

## Rhizobial Polysaccharides Involved in the Symbiosis with *Glycine max*

JOHN G. STREETER

*Department of Horticulture & Crop Science, The Ohio State University and Ohio Agricultural Research & Development Center, 1680 Madison Ave., Wooster, OH 44691, USA, Tel. +330-263-3883, Fax. +330-263-3887, E-mail. streeter.1@osu.edu*

Received May 1, 1999; Accepted August 10, 1999

### Abstract

Polysaccharides produced by the nitrogen-fixing microsymbionts of soybean represent a significant commitment of reduced carbon by the organism. This does not assure that they play some role in the symbiosis but it would be logical if one or more of the types of polysaccharide was important. For exopolysaccharide (EPS) – the extracellular polysaccharide produced in culture, the situation is still confusing and is complicated by the fact that many EPS-negative mutants have complex phenotypes. For "nodule polysaccharide" (NPS) – the extracellular polysaccharide produced in nodules, the situation is also unclear; extensive studies suggest that NPS does not play a role in the nodule – where it was discovered and is easiest to document. Some role of NPS in the infection process under certain soil conditions is still a possibility. For the cyclic  $\beta$ -glucans, recent evidence indicates that these molecules are crucial for the establishment of an effective symbiosis and this is probably true because of their ability to "disarm" plant defense mechanisms.

Keywords: EPS, NPS, cyclic glucans, *Bradyrhizobium japonicum*, *Bradyrhizobium elkanii*, *Sinorhizobium fredii*

Presented at the 8th Congress of the African Association for Biological Nitrogen Fixation, November 23–27, 1998, Cape Town, South Africa

## 1. Introduction

Soybean (*Glycine max* (L.) Merr.) forms active nitrogen-fixing root nodules with three rhizobial species: *Bradyrhizobium japonicum*, *B. elkanii*, and *Sinorhizobium fredii*. This topic is reviewed here because at least three different types of polysaccharides are produced by *B. japonicum*, resulting in a nomenclature that is confusing to many workers. Also, there is increasing evidence that at least some of these polysaccharides are important components for the establishment of effective symbioses. Future efforts to control the specificity of legume/rhizobial systems may require manipulation of the polysaccharides produced by these bacteria. A more comprehensive review relevant to the polysaccharides involved in plant-microbe interactions was published recently (Leigh and Coplin, 1992).

## 2. Extracellular Polysaccharide (EPS)

The EPS are the polysaccharides produced in liquid cultures and on agar plates – slimy materials familiar to all who have worked with rhizobia. The term "capsular polysaccharide" is used to refer to the fraction of the EPS that sticks tightly to the bacterial outer membrane; its composition is not different from the material excreted and dissociated from the cell wall (Mort and Bauer, 1982). These molecules have molecular weights in the millions; i.e., they are long chains with repeating units that are relatively simple in composition. The repeating units of EPS are different for the three species of rhizobia infecting soybean (Mort and Bauer, 1982; Lim and Tan, 1983; Huber et al., 1984). The basic structure of the three types of EPS are shown below (for simplicity, detailed features of the linkages and possible pyruvyl or acetyl substituents are not shown):

<i>B. japonicum</i>	O-methyl-galactose or galactose   (- glucose - glucose - galacturonic acid - mannose -)
<i>B. elkanii</i>	4-O-methyl-glucuronic acid   (- rhamnose - rhamnose - rhamnose -)
<i>S. fredii</i>	(- glucuronic acid - mannose - galactose - glucose -)

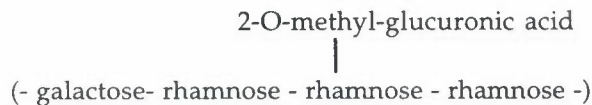
Although the exact function of these sinks for reduced carbon is not known, possibilities include protection from predators, protection from desiccation, or a

source of reserve carbon (see Leigh and Coplin, 1992). There is little evidence for or against the first two possibilities, but the latter possibility seems unlikely because, although *B. japonicum* can degrade its own EPS, *B. elkanii* cannot (Dunn and Karr, 1992; Streeter et al., 1995). (The situation in *S. fredii* has not been determined.)

Although, for some rhizobia – *R. meliloti* in particular – EPS is required for infection (Leigh and Coplin, 1992), the situation for the soybean rhizobia (and other rhizobia forming determinate nodules) is probably different. Most studies with putative EPS-mutants show unimpaired infection (see Leigh and Coplin, 1992; Gray and Rolfe, 1990). However, studies with mutants of *B. japonicum* USDA 110 having EPS with an altered structure showed delayed nodulation of *Glycine max*, accumulation of phytoalexin in roots during early stages of infection or no nodule formation on *Glycine soja* (Kosch et al., 1994; Parniske et al., 1994). Thus, the role of EPS may depend on the host genotype.

### 3. "Nodule Polysaccharide" (NPS)

Certain strains of *B. japonicum* and *B. elkanii* also produce extracellular polysaccharide in nodules – named "NPS" to distinguish it from the EPS produced in culture (Streeter et al., 1992). Although copious quantities of very viscous, high molecular weight NPS are deposited in the symbiosome space of soybean nodules, nitrogen-fixing activity is not seriously impaired (Streeter and Salminen, 1993a). Curiously, the structure of *B. elkanii* NPS is the same as its EPS (see above), whereas *B. japonicum* produces a totally different polysaccharide (An et al., 1995):



Equally curious is the fact that *B. japonicum* cannot degrade its NPS whereas it can degrade its EPS (Streeter et al., 1995). Also, the structure of *B. japonicum* NPS is remarkably similar to the structure of *B. elkanii* EPS/NPS (An et al., 1995).

A survey of strains of *B. japonicum* and *B. elkanii* revealed that strains that dominate nodule formation in soybean fields in the U.S. are most likely to be NPS-positive strains (Streeter et al., 1992; Streeter and Salminen, 1993b). Specifically, about 70% of the strains tested in serogroups 31 and 123 – bacterial types common in U.S. soils – were NPS-positive, whereas the synthesis of NPS was rare among members of serogroups that are not commonly found in soybean fields. This suggested that NPS might play some role in improving the

survival of nodule occupants. Tn5 mutants of *B. japonicum* lacking the ability to produce NPS have been isolated (Streeter et al., 1995). These mutants are stable, are EPS<sup>+</sup>, Nod<sup>+</sup>, and Fix<sup>+</sup>, and, unlike EPS mutants, do not appear to be pleiotropic. These mutants were used to study the role of NPS in protecting *B. japonicum* during nodule senescence, but the NPS<sup>+</sup>, wild-type bacteroids died at a rate similar to the NPS<sup>-</sup> mutants (Streeter et al., 1995).

Other studies indicated that NPS-positive strains are most common in soils having high calcium concentration and high pH (Streeter et al., 1994). In an analysis of the NPS phenotype of 450 nodules from field grown plants in Western Ohio, nodule occupancy by NPS-forming bacteria was correlated as follows:

%NPS <sup>+</sup> vs. pH:	$r = 0.758^{**}$ (range of pH values from 5.3 to 7.7)
%NPS <sup>+</sup> vs. Ca conc.:	$r = 0.743^{**}$ (range from 895 to 3190 mg Ca/kg dry soil)
%NPS <sup>+</sup> vs. Ca% base saturation:	$r = 0.703^{*}$ (range from 53% to 90%)

Because calcium may inhibit ammonium transport from bacteroids through the symbiosome membrane (Tyerman et al., 1995), it was thought that deposition of the anionic NPS in the peribacteroid space might sequester Ca<sup>++</sup> in the nodule. However, studies showed that NPS accumulation in nodules did not protect N<sub>2</sub> fixation against the inhibitory effects of high calcium (Streeter, 1998).

Although NPS was discovered as a polysaccharide deposited inside of nodules, NPS might play some role in infection and, in recent unpublished studies, this possibility has been investigated by analysis of nodule occupancy by isogenic NPS<sup>+/-</sup> strains in response to adjustment of soil pH. For this study a local soil that is essentially void of *B. japonicum* was inoculated with mixtures of isogenic NPS<sup>+</sup> and NPS<sup>-</sup> bacteria. Lowering of soil pH significantly decreased nodule occupancy by the NPS<sup>+</sup> strains, as would be predicted by the correlations from field studies (Table 1). Because Ca levels in soils and soil pH are correlated, we still do not know whether pH or Ca is most important in affecting NPS formation.

#### 4. Cyclic $\beta$ -Glucans

The third class of polysaccharides consists of lower molecular weight cyclic molecules containing about 13  $\beta$ -1-3 and  $\beta$ -1-6-linked glucose molecules per ring (Miller et al., 1990; Tully et al., 1990). *S. fredii* also produces a cyclic periplasmic glucan, but the glucose linkages are  $\beta$ -1-2 linked, similar to the glucans found in other rhizobia (Bhagwat and Keister, 1992; Breedveld and Miller, 1994). In the bradyrhizobia, synthesis of these glucans is stimulated by

Table 1. Nodule occupancy by NPS-forming *B. japonicum* bacteria ("%NPS+") following pH adjustment of soil samples containing mixtures of NPS+ and NPS- strains<sup>a</sup>

Treatment	Number of nodules analyzed	Soil pH	%NPS+
Native pH	89	6.68 <sup>0.16</sup>	59.45.4
Altered pH	87	5.87 <sup>0.14</sup>	41.94.4

<sup>a</sup>Nodule occupancy determined by viscometric analysis of individual nodules (unpublished method). Standard error values (superscripts) for %NPS+ represent 8 different plants; i.e., plants = replicates. (The number of nodules analyzed per plant varied slightly between plants.) Data for soil pH represent three observations.

Table 2. Suppression of glyceollin synthesis by *B. japonicum* cyclic  $\beta$ -glucans in soybean cotyledons<sup>a</sup> (Adapted from Mithöfer et al., 1996)

Treatment	Glyceollin concentration (nmole per cotyledon)
Control	2.2
Fungal $\beta$ -glucan	
200 $\mu$ g/ml	35.4
2 $\mu$ g/ml	23.2
0.02 $\mu$ g/ml	6.6
<i>B. japonicum</i> $\beta$ -glucan	
200 $\mu$ g/ml	1.4
2 $\mu$ g/ml	3.2
0.02 $\mu$ g/ml	5.2
Fungal + rhizobial $\beta$ -glucans	
2 $\mu$ g/ml + 200 $\mu$ g/ml	5.8
2 $\mu$ g/ml + 20 $\mu$ g/ml	5.0
2 $\mu$ g/ml + 2 $\mu$ g/ml	8.0

<sup>a</sup>Fungal  $\beta$ -glucan was used to induce glyceollin synthesis in a soybean cotyledon assay.

low osmotic potentials (Tully et al., 1990; Pfeffer et al., 1994) and the *B. japonicum* glucan is "decorated" with phosphocholine (Pfeffer et al., 1994).

The biochemistry (deIannino and Ugalde, 1993) and molecular genetics (Bhagwat et al., 1993; Bhagwat et al., 1996) underlying cyclic glucan synthesis have been studied. These latter studies indicate that these periplasmic glucans play an important role in nodule formation; for example, a mutant of *R. fredii* unable to produce the  $\beta$ -1,2-glucan formed ineffective nodules on soybean

(Bhagwat et al., 1992). Furthermore, it appears that the  $\beta$ -1-6 linkages in the *B. japonicum* cyclic glucan are of particular importance because only small nodule-like structures devoid of bacteroids were formed by mutants of *B. japonicum* that produce glucans consisting only  $\beta$ -(1-3)-glycosyl linkages (Bhagwat et al., 1996).

Recent studies by Mithöfer and colleagues indicate that the *B. japonicum* glucans play a role in suppression of the defense responses of the soybean plant (Mithöfer et al., 1996). When purified *B. japonicum*  $\beta$ -glucan was added to an assay in which fungal  $\beta$ -glucan is used to elicit the synthesis of the phytoalexin glyceollin, only low concentrations of rhizobial glucan were required to suppress glyceollin accumulation (Table 2). The mechanism by which the fungal  $\beta$ -glucans elicit the defense response in soybean probably involves rapid formation of  $\text{Ca}^{2+}$  gradients and, interestingly, the *B. japonicum* cyclic glucans also suppress this the  $\text{Ca}^{2+}$  response (Mithöfer et al., 1999). Because the *B. japonicum*  $\beta$ -glucans are produced by bacteroids (Gore and Miller, 1993), these rhizobial glucans could be employed to continue to suppress the plant defense response in the functional nodule.

## 5. Genetic Complexity of Mutants

To conclude, the study of rhizobial polysaccharides is complicated by the pleiotropic nature of some mutations. For example, mutants of *R. leguminosarum* selected for altered synthesis of one polysaccharide, were found to show some defect with respect to synthesis of one or more other polysaccharides (Breedveld et al., 1993). Presumably, this is due to the interdependence of the biosynthetic pathways or transport of these polymers. The effect of this complication is that it is very difficult to draw firm conclusions about the role of a polysaccharide without carefully checking on all of the facets of the potentially pleiotropic phenotype. The extent to which these complications documented for *R. leguminosarum* relate to *B. japonicum* is not known. However, my experience with putative EPS- mutants of *B. japonicum* is that most produce reduced, but nevertheless detectable, quantities of EPS, depending on the medium used (unpublished).

## REFERENCES

- An, J., Carlson, R.W., Glushka, J., and Streeter, J.G. 1995. The structure of a novel polysaccharide produced by *Bradyrhizobium* species within soybean nodules. *Carbohydrate Research* **269**: 303-317.
- Bhagwat, A.A. and Keister, D.L. 1992. Synthesis of  $\beta$ -glucans by *Bradyrhizobium japonicum* and *Rhizobium fredii*. *Canadian Journal of Microbiology* **38**: 510-514.

- Bhagwat, A.A., Tully, R.E., and Keister, D.L. 1992. Isolation and characterization of an *ndvB* locus from *Rhizobium fredii*. *Molecular Microbiology* **6**: 2159–2165.
- Bhagwat, A.A., Tully, R.E., and Keister, D.L. 1993. Identification and cloning of a cyclic  $\beta$ -(1-3),  $\beta$ -(1-6)-D-glucan synthesis locus from *Bradyrhizobium japonicum*. *FEMS Microbiology Letters* **114**: 139–144.
- Bhagwat, A.A., Gross, K.C., Tully, R.E., and Keister, D.L. 1996.  $\beta$ -Glucan synthesis in *Bradyrhizobium japonicum*: Characterization of a new locus (*ndvC*) influencing  $\beta$ -(1-6) linkages. *Journal of Bacteriology* **178**: 4635–4642.
- Breedveld, M., Canter Cremers, H.C.J., Batley, M., Posthumus, M.A., Zevenhuizen, L.P.T.M., Wijffelman, C.A., and Zehnder, A.J.B. 1993. Polysaccharide synthesis in relation to nodulation behavior of *Rhizobium leguminosarum*. *Journal of Bacteriology* **175**: 750–757.
- Breedveld, M.W. and Miller, K.J. 1994. Cyclic  $\beta$ -glucans of members of the family Rhizobiaceae. *Microbiological Reviews* **58**: 145–161.
- Gore, R.S. and Miller, K.J. 1993. Cyclic  $\beta$ -1,6-1,3 glucans are synthesized by *Bradyrhizobium japonicum* bacteroids within soybean (*Glycine max*) root nodules. *Plant Physiology* **102**: 191–194.
- Gray, J.X. and Rolfe, B.G. 1990. Exopolysaccharide production in *Rhizobium* and its role in invasion. *Molecular Microbiology* **4**: 1425–1431.
- delannino, N.I. and Ugalde, R.A. 1993. Biosynthesis of cyclic  $\beta$ -(1-3),  $\beta$ -(1-6) glucan in *Bradyrhizobium* spp. *Archives of Microbiology* **159**: 30–38.
- Huber, T.A., Agarwal, A.K., and Keister, D.L. 1984. Extracellular polysaccharide composition, ex planta nitrogenase activity, and DNA homology in *Rhizobium japonicum*. *Journal of Bacteriology* **158**: 1168–1171.
- Ko, Y.H. and Gayda, R. 1990. Nodule formation in soybeans by exopolysaccharide mutants of *Rhizobium fredii* USDA 191. *Journal of General Microbiology* **136**: 105–113.
- Kosch, K., Jacobi, A., Parniske, M., Werner, D., and Müller, P. 1994. The impairment of the nodulation process, induced by a *Bradyrhizobium japonicum* exopolysaccharide mutant is determined by the genotype of the host plant. *Zeitschrift für Naturforschung* **49c**: 727–736.
- Leigh, J.A. and Coplin, D.L. 1992. Exopolysaccharides in plant-bacterial interactions. *Annual Review of Microbiology* **46**: 307–346.
- Lim, S.T. and Tan, E.L. 1983. Exopolysaccharides and lipopolysaccharides from a fast-growing strain of *Rhizobium japonicum* (USDA 191). *FEMS Microbiology Letters* **22**: 53–56.
- Miller, K.J., Gore, R.S., Johnson, R., Benesi, A.J., and Reinhold, V.N. 1990. Cell-associated oligosaccharides of *Bradyrhizobium* spp. *Journal of Bacteriology* **172**: 136–142.
- Miller, K.J., Hadley, J.A., and Gustine, D.L. 1994. Cyclic  $\beta$ -1,6-1,3-glucans of *Bradyrhizobium japonicum* USDA 110 elicit isoflavonoid production in the soybean (*Glycine max*) host. *Plant Physiology* **104**: 917–923.
- Mithöfer, A., Bhagwat, A.A., Feger, M., and Ebel, J. 1996. Suppression of fungal  $\beta$ -glucan-induced plant defence in soybean (*Glycine max* L.) by cyclic 1,3-1,6- $\beta$ -glucans from the symbiont *Bradyrhizobium japonicum*. *Planta* **199**: 270–275.
- Mithöfer, A., Ebel, J., Bhagwat, A.A., Boller, T., and Neuhaus-Url, G. 1999. Transgenic aequorin monitors cytosolic calcium transients in soybean cells challenged with  $\beta$ -glucan of chitin elicitors. *Planta* **207**: 566–574.

- Mort, A.J. and Bauer, W.D. 1982. Application of two new methods for cleavage of polysaccharides into specific oligosaccharide fragments: Structure of the capsular and extracellular polysaccharides of *Rhizobium japonicum* that bind soybean lectins. *Journal of Biological Chemistry* **257**: 1870-1875.
- Parniske, M., Schmidt, P.E., Kosch, K., and Müller, P. 1994. Plant defense responses of host plants with determinate nodules induced by EPS-defective *exoB* mutants of *Bradyrhizobium japonicum*. *Molecular Plant Microbe Interactions* **7**: 631-638.
- Pfeffer, P.E., Bécard, G., Rolin, D.B., Uknalis, J., Cooke, P., and Tu, S.-I. 1994. In vivo nuclear magnetic resonance study of the osmoregulation of phosphocholine-substituted  $\beta$ -1,3;1,6 cyclic glucan and its associated carbon metabolism in *Bradyrhizobium japonicum* USDA 110. *Applied and Environmental Microbiology* **60**: 2137-2146.
- Streeter, J.G. 1998. Effect of elevated calcium concentration in infected cells of soybean (*Glycine max* (L.) Merr.) nodules on nitrogenase activity and N input to the plant. *Journal of Experimental Botany* **49**: 997-1003.
- Streeter, J.G., Peters, N.K., Salminen, S.O., Pladys, D., and Zhaohua, P. 1995. Fate of nodule-specific polysaccharide produced by *Bradyrhizobium japonicum* bacteroids. *Plant Physiology* **107**: 857-864.
- Streeter, J.G. and Salminen, S.O. 1993a. Effect of polysaccharide deposition by *Bradyrhizobium japonicum* bacteroids in soybean nodules. *Plant Physiology and Biochemistry* **31**: 73-79.
- Streeter, J.G. and Salminen, S.O. 1993b. Distribution of the two types of polysaccharide formed by *Bradyrhizobium japonicum* bacteroids in nodules on field-grown soybean plants (*Glycine max* (L.) Merr.). *Soil Biology and Biochemistry* **25**: 1027-1032.
- Streeter, S.O., Salminen, S.O., Beuerlein, J.E., and Schmidt, W.H. 1994. Factors influencing the synthesis of polysaccharide by *Bradyrhizobium japonicum* bacteroids in field-grown soybean nodules. *Applied and Environmental Microbiology* **60**: 2939-2943.
- Streeter, J.G., Salminen, S.O., Whitmoyer, R.E., and Carlson, R.W. 1992. Formation of novel polysaccharides by *Bradyrhizobium japonicum* bacteroids in soybean nodules. *Applied and Environmental Microbiology* **58**: 607-613.
- Tully, R.E., Keister, D.L., and Gross, K.C. 1990. Fractionation of the  $\beta$ -linked glucans of *Bradyrhizobium japonicum* and their response to osmotic potential. *Applied and Environmental Microbiology* **56**: 1518-1522.
- Tyerman, S.D., Whitehead, L.F., and Day, D.A. 1995. A channel-like transporter for  $\text{NH}_4^+$  on the symbiotic interface of  $\text{N}_2$ -fixing plants. *Nature* **378**: 629-632.