Review
Rhizobium sp. NGR234: Molecular Microbial Ecology and Multiple Symbiosis

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Abstract

Rhizobium bacteria generally exhibit a specific symbiotic relationship with their host legume species, and this has classically formed the basis for the description of rhizobial species. *Rhizobium* NGR234 provides an exception to this paradigm since it can form nitrogen-fixing nodules on at least 12 genera of legumes. This broad host-range is a biologically significant phenomenon among microbial-plant interactions, and it is possible that an understanding of its mechanism could have significant agronomic applications. NGR234 offers the possibility of a molecular genetic analysis of early, developmental and late symbiotic interaction with a wide variety of legume plants. These symbioses encompass a range of tropical legumes which includes both persistent meristem (indeterminate) nodule plants like *Leucaena*, and many nonpersistent meristem (determinate) nodule plants like *Lablab* and *Vigna*. For instance comparative symbiotic data exist already for null mutations in NGR234 genes encoding the alternative sigma factor RPON, aminolaevulinic acid synthase and capsular exopolysaccharide. Fundamental scientific findings that have emerged from studies of NGR234 include the key symbiotic-regulatory roles of the genes *nodD* and *rpoN*.

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1. Microbial Ecology of NGR234

The symbiotic bacteria which cooperatively infect and induce formation of nitrogen-fixing nodules on the roots of leguminous plants belong to the family Rhizobiaceae, and fall into very divergent genera, the fast-growing *Rhizobium* and the slow-growing *Bradyrhizobium* (Jordan, 1984). *Bradyrhizobium* species infect and fix nitrogen in tropical legumes. *Rhizobium* species are symbiotic on temperate zone legumes. Temperate-zone rhizobia have a narrow host range; for example, *Rhizobium leguminosarum bv. viciae (Rlv)* is symbiotic only on peas, lentils and vetches. *Rhizobium* NGR234 is an unusual intermediate strain because it combines a very wide tropical legume host range with the growth rate and general genetic organization (see below) of fast-growing rhizobia.

Trinick (1980) described the bacteriological characteristics and host range relationships among fast-growing rhizobia, resembling *R. meliloti (Rm)*, isolated from the tropical legumes *Leucaena*, *Mimosa*, *Acacia*, *Sesbania* and *Lablab*. Effective nitrogen-fixing rhizobial cross-infections between these divergent plants were common, except that *Lablab* could only be nodulated effectively by one fast-growing strain, its own isolate, a *Rhizobium* strain from Papua, New Guinea (*New Guinea Rhizobium*: NGR). The *Lablab* isolate was the only fast-growing isolate from this plant. *Lablab*, like other tropical legumes is normally nodulated by bradyrhizobia. The strain, NGR234, was able to nodulate Cowpea (*Vigna unguiculata*) and to fix nitrogen on this host as efficiently as did "homologous" bradyrhizobia. Furthermore NGR234 formed an effective symbiosis with seven other plants of the "Cowpea miscellany". These all normally have *Bradyrhizobium* microsymbionts, and were *Calopogonicum caeruleum*, *Flemingia congesta*, *Macroptilium atropurpureum* (Siratro), *Macroptilium lathyroides* (Phasey bean), *Tephrosia candida*, *Vigna sequipedalis* (Snakebean), and *Glycine max*. In contrast NGR234 could not nodulate lupin, clover, pea or tested species of *Phaseolus*, and poorly nodulated *Medicago*. Trinick concluded that NGR234 represented an intermediate evolutionary form between the (brady)rhizobia of tropical soils and the specialized fast-growing rhizobia typical of temperate zones such as *Rm* or *Rlv*. The tradition of naming rhizobia or other plant-associated bacteria after the plant-host from which they were originally isolated would define NGR234 as *Rhizobium lablab*, but, in fact, NGR234 escapes the classical "cross-inoculation group" concept of *Rhizobium* speciation. Figure 1 summarizes the ecological and host-range relationships of NGR234 described in this review. NGR234 is maintained in the C.S.I.R.O (Canberra, Australia) strain collection of M.J. Trinick.

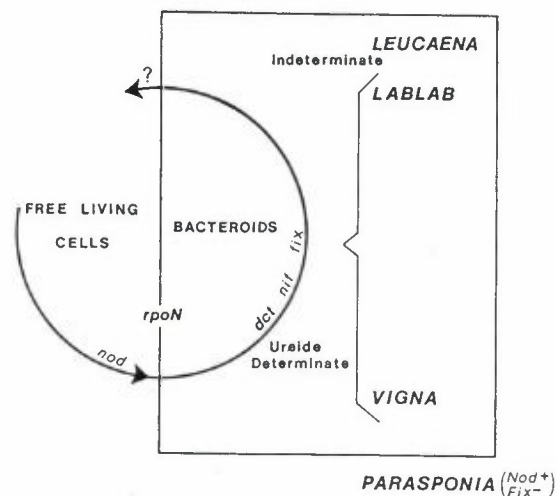Legume root-nodule morphology is of two types: indeterminate (nodules

Figure 1. Ecology of NGR234: The box represents the large extent of legume host-range. NGR234 fixes nitrogen with (is Fix$^+$ on) at least 12 legume genera, and forms ineffective nodules on the nonlegume *Parasponia*. Effectively nodulated legume host genera are: *Lablab, Calopogonium, Desmodium, Flemingia, Glycine, Leucaena, Macroptilium, Pachyrhizus, Phaseolus* (sp. *coccineus*), *Psophocarpus, Tephrosia,* and *Vigna*. Ineffective (Fix$^-$) nodules are formed on at least eight other legume genera: *Acacia, Arachis, Centrosema, Lotus, Medicago, Mimosa, Sesbania* and *Styloxanthes*. The incomplete circle in the diagram represents the life-cycle of *Rhizobium*; transition from bacteroids to free-living soil bacteria remains to be definitively established. The root nodule, a plant organ, is progressively established as an intracellular symbiosis, and during this process key bacterial genes are temporally expressed (written inside circle). The gene *rpoN* encodes an alternative sigma factor which is a fundamental regulator of endosymbiosis.

remain meristematic and continue to elongate as the plant grows) and determinate (nodules are spherical with no meristematic tissue at maturity). Furthermore indeterminate-nodule legumes from temperate zones export symbiotically fixed nitrogen from the nodule as the amides, glutamine and asparagine; whereas tropical legumes employ the ureides allantion and allantoic acid for this transport role. The capacity of NGR234 to give nitrogen-fixing nodules on plants forming either indeterminate (*Leucaena*) or determinate *Vigna, Macroptilium,* etc.) nodules is very useful in comparing nodule organogenesis and physiology. In this context, one may analyse the differential expression of "late" bacterial symbiotic genes whose products are necessary for functionality of the nitrogen-fixing nodule. Genes in NGR234 which encode the generalized symbiotic phenotypes Bad (Bacteroid Development), Fix

(Fixation), and Cof (Complementary Functions) are interesting subjects of investigation. The mature nodule is a mosaic of two types of plant cells. Infected cells contain the bacteroids within "peri-endosymbiotic membrane"; the site of nitrogen fixation. Uninfected plant cells participate in assimilation and transport of the nitrogenous reduction products. It is evident that *Rhizobium* contributes to subcellular compartmentalization of the infected plant cells since the plant-synthesized peribacteroid membrane, the primary "late" symbiotic interface, is not made in symbiosis with certain *Rhizobium* mutants (see below).

Fast-growing rhizobia are considerably more "workable" for bacterial geneticists than are the slow-growing bradyrhizobia. NGR234 is thus a natural choice for the investigation of broad symbiotic host-range. Fast-growing rhizobia from temperate zone legumes possess large symbiotic (pSym) plasmids encoding genes essential for nodulation (*nod* genes), nitrogenase (*nif* genes) and associated proteins necessary for *in planta* nitrogen fixation (*fix* genes). These large Sym plasmids, which can be transferred conjugatively in bacterial plate genetics experiments (e.g. Hooykaas et al., 1981), simplify the initial analysis of the symbiotic genes of rhizobia. They have not been detected in bradyrhizobia, which have chromosomal Sym genes.

In the molecular microbiological analysis of NGR234 and its symbiosis with divergent legumes, research groups at the Australian National University (Canberra) and the Max Planck Institute (Köln) have contributed significant genetic results. Consequently there exists in the literature two series of publications using either the Australian National University (ANU) strains of NGR234 (identical with the CSIRO Canberra strain, described above), or the derivative strain, MPIK (Max Planck Institute Köln) 3030. These strains are similar with respect to many tested host-ranges: for instance, *M. atropurpureum* and *V. unguiculata* have been employed by all groups analyzing the genetics of symbiotic host-range. It is clear that the Sym plasmids of NGR234/ANU240 on one hand and MPIK3030 on the other are well conserved over most of their length. However, published data (Nayudu et al., 1988) indicate that MPIK3030 is not genetically identical with NGR234/ANU240. In particular, the host-range of MPIK3030 does not extend to the nonlegume *Parasponia*, and its pSym contains a single copy of the nitrogenase operon *nifHDK* (Pankhurst et al., 1983), rather than the two copies (Badendoch-Jones et al., 1989) found in NGR234. There are other differences in the physical-genetic maps of their Sym plasmids, such as the spacing between the essential nodulation and nitrogen fixation genes (*nodC* and *nif* loci, see following sections). The strains of NGR234 described in this Review are detailed in Table 1.

Table 1. Strains of NGR234 described in this Review

Strains	Characteristics	origin
NGR234	New Guinea Rhizobium C.S.I.R.O. collection	Trinick, 1980
ANU240	Australian National University strain of NGR234, Str$^{®}$	Badendoch-Jones et al., 1989
ANU280	Rif$^{®}$-derivative of ANU240	Chen et al., 1985
ANU264/265	NGR234 cured of the Sym plasmid	Morrison et al., 1983
ANU1255	NGR234 *nodD1* mutant	Morrison et al., 1984, Honma et al., 1990
MPIK3030	Max Plank Institute Köln derivative of NGR234	Broughton et al., 1984

2. Genetic Organization of NGR234

The general circularity and organization of the bacterial chromosome in NGR234 has been partially elucidated by mapping genetic markers generated by random mutagenesis with transposon Tn*5-mob* (Osteras et al., 1989). In addition, NGR234 contains four plasmids, of sizes 20, 25, 300 Mdal and a cryptic megaplasmid of over 400 Mdal (Morrisson et al., 1983; 1984). Heat curing (37°C) eliminated the Sym plasmid producing concomitant loss of nodulation of *Lablab, Vigna, Macroptilium, Leucaena* and *Parasponia*. The *nif* genes were located on this 300 Mdal Sym plasmid, as in temperate zone fast-growing strains, and absent from ANU265, a derivative of NGR234 from which it was eliminated by a high growth temperature regime. Pankhurst et al. (1983) identified the Sym plasmid of MPIK3030, sized it at 300Mdal ($\approx$ 460 kb), and demonstrated regions of homology with *nod* gene probes of *Rm*. They isolated cosmids hybridizing to *nif* and nodulation (*nod*) gene probes; these regions are separated by 20–25 kb in MPIK3030. The Sym plasmids of NGR234 and MPIK3030 are not self-transmissible via bacterial conjugation. However, a cointegrate of pSym NGR234 with a vector plasmid (Morrison et al., 1984) was transferred to ANU265 in matings using the plant host (*Macroptilium*) to provide selection pressure detecting plasmid cotransfer. This restored nodulation ability to Nod^{-} (deletion) mutants of the temperate zone rhizobia *Rm* and *Rlt*, but only on a NGR234 host plant, siratro.

In order to facilitate genetic analysis of NGR234, R-prime plasmids have

been constructed genetically that contain large regions of NGR234 genomic
DNA recombined with a conjugative enterobacterial antibiotic-resistance (R)
plasmid.   Three such R-primes containing large regions of pSym NGR234
(Nayudu and Rolfe, 1987) were characterized.   Only the largest, 330 kb in
size, conferred the whole tested spectrum of NGR234 nodulation host range
on ANU265 (*Lablab, Macroptilium, Desmodium, Vigna, Leucaena, Glycine
max, Parasponia*, and *Sesbania rostrata*). This R-prime contained restriction
fragments hybridizing to gene-specific DNA fragment probes for the *Rm* Sym
genes *nodA, B, C, D, I*, and *J* and *nifH, D*, and *K*. Similarly the analysis of
three non-overlapping cosmids which conferred *Vigna*-specific nodulation on
R. loti, (Broughton et al., 1986) indicated that host-specific nodulation loci
are widely dispersed on the pSym of MPIK3030.

3. Molecular Genetic Analysis of Broad Host-Range of NGR234

Host-plant infection and nitrogen-fixing nodules result from multiple inter-
actions between *Rhizobium* and the plant. The Nodulation (*nod*) genes of
NGR234 are the determinants of symbiotic infection and its host-range. Strains
of *Rm* and *Rlv* have been used classically as models for the study of nodu-
lation genes. Nodulation gene function in a broad host-range strain such as
NGR234 elucidates the genetic mechanisms of nonspecific host-range. This is
distinct from the very specific interaction represented by *Rlv/Rlt/Rm* and their
host legumes. Common elements of *nod* gene function among these divergent
endosymbiotic rhizobia are of general significance for microbe-plant interac-
tions. Nodulation genes were first cloned from the alfalfa endosymbiont, *Rm*,
in a classic experiment employing genetic complementation of a noninvasive
(Nod⁻) mutant obtained by random Tn5 mutagenesis (Long et al., 1982).
Much work has subsequently been devoted to their organization, nucleotide
sequences, and recently to the physiological roles of the products of key *nod*
genes.

Coherent results have been obtained with respect to operon structure and
regulation in three temperate-zone narrow host-range species, *Rm, Rlv,* and
Rlt. The "common" *nod* genes, *nodABC* are essential for legume nodula-
tion and linked in these species and in all other rhizobia analyzed except
NGR234/MPIK3030. The *nodABC* genes code for production of extracellular
Nod signals. In *Rm*, the major compound among these signals is a sulphated
β-1, 4-tetrasaccharide of D-glucosamine, which specifically elicited root hair
deformation on alfalfa (Lerouge et al., 1990). Mutants in *nodA, B* or *C* are
completely non-nodulating. *nodC* was located in MPIK3030 (Pankhurst et al.,
1983) and partial DNA sequencing confirmed its homology to *nodC* genes of

Rm and *Rlv* (Bachem et al., 1985). Mutants in this gene lost the ability to nodulate *Macroptilium* and could be complemented by *nodC* genes of *Rm* and MPIK3030 (Bachem et al., 1985). Insertion mutants at both sides of *nodC* retained nodulation ability; hence, *nodA* and *nodB* are not contiguous with *nodC* in MPIK3030 (Bachem et al., 1985). However, *nodA* and *nodB*-hybridizing regions are found on the same 8 kb *EcoR*I fragment as *nodC* (Kondorosi et al., 1986). Some thirty *nod* genes have been identified to date in *Rm, Rlv*, and *Rlt*, though their physiological functions in symbiosis remain mostly uncharacterized, and many mutations do not have clearly definable null phenotypes for symbiotic infection. A description of these genes is beyond the scope of the present article, and the reader is referred to the recent review by Long (1989).

The expression of all *nod* operons requires the product of *nodD*, a gene which is linked to *nodABC* in *Rm, Rlv, Rlt*, and *Bradyrhizobium* (Rossen et al., 1985; Djordjevic, M.A. et al., 1987; Göttfert et al., 1989). However, *nodD* was found some 50 kb away from *nodC* in MPIK3030 (Kondorosi et al., 1986), and this non-linkage is unique among rhizobia. The *nodD* gene product (NODD) belongs to a family of transcriptional activators. Although initially conceived to be a conserved *nod* gene of "common" functionality, evidence has accumulated that it is a determinant of host-plant specific interaction. The NODD protein binds to the *nod* operon promoters at conserved regions of ca. 40 nucleotides which are termed *nod*-boxes (Rostas et al., 1986). Induction of these promoters requires reaction of NODD with regulatory molecules (flavonoids) of plant origin. A given rhizobial NODD protein is activated or inhibited by different flavonoids. Thus the different chemical compositions of a host plant root exudate produce overall activation or repression of the *nod* operons of its "homologous" rhizobia (Firmin et al., 1986). *Rlv* and *Rlt* each have a single copy of the gene *nodD*, and mutations therein result in a Nod⁻ phenotype (Rossen et al., 1985; Djordjevic, M.A. et al., 1987). *Rm* carries three functional *nodD* genes (*nodD1, nodD2*, and *nodD3*) which react differentially with root exudates of the three host plants of this bacterium (Honma and Ausubel, 1988). NGR234 contains two *nodD* loci, only one of which, *nodD1*, is currently proven to be functional. It is characteristic of *nodD1* in NGR234 that it shows no autoregulation (Bassam et al., 1988). The *nodD1* mutant of NGR234 (ANU1255) can be complemented interspecifically for siratro nodulation by the *nodD3* gene but not the *nodD1* or *nodD2* genes of *Rm* (Honma et al., 1990) and not the *nodD* gene of *Rlt* (Djordjevic, M.A. et al., 1985; McIver et al., 1989). When the cloned *nodD1* gene is re-introduced to ANU1255 it reconstitutes nodulation of the nonlegume *Parasponia* (Rolfe, pers. comm.).

Various DNA cloning experiments have defined the significance of *nodD1* for the broad host-range of NGR234. A cloned DNA fragment from pSym

MPIK3030 extended the host range of *Rm* transconjugants to *Macroptilium atropurpureum* (Bachem et al., 1986). A cosmid clone of pSym MPIK3030, HsnI (Broughton et al., 1986) conferred ability to nodulate *Vigna unguiculata* on *Rhizobium loti*. A cloned 6.7 kb DNA fragment containing *nodD1* of NGR234 conferred siratro nodulation on *Rlt* transconjugants (Bassam et al., 1986). All these cloned DNAs had in common the presence therein of the *NodD1* allele from the respective Sym plasmids. Horvath et al. (1987) transferred the cloned *nodD1* genes of MPIK3030 to *Rm*, obtaining transconjugants capable of nodulating siratro. They sequenced the gene and compared the derived amino acid sequence of NODD (MPIK3030) with other rhizobial species, showing that the amino terminal region is highly conserved. By constructing a chimaeric NODD protein, they demonstrated that the last 75% of the gene is responsible for the interaction with plant factors, and that this interaction regulated the expression of a *nodA* gene fusion in MPIK3030. Other data (McIver et al., 1989) suggested that flavone recognition may not be restricted only to the C-terminal domain of the NODD protein. Nayudu et al. (1988) and Bassam et al. (1988) reported the cloning and sequencing of the *nodD1* gene of NGR234. It is constitutively expressed and the encoded NODD protein can activate *nod* gene expression in response to an extraordinary variety of plant molecules. These include all the conventional flavonoid activators for *Rm*, and *Rlv*, compounds inhibitory for the narrow host-range *nod* systems, and even non-legume root exudates such as those of cotton or sunflower. Cloned DNA of the *nodD1* gene alone extended the host range of *Rlt*, to siratro, cowpea, *Vigna unguiculata, Glycine usuriensis* and the non-legume *Parasponia andersonii* (Bassam et al., 1988; Bender et al., 1988).

In summary, the above experiments indicate that *nodD1* is a key gene in determining the capacity of NGR234 to nodulate a broad spectrum of legume plants. Further clarification of its role as a primary specificity determinant of symbiotic infection has been provided by the phenotype of recombinant *nodD* genes reconstructed *in vitro* from genes of two narrow host-range species (Spaink et al., 1989). The chaemeric gene, *nodD604*, consists of 75% of *nodD1* from *Rm* and 25% of *nodD* of *Rlt*. It encodes an inducer or antagonist-independent NODD protein which activates *nod* operons in *Rm* and *Rlt* even without added plant inducers. Interspecies complementation of *nodD* mutants of these two rhizobial species by *nodD604* extends their host-range to their own host plants but also to the tropical legumes *Macroptilium, Lablab* and *Leucaena*; plants which are natural hosts of NGR234. The result suggests that *nodD1* of NGR234 may resemble this *nodD604* chimaeric gene. On the other hand, transfer of *nodD1* of NGR234 to *Rlt* did not confer ability to nodulate an entire spectrum of NGR234 host plants on this recipient (Bender et al.,

1988). Thus, additional (NGR234) genes might be necessary for formation of root nodules on some host plants such as *Desmodium* or *Leucaena*, or alternatively some native host-specific genes of *Rlt* restricted the host range of these transconjugants.

Apart from the regulatory gene *nodD*, the extent of our current knowledge of *nod* genes in NGR234/MPIK3030 cannot compare with that in *Rm/Rt/Rlv*. In these species, extensive generalized mutagenesis and plant tests originally provided a broad basis for analysis, whereas this has not been reported for NGR234/MPIK3030. However, host-range extension experiments with *R. loti*, similar to those with *nodD*, have identified at least one other locus. A *nod*-box promoter element was located in an MPIK3030 cosmid clone HsnII (Lewin et al., 1987) and the downstream region was mutated and analysed, identifying two genes *nodS* and *nodU*. Mutated DNA was recombined into NGR234 confirming that one of these genes (*nodS*) is required for nodulation of *Leucaena*. The *nodS* gene was sequenced and showed no homology to other genes in current databanks (Lewin et al., 1990).

It should be noted that symbiotic host-range is a reciprocal phenotype and not simply determined by microsymbiont genotype. For instance Diaz et al. (1989) have demonstrated that one plant component of specificity of symbiotic infection is the root lectin, presumably recognized by bacterial receptor molecules as a determinant of plant specificity. Thus if the pea lectin gene (*psl*) is expressed in the roots of transgenic white clover (a plant exclusively noduled by *Rlt*) infection of transgenic clover roots by *Rlv* occurs. Thus root lectin represents a mechanism by which the legume host selectively allows a "homologous" *Rhizobium* species to penetrate root cells and induce infection threads.

4. Nodule Organogenesis and Symbiotic Nitrogen Fixation

In the final section, we discuss some relevant symbiotic genes of NGR234 expressed "late" during symbiosis. We attempt to provide an overview of linked symbiotic metabolism in this context. We discuss the role of bacterial capsular exopolysaccharide and the genetics of the nitrogen-fixing enzyme complex. We also discuss symbiotic relations between the host and the NGR234 symbiont during the nitrogen-fixation phase: oxygen regulation, bacterial uptake of plant-supplied substrate and a key regulator of bacterial symbiotic genes, the sigma factor RPON. These relations are diagramatically represented in Fig. 2.
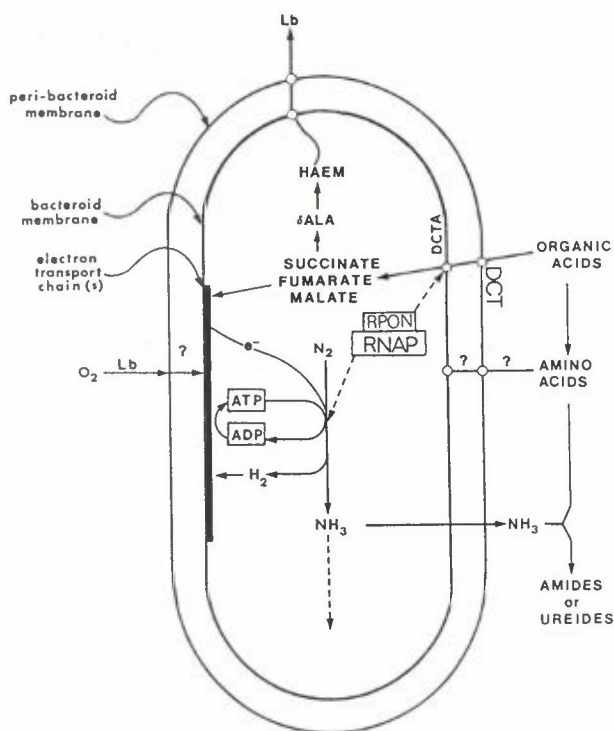
Figure 2. Linked symbiotic metabolism. The bacteroid is shown enclosed in peribacteroid plant membrane forming a nitrogen-fixing "plastid" within the infected nodule cells of the host (modified from Dilworth and Glenn, 1984).

Rhizobium mutants defective in the production of capsular exopolysaccha-ride (EPS) may be (a) unaffected in symbiotic nitrogen fixation, (b) "uncou-pled" for development of normal nodules (Finan et al., 1987) or (c) defective in both nodulation and nitrogen fixation (Chen et al., 1985). The major acidic exopolysaccharide produced by *Rm* is termed succinoglycan, an octosaccharide consisting of repeating units of β-linked glucose and galactose with acidic side-groups. The structure of the EPS unit of NGR234 determined by Djordjevic, S.P. et al. (1987) is shown in Fig. 3. This unit lacks succinate substituents, as is the case with that of *Rlt.* Chen et al. (1985; 1988), isolated EPS-defective *(exo)* mutants of NGR234. Their phenotypes varied in the type and quantity of capsular EPS. Differential effects on symbiosis with indeterminate or deter-minate nodule plants existed for some of these *exo* mutants, but for most a clear pattern of relationships is not discernible. One NGR234 *exo* mutant pro-duced normal nodules on plants that form determinate nodules, but produced only calli-like growths on *Leucaena*, an indeterminate nodule host. Chen et al.

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          β            β            β            β            β
glc-1 ──→ 6-glc-1 ──→ 6-glc-1 ──→ 4-glc-1 ──→ 4-glc-1 ──→ 3-gal
                │
                4  β
                ↑
                1
                │
              glcA
                │
                3  α
                ↑
                1
                │
              glcA
                │
                4  α
                ↑
                1
          6     │
R-pyr  <        gal-2or3-OAc
          4
```
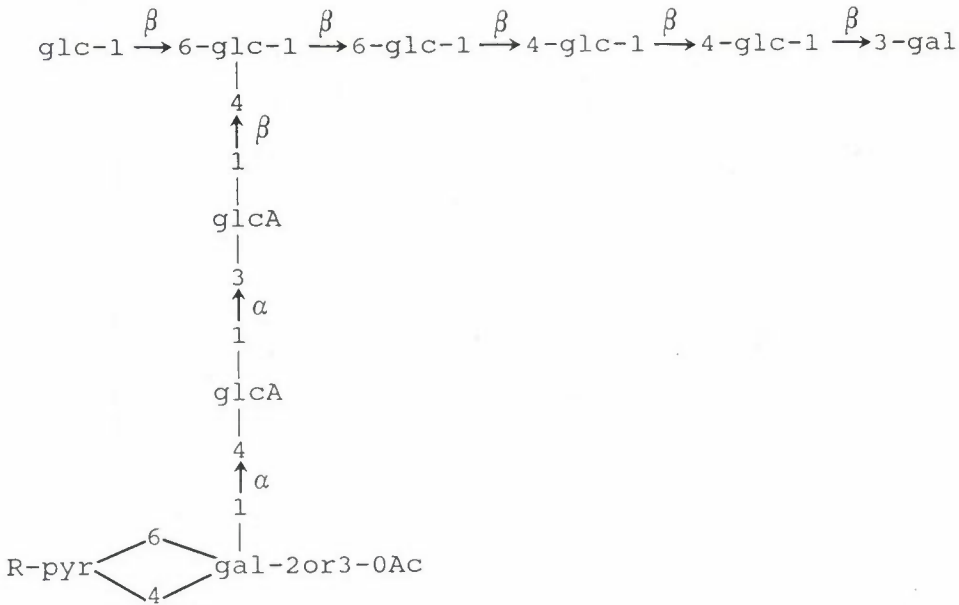
Figure 3. Structure of the extracellular polysaccharide of NGR234 (after Djordjevic, S.P. et al., 1987).

(1988) mapped five linked *exo* genes of NGR234 involved in acidic EPS synthesis. Symbiotic infection of siratro and *Leucaena* by some of these mutants was corrected by the addition of EPS from wildtype NGR234 purified through a hollow-fibre filtration unit, or the oligosaccharide repeat unit from which the polymer is built (Djordjevic, S.P. et al., 1987). Two genes, *exoX* and *exoY*, which regulate exopolysaccharide synthesis in NGR234 have been analyzed to the DNA sequence level (Gray et al., 1990). The phenotype of *exoX* is analagous to an *Rlv* gene called *psi* which is a regulator of both exopolysaccharide synthesis and symbiotic nitrogen fixation (Borthakur and Johnston, 1987).

The enzyme nitrogenase catalyzes the *in planta* reduction of nitrogen gas to ammonia. The three polypeptides of this enzyme complex are encoded by the genes *nifH, D, K*, first analysed in the free-living bacterium *Klebsiella pneumoniae*, and conserved in all other diazotrophic bacteria (Ruvkun and Ausubel, 1980). Badendoch-Jones et al. (1989) have analyzed the *nif* genes of NGR234 to the DNA sequence level. A contiguous operon structure *nifHDK*, was found, typical of the fast growing rhizobia. In *Bradyrhizobium* species on the other hand, *nifH* is separated from *nifDK* by some 20 kb. In NGR234 the
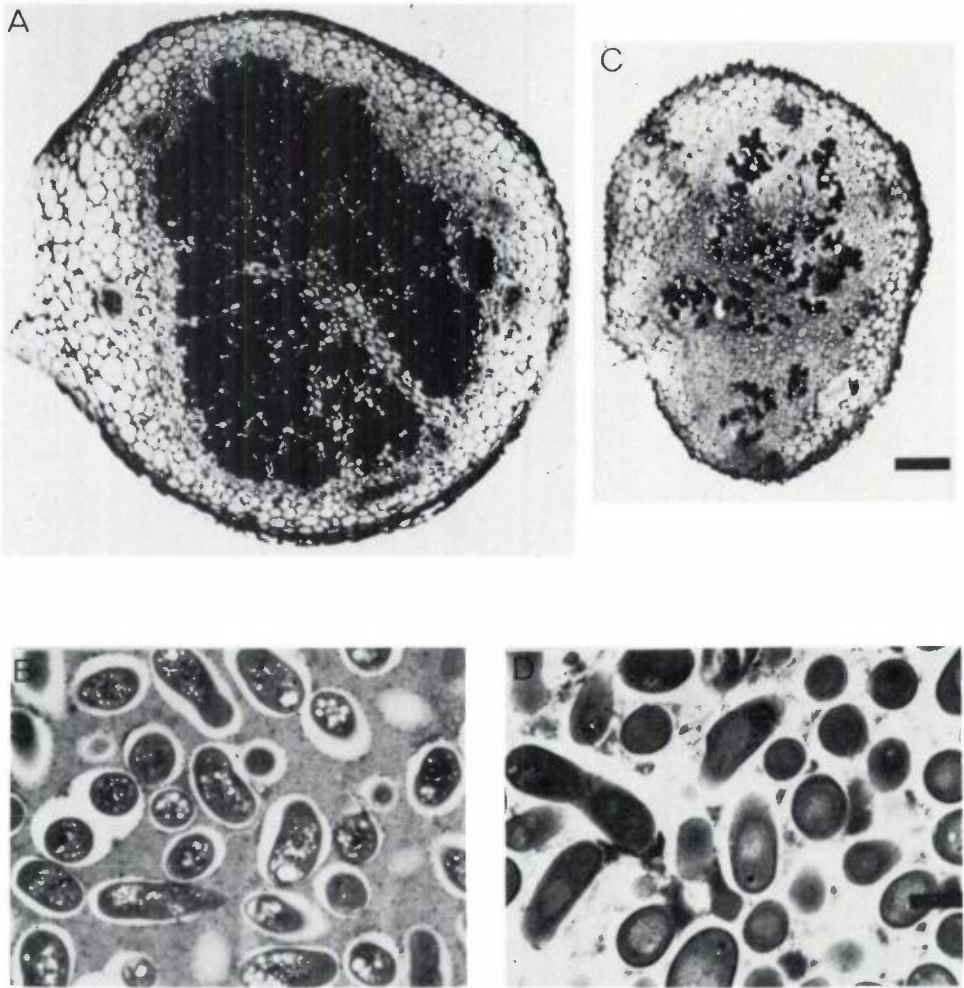
Figure 4. Structure of *Vigna ungiculata* determinate nodules formed by *Rhizobium* NGR234 and its *rpoN* mutant. A and C are tissue maps by light microscopy (magnification ×50). The wild-type Fix⁺ nodules are composed of a mosaic of infected plant cells (dark areas) and uninfected plant cells (light areas). Bar represents 200 μm. B and D are electron microscope studies of the infected plant cells (magnification ×10000) where bar represents 1 μm. In B, NGR234 bacteroids are partitioned by p.b.m. from metabolically active plant cell cytoplasm. In D, *rpoN* mutant bacteroids lack surrounding p.b.m., and plant cell cytoplasm is electron-lucent (inactive): symbiosis has degenerated to an essentially parasitic state.

operon is precisely duplicated, and within the coding region there is complete conservation of restriction sites. NGR234 shares this *nif* gene duplication with Central and South American biotypes of *R. phaseoli* (Quinto et al., 1982; 1985) and with *R. fredii* (Prakash and Atherly, 1984). Several other *nif* genes (*Q, B, V, N, E, M*) which have not been characterized in NGR234, code for products physiologically essential for nitrogenase activity. *nifA*, and *nifL* are key regulatory genes. NIFA is a protein which is an Activator of expression of the *nif* operons while NIFL is a negative regulator. In *K. pneumoniae, nifA* and *nifL* are regulated indirectly by availability to the (free-living) cell of fixed nitrogen. The latter regulation is mediated by genes termed *ntr*, which are part of the cellular machinery for sensing environmental nitrogen. Another gene product for the transcription regulation of the *nif* genes is the RPON protein. This is an alternative sigma factor which allows the transcription machinery to initiate the expression of *nif* operons. RPON is also required for expression of diverse metabolic genes in gram-negative bacteria (see review by Kustu et al., 1989). The NGR234 *nif* operons are transcribed in bacteroids in *Lablab* nodules, and show typical promoters for *nif* gene transcription regulation by NIFA and RPON (Beynon et al., 1983; Badendoch-Jones et al., 1989).

Bacterial nitrogenase is sensitive to oxygen but since *Rhizobium* is an aerobe there are seemingly contradictory elements in root nodule physiology. These are resolved by "facilitated diffusion" of oxygen within the microaerobic nodule environment (Fig. 2). It is mediated by a predominant nodule-specific plant protein called Leghaemoglobin (Lb) whose red colour is diagnostic for effective (nitrogen-fixing) nodules. Evidence that the globin apoprotein of Lb is plant-encoded was provided by comparative analysis of snake bean nodules induced by NGR234 and an evolutionarily divergent snake bean *Bradyrhizobium* strain called NGR46 (Broughton and Dilworth, 1971). Biochemical tracer experiments have indicated that the haem prosthetic group of Lb is synthesized by rhizobial bacteroids (Cutting and Schulman, 1972), via their haem biosynthetic pathway, whose first committed step is catalyzed by aminolaevulinic acid synthase (ALAS). The gene coding ALAS is termed *hemA*. In *R. meliloti hemA* mutants lack ALAS and are Fix$^-$ on their indeterminate nodule host plant, *Medicago sativa* (Leong et al., 1982). However a similar site-directed *hemA* mutant of *Bradyrhizobium japonicum* is Fix$^+$ on its determinate nodule host plant, *Glycine max* (Guerinot and Chelm, 1986). Either plant (alfalfa vs. soyabean) or bacterial (*Rm* vs. *B. japonicum*) differences could account for the Fix phenotype of *hemA* mutants. NGR234 was employed (Stanley et al., 1988) to resolve this contradiction, by cloning its *hemA* gene and constructing a site-directed *hemA* mutant. The NGR234 mutant produced Fix$^-$ nodules

on either determinate (*Lablab, Vigna, Macroptilium, Desmodium*) or indeterminate (*Leucaena*) nodule legumes, consistent with the general concept of a requirement for bacteroid haem synthesis to generate the haem group of Lb in nodules. The Fix$^+$ phenotype of *hemA* in *Bradyrhizobium* therefore suggests the existence in the slow-growing genus of a second haem synthetic pathway, which can be specifically used *in planta*.

Plant physiology and bacterial molecular genetics both suggest that the C$_4$-dicarboxylic acids succinate, fumarate and malate are the plant-supplied substrate for symbiotic nitrogen fixation. C$_4$-dicarboxylates are one of the major compounds in legume nodule-cell cytoplasm. A plant dicarboxylate transporter has been located in the peribacteroid membrane (Udvardi et al., 1988), but amino acids have not been shown to cross this membrane. Both free-living bacteria and bacteroids take up succinate, fumarate or malate via a common permease (DCTA) and metabolise them via their TCA cycle. Succinate is also required for haem synthesis (see Fig. 2). The rhizobial transport system for dicarboxylates (Dct) is essential to support bacteroid nitrogenase activity in indeterminate amide-exporting nodules of *Pisum* and *Medicago*. In *Rlv* and *Rm*, the bacterial Dct regulon is composed of three genes *dctA, B* and *D* (Ronson et al., 1984; Yarosh et al., 1989). They encode, respectively, a membrane-bound transporter (DCTA), a sensor of dicarboxylates (DCTB) and an activator of transcription (DCTD) which co-regulates *dctA* expression with the alternative sigma factor, RPON (Ronson et al., 1987). The *dct* genes of NGR234 have been cloned in the authors' laboratory. The phenotype of site-directed mutants therein will be of interest with respect to the plant-supplied substrate for nitrogen fixation in agronomically important determinate-nodule legumes such as *Vigna* and *Glycine max*. DNA sequencing of the regulatory region which precedes the NGR234 permease gene *dctA* identified a consensus promoter (cf. Beynon et al., 1983), which in other nitrogen-fixing bacteria is unique to genes regulated positively by the product of the gene *rpoN*.

In *Rm, rpoN* product is essential for expression of *dctA*, the *nif* operons and for the capacity to grow on low concentrations of nitrate (Ronson et al., 1987). The gene encodes an alternative sigma factor which confers a new set of promoter specificities on RNA polymerase, such that genes preceded by the consensus promoter element (GG-N$_{10}$-GC) are expressed in the presence of specific Activator proteins. An Activator of the nitrogenase (*nifKDH*) operon of diazotrophic bacteria is the NIFA protein. An Activator of the *Rm dctA* gene is the protein *DCTD*. The *rpoN* gene has been cloned and analyzed from NGR234 (Stanley et al., 1989). A site-directed *rpoN* mutant of NGR234 formed Fix$^-$ nodules on all tested legume hosts. The mutant strain could not transcribe *nif* gene fusions, or transport succinate. Expression of

a nodulation gene (*nodC*) fusion was also reduced. van Slooten et al. (1990) quantified delayed kinetics of nodulation in the *rpoN* mutant of NGR234 using *Vigna unguiculata* in the growth-pouch system. They sequenced the gene, and compared the deduced amino acid sequence with the homologous *Rm* and dissimilar *Pseudomonas putida* proteins, showing the existence in all RPON proteins of a conserved block of 9 amino acids at the carboxy terminus (an "RPON box"). In these divergent bacteria RPON has a similar polypeptide "fingerprint" (domain structure), but regulates diverse metabolic functions. Examination of determinate nodules produced by NGR234 and its *rpoN* mutant, show that the peribacteroid plant membrane, a critical symbiotic interface which partitions bacteroids from host cell cytoplasm, is probably not formed or defective in *rpoN* mutant symbioses: Fig 4 shows results obtained for determinate nodules of *Vigna unguiculata*. As a whole, the above results indicate that the RPON sigma factor plays a key regulatory role in expressing symbiosis-related genes of *Rhizobium* from the early stage of infection (delay of nodulation), until the establishment of effective nitrogen fixation (synthesis of peri-endosymbiont membrane by the host, transport of host-supplied substrate by the bacteria, and bacterial nitrogenase activity).

We have attempted to correlate in this Review a variety of bacterial molecular genetics findings which have a direct bearing on the ecological relationships of *Rhizobium* NGR234. This organism exhibits fast growth rate, relative genetic workability and extraordinarily nonspecific host range. It has therefore a special claim to be the *Rhizobium* species which could yield data of very general relevance to studies of the legume symbiosis.

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