Rhizobium-Legume Nodulation: Life Together in the Underground

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Introduction

How good and how pleasant it is for organisms to dwell together in unity! And, probably, how common it is in the biological world. This review concerns a dramatic association, one of the few that has been studied in detail: the nitrogen fixing symbiosis between certain plants and microbes. Rhizobium bacteria stimulate leguminous plants to develop root nodules, which the bacteria infect and inhabit. Ultimately, the two organisms establish metabolic cooperation: the bacteria reduce (fix) molecular nitrogen into ammonia, which they export to the plant for assimilation; the plant reduces carbon dioxide into sugars during photosynthesis and translocates these to the root where the bacteria use them as fuel.

The plant family Leguminosae (Fabaceae) is the third largest family in the Angiosperms, spreads from the tropics to arctic regions, and includes forms varying from annual herbs to large trees. It doubtlessly owes at least some of this diversity and success to its ability to grow independently of often scarce soil nitrogen. Only one non-legume plant, Parasponia, has been found to form symbiotic root nodules with Rhizobium. The question of what makes the legumes unique is an important and provoking one. There is also considerable specificity of individual strains or species of Rhizobium for particular groups of plants, as shown in Table 1. The ecological and economic importance of nitrogen fixation has justified research attention for the Rhizobium-legume symbiosis. The system has an additional, fundamental attraction. During a complex series of developmental steps, the bacteria and the plant each influence in the other such fundamental activities as cell division, gene expression, metabolic function, and cell morphogenesis. Analysis of the bacterial influence on these processes may lead to identification of otherwise elusive components that are parts of the indigenous plant systems for signal transduction, gene regulation, cell division, and cell wall formation.

The driving forces for recent study of Rhizobium-plant symbioses include bacterial genetics, plant molecular biology, and detailed microscopy of the bacteria-plant interaction. This review highlights several questions of recent interest, with the focus on genetics and molecular biology; the references are representative, not exhaustive. A more complete view of the field can be found in two recent symposium volumes (Bothe et al., 1988; Verma and Palacios, 1988).

How Nodules Form

Nodules develop in a complex series of steps (Vincent, 1974; Newcomb, 1981). Rhizobium are chemotactic toward plant roots, probably due in part to specific plant attractants (Bergman et al., 1988; Caetano-Anolles et al., 1988). At the surface of the root, bacteria alter the growth of the epidermal hairs on the root, such that they grow deformed, even curled (Yao and Vincent, 1969; Dazzo and Gardiol, 1964). As this happens, the cells of the root cortex, under the epidermis, begin dividing (Libbenga and Harkes, 1973: Newcomb, 1981). Bacteria trapped in a curled root hair, or between a hair and another cell, proliferate and begin to infect the outer plant cells; as they do, the invaded plant cell is stimulated to produce a cell wall sheath, "infection thread" (Callaham and Torrey, 1981). As cell divisions in the plant root establish the body of the nodule, infection threads ramify and penetrate individual target cells within the nodule. Bacteria are released into the plant cytoplasm itself, enveloped in plant plasma membrane (Robertson et al., 1978). The bacteria and plant cells differentiate and begin symbiotic nitrogen fixation and metabolite exchange (reviewed by Sutton et al., 1981; Verma and Long, 1983).

Genetics of Rhizobium and Plants

Rhizobium genetics has been greatly advanced by transposon mutagenesis, recombinant cloning, and plasmid transfer experiments (reviewed by Kondorosi and Johnston, 1981; Denarie et al., 1981; Long, 1984). The fast-growing Rhizobium species typically have large plasmids, one or more of which carry symbiotic genes and are designated pSym. These vary from R. leguminosarum plasmids of about 200–300 kilobases (kb) to the large "megaplasmids" (1200–1500 kb) of R. meliloti. In some other symbionts, such as Bradyrhizobium, symbiotic genes are apparently not located on plasmids. Several groups of symbiotic genes—nod, exo, nif, and fix—are defined and discussed below.

Genetic analysis of plants also has been used successfully to define some host symbiotic loci (Rolfe and Gresshoff, 1988), although not yet at the molecular level. What molecular understanding we have so far of nodulation is due to both traditional biochemistry and physiology and also to direct molecular genetic analysis. This latter approach focuses on leghemoglobins and on nodulins, which are defined as additional gene products uniquely expressed in nodules. A comprehensive list of cloned nodulins has recently been published by Delauney and Verma (1988).

Early Events: Signals, Gene Expression, and Cell Behavior

In the initial stages of the symbiosis, the plant root surface and cortex are dramatically altered by Hizobium, and it is here that host specificity is first evident. Among the key questions are: How does the bacterium cause all of these changes in the plant? Given that the events are so similar on various host plants, why are they carried out by different bacteria for each group of hosts? The search for an-
swers to these questions has focused largely on the bacterial genetics, using plant microscopy and molecular biology to assay the success of the interaction.

Nodulation Genes
Rhizobium nodulation (nod) genes have been defined by sequencing, transposon mutagenesis, and, in some cases, protein analysis. Most gene definitions have emerged from the study of R. meliloti, R. leguminosarum biovar viciae, and R. leguminosarum biovar trifolii (Figure 1). Nodulation genes are defined by their effect on the bacteria's ability to cause prompt nodulation on the correct host, but they differ in the degree of severity of mutant phenotype. Mutants in nodA, nodB, or nodC are completely Nod- (no nodules form); these genes are required for bacteria to cause cell division (Dudley et al., 1987) and for deformation of root hairs (Djordjevic et al., 1985b; Kondorosi et al., 1984; Rossen et al., 1985; Bender et al., 1987). The nodABC genes appear to be functionally interchangeable among all Rhizobium (Kondorosi et al., 1984; Fisher et al., 1985; Marvol et al., 1985; Djordjovic et al., 1985a). Bacteria carrying mutations in other genes, such as nodEF, nodG, nodH, and nodLMN, eliciting normal root hair reactions on their usual hosts and sometimes eliciting root hair deformation and even curling on hosts they normally ignore (Debelle et al., 1986; Djordjovic et al., 1985b). These genes are not conserved, since alleles from different Rhizobium cannot substitute for each other on different host plants (Kondorosi et al., 1984; Djordjevic et al., 1985b). Based on this, nod genes are tentatively grouped as "common" and "host-specific" nodulation genes (Horvath et al., 1986). Until the biochemical functions of these genes are elucidated, it will be difficult to specifically assign them to genetic categories besides "nod" or to explain the pattern of conservation and divergence.

Early Plant Nodulins
A distinct category of plant nodule genes has been revealed by studies of early plant responses and of mutant nodule structures. These are the early nodulins, which are expressed in roots within a few days of exposure to Rhizobium. Among the most characterized is soybean nodulin N-75, carried in part on a cDNA clone pENOD2 (Franssen et al., 1987). The sequence of this gene reveals a highly proline-rich repeat motif, [Pro-Pro-Pro-X-Glu-Lys-Pro-Pro], which suggests that it may be related to the (hydroxy)proline-rich glycoproteins characterized as plant cell wall components.

The N-75 component is expressed in soybean roots within 6-7 days of inoculation by Bradyrhizobium japonicum, and, unlike the late nodulins (see below), N-75 and other early nodulins are expressed in "empty" (uninfected) nodules such as those elicited by certain bacterial mutants (see below; Govers et al., 1986; Norris et al., 1988; Dickstein et al., 1988). This suggests two things: first, that the trigger for early nodulin expression is produced by plant-bacteria interactions at the surface, probably involving the function of the known nod genes (Govers et al., 1986); second, that the function of N-75 and other early nodulins is most likely associated with nodule development, rather than with later differentiation (Franssen et al., 1987).

nod Gene Regulation
It has been observed in many systems that many Rhizobium nod genes are not expressed in cultured cells; an exception is nodD, which is constitutive. Most other nod genes are induced when cells are exposed to plant exudates or extracts (Mulligan and Long, 1985; Rossen et al., 1985; Innes et al., 1985; Horvath et al., 1987; Zaat et al., 1988; Davis et al., 1988; Surin and Downie, 1988). This induction depends on the nodD gene product. The increase in nod gene expression can be seen at the transcriptional level (Fisher et al., 1987). In some cases, such as R. leguminosarum, nodD also autogenously regulates its own expression (Rossen et al., 1985). In several plant-Rhizobium systems, the inducing molecules have been purified from plant exudates and identified as flavonoids, three-ring aromatic compounds derived from phenylpropanoid metabolism (Figure 2). In alfalfa and clover, the most active inducers were flavones, such as luteolin (3',4',5,7-tetrahydroxylavone) (Peters et

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**Table 1. Rhizobium-Plant Associations**

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<thead>
<tr>
<th>Rhizobium</th>
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<tr>
<td>Rhizobium melloti</td>
<td>Alfalfa</td>
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<tr>
<td>Rhizobium leguminosarum biovar viciae</td>
<td>Pea, vetch</td>
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<td>Rhizobium leguminosarum biovar trifolii</td>
<td>Clover</td>
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<td>Rhizobium leguminosarum biovar phaseoli</td>
<td>Bean</td>
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<td>Rhizobium fredii</td>
<td>Soybean</td>
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<td>Bradyrhizobium japonicum</td>
<td>Lotus</td>
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<tr>
<td>Rhizobium loti</td>
<td>Lotus</td>
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<tr>
<td>Azorhizobium caulinodans</td>
<td>Lotus</td>
</tr>
<tr>
<td>Bradyrhizobium spp.</td>
<td>Sesbania (stem)</td>
</tr>
<tr>
<td>Bradyrhizobium spp.</td>
<td>Parasponia (a non-legume)</td>
</tr>
</tbody>
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**Figure 1. Map of Nodulation Genes in Two Rhizobium Species**

(A) R. meliloti. (B) R. leguminosarum. Definition of genes by sequence or by complementation and/or mutagenesis can be found in the following references: Torok et al., 1984; Jacobs et al., 1985; Eglihoff et al., 1985; Eglihoff and Long, 1985; Schofield and Watson, 1986; Djordjevic et al., 1986; Debellé and Sharma, 1986; Horvath et al., 1988; Shearman et al., 1986; Fisher et al., 1987; Evans and Downie, 1986; Surin and Downie, 1988; and Davis et al., 1988. nodPGQ is described by J. Schwedock and S. Long (submitted). Note: nodX is found only in some strains of R. leguminosarum biovar viciae. Other nod genes are reported for R. leguminosarum biovar phaseoli (Lamb et al., 1985), Bradyrhizobium japonicum (Lamb and Hennecke, 1986; So et al., 1987), Azorhizobium caulinodans (Van den Eede et al., 1987), and broad host range Rhizobium and Bradyrhizobium strains (Scott, 1986; Scott et al., 1986; Bender et al., 1987; Backem et al., 1986; Lawton et al., 1987).
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Figure 2. Three Compounds Active as nod Gene Inducers in Different Systems.

(Top) Luteolin (3',4',5,7-tetrahydroxyflavone), the most active inducer of R. meliloti nod genes (Peters et al., 1986). (Center) Naringenin (4',5,7-trihydroxyflavanone), active in R. leguminosarum biovar viciae (Firmin et al., 1986; Zaat et al., 1987). (Bottom) Daidzein (4',7-dihydroxyisoflavone), a naturally occurring inducer of Bradyrhizobium japonicum nod genes (Kosslak et al., 1987).

That different compounds act as inducers or inhibitors in various legume-Rhizobium systems has been substantiated by studies using a variety of purified natural or synthetic compounds (Firmin et al., 1986; Zaat et al., 1987). Intriguingly, this has been shown to be an inhibitor of induction in the clover system (Djordjevic et al., 1986). Furthermore, the distinguishable responses of various Rhizobium to different compounds have been genetically linked to the particular nodD allele. In several cases, it has been shown that different nodD genes confer distinctive patterns of nod gene response to a variety of pure flavonoids or plant exudates (Spank et al., 1987; Horvath et al., 1987; Bassam et al., 1988). The broadness or specificity of Rhizobium response to inducers correlates with their nodD alleles. In R. meliloti and some other species, there are multiple nodD genes (Appelbaum et al., 1986; Göttert et al., 1986; Honma and Ausubel, 1987; Mulligan and Long, in press). It has been proposed that this confers broad responsiveness to various plant hosts (Györgypal et al., 1988; Honma and Ausubel, 1987).

This association of inducer specificity with naturally occurring nodD gene variants is sustained by genetic analysis of mutant nodD strains. Altered nodD genes created by mutagenesis or by recombination display several categories of mutant behavior, such as causing nod gene activation with no inducer and responding to a broader spectrum of inducer (Burn et al., 1987; H. Spaink and M. Djordjevic, personal communication). These genetic data all support a model in which the nodD gene product interacts directly with the plant signals.

Biochemical studies have been partially successful at defining NodD's role. NodD is a 33 kd protein, which by sequence homology and behavior is part of a newly defined family of transcriptional, activating proteins, including iesR, metR and others (discussed by Henikoff et al., 1986). The NodD protein binds the promoters of inducible nod genes, as detected by a shift in promoter electrophoretic mobility caused by Rhizobium extracts (Hong et al., 1987) or by purified preparations of R. meliloti NodD1 and NodD3 (Fisher et al., 1989). These inducible promoters are characterized by a long (about 50 base pairs [bp]), highly conserved sequence, the "nod box," first defined by Rostas et al. (1986) and now noted in many Rhizobium systems. The NodD protein displays a footprint on nod gene promoters of about 55-60 bp, which corresponds very closely to the extent of the nod box (R. Fisher and G. Long, submitted; A. Kondorosi, personal communication). These biochemical studies thus support the proposal that NodD is a DNA binding transcriptional activator. But these assays have not yet revealed the action of the inducer, and the involvement of additional proteins or factors also cannot be ruled out.

NodD should be an exciting protein to study, given that information on how it works may also be applicable to an entire series of positive activator proteins. In addition, it is at the center of a critical communication between two organisms: Is it the receptor for the plant signal? How did Rhizobium evolve a response to a compound that it cannot itself synthesize? At what level does the plant flavonoid signal have its effect? The goals of research in this area will include reconstitution of inducible nod promoter function in vitro, with determination of the role of RNA polymerase(s). NodD, inducers, and other components that may not yet have been identified.

nod Gene Function: Invasion and Plant Cell Division

How do the bacteria initiate infection? How is the infection thread built? And what signal(s) cause cell division, infection, and for cell division, implies that there may be common antecedent causes for the two events (reviewed by Dudley and Long, 1989). Other genes, which are involved in host selection (such as nodFE, nodH, and nodLMN), affect the location and tightness of root hair curling and the efficiency and persistence of cell division.

There are no known biochemical functions for the nod genes. Sequence and protein analysis give some intriguing hints but permit no firm conclusions (see references for Figure 1, and John et al., 1988). Because no specific mechanisms are known for any of the nod genes or the events they control, many researchers are working on the development of new assays for nod gene action. Rhizobium cells induced to express nodABC export a factor(s) that al-
fects root morphology and root hair growth (Van Brussel et al., 1988; Cantor-Cremers et al., 1988; J. Dénaire, personal communication). This factor may represent a soluble product of the nodABC gene action or a breakdown or by-product. Some lines of evidence have shown that nod genes, specifically nodABC, are sufficient to cause expression of early nodulins such as pENOD2 (Govers et al., 1988). This should present the opportunity to use reporter gene fusions to assay for purification of bacterial signals to the plant.

Two recent experiments have approached the nodule morphogenesis question from another angle. Long and Cooper (1988) reported that a plasmid causing constitutive synthesis of the cytokinin, zeatin, allowed Nod-(nodABC) Rhizobium meliloti mutants to cause primitive nodule formation on alfalfa. Because these mutants could not curl root hairs, cytokinin may imitate a secondary effect of the nodABC genes. That hormone balances may be central to nodule morphogenesis is also indicated by the report from A. Hirsch and T. Bisseling (submitted) that "anti-auxins" such as NPA (naphthylphthalamic acid) also cause alfalfa to elaborate nodules on its roots and that these nodules express early nodulins. This observation is made doubly interesting by the report of Jacobs and Rubery (1988) that flavonoids are also natural ligands of the NPA receptor and act as anti-auxins. Could flavonoids have a role beyond nod gene induction?

Accompanying cell division is the infection process, which is highly host-specific due to the action of several nod genes (Djordjevic et al., 1985a, 1985b, 1986; DeWelle et al., 1985; Horvath et al., 1986). How does plant-microbe recognition occur? When a wild-type Rhizobium invades, why does it not provoke a host defense response? The successful interaction of a Rhizobium and its host needs to be considered both in terms of successful presentation of positive factors and successful disguise of negative factors (Long, 1984; Djordjevic et al., 1987). Some of the nod genes, such as nodFE, may play a role in both (Djordjevic et al., 1985b; DeWelle et al., 1986). Working out the biochemical mechanism for the action of the nod genes may give insights not only into the basis for Rhizobium specificity but into the general nature of plant recognition and resistance.

Surfaces: Exopolysaccharides and the Infection Thread

After bacteria initiate infection, they must complete penetration and release into host cells. This requires correct bacterial surface components (acidic heteropolysaccharides, neutral glucans, and lipopolysaccharide) and correct plant components as well. The actions of some surface components occur not at the earliest host-specific nodulation stages but slightly later in the symbiotic interaction, specifically during infection itself.

Bacterial Polysaccharides

The bacterial extracellular polysaccharides include charged heteropolysaccharides, neutral β-glucans, and lipopolysaccharides (reviewed by Carlson, 1982). Among the possible roles for extracellular polysaccharides are signals or substrates for signal production, osmotic materials necessary during invasion, and recognition factors that act to present and/or to disguise the bacterium during invasion.

It is interesting to note that although the complete absence of certain polysaccharide structures often leads to "empty nodules" (uninfected), nonetheless, the host range of the mutant bacteria often remains the same (Finan et al., 1988; for a counterexample, see Chen et al., 1985). In such cases, the polysaccharides are apparently not the reservoirs (or at least, not the unique ones) of host-range determinants.

Genetic evidence for polysaccharide involvement in infection is very strong, and genetic studies should make it increasingly possible to identify which polysaccharides are involved in the Rhizobium symbioses. The exopolysaccharide synthesis (exo) genes are defined by their effect on the synthesis of extracellular or capsular polysaccharides. These have been most extensively defined in Rhizobium meliloti, which produces an acidic heteropolysaccharide (Leigh et al., 1985). An extensive, comprehensive map of one linkage group of exo genes has been recently published by O. Walker and colleagues (Long et al., 1988). Either the absence or the alterations of surface polysaccharides (Leigh et al., 1987; Müller et al., 1988) results in a symbiotic defect; the bacteria cannot invade the plant normally, although they do provoke the typical plant cell division pattern and often cause some root hair curling (Finan et al., 1985; Leigh et al., 1987; Müller et al., 1988). Such "empty nodules" were those used to discriminate expression of early and late nodulins.

Another set of genes are essential for the production and secretion of neutral β-1,2-cyclic glucan (Dylan et al., 1986). These genes affect nodule development, in that bacterial mutants fail to infect properly, and they are referred to as ndv loci. Two chromosomal loci, ndvA and ndvB (Stierlin et al., 1988), are highly conserved with the chv (chromosomal virulence) loci of Agrobacterium (Dylan et al., 1986) and are needed for cyclic glucan production. A role for small neutral glucans in osmotic adaptation has been proposed (Miller et al., 1986), which may have direct relevance to the function of bacteria in close association with plant cells.

A direct genetic analysis has been applied to lipopolysaccharides (LPSs) R. leguminosarum biovar phaseoli. Tn5 mutagenesis yielded an Ndv- mutant unable to establish normal infection threads on bean (Noel et al., 1986) and in which the LPS antigenic O-saccharide is defective (Carlson et al., 1987). For infection of bean, then, a complete LPS is not required for recognition, root hair curling, or the beginning of infection but is required for sustaining the infection process. Genes for LPS synthesis have recently been cloned (Cava et al., 1989).

It has been suggested that one or more of these polysaccharides interact with plant lectin proteins (reviewed by Dazzo and Gardiol, 1984; Graman, 1981; Halverson and Stacey, 1986), but the mechanistic role of such binding in the symbiosis is not known, and there is disagreement on whether the initial model for lectin-mediated recognition is generally applicable to all Rhizobium symbiotic systems.
Other adhesins have also been described, including a calcium-dependent pea-Rhizobium leguminosarum root hair adhesin (Smit et al., 1987). It is likely that multiple attachment mechanisms exist, some host-specific, others more general (Kijne et al., 1988).

Because these examples are so few, it is important to keep in mind a point stated by E. Signer, G. Walker, and others: namely, that the "information" carried in a surface polysaccharide is not necessarily exclusive to that one type of molecule. Other complex surface components may have equivalent information, mounted in a distinct, molecular setting. Different Rhizobium species, too, may display their information in various surface components (Borthakur et al., 1986).

**Plant Surfaces**

The plant surfaces—the cell wall, other secretions, and the underlying plant membrane—are also implicated in correct invasion and differentiation of bacteria into plants. The inside of the infection thread is packed not only with bacteria but with a matrix, whose origin has been a puzzle. Recent evidence presented by Bradley et al. (1988) shows for the first time that one component of the infection thread matrix is a plant-derived glycoprotein. Because of the intimate contact that is therefore occurring between this plant protein and the invading bacteria, this may be a key element in the recognition process.

The role of the plant cell wall itself is not well understood. Clearly, Rhizobium interacts with the plant wall and must have a means of recognizing and penetrating it. Whether or not penetration involves active degradation has been debated (Callaham and Torrey, 1981; Turgeon and Bauer, 1985; Ridge and Rolfe, 1986). Because the infection thread is new wall, however, it is clear that Rhizobium also alters wall synthesis itself, and therefore, must interact during penetration with the systems in the plant membrane or cytoplasm that deposit and synthesize various wall constituents (Robertson et al., 1978; Bauer, 1981).

**Differentiation and Symbiotic Function**

The events in the final stages leading to full nodule function include correct release and controlled proliferation of bacteria, followed by their differentiation and the differentiation of the host. Molecular analysis of this stage, more than any other, benefits from a wealth of background information about biochemistry and physiology. The partnership of these approaches is especially relevant to these questions: How does nitrogen fixation work in endosymbiotic Rhizobium? With what molecular components does the plant participate in the physiology of fixation and assimilation, and how are they produced? Combining these two questions, what signals and mechanisms are exchanged by the two partners to control each other?

The definition of Rhizobium genes and plant molecular components has been critical to the study of differentiation. In all nitrogen-fixing systems, there are predictable components and constraints (see Postgate, 1982). First, nitrogenase enzyme is irreversibly inactivated by oxygen; preventing oxygen damage is a physiological challenge universal to nitrogen-fixing organisms. The bacteria face the additional challenge of generating enough ATP to satisfy a massive requirement for it during nitrogenase function. Furthermore, it is necessary for the cell to assimilate ammonia efficiently; nitrogen-fixing organisms typically do this by the glutamine synthase-GOGAT pathway. The combination of these constraints leads to some interesting and unusual microbial adaptations. In the case of the symbiosis, the plant assists with bacterial physiology.

**Rhizobium nif and fix Genes**

Genes for nitrogen fixation in Rhizobium are generally divided into two groups: those with homologs in free-living nitrogen fixation systems, such as Klebsiella, are referred to as nif genes; those that are shown to be required for symbiotic nitrogen fixation, but whose function is not known to be analogous to a free-living function, are referred to as fix genes. Both nif and fix gene mutants are able to cause nodule development, but the nodules do not fix nitrogen (Nod+ Fix-). The nif and fix loci in three systems are shown in Figure 3.

Several interesting systems are not shown in Figure 3, including Azorhizobium caulinodans, a stem-nodulating bacterium that is unique in being able to grow by fixing nitrogen in the free-living state. This system has therefore been useful for the study of nif genes, nif and ntr regulatory genes, and the metabolic infrastructure supporting Rhizobium nitrogen fixation (Ludwig, 1986; Norel et al., 1985; Pawlowski et al., 1987). Another bacterium with interesting genetic features is R. phaseoli, which contains multiple copies of nif genes and complex patterns of symbiotic plasmids (Quinto et al., 1985; Soberon-Chavez et al., 1988).

**Background on Nodule Differentiation and Function**

Enzymology and cell studies have shown that plant cell physiology is modulated to accompany bacterially dif-
differentiated function (see Schubert, 1986; and volumes edited by Giles and Aherne, 1981; Verma and Hohn, 1984). Leghemoglobin (Lb) proteins bind oxygen then release it when the local concentration drops below a certain level, thus providing high flux for the Rhizobium to use in respiration, but in an environment with low free oxygen (Appleby, 1984). The plant supplies carbon compounds, derived from photosynthesis in the shoot, to the bacteria for generation of ATP and reduced electrons. The plant itself assimilates the primary fixation product (ammonia) into glutamine and other amino acids. Further conversion into ureides occurs in some plants such as soybean in specialized uninfect ed nodule cells. Enzymes for nitrogen assimilation (glutamine synthase, uricase) are thus prominent in nodules.

The final release of bacteria into plant cells is not well understood, but microscopic studies indicate that the plant plasma membrane envelops bacterial cells as they emerge from discontinuities in the infection thread wall (Robertson et al., 1978; Mellor and Werner, 1987). The bacteria undergo limited DNA replication and division, then cease both processes. They display distinct morphology and gene expression and are referred to as bacteroids (Sutton et al., 1981). The plant-derived “peribacteroid membrane” (PBM) undergoes changes in quantity and content (Verma et al., 1978; Brewin et al., 1985; Mellor and Werner, 1987; Bradley et al., 1988). The specialization of the PBM may include specific transport or permeability functions.

**Plant Genes: Leghemoglobin, Late Nodulins**

Leghemoglobins are interesting as proteins and as genes (reviewed by Appleby, 1984; Verma et al., 1986). They show considerable homology with and structural similarities to animal oxyhemoproteins such as hemoglobin (Appleby, 1984). They have been identified by either antibody or sequence analysis as being expressed uniquely in nodules (Legocki and Verma, 1980; Fuller et al., 1983; Dunn et al., 1988; Lullien et al., 1987). The plant-derived “peribacteroid membrane” (PBM) undergoes changes in quantity and content (Verma et al., 1978; Brewin et al., 1985; Mellor and Werner, 1987; Bradley et al., 1988). The specialization of the PBM may include specific transport or permeability functions.

**Patterns of Plant Gene Regulation**

Leghemoglobin (Lb) genes and most nodulins are transcribed uniquely in nodules (Fuller et al., 1983). Leghemo- globins in soybean typically appear within about 8–10 days after inoculation. The trigger for expression probably fires fairly early; for example, R. meliloti Exo- mutants that stimulate “empty nodules” (morphogenesis without invasion) do not stimulate the expression of leghemoglobin (Dickstein et al., 1988; Norris et al., 1988; Lullien et al., 1987). However, most bacterial mutants, such as those lacking nitrogenase genes, express leghemoglobin genes, although at lower levels (Lang-Unnasch and Ausubel, 1985). Most nodulins are expressed coordinately with leghemoglobin and are referred to as “late nodulins.” The mutant studies thus indicate that the signal for transcription of these nodulins occurs when the plant is first infected and results in expression of almost all nodulins. There may be some plant genes, however, whose correct expression depends on final release of bacteria into target cells (Morrison and Verma, 1987; Norris et al., 1988).

This system obviously provides very attractive opportunities for studying molecular aspects of gene expression. Analysis of transgenic constructs showed that promoter of the soybean leghemoglobin gene was shown to express appropriately when transformed into a Lotus corniculatus plant, although the Lotus nodules are formed by a different bacterium than that for soybean (Stougaard et al., 1987). This supports the idea of a nodule as a predictably differentiated plant tissue conserved across the legumes. The cloned genes and transgenic constructs should lead to a molecular analysis of gene regulation. Segments of conserved sequences upstream of several Lb and nodulin genes (Mauro et al., 1985) appear to be essential for regulation and functionally conserved across several legume groups (Stougaard et al., 1987). The next steps will be to identify the trans-acting factors that interact with these nodulin promoters (Jensen et al., 1988; Mauro and Verma, 1988) and to back-track through these proteins to the signal transduction pathway.

**Regulation of Bacterial Nitrogen Fixation**

Questions abound concerning the molecular regulation of nitrogen fixation in the symbiotic state. Free-living Klebsiella pneumoniae regulates transcription of nif genes via the nifA gene product and the ntr-encoded sigma factor. nifA itself is in turn activated by ntrC acting with ntrA (reviewed by Gussin et al., 1986). In Klebsiella pneumoniae, the regulatory genes include nifL, which mediates the physiological effect of O2.

While many details of nif regulation in Rhizobium are unknown, the knowledge to date about R. meliloti provides an example of how different it may be than Klebsiella. The symbiotic activation of nif genes is indeed dependent on nifA, but nifA is not activated by ntrC (Gussin et al., 1987). nifA expression is dependent upon low oxygen concentration (Ditta et al., 1987), whose effect is mediated by distantly linked fix genes (David et al., 1987, 1988; Virts et al., 1988). These loci have been sequenced and identified as two genes, fixL and fixU (David et al., 1988), and their sequence reveals them to fall into a newly defined pattern of two-component regulatory systems (Nixon et al., 1986). No nifL homolog in any Rhizobium or Bradyrhizobium has been found. In B. japonicum and R. meliloti,
the function of the nifA gene product itself is affected by oxygen concentration (Fischer and Hennecke, 1987; Beynon et al., 1988) nifA protein. Thus, nifA may incorporate functions of nifL in itself, and comparison of Bradyrhizobium and Rhizobium with other nifA sequences has provided clues as to structure-function relationships (Fisher et al., 1988). The regulatory patterns may differ in other Rhizobium systems (Pawlowski et al., 1987).

The Symbiotic State: Import-Export Controls and Nonproliferation

The universal problems of metabolic and cellular life—energy, reducing power, compartmentation, and growth regulation—are posed in a uniquely interesting way when two cells cooperate as intimately as Rhizobium and its host cell.

The nature of the carbon source supplied by the plant to the bacteria has been debated, and a prominent theory predicts that it is succinate or other organic acids (Dilworth and Glenn et al., 1984). This proposal is supported by the necessity of the Rhizobium dicarboxylate transport (dct) genes for nitrogen fixation (Ronson et al., 1981, Finan et al., 1983), by the fact that they are carried in R. meliloti on pSymb (Finan et al., 1988), and by the dependence of dct expression on the rpoN (ntrA) sigma factor (Ronson et al., 1987). Kahn et al. (1985) proposed that exchange of amino acids and/or other organic acids across the bacteria/plant interface could be a crucial driving force for nitrogen fixation. In this model, the bacterial export of ammonia is coupled to the import of carbon-skeleton compounds. A prominent role for amino acids is also indicated by the recent discovery (Kohl et al., 1988) that soybean symbiotic nodules contain highly elevated levels of pyroline-5-carboxylate reductase. The study of metabolite exchange is exciting because the interface of bacteroid and plant is a de novo boundary, the creation of which can be experimentally observed and manipulated, and which may yield insights related to the whole issue of cellular compartmentation and its evolution.

Accompanying the active transcription, translation, and metabolism of the nitrogen fixing state is another intriguing behavior: the bacteria stop dividing after an initial phase of replication. In some cases, the bacteria have on reduplicated their DNA (Van den Bos et al., 1978; Paau et al., 1978). At what stages in the bacterial cell division cycle are controls exerted? This relates to the central question of what makes a bacterium a symbiont instead of a pathogen; one of the ultimate criteria is that the bacterium must come under control of the host, now grow unchecked at its expense. Finding out how Rhizobium is controlled by its host may suggest ways in which other not-so-benign bacteria might be made subject to biocontrol.

Bacterial colony forming units are recovered from individual nodule plant cell protoplasts (Gresshoff et al., 1977), which probably represent an undifferentiated subpopulation (Zhou et al., 1985).

Those final topics are significant because they relate to the question of what is the evolutionary driving force, or selective advantage, of this association. Exploring all of the aspects of the symbiosis may yield new ideas on how the two partners balance sacrifice and gain. A taste of the insights to come is offered by the recent work of Murphy et al. (1987, 1988). They discovered that a particular strain of Rhizobium meliloti has genes, mos, that synthesize a unique carbon- and nitrogen-containing compound. These genes, which are carried on an auxiliary plasmid, are expressed in differentiated bacteroids and in fact are controlled by the ntrA-nifA system of regulation. The free-living bacteria express a set of genes for catabolism of the compound, these being designated moc. Thus, bacteria of the same strain occupying the outside of the nodule benefit from the activity of their descendents inside, since they uniquely can use this compound—a "rhizopine"—for carbon and nitrogen nutrition.

The psalmist sang that dwelling in unity is like precious oil running down from the head to the robe. While the symbiosis of Rhizobium and legume involves the earthward transport of more oxidized compounds, the image of abundance arising from togetherness is an appropriate one. It reflects both the benefits of symbiosis for organisms in nature and the growing understanding that has come with the cooperation of diverse approaches to the study of symbiosis.

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Note Added in Proof
