasmid of *Rhizobium* sp. NGR234 (Freiberg et al., 1997). Walshaw and Poole (1996) have described an unusual general amino acid permease (AAP) in *R. leguminosarum* with broad specificity for L-amino acids with diverse side chain structures. Mutational analysis of the four genes encoding this ABC transporter system, *aapJQMP*, confirms that these genes are required for amino acid import and export in free-living bacteria; however, as bacteroids, *aap*

mutants are Fix^+ within pea nodules. In culture and in bacteroids, the transport activity of AAP appears to be regulated by the availablity of amino acids, particularly aspartate and glutamate, synthesized by the tricarboxylic acid (TCA) cycle enzymes that are encoded by *mdh-sucCDAB* (Walshaw et al., 1997). Amino acid uptake is also inhibited in *phaC* mutants, resulting in the absence of poly-β-hydroxybutyrate, a storage source for carbon and/or electron sink (Cevallos et al., 1996; Walshaw et al., 1997).

In addition to these general uptake pathways, a second transport system (Dct) has been identified in a number of rhizobia that is specific for C_4-dicarboxylic acids, namely aspartate, malate, succinate, fumarate (Ronson et al., 1987a; Watson, 1990; van Slooten et al., 1992). Indeed, in *R. meliloti*, Robinson and Bauer (1993) have shown that at least two chemoreceptors are involved for taxis to aspartate and other C_4-dicarboxylic acids in conjunction with the Dct transport system. Uptake of these amino acids require three gene products, DctA, a membrane-associated transport protein, and DctB and DctD, a sensor kinase/response regulator pair of regulatory proteins. Indeed, Reid et al. (1996) have shown that the AAP transport system is inhibited in the presence of aspartate, allowing uptake via DctA. *dct* gene expression is regulated by RpoN and is essential for symbiotic nitrogen fixation; mutations in *dctA* and *dctB* lead to the formation of Fix^- nodules (Ronson et al., 1981; Jiang et al., 1989). *Rhizobium* sp. NGR234 *dctA* mutant nodules contained few bacteroids, whereas *R. meliloti dctA*-induced nodules exhibited early senescence (Engelke et al., 1989; van Slooten et al., 1992). However, despite the loss of nitrogen-fixing capacity, the overall morphology of nodules induced by *B. japonicum* and *R. trifolii dct* mutants is not dramatically altered (El-Din, 1992). Interestingly, two *dct* loci have been isolated from *Rhizobium* sp. NGR234, one corresponds to the plasmid-borne *dctA*, and the other to a chromosomal locus, *dctII* (Engelke et al., 1989; van Slooten et al., 1992). Unlike other *dct* mutants, *Rhizobium* sp. NGR234 mutants containing only *dctII* were capable of growing on dicarboxylates in culture. *dctA* is regulated independently of the chromosomally-encoded gene, lacks flanking *dctBD* genes, and appears to be exclusively required during nodule development; *dctA* mutants are unable to form nitrogen-fixing nodules on host plants.

During nodule development, intermediates of the TCA or Krebs cycle are utilized as energy sources (Kahn et al., 1995; for review, see Streeter, 1995). Several enzymes of the TCA cycle are essential for enabling bacteroid differentiation and nitrogen fixation. Accordingly, rhizobial mutants lacking these enzymes have symbiotic defects, often inducing Fix^- nodules on their hosts (Ronson et al., 1981). Early reports on *R. meliloti* TCA cycle mutations indicate that loss of both succinate dehydrogenase and α-ketoglutarate dehydrogenase (KGDH) resulted in Fix^- nodules; this has recently been

confirmed with *R. leguminosarum sucA* and *sucD* mutants (Gardiol et al., 1982; Duncan and Fraenkel, 1979; Walshaw et al., 1997). At least one other component that complexes with KGDH, lipoic acid, is not involved in nodule function because *lipA* transcription is down-regulated in bacteroids and nondividing cells (Taté et al., 1997). Green and Emerich (1997a) determined that *B. japonicum sucA* mutants lacking KGDH activity can still grow on succinate or malate, implying that the gene is not essential for growth. In addition, the soybean nodules elicited by *B. japonicum sucA* mutants are Fix^+ (Green and Emerich, 1997b). In contrast, *R. leguminosarum sucA* mutants formed small, Fix^- nodules that are absent of bacteroids (Walshaw et al., 1997). However, the soybean nodules did exhibit a symbiotic defect, namely, a lag in the onset of nodulation and formation of only ~20% of the normal number of bacteroids per nodule (Green and Emerich, 1997b). The authors suggest that the defect occurs before the release of the rhizobia from the infection threads, perhaps during growth within the threads. They further note that the mutant nodules resemble *sipS*-induced nodules (discussed below).

The TCA cycle enzyme phosphoenolpyruvate carboxykinase (PEPCK), catalyzing the first step in the conversion of TCA intermediates into hexose sugars, is also required for effective nodule formation. Encoded by *pckA* in *R. meliloti* and *Rhizobium* sp. NGR234, PEPCK activity has been measured in symbiotic and free-living bacteria (Osterås et al., 1991, 1995a). Fusions of *pckA* to *lacZ* indicate that the gene is induced in succinate-supplemented media, under nutrient limiting conditions, and as rhizobia enter stationary-phased growth (Osterås et al., 1995a). Transcriptional regulation of *pckA* expression involves RpkA, a putative repressor that has not yet been fully characterized (Osterås et al., 1997). However, despite detectable levels of PEPCK activity in *Rhizobium* sp. NGR234 bacteroids, nodules elicited by *pckA* mutants contain few bacteroids, are Fix^-, and show early senescence of infected cells (Osterås et al., 1991). Interestingly, different hosts of *Rhizobium* sp. NGR234 are capable of varying degrees of nitrogen fixation, suggesting that host factors may influence gluconeogenesis in bacteroids.

R. meliloti mutants lacking isocitrate dehydrogenase (ICD), the enzyme acting at a branch point in the TCA cycle, also elicit Fix^- nodules on alfalfa (McDermott and Kahn, 1992). In culture, however, a fraction of the mutants are glutamate auxotrophs, developing small colonies on plates. Loss of a second TCA cycle enzyme, citrate synthase (CS, encoded by *gltA/ccsA*), and the inability to produce citrate also characterizes these mutants. During nodule development, CS is yet another essential enzyme for normal bacteroid function because *gltA/ccsA* mutants induce small, white nodules devoid of bacteroids (Kahn et al., 1995; Hernández-Lucas et al., 1995). ICD^-CS^- double mutants elicit callus-like bumps on alfalfa, indicating an involvement of the gene

products early in nodule development (McDermott and Kahn, 1992). In *R. tropici*, two *gltA/ccsA* genes are present, a chromosomal and a pSym copy, each with distinct regulatory sequences (Hernández-Lucas et al., 1995). Although mutants lacking either the chromosomal copy or the plasmid-encoded copy (*pcsA*) exhibit diminished CS activity and form up to 50% fewer nodules on bean, the nodules elicited by these mutants are Fix^+ (Hernández-Lucas et al., 1995; Pardo et al., 1994). PcsA appears not to be required for free-living growth, because *pcsA* mutants show wild-type levels of growth and CS (Pardo et al., 1994). However, loss of both copies of the genes encoding CS severely affects the ability of the rhizobia to nodulate; nodules are Fix^- and devoid of bacteroids (Hernández-Lucas et al., 1995). These observations suggest that *pcsA* is required early in development prior to nitrogen fixation, perhaps in the infection thread during nutrient acquisition (Pardo et al., 1994).

A key enzyme linking carbon and nitrogen metabolism in rhizobia is aspartate aminotransferase (AAT), which is involved in metabolism of aspartate and conversion of TCA cycle intermediates into other amino acids. AAT catalyzes the reversible conversion of aspartate and 2-oxoglutarate to glutamate and oxaloacetate (Alfano and Kahn, 1993). Several homologous genes encoding this enzyme have been isolated from *R. meliloti* (*aatA*, *aatB*, *tatA/aat1*, and *batA/aat2*) (Watson and Rastogi, 1993; Alfano and Kahn, 1993; Kittell et al., 1989). *aatA* is capable of complementing an *E. coli* aspartate auxotroph and appears to be required for symbiosis, unlike *aatB* and *tatA* (Rastogi and Watson, 1991; Alfano and Kahn, 1993). *tatA* appears to be required to maintain wild-type levels of growth in minimal medium and, therefore, may play a role in the rhizosphere (Alfano and Kahn, 1993).

Malic acid enzymes convert the TCA intermediate malate to pyruvate. In many organisms, including rhizobia, two malic acid isozymes, DME and TME – containing an NAD^+ and an $NADP^+$ cofactor, respectively – are present. Both enzyme activities have been isolated from free-living cells and nitrogen-fixing bacteroids (Driscoll and Finan, 1993). To determine whether DME or TME has a specific role during nodulation, mutants in the genes encoding these enzymes (*dme* and *tme*) have been isolated from *R. meliloti*; only *dme* mutant-induced nodules are Fix^- (Driscoll and Finan, 1993, 1996, 1997). The symbiotic defects in these nodules arise at the onset of nitrogen-fixation, affecting bacteroid development within zone III. It appears that in nodules, *tme* mutations can be compensated for by high *dme* expression and by the ability of DME to utilize $NADP^+$ (Driscoll and Finan, 1993, 1997). A plasmid carrying *tme*, however, cannot complement *dme* mutants (Driscoll and Finan, 1996). Interestingly, only low levels of TME are detected in *R. meliloti* bacteroids in alfalfa nodules, whereas considerably higher levels have been reported in bacteroids of *B.*

japonicum-induced nodules. This suggests that *B. japonicum tme* mutants may have symbiotic phenotypes (Driscoll and Finan, 1996; Copeland et al., 1989).

Respiration complexes

The enzyme nitrogenase is sensitive to oxygen; consequently, the environment surrounding bacteroids is microaerobic (Hennecke et al., 1993). Within the low oxygen environment of the peribacteroid membranes and differentiated rhizobia, energy for nitrogen fixation (at least 18 ATPs/N_2 fixed) is obtained by respiration-driven metabolism from membrane-associated cytochrome complexes with terminal oxidases having different affinities for oxygen. As rhizobia make the transition from an aerobic, free-living organism to a microaerobic symbiont, the composition of these complexes change. It is possible that these changes are initiated within the infection threads prior to differentiation (Hennecke et al., 1993). To date, a number of different cytochrome complexes with covalently-attached heme or heme/copper cofactors that are required at different stages of nodule development have been well-characterized (Appleby, 1984; Hennecke et al., 1993; Thöny-Meyer et al., 1994; Surpin et al., 1996). In addition to the genes encoding the cytochromes, there are a host of accessory genes required for the proper regulation, assembly, co-factor association, and membrane localization of these respiratory complexes.

The cytochrome complex aa_3 (partially encoded by *coxA*) is required under aerobic conditions (Bott et al., 1990, 1992; Gabel et al., 1994). Even though the protein is present within *B. japonicum* and *R. tropici* bacteroid membranes, the loss of this cytochrome has been shown not to have an effect on nitrogen-fixing ability of soybean nodules (Keister and Marsh, 1990; Gabel et al., 1994). Another operon, *coxMNOP*, with homology to *coxA*, is present in *B. japonicum*, but does not appear to be essential in free-living or symbiotic cells (Bott et al., 1992). The role of any of these components within this microaerobic environment is unknown. Similarly, three other cytochromes, c_{550}, c_{552}, c_{555}, designated by their absorption spectral peak characteristic for *c*-type cytochromes, also appear not to be required during nodule development, even though the proteins have been isolated from bacteroids. Mutations in *cycA*, *cycB*, or *cycC*, genes encoding these three cytochromes, have no effect on nitrogen fixation for soybean (Bott et al., 1995; Rossbach et al., 1991; Tully et al., 1991). And finally, loci encoding cytochrome P-450 have been identified in *B. japonicum* and *Rhizobium* sp. NGR234 (Tully and Keister, 1993; Freiberg et al., 1997). Expressed in *B. japonicum* cells grown anaerobically and detected within nodules, cytochrome P-450 is not essential for bacteroid function, and its role in respiration has not yet been defined (Tully and Keister, 1993).

Genes for *c*-type cytochromes have been identified in *R. meliloti*, *R. etli*, and *B. japonicum* that are important in both free-living and symbiotic cells (*cycH*, *cycM*, and *cycVWX*) (Bott et al., 1991; Ramseier et al., 1991; Ritz et al., 1995; Delgado et al., 1995; Kereszt et al., 1995; Tabche et al., 1996). Mutations in *cycHJKL* genes, encoding integral membrane and membrane-anchored components of the cytochrome *c*-heme lyase complex, have pleiotrophic effects (Ritz et al., 1993, 1995; Delgado et al., 1995; Kereszt et al., 1995). *cycVWX*, encoding putative ABC translocator proteins, and a neighboring gene, *tlpB*, encoding a thioredoxin-like protein, are also required for cytochrome *c* biogenesis in *B. japonicum* (Ramseier et al., 1991). Recently, homologs to *B. japonicum cycVW*, designated *ccmA* and *ccmB*, have been found in *R. etli* (Aguilar and Soberón, 1996). A *cycX* homolog has been isolated from *R. leguminosarum* bv. *viciae* as well as *cycY*, which encodes a thioredoxin-like protein (Vargas et al., 1994). In free-living *R. meliloti*, *cycHJKL* mutants are unable to maintain high nitrate reductase activity (Rnr^+) during exponential growth. Yet in *B. japonicum*, such mutants are unable to grow anaerobically on nitrate (Ritz et al., 1995; Kereszt et al., 1995). Within bacteroids, mutations in these genes result in Fix^- nodules on host plants (Hennecke et al., 1993; Soberón et al., 1993). In *R. meliloti*, *R. etli*, and *B. japonicum*, the loss of these respiration complexes is dramatic – few bacteroids are found within the nodules induced by these mutants, whereas in *R. leguminosarum* bv. *viciae*-induced nodules, only a minimal loss of bacteroids occurs. None of the bacteroids, however, are able to fix nitrogen (Soberón et al., 1993; Ritz et al., 1993, 1995; Delgado et al., 1995; Kereszt et al., 1995).

The *c*-type cytochrome CycM is membrane-anchored and appears to be required for assembly of a supercomplex in which electrons are transferred from the cytochrome bc_1 through CycM to aa_3 to oxygen (Bott et al., 1991; Hennecke et al., 1993; Wu et al., 1996). Proper assembly of cytochrome aa_3 requires numerous accessory proteins, including *tlpA* (Loferer et al., 1993). *B. japonicum tlpA* mutations lead to defects in developing bacteroids, including a loss of nitrogen fixation and low levels of oxidase activity due to the loss of functional holocytochrome aa_3. This occurs even though at least one of the three structural proteins (encoded by *coxA*) is synthesized and exported to the cytoplasmic membrane. Loferer et al. (1993) suggested that the unusual location of TlpA, anchored to the cytoplasmic membrane with its active site exposed to the periplasmic space, enables this enzyme to function as a periplasmic protein disulfide oxidoreductase. While TlpA is required in free-living *B. japonicum* grown under aerobic conditions for assembly of cytochrome aa_3, the loss of the protein does not appear to affect growth rate. These cells are capable of infecting soybean roots similar to wild-type cells, and in addition, infection threads develop normally. However, *tplA* mutant-induced

nodules are devoid of bacteroids (Loferer et al., 1993). Hence, although cytochrome aa_3 itself is not required for nitrogen fixation, the passing of electrons from cytochrome bc_1 to aa_3 makes the assemblage of this supercomplex crucial during nodule development and nitrogen fixation.

In *B. japonicum*, cytochrome bc_1 is encoded by *fbcH*, a fusion gene incorporating both *b* and c_1 activities (Thöny-Meyer et al., 1989, 1991). In *R. leguminosarum*, these gene products are encoded by *fbcB* and *fbcC* (Wu et al., 1996). Loss of either FbcH or FbcF, the Rieske iron sulfur protein, has no affect on aerobically grown *B. japonicum* but leads to the formation of Fix$^-$ nodules on soybean (Thöny-Meyer et al., 1989). This complex is the pivotal branch point for electron transfer to a cytochrome *c* of the cbb_3-type (Preisig et al., 1996). In studies measuring respiration and nitrogenase activities in isolated *R. leguminosarum* bacteroids, several groups have argued that a cytochrome with high affinity for nanomolar concentrations of free oxygen, like cytochrome cbb_3, is crucial for maintaining nitrogen fixation (for review, see Appleby, 1984; Haaker et al., 1996). The *fixNOQP* genes, encoding the cbb_3 complex, are specifically induced within nodules, and are required for nitrogen-fixation (Batut et al., 1989; Preisig et al., 1993). A second complex with high affinity for O_2, cytochrome bb_3, that is encoded by *coxWXYZ*, is also expressed microaerobically in *B. japonicum*-induced nodules (Surpin et al., 1996). Two *fixNOQP* operons have been identified in *R. leguminosarum bv. viciae* (Patschkowski et al., 1996; Weidner et al., 1996). Although the regulation of each operon has not been defined, the transcriptional activator Fnr may be involved (Gutierrez et al., 1997). Recently, a gene encoding PurF, which represses cytochrome cbb_3 production in free-living cells has been identified in *R. etli* (Soberón et al., 1996). *purF* mutants, like Pur$^-$ *R. etli* are defective in infection. These observations reiterate the requirement for this respiration pathway during nodule development.

Other genes

Characterization of an increasing number of rhizobial gene products indicate that the bacterial symbionts and hosts exchange numerous signals during nodule development. Mutations in a number of these genes hamper molecular communication, alter complexes spanning the peribacteroid space, disupt nodule development, and block nitrogen fixation.

mel/mep

Rhizobia grown for extended periods in laboratory media supplemented with L-tyrosine and $CuSO_4$ become pigmented due to the production of melanin by the enzyme tyrosinase (Borthakur et al., 1987; Mercado-Blanco et al., 1993).

Encoded by *melA/mepA* (for melanin production), melanin pigmentation occurs in free-living bacteria and may play a role in symbiosis. Although *mepA* from *R. meliloti* appears not to be regulated by σ^{54} or NifA, an *R. etli* gene required for melanin synthesis is regulated by NifA (Mercado-Blanco et al., 1993; Hawkins and Johnston, 1988). Indeed, one of the originally isolated genes required for melanin production, the class II *mel*, encodes *nifA* (Borthakur et al., 1987; Hawkins and Johnston, 1988). A second regulatory gene, *melC*, with similarities to *ntrA/rpoN* also appears to be required for nitrogen fixation and melanin production in *R. leguminosarum* (Hawkins et al., 1991). Melanin production appears to be required in *R. etli* for the induction of Fix^+ nodules, but is not required in *R. meliloti*. While the contribution of melanin during nodule development is unclear, it has been suggested that in senescent nodules, melanin may provide a protective effect by detoxifying phenolic compounds (Borthakur et al., 1987; Hawkins and Johnston, 1988). Indeed, various phenolics have been measured in alfalfa nodules and are increased in senescent tissues (Vance, 1978).

Host-specific genes

The contribution of host-specific genes during nodule and bacteroid development has mostly focused on *nod/nol/noe* and *exo/exp* gene products (see van Rhijn and Vanderleyden, 1995). However, several rhizobia appear to nodulate hosts differentially, producing Fix^- or Fix^+ nodules on different host legumes. For example, a Tn*5* insertion at one chromosomal locus in *Rhizobium* sp. TAL1145, located ~15 kb from *ndvAB*, elicits the formation small, Fix^- nodules on bean, similar to *ndv* mutants; nodules on leucaena, however, appear normal (Pooyan et al., 1994). The authors suggest that the gene product(s) encoded at this locus may be requred for functions essential for the development of determinate nodules but not for indeterminate hosts.

Chun et al. (1994) identified a gene (*hsfA* for host-specific fixation) in *B. japonicum* which, when mutated, results in bacteria that induce the delayed development of small, white, ineffective nodules on cowpea host plants, but elicit normal, Fix^+ nodules on two other hosts, soybean and siratro. *hsfA*, encoding a putative 11 kD protein with no known homologies, is expressed exclusively in bacteroids under the regulation of σ^{54} but not NifA. *hsfA* nodulation defects occur after the release of the bacteria from infection threads. The appearance of highly vacuolated plant cells with few differentiated rhizobia at this stage in nodule development is characteristic of early senescence. The authors propose that HsfA recognizes or interacts with host-specific factors, perhaps signaling molecules or carbon substrates to ensure correct expression of the nitrogen fixation machinery.

Another set of genes that have host-specific effects is *rtx*, encoding rhizobitoxine, a toxin that induces chlorosis on the aerial portions of plants

(Ruan and Peters, 1992). The toxic effects of rhizobitoxine arise from its inhibition of ethylene biosynthesis. Produced by *Bradyrhizobium* sp. under free-living as well as symbiotic conditions, this toxin appears to have nodulation and nitrogen fixation defects on different hosts. For example, *B. japonicum rtx* mutants, even those unable to produce rhizobitoxine *in planta*, form Fix^+ nodules on soybean (Ruan and Peters, 1992). On mungbean, however, *B. elkanii rtxA* mutants elicit only a few normal nodules and many bumps containing aborted infection threads and collapsed host cells (Peters et al., 1997). While the contribution of rhizobitoxine to the *B. elkanii*-mungbean symbiosis is not well understood, it appears to be required for effective nodulation and, like HsfA and Lcr (see below), may be involved in suppressing the regulatory actions of phytohormones (Peters et al., 1997).

eff

Recently, 21 *R. meliloti* Tn*5* insertion mutants, termed Eff^{++} (for enhanced symbiotic effectiveness) have been identified by Sharypova et al. (1994). These mutants are capable of increasing plant nitrogen up to 27% without altering nodule number or rate of acetylene reduction. Accelerated growth of the free-living cells is not the reason for increased effectiveness because the mutants grow at the same rate as their parent strain in liquid culture. *eff* genes are present on the chromosome and symbiotic megaplasmids but not on cryptic plasmids, the site of many other genes involved in nodule competitiveness (Sharypova et al., 1994; Mercado-Blanco and Toro, 1996). To date, although the genes have not been isolated, their role in nodulation appears to be involved in augmentation of nitrogen assimilation.

lcr

Tn*5* insertions in two of five chromosomal and symbiotic plasmid loci in *Rhizobium* strain IC3342, a tropical legume isolate, result in mutants that form Fix^- nodules on their host legumes (Upadhyaya et al., 1992). These insertion mutants have a second phenotype that affects the aerial portions of the plant – the loss of the ability to curl leaves. Interestingly, the $Curl^+$ phenotype arises only after infection of plants, suggesting that a nodule or bacterially-produced compound, perhaps a cytokinin derivative, is transported from the roots to the leaves (Upadhyaya et al., 1991). Indeed, cytokinins have been detected in free-living cultures of rhizobia (see Taller and Sturtevant, 1991) and also in nodules (see references in Hirsch et al., 1997). To date, genes from only one of the chromosomal loci, one that does not affect nitrogen fixation ability, have been characterized. Two genes, *lcrB* and *lcrD*, encode putative transcriptional activators homologous to the global regulators *ompR*, *fnrN*, and *fixK* in *E. coli* and *R. meliloti*; these may be involved in the transport or synthesis of the curl-

inducing principle (Upadhyaya et al., 1991, 1992). It is intriguing that *lcr* loci affecting both Curl and Fix phenotypes link the synthesis and export of fixed nitrogen from nodules to the import of photosynthate from the leaves. It appears that rhizobia can influence the exchange of these substances not only within the nodule but also in other portions of the plant.

hem

The peribacteroid membrane and the spaces surrounding the differentiated bacteroids contain outer membrane proteins that are derived from the host plant and the bacteroid (see Verma, 1992). Heme cofactors participate in a number of these complexes required for the microaerobic environment of bacteroids. For example, within the inner membrane, cytochrome respiration complexes contain heme cofactors. FixL is a heme-containing oxygen sensor protein, whereas leghemoglobin, with heme cofactors supplied by the plant or symbiont, facilitates the diffusion of oxygen (O'Brian, 1996). Heme biosynthesis involves several key steps, including the formation of δ-aminolevulinic acid (ALA), a reaction that is catalyzed by ALA synthase, a subsequent step catalyzed by ALA dehydratase, and the penultimate step involving the incorporation of Fe^{2+} into protoheme via ferrochelatase (O'Brian, 1996). Despite the significant need for heme in bacteroids, *B. japonicum* mutants lacking ALA synthase (*hemA*) form Fix^+ nodules on soybean, suggesting that *hemA* is not essential for nitrogen fixation (Guerinot and Chelm, 1986). Interestingly, *hemA* mutants of *R. meliloti* and *Rhizobium* sp. NGR234 are Fix^- on their hosts (Leong et al., 1982; Dickstein et al., 1991; Stanley et al., 1988). This seeming discrepancy arises because *B. japonicum* is able to utilize plant-derived ALA for heme biosynthesis, such that *hemA* is not essential (Sangwan and O'Brian, 1991). Subsequent steps in heme biosynthesis in *B. japonicum* involving ALA dehydratase and ferrochelatase, however, do appear to be essential for bacteroid function. Mutations in the genes encoding these enzymes, *hemB* and *hemH*, respectively, lead to the formation of severely defective nodules containing few viable bacteroids (Chauhan and O'Brian, 1993; Frustaci and O'Brian, 1992). In free-living *B. japonicum*, uptake of ALA is negatively regulated by Lrp (for leucine-responsive regulatory protein), which acts as a global transcriptional regulator in *E. coli* (King and O'Brian, 1997). Symbiotic defects of *B. japonicum lrp* mutants have not been described. A *lrp* homolog has also been isolated from *Rhizobium* sp. NGR234 by subtractive hybridization screening; this gene is induced by plant-secreted flavonoids, suggesting that these regulatory genes play a role in the rhizosphere and during infection of the host plant (Perret et al., 1994; King and O'Brian, 1997).

sipS

A Tn*phoA* insertion within *sipS*, a gene encoding a signal peptidase, leads to the incorrect expression of numerous outer membrane proteins within bacteroid peribacteroid membranes (Müller et al., 1995a, 1995b). *sipS*, one of two homologous genes in *B. japonicum*, encodes a cytoplasmic membrane protein that can complement temperature-sensitive *E. coli lep* mutants. The SipS peptidase appears to be specifically required in bacteroids, although *sipS-phoA* fusions are also expressed in free-living cells (Müller et al., 1995a). Indeed, the loss of SipS becomes apparent in mutants during the transition from vegetative bacteria to differentiated nitrogen-fixing bacteroids. The nodule cells become highly vacuolated and devoid of bacteroids, suggesting lysis of the rhizobia. Perhaps most intriguing is the observation that certain nodulins, plant-encoded proteins that are expressed in nodules, are present at reduced levels within the peribacteroid membranes of *sipS* mutants (Müller et al., 1995b). Leghemoglobin levels were also reduced in mutant-induced nodules. This implies that accurate processing of outer-membrane proteins, from both the bacteroid and the plant, is necessary for proper nodule development. Indeed, the Fix$^-$ phenotype of *sipS* mutants occurs only *in planta* because *B. japonicum* mutants are capable of wild-type levels of nitrogen fixation under free-living microaerobic conditions. Müller et al. (1995b) also described a second *B. japonicum* mutant (184) with similar nodulation defects. The site of the Tn*phoA* insertion in this strain appears to lie in a different signal peptidase gene than *sipS*; the gene, however, has not yet been described.

5. Concluding Remarks and Future Prospects

The genes described in this review represent genes that, in addition to the commonly reviewed symbiotic genes (*nod/nol/noe, exo/lps/exp* and *nif/fix*), are involved in *Rhizobium* invasion and legume nodule development. These "other" genes encode products which are directly or indirectly important for normal nodulation and nitrogen fixation. Largely, we have restricted this review to those genes which, when mutated, have a symbiotic phenotype. However, some genes may play significant roles in nodule development but yet, in the laboratory, exhibit no symbiotic phenotype when mutated. We have included some of these genes because it is likely that their gene products are necessary for establishing a successful symbiosis under natural conditions. Nevertheless, we have ignored a large number of genes which are important for rhizobial growth and survival in the field, such as rhizopine/opine catabolism, carbon reserve storage, iron acquisition, and so on, because they do not appear to be directly involved in the symbiotic interaction.

The discovery that several "housekeeping genes" are within this group of so-called "other symbiotic" genes suggests that the gene products play important regulatory roles in acclimating the bacteria to new environments, particularly those within the plant host. In spite of the diversity of proteins encoded, however, only two distinctive symbiotic phenotypes – Nod^- and Fix^- – are usually noted, perhaps because they are so easily scored. Rhizobia exhibiting mutations in genes which are expressed in the rhizosphere usually induce a symbiotic phenotype that is Nod^-, whereas mutants in genes that are expressed in either the infection thread niche or peribacteroid membrane compartment elicit Fix^- nodules. With detailed examination, these descriptors can be characterized more accurately. For example, inoculation with rhizobia having mutations in various rhizosphere-expressed genes (*pur*, *hem*, and *ndv*, among others) elicits a Nod^+Fix^- phenotype. The classification "Fix^-" describes nodules of varying phenotypes, ranging from small "bumps" having limited cell division activity and few or no bacteria to normal-appearing nodules which are fully infected and in which bacteroid forms differentiate but yet do not fix nitrogen. The likely explanation for these varying phenotypes is that a particular gene product is required for a specific stage in nodule development, i.e., *hem* for the production of leghemoglobin and *ndv* for proper infection, and so on.

Fig. 2 summarizes the various stages of indeterminate nodule development and the points at which these various "other" genes are believed to affect the various stages of nodule formation. Genes for which a mutation does not lead to an obvious symbiotic phenotype are also included in the figure because these are presume to influence the symbiosis in some way. When a particular gene has been described for only determinate-nodule infecting rhizobia, we have extrapolated its potential effect on nodule formation to the indeterminate nodule situation where appropriate. Patriarca et al. (1996) have made similar correlations experimentally using *R. leguminosarum* biovars that elicit both determinate and indeterminate nodules.

The question now is: how many genes have been missed in various screens and studies, especially for the earliest stages of the interaction between the two symbiotic partners? These are the stages where we still see the largest gaps in our knowledge. Must rhizobia alter their global physiology to become primed for infection of plant roots? Do various rhizobial surface membrane proteins, such as those on fimbriae or pili, play an essential role in establishing the symbiosis? Which rhizobial genes are required for establishment of the infection thread? Are there back-up systems or gene redundancies that mask the effects of mutations in certain genes?

Several rhizobia have been utilized to generate genetic and physical maps (Osterås et al., 1989; Charles and Finan, 1990; Honeycutt et al., 1993; Kündig et

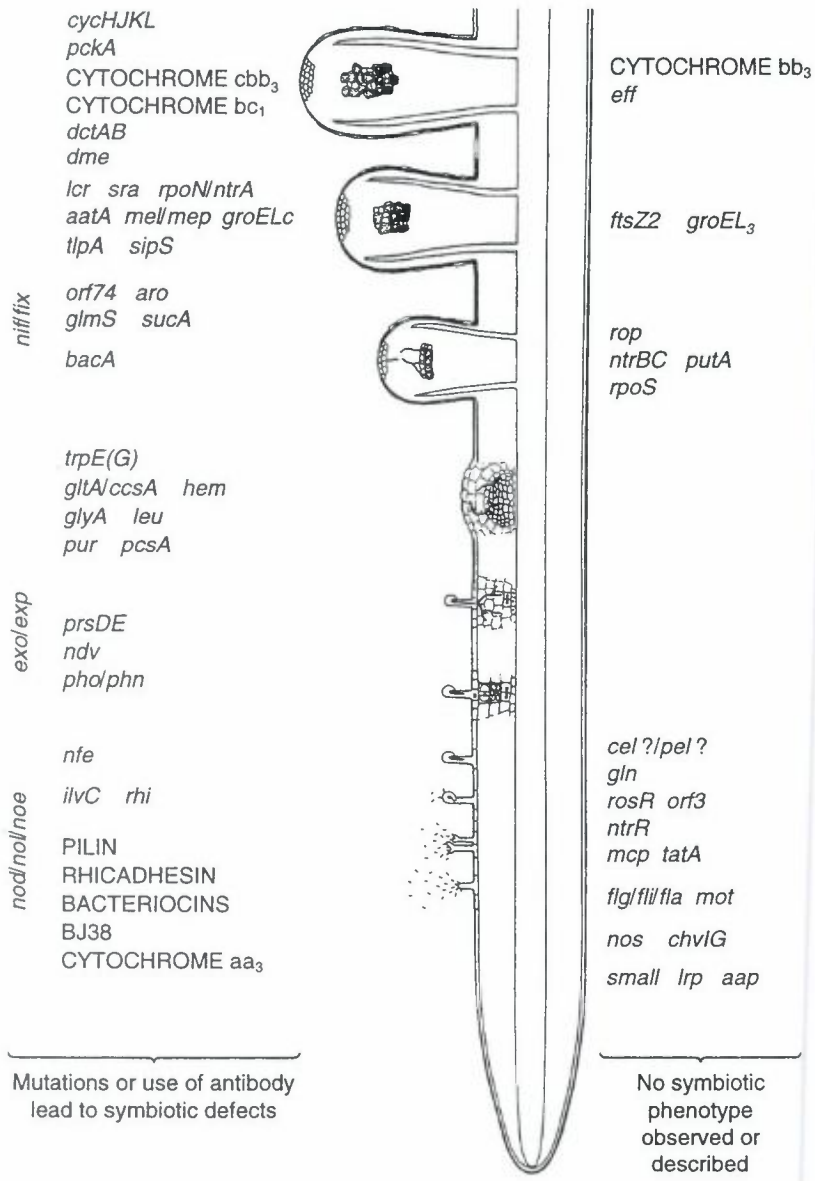

Figure 2. Diagram of the steps leading to nodule formation in a nitrogen-fixing indeterminate legume nodule. The genes (italicized) and proteins (capitalized) on the left-side of the diagram are indicated at a level in the developmental scheme where a mutation (or an antibody to a particular protein) affects the symbiosis. A loss-of-function phenotype has not been observed or described for genes and proteins on the right-side of the root. Modified from Akkermans and Hirsch (1997).

al., 1993), and recently, the complete nucleotide sequence of pSyma from *Rhizobium* sp. NGR234 has been determined (Freiberg et al., 1997). In spite of these important advances, we still know very little about the genomes of these agriculturally important bacteria. A *Rhizobium* genome project that is directed not only towards sequencing the symbiotic plasmids but also the chromosomes of diverse rhizobia will help us answer questions about the evolution of the symbiosis, as well as find new genes whose products may enhance the symbiotic interaction. In this way, we can come to a better understanding of how *Rhizobium* species orchestrate their close encounters with legume roots. With an ever-growing human population and continued loss of arable land as part of our collective futures, we should endeavor to discover effective strategies to augment crop productivity through biological nitrogen fixation as well as to develop methods to recover nitrogen-depleted soils. Not to do so will have serious consequences.

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