PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Molecular Analysis of the Rhizobium Genes Involved in the Induction of Nitrogen-Fixing Nodules on Legumes [and Discussion]

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Molecular analysis of the *Rhizobium* genes involved in the induction of nitrogen-fixing nodules on legumes

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Recent developments in the molecular genetics of *Rhizobium spp.* are presented, and the use of mutant bacterial strains to determine which properties are required for symbiotic nitrogen fixation and nodulation of legumes is described. Both the lipopolysaccharide and the exopolysaccharide of *Rhizobium spp.* are implicated in infection. Recent studies have identified several genes involved in the early steps of this process and in the determination of host-range specificity. Analysis of their products has given some indications of their functions. The expression of most of these nodulation (*nod*) genes is controlled by the regulatory gene *nodD*, which is itself expressed constitutively, whereas other *nod* genes are transcribed only when the cells are exposed to compounds present in the rhizosphere of legumes. These compounds were identified as various flavones and flavanones. Other plant-specified aromatic molecules, such as isoflavonoids, antagonize this induction.

1. Introduction

The symbiotic interaction between leguminous plants and bacteria of the genera *Rhizobium* and *Bradyrhizobium* is the single most important beneficial association between plants and bacteria in agriculture. Over and above its agronomic significance, the symbiosis is of interest because it represents a complex programme of biochemical and morphological differentiation in the two different partners. It thus offers potential as a model system for the analysis of differentiation both in bacteria and in plants and for the study of the signals that pass between the two kinds of organism.

The symbiosis has accordingly been the subject of increasing study, the greatest growth area being on the molecular biology of this interaction. As described in the paper by Evans *et al.* (this symposium), several plant genes whose expression is specific to nodule tissue have been identified and in some cases the functions of their products, the so-called nodulins, have been determined (for example uricase, leghaemoglobin and glutamine synthetase). Rapid progress has also been made on the molecular biology of the bacterial partner; here we will consider the use of molecular genetics to study the structure, function and regulation of *Rhizobium* symbiotic genes.

2. Genetic analysis of symbiotic functions in *Rhizobium*

(*a*) *Background*

During the 1970s, several of the tools and techniques required for a molecular analysis of *Rhizobium* genes were developed (Beringer *et al.* 1980). Systems for gene transfer by conjugation and transduction were developed; these systems allowed the construction of circular chromo-

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somal linkage maps for *R. leguminosarum* (which nodulates peas) and *R. meliloti* (whose host is alfalfa). Later, several important observations were made and techniques developed which greatly extended the powers of genetic analyses.

These developments included the use of such mutagens as the transposon Tn*5* in *Rhizobium* (Beringer *et al.* 1978). The advantage of a transposon as a mutagen is that transposons 'mark' the gene into which they insert both physically and genetically with an easily scored phenotype; for example, the transposon Tn*5* specifies resistance to kanamycin. This facilitates both the genetic mapping of the mutation and the cloning of the relevant gene and is of particular advantage in cases such as non-nodulating mutants of *Rhizobium*, where the mutant phenotype is difficult to score.

A second important development was the construction of wide-host-range cloning vectors which could be mobilized from *Escherichia coli* to *Rhizobium*, thus enabling the function of DNA cloned in *E. coli* to be studied in *Rhizobium*. Most of these vectors were derived from the wide host-range P1 and Q group plasmids (Figurski & Helinski 1979; Bagdasarian *et al.* 1981; Friedman *et al.* 1982). Further, Ruvkun & Ausubel (1981) developed an elegant means of coupling the powers of transposons and recombinant DNA. This allowed mutagenesis of relatively small regions of cloned DNA and, by marker exchange, the introduction of the transposon into the corresponding region of the genome of *Rhizobium*; the phenotypes of the resultant mutant strains could then be determined. Thirdly, the observation that certain of the nitrogen fixation (*nif*) genes of *Klebsiella pneumoniae* were sufficiently similar to those of *Rhizobium* for the *nif* genes of *K. pneumoniae* to be used as hybridization probes for the corresponding genes in *Rhizobium* (Nuti *et al.* 1979; Ruvkun & Ausubel 1980), has greatly facilitated the identification and subsequent analysis of *nif* genes and flanking DNA of any *Rhizobium* strain.

Fourthly, it was shown, by genetical and physical criteria, that in fast-growing *Rhizobium* species many genes required for nitrogen fixation, nodulation and the determination of host-range specificity are on large 'symbiotic' plasmids. By probing with *K. pneumoniae* DNA, *nif* genes were shown to be located on such plasmids (Nuti *et al.* 1979). Certain mutations in such plasmids abolished nitrogen fixation or nodulation (see, for example, Banfalvi *et al.* 1981). Some of these plasmids can be transferred by conjugation; for such cases it was shown that transfer of the plasmid to a *Rhizobium* with different host-specificity, or even to *Agrobacterium tumefaciens*, allowed the transconjugants to nodulate the host plant of the donor strain (Johnston *et al.* 1978; Hooykaas *et al.* 1981; see Long 1984 for a review).

How then have these background observations helped us to understand the genetic basis of what makes *Rhizobium* so special in its ability to nodulate legumes and to fix nitrogen? Attempts to answer this question have used two different but overlapping approaches. In the first ('indirect') approach, mutant strains with phenotypes easily scored on Petri dishes are isolated and the effects of such mutations on symbiotic nitrogen fixation are then determined. By definition, only a limited range of types of mutants can be isolated by such means but the advantage is that, if such a mutant strain is aberrant in symbiosis, there is an immediate indication of the biochemical basis of the defect. Various antibiotic resistant or auxotrophic mutants that have been isolated in different *Rhizobium* species have been shown to be unable to nodulate (Nod^-) or to fix nitrogen (Fix^-) (see Beringer *et al.* (1980) for review). Mutant strains defective in the uptake of organic acids (such as succinate or α-ketoglutarate) induce non-fixing nodules (Ronson *et al.* 1981; Finan *et al.* 1983); these results support the idea that these compounds are major source of energy used for the energy-expensive nitrogen reduction

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

reactions. It had been postulated several years ago (Nadler & Avissar 1977) that *Rhizobium* bacteroids made the haem moiety of leghaemoglobin and the finding that a mutant of *R. meliloti* defective in δ-aminolaevulinic acid synthetase (the first step in the biosynthetic pathway for haem synthesis) was Fix^- on alfalfa seemed to support this hypothesis (Leong *et al.* 1982). However, Guerinot & Chelm (1986) isolated a mutant of *B. japonicum* defective in the same enzyme but this mutant was Fix^+ on soybeans. At present the reason for the difference in the two species is not clear.

(*b*) *The role of the* rhizobium *cell surface*

A priori it might be predicted that the cell surface of *Rhizobium* would be implicated in the recognition of legumes and in the subsequent stages of the infection which require contact between the bacterium and the host.

The evidence from immunology, biochemistry and cytology indicates that the bacterial cell wall is radically changed during the transition from the free-living to the bacteroid form of *Rhizobium*. Presumably thse changes are in some way related to the mechanism of infection and are hence under the control of bacterial genes that affect nodule development. To identify subtle changes in biochemical composition of cell walls from nodule bacteria, monoclonal antibodies (McAbs) were isolated and used as affinity probes for cytological and biochemical analyses. Many of the McAbs that were isolated reacted with bacteroid lipopolysaccharides, which appear to be predominantly of the 'rough' form, lacking the O-antigen side chains that are commonly found in free-living cultures (Brewin *et al.* 1986). This observation, taken together with reports that the bacteroid forms do not produce capsular or exopolysaccharide (Tully & Terry 1985), may suggest that the bacteroid outer membrane is naked and is thus capable of close physical association with the adjacent peribacteroid membrane, which is of plant origin (Brewin *et al.* 1985; Bradley *et al.* 1986). Another monoclonal antibody has identified an LPS antigen which, although not expressed in free-living bacteria, appears to be particularly abundant in bacteria contained in the infection thread. These infection-thread bacteria are embedded in a matrix material composed of a plant glycoprotein, which can also be identified by a separate monoclonal antibody (figure 1). Hence, by a combination of bacterial genetics and immunocytochemistry, it should be possible to investigate the mechanism whereby individual bacteria from the infection tube are engulfed by the plant cell plasma membrane and subsequently proceed to differentiate into N_2-fixing endosymbiotic bacteroids within the infected plant cells of the central tissue of the nodule.

Genetic evidence also supports the view that bacterial surface polysaccharides are important in the infection process but the conclusions from such studies are not completely straightforward. Strains of *Rhizobium* make large amounts of exopolysaccharides (EPS) of different types, such as capsular and soluble exopolysaccharides, lipopolysaccharides and a β-1-2 linked glucan that, interestingly, is unique to the Rhizobiaceae, in which both *Rhizobium* and *Agrobacterium* are classified (Dell *et al.* 1983). Mutant strains of *A. tumefaciens* that fail to make this cyclic glucan are unable to induce tumours (Puvanesarajah *et al.* 1985). Significantly, these mutants could be corrected for tumourigenesis by cloned DNA from *R. meliloti*, showing that the genes required for the synthesis of this polymer are functionally equivalent in the two genera (Dylan *et al.* 1986). Mutations that abolish or reduce the production of the soluble, high-molecular-mass, acidic exopolysaccharide have been isolated in different *Rhizobium* species. Some such mutant strains are apparently unaffected in symbiotic nitrogen fixation (Sanders *et al.* 1981); others

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

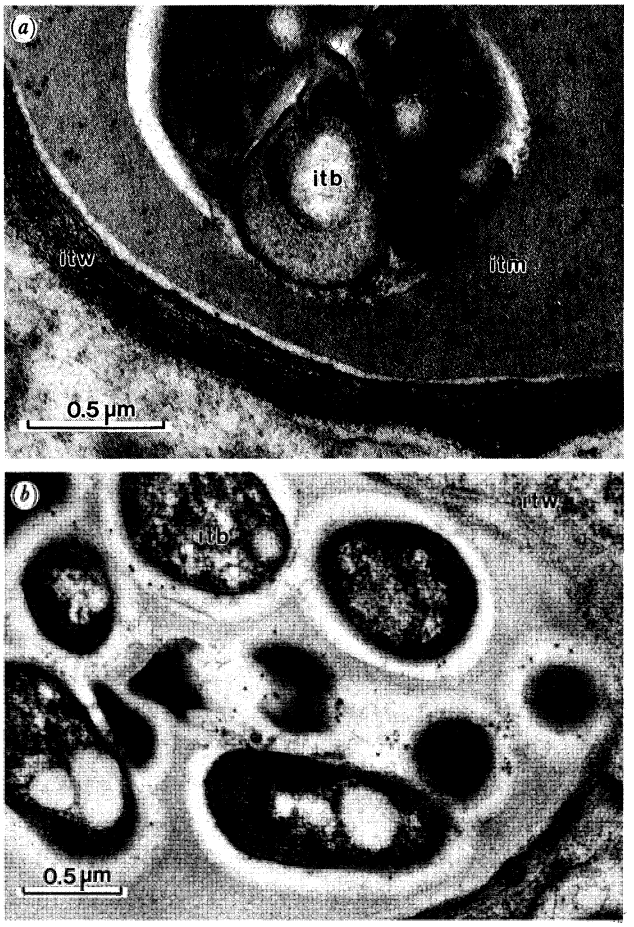


FIGURE 1. Expression of plant and bacteroid antigens within the infection thread during legume root nodule infection. Electron micrographs of thin sections of a pea nodule showing *R. leguminosarum* bacteria (itb) inside the infection thread and separated from the plant cell cytoplasm by the infection-thread wall (itw). The section shown in (*a*) has been immunogold stained with a monoclonal antibody that reacts with a *Rhizobium* lipopolysaccharide antigen that is expressed within the infection thread but not in free-living bacteria, nor in bacteroids; (*b*) shows immunogold staining with another monoclonal antibody which recognizes a plant glycoprotein component of the infection-thread matrix (itm). (Bar = 0.5 μm; colloidal gold particles are 10 nm in diameter.)

either nodulate but fail to fix nitrogen (Chakravorty *et al.* 1982) or fail to nodulate at all (Sanders *et al.* 1978). The situation appears even more complex since Borthakur *et al.* ((1986) showed that a mutation that abolished exopolysaccharide synthesis had no observable effect on the ability of *R. phaseoli* to induce nitrogen-fixing nodules on its host *Phaseolus* beans, whereas the same allele, present in a near-isogenic strain of *R. leguminosarum*, prevented nodulation of peas. Likewise Chen *et al.* (1985) isolated exopolysaccharide-defective mutants of a *Rhizobium* species which could nodulate a range of tropical legumes; different mutations were found to block nodulation or nitrogen fixation in one species of host plant, but not in another. These observations show that the role of EPS in the nodulation and/or nitrogen fixation processes may be very subtle and that it can depend on the particular host legume.

A recent approach to the isolation of mutants with altered abilities to make EPS has exploited the fact that the dye calcoflor binds to *Rhizobium* EPS polymer and causes the colonies to

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

fluoresce when viewed under UV light. Mutants of *R. meliloti* which no longer bind the dye were found to form non-fixing nodules on alfalfa (Finan *et al.* 1985; Leigh *et al.* 1985) which were devoid of *Rhizobium* cells; this is an important observation because it shows that the development of a nodule by the plant does not require the presence of *Rhizobium* within it. Genetic analysis of these types of EPS-deficient mutation has shown that they are located in at least six different gene clusters and that at least one set of these genes in *R. meliloti* is on a large indigenous plasmid. This plasmid is not the so-called symbiotic plasmid (see below) that contains genes for nitrogenase and for nodulation ability (Hyne *et al.* 1986).

A second general approach used in the isolation and characterization of symbiotic genes has been to identify them directly on the basis that mutations affected nodulation or nitrogen fixation ability. Several mutations that abolish nitrogen fixation have been located on the chromosomes of *Rhizobium* (Beringer *et al.* 1977; Forrai *et al.* 1983) but the most detailed studies have been on the symbiotic genes on the large indigenous symbiotic plasmids. In these studies, the term *nif* refers to genes which have homology with defined *nif* genes in *Klebsiella*; *fix*, to genes which are required for symbiotic nitrogen fixation but which have not been shown to be homologous to *Klebsiella nif* genes; and *nod*, to genes required for normal nodule development.

(*c*) *Identification of symbiotic genes on* Rhizobium *plasmids*

Representatives of *nif*, *fix* and *nod* genes have been located on the symbiotic plasmids of several different *Rhizobium* species. Their analysis has been facilitated by the fact that they are clustered and, to a greater or lesser extent, their locations relative to each other are conserved in different species. We will describe recent studies on a symbiotic plasmid from *R. leguminosarum*, the species that nodulates peas, and will relate these findings to those obtained in other species.

pRL1JI is a 220 kilobase (kb), transmissible plasmid which, when transferred to other *Rhizobium* species, confers the ability to nodulate peas (Johnston *et al.* 1978; Hirsch *et al.* 1980). After Tn*5* mutagenesis of pRL1JI, various non-fixing and non-nodulating mutant strains were isolated (Ma *et al.* 1982; Downie *et al.* 1983*b*), the corresponding wild-type DNA was cloned in the wide host-range cosmid vector pLAFR1 and the locations of the mutations were determined. The *nod*::*Tn5* mutations lay between two regions required for nitrogen fixation (Downie *et al.*, 1983*a*, *b*) (see figure 2), the whole symbiotic region spanning approximately

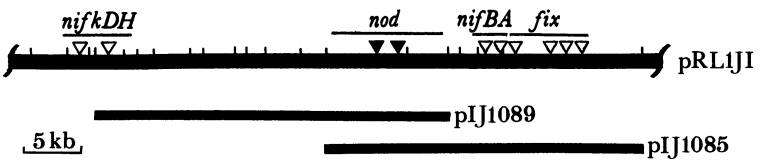


FIGURE 2. Representation of the symbiotic region of the *R. leguminosarum* plasmid pRL1JI. Open and closed triangles show the sites of mutations that abolish nitrogen fixation and nodulation ability, respectively. Locations of *nif* genes corresponding to defined *nif* genes of *K. pneumonaie* are shown. The dimensions of DNA cloned in the vector pLAFR1 to form recombinant plasmids pIJ1085 and pIJ1089 are indicated. Thin vertical lines show the positions of *EcoR*1 sites.

60 kb. One of the regions in which mutations abolished nitrogen fixation contained genes that corresponded to the *nifHD* and *K* genes of *K. pneumoniae*, which specify the structural polypeptides of nitrogenase reductase and nitrogenase (Downie *et al.* 1983*b*) and in the other were genes that corresponded to the *nifB* gene (required for the synthesis of the nitrogenase

cofactor FeMoco); and the regulatory *nifA* gene of *K. pneumoniae* (Rossen *et al.* 1984*b*). Upstream of *nifA*, Tn*5* insertions also abolished nitrogen fixation but DNA in this region had no detectable homology to any *K. pneumoniae nif* genes (Downie *et al.* 1983*b*).

In symbiotic plasmids of *R. trifolii* and *R. meliloti*, *nif* genes were also found to be linked to *nod* genes (Long *et al.* 1982); in the latter species, the *nif* genes have been analysed in detail. In contrast to the situation in *R. leguminosarum*, the *nifHDK* genes are closely linked to *nifA* and *B*, these two sets of defined *nif* genes being separated by three genes (*fixAB* and *C*) which are not detectably homologous to any *K. pneumoniae nif* genes (Buikema *et al.* 1985). The precise functions of these three *fix* genes are not known.

(*d*) *Regulation of* nif *gene expression*

A major unresolved question concerning the *nif* genes of *Rhizobium* is: why do these bacteria, in contrast to (for example) *K. pneumoniae* and *Azotobacter*, fail to fix nitrogen in free-living culture? As described in the paper by Dixon *et al.* (this symposium), when *K. pneumoniae* is grown under conditions of low oxygen tension and low concentrations of favoured sources of fixed nitrogen, transcription of the *nif* operons is activated by the regulatory *nifA* gene. Despite the inability of most strains of *Rhizobium* to fix *ex planta*, several lines of evidence show that the regulation of *nif* gene expression in *Rhizobium* is similar to that in *K. pneumoniae*. Firstly, a *nifA*-like gene, as judged by DNA sequence comparisons (Buikema *et al.* 1985) has been found on the symbiotic plasmid of *R. meliloti* and mutations in this gene abolish symbiotic nitrogen fixation. Further, these mutants synthesize none of the *nif*-specific polypeptides in the nodule, a property that is consistent with a regulatory role for the *nifA* gene in this species (Szeto *et al.* 1984). Secondly, the promoter sequences of *nif* genes in *Rhizobium* are very similar to those found in *K. pneumoniae* (see, for example, Better *et al.* 1983, 1985) and both the *K. pneumoniae nifA* gene and the *ntrC* gene can activate transcription from these *Rhizobium* promoters (Sundarasan *et al.* 1983*a*, *b*). Despite these similarities, though, there must be some unknown difference in the regulatory circuitry in the two genera to explain the reluctance of *nif* genes of *Rhizobium* to be induced outside the nodule.

However, it has been known for some ten years that many slow-growing *Bradyrhizobium* strains can fix nitrogen, albeit at low levels, in defined media. Strains of *B. japonicum*, which nodulate soybeans, have also been found to contain a *nifA*-like gene that is linked to other *nif* and *fix* genes (Fischer *et al.* 1986), and the basis of the differences between strains of *Rhizobium* and *Bradyrhizobium* with regard to their capacity to fix nitrogen in free-living culture is not clear. There are other striking differences in the organization of symbiotic genes in *Bradyrhizobium* compared with the fast-growing *Rhizobium* species. For example, in *Bradyrhizobium*, *nod* and *nif* genes are chromosomally located as opposed to being on symbiotic plasmids in *Rhizobium* strains. Secondly, the *nif* and *nod* genes appear to be more dispersed in *Bradyrhizobium* than the fast-growing species; for example *nifH*, which specifies nitrogenase reductase, is separate from, though linked to, the *nifD* and *K* genes, which specify nitogenase (Fischer & Henneke 1984) whereas in the fast-growing species examined these genes are in the same transcription unit, just as they are in *K. pneumoniae*. As yet, the significance of the differences in the arrangement of *nod* and *nif* genes in the two genera is not apparent.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

(*e*) *Nodulation genes*

As mentioned above, the nodulation genes in the *R. leguminosarum* plasmid pRL1JI lie between two regions of *nif* DNA (figure 2). A relatively small region, approximately 10 kb in size, of pRL1JI is involved in nodulation ability and the determination of host-range specificity for peas as shown by the fact that two recombinant plasmids (pIJ1085 and pIJ1089) which share only this DNA, each conferred the ability to nodulate peas when transferred to other *Rhizobium* species or to strains lacking their symbiotic plasmid (Downie *et al.* 1983*a*, *b*). Such transconjugants induced nodules which, although they did not fix nitrogen, were normal during the early stages of nodule development; infection threads were formed and the bacteria were liberated to form bacteroids. Similarly, a relatively small region of an *R. trifolii* symbiotic plasmid conferred the ability to nodulate clover, the host of this species, when transferred to other *Rhizobium* species (Schofield *et al.* 1984); in *R. meliloti* two small regions separated by 6 kb appear to be sufficient to specify the early stages of nodulation of alfalfa (Putnocky & Kondorosi 1986).

The nodulation genes of *R. leguminosarum*, *R. trifolii* and *R. meliloti* have recently been analysed in great detail. One of the striking features to emerge from these studies is that these *nod* genes, although in different *Rhizobium* species are very similar with regard to their sequences, their locations relative to each other, their function and their regulation. This conservation of *nod* genes even extends to strains of the slow-growing *Bradyrhizobium* (Scott 1986).

In the *R. leguminosarum* plasmid pRL1JI, genetic characterization and DNA sequence determination of the nodulation region revealed the presence of eight genes, *nodEFDABCI* and *J* (Rossen *et al.* 1984*a*; Downie *et al.* 1985; Shearman *et al.* 1986; Evans & Downie 1986 (figure 3; table 1). Mutations in *nodD*, *A*, *B* and *C* abolish nodulation ability and root-hair curling whereas mutations in the other *nod* genes merely delay the onset of nodulation and reduce the numbers of nodules formed.

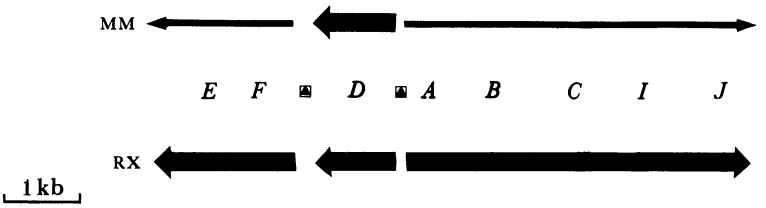


FIGURE 3. Representation of the dimensions and states of expression of *R. leguminosarum nod* genes in different growth conditions. The thickness of the arrows indicates the levels of transcription of the different *nod* genes; thus in minimal medium (MM) only *nodD* is transcribed but when root exudate (RX) is added, all the genes are expressed. This induction of *nodABCIJ* and *nodFE* requires the presence of *nodD*, which is regulatory. The boxed arrows indicate the location of conserved sequences involved in *nodD*-mediated activation of transcription.

Studies on the *nod* genes of other *Rhizobium* species (*R. meliloti* and *R. trifolii*) and one strain of *Bradyrhizobium* revealed genes corresponding (as judged by DNA sequence comparisons) to *nodEFDABC* and *I* (Schofield & Watson 1986; Torok *et al.* 1984; Egelhoff *et al.* 1985; Horvath *et al.* 1986; Scott 1986) with locations relative to each other similar to those in *R. leguminosarum* (although in *R. meliloti nodFE* are separated from *nodDABC*). Some of these genes (*nodD*, *A*, *B* and *C*) are functionally equivalent, i.e. the nodulation defects of a mutant in one species can be corrected by the corresponding gene of another species (Djordjevic *et al.* 1985*a*; Fisher *et al.*

Table 1. Characteristics of *nod* genes

gene	phenotype of mutations		comments
nodA	Nod^{-b}	Rhc^{-b}	membrane-bound[a]
nodB	Nod$^-$	Rhc$^-$	—
nodC	Nod$^-$	Rhc$^-$	membrane-bound[a]
nodI	Nod delay	Rhc$^+$	transport protein
nodJ	Nod delay	Rhc$^+$	membrane-bound
nodD	Nod$^-$	Rhc^{-c}	regulatory
nodF	Nod delay	Rhc$^+$	similar to acyl carrier protein
nodE	Nod delay	Rhc$^+$	—

[a] Evidence obtained from *R. meliloti* (John *et al.* 1986; S. R. Long, personal communication).
[b] Nod$^-$, unable to nodulate; Rhc$^-$, unable to curl root hairs.
[c] In *R. meliloti*, *nodD* mutant strains are only delayed for nodulation ability.

1985). In contrast, the *nodF* and *nodE* genes appear to be involved in the determination of host-range specificity and mutations in these genes cannot be corrected by the *nodF* or *E* genes from different species (Kondorosi *et al.* 1984). It is surprising to find, though, that the *nodE* gene is the most highly conserved of the *nod* genes so far examined (Shearman *et al.* 1986; Horvath *et al.* 1986; Schofield & Watson 1986); this implies that the determination of a particular host range depends on some subtle distinction between the *nodE* (and perhaps other) genes rather than there being completely unrelated host-range genes in different species. Moreover, it seems that a functional *nodE* gene may actually prevent nodulation of 'non-host' legumes. This was deduced from the observations of Djordjevic *et al.* (1985*b*, 1986) that mutations in the *R. trifolii* *nodE* gene, while severely inhibiting the nodulation of white clover, allowed the mutant strains to nodulate peas, albeit poorly.

Despite the detailed physical analyses of these *nod* genes, there is still no clear understanding of their functions, but some indications are beginning to emerge. It appears that the *nodABCI* and *J* genes, which are cotranscribed (see below) specify proteins that are associated with the membranes of *Rhizobium*. The *nodC* and *nodA* gene products of *R. meliloti* have been purified, and antibodies raised against them reacted with the bacterial membrane (John *et al.* 1985; S. R. Long, personal communication). Further, computer-assisted analyses revealed that the predicted gene product of the *R. leguminosarum* *nodI* gene was similar to those of several inner membrane ATP-dependent transport proteins, such as *malK*, *hisP*, *oppD*, *pstB* of enteric bacteria, which are required for the transport of maltose, histidine, oligopeptide and phosphate respectively (Evans & Downie 1986; Higgins *et al.* 1986) and that the predicted *nodJ* gene product is very hydrophobic, consistent with its being a membrane-associated protein. Comparison of the amino acid sequence of the deduced *nodF* gene product with sequences in a data bank of polypeptide sequences showed that it was similar to acyl-carrier protein, a protein that is involved in the synthesis of lipids (Shearman *et al.* 1986) but direct evidence that *nodF* is involved in lipid synthesis remains to be established. No functions have yet been allocated to the *nodB* and *nodE* genes. As described below, the *nodD* gene has been shown to be regulatory, being required for the transcription of the other *nod* genes.

(*f*) Nod *gene regulation*

Studies on *nod* gene regulation in *R. leguminosarum*, *R. meliloti* and *R. trifolii* have been facilitated by the construction of fusions in which the *lacZ* gene of *E. coli* was fused either translationally or transcriptionally to *nod* genes. These studies showed that *nodD* was transcribed constitutively in normal free-living culture but that under these conditions the *nodABCIJ* and

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

nodFE transcription units were transcribed at low levels, if at all (Mulligan & Long 1985; Innes *et al.* 1985; Rossen *et al.* 1985; Shearman *et al.* 1986). However, when cells of these strains were grown in the presence of exudate from the roots of their host plants, the *nodFE* and *nodABCIJ* transcripts were expressed at high levels (up to 70-fold above background); this induction was dependent on the presence of *nodD*, which was thus shown to be a regulatory gene. In *R. leguminosarum* (Rossen *et al.* 1985), but not in *R. meliloti* (Mulligan & Long 1985) *nodD* was autoregulatory, i.e. *nodD* repressed its own expression. The implications of this difference are not clear but it may be significant that the phenotypes of *nodD* mutants in the two species differ; in *R. leguminosarum* such mutants fail to nodulate but in *R. meliloti* mutations in *nodD* have only a slightly detrimental effect on nodulation ability (Fisher *et al.* 1986).

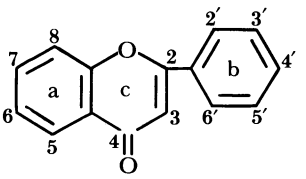
Upstream of the *nod* transcripts (i.e. *nodFE* and *nodABCIJ*) that are activated by *nodD* plus factors in legume root exudate is a sequence of approximately 35 bp, which is highly conserved both within and between *Rhizobium* species (Schofield & Watson 1986; Shearman *et al.* 1986; Rostas *et al.* 1986) and it seems likely that this sequence (the 'nod-box') is implicated in the induction of transcription of these operons.

2. Identification of the molecules that activate *nod* gene transcription

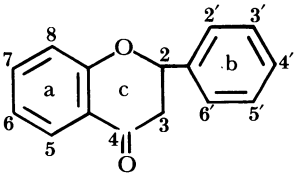
Recent studies have identified plant-specified molecules which, at very low concentrations (200 nM) cause *nodD*-dependent activation of transcription of the other *nod* genes of *R. meliloti*, *R. trifolii* and *R. leguminosarum* (Peters *et al.*, 1986; Redmond *et al.* 1986; Firmin *et al.* 1986); in all these reports flavonoid molecules, either flavones or flavanones, were shown to be responsible for *nod* gene expression. Thus, the most potent inducer of *R. trifolii nodABC* and *nodFE* isolated from clover seedlings was 7-4′-dihydroxyflavone (Redmond *et al.* 1986) and the flavone luteolin was shown to be the most active inducer of *nodABC* of *R. meliloti* (Peters *et al.* 1986). Three flavones, one flavone glucoside and three flavanones had inducer activity for the *nodABCIJ* and *nodFE* transcripts of *R. leguminosarum* (table 2) and one of the inducer molecules isolated from the root exudate of peas was the flavone glucoside, apigenin 7-*O*-glucoside (Firmin *et al.* 1986). By comparing the molecular structures of those compounds with inducer activity with those of chemically related inactive compounds, it was concluded that both flavones and flavanones acted as inducers provided that they had hydroxyl groups on the 3′ or 4′ position of the B ring and that they contained either a hydroxy or a glucoside at the 7-position of the A ring (see figure 4). Because root exudates of alfalfa and clover (two species not nodulated by *R. leguminosarum*) were able to activate transcription of the *R. leguminosarum* *nodABCIJ* transcript (Firmin *et al.* 1986), it is clear that the determination of host-range specificity cannot depend on the identity of the inducer molecule made by a specific host legume.

In fact, the molecules found to activate *nod* gene transcription are not confined to the family Leguminosae, being widespread among angiosperms. However, *nodABC* transcription was not induced by root exudates of a range of non-leguminous plants (J. L. Firmin, unpublished observations); this result suggests that legumes may be unusual in that flavones and/or flavanones are synthesized and secreted by the roots. Firmin *et al.* (1986) also found that other plant-specified phenolic compounds antagonized the activation of *nod*-gene expression by the inducing flavones and flavanones (table 3). These antagonists included various acetophenones which, interestingly, have been shown to be potent *inducers* of the virulence genes of *Agrobacterium*

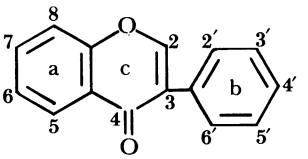
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES



flavones



flavanones



isoflavones

FIGURE 4. Structures of core flavone and flavanone molecules, some of which are *nod*-gene inducers, and of isoflavones, some of which antagonize this induction.

TABLE 2. EFFECTS OF SELECTED FLAVONOIDS ON THE *nodD*-DEPENDENT ACTIVATION OF *nodABCIJ* OF *R. LEGUMINOSARUM*

(*Rhizobium* cells containing pIJ1477, a *nodC–lacZ* fusion plasmid (Rossen *et al.* 1985) plus a functional *nodD* gene were grown in the presence of the different compounds.)

compound	level of expression[a]			
	10 μM	1 μM	100 nM	10 nM
flavones				
4′,5,7-trihydroxyflavone (apigenin)	5163	4649	541	59
3′,4′,7-trihydroxyflavone	6814	1069	109	64
3′,4′,5,7-tetrahydroxyflavone (luteolin)	5120	1437	77	73
flavone glucoside				
4′,5,7-trihydroxyflavone 7-*O*-glucoside (apigenin 7-*O*-glucoside)	5964	3642	239	55
flavanones				
4′,5,7-trihydroxyflavanone (naringinin)	4552	3154	100	58
3′,5,7-trihydroxy-4′-methoxyflavanone (hesperitin)	9207	6409	1036	250
3′,4′,5,7-tetrahydroxyflavanone (eriodictyol)	7144	5066	523	75

The following flavonoids had no detectable activity in the concentration range 10 nM–10 μM.

Flavones: 5,7-dihydroxy-; 5,7-dihydroxy; 3′,4′-dimethoxy-; 7-hydroxy-4′-methoxy-; 3,5,7-trihydroxy-; 3,4,5,7-tetrahydroxy-.

Flavone glycosides: 4′,5,7-trihydroxyflavone 8-*C*-glucoside; 3′,4′,5,7-tetrahydroxyflavone 3-*O*-rutinoside.

Flavanone: 5-hydroxyflavanone.

Isoflavones: 4′,5,7-trihydroxy- (genestein); 4′,7-dihydroxy.

Flavanone glycosides: 3′,5-dihydroxy-4′-methoxyflavanone 7-*O*-rhamnoglucoside; 4′,5-dihydroxyflavanone 7-*O*-rhamnoglucoside.

[a] The level of expression is presented as units of β-galactosidase activity. In the absence of inducer, or in a strain with a mutation in *nodD*, the background levels were approximately 60 units.

tumefaciens (Stachel *et al.* 1985). Among the most potent of these antagonists of *nod*-gene induction were isoflavonoids (figure 4); an apparent paradox is that this class of flavonoid is very widespread among legumes (in which they act as antifungal phytoalexins) but are found only very sporadically in other families of plants (Harbourne 1977).

It is apparent that the regulation of the amount of *nod* gene transcription is a subtle process

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

TABLE 3. ANTAGONISM OF *nodABC* TRANSCRIPTION (AS PERCENTAGE INHIBITION) BY FLAVONOIDS AND ACETOPHENONE ANALOGUES

(*Rhizobium* cells containing the *nodC–lacZ* fusion plasmid pIJ1477 plus a functional *nodD* gene were grown in pea exudate such that in the absence of antagonistic molecules the level of β-galactosidase activity was approximately 2000 units. To examine the effects on expression of the antagonists the cells were grown for 18 h with both pea extract and the compounds shown above were also present in the growth media before assaying for β-galactosidase.)

antagonistic compound	concentration/μM				
	500	100	50	10	5
3,4′,5,7-tetrahydroxyflavone (kaempferol)	82	82	65	69	60
4′,7-dihydroxyisoflavone (daidzein)	85	88	89	85	75
4′,5,7-trihydroxyisoflavone	91	82	58	78	87
4-hydroxy-3-methoxyacetophenone (acetovanillone)	95	91	85	20	ND
4-hydroxy-3,5-dimethoxyacetophenone (acetosyringone)	94	95	46	5	ND

and it will be of interest to determine if the numbers of infections and of nodules are regulated by the relative concentrations of *nod* gene inducers and anti-inducers in the plant and its rhizosphere.

3. CONCLUDING REMARKS

In conclusion, the molecular genetics of the *Rhizobium*–legume symbiosis has progressed considerably in recent years. A major challenge now is to translate the information at the level of DNA into meaningful biological terms that will allow a real understanding of the biochemical bases of at least some of the steps in the remarkable association whose anniversary of discovery we are celebrating in this meeting.

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Discussion

J. M. Vincent (*Emeritus Professor, University of New South Wales, Sydney, Australia*). Has any relationship been established between infection specificity and the effects of flavanones and flavones in triggering the expression of *nod* genes?

A. W. B. Johnston. Apparently not; exudate of the roots of clover or alfalfa are very effective at inducing the *nod* genes of *R. leguminosarum* and luteolin, the flavone identified by Long and her colleagues as being 'the' inducer of *R. meliloti nod* genes is also a potent inducer in *R. leguminosarum*. However, we found that flavanones were very potent for *R. leguminosarum nod* gene induction but the Stanford studies indicated that these compounds were not active inducers for *R. meliloti*.

R. Haselkorn (*Department of Biophysics and Theoretical Biology, University of Chicago, Illinois, U.S.A.*). Are any of the effects that Dr Johnston described in the initial reactions of the expression of *nod* genes attributable to chemotactic responses?

A. W. B. Johnston. I am not aware of any studies on the chemoattractiveness of *nod*-inducing molecules; it's something that I am sure people will be looking for.

D. G. Jones (*Department of Experimental Botany, University College of Wales, Aberystwyth, U.K.*). Bearing in mind the fact that *Rhizobium* geneticists only appear to distinguish between strains as being either Fix^+ of Fix^-, can Dr Johnston give any information on the genetic status of the

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

strains traditionally referred to as Intermediate? In addition, I would like a comment on the most likely genetical approaches to increasing the efficiency in nitrogen fixation of the *nif*$^+$ strains.

A. W. B. Johnston. I agree that this is an important point, but I don't think anyone has an explanation, at a genetic or biochemical level, for the basis of the intermediate strains referred to.

F. R. Minchin (*Animal and Grassland Research Institute, Hurley, Maidenhead, Berkshire, U.K.*). Has anyone tested roots of *Parasponia* spp. for the production of flavones and flavanones?

A. W. B. Johnston. I think that so far no one has been able to demonstrate *nod* gene induction by root exudates from *Parasponia*.

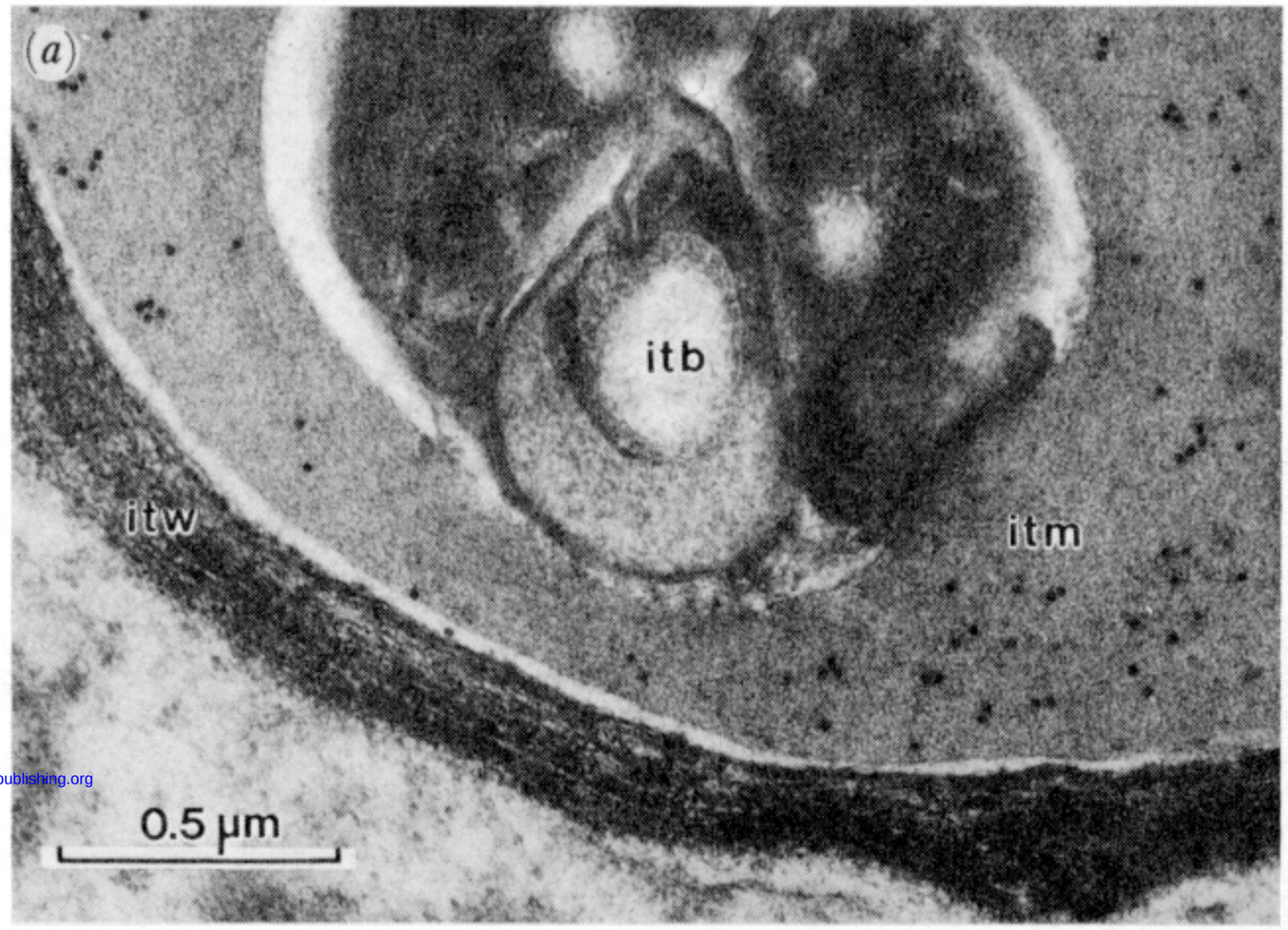
J. Döbereiner (*EMBRAPA-UAPNBS, Seropédica, Rio de Janeiro, Brazil*). In our experience in Brazil *Rhizobium phaseoli* strains are frequently unstable genetically. How does this affect the work described with *R. phaseoli* and *R. leguminosarum* sym-plasmid DNA?

A. W. B. Johnston. With the strains which we have used, instability has not been a problem.

Janet I. Sprent (*Department of Biological Sciences, University of Dundee, U.K.*) Could Dr Johnston or Dr Brewin please expand on the immunogold labelling of infection thread components? In particular, were they specific to this stage or did they persist into the bacteroid stage?

N. J. Brewin. Using monoclonal antibodies in conjunction with immunogold localization techniques, we have identified two components present within infection threads. The first is a plant glycoprotein present in the matrix material of infection threads and infection droplets, but not found in the peribacteroid space or elsewhere within nodule tissue. The second antigen, identified by monoclonal antibodies, reacts with a component of *Rhizobium* lipopolysaccharide. This antigen is not found in free-living cultures of *Rhizobium* and is most abundant in infection thread bacteria, although it is also occasionally found in bacteroids.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES



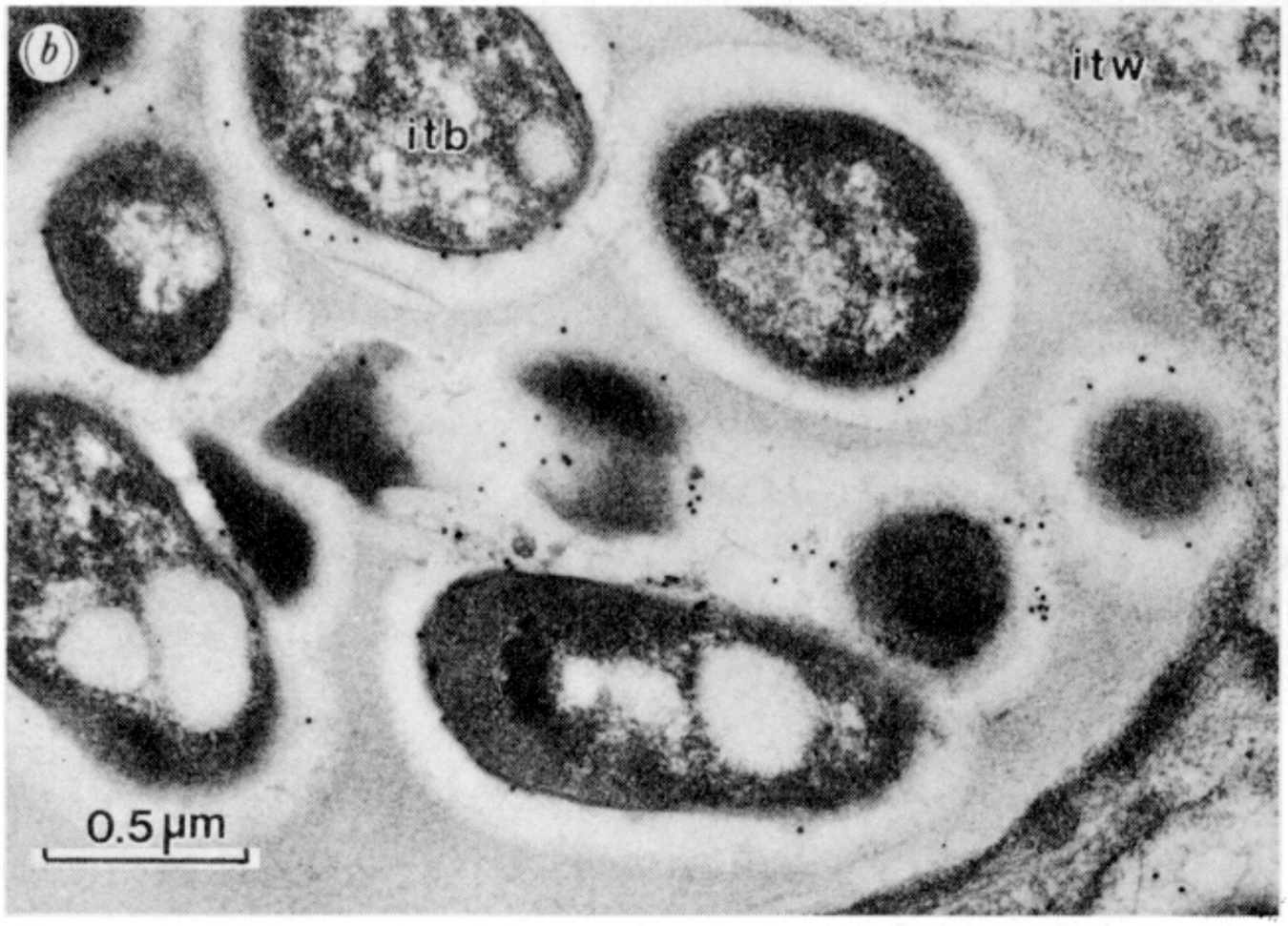


FIGURE 1. Expression of plant and bacteroid antigens within the infection thread during legume root nodule infection. Electron micrographs of thin sections of a pea nodule showing *R. leguminosarum* bacteria (itb) inside the infection thread and separated from the plant cell cytoplasm by the infection-thread wall (itw). The section shown in (*a*) has been immunogold stained with a monoclonal antibody that reacts with a *Rhizobium* lipopolysaccharide antigen that is expressed within the infection thread but not in free-living bacteria, nor in bacteroids; (*b*) shows immunogold staining with another monoclonal antibody which recognizes a plant glycoprotein component of the infection-thread matrix (itm). (Bar = 0.5 μm; colloidal gold particles are 10 nm in diameter.)