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RHIZOBIUM GENETICS

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CONTENTS

INTRODUCTION

Nitrogen fixation, the reduction of molecular dinitrogen to ammonia, is exclusively a prokaryotic feature. Numerous plants nonetheless benefit through symbiotic plant-prokaryote associations (141). One such association is that between the *Rhizobiaceae* bacteria (*Rhizobium, Bradyrhizobium,* and *Azorhizobium),* which infect plant hosts in a particular taxonomic family, the Leguminosae. The rapid pace of study on this symbiosis is due largely to the increasingly sophisticated genetics in the system. In turn, *Rhizobium* studies have produced interesting surprises for geneticists, such as: the largest plasmids yet described which may carry required housekeeping functions (pSym a and b, 1200 and 1500 kb, in *Rhizobium meliloti*); multiple genes for some

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functions, such as two glutamine synthetases, one of which resembles the eukaryotic GS gene; and a founding member of a new family of positive gene activator proteins *(nodD)*. This review highlights recent progress in *Rhizobium* genetics, and aims to point out opportunities for genetic studies both on symbiosis and on fundamental questions of bacterial function and inheritance. Examples are chosen largely from papers since 1986, and to emphasize study of genes rather than physiological or developmental aspects of mutant analysis. Other reviews of *Rhizobium* present more extensive discussions of general genetics (10, 11, 98), plasmids (32, 142) and symbiosis (42, 112, 113, 146).

RHIZOBIUM BEHAVIOR AND CLASSIFICATION

The symbiosis of *Rhizobium* and its host requires recognition of the bacteria and the plant root. The *Rhizobium* bacteria associate with the host's epidermal root hairs, and usually penetrate by deformation of the hair and subsequent formation of a specialized invasion structure, the "infection thread." Mitoses and cell growth in the plant root cortex lead to the formation of a root nodule, in which bacteria infect host cells and differentiate into "bacteroids" that fix nitrogen. This is of considerable physiological benefit to the host plant in nitrogen-limited conditions.

The *Rhizobium* bacteria were formerly in one genus, but have been reclassified in three genera within the Rhizobiaceae, *Rhizobium, Bradyrhizobium,* and *Azorhizobium* (43, 88). These form nodules on roots (and sometimes on stems) of various host plants. Species or subspecies (biovars) within these genera are defined to an extent by host range. Taxonomic questions continue to be important in *Rhizobium* research. It is striking that the symbiotic interactions, which are highly conserved functions, are directed by diverse genes whose location and organization vary, and that are borne by numerous different bacteria. The major *Rhizobium* groups discussed in this review are presented in Table 1. When I am referring to all three genera of organisms as a group, I use "*Rhizobium*" with no specific epithet. The phenotype of a strain in the free-living state is designated in a standard way, e.g. Trp^-. The phenotype for symbiotic gene changes are assigned by the properties of the plant and bacteria together: Nod^-: no nodules; Fix^-: nodules form, but no nitrogen fixation. Further refinements for naming symbiotic phenotypes have been proposed (133, 175).

RHIZOBIUM AS A GENETIC SYSTEM

Genetic analysis of all types depends upon finding or generating genetic variability, and upon having methods to move and map genes. Although there have been some innovations in the ten years since the use of broad host range

Table 1 Major groups of *Rhizobiaciae* bacteria used for genetic study

Bacterial species	Symbiotic host
Rhizobium leguminosarum	
biovar viciae	pea, vetch
biovar phaseoli	bean
biovar trifolii	clover
Rhizobium meliloti	alfalfa
Rhizobium fredii	soybean
Bradyrhizobium japonicum	soybean
Azorhizobium caulinodans	sesbania

R-factors and transposons gave *Rhizobium* genetics its initial push (10, 98), there remain several significant technical barriers to genetic analysis.

GENE TRANSFER AND VECTORS Transfer of genes between *Rhizobium* cells by transduction or conjugation has been reviewed (11, 98, 112). Gene transfer into *Rhizobium* from *Escherichia coli* is carried out by conjugation, and useful stable vectors therefore must be mobilizable, and broad host range (e.g. inc-P, incQ, or native plasmid origins; see 98, 142, and 41, 63, 150). Modifications of *Rhizobium* so that it is infectible by λ phage has been described (115).

R-prime construction has special usefulness for symbiotic gene mapping in *Rhizobium,* since broad host range inc-P plasmids such as RP4 (R68, RK2) can stably bear extremely large inserts. Although more difficult to work with biochemically than the smaller cloning vectors derived from them, such as pRK290, the R-primes permit a wider survey of genetic material that is useful for dispersed sets of genes (24, 89, 127, 161).

MUTAGENESIS AND MARKERS Chemical mutagens and UV mutagenesis have been widely used both for general genetic study and for symbiotic study. More commonly though, studies of *Rhizobium* genetics have used transposable element mutagenesis, particularly with Tn*5* and derivatives such as Tn*5-mob* (162), which includes the transfer origin for RP4, and Tn*5*s with altered markers (34, 79) or with reporter fusions (38, 110). Other transposons have also been used (16a, 122, 123, 153), as have standard reporter fusions for study of gene expression including *lacZ* (92, 172); *lux* (103); and *uidA* (125). Transposon delivery methods typically involve unstable plasmids that cannot replicate, or that can be eliminated by incompatibility after transfer into *Rhizobium* (10, 89, 160, 163). Similar plasmids are used to introduce directed or random interrupted gene mutations through double crossover,

cointegration into the genome, or transposon-mediated vector insertion (40, 72, 87, 151, 163).

Loss of vectors and cloned inserts during nodulation is a problem for functional studies in many strains of *Rhizobium* (41, 102). Marker exchange of insertions flanked by a neutral sequence resident in the chromosome is proposed as a method for introducing stable genomic copies of new or added genes. Among targets proposed for this job are the repeat sequences of *Bradyrhizobium japonicum* (1) and the *int-att* site for lysogenic phage 16-3 (137), which infects *R. meliloti* strain 41 and whose repressor bears an intriguing resemblance to that of phage λ, despite the difference in their target sites (26). In addition to families of repeat sequences (1, 73, 119), native transposable elements have been identified through insertions into symbiotic genes (44, 152, 178).

PLASMIDS Most strains in the *Rhizobiaceae* have large plasmids and in *Rhizobium* and *Azorhizobium* these appear to be the primary sites of genes for symbiosis; this topic has been reviewed (11, 32, 98, 142). Current studies indicate diversity and fluidity of plasmid-genome arrangements (62, 75, 156, 165, 180). The arrangement of symbiotic genes differs in various species. Symbiotic plasmids sort into incompatibility groups, but having the same origins does not necessarily relate plasmids in terms of markers such as *sym* genes (reviewed in 32, 98; 17, 75). It is thus genetically possible to introduce two different p*Sym* into the same strain, and such strains are also recovered occasionally in natural populations (75, 176a); the symbiotic behavior of such strains is not stable, and often one of the plasmids is lost during plant infection (176a). *R. meliloti* carries genes necessary for symbiois on two extremely large plasmids, termed megaplasmids, of about 1200 and 1500 kilobases (18), designated p*Syma* and p*Symb*. These megaplasmids also carry genes for metabolic functions including thiamine biosynthesis *(thi)* (54) and dicarboxylic acid transport (*dct*; 49, 55, 177). By contrast, a single *Rhizobium leguminosarum* bv. *phaseoli* 410-Kb plasmid (pCFN299) bears all the genes needed to confer nodulation and nitrogen fixation on recipient *Agrobacterium tumefaciens* (117). Even tighter clustering is shown by *Rhizobium* bv. *trifolii:* a 32-Kd segment of the symbiotic plamid (pSym) has all the genes sufficient for host-specific nodulation and nitrogen fixation if placed in a pSym-cured *Rhizobium* background (86). This observation could provide a new impetus to defining a minimum set of symbiotic genes, as genetic studies of *nif* in free-living systems were accelerated by the initial discovery of a *nif*-sufficient *Klebsiella pneumoniae* clone (36).

BASIC MOLECULAR BIOLOGY Few studies have been carried out on replication, recombination, transcription, and translation in *Rhizobium*. A *recA*$^-$ *R.*

meliloti has been produced by cloning the *R. meliloti recA* gene through functional complementation of the *E. coli* locus, and by subsequent site-directed mutagensis (12). Other recombination-defective strains have been described (136). RNA polymerase activity has been obtained in vitro from strains of *B. japonicum* (144), *Rhizobium leguminosarum* bv. *viciae* (114), and *R. meliloti* (59, 129). In vitro coupled transcription-translation systems in *R. meliloti* have been used to define protein products from symbiotic genes (45, 61). Basic studies on transcription are needed to show what rules govern the general activity of promoters in *Rhizobium;* to date, transcripts have been characterized for relatively few constitutive genes (*nodD:* 59; *hemA:* 107, 121; *trpE:* 6; *glnA:* 21; *flaA* and *flaB:* 140), and little relationship can be seen among these promoters. It appears that many *Rhizobium* promoters do not function in *E. coli* (45, 59, 80), although enough genes have been cloned by complementation of *E. coli* mutants *(recA, glnA, cya)* that this approach should probably be tried routinely. It should be noted that these interspecific complementation tests sometimes yield very unexpected results, for example *glnT* (31).

Much room remains for genetic innovations that will facilitate analysis of *Rhizobium*. Restriction-deficient bacterial strains that allow direct transformation by DNA prepared from *E. coli* would be very useful, as they would permit use of non-conjugatable, smaller plasmid vectors. Transducing phage are still not available for most strains of *Rhizobium,* and the phage that exist would be more useful if temperature-sensitive or other conditional variants were obtained. Genetic and regulatory analyses would benefit from improved vectors, such as plasmids with regulatable promoters for conditional expression. Basic studies on and mutants in replication, transcription, translation (e.g. suppressors) and other processes are needed. For example, it is intriguing that some symbiotic genes are transcriptionally sensitive to gyrase inhibitors (30, 134). Such observations can be pursued in more detail when more analyses of basic molecular functions are carried out in *Rhizobium*.

METABOLIC GENES

The genetic study of *Rhizobium* metabolism is significant both because metabolism is directly involved in symbiotic processes, and because work with easily scored metabolic phenotypes permits rapid progress in analyzing underlying genetic processes. Our understanding of the genetics and regulation of *Rhizobium* symbiosis will be limited unless we develop a more extensive knowledge base about metabolism and general genetics. It would be a great boon to the field if more laboratories each took a so-far unelucidated pathway and thoroughly analyzed its genes and regulation.

Amino acid metabolism has been of interest on general principles, and because of the possible role of amino acids as biosynthetic precursors to plant

hormones such as auxin and ethylene. Numerous studies have also found that certain amino acid pathway mutations affect ability to fix nitrogen symbiotically, although these symbiotic effects vary (10, 95, 97, 154, 158). Such studies will be more easily interpreted when specific metabolic defects are defined.

The pathway for tryptophan biosynthesis has received most genetic scrutiny and is of particular interest because tryptophan is the metabolic precursor to the plant hormone indole-3-acetic acid (IAA). Trp^- mutants of *R. l. viciae* were identified with pathway steps by Johnston and colleagues (reviewed in 98), by isolating *R. meliloti* R-primes that complemented each mutation, and sorting these according to complementation of known *Pseudomonas aeruginosa* mutants. The *R. meliloti trpE* gene identified in that study was cloned and sequenced by Crawford and colleagues (80); transcript analysis showed that transcription initiation occurs constitutively at a low rate, and that expression is regulated primarily by attenuation (6). Mutations in some but not all *trp* pathway steps cause symbiotic defects (G. Walker, personal communication).

Glutamine synthetase (GS) is the primary enzyme for ammonia assimilation in *Rhizobium,* as in other organisms. While enteric bacteria typically carry a single structural gene for GS, *glnA, Rhizobium* and *Bradyrhizobium* bacteria have two forms of GS, one of which resembles enteric GS *(glnA),* in that it functions as a 12-mer and is subject to adenylyl control, while the other GSII *(glnII)* resembles eukaryotic GS both in its gene structure and its function as an octomer, displaying no posttranslational regulation (27). The *glnA* and/or *glnII* genes have been studied in several organisms (21, 31, 51, 166). A third *R. meliloti*-DNA region able to complement glutamine synthetase function, *glnT,* has been identified; it appears to be a complex locus (31). In *R. meliloti,* both *glnA* and *glnII* are dispensible for normal symbiotic function, in that *glnA-glnII* auxotrophs still make Fix^+ nodules. What bacteroids do to obtain amino acids during nitrogen fixation is obviously an interesting question, and relates to the models for metabolite exchange discussed below. The various *gln* loci are differentially regulated in *B. japonicum* and *R. meliloti,* in that *glnA* is constitutively transcribed and subject to posttranslational regulation (21, 31), while *glnII* displays a typical *"ntr"*-type promoter and is transcriptionally regulated through the *ntrA-ntrC* circuitry (31) in response to nitrogen nutrition and oxygen level (2, 21, 31). *Azorhizobium caulinodans* is distinctive in having only one glutamine synthetase but two different glutamate synthases, one of which (the NADPH-dependent form, *glt*) has been cloned (38, 78) and is expressed independently of the *ntr*-regulatory circuit (78).

Some early mutant studies (10) proposed that purines and pyrimidines were essential for symbiotic function, which again might be suggested because of

their relationship to plant hormones. Some purine-requiring *Rhizobium* have symbiotic defects (94, 97, 132). In *R. phaseoli,* seven purine auxotrophs made Ndv^- nodules. Pathway analysis showed that all seven could be rescued by IACAR, two steps from synthesis of inosine, and four were blocked early in the pathway. The bacteria with such lesions were able to provoke nodule initiation on plants, but were unable to invade (132).

Some strains of *Rhizobium,* along with other soil microbes, are able to synthesize melanin. The Mel^+ phenotype sometimes is apparent only as colonies age; this dramatic result has provided the occasion for startling many a novice at *Rhizobium* genetics who revisits plates stored for a few weeks in the cold room. Melanin is derived from tyrosine, and the *mel* synthetic enzymes are typically encoded by *Rhizobium* plasmids (25). In a study of *R. l. phaseoli* (15), *mel* genes fell into two classes, one apparently synthetic (*mel* Class I); the other, *mel* class II, was regulatory on both *nif* and *mel* genes, and turned out to be *nifA* itself (A. W. B. Johnston, personal communiction). That *mel* is coregulated with some *nif* or *fix* functions implies that the pigment chemical or its derivative may have some symbiotic function.

The function of nitrogenase enzyme is dependent upon a low-oxygen environment, maintained in the symbiosis by a plant-encoded oxygen-buffering protein, leghemoglobin (Lb), which gives nodules a characteristic pink color. Genetic analysis has been used to track the source of the heme cofactor. The *R. meliloti hemA* gene, encoding amino levulinic acid synthase, the committed step leading to heme synthesis, is transcribed in both free-living cells and bacteroids from two different promoters with similar -10 sequences (106, 107). *R. meliloti hemA* mutants were Fix^-, implying that *Rhizobium* heme synthesis was necessary for production of functional leghemoglobin.

By contrast, *B. japonicum hemA*::Tn*5* mutants were Fix^+ and leghemoglobin was formed at normal levels (69). A further complication was the observation that *B. japonicum* mutants defective in cytochrome and possibly in heme synthesis, in some cases also lacked leghemoglobin (135). These varied results leave unresolved the exact *Rhizobium* genetic requirement for support of leghemoglobin and at the very least imply that various host-*Rhizobium* pairs may differ.

Another peculiarity of the nitrogenase enzyme is its high ATP requirement, and its obligate evolution of molecular hydrogen (H_2) during nitrogen reduction, with consequent loss of energy and reducing equivalents (138, 141). Energetics is thus a particularly interesting topic in *Rhizobium*. Genes for adenylcyclase *(cya)* have been identified in *R. meliloti* and in *B. japonicum* (68, 96) by complementation of *E. coli* mutants or by mutagenesis. There may be redundant genes for *cya,* which has complicated efforts to test its symbiotic function. In some *Rhizobium,* an uptake hydrogenase enzyme is

produced (Hup^+), which recycles the H_2 back to protons, regaining reducing power and saving some of the energy (141). The ability of *B. japonicum* to grow chemoautotrophically on CO_2 and H_2 through this uptake hydrogenase allows direct genetic study of Hup in this system (84, 101). Growth can also occur on CO_2 and formate, and genetic analysis of this trait has led to definition of a regulatory locus (120).

Genes for *hup* itself were cloned from *B. japonicum* by complementation of a Hup^- mutant. A 20-Kb DNA insert was sufficient to confer hydrogenase activity on *Rhizobium* and *Bradyrhizobium* strains with Hup^- background (101 and references therein). Deletion analysis of the cloned *hup* DNA indicated that a slightly different set of functions were required for Hup activity in free-living vs symbiotic *B. japonicum* cells, but functions for individual protein products (182) in this clone have not yet been biochemically demonstrated. Additional genes may be located elsewhere in the *B. japonicum* genome (84). Less is known about *hup* genes in other species of *Rhizobium*. Random mutagenesis and screening of a Hup^+ *R. l. viciae* strain yielded Hup^- mutants; all of these were phenotypically restored to Hup^+ by the cloned *B. japonicum* DNA, indicating functional and regulatory conservation of the trait (90). A general correlation is seen among numerous *R. l. viciae* strains between hydrogen uptake and DNA homology with the cloned *B. japonicum hup* DNA (109, 128).

NODULATION GENES

For phenotypic and genetic designation, nodulation is taken to mean emergence of nodules, functional or not. The bacterial genetics of nodulation has been most studied in *R. meliloti*, *R. leguminosarum* bv. *viciae* and bv. *trifolii*, and *B. japonicum* (reviewed in 42, 100, 112, 113, 146; additional references not covered in those reviews are indicated below). Mutations resulting in completely non-nodulating bacteria have been recovered in these and other *Bradyrhizobium*, *Rhizobium*, and *Azorhizobium* bacteria, and found to map in a cluster of four genes, *nodDABC* (see reviews; 118, 159, 164, 174, 179). Of these, *nodD* is read divergently and appears to be regulatory on *nodABC* and other *nod* operons. The *nodABC* genes function as allelic equivalents across some species with no effect on host range. Mutations in *nodA*, *nodB*, and *nodC* all have similar phenotype: almost no root hair curling or cell division in plants are caused by such bacteria.

Additional *nod* genes appear to influence nodulation efficiency on a given plant host, and to control host-plant selectivity (42, 100). Mutations in these genes are not complemented by symbiotic plasmids or clones from other *Rhizobium* species or biovars. Host-range determinants may act in combination with each other and with the common *nod* genes; they may function both

positively (required for nodulation of a plant) and negatively to prevent nodulation of nonhosts (146). Mutant analyses imply the *nodABC* genes act epistatically to the host-range genes (50). Because mutations in some *nod* genes cause rather subtle changes in phenotype, many were not identified by random mutagenesis and screening, but by transposons and or deletion mutagenesis of DNA segments linked to *nodABC,* or otherwise shown to affect nodulation and host range (see reviews; 5, 65, 74, 108, 130, 170). Nucleotide sequencing of these cloned segments has revealed numerous genes. Downstream of *nodABC* are *nodI, nodJ,* and sometimes *nodX* or other genes. In *R. l. trifolii* and *viciae, nodFEL* and *nodMN* are downstream of *nodD,* and regulated by it (42). In *R. meliloti nodFE,* a downstream gene *nodG,* and a divergent gene, *nodH,* are present in a distinct cluster, instead of being adjacent to *nodD* (100, 113). Two newly defined genes, *nodP* and *nodQ,* are downstream from *nodFEG* (23, 157). Additional *nod* genes may be present in *R. meliloti* and *R. L. viciae/trifolii,* and are also being defined in *B. japonicum* and broad host range tropical *Rhizobium* strains (5, 65, 74, 108, 127, 130, 161, 174).

REGULATION Although *nodD* is constitutive, most *Rhizobium nod* genes are not expressed in free-living cells. Exposure to plants or to plant exudates induces expression of these genes (reviewed in 42, 113); regulation occurs at least at the level of transcript abundance (61, 125). Regulation by plants may provide a route to discovery of new *nod* genes (7, 65, 149, 153). The factors in plant exudates that cause induction are small aromatic molecules, identified as various flavonoids (146 42). Induction of *nodABC* by plant exudates depends on *nodD;* the ability of *Rhizobium* to induce *nod* genes in response to specific flavonoid molecules, or to extracts of different plants, varies according to the species source of *nodD* gene placed in the strain (8, 83, 169). Some mutations in *nodD* create alleles that activate inducible *nod* gene expression in response to a broader range of plant-derived compounds, or at a higher basal level, or both (19, 167). In *R. l. viciae* and some other *Rhizobium,* the *nodD* gene product also regulates its own expression (42). Most *Rhizobium* strains have two or more loci showing strong physical homology to *nodD* probes (145). In a few cases, sequence analyses have been carried out that show these to be highly homologous to *nodD,* but only in one *Rhizobium* species, *R. meliloti,* have functional studies demonstrated that all the *nodD*-homologous loci display the NodD function of activating *nod* genes. In *R. meliloti,* there are three copies of *nodD* (71, 82, 125); the one linked to *nodABC* is *nodD1,* which activates transcription of inducible genes in response to luteolin; *nodD2* also responds to an unknown plant inducer distinct from luteolin (71, 82, 125). The *nodD3* product constitutively activates high rates of *nod* gene transcription, but is itself not expressed except under control of another

nodD-like locus, *syrM* (125; M. Barnett, M. M. Yelton & S. Long, unpublished observations).

The inducible *nod* gene operons contain a highly conserved sequence, designated as the "*nod* box" (149, 168), which probably represents a transcriptional control region for the *nod* genes (reviewed in 42, 113). The *nod* box is located approximately 26–28 bases upstream of the mapped transcript start sites for *nodA, nodF* and *nodH* in *R. meliloti* (61, 125). NodD proteins bind to the promoters for inducible *nod* genes (60, 81) and establish a 50–60 base pair "footprint" from about −25 to −85 upstream of the $^{+}1$ mRNA start sites (58, 99). Neither overall binding nor the footprint has so far been observed to be affected by inducer.

GENETICS OF CELL SURFACE COMPONENTS

The *Rhizobium* cell surface has long been of interest because it was hypothesized to have a role in early recognition. Recent genetic studies complement biochemical and cellular analyses. The primary subjects of interest are the extracellular polysaccharides (of which there are several types), the chemotactic and motility apparatus, and other components of undetermined function, which may contribute to bacterial surface–plant surface interactions.

EXOPOLYSACCHARIDES The categories and characteristics of cell surface carbohydrates in *Rhizobium* have been reviewed by Carlson (20). They are, briefly, the extracellular (or exo-) polysaccharide (EPS), capsular polysaccharide (CPS), cyclic β-glucans, and lipopolysaccharide (LPS). A protoype for genetic analysis of a *Rhizobium* surface is a series of studies by *R. meliloti* exopolysaccharides. Leigh et al (105) analyzed a series of *R. meliloti* Exo^{-} Tn*5* mutants both by genetics and by NMR analysis of purified EPS-I, an acidic hetero-polysaccharide. Six complementation groups were initially defined, in which mutations abolished production of EPS-I (Exo^{-}). These strains were able to cause nodule emergence, on the correct host, but were unable to invade (53, 105) (Ndv^{-}, *n*odule *dev*elopment, phenotype; 133). Similar approaches have yielded mutants and mapped, cloned genes in other *Rhizobium* systems, although genetic analysis is more preliminary (14, 24, 124).

The number of *R. meliloti* genes known to affect the extracellular polysaccharides has been increased through further mutagenesis of DNA linked to the six original *exo* genes, and through further screening for altered structure or regulation of polysaccharides (52, 110, 111). Mutations in at least eight genes *(exoA, exoB, exoC, exoF, exoL, exoM, exoP, exoQ)* result in an exo^{-} phenotype, that is, no acidic extracellular polysaccharide EPS-I is produced (111). Two genes have been defined that affect acidic substitution, *exoH* for succinylation (104) and another gene needed for pyruvylation (124); defects

in these genes also cause Ndv$^-$ phenotype. Additional genes, *exoN, exoG, exoJ, exoD* and *exoK*, have quantitative effects on exopolysaccharide production, but do not in every case affect symbiotic properties (111; G. Walker, personal communication). *Exo* genes are expressed during nodulation (93). Control of polysaccharide genes appears to be critical, as regulatory mutants in *R. meliloti* and also in *R. l. phaseoli* are in some cases Fix$^-$ (14, 37). Two negative regulatory elements in *R. meliloti* have been identified. The *exoR* locus is involved in the down-regulation of *exo* gene expression in response to nitrogen sufficiency; the *exoS* locus also affects *exo* gene expression, but to a lesser degree and its effect is not sensitive to nutritional status (37). Regulatory mutation *expR101* had the remarkable effect of derepressing synthesis of a second, structurally different exopolysaccharide termed EPS-II (64), through activation of at least seven genes, *expA, expC, expD, expE, expF, expG,* and the previously identified *exoB* (64; J. Leigh, personal communication). The second polysaccharide could functionally replace the EPS-I on one host plant, but not on several others.

LIPOPOLYSACCHARIDES A series of noninfective Tn*5* mutants of *R. l. phaseoli* were capable of deforming root hairs and stimulating cell divisions, but either did not invade or formed aborted infection threads (133). In one of these mutants, the lipopolysaccharide (LPS) structure was defective, in that the LPS lacked the O-antigen side chain. NTG mutagenesis yielded eight more strains with correlated defects in LPS and in symbiotic behavior. DNA segments containing two distinct sets of *R. l. phaseoli lps* genes have been cloned (22).

β-1, 2-GLUCANS The genes encoding functions required for β-1,2-glucans synthesis were identified in *R. meliloti* DNA that was homologous to, and able to replace functionally, the *chvAB* virulence genes of *A. tumefaciens*. In *R. meliloti* two genes were identified as *ndvA* and *ndvB* (46). The *chvAB* and *ndvAB* loci are correlated with the ability of the bacteria to synthesize and export the neutral, cyclic Beta-1,2-glucan, which is characteristic of the Rhizobiaceae (20). *R. meliloti* mutated in *ndvA* or *ndvB* are noninfective, or form aborted infection threads (46). The *ndvB* gene product appears to be a 235-kDa inner membrane protein reported to be involved in the synthesis of the glucan (181); the *ndvA* gene product is homologous to the *E. coli* export protein HlyB, and is postulated to be involved in export of the glucan to the cell exterior (171).

CHEMOTAXIS *Rhizobium* are chemotactic, and *R. meliloti* with mutations affecting flagellae, motility, and chemotaxis have been isolated and characterized (3). All such mutants are able to form normal nodules, perhaps with less

competitive ability. Among chemotactic mutants, most were generally deficient in reponse to all stimuli; however, two had lost the ability to swim towards general attractants such as amino acids, but retained the ability to be attracted by plant exudates. The genetic analysis implies the existence of a second, plant-specific pathway for chemotaxis, which shares some components with the general chemotactic pathway (9). The *Rhizobium meliloti* flagellae are complex in morphology (140), and there are in fact two closely linked genes for flagellae, *flaA* and *flaB*. These two flagellins are 87% similar to each other, and show very little resemblance to other flagellins; it is proposed that the two distinct proteins assemble as heterodimers to produce the complex filament form of *R. meliloti* flagellae.

NIF, FIX, AND *DCT* GENES

Genes used in the final stages of symbiosis are generally identified by the observation that mutations in them make the *Rhizobium* unable to fix nitrogen, thus are Fix^- (10). Where the lack of nitrogen fixation is due to a gross failure in the development of nodules, such as a defect in bacterial invasion into the nodule, the phenotype is described as Ndv^-, for nodule development (133). Although some genetic loci named *ndv* have been identified as affecting the bacterial surface (46; see above), there does not seem to be any reason why the phenotype designation could not also continue to be used for newly found genes, of initially unknown function, affecting gross developmental processes prerequisite to nitrogen fixation. The Fix^- phenotype relates more particularly to the differentiation of bacteria into bactcroids, and to the functions of nitrogen fixation and interchange with the plant cytoplasm. Loci specifically affecting symbiotic nitrogen fixation (i.e. with no other metabolic effect) are designated by either of two names: those shown to be equivalent to a *Klebsiella pneumoniae nif* gene are assigned this same name; other genes are designated as *fix*.

The location and organization of *nif* and *fix* genes vary a great deal (16 and references therein). The structural *nif* genes of *R. meliloti,* and other *nif* and *fix* genes, are located in several clusters dispersed along more than 200 Kb of DNA on the megaplasmid pSym-a. The *R. leguminosarum* biovars appear to have more tightly linked *nif* and *fix* genes. In *Bradyrhizobium,* all the symbiotic genes appear to be located on the chromosome. The *nod* genes are typically linked to at least one cluster of *nif-fix* genes.

Nif genes have been identified through two main approaches: first, homology with the identified *nif* genes of *K. pneumoniae;* secondly, sequence analysis of DNA regions believed to carry *nif* genes because of linkage to known *nif* genes, or because of preliminary genetic evidence. In some systems it is possible to assay nitrogen fixation independently of the symbiotic

state, and this can be used as a criterion to identify *nif* genes. For example, *A. caulinodans* can grow on the nitrogen that it fixes (33, 39, 139); *B. japonicum* derepresses *nif* enough to show enzyme activity (67). *R. meliloti* and other *Rhizobium* do not show colony growth or enzyme activity, but *nif* expression can be monitored by gene fusions (35, 172).

Of the more than 17 identified *nif* genes in *Klebsiella* (some counts now go as high as 21), at least 8 have been identified in various *Rhizobium* species (reviewed in 16, 30, 70, 76; see also 2, 33, 39, 66, 143, 155). The earliest to be studied were the genes for the nitrogenase enzyme peptides, *nifHDK*. These are found in all *Rhizobium, Azorhizobium* and *Bradyrhizobium* strains, although their organization varies in interesting ways. For example, the structural genes for nitrogenase, *nifHDK,* form an operon in *R. meliloti* and *R. leguminosarum,* but are split into two unlinked operons, *nifH* and *nifDK,* in *B. japonicum*. Other genes found in *R. meliloti, R. leguminosarum,* and *B. japonicum* are *nifN, nifE, nifB;* these are involved in the synthesis of the molybdenum-iron cofactor (FeMoco) required for activity of the nitrogenase component 1, or molybdenum-iron protein. The *nifS* gene, identified in *B. japonicum,* has no currently proven function.

Fix genes have been identified in several ways: through random mutagenesis and screening; through mutagenesis and sequencing of DNA adjacent to the identified *nifHDK* or other *nif* genes; and by mapping DNA regions represented in differentiated bacteroid RNA (e.g. reference 29, 116, 151; and examples below). The functions of most *fix* genes have not yet been biochemically demonstrated, although sequence comparisons are suggestive. Some *fix* genes resemble bacterial ferredoxins or other metal-binding proteins; examples are *frxA* and *fixX* of *Bradyrhizobium* (48, 76), and *fixX* of *R. trifolii, R. meliloti* (47, 85). It has been proposed that these genes encode specialized components for electron transfer in the symbiosis, perhaps as assemblies with other *fix* gene products (47, 67). *FixGHIS* form an operon in *R. meliloti* (91); the sequences of all four indicate the proteins are likely to be membrane-bound. *FixG* is indicated to have an iron-sulfur center; the *fixI* sequence is similar to cation-transporting ATPases. A membrane-bound complex that couples an oxidation-reduction reaction to ion transport is proposed to be a likely function for the products of *fixGHIS* (91), and by DNA homology these genes appear to be widely conserved among different *Rhizobium*.

REGULATION The study of regulation is often most revealing of the overall biology of a system, and symbiotic *nif* gene regulation has exemplified this nicely. The nitrogenase enzyme is expensive to operate, in that it takes up to 28 moles of ATP to reduce one mole of molecular nitrogen (141), and is very oxygen-labile. Nitrogen fixing bacteria thus typically regulate the expression of the *nif* genes in response to nitrogen availability, not using molecular

nitrogen as a source except as a desperation measure, and even then only if ambient oxygen tension is appropriate.

In the general nitrogen regulatory circuit (70) transcription initiation at specific *ntr*-type promoters occurs only when nitrogen availability is low, and in the presence of the protein products of two genes, *ntrA* and *ntrC*. The *ntrA* gene product is a sigma factor interacting with *ntr*-type promoters. Regulatory circuits in *R. meliloti, B. japonicum* and *A. caulinodans* all involve the *ntrA* sigma factor. It has been directly shown to be required for *nif, nar* and *dct* expression in *R. meliloti* (148). The conservation of *ntrA*-type promoter structures for various *nif* and *fix* genes is striking; a consensus "−24/−12" promoter sequence (e.g. −26 CTGGYAYR-N_4-TTGCA-10; see 70) is found upstream of *nifHDK(E), FIXABCX, nifN, dctB,* and *ros* in *R. meliloti* (47, 126, 148, 172), upstream of *nifDKEN, nifS, nifBfrxA, nifH, fixBCX,* and *fixRnifAfixA* in *B. japonicum* (reviewed in 76), and upstream of *nifA* and *nifH* in *A. caulinodans* (30, 33). New work in progress on *ntr*-type genes in these and other systems is presented in (16).

Differences arise in various systems because the sigma factor encoded by *ntrA* works along with an upstream activator protein, and the identity and regulation of this activator may vary. The standard for comparison is the circuitry known for *K. pneumoniae* (70): the *ntrC* protein (NR-I) is activated, in nitrogen-limited conditions, by the kinase function of the *ntrB* gene product (NR-II). The *ntrC* and *ntrB* genes belong to a family of two-component regulatory systems (70, 131). The *ntrC*-type gene products are gene activators, which display domains for DNA binding and for interaction with the other, *ntrB*-type gene products, which function as sensors for environmental conditions (in the case of NR-II, response to nitrogen nutrition). In *K. pneumoniae,* the products of *ntrA* and *ntrC* together activate transcription of *nifA;* the *nifA* protein then replaces NR-I, as the *ntrA* and *nifA* proteins together activate expression of the other *nif* genes. Response to oxygen and possibly to other factors is mediated by the product of the nifL gene (70, 138).

Rhizobium systems have uncoupled to some extent the *ntrC-nifA* connection. The symbiotic *nif* and *fix* genes are, as expected, transcriptionally controlled by *nifA* and *ntrA*. However, the exact mechanisms for controlling *nifA* appear to vary among *Rhizobium, Bradyrhizobium* and *Azorhizobium*. In *R. meliloti,* the transcription of *nifA* itself is controlled by oxygen (35, 176), whose effect is mediated by two genes, *fixL* and *fixJ* located in a distantly linked cluster of symbiotic genes on pSym a (28). The sequences of *fixL* and *fixJ* suggest that they also belong to the family of two-component regulatory genes (*fixL* is *ntrB*-like; *fixJ* is *ntrC*-like). Furthermore, *R. meliloti* has a parallel regulatory system, in which *fixL* and *fixJ* activate a second regulatory gene, *fixK; fix* genes may then be turned on by the FixK-NtrA combination (77).

In *Azorhizobium,* the diazotrophic growth habit of the free-living cells allows parallel phenotypic analysis in culture and in nodules (33, 39, 139). Here, *nifA* is autoregulatory; *nifA* transcription is repressed by available nitrogen, but this is partly independent of *ntrC,* suggesting further regulatory genes (139). At least two more regulatory genes, *ntrYX,* have been identified at a site where a second site mutation rescues growth of an *hemA*::Tn*5* mutant (30). Oxygen control may be mediated in part by one of these genes.

In *B. japonicum,* it is not known what exactly controls the expression of *nifA,* which is the second gene in the *fixRnifA* operon. The *nifA* locus is not autoregulatory (173); whether the *ntrC* locus plays a role in *nifA* control is not known. The ability of *nifA* to regulate other *nif* genes is decreased by oxygen, but the effect seems to occur as a result of direct oxygen inactivation of the NifA protein itself (57). The *B. japonicum* and *R. meliloti* NifA protein sequences include an inserted stretch of amino acids rich in cysteine, not found in other *Nif*As. Site-directed mutagenesis of the *B. japonicum nifA* gene showed that each of several cysteines was indispensible for NifA activity. This may represent a metal-binding and/or oxygen-sensitive domain (56). Action of the *R. meliloti nifA* protein is not as sensitive to oxygen (35, 56).

GENES FOR BACTEROID METABOLISM The loci for transport of dicarboxylic acids such as fumarate, malate, and succinate appear to be strictly required for successful symbiotic function. Mutants in *dct* in diverse *Rhizobium* systems have been found to establish non-fixing nodules (for example, see 13, 49, 55, 147, 177); and previous work cited therein). Metabolic studies have shown that enzymes for most carbohydrate pathways are not present in bacteroids; Dilworth and colleagues have postulated that symbiotic bacteria are not exposed to the carbohydrate substrates that would induce the expression of these pathways; rather the plant-derived peribacteroid membrane allows only dicarboxylic acids to get through to the bacteria (34a). The *dct* loci of *R. l. viciae* include the structural gene *dctA,* and two tandem regulatory genes *dctB* and *dctD* that are read divergently from *dctA*. The *dctB-dctD* pair also belong to the two-component regulatory system family and *nifA* can substitute for *dctB* (148). The relevance of the *dct* loci to the symbiosis is supported by several recent discoveries: first, the genes for the *dct* proteins are encoded not on the chromosome, but on one of the pSym megaplasmids of *R. meliloti* (49, 55, 177). Second, the expression of these genes is controlled by the *ntrA* sigma factor, which also is required for the expression of *nif* genes (148). Further expression studies of the genes for both catabolic and synthetic carbohydrate and amino acid pathways may reveal more on the relationship of plant signals to the regulation of bacteroids. In turn, the observation that genes for *dct, mel,* and *ros* (a

unique *Rhizobium* symbiotic metabolite) are subject to *ntrA* type expression control suggests that a global search for genes expressed by *ntrA* and *nifA* might be a useful approach to defining a more complete set of the genes needed for symbiosis.

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