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Reference


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X. PERRET & W.J. BROUGHTON

Abstract

Formation of nitrogen fixing symbioses between *Rhizobium* and legumes requires the co-ordinated expression of many bacterial and plant genes. Exchange of a series of molecular signals between the symbionts modulates transcription of nodulation and nitrogen fixation loci. Signal exchange also permits the selection of compatible partners from the many bacteria found in the rhizosphere of potential hosts. Amongst the signals exchanged, flavonoids released by host-plants act in conjunction with bacterial NodD transcriptional regulators to activate transcription of rhizobial nodulation genes. In turn, the micro-symbionts secrete families of lipo-chitooligosaccharide molecules called Nod-factors which induce formation of root nodules as well as permit entry of rhizobia into root-hairs. Once the bacteria are within the plant, expression of nitrogen fixation genes is partly controlled by low levels of free oxygen. Other signals have been recently discovered that also control the development of symbiotic interactions. Mostly because of an extremely broad host-range, *Rhizobium* sp. NGR234 is an ideal candidate to study these supplementary keys to the legume doors. Genetic analysis of the 536 kb symbiotic plasmid of NGR234 revealed a functional type three secretion system (TTSS) responsible for the flavonoid-dependent secretion of a number of proteins. Disruption of the TTSS machinery or the genes coding for the secreted proteins modifies the symbiotic properties of the NGR234 mutants.

1. Introduction

Many dissimilar Gram-negative bacteria colonise the nutrient-rich rhizospheres of plant roots. Some bacteria are pathogenic and propagate plant diseases while others form beneficial associations with legumes. In nitrate poor soils, strains of *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, and *Rhizobium* (collectively known as rhizobia), form nitrogen-fixing symbioses with leguminous plants. In compatible interactions, invading rhizobia penetrate their hosts through infection-threads that develop towards the root cortex. At the same time, new structures called nodules
emerge from meristems induced in the cortex of infected roots. Rhizobia are released from infection threads into nodule cells, where they stop dividing, enlarge and finally differentiate into nitrogen fixing bacteroids. In exchange for fixed-nitrogen, host plants provide for the bacteria a micro-aerobic environment that is rich in carbohydrates and permits efficient nitrogen fixation.

Thus, nodulation leads to controlled colonisation of plant cells by invading bacteria. Although many host-plants and effective rhizobia have the ability to enter into symbiosis with more than one partner, only certain combinations of symbionts result in the formation of nitrogen-fixing nodules (i.e. the Fix+ phenotype). Ineffective associations (Fix− lead to empty or non-fixing bacteroid containing nodules. Specificity minimises the chances of infection by pathogens as well as formation of ineffective associations that are detrimental to both symbionts. Interestingly, some rhizobia nodulate many hosts (i.e. have a broad host-range), while strains such as _R. meliloti_, _R. leguminosarum_ bv. _viciae_ and _R. leguminosarum_ bv. _trifolii_, associate with only few plants (narrow host-range rhizobia). Similarly some host-plants are nodulated by few strains whereas _Vigna unguiculata_ is one of the most promiscuous legumes known [12]. Although the mechanism responsible for such specificity (or its absence) is not fully understood, some of the molecular keys required for nodulation have been identified. Experimental evidence suggests that the progression of invasive rhizobia towards nodule primordia is challenged at various “check points”, and that molecular codes open these “gates”. In fact, it is through a continuous exchange of molecular signals that micro- and macro-symbionts co-ordinate expression of symbiotic genes [2, 17]. Although _Rhizobium_-legume symbioses develop continuously, it is convenient to divide the process into two steps. Nodulation refers to the invasion process that leads to the formation of root- or stem-nodules, whereas nitrogen fixation begins after differentiation of rhizobia into nitrogen-fixing bacteroids.

2. Nodulation

During the initial phases of nodulation (root-hair curling, bacterial entry), flavonoids and Nod-factors are the key molecular codes exchanged by the symbionts.

2.1. Flavonoids and regulation of rhizobial nodulation genes

Plants jettison large amounts of organic matter into the rhizosphere, most of which supports the growth of micro-organisms. Exuded compounds include carbohydrates, organic acids, vitamins, amino acids, phenolic derivatives, etc. Of these, flavonoids (2-phenyl-1,4-benzopyrone derivatives) are the most important from the symbiotic perspective since they specifically modulate the expression of the rhizobial genes required for nodulation (nod, nol, noe). Inducing capacity varies with flavonoids and rhizobia, and in some cases may also inhibit induction. Regulation of nod-gene expression in rhizobia varies from strain to strain, but is almost always mediated by NodD [for review see 23]. NodD proteins belong to a family of LysR-like transcriptional regulators that bind to highly conserved 49 bp DNA motifs (nod-boxes) found in the promoter regions of many nodule loci. Although NodD proteins bind to nod-boxes even in the absence of an inducer, flavonoids are generally required for a NodD-dependent expression of nod-genes (Fig. 1). Thus, NodD acts both as a sensor of the plant signals and an activator of transcription of nod-loci.
Exchange of molecular signals coordinates gene expression

**Figure 1.** NodD- and flavonoid-controlled expression of nodulation genes. Expression of most nodulation genes is controlled by NodD proteins. NodD proteins are members of the LysR family of transcriptional regulators and recognize *nod-box* consensus sequences found upstream of many *nod* loci. Although binding of NodD to *nod-boxes* occurs even in the absence of inducer, activating flavonoids are required for initiation of transcription.

Although *nodD* genes are ubiquitous in rhizobia, their symbiotic characteristics vary from one species to another. Some strains, such as *R. leguminosarum* bv. *trifolii*, have only one *nodD*-gene and in these cases, its mutation renders the strain incapable of nodulation (Nod'). In contrast, *B. japonicum*, *Rhizobium* sp. NGR234, *R. meliloti* and *R. tropici* possess two to five copies of *nodD*. In *R. meliloti*, mutation of all three copies of *nodD* is required to abolish nodulation [11], whereas inactivation of *nodD1* is sufficient to render NGR234 Nod' [21]. *nodD* products of various *Rhizobium* species also vary in that they respond to different sets of flavonoids [5,25], and NodD homologues from the same strain may have varying flavonoid preferences [9,10]. In addition, other proteins such as NolR, NodV, NodW and SyrM help regulate *nod* genes expression in various rhizobia (Table 1). Together with NodD proteins, they form complex regulatory networks that modulate transcription of nodulation loci in a host-specific manner.

**2.2. Nodulation genes and the synthesis of Nod-factors**

In response to the release by host-plants of appropriate inducers, rhizobia synthesise and secrete a family of lipo-chito-oligosaccharides (LCOs), called Nod-factors [for recent reviews see 3,17]. The first step in Nod-factor biosynthesis is performed by NodC, which assembles oligomers of β 1,4-linked N-acetyl glucosamine residues. Then, an N-linked acyl-chain is added to the non-reducing terminus of the N-
X. Perret & W.J. Broughton

Table 1. Function of transcriptional regulators known to regulate nod-gene expression in different rhizobia:

<table>
<thead>
<tr>
<th>Transcriptional Regulators</th>
<th>Micro-symbionts</th>
<th>Bradyrhizobium japonicum</th>
<th>Rhizobium meliloti</th>
<th>Rhizobium sp. NGR234</th>
</tr>
</thead>
<tbody>
<tr>
<td>NodD1</td>
<td>activator</td>
<td>activator</td>
<td>activator</td>
<td>activator</td>
</tr>
<tr>
<td>NodD2</td>
<td>repressor</td>
<td>activator</td>
<td>activator / repressor</td>
<td>not found</td>
</tr>
<tr>
<td>NodD3</td>
<td>not found</td>
<td>activator</td>
<td>not found</td>
<td>not found</td>
</tr>
<tr>
<td>NodV/NodW</td>
<td>sensor / activator</td>
<td>not found</td>
<td>not found</td>
<td>not found</td>
</tr>
<tr>
<td>NolR</td>
<td>not found</td>
<td>repressor</td>
<td>repressor</td>
<td></td>
</tr>
<tr>
<td>SyrM1</td>
<td>not found</td>
<td>activator</td>
<td>activator</td>
<td></td>
</tr>
<tr>
<td>SyrM2</td>
<td>not found</td>
<td>not found</td>
<td>unknown</td>
<td></td>
</tr>
</tbody>
</table>

Acetylglucosamine oligosaccharides by the sequential action of NodB (an N-deacetylase) and NodA (an acyl transferase). Later, the products of other nod-genes modify this basic structure by adding various chemical substituents to the acylated di- to penta-mers of N-acetyl-D-glucosamine. Since these substituents enhance rather than support the basic structure of Nod-factors, they can be likened to "baroque decorations". Although all known rhizobia possess nodABC genes, other nod-genes required for further Nod-factor modifications are found only in certain strains. As a direct consequence, specific modifications present on Nod-factors secreted by a given isolate are not necessarily found on LCOs produced by different rhizobia.

Purified Nod-factors provoke extreme curling of the root-hairs (shepherd's crook formation), which is thought to be the most specific of the early morphological responses induced by rhizobia. Nod-factors also induce cortical cell division [27], and permit rhizobia to penetrate host-tissues [20]. Since rhizobia that are unable to synthesise Nod-factors cannot nodulate, these signal molecules are obviously essential to the symbiosis. It was suggested that structural variations in Nod-factors are the determinants of host-specificity. Indeed, mutation of nodH, which encodes a sulphotransferase, leads to the absence of the sulphate group from R. meliloti LCOs, and loss of nodulation of its homologous host M. sativa. Furthermore, these nodH mutants are able to nodulate the non-homologous host V. sativa [22]. Few other direct correlations between Nod-factor substituents and host-range have been reported however, while various examples clearly show that Nod-factors alone are not sufficient to determine host-specificity [see 2]. Instead, recognition of compatible partners seems more likely to be mediated by a series of molecular codes (e.g. flavonoids, NodD proteins, Nod-factors, exo-polysaccharides, etc.) that are exchanged throughout nodulation [for reviews see 2,17].

3. Nitrogen fixation

During release from infection threads into the cytoplasm of nodule cells, rhizobia
become surrounded by a peri-bacteroid membrane. These incompletely evolved symbiotic organelles are called symbiosomes. Within them micro-aerobic conditions develop that are necessary for nitrogen fixation. It is, the combined effects of specialised plant cells acting as oxygen diffusion barriers around the nodule, as well as leghaemoglobin which reversibly binds oxygen that result in concentrations of free oxygen as low as 3 to 30 nM. As biological nitrogen fixation demands high levels of ATP however, leghaemoglobin also maintains sufficient levels of free O$_2$ to ensure an efficient oxidative phosphorylation in bacteroids. Unlike the nif-genes of Klebsiella pneumoniae that are mainly regulated by nitrogen and oxygen conditions, nitrogen fixation in symbiotic diazotrophs is primarily controlled by levels of cellular oxygen [for review see 6]. Although the regulation of nif- and fix-loci differs between rhizobial strains, it is always controlled by complex regulatory networks that include FixL/FixJ and NifA as prime components (Fig. 2).

3.1. FixLJ and the oxygen-dependent regulation of nif and fix genes

FixL and FixJ are members of a large family of two-component regulatory systems that allow bacteria to respond to environmental stimuli through the transfer of phosphoryl groups from a sensor kinase to a response regulator. Under anoxic conditions, autophosphorylation of the membrane bound FixL (the sensor) is enhanced, while the phosphatase activity of FixL is repressed. In turn, the aspartate-54 of FixJ is phosphorylated via the kinase activity of the cognate phosphorylated-FixL. This enhances the transcriptional activity of FixJ by at least 100 fold. Conversely, in conditions of higher levels of free O$_2$, the transcriptional-activation domain (C-terminus) of FixJ is masked by the non-phosphorylated N-terminal part, which prevents activation of the nif-fix regulatory cascade.

3.2. NifA-σ$^{54}$ dependent regulation of nitrogen fixation genes

NifA, which is present in all rhizobia as well as in K. pneumoniae, is the next key element in the regulation of nif- and fix-loci. Although FixJ in R. meliloti mainly controls transcription of nifA, other regulators modulate NifA expression in B. japonicum and A. caulinodans. All rhizobial NifA proteins are highly conserved however, especially in their central and C-terminal domains. The carboxy-terminus of NifA proteins contains a helix-turn-helix (HTH) motif responsible for binding to upstream activator sequences (UAS) (5'-TGT-N$_{10}$-ACA-3') found 80 to 150 nucleotides upstream of the transcription start point of NifA-controlled genes. The central domain, which consists of 240 amino acids, is conserved in all NifA proteins as well as in over 30 additional transcriptional regulators from at least 20 different species [24]. Activation of gene expression by this family of proteins requires RNA polymerase containing the alternative sigma factor σ$^{54}$. Sigma-54 confers to the core enzyme the ability to recognise and initiate transcription from a distinct class of -24/-12 bacterial promoters (5'-TGGCAC-N$_5$-TTGC-3'). Interaction of UAS-bound NifA with the σ$^{54}$-RNA polymerase complex also involves the formation of DNA loops induced by the integration host factor (IHF) (Fig. 2). Thus nif- and fix-loci controlled by NifA have promoter regions that include characteristic UAS as well as σ$^{54}$ -24/-12 motifs, and often conserved IHF binding sequences.
Figure 2. Part of the oxygen-dependent regulatory cascade responsible for the activation of nitrogen fixation genes in *Rhizobium meliloti*. Under conditions of low free oxygen, the two component regulatory system FixL (sensor)/FixJ (activator) activates expression of *nifA*. In turn, NifA proteins in association with $\sigma^{54}$-RNA polymerase complexes control transcription of most *nif* and *fix* genes.
4. **Rhizobium sp. NGR234, a promiscuous symbiont**

*Rhizobium* sp. NGR234 was selected for its ability to associate with an unusually broad spectrum of host plants. It is now known that NGR234 can nodulate more than 112 genera of legumes [19] as well as the non-legume *Parasponia andersonii* [26]. Since it combines the attributes of fast generation time [13], extremely broad host-range, as well as plasmid-born nodulation (*nod, nol, noe*) and nitrogen-fixation (*nif, fix*) genes [14], it is an ideal model for studying the bacterial determinants of symbiotic promiscuity. Early host-range extension experiments showed that most loci involved in host-specificity of nodulation (*hsn*) are carried by the 536 kb symbiotic plasmid pNGR234a [1]. More recent hybridisation and genetic data indicate that a mega-plasmid larger than 2 Mb (pNGR234b) and a chromosome of c.a. 3.5 Mb [18] also carry symbiotic genes [7,15].

4.1. A type III secretion machine contributes to NGR234 host-range

Sequencing and computational analyses of the 536'165 bp pNGR234a revealed 416 putative open reading frames (ORFs), many of which are homologous to symbiotic genes, as well as 19 *nod*-boxes and 16 conserved NifA-σ^54^ regulatory sequences [8]. A high resolution transcription map of pNGR234a was constructed to see which of these ORFs were symbiotically expressed [16]. Interestingly, transcription of a cluster of genes coding for components a type three secretion system (TTSS) was shown to be flavonoid- and NodD1-dependent [4,16]. In many plant and animal pathogens, type three secretion machines target virulence proteins directly into the cytoplasm of host-cells. Genetic analyses confirmed that a functional TTSS was required for the secretion of several flavonoid-inducible proteins of NGR234, and that type III mutants showed altered symbiotic properties on various host plants [28].

5. **Conclusion**

Although flavonoids, NodD proteins and Nod-factors play an important role in nodulation, it is now clear that a number of additional signals are also involved in the control of host-specificity. Early work showed that the relationship between the numbers of infection-threads initiated and nodules that develop from them varies from 1.5% to almost 100%, suggesting that abortion of inappropriate infections helps control nodulation [for review see 17]. Mounting evidence also suggests that there is a fine line between symbiosis and pathogenicity, since successful micro-symbionts must also either evade or neutralise plant defence systems. As TTSS genes are expressed later than loci involved in Nod-factor biosynthesis, it is possible that proteins secreted by the TTSS are involved in development of infection-threads rather than bacterial entry into root-hairs. Type three secretion systems are not ubiquitous in rhizobia however [28], suggesting that some sets of "symbiotic-keys" are specific to certain associations. If this is true, the mosaic genetic structure of pNGR234a [8] suggests that NGR234 has laterally accumulated genes and integrated them into a functional network of symbiotic signals.
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References
Exchange of molecular signals coordinates gene expression


