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Spaink, H.P.

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ROOT NODULATION AND INFECTION FACTORS PRODUCED BY RHIZOBIAL BACTERIA

Herman P. Spaink
Leiden University, Institute of Molecular Plant Sciences, 2333 AL Leiden, The Netherlands; e-mail: Spaink@RULBIM.leidenuniv.nl

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■ **Abstract** Rhizobia are soil bacteria that can engage in a symbiosis with leguminous plants that produces nitrogen-fixing root nodules. This symbiosis is based on specific recognition of signal molecules, which are produced by both the bacterial and plant partners. In this review, recognition factors from the bacterial endosymbionts are discussed, with particular attention to secreted and cell surface glycans. Glycans that are discussed include the Nod factors, the extracellular polysaccharides, the lipopolysaccharides, the K-antigens, and the cyclic glucans. Recent advances in the understanding of the biosynthesis, secretion, and regulation of production of these glycans are reviewed, and their functions are compared with glycans produced by other bacteria, such as plant pathogens.

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INTRODUCTION

Various bacterial species that belong to the α-proteobacteria and the order Rhizobiales (112) can engage in a symbiosis with plants of the leguminous family (160). These bacteria, which, based on this symbiotic behavior, are collectively called rhizobia, have the unique capacity to induce the formation of root nodules in the host plant by the production of specific signal molecules called Nod factors (101, 162, 174). The rhizobia are also able to invade the plant tissue via so-called infection threads, resulting in a differentiated root nodule-inhabiting form of the bacteria that exists inside the cells of the host plant (27). This differentiated form of the bacteria, called bacteroids, can fix gas phase nitrogen into ammonia (88). The fixed nitrogen, in the form of ammonia or alanine (183), is supplied to the host plant, which, in turn, supplies the bacteria with various nutrients (87). The rhizobial species are genetically a very diverse group (175), as is illustrated by their recent division into four different families, the Rhizobiaceae, the Phyllobacteriaceae, the Hyphomicrobiaceae, and the Bradyrhizobiaceae (112). Within these four families, only a limited number of genera have the capacity to engage in a nitrogen-fixing symbiosis with leguminous plants. Currently these genera include *Rhizobium, Sinorhizobium, Mesorhizobium, Bradyrhizobium, Azorhizobium,* and *Allorhizobium* (40).

Despite this genetic diversity, it has become clear that these genera of bacteria have many common genetic and biochemical characteristics related to their capacity to establish a successful symbiosis. These common factors include the capacity to recognize specific signal molecules, such as flavonoids (153), from the host plants and to produce special signal molecules, such as the Nod factors, which apparently are not produced by other related genera. In addition, common factors include specialized structural adaptations and special regulation of classes of molecules also occurring in most other soil bacteria. These molecules serve specialized functions during symbiotic conditions, such as (*a*) different growth conditions, (*b*) the presence of many potentially toxic compounds (e.g. phenolics and enzymes), and (*c*) the need to avoid a defense response of the plant host. For instance, it can be expected that a wide variety of cell surface characteristics of the rhizobia are different from those of other related soil bacteria. However, the identification of common adaptations is hampered by the fact that different plant-host species offer very different habitats for the guest bacteria (leading to host specificity) and that the evolution of various bacterial traits has occurred convergently from many different genetic backgrounds, as shown by the genetic diversity mentioned above. The identification of various common rhizobial characteristics that are involved in symbiosis was facilitated by the apparent clustering of various groups of genes encoding these characteristics on transmissible genetics elements, such as large plasmids in all rhizobial genera (28, 57, 83), and large transposable elements, such as the so-called symbiosis islands, in *Mesorhizobium* spp. (171). Other common rhizobial characteristics have been identified by a detailed analysis of factors that were expected to be involved in a symbiotic capacity, such as specialized nitrogen and carbon metabolism (87, 88), cell surface characteristics

(27, 94), secreted factors (54, 55, 181, 186), and specialized uptake systems (87, 111, 184). Regarding the recognition processes underlying the specialization of various subgroups of rhizobia for different host plants (i.e. host specificity), the rhizobia as a group are specialized for the structure and regulation of production of their cell surface and secreted glycans, including glycolipids, which are the major focus of this review. This statement applies, namely, for the characteristics of the Nod factors (48), the extracellular polysaccharides [EPSs (13)], the K-antigens (94), the cyclic glucans (25), and the lipopolysaccharides [LPSs (94)]. Although the extent of the specialization, compared with that of non-rhizobial species from the α-proteobacteria, is not always clear (except perhaps for the Nod factors, which seem to be uniquely produced by the rhizobia), there are various unique chemical features or unique combinations of chemical features that seem to be associated with the capacity to engage in a symbiosis with leguminous plants. This review highlights such common chemical features of the glycans listed above and links these with biological functionality. Owing to the constraints of space and to many excellent, recently published reviews in various books (160, 167, 168) and journals (22, 28, 35, 45, 78, 80, 104, 114, 120, 131, 156, 166, 178), this review mainly refers to new data that have been published in the last 2 years. The reader is also referred to the summary of the recent meeting of the International Society of Molecular Plant-Microbe Interactions (95), the proceedings of this meeting (45), and the proceedings of the 12th International Congress on Nitrogen Fixation (129).

NOD FACTORS

Structure, Biosynthesis, and Secretion of Nod Factors

The chemical structures of Nod factors produced by >30 rhizobial strains have been studied in detail (Figure 1, Table 1). Because the set of strains analyzed is quite representative for all rhizobial genera and geographic regions where they

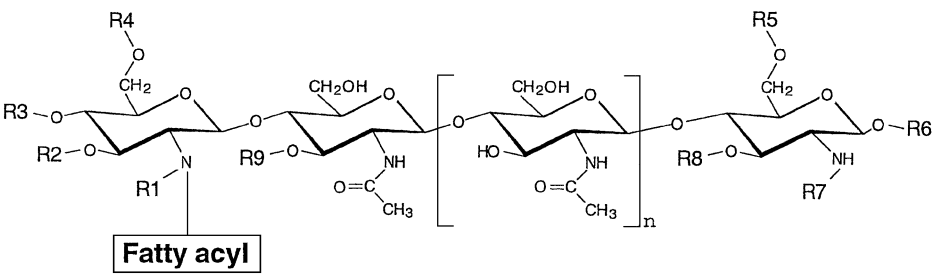

Figure 1 General structure of the Nod factors produced by rhizobia. The presence of substituents numbered R1–R9 is variable within various strains of rhizobia. For identities of these substituents and references, see Table 1. In the absence of specific substituents, the R groups stand for hydrogen (R1), hydroxy (R2, R3, R4, R5, R6, R8, and R9), and acetyl (R7).

TABLE 1 Modifications of Nod factors and gene products involved in their incorporation into the common backbone structure[a]

Bacterial strain	Nodulated plant tribes	GlcNAc residues (n)[b]	Special substituents[c]	Specialized genes[d]	Reference(s)
S. meliloti	Galegeae	3,4,5	R4:Ac, R5:S, C16:2, C16:3, C26(ω-1)OH	R4:NodL, R5:NodH, FA:NodAFEG	42
R. leguminosarum					
bv. *viciae* RBL5560	Galegeae	3,4,5	R4:Ac, C18:4	R4:NodL, FA:NodAFE	159
bv. *viciae* TOM	Galegeae	3,4,5	R4:Ac, R5:Ac, C18:4	R4:NodL, R5:NodX, FA:NodAFE	56
bv. *viciae* Al	Galegeae	3,4,5	R4:Ac, R5:Ac, C18:4, C18:3	R4:NodL, R5:NodX, FA:NodAFE	126
bv. *trifolii* ANU843	Galegeae	3,4,5	R4:Ac, R5:Ac, R6:Et, C20:4, C20:3, C18:3	R4:NodL, R5:NodX, FA:NodAFE	177
R. galegae	Galegeae	4,5	R4:Cb, R9:Ac, C18:2, C18:3, C20:2, C20:3	R4:NodU, FA:NodAFE	185
M. huakuii	Galegeae	3,4,5	R5:S, R7:G, C18:4	R5:NodH, FA:NodAFE	185
M. loti					
E1R,NZP2235, NZP2238	Loteae, Genisteae	4,5	R1:Me, R3:Cb, R5:AcFuc	R1:NodS, R3:NolO, R5:NodZ & NolL	107
NZP2037	Loteae, Genisteae	4,5	R1:Me, R2:Cb, R3:Cb, R5:AcFuc	R1:NodS, R3:NolO, R5:NodZ & NolL	108
NZP2213	Loteae	2,3,4,5	R1:Me, R3:Cb, R5:AcFuc, R9:Fuc	R1:NodS, R3:NolO, R5:NodZ & NolL	122
B. aspalati bv. *carnosa*	Crotalarieae	3,4,5	R1:Me, R3:Cb, R4:Cb	R1:NodS, R3:NolO, R4:NodU	24
B. japonicum USDA110	Phaseoleae	5	R5:MeFuc	R5:NodZ & NoeI	149

B. japonicum USDA135	Phaseoleae	5	R4:Ac, R5:MeFuc	R4:NodL, R5:NodZ & NoeI	33
B. elkanii USDA61	Phaseoleae	4,5	R1:Me/R4:Ac, R3:Cb, R5:MeFuc, R6:Gro	R1:NodS, R3:NolO, R5:NodZ & NoeI	33
R. etli	Phaseoleae	4,5	R1:Me, R3:Cb, R5:AcFuc	R1:NodS, R3:NolO, R5:NodZ & NolL	31,133
R. tropici	Phaseoleae, Mimoseae	4,5	R1:Me, R5:S, R6:Man	R1:NodS, R5:NodH	58, 134
S. fredii					
USDA257	23 tribes	3,4,5	R5:MeFuc	R5:NodZ & NoeI	10, 67
NGR234	26 tribes	4,5	R1:Me, R3:Cb, R4:Cb, R5:MeFuc/AcMeFuc/ SMeFuc	R1:NodS, R3:NolO, R4:NodU, R5:NodZ & NoeI/NolL/NoeE	135
Rhizobium sp. GRH2	Acacieae	4,5,6	R1:Me, R5:S	R1:NodS, R5:NodH	106
S. teranga bv. *acaciae*	Acacieae	5	R1:Me, R3/4:Cb, R5:S	R1:NodS, R5:NodH	110
Mesorhizobium ORS1001	Acacieae	5	R1:Me, R3/4:Cb, R5:S	R1:NodS, R5:NodH	110
A. caulinodans	Robinieae	4,5	R1:Me, R4:Cb, R5:Fuc, R8:Ara	R1:NodS, R4:NodU, R5:NodZ, R8:NoeC	114
S. saheli	Robinieae	4,5	R1:Me, R3/4:Cb, R5:Fuc, R8:Ara	R1:NodS, R5:NodZ, R8:NoeC	109
S. teranga bv. *sesbaniae*	Robinieae	4,5	R1:Me, R3/4:Cb, R5:Fuc, R8:Ara	R1:NodS, R5:NodZ, R8:NoeC	109

[a]For backbone structure, see Figure 1.

[b]The underlined numbers of *N*-acetylglucosamine (GlcNAc) residues indicate the most abundant species.

[c]The indicated substituents do not always occur in all lipochitin oligosaccharides (LCOs) produced, leading to a mixture of LCOs, which do or do not contain all possible substituents. Abbreviations: Me, *N*-methyl; Cb, *O*-carbamyl; Ac, *O*-acetyl; S, *O*-sulfyl; Fuc, α-linked fucosyl; MeFuc, 2-*O*-methylfucosyl; AcMeFuc, 4-*O*-acetyl-2-*O*-methylfucosyl; SmeFuc, 3-*O*-sulfate-2-*O*-methylfucosyl; Et, ethyl; Gro, glyceryl; Man, mannosyl; G, *N*-glycolyl; FA, fatty acyl.

[d]The genes encoding the indicated enzymes have not always been identified for each organism and are therefore in several cases hypothetical. Functions of proteins are according to Downie (48).

occur, the discovery of many new Nod factor structures is not expected. All Nod factors produced by rhizobia, with the exception of one minor Nod factor produced by *Sinorhizobium fredii* USDA191 (11), consist of an oligosaccharide backbone of ß-1,4-linked *N*-acetyl-D-glucosamine. A fatty acyl group is always attached to the nitrogen of the non-reducing saccharide. Because of the resemblance of the oligosaccharide backbone to a fragment of chitin, the Nod factors are often called lipo-chitin oligosaccharides (LCOs). All rhizobia appear to produce complex mixtures of LCO species. Differences in structures occur as result of the following variations:

1. Variation in the number of *N*-acetyl-D-glucosamine units. Most commonly, LCOs that vary in length from three to six *N*-acetyl-D-glucosamine units are produced; however, a strain of *M. loti* was shown to produce a dimeric LCO species (122).
2. The presence or absence of strain-specific substituents, indicated as R1 to R9 in Figure 1. In terms of the number of substituents found, one can distinguish bacterial strains that produce LCOs with only a few modifications, such as *R. leguminosarum* bv. *viciae* strains, which contain only an acetyl substituent at position R4, or many modifications, such as *S. fredii* strain NGR234.
3. Variation of the structure of the fatty acyl moiety attached. LCOs can contain one of a broad variety of fatty acyl groups that also occur commonly as moieties of the phospholipids. It is thought that the ratios of the common types of fatty-acyl substituents reflect the composition of the fatty acyl pool that is present as components of the phospholipids. C18–C22 (ω-1)-hydroxy fatty acyl, which are possible intermediates in the synthesis of C23 (ω-1) hydroxy fatty acyl groups found in the rhizobial LPS, can be present in the LCOs produced by *S. meliloti* (41).
4. The presence or absence of special α,β-unsaturated fatty acyl moieties. These can be present in the LCOs produced by *S. meliloti, R. leguminosarum* biovars *viciae* and *trifolii, R. galegae*, and *M. huakuii*. The relative abundance of LCOs containing a special fatty acyl (as compared with common fatty acyl moieties) in the mixtures produced varies considerably in the different strains tested (101, 159, 162, 185). Some researchers have not been able to detect LCOs that contain highly unsaturated fatty acyl moieties in *R. leguminosarum* biovar *trifolii* (124). This difference from earlier results was suggested, by van der Drift et al (177), to result from a difference in the tested strain or in the laboratory conditions used to grow the bacteria.

The biosynthesis of Nod factors has been studied extensively (48, 92). Several proteins encoded by the so-called *nod, nol,* and *noe* genes have been shown to play a role in the biosynthesis of LCOs (Figure 1). For some of these proteins, detailed biochemical analyses have indicated their position in the biosynthetic pathway

leading to the production of LCOs, including various strain-specific modifications (48, 92, 158). The NodC, NodB, and NodA proteins play a pivotal role in the synthesis of the LCO-backbone structure, by their function as chitin oligosaccharide synthase, chitin oligosaccharide deacetylase, and acyl transferase, respectively (3, 66, 86, 145, 165). These functions were demonstrated by in vitro enzymatic activity of purified protein for the NodB protein only (86). Further biochemical studies on the function of the NodC protein are still in progress and will lead to further insights into the mechanism of chitin synthesis, a process that is still poorly understood (89, 91, 93). The acyl group used as a substrate for the acyltransferase NodA, which biochemical function is the least understood, has been shown to be delivered directly by an acyl carrier protein (141). The functions of the NodC and NodA proteins have been shown to be specialized in several rhizobial species. Namely, the NodC proteins of *S. meliloti*, *M. loti*, and *R. leguminosarum* are different in that the average chain lengths of the chitin oligosaccharides produced are different (90, 143). The NodA proteins of *R. leguminosarum* and *S. meliloti* have been shown to be specialized for the transfer of α,β-unsaturated fatty acyl moieties (64, 142, 143).

In most studies, LCOs were isolated from the spent culture for structural analysis, which indicates the existence of a secretion mechanism (158). Because LCOs are hydrophobic molecules, they might be present in the medium as multimeric forms or are attached to carrier compounds such as extracellular cyclic glucans or one of the many secreted rhizobial proteins (15, 54, 181). However, proof for this hypothesis is still lacking. The *nodI* and *nodJ* genes, which are members of the type-I transport protein family (48), have been shown to play a role in the secretion of LCOs (32, 52, 164). However, in knockout mutants of the *nodI* and *nodJ* genes, substantial amounts of LCOs are still found to be secreted in the medium (32, 52, 164), indicating that other transport mechanisms are also operational. Because the LCOs are unlikely to be able to flip-flop over the membrane bilayer (69), such alternative transport mechanisms are likely mediated by proteins. Candidates for such proteins are ABC-type transport proteins related to NodI and NodJ (23) or the RND type of efflux pump proteins, such as NolF, G, H, and I, which are found in *S. meliloti* (4, 148).

Regulation of Nod Factor Biosynthesis

Nod factors are produced in response to inducers that are secreted from the plant roots. The most potent of these inducers belong to the group of flavonoids (153). Other molecules, such as the betaines (e.g. stachydrine and trigonelline) and the aldonic acids (e.g. erythronic acid and tetronic acid), are active as inducers in some rhizobial species at much higher concentrations (61, 153). The induction of Nod factor production is specific for the structure of the flavonoid. The specificity of this process has been shown to be mediated by the protein NodD, which is a positive transcriptional regulator belonging to the LysR family and found in all rhizobial species (82, 153, 163). In several rhizobial strains, multiple isoforms

of the *nodD* genes are found which, for *S. meliloti*, have been shown to be specialized in their response to different groups of flavonoids. This multiplication of the *nodD* genes is thought to have evolved so that the bacteria could adapt the structures of the Nod factors to their interactions with multiple hosts that secrete different flavonoids (41). The transcriptional regulation by NodD in several rhizobia is further complicated by the occurrence of one or more other LysR family members, called *syrM* genes, that coregulate the production of Nod factors apparently independently from flavonoids (6, 77, 153). It is interesting that the *syrM* gene is also involved in the production of the extracellular polysaccharides (see below; 50, 118). In *B. japonicum*, the NodV and NodW proteins belonging to the two-component type of regulatory proteins are involved in the recognition of the iso-flavonoid genistein (102). Nod factor production in *B. japonicum* is also regulated by the *nolA* gene (103). *nolA*, which encodes three functionally distinct proteins, is probably involved in mediating specificity toward different soybean genotypes via the regulation of *nodD2* (63).

In addition to positive regulators of Nod factors production, negative regulation is observed. In the genera *Sinorhizobium* and *Rhizobium*, a repressor called *nolR* has been identified that can bind to particular target sequences in the promoter regions of genes involved in Nod factor synthesis (98).

Nod factor production might also be regulated at the posttranscriptional level, which was suggested by the results of a recent study showing that the presence of several modifications of the Nod factors is regulated by growth temperature (123). A likely mechanism for the findings reported by Olsthoorn et al is that the activity of the acetyltransferase NodX, which is involved in the substitution of an acetyl at position R5 (Figure 1, Table 1), is strongly temperature dependent.

Little is known about the regulation of Nod factor synthesis in soil or in planta after the initial infection steps. Although it has been shown that the *nod* genes that are essential for Nod factor synthesis are switched off at later stages of the symbiosis, there is no information on the regulatory mechanisms responsible for this down-regulation (152, 157).

Function of the Nod Factors

The Nod factors are pivotal for the capacity of rhizobia to induce root nodules and various other responses that are related to the infection process in the host plant (38, 76, 158). The recognition mechanism underlying this induction process is currently unknown, but it is the subject of several current intense investigations (46, 51, 75, 76, 80, 156). It will be of particular interest to find out whether the signal recognition of the structurally analogous chitin oligosaccharides, which play a role in plant defense and vertebrate embryogenesis, shares similar molecular mechanisms (5, 169).

In conjunction with their pivotal function in the root nodule formation and infection processes, the Nod factors play a major role in the determination of host

specificity of these processes. The host-specific characteristics are based on the structural variations in the Nod factors produced by rhizobial strains that have different host ranges. Although it could be postulated that all variations found in the Nod factors reflect adaptations to the host range of the particular rhizobial strain under study, this is by no means demonstrated for all found modifications. It can also be argued that several of the variations found could merely be artifacts, which result from artificial cultural conditions that lead to overproduction of the Nod factors even in wild-type strains. Furthermore, some of the complexities of the mixtures of Nod factors produced could be the result of the loss of some of the particularly labile groups, such as *O*-acetyl or *O*-carbamyl groups, during the isolation procedure. However, in many cases it is very clear that modifications found in the structures of Nod factors are important as host range determinants (28, 35, 44, 53, 78, 104, 114, 156, 158). Most clearly this is the case for modifications at R5 of the reducing terminus of the Nod factors (Figure 1, Table 1). Three examples of the importance of modifications in Nod factor structures are the following: (*a*) The presence of the sulfyl substituent in the Nod factors of *S. meliloti* is essential for host-specific nodulation of *Medicago sativa* and prevents nodulation on other host plants such as *Vicia sativa* (101); (*b*) the *O*-acetyl substituent in the Nod factors of *R. leguminosarum* bv. *viciae* TOM and A1 is essential for cultivar-specific nodulation of pea (56, 125); (*c*) the fucosyl substituent in the Nod factors of many rhizobia is essential for determining a broad host range, for example for several plant species belonging to the tribe of Phaseoleae (105). Furthermore, modification of this fucosyl moiety is important for nodulation and infection of various host plants (18, 36, 79, 127, 136).

At the non-reducing terminus, modifications such as the carbamoyl or acetyl groups at R4 and the methyl group at R1 have also been shown to be important for establishing nodulation capacity for various host plants (39, 84, 85, 162). Of particular importance for determining host specificity is the presence of special α,β-unsaturated fatty acyl moieties in the Nod factors of various rhizobial strains (2, 43, 159, 162, 185). It recently has become apparent that the occurrence of an α,β-unsaturated fatty acyl moiety is correlated with the capacity to nodulate the leguminous species belonging to the Galegeae tribe (185). It is interesting that the plants belonging to this tribe form a particular type of indeterminate nodules (i.e. long-shaped nodules that contain a persistent meristem), which are characterized by their ontogeny from inner cortical root cells (76).

EXOPOLYSACCHARIDES

Structure, Biosynthesis, and Secretion of Exopolysaccharides

The structures of EPSs produced by >20 strains of rhizobia have been studied (13, 178). As in many other proteobacteria, the EPS consists, at least partly, of large heteropolymers formed from repeating unit structures. The carbohydrate components found in rhizobia are mainly common monosaccharides like D-glucose,

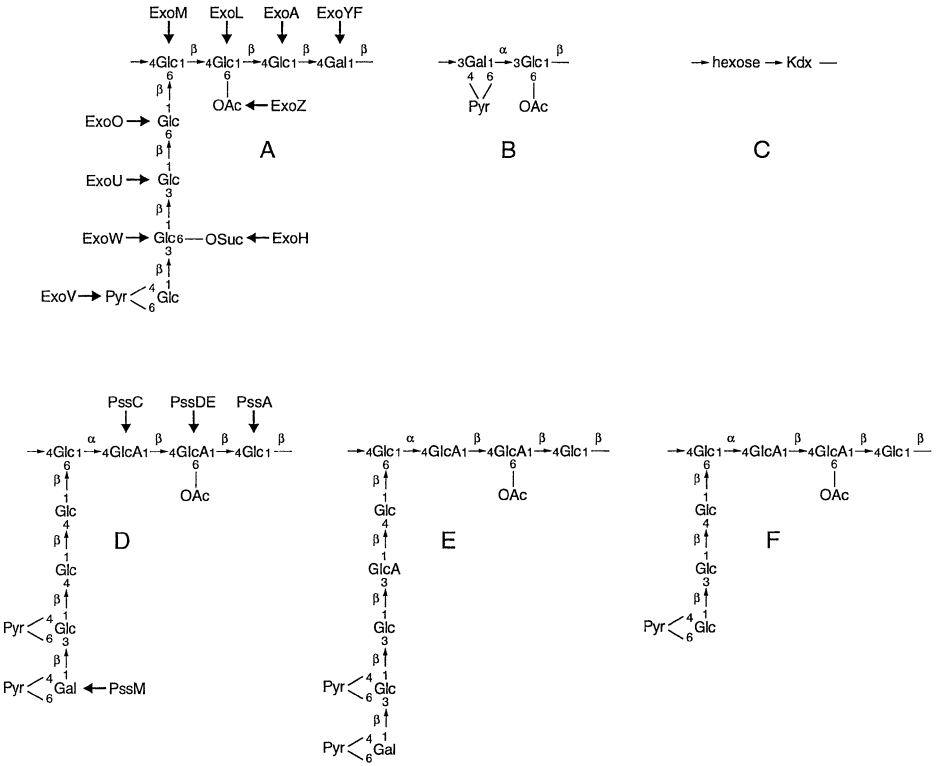

Figure 2 Structures of repeating units of exopolysaccharides of several rhizobia. Shown are the structures of the repeating units of EPS I (*A*) and EPS II (*B*) of *S. meliloti* strain SU47, the consensus structure of K-antigen found in various *Sinorhizobium* strains (*C*), the EPS structures of *R. leguminosarum* bv. *trifolii* strains LPR5 (*D*), and strain 4S (*F*), and *R. leguminosarum* bv. *viciae* strain 248 (*E*). The structures are derived from the work of Becker & Pühler (13), van Workum & Kijne (178), and Reuhs et al (137). Functions of glycosyltransferases are from the work of Becker & Pühler (13) and van Workum et al (179). Abbreviations: Glc, glucose; Gal, galactose; GlcA, glucuronic acid; Kdx, any 1-carboxy-2-keto-3-deoxy sugar; pyr, pyruvate; Suc, succinate; Ac, acetate.

D-galactose, D-mannose, L-rhamnose, D-glucuronic acid, and D-galacturonic acid. The repeat units are highly variable up to the species level, as is exemplified by the occurrence of different repeat units within the same biovarieties of *R. leguminosarum*, for example, the biovars *trifolii* and *viciae* (Figure 2). Some of the saccharide units can be modified by acetyl, pyruvyl, succinyl, and hydroxubutanoyl groups. The resulting complexity is often aggravated by the occurrence of multiple forms of EPS of the same repeat subunit or different repeat subunits. For example, in *S. meliloti*, two different EPS classes, called EPS I (a succinoglycan) and EPS II (a galactoglucan), have been identified (Figure 2). In both

of these forms of EPS, two different size classes can be distinguished: a class of high-molecular-weight molecules, consisting of thousands of saccharide units, and a class of low-molecular-weight molecules, consisting of 8–40 saccharide units (70, 188).

The biosynthesis of the rhizobial EPS has been most extensively studied for the succinoglycan of *S. meliloti*. The genes responsible for the synthesis of the precursor molecules, the repeating units, and polymerization, called *exo* and *exs*, have been identified by mutant studies and based on their homology with genes from other bacterial species (13). The identification of these genes was facilitated by their clustering on a megaplasmid (100). Unlike LCO synthesis (92, 113), the synthesis of the repeating unit of the EPS proceeds via a prenyl carrier, which results in the formation of a lipid-linked octasaccharide intermediate (173). The glycosyl transferases involved in each successive step in the synthesis of the repeating units have all been identified, except for the transferase involved in the addition of the terminal saccharide of the repeat unit (13; Figure 2). In addition, several proteins involved in secretion (ExsA) and polymerization (ExoQ, ExoT, and ExoP) have been identified (13, 14, 70, 71). Homologous gene products that are involved in the synthesis and secretion of EPS II of *S. meliloti* (15) and the EPSs of other rhizobial species (Figure 2), such as *R. leguminosarum* bv. *trifolii* (99, 132, 178) and *B. japonicum* (16), have also been reported. The prediction of the biochemical function of these homologs has been difficult in many cases (13) owing to limited sequence similarities, which is not surprising because even functionally closely related glycosyl transferases tend to have little sequence similarity. Nevertheless, biochemical function can be quite conserved, as was demonstrated by Pollock et al (132), by the functional exchange of the *R. leguminosarum pssDE* genes (Figure 2) with the *spsK* gene of *Sphingomonas* sp.

In addition to the EPS polymerization enzymes ExoP (14), ExoQ, and ExoT (71), secreted glycanases also play an important role in regulation of EPS chain length (Figure 3). Such secreted glycanases have been identified in *S. meliloti* [called ExoK and ExsH (186)] and *R. leguminosarum* bv. *viciae* [called PlyA and PlyB (55)]. Although the ExsH and PlyA/B glycanases share no obvious sequence homology, their secretions are both dependent on type I transport proteins, called PrsD and PrsE (54, 186). The PrsD and PrsE transport proteins also play a role in the secretion of the NodO protein, which has an as yet unknown function in nodulation (54). In contrast, ExoK is probably secreted by the *sec* signal peptide-dependent system (55). Detailed analysis of the activity of the ExoK and ExsH proteins has shown that their enzymatic activity is influenced by the presence of succinyl and acetyl modifications of the succinoglycan (188). The absence of the acetyl group increases the susceptibility of succinoglycan to cleavage, whereas the absence of the succinyl group decreases the susceptibility in a more dominant way (188). The apparent stringent regulation of EPS cleavage has been demonstrated by York & Walker (187), who showed that the ExoK and ExsH proteins specifically hydrolyze nascent succinoglycan only during a limited period after its synthesis.

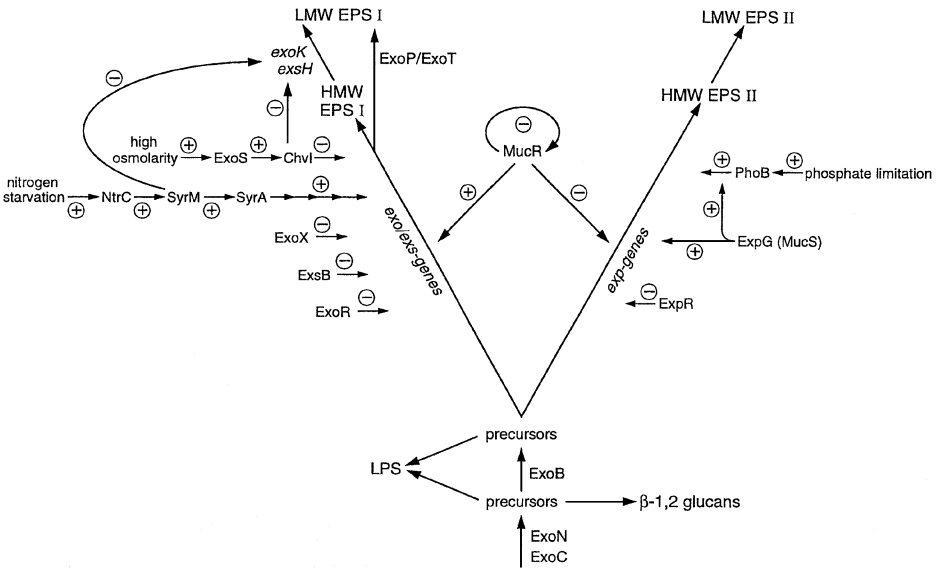

Figure 3 A model for the function of regulation of exopolysaccharide biosynthesis and depolymerization in *S. meliloti*. The figure is based on the model by Becker & Pühler (13) and recent new data that are discussed in the text (34, 50, 71, 147).

Regulation of Exopolysaccharide Biosynthesis

Various factors have been shown to regulate both the quantity and structural features of EPS. In *S. meliloti*, these factors include (*a*) osmolarity of the medium, which regulates the relative abundance of low- and high-molecular-weight forms of EPS I (26); (*b*) nitrogen starvation, which up-regulates EPS production and favors the production of low-molecular-weight EPS I forms (50); and (*c*) phosphate limitation, which stimulates production of EPS, most clearly noticeable by a relatively higher production level of EPS II (147). Various regulatory genes that regulate synthesis of EPS I and EPS II have been identified in *S. meliloti* (Figure 3); however, their interconnectedness is not yet understood, and a detailed overview of their target genes is not yet available. The identification of several key regulatory genes and several homologs in other rhizobia (60, 115, 116) is now leading to some general insights into the molecular mechanism of EPS regulation (13). In *S. meliloti*, most notable is the role of the MucR protein, which is a key regulator of the relative levels of EPS I and II (Figure 3). The MucR protein, which is very similar to the Ros proteins of *Agrobacterium* spp. and *R. etli*, exerts its positive or negative regulation of the EPS biosynthesis genes probably directly by binding to a conserved DNA sequence called the Ros box (160). Other regulatory proteins, such as ExoR, ExsB, ExoR, ExoS/ChvI, ExpG, and ExpR (Figure 3), seem to be more specialized for the production of only one of the EPS classes; however, this

picture might be misleading because cross-talk between the regulatory switches shown in Figure 3 has not yet been directly studied. Illustrative for the high level of regulatory complexity are the recent results of Dusha et al (50) on the regulation of EPS production by nitrogen starvation. These authors have shown that the SyrM protein of *S. meliloti* is involved in regulating the relative ratios of low- and high-molecular-weight forms of EPS I. Because SyrM production is negatively regulated by SyrB (6) and positively regulated by NodD3 (see the paragraph on Nod factor regulation), which in turn is regulated by flavonoids and nitrogen limitation [via NtrC (50)], this suggests many possibilities of co-regulation of Nod factor and EPS production. It is very likely that still other connections with known regulatory mechanisms that are involved in various cell surface factors will be discovered. For instance, an interesting question is whether the quorum-sensing control mediated by *N*-acyl homoserine lactones, which are probably involved in regulating infection or nodulation (74, 95, 144, 155), also plays a role in the regulation of EPS synthesis. That this might be the case is suggested by data obtained on the regulation of EPS production in the plant pathogen *Pantoea stewartii*, which apparently is under stringent control of an *N*-acyl homoserine lactone that is involved in disease symptoms (12).

Although other factors, such as sulfur limitation, have been reported to regulate EPS synthesis as well, the regulation by these factors has not been studied at a molecular level. Completely unexplored is the effect of plant-determined factors that play a role in later stages of the symbiosis, such as oxygen concentration. It can be expected that the identification of the complex regulatory networks in which SyrM and SyrB are involved will lead to further insights on the importance of late symbiotic factors in EPS production and modification. Such studies will certainly be rewarding because it has already been shown that certain rhizobia can produce EPS molecules in later stages of the symbiosis that are quite distinct from the EPS in the free-living stage, as has been shown in the *B. japonicum*-soybean symbiosis (1, 170).

Function of Exopolysaccharides

Owing to its abundance, highly charged nature, and location at the extracellular surface, EPS is expected to function in protection against environmental factors, attachment to surfaces, and osmoregulation. By its effect on the Donan potential, it also influences ion transport. However, extensive studies of the symbiotic phenotypes of many rhizobial mutants that are disturbed in EPS production have shown that EPS also plays a major role in the infection of the leguminous host plants (13). Some major complicating factors in all of these studies have been that the symbiotic phenotypes of the studied mutants in many cases differ in various rhizobia-host plant interactions and that the symbiotic phenotype was often masked by other factors, which could compensate for the loss of the capacity to produce EPS. This latter apparent redundancy of EPS has probably even been underestimated in initial studies with EPS I of *S. meliloti*, because of the stringent regulation of

compensating factors such as EPS II and K-antigens (see below) under the chosen laboratory conditions. A major breakthrough in the understanding of the symbiotic role of EPS has come through the discovery that the external addition of low-molecular-weight fractions of EPS could complement the defects in the infection phenotype of EPS mutants (9, 47). The subsequent discoveries that the *S. meliloti* low-molecular-weight forms of EPS I, as well as EPS II, are able to rescue the defects of EPS mutants at picomolar concentrations indicated the existence of a specific recognition system for EPS oligosaccharides in the plant host (9, 70). It can be suggested that this recognition system is involved in the suppression of a defense response by the plant host, based on observations that EPS mutants usually are more active in eliciting a plant defense response (119, 128). However, molecular details on the underlying recognition system remain completely obscure. Therefore, there is currently no explanation for seemingly conflicting observations, such as the possibility of complementing defects in EPS I synthesis with the structurally completely different EPS II fragments (70) and the apparent inability of heterologous EPS to complement EPS mutants of *S. fredii* strain NGR234 and *R. leguminosarum* bv. *trifolii* (47).

LIPOPOLYSACCHARIDES AND K-ANTIGENS

Structure, Biosynthesis, and Regulation of Lipopolysaccharides and K-Antigens

Rhizobial LPSs and K-antigens are often discussed as one group because they usually are both tightly linked to the cell surface and because of the common occurrence of various special saccharide residues, such as Kdo (3-deoxy-D-manno-2-octulosonic acid). However, because rhizobial K-antigens are structurally very distinct from rhizobial LPSs in all other aspects (e.g. K-antigens do not always contain Kdo, and lipid anchors have not been found at all, whereas their occurrence is standard in LPSs), their joint discussion merely reflects historical lines of investigation (94).

Considering the great complexity of LPS structures, only a few rhizobial LPS structures have been described in detail. The most complete structure, which describes all three easily separable parts of LPS (i.e. the lipid A, core chain, and repeat unit of the O-antigen chain), has been reported (59) only for the LPS of *R. etli* (Figure 4). Identification of parts of the LPS structures from several other organisms shows that the LPSs of various rhizobia are highly variable, especially for O-antigen, but also in their core region and the lipid A moiety (94). A typicality of the LPS structures of rhizobia is the occurrence of the very long chain hydroxy fatty acids, such as 27OH-C28:0 (81), which seem to be exclusively found in the LPSs of the α-proteobacteria (20, 21) and also in the Nod factors (see above). Structural details of the lipid A and core regions of LPS have been shown to be very useful markers to recognize the phylogenetic relationships of rhizobia (94).

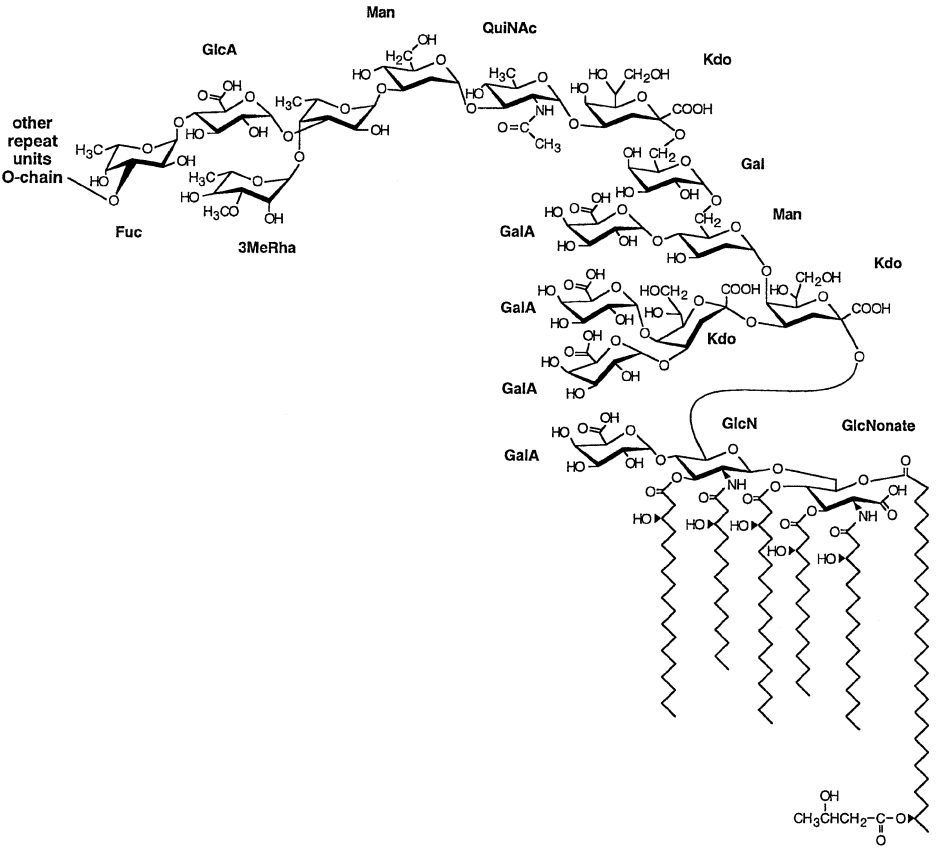

Figure 4 The structure of the core region and part of the O-antigen chain of lipopolysaccharide of *R. etli* strain CE3, from Forsberg & Carlson (59). The position of the 27-OH-C28:0 at the C5 of GlcN-onate is hypothetical. Abbreviations: GalA, galacturonic acid; GlcA, glucuronic acid; Kdo, 3-deoxy-D-manno-2-octulosonic acid; GlcN-onate, 2-amino-2-deoxygluconic acid; QuiNAc, 2-*N*-acetamido-2,6-dideoxyglucose (*N*-acetylquinovosamine); GlcN, glucosamine; Man, mannose; 3MeRha, 3-*O*-methylrhamnose; Fuc, fucose.

For instance, the core oligosaccharides of *Sinorhizobium* strains appear to be quite different from those of *Rhizobium* or *Bradyrhizobium*, in that they are the dominant antigenic region of the LPS (94).

Still very little is known about the genes that are involved in LPS biosynthesis, and progress is slow, which is not surprising considering the variability and complexity of LPS structures and the general lack of homology between functionally similar glycosyl transferases. Relatively well studied are the genes from *R. etli*, for which some of the genes involved in LPS core and O-antigen synthesis are located on a plasmid (62, 180). Concerning the genes involved in the biosynthesis

of the lipid A moiety, considerable progress has been made by a comparison with the *Escherichia coli* system (7, 8, 29). A recent model postulates that the specific structures found in rhizobia are derived from intermediates that resemble the LPS structures of *E. coli* (94).

Based on epitope mapping with monoclonal antibodies raised against O-antigen structures, evidence has been obtained that the structure of LPS is differentially regulated during symbiosis (27). Especially during the later stages of symbiosis, quite abrupt changes in LPS epitopes occur, indicating that this is the result of degradation or modification of LPS structures rather than repression of their synthesis (72). It is interesting that such rapid changes in the LPS structure during *Vicia* infection take place in the same region in which the synthesis of some outer membrane proteins of the infecting bacteria is repressed (111). Currently, no information is available on the genetic and physiological bases that underlie these observed changes. However, further analysis of the regulatory mechanisms underlying LPS modifications induced by plant exudate factors, such as the anthocyanin identified by Duelli & Noel (49), could lead to possible clues as to how rhizobial LPS synthesis is regulated in planta.

Structures of the K-antigen are as diverse as those observed with the O-antigen chains of LPS. Already within the same species, such as *S. meliloti*, the K-antigens can differ greatly in structure. Although, within the genus *Sinorhizobium*, a general consensus structure (Figure 2) has been formulated (94), two exceptions to the rather non-stringent defined repeat unit have already been identified (68, 137, 139).

Composition analysis showed that K-antigen-like acidic polysaccharides of other species of the Rhizobiaceae family are not similar to the consensus structure formulated for *Sinorhizobium* spp., with the exception of *Agrobacterium* strains (94, 139). A very interesting observation is that the host-root exudate and flavonoids that induce Nod factor synthesis are able to increase the production of K-antigen and change the minor to major K-antigen ratio in *S. fredii* (138, 140).

For a recent overview of K-antigen biosynthesis, the reader is referred to Kannenberg et al (94). The biosynthesis of K-antigens has still been studied only in *S. meliloti* Rm41 (97) and, in most detail, in a mutant derivative of strain Rm41 that is no longer able to produce EPS because of a mutation in the *exoB* gene (30). Detailed analysis of various classes of mutants affected in K-antigen biosynthesis in this strain indicates that the biochemical pathways for K-antigens and LPS may share common steps.

Function of Lipopolysaccharides and K-Antigens

As in all gram-negative bacteria, LPS is essential for their survival under all growth conditions, which makes their study very difficult. Studies of the symbiotic phenotypes of various mutants in which the LPS synthesis is altered indicate that LPS plays an important role during the infection process (27, 94). The fact that at least all nonpleiotropic LPS mutants are able to infect plant tissue to some degree indicates that LPS is not specifically involved in the initial steps of the symbiosis

up to root hair infection. However, it is very possible that other factors are able to complement the defects in LPS. The source of such a caveat is that initiation of the infection thread requires the presence of rhizobia at the site of infection, and therefore the capsule (in which LPS is dominantly present) would seem to be the required element. Nevertheless, LPS seems to play a more apparent role in the later stages of root nodule invasion, release from the infection thread, and symbiosome development. Because the infection process is tightly linked to the formation of a full-grown nodule, it is clear that defects in these infection steps also influence the nodulation phenotype. Generally, LPS seems to be less important for plants belonging to the Galegeae tribe, which form indeterminate nodules (see above). However, proof for any host-specific adaptation is still lacking, and some results strongly argue against this. For instance, *R. leguminosarum* bv. *viciae* strains that are genetically engineered to produce suitable Nod factors can successfully invade *Lotus japonicus* plants (127), whereas the natural symbiont of this plant (*M. loti*) is extremely different from these rhizobia in the composition of its LPSs (94). However, host-specific features of LPS were suggested by results obtained by Dazzo et al (37), who found that the LPSs of *R. leguminosarum* bv. *trifolii* promoted infection thread formation in clover, an effect that was not observed with the LPS obtained from heterologous rhizobia.

Microscopic studies of responses of plants inoculated with LPS mutants have indicated that rhizobial LPS is involved in suppressing a host-plant defense response (130), possibly in analogy with a role for the LPS of plant pathogens (154). The same function could be postulated as a function for the K-antigens of *S. meliloti*, because K-antigens can functionally replace EPS biosynthesis in symbiosis (30). However, K-antigen is functionally different from EPS in that it can induce the transcription of the isoflavonoid biosynthetic pathway in alfalfa leaves, which is indicative of the triggering of defense responses (17).

CYCLIC GLUCANS

Structure, Biosynthesis, and Secretion of Cyclic β Glucans

Although the occurrence of cyclic β-linked glucans is not unique for the rhizobia [they are also produced by some bacterial species that fall outside the α-proteobacteria group (172)], they are certainly best studied in these organisms. In the genera *Rhizobium* and *Sinorhizobium*, these molecules are linked solely by β-(1,2) glycosidic bonds with degrees of polymerization (25) ranging from 17 to 25 (*R. leguminosarum*) or $\leq$40 (*S. meliloti*). Species of *Bradyrhizobium* produce cyclic glucans containing both β-(1,3) and β-(1,6) glycosidic linkages. These molecules contain 10 to 13 glucose residues and appear (Figure 5) to be branched in structure (146). Cyclic β-(1,2) glucans may become charged through the addition of anionic substituents, depending on the growth phase of the cultures. The predominant substituent on the cyclic glucans from *S. meliloti* is *sn*-1-phosphoglycerol linked to C6, which is derived from the head group of phosphatidylglycerol (25).

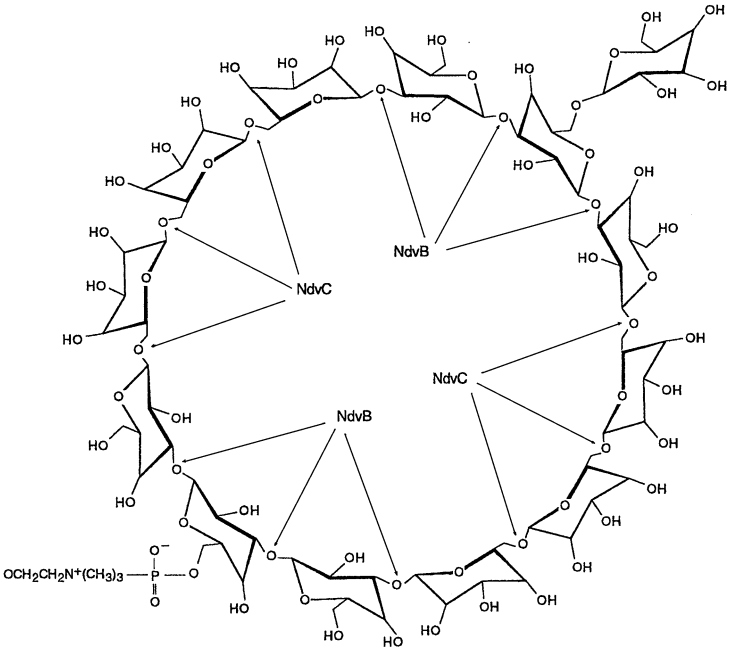

Figure 5 The structure of a cyclic β-(1,6)-β-(1,3)-glucan of *B. japonicum*. The structure is according to Rolin et al (146). The position of the phosphocholine residue is arbitrarily chosen at one of the β-(1,3)-linked saccharide units. The functions of the NdvB and NdvC proteins in the formation of the glycosidic linkages are hypothetical and based on the results of Bhagwat et al (19).

Recently, a gene from *S. meliloti* was identified that is necessary for the addition of the phosphoglycerol substituents (182). The introduction of this gene, called *cgmB*, into a *R. leguminosarum* strain, which normally synthesizes only neutral cyclic glucans, resulted in the production of phosphoglycerol-containing cyclic glucans. In *Bradyrhizobium* spp. (Figure 5), the cyclic glucans are uncharged in character but contain the zwitterionic substituent phosphocholine (146).

The biosynthesis of cyclic β-(1,2) glucans of *Sinorhizobium* and *Rhizobium* is dependent on the NdvB protein. The NdvB protein is very large (in *S. meliloti*, 319 kDa) and has been shown to form a covalent intermediate with the glucan backbone during biosynthesis (189). Also, the biosynthesis of β-(1,3)–β-(1,6)-linked cyclic glucan from *Bradyrhizobium* spp. involves a close relative of the NdvB protein. In addition, a second protein, called NdvC, has been identified, which, based on mutant studies, is probably involved in the formation of the β-(1,6) linkages (19).

During logarithmic growth, the cyclic β-glucans are predominantly localized within the periplasmic compartment. At stationary growth stage, depending on culture conditions, high amounts of cyclic glucan produced may also be found

extracellularly (65). The secretion of cyclic glucans to the periplasm and the extracellular environment is mediated by the NdvA protein, which is the ABC component of a type 1 secretion system.

Regulation and Function of Cyclic Glucans

In analogy with the role of membrane-derived β-linked oligosaccharides in the periplasm of *E. coli*, the cyclic glucans have been thought to be involved in protection against hypoosmotic conditions (96). Consistent with this hypothesis are the observations that several mutants that are defective in cyclic glucan synthesis are impaired for growth in hypoosmotic media and that cyclic glucan synthesis by most rhizobial species is regulated by osmotic conditions (25). However, because these observations do not apply to all rhizobia and because the effect of mutations leading to a defect in cyclic glucan biosynthesis are very pleiotropic, conclusive evidence for the role of cyclic glucans in osmoprotection is still lacking (25). Enigmatic with the presumed role of cyclic glucans in protection against hypoosmolarity is the observation that rhizobia also produce large amounts of cyclic glucans during the symbiosome stage, because the osmotic environment within the nodule is likely to be relatively high enough to inhibit glucan biosynthesis in free-living rhizobia (25, 73). This could indicate that cyclic glucans also have an additional function during symbiosis. A possible function of the cyclic glucans could be to serve as a means of transport for other signal molecules into the plant tissue, which is suggested by the capacity of cyclic β-(1,2)-glucan to form inclusion complexes with hydrophobic guest molecules (117). Specifically, it has been shown that the solubility of legume-derived flavonoids (117) and Nod factors (151) is greatly increased in the presence of cyclic glucans and cyclic dextrans, respectively. Recently, Bhagwat et al have obtained convincing evidence that the cyclic glucans of *B. japonicum* can function as specific suppressors of a plant defense response (19). This was shown by a detailed study of a mutant affected in the *ndvC* gene, which, consequently, produced a mutant form of its cyclic glucan that is devoid of β-(1,6) linkages. In contrast to the wild-type cyclic glucan, the mutant form was not able to suppress a fungal β-glucan–induced plant defense response and had a much lower affinity for the putative membrane receptor protein (19). Because bacteria belonging to other proteobacterial tribes that also produce cyclic β glucans are often characterized by their ability to infect eukaryotes, it is tempting to speculate that cyclic β glucans play a general role in the suppression of host plant defense responses (25).

GENERAL CONCLUSIONS AND FUTURE PROSPECTS FOR RESEARCH

From the above concise review of the recent literature, it can be concluded that rhizobial cell surface and secreted glycans play a major role in the symbiotic interaction with their leguminous host plants. It is also clear that in all rhizobia there

is an apparent redundancy as to the biological functions performed. Furthermore, mutations in genes involved in the synthesis of these glycans can have various pleiotropic effects. This redundancy and pleiotropy have greatly hampered studies that were aimed at linking structural features with biological function. The only glycans that are apparently exempt from this rule are the Nod factors, which are absolutely crucial for the capacity of rhizobia to induce the formation of root nodules and to infect the host tissue. Because of this, it has been relatively easy to identify functions of the Nod factors and to obtain detailed information on structure-function relationships. However, a major bottleneck in the study of the symbiotic function of all glycan molecules identified up to this point remains the absence of knowledge of plant factors that are involved in their recognition. Such studies are very difficult because of the very low concentrations at which various glycans (such as Nod factors and EPS oligosaccharides) are active and, as a consequence the expected high affinity of the receptor proteins involved. The technical difficulty of such studies is compensated for by their challenging nature, because still very little is known about high-affinity binding sites for carbohydrates in general. Fortunately, it can be expected that the recent rapid progress in development of molecular genetic techniques in several model legumes (95, 150) will soon lead to better insights into glycan recognition systems of leguminous plants, which will make it possible to link the great wealth of knowledge obtained on the rhizobial system with plant genetics. The fact that glycans such as EPSs, K-antigens, LPSs, and cyclic glucans are likely to be involved in suppressing a plant-defense response will guarantee a further impact of these studies on investigations of plant-pathogen interactons. Considering the structural resemblance of Nod factors to oligosaccharides, which have been shown to play a role in embryogenesis of zebrafish, such studies might even have a broad impact in the field of vertebrate development (5).

From the bacteriological viewpoint, several technical advances will also give many new possibilities for further unraveling the molecular dialog between rhizobia and leguminous plants. For instance, the use of color varieties of the green fluorescent protein as a marker for bacterial infection will enable more sophisticated competition experiments (161). Competition experiments, which can be used to simulate the natural situation in soil, might show that various rhizobial factors whose inactivation leads to only minor phenotypes can be very important in symbiosis. Future studies on the role of glycans will also be greatly helped by the big advances in new technologies that will assist in the determination of complex carbohydrate structures or biochemical function of their biosynthetic enzymes, such as new mass spectrometry instrumentation (176) and better tools for protein crystallization studies. Perhaps the most important advance can be expected to come from the large-scale analyses of the genomes of several rhizobial species and related species from the α–proteobacteria group. The identification of genetic differences and similarities among symbiotic factors of rhizobia and the virulence factors of pathogens, such as *Brucella abortus* [used as an example because this organism has already been shown (111) to share interesting analogies with rhizobia] will undoubtedly lead to a better understanding of the rhizobium-plant symbiosis

in an evolutionary context. Furthermore, the application of mRNA screening using chips coated with arrays of fragments of all known bacterial genes will provide tools to better understand the complexity of symbiotic gene regulation, which is still poorly understood, especially at the later stages of symbiosis (121).

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