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EXPERIMENTAL ARTICLES

Functional Activity of Exoglycans from *Rhizobium leguminosarum* bv. *viciae* 250a and Its Nitrogen-Resistant Mutant M-71 during the Formation of Legume–Rhizobia Symbiosis against a High-Nitrogen Background

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Abstract—The functional activity of the exoglycan complex (EGC) polysaccharides from *Rhizobium leguminosarum* bv. *viciae* 250a and its nitrogen-resistant mutant M-71, capable of inducing the formation of nitrogen-fixing nodules on pea roots against a high-nitrogen background (4.8 mM NO_3^-), was studied in vegetation tests. For this purpose, the bacterial inoculum washed free of its own exoglycans was supplemented with EGC of the same or another strain grown in the presence of 6 or 20 mM nitrate. The best symbiotic characteristics (nodule number and nitrogenase activity, mass of the roots and aerial parts of plants) were recorded when the inoculum cells and exoglycans were obtained from strain M-71 grown in the presence of 20 mM nitrate. When the plants were inoculated with the cells (grown at 6 mM nitrate) + EGC (obtained at 6 mM nitrate) of this strain, the nodulation characteristics and the effectiveness of symbiosis decreased 1.5- to 2-fold. Partial recovery of the symbiotic potential of strain M-71 was observed when EGC (obtained at 20 mM nitrate) was substituted for its exoglycans (obtained at 6 mM nitrate). In the presence of exoglycans of the parent strain 250a (obtained at 6 or 20 mM nitrate), the mutant formed a substantially lesser number of nodules with a very low nitrogen-fixing activity. In turn, the mutant exoglycans synthesized in medium with either high or low nitrate nitrogen concentration did not recover the fix^+ phenotype of strain 250a, capable of forming symbiosis with pea plants only against a low-nitrogen background. In study of the relative content of high-molecular-weight exopolysaccharide components and low-molecular-weight glycans in the exoglycan complex, it was established that, in strain 250a (grown at 6 and 20 mM nitrate), as well as in its mutant M-71 (grown at 6 mM nitrate), exopolysaccharides prevailed, accounting for 72–75% of the sum of both types of glycopolymers, while low-molecular-weight glycans accounted for 25–28%. In contrast, in the EGC of strain M-71 obtained at 20 mM nitrate, which was the most active inducer of the formation of the symbiotrophic system by strain M-71 in the presence of a high mineral nitrogen concentration, low-molecular-weight glycans were the main component, accounting for 61% of total glycopolymers, while the polysaccharide content was 39%. Low-molecular-weight exoglycans are supposed to be involved in maintaining the physiological activity and the symbiotic status of rhizobia under unfavorable environmental conditions.

Key words: *Rhizobium*, legume–rhizobia symbiosis, exoglycans, biological activity.

Rhizobial exoglycans—exopolysaccharides (EPS) and capsular polysaccharides (CPS)—are of importance in the formation of symbiosis of nodule bacteria with plants such as peas, vetch, clover, alfalfa, fodder beans, and other plants that form nodules of undetermined type [1]. The biological activity of exoglycans is supposed to be realized through their interaction with the host plant lectin [2], i.e., via the mechanism of lectin–carbohydrate regulation, exerted on many endogenous processes occurring in both the micro- and the macropartner.

One of the factors repressing the establishment of legume–rhizobia symbiosis is mineral nitrogen. The presence of more than 15 mM nitrate in the plant growth medium results in the inhibition of nodule morphogenesis [3], accompanied by a decrease in the lectin activity in plant root exudates [4] and a decrease in the specificity of rhizobial binding on the surface of the root hairs [5].

Under these conditions, microsymbionts synthesize a modified complex of cellular and extracellular polysaccharides with modified physicochemical and biological properties [6–8]. In particular, in the exopolysaccharides synthesized by pea rhizobia against a high-nitrogen background, the affinity to the host plant lectin was shown to decrease almost twofold

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[7]. Since bacterial polysaccharides are plant lectin receptors, disappearance of one of the links of the lectin–carbohydrate system of symbiont recognition may impair the regulatory mechanism controlling normal development of the legume–rhizobia symbiosis. Therefore, nitrogen-resistant rhizobial strains capable of forming effective symbiosis with leguminous plants in the presence of high mineral nitrogen concentrations are of great interest. It is absolutely unclear whether the exoglycans of such bacteria exhibit signal properties as do the polysaccharides of ordinary nitrogen-sensitive rhizobia during the establishment of relationships with the host plant at the early stages of symbiosis formation.

In this connection, the aim of this work was to conduct a comparative study of the functional activity of the exoglycans of *R. leguminosarum* bv. *viciae* 250a and its nitrogen-resistant mutant M-71 during the establishment of symbiosis with pea plants against a high-nitrogen background.

MATERIALS AND METHODS

Bacteria. Two strains of *Rhizobium leguminosarum* bv. *viciae* (microsymbionts of pea plants) were used in this work: strain 250a from the Rhizobium collection of the All-Russia Research Institute of Agricultural Microbiology, Russian Academy of Agricultural Sciences (St. Petersburg–Pushkin), and strain M-71, its nitrogen-resistant mutant obtained by the chemical mutagenesis method using ethyl metasulfonate [9].

The bacteria were grown at 26–28°C for three days in mannitol–mineral medium containing 6 or 20 mM NO_3^- (0.60 and 2.02 g/l KNO_3, respectively) [10].

Inoculum preparation for plant bacterization. The bacteria of both strains were grown in test tubes in liquid medium with different nitrate nitrogen contents as described above. The culture liquid containing the exoglycan complex (EGC) was centrifuged at 40000 *g* for 40 min; the cells were washed with phyisological saline; the supernatant fluids were pooled and adjusted to a certain volume with saline. The culture liquid was added to cells of its own or the foreign strain, depending on the test variant.

All the procedures were carried out under sterile conditions.

The following designations were used to indicate the inoculated strain and the donor strain for the EGC added to the inoculation suspension: M-71 cells or 250a cells, M-71 EGC or 250a EGC. To specify cells grown or EGC obtained at a particular nitrate concentration in the medium, we hereafter use such designations as "6 mM" cells or "20 mM" EGC.

Vegetation tests were performed using sand cultures of Uladovskii yubileinyi pea plants. Gelriegel's medium containing 4.8 mM NO_3^- in the form of $Ca(NO_3)_2 \cdot 4H_2O$ (567 mg per kilogram of sand) was the nutritive substrate for the plants.

Pea seeds were subjected to surface sterilization with strong sulfuric acid, washed thoroughly with sterile tap water, and placed in petri dishes with starvation agar for germination. Calibrated three-day pea seedlings were kept in the inoculum (10^9 cells/ml) for 1 h.

Inoculated seedlings were planted into 0.5-kg vessels. Each test variant was performed in six replicates. The crop was harvested after 30 days. Special note was taken of the number of root nodules, their nitrogenase activity [11], and the dry mass of the aerial part of a plant and its roots.

Study of the influence of mineral nitrogen on the biosynthesis of exoglycans by rhizobia. In this test, the bacteria were grown as pure cultures in petri dishes on agarized mineral medium (15 ml/dish) containing 6 or 20 mM of nitrate as described above.

Upon completion of growth, the culture was quantitatively washed off of the medium with saline. The cells were precipitated by centrifugation at 40000 *g* for 40 min and washed with saline. The weight of the cells was determined after their drying at 103–105°C. The supernatant fluids were pooled, centrifuged once again, and adjusted to a certain volume. In the pooled supernatant fluid, the amount of the following was determined: (a) total extracellular carbohydrates (by reaction with phenol and sulfuric acid [12]); (b) exopolysaccharides (by the gravimetric method after their precipitation from the supernatant fluid with three volumes of ethanol and drying at 103–105°C); and (c) extracellular low-molecular-weight glycans (LMWG) (from the difference between the total carbohydrate and EPS contents). The tests were performed in three replicates (three petri dishes in each).

Statistical processing of the data was carried out according to Dospekhov [13].

RESULTS AND DISCUSSION

The strains of pea rhizobia studied in this work differed significantly in their symbiotic properties. When forming symbiosis against the high-nitrogen background, *R. leguminosarum* bv. *viciae* 250a, similarly to the overwhelming majority of other rhizobia, was unable to induce the formation of nitrogen-fixing nodules on the roots of pea plants. At the same time, its mutant M-71 displayed symbiotic effectiveness, forming nodules with a sufficiently high level of symbiotrophic nitrogen fixation [14]. For the nitrogen-resistant strain, the best symbiosis characteristics were obtained when the inoculum bacteria were grown at a high nitrate nitrogen concentration (20 mM); for the parent strain 250a, the use of the inoculum grown at a low nitrogen concentration (6 mM) was optimal [15].

Since exoglycans belong to signal molecules functioning when the relations between bacterium–plant symbiosis partners are being established, their biological activity in the inoculum intended for plant bacterization is of importance. Therefore, we studied the

Table 1. Influence of rhizobial exoglycans (EGC) on the establishment of symbiosis between *R. leguminosarum* bv. *viciae* M-71 and pea plants against a high nitrogen background (4.8 mM NO_3^-)

Test variants		Nodules		Aerial part of the plant	Roots
variant	inoculation of plants with strain M-71 cells	number per plant	nitrogen-fixing activity, nmol C_2H_4/(plant h)	g dry mass/plant	g dry mass/plant
1	No inoculation	0	0	2.8	1.5
2	"20 mM" M-71 cells + "20 mM" M-71 EGC	28.8	336.8	3.1	1.8
3	"6 mM" M-71 cells + "6 mM" M-71 EGC	15.5	151.5	2.1	1.0
4	"6 mM" M-71 cells + "20 mM" M-71 EGC	11.0	244.6	2.6	1.5
5	"20 mM" M-71 cells + "6 mM" M-71 EGC	13.0	236.9	2.5	1.6
6	"6 mM" M-71 cells + "6 mM" 250a EGC	15.3	157.4	2.1	1.0
7	"6 mM" M-71 cells + "20 mM" 250a EGC	8.0	3.8	2.0	1.4
8	"20 mM" M-71 cells + "6 mM" 250a EGC	8.3	71.5	2.2	1.5
9	"20 mM" M-71 cells + "20 mM" 250a EGC	9.1	70.2	2.0	1.3
LSD_{05}		3.1	–	0.2	0.2

Note: Here and in Table 2, cells ("6 mM" and "20 mM") and EGC ("6 mM" and "20 mM") were obtained from bacterial cultures that were grown in media containing 6 and 20 mM NO_3^+, respectively. LSD_{05} is the least significant difference between the values at a 5% level of significance.

influence of mineral nitrogen on the symbiotic competence of the cells and exoglycans intended for making up the inoculation suspension.

As seen from Table 1, when plants were bacterized with the nitrogen-resistant strain M-71, the best symbiosis parameters were obtained in variant 2 (the use for the inoculum of bacterial cells and EGC from the rhizobial cultures grown at a high nitrogen concentration (20 mM)).

When we used the inoculum grown in the presence of the starting nitrate concentration (variant 3), the pea plants formed half as many nodules and the nitrogen-fixing activity per plant was lower by a factor of 2.2. The mass of the aerial part of the plants and the roots in variant 3 was also less than in variant 2. However, when either "20 mM" EGC was substituted for "6 mM" EGC (variant 4) or "20 mM" cells were substituted for "6 mM" cells (variant 5) in this inoculum, the nitrogen-fixing activity of the nodules increased approximately 1.6-fold and the mass of the aerial part of the plants increased 1.2-fold compared to variant 3; i.e., the symbiosis effectiveness increased.

The data obtained unambiguously indicate that not only the cell systems responsible for symbiosis formation but also exoglycans are involved in the manifestation of the symbiotic potential of strain M-71 and that the biological activity of both of them is more markedly pronounced in strain M-71 when it is grown at a high nitrogen concentration.

For comparison, the EGC of the parent strain 250a was added to strain M-71 cells (variants 6–9). Typically, the same results were obtained when the "6 mM" 250a EGC was used in the inoculum (variant 6) and on the addition of the "6 mM" M-71 EGC (variant 3). However, the "20 mM" EGC of 250a (variant 7) induced the formation of 50% fewer nodules with a negligible nitrogenase activity. These results are consistent with our earlier data on the loss of the fix^+ phenotype by strain *R. leguminosarum* bv. *viciae* 240b under the conditions favorable for symbiosis formation, i.e., when "20 mM" EPS were substituted for their own "6 mM" EPS [6]. To put it differently, pea rhizobia grown in medium with a high nitrate content synthesized "inactive" EPS unable to serve as an inducer of the nodulation process.

When the plants were inoculated with strain M-71 cells grown at high nitrogen concentrations (variants 8 and 9), the symbiosis characteristics were worse than in variant 2 irrespective of the level of nitrogen in the medium in which the 250a EGC was synthesized.

Substantially different results obtained on addition to M-71 cells of their own EGC (variants 2–5) or EGC

Table 2. Influence of rhizobial exoglycans on the establishment of symbiosis between *R. leguminosarum* bv. *viciae* 250a and pea plants against a high nitrogen background (4.8 mM NO_3^-)

Inoculation variants		Nodules		Aerial part of the plant	Roots
variant	inoculation of plants with strain 250a cells	number per plant	nitrogen-fixing activity, nmol C_2H_4/(plant h)	g dry mass/plant	g dry mass/plant
1	No inoculation	0	0	2.8	1.5
2	"6 mM" 250a cells + "6 mM" 250a EGC	0.3	0	2.6	1.2
3	"20 mM" 250a cells + "20 mM" 250a EGC	0	0	2.2	1.4
4	"6 mM" 250a cells + "20 mM" 250a EGC	0	0	2.6	1.2
5	"20 mM" 250a cells + "6 mM" 250a EGC	0.6	0	2.4	1.7
6	"6 mM" 250a cells + "6 mM" M-71 EGC	4.0	28.8	2.0	1.2
7	"6 mM" 250a cells + "20 mM" M-71 EGC	1.7	9.4	2.5	1.3
8	"20 mM" 250a cells + "6 mM" M-71 EGC	0.6	0	2.4	1.7
9	"20 mM" 250a cells + "20 mM" M-71 EGC	0.8	10.4	2.0	1.3
LSD_{05}		0.2	–	0.2	0.1

of the parent strain (variants 6–9) indicate that the nitrogen-resistant mutant M-71, retaining the capacity for symbiotic cooperation with the host plant in the presence of a high mineral nitrogen concentration, developed its own adaptation mechanisms. One of them is likely to be the synthesis of modified exoglycans, which are able to play a signal role under these conditions.

As distinct from its nitrogen-resistant mutant, the parent strain 250a did not enter into symbiosis with pea plants at high nitrogen concentrations (4.8 mM NO_3^-) (Table 2, variants 2–5). The exoglycans of the mutant exhibited an insignificant stimulatory effect on the ability of 250a cells to induce nitrogen-fixing nodules (variants 6, 7, 9), the greatest effect being exerted by the "6 mM" M-71 EGC in the presence of "6 mM" 250a cells.

The analysis of the data obtained shows that the exoglycans of both strains synthesized in a low-nitrogen medium are compatible with the cells of both parent and mutant bacteria if the cells were also grown under conditions where mineral nitrogen is not readily available. This may suggest a certain degree of similarity between the physicochemical and biological properties of these exoglycans. When grown at high nitrogen concentrations, the same bacteria synthesize exoglycans that differ in their biological activity.

To clarify the question of whether the exoglycans also differ in their physicochemical characteristics, we investigated the constituent composition of the exoglycan complexes of both strains when the bacteria were grown in pure cultures. We arbitrarily subdivided the total extracellular carbohydrates into two groups: the higher molecular weight polysaccharides usually referred to as exopolysaccharides and low-molecular-weight glycans (LMWG) [10].

In this test, regardless of the medium content of nitrate (6 or 20 mM), the parent strain 250a exhibited better growth than the mutant, showing a lower capacity for the synthesis of total exoglycans (59 and 41% relative to the weight of cells, respectively, compared to strain M-71, in which exoglycans accounted for 67 and 77%).

As seen from the data in Table 3, the parent and the mutant strain synthesize both EPS and low-molecular-weight glycans. The EPS/LMWG ratio in the exoglycan complex of strain 250a, irrespective of the concentration of nitrate in the medium, as well as that in strain M-71 grown at a low nitrogen concentration, equaled 1 : (0.3–0.4); i.e., EPS were predominant in it. Their amount accounted for about 72–75% of the sum of both types of exoglycans, while the amount of LMWG constituted only 25–28%. In contrast to these results, the mutant synthesized a considerably greater amount of low-molecular-weight exoglycans (approximately 61%) in medium with a high bound nitrogen content, whereas exopolysaccharides accounted only for 39%. The EPS/LMWG ratio was 1 : 1.5.

Vegetation tests (Table 1) showed that precisely this exoglycan complex, namely, the "20 mM" M-71 EGC (variants 2 and 4), contributed to the formation of the

Table 3. Influence of mineral nitrogen on the biosynthesis of exoglycans by two *R. leguminosarum* bv. *viciae* strains

Strain	NO_3^- concentration in the medium	Total culture biomass	Cell mass	Exoglycans								
				EPS + LMWG		EPS			LMWG			EPS/LMWG ratio
	mM	mg/dish	mg/dish	mg/dish	% of cells	mg/dish	% of cells	% of (EPS + LMWG)	mg/dish	% of cells	% of (EPS + LMWG)	
250a	6	44.7	28.1	16.6	59.1	12.0	42.7	72.3	4.6	16.4	27.7	1 : 0.4
	20	38.0	27.0	11.0	40.7	8.2	30.4	74.5	2.8	10.4	25.5	1 : 0.3
M-71	6	37.3	22.3	15.0	67.3	10.9	48.9	72.7	4.1	18.4	27.3	1 : 0.4
	20	42.9	24.3	18.6	76.5	7.3	30.0	39.2	11.3	46.5	60.8	1 : 1.5
	LSD_{05}		1.7	2.6	–	2.9	–	–	3.5	–	–	–

most effective nodules when the symbiosis between the nitrogen-resistant mutant M-71 and pea plants was formed at high nitrogen concentrations (4.8 mM NO_3^-).

The biological activity of low-molecular-weight exoglycans may be explained by different causes.

One of them may the lower molecular mass of LMWG compared to EPS and, accordingly, their lesser viscosity, which increases the activity of their interaction with the host plant lectin. Thus, according to Abe *et al.* [16], the CPS and EPS of *R. trifolii* 0403 are not identical despite the similarity between the glycosyl composition and structure. They differ from each other in the viscosity of their aqueous solutions, kinetics of depolymerization by the bacteriophage enzyme, and content of noncarbohydrate substitutes (acetic, pyruvic, and 3-hydroxybutyric acids), as well as in their biological activity. The less viscous CPS and the oligosaccharide fragments obtained from both CPS and EPS were capable of binding clover lectin and stimulating the formation of infective filaments in the root hairs of clover seedlings infected by *R. trifolii* 0403, while such properties were not revealed in EPS. The authors suggested that the biological activity of EPS might be determined by its secondary structure.

Another explanation may be as follows. Under the conditions of saline stress, owing to the increased content of low-molecular-weight glycans in the exoglycan complex, the mutant retains not only its physiological activity in general but also symbiotic competence. Using *R. meliloti* SU-47 as an example, Breedveld *et al.* [17] demonstrated that, at high medium osmolarity due to both ionic and nonionic osmolytics (e.g., NaCl, sucrose), bacteria synthesize and contain a greater amount of certain oligosaccharides in the cells. Of them, trehalose, a D-glucose disaccharide, is the most prominent in terms of content, while glucose-containing oligomers with a polymerization degree of 4–6 are present in far lesser amounts. The authors consider these substances to be the osmoprotectants of bacterial cells.

In this context, it is interesting to note that, as was established by us earlier [18], the content of glucose-containing oligosaccharides in the fraction of low-molecular-weight exoglycans of *R. leguminosarum* bv. *viciae* 250a increased with a saline load in the medium. When the bacteria were grown in mineral medium with 6, 20, 60, or 75 mM of nitrate, the glucose content in the low-molecular-weight glycan fraction constituted 16, 33, 89, and 100% of the fraction weight, respectively. Since the synthesis of glucose-containing oligosaccharides of pea rhizobia is osmosis-regulated, it may be suggested that the extracellular low-molecular-weight glycans of *R. leguminosarum* bv. *viciae* also contain as their constituents the glucose-containing components exerting a protective effect on the outer cell membrane when the pressure gradient between the periplasm and the medium is great under high osmolarity of the medium.

We established earlier that EPS, higher molecular weight representatives of the exoglycan complex of *R. leguminosarum* bv. *viciae*, are able to influence the nodulation and rhizogenesis of pea plants and to regulate the effectiveness of the symbiotic apparatus formed and the symbiotrophic system as a whole [19, 20]. The results of our present investigations allow the conclusion that extracellular low-molecular-weight glycans can also be involved in maintenance of the physiological activity of rhizobia, including their symbiotic status, under different environmental conditions.

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