Acta Biochimica Polonica

Vol. 46 No. 4/1999

1001-1009

QUARTERLY

Chemical characterization of effective and ineffective strains of *Rhizobium leguminosarum* bv. *viciae*°

Stanislav F. Izmailov[1], Genrietta Ya. Zhiznevskaya[1]✉, Larisa V. Kosenko[2], Galina N. Troitskaya[1], Nataly N. Kudryavtseva[1], Ludmila I. Borodenko[1], Pavel N. Dubrovo[1], Ryszard Russa[3], Hubert Pietras[3] and Zbigniew Lorkiewicz[3]

[1]*Timiriazev Institute of Plant Physiology, Russian Academy of Sciences, 35 Botanicheskaya, 127276 Moscow, Russia;*
[2]*Institute of Microbiology and Virology, Ukrainian Academy of Sciences, 154 Zabolotny, Kyiv-143, 252143, Ukraine;*
[3]*Department of General Microbiology, Marie Curie-Skłodowska University, 19 Akademicka, 20-033 Lublin, Poland*

Received: 03 September, 1999

Key words: *Rhizobium leguminosarum* bv. *viciae*, lipopolysaccharides, fatty acids

Chemical composition of lipopolysaccharide (LPS) isolated from an effective (97) and ineffective (87) strains of *R. l. viciae* has been determined. LPS preparations from the two strains contained: glucose, galactose, mannose, fucose, arabinose, heptose, glucosamine, galactosamine, quinovosamine, and 3-*N*-methyl-3,6-dideoxyhexose, as well as glucuronic, galacturonic and 3-deoxyoctulosonic acid. The following fatty acids were identified: 3-OH 14:0, 3-OH 15:0, 3-OH 16:0, 3-OH 18:0 and 27-OH 28:0. The ratio of 3-OH 14:0 to other major fatty acids in LPS 87 was higher that in LPS 97.

SDS/PAGE profiles of LPS indicated that, in lipopolysaccharides, relative content of S form LPS I to that of lower molecular mass (LPS II) was much higher in the effective strain 97 than in 87.

All types of polysaccharides exo-, capsular-, lipo, (EPS, CPS, LPS, respectively) examined possessed the ability to bind faba bean lectin. The degree of affinity of the host lectin to LPS 87 was half that to LPS 97.

Fatty acids (FA) composition from bacteroids and peribacteroid membrane (PBM) was determined. Palmitic, stearic and hexadecenoic acids were common components found in both strains. There was a high content of unsaturated fatty acids in bacte-

°Studies on lipopolysaccharide composition were supported by the State Committee for Scientific Research (KBN, Poland) grant to M. Curie-Skłodowska University (Lublin, Poland).

✉Author for correspondence: Genrietta Zhiznevskaya, fax: 482 1685; e-mail: ifr@ipptas.ru

Abbreviations: CPS, capsular polysaccharide; EPS, exopolysaccharide; FA, fatty acids; LPS, lipopolysaccharide; PBM, peribacteroid membrane; *R.l. viciae*, *Rhizobium leguminosarum* bv. *viciae*; UI, unsaturation index.

roids as well as in PBM lipids. The unsaturation index in the PBM formed by strain 87 was lower than in the case of strain 97. Higher ratio of 16:0 to 18:1 fatty acids was characteristic for PMB of the ineffective strain.

The ability of rhizobia to fix nitrogen in symbiosis with legumes is of great economical and ecological interest. These associations are responsible for reduction of 120 million tons of atmospheric nitrogen to ammonia per year. In agriculture, independence from nitrogenous fertilizers increases crop production and minimizes pollution of water in lakes and reservoirs (Freiberg *et al.*, 1997). Carbohydrates produced by rhizobia play an important role in the development of nitrogen-fixing symbiosis with legumes. Additional surface and extracellular components including LPS, EPS, CPS and cyclic β-glucans (Zevenhuizen, 1981) are required for infection of the nodule tissue by the microsymbiont. LPS and EPS are the necessary components of the root nodule bacteria (Borthakur *et al.*, 1986; Carlson *et al.*, 1987; 1992; Carrion *et al.*, 1990; Noel, 1992). The development of symbiosome, an organella-like structure consisting of bacteroid, peribacteroid space and a peribacteroid membrane within a root nodule cell forms an essential stage in the symbiotic process (Bradley *et al.*, 1986; Andreeva *et al.*, 1989; Agibetov *et al.*, 1991). The functioning of symbiosomes depends on mutual exchange of metabolites between bacteroid and the host plant cell. Lipids play an important role in this process due to their significant contribution to membrane permeability. The aim of our study was to characterize the LPS preparations of rhizobia, and fatty acids composition of bacteroids and PBM. Comparative analysis of LPS from effective (97) and ineffective (87) bacteria demonstrated differences between them.

MATERIALS AND METHODS

Plant and bacterial cultures. *Vicia faba var. minor* Aushra seeds were obtained from Lithuanian Institute of Agriculture. The seeds were inoculated with the effective *R. l. viciae* 97 or the ineffective (*R. l. viciae* 87) strain obtained from the Russian Institute of Agricultural Microbiology (St. Petersburg, Russia). Plants were grown in a greenhouse under natural illumination in sand culture (Zhiznevskaya *et al.*, 1997). Five pregerminated seeds were planted per pot in five independent assays. Nitrogen and carbon content was determined using Roboprep automatic C:N analyzer. Nitrogenase activity was determined by measuring acetylene reduction by gas chromatography (Hardy *et al.*, 1968; Vasilieva & Zhiznevskaya, 1988). PBM and bacteroids were isolated as described before (Izmailov *et al.*, 1989; Zhiznevskaya *et al.*, 1995).

Lipopolysaccharide and polysaccharide preparations. LPS, were extracted from dried bacterial cells with 45% phenol (Westphal & Jann, 1965) and separated by ultracentrifugation or purified by gel permeation chromatography on Sepharose 4B (Carlson *et al.*, 1978).

Determination of sugar composition. Alditol acetates derived from monosaccharides liberated by formolysis (103°C, 16 h) followed by hydrolysis of EPS with 0.5 M H_2SO_4 (100°C, 2 h) as well as by hydrolysis of LPS with 2 M trifluoroacetic acid (120°C, 2 h), were determined by gas chromatography. For analysis of peracetylated alditols from monosaccharides containing amino, carboxy or ketodeoxy groups the LPS samples were treated as before (Russa *et al.*, 1996) and the presence of deoxy, acetamido or (derived by carboxyl-reduction) dideuterio-hydroxy-methylene group were determined using electron impact (70 eV) mass-spectroscopy.

Amino sugars liberated by hydrolysis with 3 M HCl (100°C, 6 h) were determined in a Hitachi KLA-5 automatic amino acid analyzer. Uronic acids were estimated by the carbazole-sulphuric acid reaction (Dische, 1947). 2-Keto-3-deoxyoctonate was deter-

mined with thiobarbituric acid (Osborn, 1963).

Extraction of lipids and fatty acids determination. To separate lipid A from the polysaccharide part, LPS was subjected to hydrolysis with 1% acetic acid at 100°C for 4 h. Lipid A was then extracted with chloroform, allowed to dry and solvolyzed with 0.75 M HCl in methanol (103°C, 5 h) or 2 M HCl in methanol (85°C, 16 h). Concentrated (4–5-fold) samples were suspended in water and extracted with chloroform (3-fold).

The lipid extraction from (propanol prewashed) PBM or bacteroids was performed with a $CHCl_3/CH_3OH/H_2O$ (8:4:3, by vol.) mixture. The chloroform layer after dehydration and evaporation was suspended in diethyl ether/methanol (25:1, v/v) mixture containing sodium methoxide, and incubated at room temperature with stirring. Supernatants obtained from CH_3COOH neutralized samples were evaporated and fatty acid methyl esters were extracted with hexane and analyzed by gas chromatography.

Determination of host-binding activity of polysaccharides. Lectins were extracted from seeds of *V. faba* and purified by affinity chromatography using Sephadex 100 (Entlicher *et al.*, 1970). The hemagglutination titer of the lectin preparation was 1:1024. Lectin binding activity of polysaccharides was examined by the hemaglutination inhibition test with rabbit erythrocytes.

Gas chromatography and mass-spectrometry. Alditol acetates were analyzed on Chrom-5 chromatograph (with flame ionization detector) equipped with a packed column (120 cm × 0.3 cm, 3% neopentylglycol succinate on Chromosorb) at 200°C or on Hewlett-Packard chromatograph 5980 with HP-5 capillary column (30 m × 0.25 mm) operating at 150°C for 5 min, followed by 5°C/min increase to a maximal temperature of 310°C. Fatty acid methyl esters derived from LPS were analyzed under similar conditions. Analyses of fatty acid methyl ester samples obtained from bacteroids and PBM were performed on 5% polyethyleneglycol adipate on Celite-545 packed column at 200°C.

Electron impact mass spectra of alditol acetates and fatty acids methyl esters were made at ionization potential 70 eV on HP 5971 mass spectrometer combined to HP 5980 gas chromatograph.

RESULTS

Both tested strains of *R. l. viciae* produce large amounts of exopolysaccharides and much smaller quantities of capsular polysaccharides. Capsular polysaccharides (CPS) were more intensively synthesized by strain 87. Sugar composition of EPSs was the same in both strains, but the amount was slightly lowered in ineffective strain 87 (Table 1).

Lipopolysaccharide preparations constituted 0.7% and 0.92% of the cells *R.L. viciae* 87 and 97, respectively. Both preparations were heterogeneous and on their SDS/PAGE profiles two groups of bands, representing LPS I and LPS II, were observed (Fig. 1). In LPS 97

Table 1. Composition of exopolysaccharides of *Rhizobium leguminosarum* bv. *viciae* (dry mass, %).

Composition	strain 97	strain 87
Carbohydrates	59.7 ± 2.1	53.5 ± 1.8
Glucose	30.0 ± 1.2	30.5 ± 1.7
Galactose	6.0 ± 0.4	5.8 ± 0.5
Glucuronic acid	11.2 ± 1.3	10.9 ± 1.1
Pyruvic acid	5.4 ± 0.2	5.5 ± 0.1

Figure 1. Silver stained SDS/PAGE profiles of lipopolysaccharide preparations.

Lanes: **1** and **10** (1 and 1.5 μg LPS, respectively) – *Salmonella typhimurium* LT2; **2**, (4 μg) – *S. typhimurium* drd2; **3** (2 μg) – *R. l. trifolii* TA1; **4**, **6**, **8** (2, 5, 10 μg) – *R. l. viciae* 97; **5**, **7**, **9** (2, 5 and 10 μg) – *R. l. viciae* 87.

a fraction of high molecular mass components was dominating, whereas LPS II (deprived of O-chain) prevailed in lipopolysaccharide of the ineffective strain 87. Among sugar constituents, glucose and glucuronic acid were predominating in LPSs of both strains (Table 2). Galactose, galacturonic acid, glucosamine, 2-aminogluconic acid, quinovosamine, fucose, rhamnose, arabinose, and 2-keto-3-deoxyoctonate were also present in both preparations. Mannose, galactosamine and 2,3-diaminohexuronic acid were observed only in LPS of 87 cells.

3-Hydroxymyristic, 3-hydroxyisopentadecanoic, 3-hydroxypalmitic, 3-hydroxystearic and 27-hydroxyoctacosanoic acids were the major LPSs constituents of both the effective and ineffective strains of *R. l. viciae*. Relative content of two ester linked fatty acids: palmitic and 3-hydroxyisopentadecanoic acids was evidently lower in LPS 87 (Table 3).

Fatty acid methyl ester samples derived from bacteroids of the two strains did not differ evidently in respect of saturated and unsaturated fatty acids composition (Table 4) with dominating palmitic, hexadecenoic,

Table 2. Sugar composition (relative in %) of lipopolysaccharide preparations from *R. leguminosarum* bv. *viciae* strains determined by gas chromatography/mass spectrometry of alditol acetates

Sugar	87	97
Rhamnose	0.7	0.7
Arabinose	0.5	0.5
Fucose	2.1	2.0
Xylose	1.0	0
Quinovosamine	0.75	0.6
3-*N*-methyl-3,6-dideoxyhexose	0.2	0.8
Mannose	3.5	traces
Glucose*	53.6	48.2
Glucuronic acid **	12.3	17.4
Galactose*	5.5	2.9
Galacturonic acid **	5.5	2.2
Glucosamine ***	2.5	1.0
2-Aminogluconic acid ***	1.3	0.5
Galactosamine	3.6	0
Heptose	1.2	1.9
2,3-Diamino-glucuronic acid	1.0	0
2-Keto-3-deoxyoctonic acid	1.9	2.0

*Determined by integration of peaks formed by ions at 217 *m/z*; **Determined by integration of peaks formed by ions at 219 *m/z*; ***Ratio GlcN to 2-aminogluconic acid was found from integration of peaks of ions at 145 and 146 *m/z*, respectively.

Table 3. Lipopolysaccharide fatty acid composition (% of molar ratio) of two *R. leguminosarum* bv. *viciae* strains

Fatty acid	Linkage*	87	97
3-OH 14:0	A + E	38.8	35.3
16:0	E	1.2	6.3
3-OH 15:0	E	2.3	4.3
3-OH 16:0	A	6.6	8.3
18:1	E	3.2	0
18:0	E	1	0.9
3-OH 18:0	A	8.6**	9.5
27-OH 28:0***	E	38.3	34.4

*A, amide bound; E, ester linked; **A small amount 3-OH 18:1 was also found; ***Presence of 27-oxo-28:0 as a minor constituent was observed.

octadecenoic, octadienoic and octatrienoic acids.

Contrary to bacteroids, peribacteroid membranes of root nodules formed by strains differed significantly in their fatty acid composition. In PBM 87, fatty acids were mainly saturated (60.7% of total FA). In PBM 97 dominated the unsaturated acids (68.7%), half of which were polyunsaturated. The unsaturation index (UI), as well as the ratio of two

Table 4. The comparison of relative fatty acid composition (%) of bacteroids and peribacteroid membrane (PBM) from broad bean root nodules

Fatty acids	Bacteroids		PBM	
	97	87	97	87
i-14:0	0.7	0.9	0.8	3.7
i-15:0	0.7	-	-	-
i-16:0	1.1	0.8	-	-
ai-17:0	0.1	-	1.7	-
14:0	0.2	-	3.6	2.2
15:0	0.2	-	1.5	1.1
16:0	10.4	10.2	21.5	33.7
17:0	0.2	-	-	1.1
cyc-17:0	1.4	1.3	-	3.0
18:0	1.6	2.1	2.3	15.9
Δ^9 16:1	11.2	13.3	-	3.2
Δ^7 16:1	0.3	-	10.5	4.6
Δ^{11} 18:1	50.0	49.4	3.8	7.9
Δ^9 18:1	2.0	1.8	16.8	11.3
18:2	14.2	15.0	30.1	9.7
18:3	5.6	5.2	7.5	2.6
UI			1.1	0.5
18:2/18:3			4.0	3.7
18:2/16:0			1.4	0.3

characteristic FA 18:2/16:0 in PBM 97 surpassed that in PBM 87. On comparing FA spectra for bacteroids with those for PBM it was found that acids: 16:0, 18:0, 18:1, 18:2 and 18:3 are their obligatory, common components.

LPS, EPS and CPS bound actively plant lectin isolated from *V. faba* seeds (Table 5).

Table 5. Affinity degree (%) of *Vicia faba* lectin with rhizobial polysaccharides

Polysaccharide	Strain 97	Strain 87
LPS	62	35
CPS	58	60
EPS	30	28

The degree of affinity of LPS 97 was twice as high as that of LPS 87. Lectin binding activity of LPS preparations isolated from either strain was twice as high as that of EPS binding.

DISCUSSION

Lipopolysaccharide consists of lipid A bound to a small core oligosaccharide, to which the O-side polysaccharide chain is attached. Within *Rhizobium spp.* the comparison of the composition of LPS shows as many differences between strains of a single species as among different species (de Maagd *et al.*, 1989). LPS are supposed to be signal molecules in the development of the infection thread upon initiation of its formation, or release of bacteria from the infection thread (Noel *et al.*, 1986; Carlson *et al.*, 1987; Hancock, 1991). The rhizobial mutants with defective LPS or lacking some EPS cause formation of ineffective root nodules or are unable to form them (Lorkiewicz *et al.*, 1981; Noel *et al.*, 1986; 1992; Carlson *et al.*, 1987; Geremia *et al.*, 1987; Carlson *et al.*, 1992; Kannenberg *et al.*, 1994).

During the symbiosis between *Rhizobium* and the plant, the bacteria elicit the formation of nodules on the roots of the host plant. The bacteria infect the nodule, enter the cytoplasm of the plant and create a distinct cell type called a bacteroid, which is capable of fixing atmospheric nitrogen. During the transition of bacteria into the endosymbiotic form – bacteroids, rhizobia are released from the infection thread and surrounded by the peribacteroid membrane, form symbiosomes (Andreeva *et al.*, 1989; Agibetov *et al.*, 1991). Peribacteroid membrane fulfills various functions: protection of bacteroids against excess of O_2, exchange of metabolites between host cell and that of microsymbiont, resulting in the compartmentation of the prokaryotic and eukaryotic metabolism (Izmailov, 1996; Oke & Long, 1999). Lipids in cooperation with proteins play an important role in the transport function of membranes. Nevertheless, the data on the FA composition of PBM are still limited (Bassarab *et al.*, 1989; Genina *et al.*, 1990; 1993; Kudryavtseva *et al.*, 1995). Unlike bacteroids, PBM of root nodules formed by the two strains (97, 87) studied differed significantly in the qualitative composition of FAs and the ratio of saturated to unsaturated FAs. In PBM-87 the predominating lipid components were saturated FAs (60.7%), while in PBM-97 dominated unsaturated FAs (68.7%), half of which belonged to polyunsaturated FAs. The unsaturation index as well as the ratio of two characteristic FAs 18:2/16:0 in PBM-97 was higher than in PBM-87. In PBM of *R. l. viciae*, as compared with bacteroids, the content of palmitic and oleic FA is high with a low level of vaccenic acid. These results agree with the data obtained in experiments on *Lupinus luteus* (Genina *et al.*, 1993,

Kudryavtseva *et al.*, 1995). The content of palmitic acid in the PBM of effective root nodules of yellow lupin was three times as high as in bacteroids but that of octadecenoic acids (oleic + vaccenic) was half that in bacteroids where stearic acid predominated (Genina *et al.*,1993). Unsaturation index (UI) in *R. l. viciae* was lower in PBM than in bacteroids of both strains. UI of PBM-87 was half that of PBM-97. The characteristic feature of nodule effectiveness is the increased ratio of 18:2/16:0 which for PBM 97 was 1.4 and for PBM-87 0.3. However, it is interesting to note that the ratio of 18:2/18:3 was similar in PBM-87 and PBM-97. It is known that polyunsaturated acids increase the fluidity of membrane and its permeability. This is why the revealed differences between PBMs in content of polyenic acids under effective and ineffective symbiosis may have a functional significance (Kudryavtseva *et al.*, 1995). On comparing the FA spectra for free living rhizobia, their bacteroids and PBM, it was found that 16:0, 18:0 and 18:1 FA are their obligatory components.

Plant lectins have been implicated as playing an important role in mediating recognition and specifity in *Rhizobium* – legume nitrogen fixing symbiosis (Planque & Kijne, 1977; van Rhijn *et al.*, 1998). The data obtained support the idea that each of the surface rhizobial glycopolymers performs a particular role in the nodulation process. Both studied strains were virulent forms in nodulation of *V. faba* and they exhibited LPS, EPS and CPS lectin-binding activity. The capability of CPS to interact more actively with pea lectin may be explained by the smaller molecular mass and lower viscosity of CPS in comparison with EPS (Abe *et al.*, 1984). Twice as large lectin binding capability of LPS-97 than that of the almost O-chain deprived LPS-87 could confirm the supposition that LPS lectin recognition determines some specific crucial stages of nodule development.

The authors thank Dr. Ken Giller (Wye College, University of London, U.K.) for the assistance with C:N analysis and also Dr. Garry Dobson (Scottish Crop Research Institute, Dundee, U.K.) for the assistance in the fatty acid analysis of PBM and bacteroids, as well as Jadwiga Dolecka for SDS/PAGE analysis of LPS.

REFERENCES

Abe, M., Sherwood, J.E., Hollingsworth, R.J. & Dazzo, F.B. (1984) Stimulation of clover root hair infection by lectin-binding oligosaccharides from capsular and extracellular polysaccharides of *Rhizobium trifolii*. *J. Bacteriol.* **160**, 517–520.

Agibetov, K.A., Genina, O.S., Dubrovo, P.N., Livanova, G.J., Zhiznevskaya, G.Ya. & Izmailov, S.F. (1991) Nodulins, bacteroids and their intracellular localization in root nodules of yellow lupine. *Sov. Plant Physiol.* **38**, 71–79 (English edition).

Andreeva, I.N., Kozlova, G.I., Livanova, G.I., Zhiznevskaya, G.Ya. & Izmailov, S.F. (1989) Electron microscope study of the peribacteroid space in legume root nodules. *Sov. Plant Physiol.* **36**, 447–459 (English edition).

Bassarab, S., Schenk, S.U. & Werner, D. (1989) Fatty acid composition of the peribacteroid membrane and the ER in nodules of *Glycine max* varies after infection by different strains of the microsymbiont *Bradyrhizobium japonicum*. *Botanica Acta* **102**, 196–201.

Borthakur, D., Barbur, C.E., Lamb, J.W., Daniels, M.J. & Downie, J.A. (1986) A mutation that blocks exopolysaccharide synthesis prevents nodulation of peas by *Rhizobium leguminosarum* but not of beans by *R. phaseoli* and is corrected by cloned DNA from *Rhizobium* or the phytopathogen *Xanthomonas*. *Mol. Gen. Genet.* **203**, 320–323.

Bradley, D.J., Butcher, G.W. & Galfre, G. (1986) Physical association between the peribacteroid membrane and lipopolysaccharide from the bacteroid outer membrane in *Rhizo-*

bium-infected pea root nodule cells. *J. Cell Sci.* **85**, 47–61.

Carlson, R.W., Saunders, R.E., Napoli, C. & Albersheim, P. (1978) Host-symbiont interactions. 111. Purification and partial characterization of *Rhizobium* lipopolysaccharides. *Plant Physiol.* **62**, 912–917.

Carlson, R.W., Kalembasa, S., Turowski, D., Pachori, P. & Noel, K.D. (1987) Characterization of the lipopolysaccharide from a *Rhizobium phaseoli* mutant that is defective in infection thread development. *J. Bacteriol.* **169**, 4923–4928.

Carlson, R.W., Bhat, U.R. & Reus, B. (1992) *Rhizobium* lipopolysaccharides: Their structure and evidence for their importance in the nitrogen-fixing symbiotic infection of their host legumes; in *Plant Biotechnology and Development*, pp. 33–44, CRC Press, Boca Raton.

Carrion, M., Bhat, R.U., Reus, B. & Carlson, R.W. (1990) Isolation and characterization of the lipopolysaccharides from *Bradyrhizobium japonicum*. *J. Bacteriol.* **172**, 1725–1731.

Dische, Z. (1947) A new specific color reaction of hexuronic acid. *J. Biol. Chem.* **167**, 189.

De Maagd, R.A., de Rijk, R., Mulders, J.H.M. & Lugtenberg, B.J.J. (1989) Immunological characterization of *Rhizobium leguminosarum* outer membrane antigens by use of polyclonal and monoclonal antibodies. *J. Bacteriol.* **171**, 1136–1142.

Entlicher, G., Kostir, J.V. & Kocourek, J. (1970) Studies on phytohemagglutinins. 111. Isolation and characterization of hemagglutinins from the pea (*Pisum sativum* L.) *Biochim. Biophys. Acta* **221**, 272–281.

Freiberg, C., Fellay, R., Bairock, A., Broughton, W.F., Rosenthal, A. & Perrot, X. (1997) Molecular basis of symbiosis between *Rhizobium* and legumes. *Nature* **387**, 394–401.

Genina, O.S., Borodenko, L.I., Fedorova, E.E., Agibetov, K.A., Zhiznevskaya, G.Ya. & Izmailov, S.F. (1993) The fatty acid composition of the peribacteroid membrane, the root nodule bacteroids and the plasmalemma of uninfected yellow lupin roots. *Sov. Plant Physiol.* **40**, 79–83.

Genina, O.S., Radukina, N.L., Agibetov, K.A., Zhiznevskaya, G.Ya., Vereschagin, A.G. & Izmailov, S.F. (1990) The fatty acid composition of the peribacteroid membrane and of the peribacteroid space material from *Lupinus luteus* L. root nodules. *8th Intern. Congress on Nitrogen Fixation*. Knoxville, Tennessee, U.S.A., 1990, Abstracts G-06.

Geremia, R.A., Cavaignas, S., Zorrequieta, A., Tor, N., Olivares, J. & Ugalde, R.A. (1987) A *Rhizobium meliloti* mutant that forms ineffective pseudonodules in alfalfa produces exopolysaccharide but fails to form β-(1-2)-glucan. *J. Bacteriol.* **169**, 880–884.

Hancock, R.E. (1991) Bacterial outer membranes, evolving concepts. *Am. Soc. Microbiol. News* **57**, 175–182.

Hardy, R.W.F., Holsten, R.D., Jackson, E.K. & Burns, R.C. (1968) The acetylene-ethylene assay for N_2-fixation: Laboratory and field evaluation. *Plant Physiol.* **43**, 1185–1207.

Izmailov, S.F. (1996) Physiology of symbiotic relations in legume nodules: Biogenesis and functions of the peribacteroid membrane. *Russian J. Plant Physiol.* **43**, 672–687 (English edition).

Izmailov, S.F., Radukina, N.L., Agibetov, K.A., Kadyrov, R.M., Andreeva, I.N., Fedorova, E.E. & Zhiznevskaya, G.Ya. (1989) Peribacteroid space of the yellow lupin nodule: Isolation, its content and inactness of the peribacteroid membrane. *Sov. Plant Physiol.* **36**, 257 (English edition).

Kannenberg, E.L., Perotto, S., Blanciotto, V., Rathbun, E.A. & Brewin, N.J. (1994) Lipopolysaccharide epitope expression of *Rhizobium* bacteroids as revealed by *in situ* immunolabelling of pea root nodule section. *J. Bacteriol.* **176**, 2021–2032.

Kudryavtseva, N.N., Tsydendambaev, V.D., Dubrovo, P.N., Krylova, V.V., Borodenko, L.I., Zhiznevskaya, G.Ya. & Izmailov, S.F. (1995) Role of the peribacteroid membrane in the interaction of the symbiotic partners in the nitrogen-fixing root nodules of *Lupinus luteus* L.; in *Nitrogen Fixation: Fundamentals and Applications* (Tikhonovich, I.A., *et al.*, eds.) pp. 551–556, Kluwer Acad. Publ., Netherlands.

Lorkiewicz, Z., Deryło, M., Głowacka, M., Kowalczuk, E., Russa, R. & Skorupska, A. (1981) Plasmids controlling symbiotic properties; in *Current Perspectives in Nitrogen Fixation* (Gibson, A.H. & Newton, W.E., eds.) pp. 409, Australian Academy of Sciences, Canberra.

Noel, K.D., Vandenbosch, K.A. & Kulpaca, B. (1986) Mutations in *Rhizobium phaseoli* that lead to arrested development of infection threads. *J. Bacteriol.* **168**, 1392–1401.

Noel, K.D. (1992) Rhizobial polysaccharides required in symbiosis with legumes; in *Molecular Signals in Plant Microbe Communication* (Verma, D.P.S., ed.) pp. 341–357, CRC Press, Inc. Boca Raton.

Osborn, M.J. (1963) Studies on the Gram-negative cell wall, I. Evidence for the role of 2-keto-3-deoxyoctonate in the lipopolysaccharide of *Salmonella typhimurium*. *Proc. Natl. Acad. Sci. U.S.A.* **50**, 499–506.

Oke, V. & Long, S.R. (1999) Bacterial genes induced within the nodule during the *Rhizobium* – legume symbiosis. *Molec. Microbiol.* **32**, 837–849.

Planque, K. & Kijne, J.W. (1977) Binding of pea lectin to a glucan-type polysaccharide in the cell walls of *Rhizobium leguminosarum*. *FEBS Lett.* **73**, 64–66.

van Rhijn, P., Goldberg, R.G. & Hirsch, A.M. (1998) *Lotus corniculatus* nodulation specificity is changed by the presence of a soybean lectin gene. *Plant Cell* **10**, 1233–1249.

Russa, R., Bruneteau, M., Shashkov, A.S., Urbanik-Sypniewska, T. & Mayer, H. (1996) Characterization of the lipopolysaccharides from *Rhizobium meliloti* strain 102F51 and its nonnodulating mutant WL 113. *Arch. Microbiol.* **165**, 26–33.

Vasilieva, A.V. & Zhiznevskaya, G.Ya. (1988) Nitrogen-fixing activity in broad bean root nodules in ontogeny. *Sov. Plant Physiol. (Moscow)* **35**, 386–392 (English edition).

Westphal, O. & Jann, K. (1965) Bacterial lipopolysaccharides. Extraction with phenol- water and further application of procedure. *Methods Carbohydr. Chem.* **5**, 83–91.

Zevenhuizen, L.P.T.M. (1981) Cellular glycogen, β-1,2-glucans, polyhydroxybutyric acid and extracellular polysaccharides in fast-growing species of Rhizobium. *Antonie van Leeuwenhoek J. Microbiol. Serol.* **47**, 481–497.

Zhiznevskaya, G.Ya., Troitskaya, G.N., Dubrovo, P.N. & Kosenko, L.V. (1997) Nitrogen-deficiency stress in *Vicia faba* L., resulting from ineffective symbiosis. *Russian J. Plant Physiol.* **44**, 585–591.

Zhiznevskaya, G.Ya., Klasens, V., Troitskaya, G. N., Borodenko, L.I., Kudryavtseva, N.N., Bobylev, G. & Sorokin, E.M. (1995) The effect of *Rhizobium leguminosarum*, strains on the growth of *Vicia faba* plants and the properties of root nodule symbiosis, *2nd Europ. Conf. on Grain Legumes*, Copenhagen, Proceedings, pp.70–71.