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EXPERIMENTAL ARTICLES

The Influence of Lipopolysaccharides and Glucans from Two *Rhizobium leguminosarum* bv. *viciae* Strains on the Formation and Efficiency of Their Symbioses with Pea Plants

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Abstract—The influence of lipopolysaccharides (LPS), glucans, and their unseparated complexes on nodulation activity of rhizobia and efficiency of their symbioses with pea plants was studied in vegetation tests. Two *Rhizobium leguminosarum* bv. *viciae* strains which differed in their symbiotic properties were used: strain 31 (fix^+, efficient, moderately virulent, and moderately competitive) and strain 248b (fix^-, inefficient, highly virulent, and highly competitive). Preparations of LPS–glucan complex and the respective LPS from the highly virulent strain 248b increased the nodulation activity of both strains by 10–26%. Analogous preparations from a less virulent strain 31 did not have this ability. Unseparated LPS–glucan complexes from these strains increased the productivity of plants infected with the efficient strain by 18–23% but did not change it in plants inoculated with the other, inefficient strain. No significant influence of LPS preparations on the symbiosis productivity was observed. Glucans from both strains enhanced the nodulation ability of the highly virulent strain by 36–56%. In addition, treatment of pea plants with glucan from strain 248b increased nitrogen fixation by root nodules by 27% in plants inoculated with strain 31. In general, the formation and efficiency of the symbiosis of *R. leguminosarum* bv. *viciae* with pea plants was more influenced by preparations from strain 248b, highly virulent but deficient in nitrogen fixation, than by preparations from the nitrogen fixation–proficient but less virulent strain 31.

Key words: Rhizobium, lipopolysaccharide, glucan, legume–rhizobial symbiosis.

Rhizobia produce a rich complex of cellular and extracellular glycopolymers, which control the establishment of relationships between the bacteria and the host plant at various stages of the development of the legume–rhizobial symbiosis. Extracellular glycopolymers, or exopolysaccharides (EPS), and capsular polysaccharides participate in the earliest preattachment stages of symbiont cooperation.

Polysaccharides of the cell surface of rhizobia include lipopolysaccharides (LPS) and (chito)lipooligosaccharides. Despite their cellular localization [1], lipooligosaccharides serve as extracellular signal molecules during the formation of symbiosis. They are excreted from the rhizobial cell membrane in small amounts in response to certain phenolic compounds secreted by the host plant and specifically induce nodule formation, thus serving as *Nod factors* [2].

Unlike lipooligosaccharides, two other cellular polysaccharides, cell wall LPS and a periplasmic glucan associated with it, play their roles in symbiosis as intracellular bacterial components [3–5]. Although several bacterial species are capable of LPS secretion [6], it is currently believed that LPS determine the specificity of a forming symbiotic system together with Nod factors but act later in the symbiosis development [7]. The glucan part also influences the symbiotic competence of rhizobia. The mere ability of a rhizobial cell to synthesize the glucan is insufficient to achieve competence; glucan transport across the cell membrane into the periplasm is required [5]. LPS-deficient and glucan-deficient mutant rhizobial strains can be used to study major functional roles of LPS and the periplasmic glucan. Considering the ability of bacteria to secrete these glycopolymers into the environment, their biological activity towards both bacteria and plants needs to be understood better. Such data are very scarce in the literature [8–11].

Earlier, we investigated the role of EPS in the nodulation activity of pea rhizobia and in the formation of the legume–rhizobial symbiosis [12–14]. The present study addresses the influence of cellular polysaccharides (LPS and glucan) on the nodulation activity of bacteria and on the efficiency of symbiosis formation between pea plants and *Rhizobium leguminosarum* bv. *viciae*.

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MATERIALS AND METHODS

Bacteria. Two strains of the pea microsymbiont *Rhizobium leguminosarum* bv. *viciae* were used: strain 31, fix$^+$, efficient, moderately virulent, and moderately competitive (Ukrainian Collection of Microorganisms, Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine, Kiev) and strain 248b, fix$^-$, inefficient, and highly virulent, highly competitive (Rhizobium Collection of the Research Institute for Agricultural Microbiology, St. Petersburg–Pushkin, Russia).

Isolation of LPS and glucan. The bacteria were grown at 26–28°C for three days on pea agar containing 1% mannitol. The bacterial mass was washed from the agar surface with saline; the cells were pelleted by centrifugation at 5500 *g* for 40 min, washed five times with saline, and dried with acetone and ethyl ether. LPS was isolated according to Westphal and Jann [15]. To remove acidic exoglycans and nucleic acids, the preparation was treated with 2% Cetavlon in 0.5 M NaCl. Since in rhizobia LPS is usually associated with periplasmic glucan, the LPS–glucan complexes were separated by ultracentrifugation at 105000 *g* (three 5-h spins). Pellets (LPS preparations) and supernatants (glucan preparations) were lyophilized.

Vegetation tests used pea plants (*Pisum sativum* L.) of the Uladovskii Yubileinyi variety grown in sand culture at 60% humidity. Coarse-grained stream sand was repeatedly washed and baked before filling the cultivation pots. Crushed gravel was used to ensure good drainage. The plants were irrigated with Hellriegel solution containing 0.1 norm of mineral nitrogen (0.6 mM $Ca(NO_3)_2 \cdot 4H_2O$). The seeds were surface-sterilized with concentrated sulfuric acid for 5 min, rinsed repeatedly with sterile tap water, and allowed to germinate in Petri dishes containing nutrient-deficient agar. Three-day pea germs were gauged, soaked in the inoculation suspension for 1 h, and planted in 1-kg vegetation pots.

The inoculation suspension consisted of a two-day bacterial culture washed free of EPS and supplemented with either isolated LPS or glucan or LPS–glucan complex, depending on the type of experiment. The final concentration of the polysaccharide preparations in the inoculation suspension was 1.5 mg/ml, and the cell titer was 1×10^9 cells/ml. Before the experiment, the polysaccharide solutions were heated for 1 h at 80°C. Each experiment was repeated eight times. The results were recorded 35 days after planting, when the plants were at the late vegetative to early bud stage.

Nitrogen fixation activity was assayed by the acetylene reduction method [16]; the nitrogen content in the green material was measured by micro-Kjeldahl technique [17], using a 6.25 conversion factor to calculate the protein content.

The statistical treatment was done according to Dospekhov [18]. Mean values are presented in the tables.

RESULTS AND DISCUSSION

Two strains of *R. leguminosarum* bv. *viciae*, differing strongly in their symbiotic properties, were used in this work to study the biological activity of rhizobial cellular polysaccharides. In our experiments, we used unseparated LPS–glucan complexes of both strains, as well as LPS and glucan preparations purified free of the other compound. LPS of the strains used were characterized in our laboratory previously [19]. They contain neutral sugars (glucose, galactose, mannose, fucose, and rhamnose; LPS from strain 31 also contains arabinose), uronic acids (glucuronic, galacturonic), the amino sugar glucosamine, unidentified amino compounds, and 2-keto-3-deoxyoctonic acid (KDO). Glucan preparations contained no sugars other than glucose, as indicated by gas–liquid chromatography and chemical analysis.

We found that treatment of the cells in the inoculum with the combined LPS–glucan preparation from the highly virulent strain 248b caused an increase in the nodulation activity of both strains under study (Table 1). The mean number of root nodules was 26% higher than in the control when strain 31 was used for infection and 10% higher if strain 248b was used. On the other hand, the LPS–glucan complex from the less virulent strain 31 caused no detectable change in the nodulation ability of either of the *R. leguminosarum* bv. *viciae* strains.

The influence of the individual components of the LPS–glucan complex on nodulation depended on the virulence of the infecting strain. Strain 31 formed less nodules when combined with its own LPS or glucan then when combined with its unseparated LPS–glucan complex. LPS of the highly virulent strain 248b stimulated nodulation activity of strain 31 better (by 36%) than did strain 31's own LPS. The virulence of strain 248b did not change significantly after treatment with LPS from either of the strains. In the presence of glucans, the number of nodules formed by strain 248b increased by 35–56% over the control, while the number of nodules induced by strain 31 decreased by 11–16%. Therefore, our data indicate that the virulence and nodulation activity of rhizobia are regulated by both LPS and glucans of these microorganisms.

Similarly to EPS of microsymbionts [20], polysaccharides of their LPS–glucan complex were able to control the nitrogen fixation activity of the pea plant root nodules, which require microsymbiont EPS for their formation [20]. When the seeds were inoculated with the cells of strain 31 washed free of EPS, the resulting nodules were impaired in nitrogen fixation, which could not be rescued by adding the strain's own LPS, glucan, or their unseparated complex (experiments 2–4). However, if washed cells of strain 31 were supplemented with preparations derived from the highly virulent strain 248b (experiments 5–7), the negative consequences of the lack of EPS were abolished, and the active fix$^+$ phenotype of the inoculating strain was

Table 1. Effect of polysaccharides of the *R. leguminosarum* bv. *viciae* LPS–glucan complex on the formation of nodules and their nitrogen fixation activity

Experiment no.	Strain used for inoculation	Culture pretreatment	Nodules			Nitrogen fixation activity	
			Number		Nodule mean weight, mg	μmol C_2H_4/(plant h)	μmol C_2H_4/(g nodules h)
			nodules/plant	% of control			
1	31	Native culture, no pretreatment	57	100	161	1.1	3.4
2	31	strain 31 LPS–glucan	60	105	156	0.7	2.3
3	31	strain 31 LPS	47	82	143	0.8	2.6
4	31	strain 31 glucan	48	84	133	0.7	3.0
5	31	strain 248b LPS–glucan	72	126	185	1.3	3.6
6	31	strain 248b LPS	64	112	189	1.1	3.0
7	31	strain 248b glucan	51	89	185	1.4	3.6
8	248b	Native culture, no pretreatment	90	100	ND	0	0
9	248b	strain 248b LPS–glucan	99	110	ND	0	0
10	248b	strain 248b LPS	96	107	ND	0	0
11	248b	strain 248b glucan	122	136	ND	0	0
12	248b	strain 31 LPS–glucan	91	101	ND	0	0
13	248b	strain 31 LPS	95	106	ND	0	0
14	248b	strain 31 glucan	140	156	ND	0	0
15		No inoculation	0	0	0	0	0

Note: ND stands for “not determined”.

restored, thus confirming the regulating role of LPS–glucan polysaccharides in nitrogen fixation. Interestingly, treatment with glucan or the unseparated complex of strain 248b produced nodules with higher nitrogen fixation activity than did control inoculation with untreated strain 31. In general, nitrogen fixation activity in nodules correlated with their number and mass.

Signalling functions of the glycopolymers are further supported by the observation that a 1-h incubation of pea seedlings with any preparation employed here caused formation of nodule clusters in the basal part of the taproot, with the eliciting activity of glycopolymers closely correlating with the virulence of the strain from which the preparation was derived.

A comparison of the data on the yield and nutritive value of the pea plant crop showed (Table 2) that inoculation with strain 31 combined with treatment with the LPS–glucan complex from either of the rhizobial strains led to a 18–23% increase in the mass of green material and to a 15–26% increase in its protein content (experiments 1, 2, 5). Inoculation with strain 248b was effective only if combined with the treatment with the LPS–glucan complex from the efficient strain 31 (experiments 8, 9, 12). Supplementing the inoculated strains with their own LPS increased the symbiosis efficiency in terms of both of the above parameters (compare experiment 1 vs. 3 and 8 vs. 10). The addition of LPS from another strain caused only an increase in the protein content of the plants (1 vs. 6, 8 vs. 13). Preparations of glucans from both strains did not influence the symbiosis efficiency with strain 31 (experiments 1, 4, 7) but increased protein content in the plants inoculated with strain 248b (experiments 8, 11, 14).

Analysis of the data presented here allows us to suggest that when nodule-forming bacteria inoculate pea seeds, plant growth and nutrient accumulation are stimulated not only by nitrogen fixation but also through other mechanisms of plant metabolism enhancement. One such mechanism is likely to be an increase in the general level of phytohormones in response to rhizobial glycoinducers.

Glucans probably play a special ecological role. They enhance the main symbiotic properties of rhizobia, such as virulence in the virulent but inefficient strain 248b or nitrogen fixation activity in the efficient strain 31, and therefore increase their fitness in natural environments.

We propose the following model to explain the mechanism of glucan-mediated symbiosis induction. Infection provides a plant with a number of primary signals (Nod factors, EPS, etc.) that trigger the program of nodule development. When ineffective highly virulent nodule-inducing bacteria (such as strain 248b) are used for inoculation, the low affinity of bacterial LPS to pea lectin [19] prevents the plant from obtaining the information required for the continuation of symbiosis formation and for timely development of resistance to the rhizobial infection (usually, within 1–2 days). This

Table 2. Effect of polysaccharides of the *R. leguminosarum* bv. *viciae* LPS–glucan complex on the efficiency of the host pea plant symbiosis with rhizobia

Experiment no.	Strain used for inoculation	Culture pretreatment	Yield		Protein content	
			mg/plant	% of noninoculated control	mg/plant	% of noninoculated control
1	31	Native culture, no pretreatment	560	144	140	192
2	31	strain 31 LPS–glucan	660	169	161	221
3	31	strain 31 LPS	620	159	151	207
4	31	strain 31 glucan	510	131	121	166
5	31	strain 248b LPS–glucan	690	177	177	242
6	31	strain 248b LPS	550	141	158	216
7	31	strain 248b glucan	570	146	146	200
8	248b	Native culture, no pretreatment	470	121	59	81
9	248b	strain 248b LPS–glucan	460	118	66	90
10	248b	strain 248b LPS	510	131	73	100
11	248b	strain 248b glucan	430	110	67	92
12	248b	strain 31 LPS–glucan	500	128	84	115
13	248b	strain 31 LPS	480	123	75	103
14	248b	strain 31 glucan	450	115	79	108
15		No inoculation	390	100	73	100

Note: LSD_{05} of the yield = 25 mg/plant.

"lack of control" allows the nonfixing strain to form more nodules than happens when normal symbiosis is established. At the same time, efficient strains (strain 31 in our experiments) possess a complete set of information signals required for induction of the later stages of symbiosis development. The weak response of such strains to the addition of their own glucan is likely caused by its optimal concentration in the periplasmic space of the cell wall. Enhancement of the nitrogen fixation activity after the addition of glucan from a highly virulent strain 248b to the inoculum may be due to certain structural differences between glucans from different strains, such as the degree of anionic substitution. Thus, the ability of exogenous glucans to influence the functional activity of developing nodules suggests that glucans play the role of inducers for the program of efficient symbiosis formation.

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