Cyclic β-Glucans of Members of the Family *Rhizobiaceae*

MICHAËL W. BREEDVELD[1] AND KAREN J. MILLER[1,2,3]*

Department of Food Science[1] and Graduate Programs in Plant Physiology[2] and Genetics,[3] The Pennsylvania State University, University Park, Pennsylvania 16802

INTRODUCTION

Bacteria within the *Rhizobiaceae* family are distinguished by their ability to infect plants, and the cell surface carbohydrates of these bacteria have been shown to provide important functions during the plant infection process. While there have been numerous reviews concerning the possible roles for the extracellular polysaccharides, capsular polysaccharides, lipopolysaccharides, and lipooligosaccharides of these bacteria during plant infection (see, e.g., references 51, 53, 56, 78, 80, and 119), an additional class of cell surface carbohydrates, the cyclic β-glucans, has received comparatively little attention.

Cyclic β-glucans are unique molecules that are found almost exclusively in bacteria of the *Rhizobiaceae* family. Reaching

* Corresponding author. Mailing address: 105 Borland Laboratory, Department of Food Science, The Pennsylvania State University, University Park, PA 16802. Phone: (814) 863-2954. Fax: (814) 863-6132.

concentrations as high as 5 to 20% of the total cellular dry weight under certain culture conditions, the cyclic β-glucans are major cellular constituents. In *Agrobacterium* and *Rhizobium* species, these molecules contain glucose residues linked solely by β-(1,2) glycosidic bonds. However, in *Bradyrhizobium* species, the glucose residues are linked by both β-(1,3) and β-(1,6) glycosidic bonds. While these molecules contain glucose as the only hexose monomer, they may become highly substituted with nonsugar moieties such as *sn*-1-phosphoglycerol. The degree of polymerization (DP) of the cyclic β-glucans ranges from 10 to 13 in *Bradyrhizobium japonicum* to 17 to 40 in *Rhizobium meliloti*.

The first report of cyclic β-glucans came in 1942 with their discovery in the extracellular media of *Agrobacterium tumefaciens* cultures (83). These glucans, originally referred to as crown gall polysaccharides, were described as low-molecular-mass glucose polymers of around 3,600 Da. Since that report, cyclic β-glucans have been found in cultures of all *Agrobacte-*

TABLE 1. Classification of cyclic β-(1,2)-D-glucans based on ring size distribution[a]

Class	DP			Representative species	Main substituents	References
	Lowest	Major	Highest			
I	17	17	20–21	*Rhizobium* sp. (*Phaseolus*)[b]	Unknown	55, 58, 75
II	17	19–20	24–25	*R. leguminosarum* (all biovars)[c]	Unsubstituted[d]	18, 58, 75, 95, 109, 142
III	17	21–22	40	*R. meliloti*	Phosphoglycerol	7, 19, 76, 86, 142
IV	17	19–22	25	*A. tumefaciens*[e]	Phosphoglycerol	58–60, 75, 90

[a] This classification is adapted from reference 58.
[b] Class I has been described for only three strains, originally described as *R. phaseoli*, *R. trifolii*, and *R. lupini* (55, 58, 75).
[c] Class II has been described for many *R. leguminosarum* strains and one strain originally described as *R. japonicum* (58, 75, 142).
[d] Some *R. leguminosarum* bv. viciae strains contain small amounts of phosphoglycerol-substituted cyclic β-(1,2)-glucans (142).
[e] Class IV has been described for many *A. tumefaciens*, *A. rhizogenes*, and *A. radiobacter* strains and one *R. trifolii* strain (58, 75).

rium, *Rhizobium*, and *Bradyrhizobium* species thus far examined. Furthermore, recent studies conducted in several laboratories reveal that these molecules provide important functions both for the free-living forms of these bacteria and during the process of plant infection.

This review will summarize the structural features and occurrence of the cyclic β-glucans in the *Rhizobiaceae*. An overview of the progress made toward identifying and characterizing the genetic loci and enzyme systems involved in cyclic β-glucan biosynthesis will also be presented. Finally, the biochemical functions of the cyclic β-glucans, as well as possible industrial applications for these molecules, will be considered.

STRUCTURE AND RING SIZE DISTRIBUTION OF THE CYCLIC β-GLUCANS

Cyclic β-(1,2)-Glucans

The (1,2)-linked D-glucosyl backbone structure of the cyclic β-(1,2)-glucans of *Rhizobium* and *Agrobacterium* species was first revealed by methylation analysis, which yielded 3,4,6-tri-O-methyl-D-glucose as the only methylated product (30, 48, 105, 136, 140). The products of periodate oxidation and Smith degradation were also consistent with a β-(1,2)-linked backbone (82, 140). A macrocyclic, unbranched form was proposed because of the absence of reducing and nonreducing terminal residues (136, 140). The cyclic character of the glucans was unequivocally established by ^{13}C nuclear magnetic resonance spectroscopy and fast atom bombardment mass spectrometry (34). The β-anomeric configuration at the C-1 carbon atoms was suggested by optical rotation and confirmed by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy (7, 34, 58).

Hisamatsu et al. (58) have compared the ring size distribution of cyclic β-(1,2)-glucans from a variety of *Agrobacterium* and *Rhizobium* species (Table 1). On the basis of these analyses, four classes of glucan producers have been identified. The cyclic β-(1,2)-glucans of *R. leguminosarum* and *A. tumefaciens* strains consist of a mixture of rings with DPs ranging from 17 to 25 (10, 34, 60, 73, 75, 76, 109), while much larger cyclic β-(1,2)-glucans (up to DP 40) have been detected within cultures of *R. meliloti* (76). In a recent study, Williamson et al. show that the size distribution of the cyclic β-(1,2)-glucans of *A. tumefaciens* appears to result from competing elongation and cyclization reactions (133). A profile of the ring size distribution of the cyclic β-(1,2)-glucans of *R. leguminosarum* is shown in Fig. 1.

Nuclear magnetic resonance spectroscopy has indicated that the neutral cyclic β-(1,2)-glucans contain a high degree of symmetry and that all glycosyl residues are chemically equivalent (34, 104). Cyclic β-(1,2)-glucans with DP below 17 have

never been found within rhizobial cultures, suggesting perhaps that there may be too much strain within the linkages of smaller cyclic β-(1,2)-glucans (102, 136a). Consistent with this hypothesis, molecular modeling studies have predicted that β-(1,2)-glucan rings with DP lower than 17 are not energetically favored (102, 136a). These studies have also predicted that the strain energy associated with the cyclic ring would decrease exponentially with increasing ring size from 17 to 24 residues. Further, the cavity diameters of the cyclic β-(1,2)-glucans were estimated to range between 0.88 nm (DP = 18) and 1.30 nm (DP = 24) (102). A proposed structure of the symmetrical cyclic β-(1,2)-glucan containing 18 glucose residues is shown in Fig. 2.

Cyclic β-(1,6)-β-(1,3)-Glucans

Species of *Bradyrhizobium*, a third genus within the *Rhizobiaceae* family, synthesize β-glucans containing both β-(1,3) and β-(1,6) glycosidic linkages (37, 87). These glucans appear to be branched and cyclic in structure with DPs ranging from 10 to 13 (87). Glucans containing 11 and 12 residues are predominant in at least two bradyrhizobial strains (87). Recently, the structure of the bradyrhizobial cyclic β-(1,6)-β-(1,3)-glucan of DP 13 has been proposed to consist of a
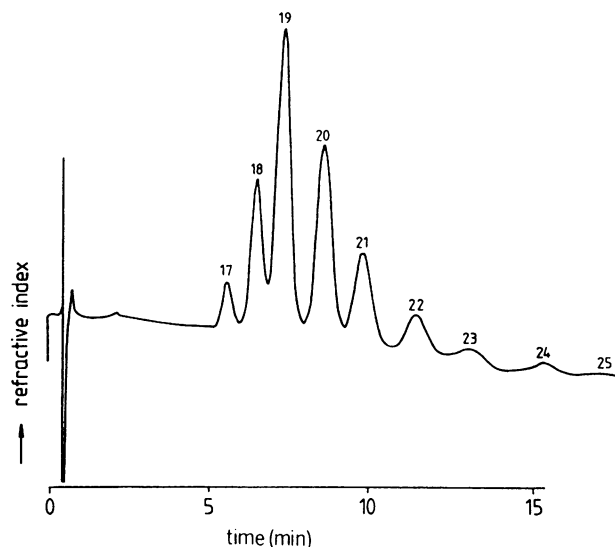

FIG. 1. High-pressure liquid chromatography elution profile of the purified cyclic β-(1,2)-glucan fraction from *R. leguminosarum* bv. trifolii TA-1. The ring sizes are indicated in the figure. Adapted from reference 18 with permission of the publisher.
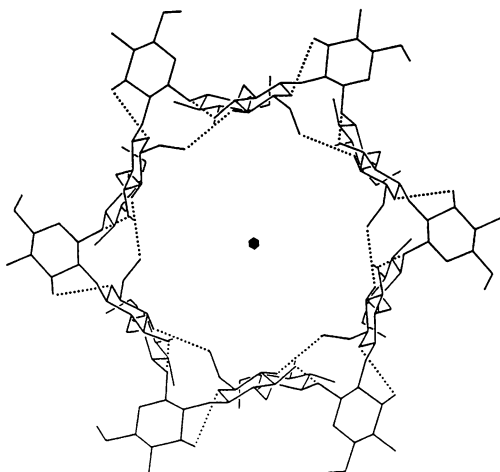
FIG. 2. Minimum-energy, maximum-symmetry conformation of a neutral cyclic β-(1,2)-glucan (DP = 18). Dotted lines indicate hydrogen bonds. Adapted from reference 102 with permission of the publisher.

backbone of 12 glucose residues containing triplets of β-(1,3)-linked glucose residues separated by triplets of β-(1,6)-linked glucose residues (110). The 13th residue is proposed to be present as a branch on the C-6 of a (1,3)-linked glucose residue (110). The proposed structure of the bradyrhizobial cyclic β-glucan (DP = 13) is shown in Fig. 3.

LOCALIZATION OF THE CYCLIC β-GLUCANS

Most of the initial studies of the cyclic β-(1,2)-glucans of *Rhizobium* and *Agrobacterium* species focused on glucan preparations isolated from the extracellular medium of these cultures. Consequently, the presence of cell-associated cyclic β-(1,2)-glucans has generally been overlooked. Zevenhuizen and Scholten-Koerselman (140) were the first to demonstrate that relatively high levels of cyclic β-(1,2)-glucans are cell associated. Typically, cellular concentrations range between 5 and 20% of the total cellular dry weight, and levels have been
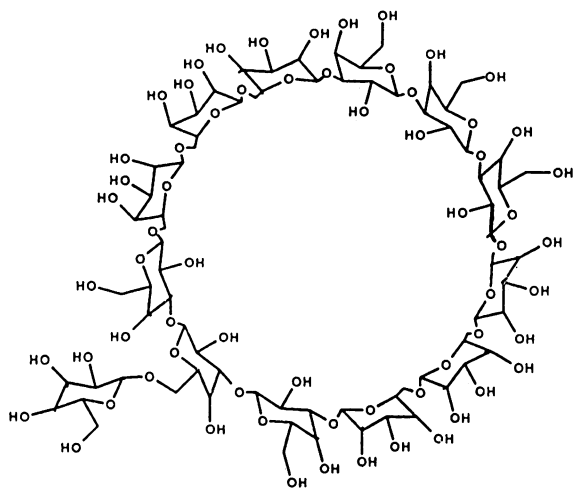
shown to follow distinct patterns depending on species, medium composition, and growth phase (137, 139, 141).

The cellular location of the cyclic β-glucans in cultures of *A. tumefaciens*, *R. leguminosarum* bv. trifolii, and *B. japonicum* has been examined (1, 85, 89). By using treatments designed to preferentially disrupt the outer membranes of these cultures, these studies have revealed that the cyclic β-glucans are localized predominantly within the periplasmic compartment. If the relative periplasmic volume of *Rhizobium*, *Agrobacterium*, and *Bradyrhizobium* cells can be assumed to be similar to that measured for *Escherichia coli* and *Salmonella typhimurium* (121), it can be estimated that these molecules reach concentrations as high as 15 mM within the periplasmic compartment (21).

The export of the cyclic β-glucans to the extracellular medium varies greatly among different species and is influenced strongly by growth stage and culture conditions. For example, high levels of extracellular cyclic β-glucans are generally detected within stationary-phase cultures (18, 20, 57, 138). The excretion of cyclic β-(1,2)-glucans has also been shown to be strongly enhanced in certain growth media (18, 20, 43) and at elevated temperatures (18).

SUBSTITUENTS ON THE CYCLIC β-GLUCANS

During early studies with the cyclic β-(1,2)-glucans, it was believed that these molecules were unsubstituted and strictly uncharged in character. In fact, in some of these early studies, when cyclic β-(1,2)-glucans were detected within anionic low-molecular-weight fractions obtained from culture media, it was suggested that the glucans had formed complexes with the anionic repeating units of the extracellular polysaccharides (8, 59). It is now known that the cyclic β-(1,2)-glucans themselves become charged through the addition of anionic substituents such as *sn*-1 phosphoglycerol, succinic acid, and methylmalonic acid (9, 15, 19, 60, 86, 89, 90, 142). The predominant substituent on the cyclic β-(1,2)-glucans of *R. meliloti* and *A. tumefaciens* strains is *sn*-1-phosphoglycerol, which is derived from the head group of phosphatidylglycerol and is linked to glucose through phosphodiester linkage at position C-6 (86). A large fraction of the glucans of these bacteria (e.g., 50 to 90%) contain between one and four *sn*-1-phosphoglycerol substituents per glucan backbone (9, 86, 90). Pulse-chase experiments have revealed that the neutral cyclic β-(1,2)-glucans are the biosynthetic precursors of the glycerophosphorylated cyclic β-(1,2)-glucans (15a, 43).

The degree of substitution of the cyclic β-(1,2)-glucans may vary greatly among different *Agrobacterium* and *Rhizobium* species. For example, whereas the cyclic β-(1,2)-glucans produced by the broad-host-range *Rhizobium* sp. strain GRH2 and by certain strains of *R. leguminosarum* apparently contain no anionic substituents (81, 142), as much as 90% of the cyclic β-(1,2)-glucan fraction of strains of *R. meliloti* and the broad-host-range *Rhizobium* sp. strain NGR234 is anionic (9, 86). The stage of growth also influences the degree of substitution of the cyclic β-(1,2)-glucans. For example, Geiger et al. (43) have shown that the conversion of neutral glucans into anionic glucans in cells of *R. meliloti* 1021 occurs most rapidly in exponentially growing cultures.

Although predominantly uncharged in character, the cyclic β-(1,6)-β-(1,3)-glucans of *B. japonicum* can also become substituted with phosphoryl substituents. Specifically, the zwitterionic substituent, phosphocholine, has been identified on the C-6 position of a (1,3)-linked glucose residue (110).

FIG. 3. Proposed structure for the unsubstituted cyclic β-(1,6)-β-(1,3)-glucan of *B. japonicum* containing 13 glucose residues. Adapted from reference 110 with permission of the publisher.

TABLE 2. Properties of membrane-associated glucosyltransferases of the *Rhizobiaceae* linked to cyclic β-glucan biosynthesis[a]

Organism	$K_{m,UDPG}$[b] (μM)	Metal requirements	Protein-linked intermediate	DP	Linkage	Reference(s)
R. meliloti	—[c]	Mn = Mg	Yes	14–25[d]	β-(1,2)	145, 147
R. phaseoli	33	Mn > Mg	—	17–20	β-(1,2)	3
A. tumefaciens	—	Mn = Mg	Yes	17–24	β-(1,2)	133, 145, 147
A. radiobacter	50	Mn = Mg	—	17–24	β-(1,2)	3
R. leguminosarum	—	Mn = Mg	—	17–24	(1,2)	21
B. japonicum	50	Mn > Mg	ND[e]	13	β-(1,3)-β-(1,6)	11, 28
B. japonicum	—	Mg > Mn	Yes	≥11	β-(1,3)-β-(1,6)	66
R. japonicum[f]	300	Mn = Mg	—	—	β-(1,2)-β-(1,3)-β-(1,6)	32

[a] Activities have been characterized within crude membrane preparations.
[b] UDPG, UDP-glucose.
[c] —, not reported.
[d] Reported as 14 to 25 glucose residues on the basis of elution behavior during gel filtration column chromatography.
[e] ND, not detected.
[f] Because of its growth properties, it was suggested by Cohen and Miller (28) that this strain of *R. japonicum* represented a fast-growing *Rhizobium* strain.

β-(1,2)-LINKED GLUCANS IN OTHER BACTERIAL GENERA

Cyclic β-(1,2)-Glucans

Bundle et al. reported that the mammalian pathogens *Brucella* spp. synthesize cyclic β-(1,2)-glucans essentially identical (with DPs between 17 and 24) to those synthesized by members of the *Rhizobiaceae* (23). Like *Rhizobium* and *Agrobacterium* species, this bacterium lives in close association with eukaryotic cells, suggesting, perhaps, a general role for the bacterial cyclic β-(1,2)-glucans during the infection of eukaryotic cells. Interestingly, recent analysis of the 16S rRNA and lipid A composition of *Brucella* species has revealed a close phylogenetic relationship with *Agrobacterium* and *Rhizobium* species (91, 132, 134).

To our knowledge, cyclic β-(1,2)-glucans have been found in only two other bacterial genera, *Xanthomonas* (4) and *Alcaligenes* (53a, 59). However, the *Xanthomonas* cyclic β-(1,2)-glucan appears to be different in structure from the cyclic β-(1,2)-glucans of members of the *Rhizobiaceae* and of *Brucella* and *Alcaligenes* species. Specifically, the *Xanthomonas* cyclic glucan has a DP of 16, contains one (1,6) glycosidic linkage, and contains one α linkage (4). It is of interest that *Xanthomonas* species are plant pathogens, again suggesting a possible role for cyclic glucans during the infection of eukaryotic cells.

Linear β-(1,2)-Glucans

Linear β-(1,2)-glucans containing 6 to 19 glucose residues have been detected within a few tropical *Rhizobium* strains (5) as well as a small number of other bacteria, most notably *E. coli*. The glucans of *E. coli* contain a β-(1,2)-linked backbone and β-(1,6)-linked branches, and they range in size from 6 to 12 glucose residues (69). Kennedy and coworkers have called these glucans membrane-derived oligosaccharides (MDO) because they become highly substituted with phosphoglycerol and phosphoethanolamine residues derived from the head groups of phosphatidylglycerol and phosphatidylethanolamine, respectively (45, 88a, 113, 114; for a review on MDO, see reference 69). It is interesting that the linear, branched MDO of *E. coli* and the cyclic β-(1,2)-glucans of *R. meliloti* and *A. tumefaciens* all contain phosphoglycerol substituents derived from phosphatidylglycerol (86, 90). Although it has been demonstrated that there are two phosphoglycerol transferases involved in the transfer of phosphoglycerol substituents to the MDO (45, 67), little is known regarding the phosphoglycerol transferases of members of the *Rhizobiaceae*.

Linear β-(1,2)-glucans have also been identified within cultures of *Acetobacter* and *Xanthomonas* species. The linear β-(1,2)-glucans of *Acetobacter* species have DPs between 6 and 42 (6). Membrane preparations derived from *Acetobacter xylinum* have also been shown to catalyze the formation of high-molecular-weight linear β-(1,2)-glucans from UDP-glucose (112). The linear β-(1,2)-glucans of *Xanthomonas* cultures are smaller, with DPs ranging from 8 to 20 (4).

BIOSYNTHESIS OF THE CYCLIC β-GLUCANS

In Vitro Biosynthesis: Membrane-Associated Glucosyltransferases

The first demonstration of the in vitro biosynthesis of β-(1,2)-glucans was made by using cell extracts from *R. japonicum* (32). Since that time, membrane preparations derived from strains of *R. leguminosarum* bv. phaseoli (3), *R. leguminosarum* (21), *R. meliloti* (145, 147), *A. tumefaciens* (133, 145, 147), and *A. radiobacter* (3) have been shown to catalyze the formation of cyclic β-(1,2)-glucans from UDP-glucose. In all of these cases, the enzyme system is activated by the metal ions Mn^{2+} and/or Mg^{2+}. The structures of the cyclic β-(1,2)-glucans produced in vitro are indistinguishable from those produced in vivo (3, 21, 133). The properties of these glucosyltransferase activities are summarized in Table 2.

A novel glucosyltransferase activity has been identified within membrane preparations derived from *B. japonicum* (28). This activity also catalyzes the biosynthesis of a glucan product from UDP-glucose and is stimulated by either Mn^{2+} or Mg^{2+} ions. Furthermore, the glucan product lacks a detectable reducing terminal residue, consistent with a cyclic structure. However, the glucan product contains both β-(1,6) and β-(1,3) glycosidic linkages and has an average molecular mass of 2,100 Da (28). From these structural properties, it was suggested that this glucosyltransferase activity is responsible for the biosynthesis of the cyclic β-(1,6)-β-(1,3)-glucans (28).

There is a notable difference between the glucosyltransferase systems identified in vitro for cyclic β-(1,2)-glucan biosynthesis and cyclic β-(1,6)-β-(1,3)-glucan biosynthesis: whereas the rhizobial β-(1,2)-glucans produced in vitro and in vivo appear to be essentially identical in structure, this is not the case for the bradyrhizobial β-(1,6)-β-(1,3)-glucans. Specifically, the cyclic β-(1,6)-β-(1,3)-glucans isolated from growing cultures of *B. japonicum* USDA 110 have been shown to contain 10 to 13 glucose residues with similar levels of β-(1,6) and β-(1,3) glycosidic linkages [e.g., the ratio of β-(1,6) to β-(1,3) glycosidic linkages ranges between 1 and 3] (87, 110).

In contrast, the cyclic β-(1,6)-β-(1,3)-glucans produced in vitro have a much higher relative proportion of β-(1,3) linkages [e.g., the ratio of β-(1,6) to β-(1,3) glycosidic linkages is 0.1] (28). Very recently, Iñón De Iannino and Ugalde (66) performed a fairly extensive structural characterization of the bradyrhizobial cyclic β-(1,6)-β-(1,3)-glucan product formed in vitro. It was proposed that the product contained a cyclic β-(1,3) backbone of 11 glucose residues with variable numbers of β-(1,6) glucose branch points. These branch points were proposed to serve as primers for the formation of β-(1,3)-linked linear branches (66).

To date, all studies examining in vitro cyclic β-glucan biosynthesis have focused only on the enzymatic systems linked to the formation of neutral, unsubstituted cyclic β-glucan backbones. We are not aware of any in vitro studies in which the transfer of substituents to the neutral cyclic β-glucan backbone has been investigated.

Two Chromosomal Loci Are Specifically Associated with Cyclic β-(1,2)-Glucan Biosynthesis

In 1985, Douglas et al. identified two genetic loci on the chromosome of *A. tumefaciens*, *chvA* and *chvB* (chv stands for "chromosomal virulence"), which were shown to be required for virulence and for attachment of the bacterium to plant cells (36). Soon after this report, Puvanesarajah et al. (106) revealed that *chvB* mutants lacked the ability to synthesize cyclic β-(1,2)-glucans, and Dylan et al. (39) demonstrated that *R. meliloti* contained two genetic loci (*ndvA* and *ndvB*; ndv stands for "nodule development") that were functionally and structurally homologous to *chvA* and *chvB*. Indeed, mutants of *A. tumefaciens* with mutations at either locus could be fully complemented with *R. meliloti* cosmid clones containing *ndvA* and *ndvB* (39). Furthermore, loci homologous to *ndvA* and *ndvB* were found in strains of *R. leguminosarum* bv. trifolii and phaseoli which were cured for their symbiosis (pSym) plasmids, indicating that the *ndv* genes were also located on the chromosome (39, 128). Interestingly, the arrangement of the *ndvA* and *ndvB* genes on the chromosome of *R. meliloti* has been shown to be similar to that for *chvA* and *chvB* of *A. tumefaciens*: in both cases, the two loci are adjacent to each other and are transcribed in a convergent fashion (36, 39). Of further interest was the finding that *R. meliloti ndvA* and *ndvB* mutants formed ineffective nodules on alfalfa (39) and that these mutants were also affected in their biosynthesis of cyclic β-(1,2)-glucans (44, 120). Thus, not only did these studies identify genetic loci involved in cyclic β-(1,2)-glucan biosynthesis (Table 3) but also they provided evidence that the cyclic β-(1,2)-glucans have roles during plant infection.

It has been suggested by Altabe et al. that the *chvA/ndvA* and *chvB/ndvB* genetic loci, alone, are responsible for the complete biosynthesis of the neutral cyclic β-(1,2)-glucan backbone from UDP-glucose (2). These researchers introduced a cosmid, containing the *A. tumefaciens chvA* and *chvB* loci, into *Azospirillum brasilense*. When membrane preparations derived from the cosmid-containing strain were incubated in the presence of UDP-[^{14}C]glucose, radiolabeled cyclic β-(1,2)-glucan products were detected. In contrast, no activity could be detected within the strain that did not contain the plasmid (2). It is to be noted, however, that *Azospirillum brasilense* strains have been reported to contain DNA sequences homologous to *A. tumefaciens chvA* and *chvB* (129). Therefore, it is possible that *Azospirillum brasilense* strains have some capacity to synthesize cyclic β-(1,2)-glucans. Furthermore, the cosmid (pCD523) used in this study contained a total insert of 25 kb, on which the *chvA* (approximately 1.8 kb)

and *chvB* (approximately 8.6 kb) loci represent less than 50% of the total length of the insert. Thus, it is possible that this insert contains other genes that also provide functions during cyclic β-(1,2)-glucan biosynthesis.

In a more recent study with the same cosmid, Lepek et al. presented evidence that the *chvA* and *chvB* genes are involved in determining the degree of substitution of the cyclic β-(1,2)-glucans (79). In this study, the cosmid was introduced into an *R. loti* strain. The cyclic β-(1,2)-glucans of this *R. loti* strain are normally more anionic (approximately 90% anionic glucans) than those of *A. tumefaciens* (approximately 50% anionic glucans). However, the cyclic β-(1,2)-glucans produced by the *R. loti* strain containing the cosmid were found to be indistinguishable in anionic character from those produced by *A. tumefaciens* (79).

NdvB/ChvB Proteins: Synthesis of Cyclic β-(1,2)-Glucans from UDP-Glucose

To date, no other genetic loci specifically associated with cyclic β-(1,2)-glucan biosynthesis have been identified in *Rhizobium* or *Agrobacterium* species. However, progress has been made toward elucidating the roles of *ndvA* (*chvA*) and *ndvB* (*chvB*). The *ndvB/chvB* genes have each been shown to encode a high-molecular-mass cytoplasmic membrane protein (e.g., 319 kDa) that is involved in the biosynthesis of the cyclic β-(1,2)-glucans from UDP-glucose (44, 64, 144, 146). Whether NdvB or ChvB is involved in several stages of glucan biosynthesis (initiation, elongation, and cyclization) remains unclear; however, these inner membrane proteins become covalently bound to the glucan chain during biosynthesis (146). The formation of a protein-linked intermediate is intriguing because lipid-linked intermediates, involved in the biosynthesis of many other bacterial polysaccharides (123), have not been detected during the biosynthesis of the cyclic β-(1,2)-glucans (146).

It would seem likely that the NdvB/ChvB protein is involved in the cyclization of the glucan chain since only cyclic forms of the β-(1,2)-glucans are detected after release from these proteins (144). It has also been proposed that the NdvB/ChvB proteins have domains involved in functions other than cyclic β-(1,2)-glucan biosynthesis (12, 64, 144). This possibility is suggested from mutagenesis studies that have indicated that up to 40% of the NdvB/ChvB protein (from the carboxyl terminus) is not required for β-glucan biosynthesis (64, 144). Although smaller quantities of glucans are synthesized by these mutants with downstream mutations, structural analysis of these glucans has revealed them to be a mixture of cyclic glucans with size distribution and phosphoglycerol substituent profile similar to those found in wild-type cells (16).

The *ndvB/chvB* loci appear to be present in all *Rhizobium* and *Agrobacterium* strains examined (11, 39, 144). However, *Bradyrhizobium* species do not contain DNA sequences homologous to *ndvB/chvB* (11, 39). This result is consistent with the finding that bradyrhizobial strains apparently lack the ability to synthesize cyclic β-(1,2)-glucans. Although an NdvB-like protein could not be detected in *Bradyrhizobium* species (11, 28), Iñón De Iannino and Ugalde provided evidence for the formation of a protein-linked intermediate during cyclic β-glucan biosynthesis in several bradyrhizobial strains (66). The formation of a protein-linked intermediate during cyclic β-(1,6)-β-(1,3)-glucan biosynthesis is intriguing, and these authors have speculated that a protein-linked intermediate is required for the synthesis of cyclic β-glucans (66).

Although the NdvB/ChvB protein has never been purified to homogeneity, Kinoshita et al. (71) reported the partial purifi-

TABLE 3. Phenotypic properties of cyclic β-(1,2)-glucan deficient mutants of the *Rhizobiaceae*

Strain	Synthesis of cyclic glucans		Other phenotypic properties	Additional comments and references
	In vivo	In vitro		
R. meliloti ndvB upstream mutants				
GRT21S	No	No	Forms empty pseudonodules; impaired growth at low osmolarity; nonmotile	44, 117
HQ50	No	No	Same as GRT21S; increased production of EPS[a]	107
TY7, TY17, TY23	No	No	Same as HQ50; reduced number of flagella; increased sensitivity toward certain (hydrophobic) antibiotics; increased resistance to certain phages	38–40, 64
R. meliloti ndvB downstream mutants				
TY28	Yes (red.)[b]	Yes (red.)	Effective nodules; vegetative functions[c] intermediate between wild type and TY-7; no stable labeled glucan-protein intermediate detectable	16, 64
TY24	Yes (red.)	Yes (red.)	Same as TY28; forms both ineffective and effective nodules on same plant; bacteroids form triskelion-like structures	64
TY26	Yes (red.)	Yes (red.)	Same as TY28; forms branched bacteroids	64
R. meliloti ndvB pseudorevertants				
TY63	No	—[d]	Effective nodules; poor attachment and infection thread initiation; osmosensitive, unrestored for other vegetative functions; less EPS production than TY17 (but more than wild type)	Spontaneous symbiotic revertant of TY17 (40, 96)
TY88	No	—	Most vegetative functions restored; symbiotic behavior not restored	Spontaneous motile revertant of TY7 (40, 96)
HQ50oR	No	—	Partially osmoresistant and partially motile; infective, but no N fixation	Spontaneous osmotic revertant of HQ50 (107)
R. meliloti ndvA upstream mutant				
LI1	Yes (neutral only)	Yes	Impaired growth at low osmolarity; forms empty pseudonodules; impaired motility; altered sensitivity toward antibiotics; no extracellular cyclic β-(1,2)-glucans; increased production of EPS	16, 38, 39, 120
R. meliloti ndvA pseudorevertants				
TY65	—	—	Effective nodules; poor attachment and infection thread initiation; no extracellular cyclic glucan; no restoration of vegetative properties; increased EPS production (compared with LI1)	Spontaneous symbiotic revertant of LI1 (40, 96)
TY122	—	—	Partial restoration of vegetative properties; symbiotic functions not restored	Spontaneous motile revertant of LI1 (40, 96)
R. meliloti exoC				
Rm6025, Rm7020, Rm7027	No	—	Severely impaired growth at low osmolarity; forms empty pseudonodules; deficient in synthesis of EPS and LPS[e]	24, 35
A. tumefaciens chvB upstream mutants				
A1011, A1020, A1038, A1045	No	No	Impaired growth at low osmolarity; avirulent; impaired motility; increased sensitivity toward hydrophobic antibiotics; attenuated attachment; no synthesis of active rhicadhesin; no conjugative transfer of certain plasmids	36, 115, 144, 146
A. tumefaciens chvB downstream mutants				
ME3, ME112, ME116	Yes	Yes	Protein-linked intermediates are of lower M_r; virulent	36, 144, 146
A. tumefaciens chvA upstream mutants				
A2505, ME66, ME45	Yes (neutral only)	Yes	Impaired growth at low osmolarity; avirulent; attenuated attachment; no extracellular glucans	25, 36, 65, 100, 106, 146

TABLE 3—*Continued*

Strain	Synthesis of cyclic glucans		Other phenotypic properties	Additional comments and references
	In vivo	In vitro		
A. tumefaciens exoC				
A5129, A5503	No	Yes	Avirulent; low attachment efficiency; nonmotile; slower growth than *chv* mutants in low-osmolarity media; deficient in synthesis of EPS and LPS	24, 127
R. fredii ndvB upstream mutants				
Rf19, Rf44	No	No	Empty nodules	12
R. fredii ndvB downstream mutants				
Rf5, Rf16, Rf33, Rf46	Yes (red.)	Yes (red.)	Effective nodules; protein-linked intermediates are of lower M_r	12
R. leguminosarum, locus unknown				
VG5	Yes	—	No extracellular cyclic glucans; nodule formation impaired, no N fixation; deficient in LPS synthesis	Possibly a *ndvA* mutant (135)

a EPS, extracellular polysaccharide.
b red., reduced.
c Vegetative properties include osmoresistance, motility, and sensitivity toward phages and antibiotics.
d —, not determined.
e LPS, lipopolysaccharide.

cation of cyclic β-(1,2)-glucan synthetases from membrane preparations of *A. radiobacter*. Two activities were identified, one that bound to an anion-exchange column (synthetase I) and a second that did not bind to the column (synthetase II). Synthetase I was further characterized, was found to have an apparent M_r of 350,000, and showed the same catalytic activity as crude membrane preparations (71). Whether synthetase I represents the ChvB protein from *A. radiobacter* remains unclear.

NdvA/ChvA Proteins: Involvement in Transport of the Cyclic β-(1,2)-Glucans

The *ndvA* and *chvA* genes have been shown to encode proteins of 67 and 65 kDa, respectively (25, 65, 120). These proteins share 76% identity at the amino acid level, and both also share homology with a number of bacterial ATP-binding transport proteins. Greatest homology is found with HlyB, an inner membrane protein in *E. coli* involved in the export of hemolysin A (25, 120). On the basis of this homology, it has been proposed that the NdvA and ChvA proteins are involved in the transport of the cyclic β-(1,2)-glucans to the extracellular medium (25, 120). Consistent with this proposal, it has been shown that the excretion of cyclic β-(1,2)-glucans to the extracellular medium is greatly impaired in *A. tumefaciens chvA* mutants and *R. meliloti ndvA* mutants (65, 100, 120). Additional experiments have provided evidence that the transport of the cyclic β-(1,2)-glucans to the periplasmic compartment is also impaired in *chvA* and *ndvA* mutants (15a, 25). Thus, it is possible that the strongly reduced excretion of cyclic β-(1,2)-glucans to the extracellular medium by these mutants is the result of strongly reduced transport to the periplasmic space.

How the NdvA and ChvA proteins might mediate the transport of the cyclic β-(1,2)-glucans is unknown; however, their homology with HlyB may provide insight regarding this process. In addition to a requirement for HlyB, the export of hemolysin A to the extracellular medium has been shown to be dependent upon other membrane proteins (i.e., HlyD, an inner membrane protein, and TolC, an outer membrane protein) and has been proposed to occur through transenvelope structures which span both the inner and outer membranes of *E. coli* (63, 130). Whether similar transenvelope structures mediate the transport of the cyclic β-(1,2)-glucans to the extracellular medium is unknown; however, partial trypsin digestion studies have indicated that ChvA and ChvB may form complexes within the inner membrane of *A. tumefaciens* (65).

An important distinction between hemolysin secretion and cyclic β-(1,2)-glucan transport must be noted. Although hemolysin A is secreted directly from the cytoplasm to the extracellular medium without accumulating in the periplasmic space (63), the cyclic β-(1,2)-glucans accumulate to relatively high concentrations within the periplasmic compartment. Thus, the export of the cyclic β-(1,2)-glucans to the extracellular medium may occur in two steps: first, the glucans are released from the cytoplasmic membrane to the periplasmic compartment, and second, the glucans are transported from the periplasmic compartment to the extracellular medium. Whether the same protein machinery mediates the transport of the cyclic β-(1,2)-glucans to the periplasm and to the extracellular medium is unknown.

In addition to their involvement in the transport/export of the cyclic β-(1,2)-glucans, NdvA and ChvA may be involved in the transport of other classes of oligosaccharides. This is suggested from observations that extracellular levels of the oligosaccharide repeating unit of succinoglycan are greatly reduced in cultures of *ndvA* mutants of *R. meliloti* 102F34 (120).

Structural characterization of the cell-associated cyclic β-(1,2)-glucans of an *ndvA* mutant of *R. meliloti* 102F34 revealed a mixture of cyclic glucans with a ring size distribution indistinguishable from that of wild-type cells (16). Thus, NdvA and ChvA are apparently not required for cyclization of the β-(1,2)-glucan backbone. It is possible, however, that they are required for the biosynthesis of the anionic cyclic β-(1,2)-glucans. This is suggested from studies which reveal that

anionic cyclic β-(1,2)-glucan biosynthesis is dramatically reduced in *chvA* and *ndvA* mutants (16, 65). However, the inability of these mutants to synthesize anionic cyclic β-(1,2)-glucans may be an indirect consequence of the failure of these cells to transport cyclic β-(1,2)-glucans to the periplasmic compartment. By analogy to the MDO of *E. coli* (68, 69), the periplasmic compartment represents the likely site where phosphoglycerol substituents are added to the cyclic β-(1,2)-glucan backbone.

BIOLOGICAL FUNCTIONS OF THE CYCLIC β-GLUCANS

Cyclic β-(1,2)-glucans of the *Rhizobiaceae* are believed to provide roles during hypoosmotic adaptation as well as during plant infection. In the following sections of this review, evidence for both functions will be discussed.

Hypoosmotic Adaptation

Cyclic β-glucans and membrane-derived oligosaccharides. Earlier in this review, the structural similarities between the MDO of *E. coli* and the cyclic β-(1,2)-glucans of the *Rhizobiaceae* were noted. These classes of compounds also share two additional properties: periplasmic localization and osmoregulated biosynthesis. Specifically, biosynthesis of both the MDO and the cyclic β-(1,2)-glucans is greatest when cells are grown in media of low osmolarity.

These two properties were first discovered for the MDO of *E. coli* by Kennedy, who proposed that the glucans functioned during osmotic adaptation (68). It was proposed that the MDO and their counterions constitute the major osmotically active solutes within the periplasmic compartment when cells are cultured in media of low osmotic strength (68). Indeed, the concentrations of the MDO (average net negative charge of 5) and their counterions within the periplasmic compartment were found to be similar to the concentrations of cytoplasmic solutes within this bacterium. The presence of high concentrations of MDO within the periplasm provided an explanation for an earlier study by Stock et al., which revealed that the periplasmic compartment of *S. typhimurium* and *E. coli* remained isoosmotic with respect to the cytoplasm over a wide range of environmental osmotic pressures and that a Donnan potential existed across the outer membranes of these bacteria (121).

The studies by Kennedy and coworkers (42, 69, 70) have indicated that the accumulation of MDO within the periplasm may be advantageous during growth at low osmolarity for the following reasons: (i) the accumulation of these molecules provides a mechanism for the cell to regulate the relative volumes of periplasmic and cytoplasmic compartments; (ii) anionic MDO contribute to the ionic strength of the periplasm, which appears to be important for porin regulation (33) and possibly other processes; (iii) high concentrations of MDO within the periplasm should lead to a reduction in turgor pressure across the cytoplasmic membrane; and (iv) the accumulation of anionic MDO within the periplasm should lead to the development of a Donnan potential across the outer membrane.

In a study by Miller et al. (89), the cyclic β-(1,2)-glucans of *A. tumefaciens* were found to be present in the periplasmic compartment at a concentration similar to that found for the MDO of *E. coli*. Furthermore, the biosynthesis of the cyclic β-(1,2)-glucans by *A. tumefaciens* was found to be osmoregulated in a manner which paralleled that found for MDO (see below). From these observations, it was proposed that the

cyclic β-(1,2)-glucans, like MDO, function as important periplasmic solutes when cells are grown in a medium of low osmolarity. Anionic forms of the cyclic β-(1,2)-glucans [which represent 50% or more of the total cyclic β-(1,2)-glucans synthesized by *A. tumefaciens* and *R. meliloti* strains] would be expected to be the most effective form of periplasmic solute because the counterions of these glucans also contribute to periplasmic osmolarity. Furthermore, only the anionic cyclic β-(1,2)-glucans would contribute to the establishment of a Donnan potential across the outer membrane.

Behavior of cyclic β-(1,2)-glucan-deficient mutants in hypoosmotic media. Studies with *ndv* and *chv* mutants of *R. meliloti* and *A. tumefaciens* have provided the most direct evidence that periplasmic cyclic β-(1,2)-glucans do, indeed, function during hypoosmotic adaptation. These mutants have been shown to be specifically impaired for growth in hypoosmotic media, with growth restored to wild-type levels on the addition of solutes to the growth medium (26, 38). Furthermore, repeated attempts to isolate second-site revertants of *ndv* mutants with restored osmotolerance have been unsuccessful (40). Instead, revertants with only partially restored osmotolerance have been identified in these studies (40, 107). Curiously, similar experiments performed with *mdoA* mutants of *E. coli* have failed to reveal impaired growth in low-osmolarity media (68).

Additional evidence for the important role of cyclic β-(1,2)-glucans during hypoosmotic adaptation is derived from studies with *exoC* mutants of *R. meliloti*. The *exoC* gene encodes phosphoglucomutase, an enzyme involved in the biosynthesis of UDP-glucose (127). Indeed, *exoC* mutants of *R. meliloti* and *A. tumefaciens* are defective for the biosynthesis of polysaccharides such as lipopolysaccharides, extracellular polysaccharides, and the cyclic β-(1,2)-glucans (24, 35, 125) (Table 3). These mutants have also been shown to be impaired for growth in hypoosmotic media (35). Significantly, no other polysaccharide-defective mutants of *R. meliloti* have been reported to be selectively impaired for growth at low osmolarity, thus providing further evidence for a role for the cyclic β-(1,2)-glucans during hypoosmotic adaptation.

Osmoregulated synthesis of cyclic β-glucans. Several studies have shown that the biosynthesis of cyclic β-glucans is osmotically regulated in a wide variety of *Rhizobium*, *Agrobacterium*, and *Bradyrhizobium* strains, with the highest levels of synthesis during growth in low-osmolarity media (19, 38, 85, 126, 143). This suggests a general role for periplasmic cyclic β-glucans in the hypoosmotic adaptation of the *Rhizobiaceae*. However, this conclusion is complicated by observations that the level of cell-associated cyclic β-glucans in several strains of *R. leguminosarum* is independent of growth medium osmolarity (20). Furthermore, these *R. leguminosarum* cells excrete large amounts of cyclic β-(1,2)-glucans (e.g., 1,600 mg/liter) during growth at elevated osmolarity, indicating, perhaps, that the integrity of the outer membrane of these bacteria is modified under these conditions (21). It is important to note, however, that these *R. leguminosarum* strains synthesize only neutral, unsubstituted cyclic β-(1,2)-glucans (142). Thus, it appears that they lack the ability to synthesize anionic cyclic β-(1,2)-glucans. Because anionic forms of the periplasmic cyclic β-(1,2)-glucans may be critical for hypoosmotic adaptation (see above), *R. leguminosarum* strains may not utilize periplasmic cyclic β-(1,2)-glucans for this purpose. Further studies on the osmoregulatory strategies used by *R. leguminosarum* strains are needed to clarify the roles of the periplasmic cyclic β-(1,2)-glucans. Such studies are particularly important since the growth of *R. leguminosarum* strains is restricted to a relatively narrow osmolarity range (0 to 300 mM NaCl) (20, 79a)

compared with that for growth of *R. meliloti* or *A. tumefaciens* strains (0 to 650 mM NaCl) (19, 79a).

Recently it has been reported that cyclic β-(1,2)-glucan synthesis is not osmoregulated in *R. meliloti* GR4 (118). However, it is currently not known whether this strain is able to synthesize anionic cyclic β-(1,2)-glucans.

Little is known about the mechanisms by which cyclic β-(1,2)-glucan levels are osmoregulated in most rhizobia. Studies by Zorreguieta et al. suggest that elevated cytoplasmic ionic strength leads to an inhibition of cyclic β-(1,2)-glucan biosynthesis (143). These researchers have shown that although cyclic β-(1,2)-glucan accumulation is strongly repressed in hyperosmotically stressed cells of *R. meliloti*, inner membrane preparations derived from these cells catalyze the synthesis of cyclic β-(1,2)-glucans from UDP-glucose at rates similar to those measured during the use of preparations derived from nonstressed cells (143). Furthermore, the in vitro reaction is inhibited in the presence of ionic osmolytes (NaCl, KCl) but not in the presence of neutral osmolytes (glucose, mannitol). A similar mechanism for the osmotic regulation of MDO biosynthesis by *E. coli* has been proposed by Rumley et al. (111), although Lacroix et al. have provided evidence that the osmotic regulation of MDO biosynthesis also occurs at the level of transcription (77).

The regulation of cyclic β-(1,2)-glucan biosynthesis shares an additional feature with that found for MDO biosynthesis: the synthesis of both compounds appears to be regulated by end product inhibition (21, 111). The concentration of cyclic β-(1,2)-glucans at which inhibition of synthesis occurs falls in the same range as their periplasmic concentrations (25 to 50 mg/ml, or 8 to 15 mM, assuming an average M_r of 3,200), indicating that this inhibitory effect has physiological significance.

Involvement of Cyclic β-Glucans in Bacterium-Plant Interactions

The associations between bacteria and the tissues of animals and plants have been the subject of intensive study for many years. Such associations can be either beneficial or detrimental and thus can have an important impact on the health of the animal or plant host. The cell surface carbohydrates of pathogenic and symbiotic bacteria are recognized to provide functions during the infection process. In the case of the *Rhizobiaceae*, several classes of cell surface carbohydrates are believed to provide functions during plant infection. These cell surface carbohydrates include extracellular polysaccharides, capsular polysaccharides, lipopolysaccharides, lipooligosaccharides, and the cyclic β-glucans. The reader is referred to several recent review articles which discuss the possible roles for the exopolysaccharides and lipooligosaccharides of *Rhizobium* species during legume nodulation (51, 53, 78, 80, 119). These two classes of cell surface carbohydrates will not be considered further in the present review. In addition, the process of plant infection by *Agrobacterium* and *Rhizobium* species will not be described in the present review. The reader is referred to other recent reviews in which these processes are described in detail (49, 56, 97, 98).

Behavior of cyclic β-(1,2)-glucan-deficient mutants during infection of the host plant. The most compelling evidence for a role for the cyclic β-(1,2)-glucans during plant infection comes from studies with *ndvA/ndvB* mutants of *R. meliloti* (39, 40) and *chvA/chvB* mutants of *A. tumefaciens* (24, 36). These studies have revealed that the *R. meliloti ndvA* and *ndvB* mutants form ineffective white pseudonodules on alfalfa and that *A. tumefaciens chvA* and *chvB* mutants are avirulent.

Closer examination of the pseudonodules induced by *ndv* mutants of *R. meliloti* reveals a small number of infection threads that abort at an early stage, and no nitrogen-fixing bacteroids are observed. The initiation of infection threads by these mutants suggests that the cyclic β-(1,2)-glucans provide functions during the later stages of alfalfa nodulation.

Additional studies with *R. meliloti ndv* mutants have revealed that although the cyclic β-(1,2)-glucans appear to provide important functions during plant infection, these molecules are not essential for legume nodulation. This is concluded because it is possible to select for symbiotic pseudorevertants of *ndv* mutants which regain the capacity to nodulate alfalfa yet remain defective for cyclic β-(1,2)-glucan biosynthesis (40). It is noted, however, that the total number of infection threads elicited by such pseudorevertants is substantially smaller (11 to 23% of wild-type levels) than that elicited by wild-type cells (40). Recently these symbiotic pseudorevertants have been more closely examined by Nagpal et al. (96). These researchers have identified a 13.5-kb DNA fragment from an *ndvA* pseudorevertant gene bank which is able to suppress the *ndv* phenotype (96). This fragment was shown to contain several genes involved in the synthesis of succinoglycan (*exo* genes) and to be derived from one of the symbiotic megaplasmids. The mechanism for suppression of the *ndv* phenotype is unclear; however, these researchers speculate that the cyclic β-(1,2)-glucans and exopolysaccharides of *R. meliloti* interact during nodule development. Alternatively, these researchers also suggest that the NdvA and NdvB proteins might form a complex with proteins involved in exopolysaccharide biosynthesis (96).

It should be considered that the cyclic β-glucans may function during several stages of plant infection. In the sections below, possible roles for these molecules are considered. These include roles in conferring host specificity, interactions with plant metabolites, roles during attachment to plant cells, and suppression of host defense responses. These roles are suggested by the structural properties of the cyclic β-glucans as well as by the phenotypic properties of *ndv* and *chv* mutants.

Cyclic β-(1,2)-glucans and host specificity. The overall ring size distributions of the cyclic β-(1,2)-glucans have been shown to be very similar among diverse species within the *Rhizobiaceae* (Table 1). Indeed, all *Rhizobium* and *Agrobacterium* species synthesize cyclic β-(1,2)-glucans containing 17 to 21 glucose residues. Thus, it is unlikely that the cyclic β-(1,2)-glucan backbones themselves confer specificity during legume nodulation. Instead, it is more probable that the cyclic β-(1,2)-glucan backbone acts as a general signaling molecule during plant infection. Evidence to support this possibility is derived from studies which have revealed that the addition of exogenous cyclic β-(1,2)-glucans to rhizobium-legume nodulation systems enhances both nodule number and the kinetics of nodule formation. This has been shown for both the *R. meliloti*-alfalfa (12, 40) and *R. leguminosarum* bv. trifolii-clover (1) systems. The enhancement of nodulation was found to occur at micromolar concentrations of cyclic β-(1,2)-glucans, providing further evidence that these glucans function as signal molecules. It is noted, however, that attempts to complement the symbiotic phenotype of *ndv* mutants of *R. meliloti* by the addition of exogenous cyclic β-(1,2)-glucans have thus far been unsuccessful (40), indicating, perhaps, that both cellular and extracellular cyclic β-(1,2)-glucans are important for successful symbiosis.

Because the cyclic β-(1,2)-glucans may become highly substituted with various moieties including phosphoglycerol, succinic acid, and methylmalonic acid, it is possible that host specificity during nodulation is determined in part by these

substituents. Indeed, substituents present on the lipooligosaccharides of *Rhizobium* species have been shown to confer host specificity during nodule development (27, 49, 119). Thus far, no cyclic β-(1,2)-glucan mutants specifically blocked at the stage of substituent addition have been identified. Such mutants are clearly needed to gain further insight concerning the possible roles for these substituents. Although the *Rhizobium ndvA* mutants and *Agrobacterium chvA* mutants have been shown to synthesize predominantly neutral, unsubstituted cyclic β-(1,2)-glucans (16, 65), the inability of these mutants to infect plants may result from the failure of cells to export these molecules.

There are two lines of evidence which suggest that the cyclic β-(1,2)-glucans are not involved in determining host specificity during legume nodulation. First, although different biovars of *R. leguminosarum* have distinct host specificities, there are no detectable differences in the structures of the cyclic β-(1,2)-glucans synthesized by these various biovars (142). Second, both *R. fredii* and *B. japonicum* are symbionts of the soybean. While *R. fredii* synthesizes cyclic β-(1,2)-glucans (12), *B. japonicum* synthesizes cyclic β-(1,6)-β-(1,3)-glucans but not cyclic β-(1,2)-glucans (11, 87, 110). Therefore, it may be the cyclic character, not the arrangement of glycosidic linkages, which represents the critical structural feature of these molecules. Further insight concerning the importance of the cyclic character of these molecules will be gained when mutants defective for cyclization are identified.

Interactions with plant metabolites. The studies described above suggest that it may be the cyclic character of the β-glucans that is important for their possible roles during plant infection. It is therefore of interest to consider properties that are unique to a cyclic glucan molecule as opposed to a linear glucan of similar size (e.g., MDO). First, it is possible that the glycosidic linkages within the cyclic glucans are more resistant to enzymatic degradation than are those present within a linear molecule. Such increased resistance could be important in the rhizosphere environment. Whether the cyclic β-glucans are substrates for plant β-glucanases has not been examined, and it is not yet known whether the cyclic β-glucans are degraded during plant infection. The Glc-β-(1,2)-Glc linkage is not widespread in nature, and only a few β-(1,2)-glucanases have been previously described. These glucanases have been detected within fungal cultures (72, 108) as well as within cultures of the soil bacterium *Cytophaga arvensicola* (84).

A second property of the cyclic β-glucans is suggested from studies with another class of cyclic glucan, the cyclodextrins. The cyclodextrins, derived from starch, are cyclic glucans containing six to eight glucose residues linked by α-(1,4) glycosidic bonds. These cyclic α-glucans have been the subject of intensive study because they have been shown to form inclusion complexes with a wide variety of hydrophobic guest molecules (124). Computer modeling studies have predicted that the cyclic β-(1,2)-glucans have internal cavity diameters similar to or slightly larger than those of the cyclodextrins (102). Thus, it is possible that the cyclic β-(1,2)-glucans also have the capacity to form inclusion complexes with hydrophobic guest molecules. Morris et al. have examined this possibility and have shown that the solubility of naringenin (a legume-derived flavonoid) is increased in the presence of cyclic β-(1,2)-glucan preparations (95). Because legume-derived flavonoids have been shown to be potent inducers of the nodulation genes of *Rhizobium* species (for a review, see reference 49), the formation of inclusion complexes between flavonoids and cyclic β-(1,2)-glucans could potentially influence the effectiveness of nodulation. This could perhaps provide an explanation for the enhancement of nodulation observed when

exogenous cyclic β-(1,2)-glucan preparations are added to rhizobium-legume nodulation systems (1, 12, 40). Interestingly, it has been shown that γ-cyclodextrin preparations (approximately 70 μM) also enhance nodule formation when added to the *R. leguminosarum* bv. trifolii-clover nodulation assay system (1).

Roles during attachment to plant cells. Studies with both *ndv* mutants of *R. meliloti* and *chv* mutants of *A. tumefaciens* have indicated that the cyclic β-(1,2)-glucans may be involved during the attachment of these bacteria to plant cells. Such a role for the cyclic β-(1,2)-glucans was first revealed with *A. tumefaciens chvA* and *chvB* mutants. These mutants were found to be defective in their ability to attach to isolated *Zinnia* leaf mesophyll cells and tobacco suspension culture cells (36, 106). Subsequently, it has been shown that *R. meliloti ndv* mutants are severely reduced in their ability to attach to the roots of alfalfa seedlings (40). From the above results, it is possible that the cyclic β-(1,2)-glucans mediate the attachment of *Rhizobium* and *Agrobacterium* species to specific sites on the plant cell surface. Although there have been no attempts to further characterize such binding sites, it is noted that an early study by Planqué and Kijne (103) suggests that the cyclic β-(1,2)-glucans have the capacity to bind to pea lectins.

Cyclic β-glucans as suppressors of plant defense responses. For successful nodule development, it is essential that a defense response not be mounted against the symbiont. How the symbiont suppresses or blocks such a defense response is unknown, although it has been suggested that cell surface carbohydrates such as lipopolysaccharides and extracellular polysaccharides provide such a function (22, 78, 97, 99). It is worth considering whether the cyclic β-glucans could also provide such a function. It would seem that the symbiont is potentially vulnerable to a defense response by the host plant throughout the entire process of nodule development. Thus, it is probable that the symbiont must continue to synthesize compounds which suppress the host defense response even after differentiation into the bacteroid form.

Consistent with a possible role for the cyclic β-glucans as suppressors of a plant defense response, recent studies have revealed that cyclic β-glucan biosynthesis continues throughout nodule development. In fact, the levels of the cyclic β-(1,6)-β-(1,3)-glucans within *B. japonicum* USDA 110 bacteroids isolated from mature soybean nodules are similar to levels present within aerobic, free-living cultures (47, 110). Similarly, cyclic β-(1,2)-glucans have been detected within mature alfalfa nodules infected with *R. meliloti* 1021 (15a). Thus, the microaerobic environment within the nodule apparently does not lead to the repression of cyclic β-glucan biosynthesis (46, 47, 110). Because oxygen is limiting and the process of nitrogen fixation is energy demanding, it would seem wasteful for the bacteroid to synthesize high levels of cyclic β-glucans unless the accumulation of these molecules is advantageous to the symbiont.

How might the cyclic β-glucans suppress a host defense response? Perhaps a mechanism for suppression can be envisioned for the cyclic β-(1,6)-β-(1,3)-glucans of *Bradyrhizobium* species. The cyclic β-glucans of *Bradyrhizobium* species share structural features with glucan fragments derived from the mycelial walls of fungal pathogens of the soybean plant (31). These fungal wall glucan fragments have been shown to be potent elicitors of a major defense response of the soybean plant, namely the production of isoflavonoid phytoalexins. Interestingly, the cyclic β-(1,6)-β-(1,3)-glucans of *B. japonicum* have also recently been shown to be elicitors of isoflavonoid production by the soybean (88). However, the profile of isoflavonoids elicited by the bradyrhizobial cyclic β-(1,6)-β-

(1,3)-glucans is different from that elicited by fungal glucan preparations. Specifically, daidzein (an inducer of the *nod* genes of *B. japonicum*) is the predominant isoflavonoid elicited by bradyrhizobial glucan preparations (88), whereas glyceollin isomers are predominant isoflavonoids elicited by fungal glucan preparations (50, 52, 88). Therefore, it is possible that the presence of bradyrhizobial glucans within the developing nodule modulates the levels of isoflavonoids produced by the plant host, thus preventing a host defense response. A similar role has been proposed for the cyclic β-(1,2)-glucans of *R. leguminosarum* (135). It was found that mutants of *R. leguminosarum* bv. viciae, defective for the secretion of cyclic β-(1,2)-glucans to the extracellular medium (as well as for the production of O-antigen-containing lipopolysaccharides), show an impaired development of nodules in the host plant (pea). Specifically, these mutants induce chalcone synthase (a key enzyme involved in the biosynthetic pathway of flavonoids) in the infected tissue, suggesting that the lack of certain polysaccharides, such as the cyclic β-(1,2)-glucans, causes a defense response in the plant host (135).

Other Functions for the Cyclic β-Glucans

Cyclic β-glucans and the cell envelope. The possibility must be considered that the failure of *ndv* and *chv* mutants to infect plants is an indirect consequence of the lack of cyclic β-(1,2)-glucan biosynthesis. For example, it may be the inability of these bacteria to properly osmoregulate that is primarily responsible for their failure to infect plants. Osmoregulatory responses may be important during nodulation, because it is likely that *Rhizobium* species encounter a range of osmotic environments within the rhizosphere, infection thread, and symbiosome. Similarly, it is likely that *Agrobacterium* species face different osmoregulatory challenges in the soil and during infection of the plant through wounds and proliferation in the intercellular spaces. Therefore, the accumulation of periplasmic cyclic β-glucans may be advantageous and/or essential for cells to adapt to these changing osmotic conditions. In fact, it has been proposed that cyclic β-(1,2)-glucan production leads to an increase in turgor pressure within the infection thread, which may be essential for infection thread growth during legume nodule development (96). Although the nature of the osmotic environment within the infection thread and symbiosome is unknown, it is interesting that trehalose accumulates within *R. meliloti* bacteroids (122). The accumulation of trehalose within bacteria, including members of the *Rhizobiaceae*, is normally induced on exposure to high osmolarity (19, 29, 131); however, trehalose accumulation is also induced within several *Rhizobium* species upon exposure to microaerobic growth conditions (62).

A second consequence of the failure of cells to synthesize cyclic β-glucans appears to be an alteration of the properties of the cell envelopes of these bacteria. Indeed, *ndv* and *chv* mutants have several altered cell surface properties, including (i) a loss of motility with reduced numbers of flagella (26, 38), (ii) a greater resistance to certain bacteriophages (26, 38), (iii) an increased sensitivity to certain antibiotics (38, 39), (iv) an increased production of extracellular polysaccharides (44, 96), and (v) modified cell surface protein composition (117). These cell surface alterations are most pronounced when cells are grown at low osmolarity and may be partially suppressed at elevated osmolarity (38). Interestingly, *mdo* mutants of *E. coli* have also been shown to have similar alterations in their cell surface properties (41, 42, 63a). A summary of the cell surface properties of a variety of *ndv* and *chv* mutants is provided in Table 3.

The pleiotropic cell surface character of the *ndv/chv* mutants suggests that the accumulation of cyclic β-glucans within the periplasmic compartment greatly influences the overall structure of the cell envelope. Furthermore, studies with mutants containing downstream *ndvB* mutations indicate that the concentration of the cyclic β-(1,2)-glucans within the periplasm has an important influence on cell envelope structure. As described above, the cyclic β-(1,2)-glucans synthesized by mutants with downstream *ndvB* mutations (lacking 15 to 40% of the C-terminal region of the NdvB protein) are similar to the glucans synthesized by wild-type cells in both size distribution and substituent profile (16). However, these mutants synthesize lower levels of the cyclic β-(1,2)-glucans (e.g., 20 to 40% of wild-type levels). The cell surface properties of the mutants with downstream *ndvB* mutations are intermediate in character between those of wild-type cells and mutants with upstream *ndv* mutations (64), and these mutants with downstream mutations have unusual symbiotic properties. For example, both ineffective pseudonodules and nitrogen-fixing-effective nodules are induced on the same host plant (64). Furthermore, the bacteroids present within the nitrogen-fixing-effective nodules have an unusual triskelion-like morphology (64).

From the above, it is possible that the cell envelope alterations of *ndv* and *chv* mutants are responsible for the failure of these bacteria to properly osmoregulate or infect plants. How the periplasmic cyclic β-glucans could influence the structure of the cell envelope is unclear; however, one possible mechanism is through interactions with other cell envelope components such as membrane proteins. Evidence to support this possibility is derived from recent studies with *E. coli* that have indicated an influence of MDO on outer membrane porin synthesis and activity (33, 42).

Perhaps the strongest evidence for an indirect role for the cyclic β-(1,2)-glucans during plant infection has been provided by Smit and coworkers, who have shown that *A. tumefaciens chvB* mutants fail to synthesize active rhicadhesin (115, 116, 123a). Rhicadhesin is a calcium-binding surface protein that has been implicated in the attachment of *Agrobacterium* species (and other members of the *Rhizobiaceae*) to plant cells (see reference 116 and references therein). Although the addition of purified cyclic β-(1,2)-glucans to *A. tumefaciens chvA* mutants (100) or *chvB* mutants (26) does not restore virulence, the addition of rhicadhesin has been shown to partially restore attachment ability and virulence, suggesting that it is rhicadhesin, not the cyclic β-(1,2)-glucans, that is essential for virulence and attachment (115, 123a). Whether rhicadhesin plays a role in infection and attachment for rhizobia remains to be determined; however, it was found that an *ndvB* mutant of *R. meliloti* still produced active rhicadhesin (123a). Possibly, the role(s) of rhicadhesin in attachment and infection will be clarified when *Agrobacterium* or *Rhizobium* mutants lacking rhicadhesin are isolated.

Cyclic β-glucans as a reserve material. A considerable amount of energy is devoted to the synthesis of the cyclic β-glucans, levels of which can reach 20% of the total cellular dry weight in several rhizobia (13, 139, 141). It does not appear, however, that these glucans function as carbon, phosphorus, or energy reserves for the cell. For example, in vivo ^{31}P and ^{13}C nuclear magnetic resonance analyses have shown that the levels of glycerophosphorylated and neutral cyclic β-(1,2)-glucans remain constant within stationary-phase cultures of *R. meliloti*, even after prolonged incubation in the presence of elevated NaCl concentrations [e.g., 0.4 M, a condition that suppresses cyclic β-(1,2)-glucan biosynthesis] or in the absence of external phosphate (15). Furthermore, carbon-starved rhi-

zobial cultures are apparently unable to utilize cyclic β-(1,2)-glucans as a source of carbon (61, 137). It is possible, however, that extracellular cyclic β-(1,2)-glucans are not available substrates for rhizobial β-glucanases. To date, there have been no reports of rhizobial or bradyrhizobial β-glucanases capable of hydrolyzing the cyclic β-(1,2) glucans or cyclic β-(1,6)-β-(1,3)-glucans.

TECHNICAL APPLICATIONS FOR AND LARGE-SCALE PRODUCTION OF THE CYCLIC β-GLUCANS

Applications as Inclusion Agents

In an earlier section of this review, the possibility was considered that the cyclic β-glucans form inclusion complexes with hydrophobic plant metabolites such as naringenin (95). In fact, this property of the cyclic β-glucans may be of central importance to their role during plant infection. Additional studies have indicated that the cyclic β-(1,2)-glucans form inclusion complexes with a variety of hydrophobic guest molecules such as amphotericin B, fluorescein, flurbiprofen, indomethacin, mefenamic acid, phenylbutazone, steroids, and vitamins (74, 92–94, 101). Further evidence that the cyclic β-glucans form inclusion complexes comes from studies on the behavior of these compounds during reverse-phase chromatography. Anomalies in the order of elution of a mixture of cyclic β-(1,2)-glucans have indicated that glucans of certain ring sizes bind strongly to hydrophobic C-18 resins (109).

The capacity of the cyclic β-glucans to form inclusion complexes suggests that these molecules may find industrial applications similar to those currently being explored for the cyclodextrins. The potential applications of the cyclodextrins as inclusion agents in the food and pharmaceutical industries as well as in the separation sciences have been the subject of many reviews (see, e.g., reference 124). Although the cyclodextrins and the cyclic β-(1,2)-glucans have different backbone structures [the cyclodextrins contain six to eight glucose residues linked by α-(1,4)-glycosidic linkages], the diameters of the cavities of these two classes of cyclic glucans appear to be similar (102). For example, the inner cavity diameter of γ-cyclodextrin is 0.85 nm and that of a cyclic β-(1,2)-glucan containing 18 glucose residues is predicted to be 0.88 nm (102).

Although the cyclic β-glucans potentially have industrial applications similar to those of the cyclodextrins, it is possible that the rhizobial glucans could find unique applications. For example, the inner cavity diameters of the larger cyclic β-(1,2)-glucans (e.g., greater than 18 glucose residues) are predicted to be greater than those of the cyclodextrins. Therefore, the larger cavity diameter of these cyclic β-(1,2)-glucans may permit the formation of inclusion complexes with larger hydrophobic guest molecules. Additionally, it is noted that the cyclic β-(1,2)-glucans have a greater solubility in water than the cyclodextrins [approximately 250 and 18 g/liter for cyclic β-(1,2)-glucans and γ-cyclodextrin, respectively] (74, 101). This property may prove to be of value for certain pharmaceutical or food-processing applications, in which high concentrations of inclusion complexes may be required.

It is noted that a recent molecular modeling study has predicted that the cyclic β-(1,2)-glucans may also adopt conformational structures containing relatively small, polar cavities (136a). On the basis of these results, the authors of that study suggest that the cyclic β-(1,2)-glucans may have the capacity to chelate or coordinate ions or form complexes with polar guest molecules (136a).

Large-Scale Production

Potential applications for the cyclic β-glucans in the food and pharmaceutical industries will depend on the development of bacterial strains and culture conditions which lead to an overproduction of these molecules. Although levels of the cyclic β-glucans within cultures vary considerably among the different species, all cultures typically produce milligram quantities per liter. For example, cellular concentrations of the cyclic β-(1,2)-glucans of R. leguminosarum have been reported to range between 50 and 100 mg/g of cellular dry weight, with the highest levels produced during logarithmic growth (13, 137). The cellular concentrations of the cyclic β-(1,2)-glucans of R. meliloti are slightly higher (reaching 200 mg/g of cellular dry weight) and appear to remain constant throughout the logarithmic and stationary phases of growth (137, 141). The cellular concentrations of the cyclic β-(1,6)-β-(1,3)-glucans within B. japonicum cultures (approximately 50 mg/g of cellular dry weight) are slightly lower (85).

The relative levels of cyclic β-glucans excreted into the culture medium have also been found to be variable among different species, although the amounts excreted are typically far smaller (milligrams per liter of culture) than the levels of anionic high-molecular-weight polysaccharides produced (grams per liter) (13, 46, 137, 139). There have been, however, several reports (primarily for R. leguminosarum) that cyclic β-(1,2)-glucan excretion can be greatly enhanced. For example, R. leguminosarum mutants defective for the biosynthesis of exopolysaccharides, lipopolysaccharides, and/or capsular polysaccharides excrete 1 to 10 g of cyclic β-(1,2)-glucans per liter (14, 17, 54, 55). Modified growth conditions such as elevated temperature, high osmotic strength, or cultivation in media which promote growth to high cell densities have also been shown to result in high levels (grams per liter) of extracellular cyclic β-(1,2)-glucan production (18, 20, 138). An example of enhanced cyclic β-(1,2)-glucan production by R. leguminosarum in a growth medium which promotes high cell densities is illustrated in Fig. 4. Perhaps even higher levels of cyclic β-(1,2)-glucan production can be achieved by using combinations of exopolysaccharide mutants and modified culture conditions. It is noted, however, that the growth of an R. leguminosarum extracellular polysaccharide mutant in a medium promoting high cell densities did not lead to further elevation of cyclic β-glucan levels compared with those in the wild-type strain, although the recovery of the cyclic β-glucans was greatly improved owing to the low viscosity of the medium (17).

CONCLUDING REMARKS

The cyclic β-glucans are major cell envelope constituents of all members of the Rhizobiaceae family, and these molecules have been shown to provide functions both for the free-living forms of these bacteria and during the process of plant infection. The ndv and chv mutants of Rhizobium and Agrobacterium species, respectively, have provided the most insight concerning the possible roles for the cyclic β-glucans of the Rhizobiaceae. Mutants with mutations at these loci are impaired for growth in hypoosmotic media and are defective in their ability to infect plants. It remains to be clarified whether the cyclic β-glucans act directly or indirectly during these processes; however, additional phenotypic properties of these mutants reveal that the accumulation of cyclic β-glucans within the periplasmic compartment strongly influences the overall cell envelope architecture of these bacteria.

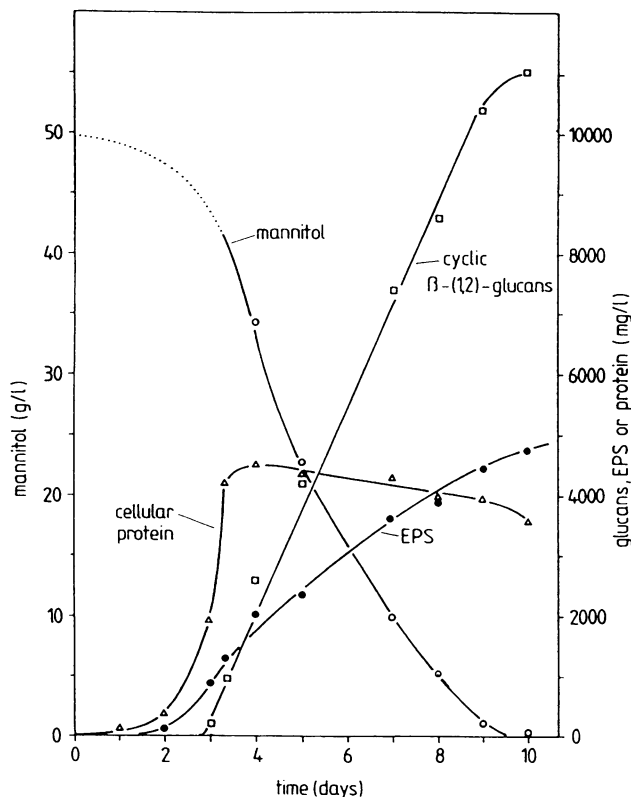Most of our understanding concerning the biosynthesis of the cyclic β-glucans is derived from studies with R. meliloti and

FIG. 4. Extracellular cyclic β-(1,2)-glucan production by *R. leguminosarum* bv. trifolii TA-1 in a mannitol-glutamic acid medium promoting growth to high cell densities. EPS, extracellular polysaccharide. Adapted from reference 18 with permission of the publisher.

A. tumefaciens. Although these studies have revealed the involvement of two proteins (NdvA/ChvA and NdvB/ChvB) during cyclic β-(1,2)-glucan biosynthesis and export, the precise roles for these proteins remain undefined. It is not known whether additional proteins are involved in cyclic β-(1,2)-glucan biosynthesis and export, but it is likely that additional proteins mediate the process of substituent addition to the cyclic β-(1,2)-glucan backbone. Much less is known concerning the biosynthesis of the cyclic β-(1,6)-β-(1,3)-glucans of *Bradyrhizobium* species. Clearly, future efforts should be made to identify additional genetic loci associated with cyclic β-glucan biosynthesis in *Rhizobium*, *Agrobacterium*, and *Bradyrhizobium* species.

The unusual structure of the cyclic β-glucans of the *Rhizobiaceae* apparently permits these molecules to form inclusion complexes with hydrophobic guest molecules. Although this property may be associated with the natural functions of these molecules, it can also be exploited for potentially many novel applications in both the food and pharmaceutical industries. Indeed, there are several laboratories in the United States, Europe, and Japan that are currently exploring potential applications for these molecules. A greater understanding of the cyclic β-glucan biosynthetic pathways among the *Rhizobiaceae* should not only provide further insight concerning the natural functions of the cyclic β-glucans but will also be critical for the development of large-scale, overproduction strategies for these fascinating molecules.

ADDENDUM

Bhagwat et al. (12a) have recently reported the cloning from *B. japonicum* USDA 110 of a 20-kb genomic DNA fragment that appears to contain loci involved in β-(1,6)-β-(1,3) glucan biosynthesis. This genomic DNA fragment was identified by complementing the motility defect of an *R. meliloti ndvB* mutant (strain TY7). The complemented strain was also reported to form effective nodules on alfalfa.

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